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TOPIC HIGHLIGHT

WJG 20th Anniversary Special Issues (2): Hepatitis C virus

Emerging therapeutic options for the management of hepatitis C infection

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Abstract

Until recently the traditional treatment for hepatitis C infection included pegylated interferon and ribavirin combination therapy. The sustained virological response (SVR) seen with this combination is poor and requires lengthy treatment to achieve. Additionally, significant side effects and numerous contraindications prevented many patients from being successfully treated with this therapy. In 2011, two new protease inhibitors, telaprevir and boceprevir, were approved for use with pegylated interferon and ribavirin in the United States by the United States Food and Drug Administration. These agents have significantly improved SVR rates; however significant problems with toxicity remain including severe skin rash and neutropenia. There are a wide range of compounds in late stage development for the future treatment of hepatitis C that exploit many different mechanisms of viral inhibition. Some of these compounds include additional protease inhibitors, like telaprevir and boceprevir, as well as inhibitors of other nonstructural proteins in the viral genome such as NS5A and NS5B, and compounds that target host proteins within the virus. Some of these agents are being developed for oral administration once daily and various combinations are being assessed for use without the need for pegylated interferon and ribavirin. This paper reviews agents in late phase development that may be commercially available within 1-2 years.

Key words: Hepatitis C; Pharmacotherapy; Interferon; Ribavirin; Protease inhibitors; Polymerase inhibitors; Cyclophilin inhibitors

Core tip: A plethora of new agents for the management of hepatitis C promising higher response rates and better tolerated side effect profiles is upon us. Many of these new drugs in development utilize novel pharmacologic mechanisms and may replace older more toxic therapies such as interferon and ribavirin. In addition, once daily dosing and shorter treatment durations should help improve adherence and optimize therapeutic outcomes for hepatitis C infection.

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INTRODUCTION

The hepatitis C virus (HCV) is a major cause of gastrointestinal morbidity and mortality worldwide. According to the World Health Organization (WHO), approximately 170 million people have chronic HCV infection with 3-4 million new infections occurring each year. In the United States, up to four million people have chronic HCV and 18-20000 new infections are diagnosed annually per the Centers for Disease Control (CDC).

Until recently treatment for HCV infection relied upon pegylated-interferon (PEG-IFN) and ribavirin combination therapy. The sustained virological response (SVR) seen with this combination is poor and requires lengthy treatment to achieve[1]. Additionally, significant side effects and numerous contraindications, prevent most patients from being successfully treated with these agents.

Recently two new protease inhibitors, telaprevir and boceprevir, have been approved by the Food and Drug Administration in the United States and the use of direct-acting antiviral (DAA) triple therapy has substantially improved SVRs[2,3]. In addition, a plethora of new chemical entities directed against various components of the nonstructural proteins in the hepatitis C virion are in late phase development and offer the promise of greater efficacy and fewer side effects, with once or twice daily oral administration and perhaps no longer requiring interferon and ribavirin. This paper will review telaprevir and boceprevir as well as agents that are in late phase development and may come available within the next 1-2 years.

TRADITIONAL TREATMENT APPROACH TO HEPATITIS C

The traditional treatment approach to hepatitis C relies heavily on combination therapy with pegylated interferon and ribavirin[4,5]. For genotype I disease, patients less than 75 kg receive peginterferon alpha-2a 180 μg by injection per week plus ribavirin 1000 mg daily in divided doses for 48 wk or peginterferon alpha-2b 1.5 μg/kg per week by injection plus ribavirin 800-1000 mg (based on weight) daily in divided doses for 48 wk. Patients greater than 75 kg receive peginterferon alpha-2a 180 μg by injection per week plus ribavirin 1200 mg daily in divided doses for 48 wk or peginterferon alpha-2b 1.5 μg/kg per week by injection plus ribavirin 1000-1400 mg (based on weight) daily in divided doses for 48 wk. Patients with genotypes 2 or 3 disease may be treated with peginterferon alpha-2a 180 μg by injection per week plus ribavirin 800 mg daily in divided doses for 24 wk or peginterferon alpha-2b 1.5 μg/kg per week by injection plus ribavirin 800-1400 mg (based on weight) daily in divided doses for 24 wk. Dose reductions may be necessary for patients with significant renal disease or those experience serious adverse reactions. For patients with contraindications or intolerance to ribavirin, monotherapy with peginterferon alpha-2a 180 μg by injection per week for 48 wk or peginterferon alpha-2b 1 μg/kg per week by injection for one year may be used.

Clinical success is greater with combination therapy, but SVR rates remain less than optimal at 54%-56%[5]. Numerous contraindications to ribavirin preclude some patients from receiving combination therapy. These contraindications include autoimmune hepatitis, decompensated liver disease, pregnancy, hemoglobinopathy, renal insufficiency, hemodialysis, thyroid disease, diabetes, rheumatoid arthritis, asthma or chronic obstructive pulmonary disease, and ischemic cardiovascular or cerebrovascular disease. Also, side effects associated with both pegylated interferon and ribavirin are substantial and include fatigue, fever, headache, nausea, arthralgia, musculoskeletal pain, insomnia, depression, neutropenia, thrombocytopenia, and anemia. For these reasons, only about 10% of patients with hepatitis C are successfully treated with traditional therapy.

TRIPLE THERAPY WITH PROTEASE INHIBITORS FOR HEPATITIS C

The hepatitis C virus contains six nonstructural HCV proteins that are processed by both viral and host proteases[6]. These nonstructural proteins (NS2, NS3, NS4A, NS4B, NS5A, and NS5B) are primarily enzymes that are essential in the HCV life cycle (Figure 1). A number of compounds have been developed which target these proteases involved in HCV polyprotein procession. They are divided into two chemical classes, macrocyclic inhibitors and linear tetra-peptide α-ketoamid derivatives. The NS3/4A protease inhibitors are potent anti-viral agents as monotherapy against HCV replication, but may cause selection of resistance species. However, this resistance appears to be attenuated when the drugs are used in combination with standard peginterferon/ribavirin therapy. The first two drugs to reach the commercial marketplace in this class are telaprevir and boceprevir.

Telaprevir

Telaprevir is an orally bioavailable NS3 protease inhibitor of the α-ketoamid class that binds the enzyme in a covalent but reversible manner with an enzyme-inhibitor complex half-life of 58 min. A phase I, placebo-controlled dose ranging study compared telaprevir 450 mg or 750 mg every 8 h with 1250 mg every 12 h in treatment-naïve genotype I patients and found the 750 mg dose to be most effective with a median reduction in HCV RNA of 4.4 $\log_{10}$ after 14 d[7]. A subsequent similar phase I study compared this dose following a 1250 mg loading dose either alone or in combination with peginterferon alpha-2a with peginterferon alpha-2a monotherapy for 14 d. The reduction in HCV RNA was 1.09 $\log_{10}$ in the peginterferon alpha-2a/placebo group, 3.99 $\log_{10}$ in the telaprevir/placebo group, and 5.49 $\log_{10}$ in the peginterferon alpha-2a/telaprevir group at the end of therapy. The development of resistant mutants was significantly lower in the peginterferon combination treatment group and no breakthrough of virus was seen throughout the study period[8]. An additional 28 d trial in treatment-naïve genotype I patients showed undetectable HCV RNA serum levels following telaprevir 750 mg every 8 h combined with peginterferon alpha-2a and weight-based dosing of ribavirin[9].

These early results were further validated in two phase Ⅱ trials, PROVE 1 (American) and PROVE 2 (European) in treatment naïve, genotype I patients. In the PROVE 1 trial, combination therapy with telaprevir

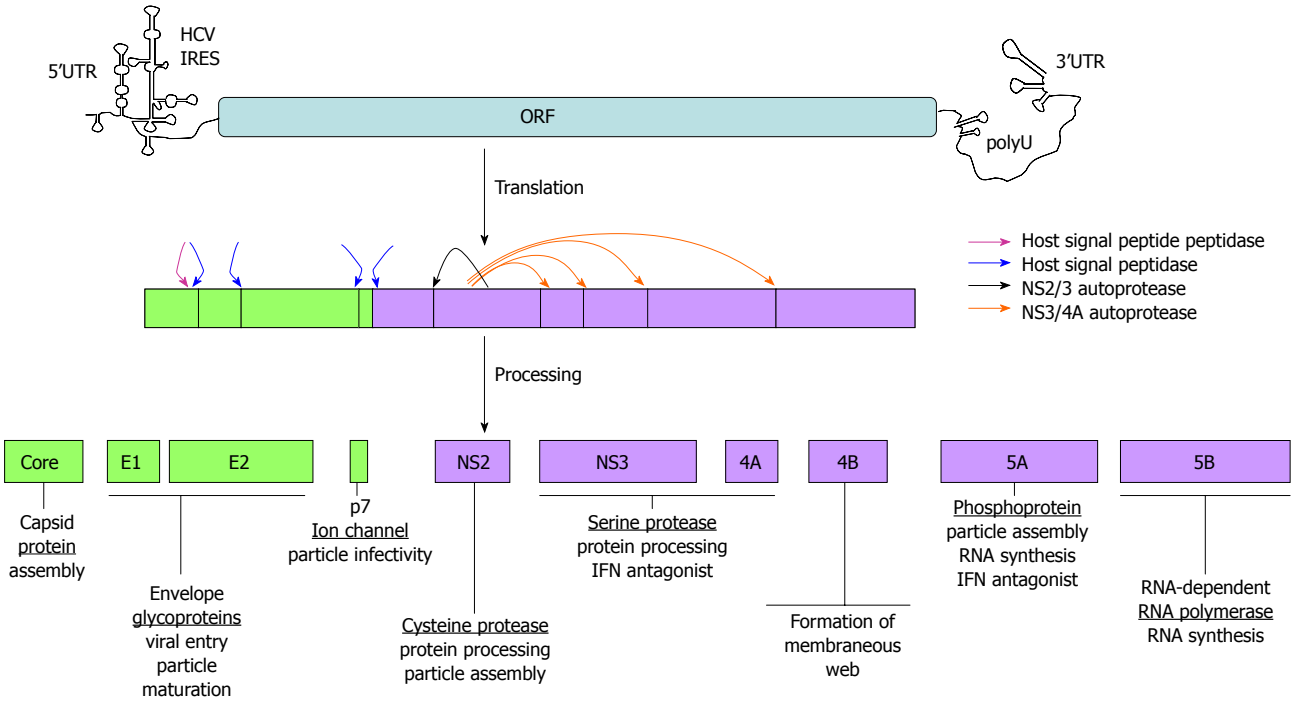

Figure 1 Hepatitis C genomic targets for drug development. HCV: Hepatitis C virus; IFN: Interferon.

1250 mg loading dose followed by 750 mg every 8 h or placebo, peginterferon alfa-2a 180 μg weekly, and ribavirin 1000-1200 mg/d based on weight for 12 wk was followed by interferon alfa-2a and ribavirin at the same dosages for an additional 0, 12, or 36 wk. Treatment was stopped after 12 or 24 wk only when a rapid virologic response (RVR) was achieved. This produced SVR rates of 35%, 61% and 67% at 12, 24, and 48 wk, respectively, *vs* 41% SVR with 48 wk standard therapy[10]. The PROVE 2 trial compared telaprevir + peginterferon alone for 12 wk, telaprevir + peginterferon and ribavirin for 12 wk, and telaprevir + peginterferon and ribavirin for 12 wk followed by an additional 12 wk of peginterferon and ribavirin alone, against standard peginterferon and ribavirin therapy. SVRs of 36%, 60%, and 69% respectively, *vs* 46% with standard therapy were documented[11]. thus, ribavirin appears important to achieve high SVRs and 12 wk of therapy appears insufficient to prevent relapse. In these trials skin rash, anemia, and gastrointestinal disorders were the most common side effects, causing up to 18% of patients to discontinue therapy.

A third trial, PROVE 3, evaluated telaprevir-based combination therapy in patients who had prior non-response or relapse with standard peginterferon and ribavirin therapy[12]. SVRs in patients retreated with telaprevir, interferon and ribavirin for 12 or 24 wk followed by peginterferon and ribavirin alone for up to 24 wk were 51% and 53%, respectively, compared with standard therapy at 14%. However, retreatment with telaprevir and interferon alone for 24 wk followed by peginterferon and ribavirin alone for an additional 24 wk gave only a 24% SVR rate, again demonstrating the need for ribavirin in initial therapy with telaprevir.

Telaprevir has since been evaluated in three large, randomized, controlled trials in both treatment naïve and standard treatment failure patients. The ADVANCE trial enrolled a total of 1095 patients with treatment naïve genotype 1 chronic hepatitis C and compared three treatment arms: telaprevir 750 mg three times daily for 8 wk followed by peginterferon and ribavirin or placebo for an additional 4 wk and then peginterferon and ribavirin in both groups for 12 subsequent weeks (or 36 subsequent weeks in patients who did not have a RVR at 24 wk); telaprevir 750 mg three times daily for 12 wk followed by peginterferon and ribavirin for 12 subsequent weeks (or 36 subsequent weeks in patients who did not have a RVR at 24 wk); and peginterferon and ribavirin or placebo for 12 wk followed by peginterferon and ribavirin for the subsequent 36 wk. SVRs were seen in 72% of the telaprevir 8-wk arm, 79% in the telaprevir 12-wk arm, but only 46% in the peginterferon and ribavirin or placebo 48-wk group ($P < 0.001$)[13]. A 24-wk treatment period appeared sufficient for patients who achieved an early RVR.

The REALIZE trial evaluated telaprevir in 662 patients of which 354 were prior relapsers and 308 were prior non-responders to standard treatment with peginterferon and ribavirin. This study compared telaprevir 750 mg three times daily with peginterferon and ribavirin for 12 wk followed by peginterferon and ribavirin for an additional 36 wk; peginterferon and ribavirin for 4 wk followed by telaprevir 750 mg three times daily with peginterferon and ribavirin for an additional 12 wk, and then peginterferon and ribavirin alone for a subsequent 32 wk; or standard therapy with peginterferon and ribavirin for a full 48 wk course. In patients who had previously relapsed, SVR rates were 84%-88% in the telaprevir

groups compared with only 22% in the placebo or peginterferon and ribavirin standard therapy groups[14]. In patients who had previously partially responded to standard therapy, SVR rates were 56%-61% in telaprevir treated patients compared with 15% in placebo or peginterferon and ribavirin treated patients. Previous nonresponders to standard therapy achieved SVRs of 31%-33% with telaprevir compared with only 5% with placebo or peginterferon and ribavirin standard therapy. The 4 wk lead-in exposure to interferon and ribavirin did not produce substantially different results than starting simultaneously with telaprevir. Patients with on-treatment virologic failure were fewer in telaprevir groups and relapse rates were lower than the control subjects for prior relapsers and prior non-responders.

A third phase Ⅲ clinical trial, ILLUMINATE, evaluated the efficacy of telaprevir therapy for 12 wk with either 24 or 48 wk of peginterferon and ribavirin based upon an achievement of extended RVR (eRVR) at 24 wk. Treatment naïve patients with genotype I chronic hepatitis C were given telaprevir 750 mg three times daily for 12 wk with peginterferon and ribavirin for at least 24 wk. Patients who achieved an extended rapid virologic response as evidenced by undetectable HCV RNA levels at weeks 4 and 12 were randomized to either stop treatment at week 24 or continue peginterferon and ribavirin therapy for a full 48 wk. Patients who did not achieve an eRVR continued peginterferon and ribavirin therapy for the full 48 wk as well. SVRs were at least 90% in both groups and total treatment for 24 wk was non-inferior to 48 wk for those achieving an extended rapid virologic response. The SVR rate was > 70% for all groups, compared with historical standards of 46%-52%, and this study population included patients with historically lower SVRs with standard therapy. In addition, the relapse rate was low for both $eRVR^+$ and $eRVR^-$ patients at an overall rate of 9.2%[15].

The safety profile of telaprevir was evaluated in a pooled analysis of adverse events reported in all five phase Ⅱ and Ⅲ placebo-controlled trials[16]. During these trials, 2012 patients received at least one dose of telaprevir and 1346 patients were randomized to receive telaprevir 750 mg three times daily for 12 wk with peginterferon and ribavirin for 24-48 wk and 764 patients were randomized to receive placebo with peginterferon and ribavirin. A total of 73% of telaprevir-treated patients and 49.1% of placebo-treated patients completed the full duration of therapy. The most frequently occurring adverse events in the telaprevir group (> 20%) included pruritus, nausea, rash, anemia, and diarrhea. Hemorrhoids, anorectal discomfort, anal pruritus, dysgeusia, and generalized pruritus occurred less frequently. Anemia caused discontinuation of participation in 2.7% of telaprevir treated patients and 0.5% of placebo treated patients. Hemoglobin concentrations decreased rapidly over the first four weeks of treatment in both groups, but continued to decrease to a greater extent thereafter in telaprevir treated patients. The initial onset of rash occurred at any time following treatment with telaprevir, but most commonly occurred within the first four weeks. Progression of severity was reported for < 10% of cases, and many cases of rash resolved over the first 24 wk of therapy. A Dermatology Expert Panel reviewed the rashes and determined that the visual appearance of rash in telaprevir-treated patients was virtually indistinguishable from that seen in peginterferon and ribavirin-treated patients. One case of rash was suggestive of Stevens-Johnson syndrome, but it was not thought to be drug related as it occurred 11 wk after the last dose of telaprevir.

Boceprevir

Boceprevir is another orally bioavailable NS3 protease inhibitor of the α-ketoamid class that binds the enzyme in a covalent but reversible manner. In an early dose ranging trial of 100-400 mg daily for 14 d as monotherapy in genotype I patients with prior treatment failure on standard therapy, the maximum dose achieved a 2.06 $\log_{10}$ reduction in HCV RNA load and was well tolerated. Viral breakthrough occurred in some patients, however[17]. A later phase I trial compared boceprevir 200 or 400 mg every 8 h for 7 d alone or in combination with peginterferon alpha 2b for 14 d with peginterferon alpha 2b monotherapy for 14 d in genotype I patients who were nonresponders to standard therapy. This approach achieved maximal reductions in HCV RNA of 2.45 and 2.88 $\log_{10}$ for boceprevir 200 and 400 mg, respectively, with peginterferon alpha 2b, 1.08 and 1.61 $\log_{10}$ for boceprevir 200 and 400 mg monotherapy, and 1.08 and 1.26 $\log_{10}$ for peginterferon alpha 2b alone in the respective boceprevir dose groups[18]. Boceprevir was well tolerated in this trial as well, both alone and in combination with peginterferon alpha-2b, but viral breakthrough was observed again, primarily in patients receiving monotherapy.

The addition of ribavirin to boceprevir and peginterferon alpha-2b was evaluated in a phase Ⅱ trial (SPRINT-1) in treatment naïve, genotype I patients. In this trial patients received 28 or 48 wk of boceprevir 800 mg three times daily, peginterferon alpha-2b, and ribavirin or a 4 wk lead-in treatment of peginterferon alpha-2b with ribavirin followed by 24 or 44 wk of boceprevir 800 mg three times daily, peginterferon alpha-2b, and ribavirin compared with standard therapy with peginterferon alpha-2b and ribavirin for a full 48 wk. The four week lead-in treatment with peginterferon alpha-2b and ribavirin boosted SVR rates from 54% to 56% at 28 wk and from 67% to 75% at 48 wk, compared with 38% with standard peginterferon alpha-2b and ribavirin therapy for 48 wk[19]. However, RVR rates with boceprevir triple therapy were only 38% compared with 70% seen in telaprevir triple therapy trials. The most common side effects seen with boceprevir in this trial were anemia, nausea, vomiting, and dysgeusia. A subsequent phase Ⅱ trial evaluated boceprevir triple therapy in HCV genotype I nonresponders, but SVR rates were poor, ranging from 2% for control to 14%

Table 1 Protease inhibitors currently under development

First generation (wave 2)
Simeprevir
Faldaprevir
Danoprevir
Vaniprevir
ABT-450/ABT-450r
Asunaprevir
Second generation
MK-5172
ACH-2684

with boceprevir[20].

Boceprevir has subsequently been evaluated in two large, well-controlled, multicenter clinical trials in both untreated and previously treated patients with genotype I chronic HCV infection. The SPRINT-2 trial enrolled a total of 1099 patients with untreated genotype I HCV infection who were randomized to receive triple therapy with boceprevir 800 mg three times daily with peginterferon alpha-2b and ribavirin for 24 or 44 wk following a lead-in treatment of 4 wk with standard peginterferon alpha-2b and ribavirin therapy and compared with a full 48 wk course of standard peginterferon alpha-2b and ribavirin therapy. One arm of the trial was allowed to discontinue therapy at 24 wk if HCV RNA levels were undetectable, while those with detectable levels received additional interferon alpha-2b and ribavirin with placebo from weeks 28 to 48. Black patients and nonblack patients were enrolled and analyzed separately. In the larger, nonblack cohort, an SVR rate of 40% was achieved with standard pegylated interferon alpha-2b and ribavirin for 48 wk, compared with 67% in patients treated with boceprevir for 24 wk and 68% in patients treated with boceprevir for 44 wk ($P < 0.001$)[21]. In the black cohort, an SVR rate of 23% was achieved with standard pegylated interferon alpha-2b and ribavirin for 48 wk, compared with 42% in patients treated with boceprevir for 24 wk ($P < 0.04$) and 53% in patients treated with boceprevir for 44 wk ($P < 0.004$). In the variable duration arm, 44% of patients had nondetectable HCV RNA levels and were able to discontinue therapy at 24 wk. Adverse events occurred in 98% of patients and were similar in number across groups. Fatigue, headache, and nausea were the most common clinical adverse events. Dysgeusia was twice as frequent in boceprevir treated patients and anemia occurred in 49% of boceprevir treated patients compared with 29% of those receiving peginterferon alpha-2b only. This led to dose reduction in 13% of the control patients and 21% of boceprevir treated patients and discontinuation of therapy in 1% and 2%, respectively.

The RESPOND-2 trial provided a similar evaluation of boceprevir in patients with chronic HCV genotype I infection, who had been previously treated with standard therapy and experienced either a nonresponse or relapse. This trial enrolled 403 patients and randomized them in a 1:2:2 fashion to standard therapy with peginterferon alpha-2b and ribavirin for 48 wk, boceprevir 800 mg three times daily with peginterferon alpha-2b and ribavirin for 32 wk, or boceprevir 800 mg three times daily with peginterferon alpha-2b and ribavirin for 44 wk. Both boceprevir triple therapy arms were preceded by 4 wk of treatment with standard peginterferon alpha-2b and ribavirin therapy. One arm of the trial was allowed to discontinue therapy at 32 wk if HCV RNA levels were undetectable, while those with detectable levels received additional interferon alpha-2b and ribavirin with placebo from weeks 36 to 48. The overall rate of SVR for boceprevir treated patients was significantly higher in this trial (59% at 32 wk and 66% at 44 wk) compared with standard therapy (21%, $P < 0.001$) for the full 48 wk[22]. Patients who had undetectable HCV RNA levels at week 8 had SVR rates of 86% after 32 wk of boceprevir triple therapy and 88% after 44 wk. Patients whose HCV RNA level decreased by less than 1 $\log_{10}$ at 4 wk had SVR rates of 33% and 34% after boceprevir triple therapy compared with 0% after standard therapy. Side effects seen in this trial were similar to those seen in SPRINT-2, with anemia being more common in boceprevir treated patients (43%-46%) compared with standard therapy (20%). Erythropoeitin was required to manage the anemia in 41%-46% of boceprevir treated patients compared with 21% with standard therapy.

DEVELOPMENTAL PROTEASE INHIBITORS

Protease inhibitors are classified as 1st generation or 2nd generation based upon resistance profiles. The generations are further subdivided into waves based on improved potency and dosing[23]. Telaprevir and boceprevir represent the first wave of the first generation of protease inhibitors. A number of agents are currently being investigated, which constitute the second wave. These agents have resistance profiles similar to telaprevir and boceprevir. Second generation protease inhibitors have pan-genotypic activity and a higher barrier for resistance than first generation PIs. Table 1 lists protease inhibitors currently under development.

Simeprevir

Simeprevir is a macrocyclic NS3/4A protease inhibitor that is active against all genotypes of hepatitis C except genotype 3. It undergoes hepatic metabolism through cytochrome p4503A with an elimination half-life of 40 h, which makes it suitable for once daily dosing[24]. In a large phase Ⅲ clinical trial, simeprevir 150 mg once daily with pegylated interferon and ribavirin for 12 wk followed by pegylated interferon and ribavirin alone for an additional 12-36 wk produced SVRs at 12 wk post-treatment (SVR_{12}) of 81% in genotype 1, treatment naïve patients[25]. Response rates were even higher in patients with the *IL28B* polymorphism and lower stage liver fibrosis. Frequently reported adverse effects included rash and indirect hyperbilirubinemia.

Table 2 Additional drugs in development for hepatitis C

NS5A hepatitis C virus replication inhibitors
Daclatasvir
Ledipasvir
ACH-3102
ABT-267
NS5B RNA dependent RNA polymerase inhibitors
Sofosbuvir
Mericitabine
ABT-333
Host targeted agents
Alisporivir
Miravirsen
Interferon-λ

Faldaprevir

Faldaprevir is another potent NS3 protease inhibitor that can be dosed on a once daily basis. However, its activity is limited to genotype 1 disease. In a large phase Ⅲ trial of genotype 1, treatment-naïve patients, faldaprevir 120 or 240 mg once daily with pegylated interferon and ribavirin for 12 wk achieved 80% SVR_{12} with even higher responses seen in patients with the *IL28B* polymorphism[26]. Indirect hyperbilirubinemia was reported in this trial as well.

Danoprevir

Danoprevir is also a potent macrocyclic protease inhibitor with activity against HCV genotypes 1, 4, and 6. Unlike simeprevir and faldaprevir, however, danoprevir requires twice daily dosing. In a recent phase Ⅱb study, danoprevir 300 mg every 8 h, 600 mg every 12 h, 900 mg every 12 h or placebo was given with pegylated interferon and ribavirin for 12 wk in genotype 1 patients and followed with pegylated interferon/ribavirin alone for an additional 48 wk[27]. Treatment was stopped at 24 wk if an extended rapid virologic response (eRVR) with HCV RNA below 15 IU/mL during weeks 4-20 was achieved. This occurred in 65% of the 300 mg group and 70% of the 600 mg group. Unfortunately, the 900 mg group was discontinued early due to reversible, grade 4 increases in alanine aminotransferase in three patients. SVR at 24 wk post-treatment was 68%, 85%, and 76% in the 300 mg, 600 mg, and 900 mg groups, respectively, *vs* 42% in the placebo group. Serious adverse effects occurred in 19% of patients in the placebo group *vs* 7%-8% of patients in the active treatment groups.

Vaniprevir

Vaniprevir is a potent NS3/4A protease inhibitor in genotypes 1 and 2 that has shown efficacy in phase Ⅱ trials when given twice daily to non-cirrhotic patients who failed previous pegylated interferon/ribavirin therapy. Vaniprevir 300 mg and 600 mg twice daily for 24-48 wk with pegylated interferon/ribavirin therapy produced SVR rates at 24 wk post-treatment ranging from 66.7%-78% *vs* only 19% with placebo and pegylated interferon/ribavirin[28]. Higher rates of gastrointestinal adverse events were seen with vaniprevir, but no difference in rash or anemia occurred between the two groups.

ABT-450/ABT-450r

ABT-450 is an NS3/4A protease inhibitor that is metabolized by cytochrome p4503A and coadministered with ritonavir 100 mg (ABT-450r) to allow once daily dosing. It has been studied in combination with ABT-333, an NS5B nonnucleoside polymerase inhibitor, as part of an interferon-free regimen[29]. Clinical trial results of this combination will be discussed later.

Asunaprevir

Asunaprevir is also a potent NS3 inhibitor that is dosed twice daily, but is limited in efficacy to genotype 1 disease. It has been studied in combination with daclatasvir, a potent NS5A replication complex inhibitor, as a part of an interferon-free regimen[30]. Clinical trial results of this combination will be discussed later.

MK-5172

MK-5172 is a second generation protease inhibitor with pan-genotypic activity. In a phase Ⅱ trial, MK-5172 100, 200, 400 and 800 mg once daily combined with pegylated interferon and ribavirin for 12 wk gave SVRs of 86%, 92%, 91%, and 87%, respectively, compared with 54% in the control group receiving triple therapy with pegylated interferon/ribavirin and boceprevir[31]. Elevations in bilirubin and serum transaminases were seen mostly in the higher dose groups. Rates of serious adverse events were similar between all groups but half as many patients discontinued therapy due to adverse events in the MK-5172 treatment arms (7% *vs* 14%).

ACH-2684

ACH-2684 is also a pan-genotypic, highly potent second generation protease inhibitor. This compound can be dosed orally once daily and does not inhibit cytochrome p450 microsomal enzymes or activate transcription[32]. ACH-2684 has completed phase I trials and is currently being evaluated in phase Ⅱ.

NS5A HCV REPLICATION INHIBITORS

Daclatasvir

Unlike protease inhibitors that generally interfere with protein processing within the HCV genome, a number of compounds in development target nonstructural proteins involved in viral replication. (Table 2) Daclatasvir works by inhibiting the function of a viral replication complex by binding to the NS5A protein. It is highly potent orally, pan-genotypic in coverage and can be dosed once daily, but offers a lower barrier to resistance so will likely be used as combination therapy. Initial studies in combination with pegylated interferon/ribavirin showed 10 and 60 mg doses once daily produced SVRs at 24 wk post-treatment of 83% compared to 25% with standard therapy alone[33]. More recently, daclatasvir combined with sofosbuvir, an NS5B polymerase inhibitor discussed

below, was shown to produce a 100% SVR at 24 wk in genotype 1 patients who had failed triple therapy with pegylated interferon/ribavirin and telaprevir or boceprevir without ribavirin[34]. The combination was generally well tolerated without serious adverse events or discontinuations related to adverse events. Daclatasvir has also been studied in combination with asunaprevir and another NS5B polymerase inhibitor (BMS-791325) in treatment-naïve, genotype 1 non-cirrhotic patients for 12 and 24 wk with SVRs of 88% and 94%, respectively[30].

Ledipasvir

Ledipasvir is also an NS5A replication inhibitor that has been studied in combination with sofosbuvir, an NS5B polymerase inhibitor, in phase Ⅱ trials. Ledipasvir 90 mg orally once daily in combination with both sofosbuvir and ribavirin in genotype 1 treatment-naïve and prior nonresponse patients increased SVRs to 100% at 12 wk post-treatment *vs* 84% in naïve patients given sofosbuvir and ribavirin alone and 10% in sofosbuvir/ribavirin nonresponders[35]. These agents are currently in phase Ⅲ trials in a fixed-dose, once daily oral combination formulation.

ACH-3102

ACH-3102 is a structurally distinct, pan-genotypic, second generation NS5A replication inhibitor with a high barrier to resistance. An ongoing phase Ⅱ clinical trial is evaluating an oral, interferon-free combination regimen of ACH-3102 and sovaprevir with and without ribavirin for 12 and 8 wk durations of treatment in genotype 1 patients with HCV[36]. ACH-3102 has been granted fast-track status from the United States Food and Drug Administration.

ABT-267

ABT-267 is an additional NS5A replication inhibitor that has been studied at a dose of 25 mg once daily in combination with ABT-450/r and ABT-333, an NS5B polymerase inhibitor, and ribavirin in non-cirrhotic, treatment-naïve patients with genotype 1 disease and prior pegylated interferon/ribavirin nonresponders. Phase Ⅱ trial data showed a greater than 90% SVR in both groups at both 12 and 24 wk post-treatment[37]. This combination regimen is currently being evaluated in a large phase Ⅲ trial.

NS5B RNA DEPENDENT RNA POLYMERASE INHIBITORS

Nucleoside/tide inhibitors (NIs) block HCV RNA transcription and elongation by acting as chain terminators. Because of the highly conserved nature of the polymerase catalytic site, NIs as a class are pan-genotypic in coverage and they have the highest barrier to resistance. Non-NIs (NNIs) bind allosteric polymerase sites away from the catalytic site and, while potent, have a much lower barrier to resistance.

Sofosbuvir

Sofosbuvir is an NS5B nucleoside inhibitor that is pan-genotypic, highly potent, and suitable for once daily oral dosing. This compound has now completed study in four large, phase Ⅲ trials and has been awarded priority review status by the United States Food and Drug Administration. Additionally, phase Ⅱ studies of sofosbuvir combination therapies as interferon-free regimens are currently underway.

Sofosbuvir 400 mg once daily was initially studied in combination with pegylated interferon/ribavirin therapy in 327 HCV patients with genotypes 1, 4, 5, or 6 for 12 wk (98% of patients were genotype 1 or 4)[38]. A SVR of 90% was achieved in these patients. A subsequent follow up study compared 499 patients with HCV genotypes 2 or 3 receiving sofosbuvir 400 mg once daily plus ribavirin or pegylated interferon plus ribavirin for 12 wk. The sofosbuvir/ribavirin regimen was shown to be non-inferior to the pegylated interferon/ribavirin regimen achieving as SVR of 67%[38]. Adverse events were less common with sofosbuvir than pegylated interferon and consisted primarily of headache, fatigue, nausea, and neutropenia.

Sofosbuvir has since been evaluated in HCV patients with genotype 2 or 3 who were not eligible to receive pegylated interferon or who had previous failed therapy with this agent. Sofosbuvir 400 mg once daily in combination with ribavirin or matching placebos was studied in 278 patients who had previously discontinued pegylated interferon therapy secondary to side effects, who had a current medical condition precluding treatment with pegylated interferon, or who decided against treatment with pegylated interferon for other reasons. Sofosbuvir produced a SVR at 12 wk of 78% *vs* 0% with placebo in these patients[39]. In a study of 201 similar patients who had failed previous therapy with pegylated interferon, sofosbuvir 400 mg once daily plus ribavirin was compared in 12 and 16 wk treatment groups. Sofosbuvir achieved SVR rates of 50% and 73% in these treatment arms respectively, compared with historical controls of 25%[39]. The drug was well tolerated in both of these trials with primary side effects of fatigue and insomnia occurring in 3%-5% of patients and few patients discontinuing use because of adverse effects.

Mericitabine

Mericitabine is also an NS5B nucleoside inhibitor with pan-genotypic activity, but requiring twice daily dosing. Mericitabine 1000 mg twice daily or placebo was given with pegylated interferon/ribavirin to 166 patients with HCV genotypes 1 or 4 for 24 wk. Patients who achieved an HCV RNA level < 15 IU/mL (eRVR) from weeks 4 to 22 stopped all treatment at that time, but all other patients continued to receive pegylated interferon/ribavirin for a full 48 wk of therapy. Mericitabine-treated patients achieved a SVR at 24 wk post-treatment of 56.8%, compared with 36.5% of placebo-treated patients[40]. Relapse rates were 27.7% and 32% in patients treated with mericitabine and placebo, respectively. The safety profile was similar in both groups, but fewer patients in the meric-

itabine group discontinued therapy for reasons of safety.

ABT-333

ABT-333 is a NS5B non-nucleoside polymerase inhibitor that has been evaluated in combination with the protease inhibitor, ABT450/r, and ribavirin in HCV genotype 1 treatment-naïve patients, as well as null or partial prior responders to pegylated interferon and ribavirin. ABT-333 at a dose of 400 mg twice daily plus ABT-450/r 150 or 250 mg and ribavirin produced SVRs of 93%-95% at 12 wk post-treatment in previously untreated patients[41]. Only 47% of prior null or partial responders to pegylated interferon and ribavirin achieved an SVR_{12}, however. As mentioned previously, this drug is currently being studied in a phase Ⅲ trial in combination with ABT-450/r and ABT-267, with or without ribavirin in genotype 1b patients with HCV.

HOST TARGETED AGENTS FOR HEPATITIS C

Alisporivir

Alisporivir is a nonimmunosuppressive form of cyclosporine that blocks HCV replication by neutralizing the peptidyl-prolyl isomerase activity of the host protein, cyclophilin, which is required by NS5B for maximum RNA binding[42]. Alisporivir 600 mg twice daily for one week followed by 600 mg daily thereafter or placebo combined with pegylated interferon and ribavirin was evaluated in 288 patients with treatment-naïve genotype 1 HCV for 24 and 48 wk of therapy[43]. SVR rates at 24 wk post-treatment were 76% in patients receiving triple therapy with alisporivir and pegylated interferon/ribavirin for 48 wk *vs* 55% in the control arm. The drug was well tolerated overall with serious adverse events occurring in 7% of patients treated with alisporivir for 24 wk and 10% of patients treated with alisporivir for 48 wk. Subsequent to this trial, 3 patients developed pancreatitis, including one who died, and the FDA asked the manufacturer to place further trials on clinical hold until it can be determined if alisporivir potentiates the risk of hepatitis that is known with interferon therapy.

Miravirsen

Miravirsen is an antisense oligonucleotide that works by inhibiting miR-122, a micro-RNA found in the liver essential to the stability and propagation of HCV RNA. Miravirsen can be dose subcutaneously once daily, is pangenotypic in activity, and has a high barrier to resistance. In a phase Ⅱa dose-ranging trial, miravirsen resulted in dose-dependent reduction in HCV RNA levels that were still not present 14 wk post-treatment[44]. No dose-limiting adverse events or escape mutations were noted in this small trial.

Interferon-λ

Pegylated interferon lambda is a type 3 interferon that signals through a different receptor than type 1 interferon, but can inhibit HCV viral replication in vitro. In early trials it was well tolerated without the usual flu-like syndrome and hematopoietic effects typically seen with interferon alpha. A phase Ⅱ trial comparing pegylated interferon lambda with pegylated interferon alpha each in combination with ribavirin found comparable SVR rates but less interferon side effects with pegylated interferon lambda[45]. This could prove to be a useful option for patients who do not respond to interferon-free regimens currently in development.

PLACE IN THERAPY OF NEW AGENTS FOR HEPATITIS C

Clearly the availability of both telaprevir and boceprevir, as well as other anti-HCV drugs in development, will vastly improve our antiviral armamentarium for patients with hepatitis C. Telaprevir and boceprevir must be used in conjunction with standard pegylated interferon and ribavirin therapy. However, the duration of treatment may be reduced substantially for many patients, based upon the clinical trial results from patients receiving a rapid virologic response. Substantial improvement in the SVR should be seen with these new agents for both treatment-naïve as well as prior relapsed or non-responsive patients. The further development of non-protease inhibitor based therapy offers the potential for interferon and ribavirin free therapy with once or twice daily oral dosing and a better tolerated side-effect profile. Additional studies are needed to determine which agent or agents should be used initially and the optimal combination regimen for patients needing additional therapy.

At this point in time, it appears that second generation protease inhibitors will supplant current protease inhibitor therapy on the basis of broader genotypic coverage and a higher barrier to resistance. Both NS5A replication inhibitors and NS5B RNA dependent RNA polymerase inhibitors offer greater potency than currently available drugs, but a lower barrier to resistance with the NS5As will likely require combination therapy. The availability of these new agents should preclude the continued need for interferon therapy and possibly for ribavirin therapy as well.

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TOPIC HIGHLIGHT

WJG 20th Anniversary Special Issues (2): Hepatitis C virus

Pathogenesis and significance of hepatitis C virus steatosis: An update on survival strategy of a successful pathogen

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Abstract

Hepatitis C virus (HCV) is a successful pathogen on the grounds that it exploits its host's metabolism to build up viral particles; moreover it favours its own survival by inducing chronic disease and the development of specific anatomic changes in the infected organ. Steatosis, therefore, is associated with HCV infection by necessity rather than by chance alone. Approximately 6% of HCV patients have steatohepatitis. Interestingly, HCV steatosis occurs in the setting of multiple metabolic abnormalities (hyperuricemia, reversible hypocholesterolemia, insulin resistance, arterial hypertension and expansion of visceral adipose tissue) collectively referred to as "hepatitis C-associated dysmetabolic syndrome" (HCADS). General, nonalcoholic fatty liver disease (NAFLD)-like, mechanisms of steatogenesis (including increased availability of lipogenic substrates and *de novo* lipogenesis; decreased oxidation of fatty substrates and export of fatty substrates) are shared by all HCV genotypes. However, genotype 3 seemingly amplifies such steatogenic molecular mechanisms reported to occur in NAFLD *via* more profound changes in microsomal triglyceride transfer protein; peroxisome proliferator-activated receptor alpha; sterol regulatory element-binding proteins and phosphatase and tensin homologue. HCV steatosis has a remarkable clinical impact in as much as it is an acknowledged risk factor for accelerated fibrogenesis; for impaired treatment response to interferon and ribavirin; and development of hepatocellular carcinoma. Recent data, moreover, suggest that HCV-steatosis contributes to premature atherogenesis *via* both direct and indirect mechanisms. In conclusion, HCV steatosis fulfills all expected requirements necessary to perpetuate the HCV life cycle. A better understanding of the physiology of HCADS will likely result in a more successful handling of disease with improved antiviral success rates.

Key words: Atherosclerosis; Fibrosis; Hepatitis C-associated dysmetabolic syndrome; Hepatocellular carcinoma; Steatohepatitis; Sustained virological response; Hepatitis C virus

Core tip: Hepatitis C virus (HCV) steatosis occurs in the setting of multiple abnormalities collectively referred to as "hepatitis C-associated dysmetabolic syndrome". General, nonalcoholic fatty liver disease-like, mechanisms of steatogenesis are shared by all HCV genotypes. However, genotype 3 seemingly amplifies such steatogenic molecular mechanisms. HCV steatosis has a remarkable clinical impact in accelerating fibrogenesis; impairing treatment response to interferons and ribavirin; and favouring the development of hepatocellular carcinoma and atherosclerosis. In conclusion, steatosis

fulfills all expected requirements necessary to perpetuate the HCV life cycle and is associated with HCV infection by necessity rather than by chance.

Lonardo A, Adinolfi LE, Restivo L, Ballestri S, Romagnoli D, Baldelli E, Nascimbeni F, Loria P. Pathogenesis and significance of hepatitis C virus steatosis: An update on survival strategy of a successful pathogen. *World J Gastroenterol* 2014; 20(23): 7089-7103 Available from: URL: http://www.wjgnet.com/1007-9327/full/v20/i23/7089.htm DOI: http://dx.doi.org/10.3748/wjg.v20.i23.7089

BACKGROUND

Steatosis was reported to occur in acute and chronic "non-A, non-B hepatitis" several years before hepatitis C virus (HCV) testing was available[1,2] suggesting a direct steatogenic role of HCV. Over time, a large body of evidence has exhaustively demonstrated HCV to be strongly associated with both metabolic derangements and steatosis.

The biological significance of such metabolic derangements and histological changes is unknown. However, it is intriguing to speculate that they occur as a result of an evolutionary strategy developed by HCV to ensure its survival. Indeed, in order to be successful in its struggle for life, a pathogen virus may be expected to: (1) exploit its host's metabolic pathways to build up viral particles; (2) favour its own survival by inducing chronic disease as opposed to self-limiting infection; and (3) induce the development of specific anatomic changes in the infected organ more often than by chance.

The aim of this article is to review the evidence that steatosis associated with HCV infection fulfills each and all the above theoretical requirements for HCV to be a successful pathogen. The existence of such a survival strategy suggests that steatosis is associated with HCV infection by necessity rather than by chance.

STEATOSIS OCCURS IN THE SETTING OF MULTIPLE METABOLIC ABNORMALITIES

Steatosis is a common finding in many hepatic and extrahepatic disorders notably including visceral obesity, a component of metabolic syndrome (MS). Over time, the initial theory that steatosis represents the hepatic manifestation of MS has evolved into the paradigm that nonalcoholic fatty liver disease (NAFLD) is an essential requirement for the development of the MS[3]. MS is a cluster of cardio-metabolic risk factors which, triggered by the expansion of adipose tissue, tend to self aggregate and to self perpetuate[4].

The prevalence of steatosis, in patients with chronic hepatitis C, is higher than expected by simple chance association[5]. When our group first suggested that HCV-related steatosis should probably be interpreted as an equivalent of NAFLD[5], the extent of such a similarity remained poorly defined. Since then, increasing evidence has been reinforcing the view that steatosis is only one of the many analogies linking these two conditions. In particular, HCV infection is associated with multiple metabolic derangements which, collectively, are referred to as hepatitis C associated dysmetabolic syndrome (HCADS)[6-10]. Figure 1 illustrates our present understanding of HCADS (modified from[10]). Some of such metabolic derangements, *e.g.*, insulin resistance (IR) and hypocholesterolemia had been earlier identified[11], others (*e.g.*, hyperuricemia and altered body fat distribution) have been recognized more recently.

Viral (including genotype, HCV RNA load and gene mutations) and host features [such as body mass index (BMI), type 2 diabetes (T2D) and alcohol consumption] affect the risk of steatosis[9-11]. Little is known about the impact of HCADS on HCV life cycle. However, it is increasingly being recognized that the individual components of the HCADS tend to facilitate HCV survival, as discussed below.

Insulin resistance

In the transgenic mouse model HCV core protein induces IR which predates the development of steatosis[12]. The specific cellular and molecular bases of IR associated with HCV infection have been extensively reviewed elsewhere[13-15]. Given that HCV infected patients are at greater risk of developing IR, it is not surprising that HCV should be considered a diabetogenic virus in predisposed individuals[16]. In their meta-analytic review, White *et al*[17] reported that the presence of HCV infection significantly increased the risk of T2D compared to both HCV-negative [odds ratio (OR) 1.68 and 1.67 in retrospective and prospective studies, respectively] and HBV-infected controls (OR 1.80). Such findings indicate that HCV infection *per se*, irrespective of chronic liver disease, directly increases T2D risk. Further evidence for such a diabetogenic action of HCV comes from the finding that human immunodeficiency virus (HIV)-HCV co-infected patients showed a higher risk of T2D when compared with HIV mono-infection (OR 1.82)[18], a condition which is often associated with hepatic steatosis and other metabolic derangements[19]. Similar results were obtained by our group by evaluating IR in different groups of mono- and co-infected patients (HIV, HCV, and HIV-HCV) with virus-associated steatosis, compared to NAFLD cases. We found that IR was predicted by HCV infection, a finding representing direct evidence for HCV infection *per se* to be associated with increased IR[20].

On these grounds, it is plausible to hypothesize that successful HCV eradication should be associated with the prevention of T2D. Although studies on this topic have yielded conflicting results (reviewed in[14]), recent research on a large cohort of HCV patients showed that sustained virological response (SVR) to treatment with interferon based regimen prevents the development of IR, whereas treatment failure was an independent risk factor for the *de*

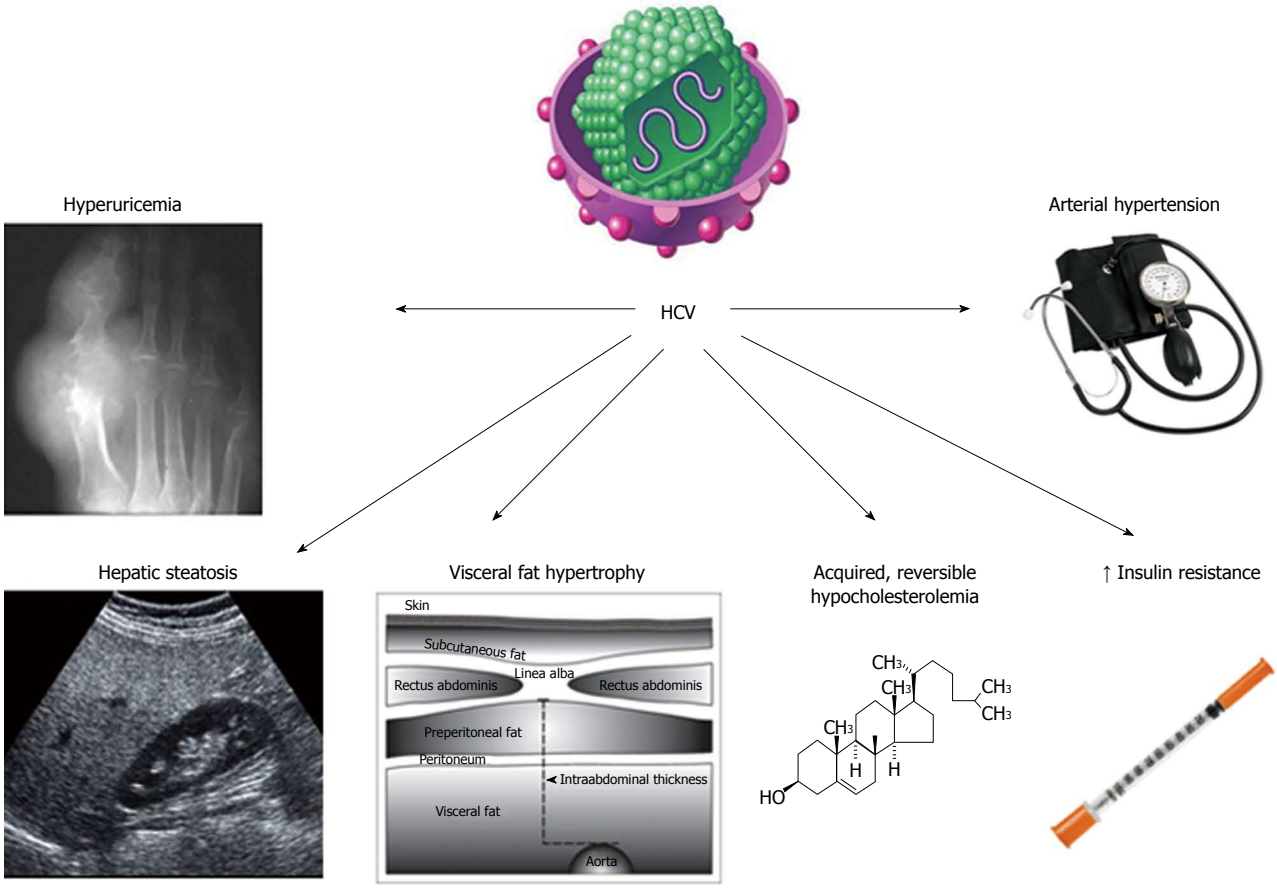

Figure 1 The hepatitis C virus-associated dysmetabolic syndrome (modified from ref[10]). HCV: Hepatitis C virus.

novo development of IR[21].

Taken collectively, these findings support the view that HCV is a strong risk factor for IR, as well as T2D in predisposed individuals. Accordingly, HCV eradication may potentially reverse IR thus helping to prevent the development of T2D in these patients.

Hypocholesterolemia and arterial hypertension

In a previous study from our group, both cirrhotic and chronic hepatitis C patients showed lower serum levels of cholesterol than those observed in both healthy controls and NAFLD individuals[8]. Of interest, normalization of serum cholesterol levels occurs following achievement of SVR[22] suggesting that HCV itself reversibly perturbs the cholesterol biosynthetic pathway.

Recent research has promoted our understanding of the fine molecular mechanisms eventually leading to the development of hypocholesterolemia in the setting of HCV infection. Lipids play a key role on virion structure, target membrane, cell receptor recognition, viral membrane fusion, viral replication, assembly and egress[23]. Research has particularly focused on the key steps of HCV entry into and egress from infected hepatocytes[23,24] which represent potential targets for antiviral strategies. HCV-associated hypocholesterolemia results from the inhibition of apolipoprotein B100 secretion and, selectively, by the perturbed distal cholesterol synthesis pathway[25,26].

Altered body fat distribution

Contrary to common belief, HCV infects and replicates not only in hepatocytes but in many extra-hepatic sites (reviewed in[27]). Nevertheless, evidence for adipocyte infection by HCV is still lacking, although clinical data seem to suggest an interaction between HCV and adipose tissue. Evidence for such an interaction comes from two recent studies[28,29].

Mostafa *et al*[28] showed that mesenteric fat was significantly thicker in HCV-infected patients than in those never infected. Although, these authors did not provide direct evidence for mesenteric fat colonization by HCV, these findings seem to suggest a possible interaction of HCV with mesenteric fat. A possible interaction of HCV with visceral adipose tissue is also suggested by the data published by Zampino *et al*[29]. These Authors reported that, in chronic hepatitis C patients, an increased amount of abdominal fat is associated with patatin-like phospholipase domain-containing 3 gene (*PNPLA3*) p.I148M and with hepatic steatosis).

Although the full pathogenic scenario is far from being elucidated, the above pioneer studies[28,29] envisage the possibility of an interaction of HCV with visceral fat accumulation and host genetics.

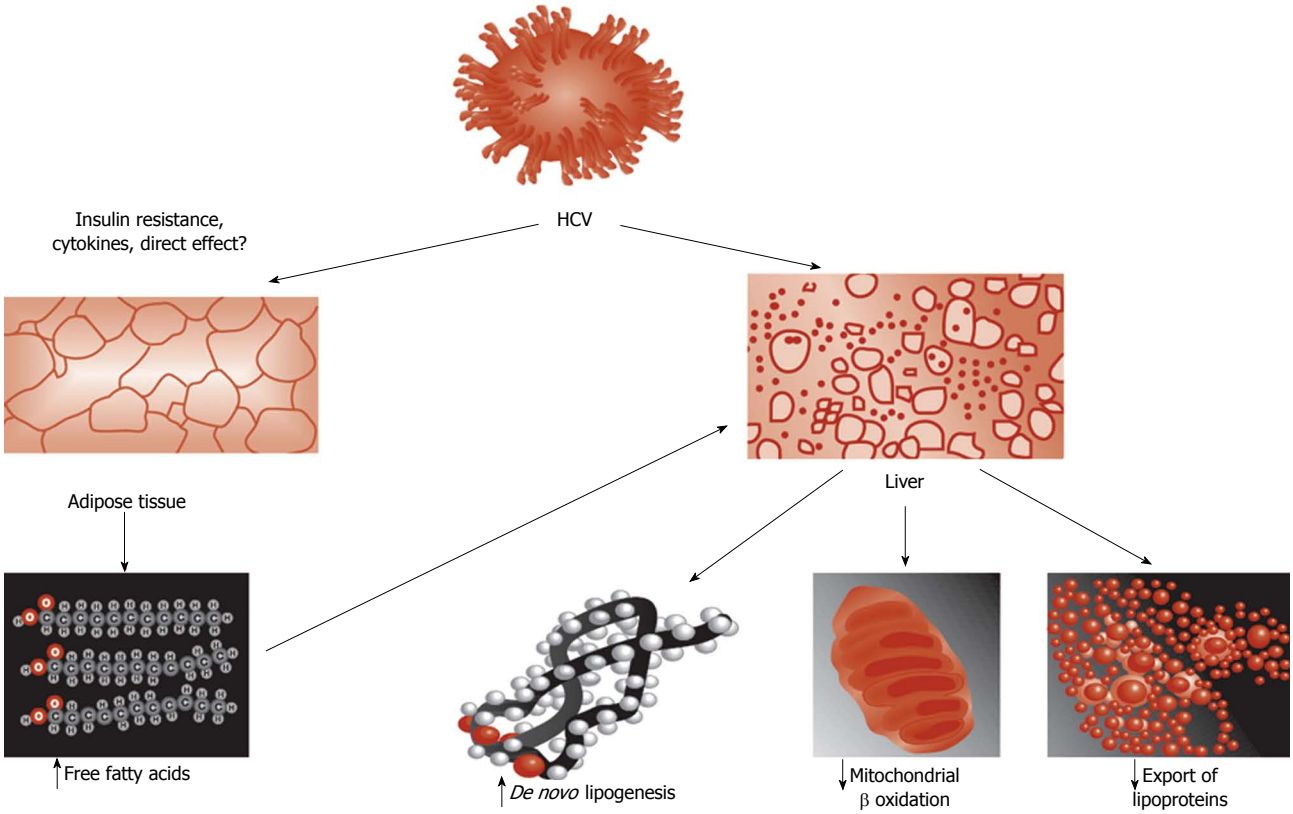

Figure 2 General steatogenic pathways associated with hepatitis C virus infection (reprinted from ref[9]). HCV: Hepatitis C virus.

Hyperuricemia

This recent line of research has been successfully conducted exploiting in depth the concept that HCV infection closely recapitulates naturally occurring MS in humans. Petta *et al*[30], showed that hyperuricemia, defined as serum uric acid either > 7 mg/dL or > 6 mg/dL in men and woman, respectively, was present in 7.5% of 496 consecutive patients with biopsy-proven chronic hepatitis C. Not surprisingly, such a metabolic feature tended to cluster with low HDL cholesterol, high blood pressure, estimated glomerular filtration rate and severity of steatosis. Moreover, hyperuricemia was among the independent predictors of steatosis severity.

Younossi *et al*[31] in their United States population study reported that at multivariate analysis HCV-RNA positivity (found in in 173 individuals out of 19741 eligible participants) was independently associated with the presence of arterial hypertension (further to IR and T2D).

The findings in HCADS of hyperuricemia and arterial hypertension are closely reminiscent of those occurring in NAFLD[32-34].

MOLECULAR MECHANISMS

General mechanisms

Based on the analogy of HCV-steatosis with NAFLD paradigm, it may be anticipated that HCV-related steatogenesis occurs *via* the following four steps, illustrated in Figure 2[9]: increased availability of lipogenic substrates; increased *de novo* lipogenesis; decreased oxidation of fatty substrates and decreased export of fatty substrates from the hepatocyte into the blood stream.

Increased availability of lipogenic substrates: The state of IR which is a typical feature of HCV infection[14-16,35] represents the biological background providing the hepatocyte with abundance of lipogenic substrates (such as glucose and non-esterified fatty acids) and hormones (hyperinsulinemia).

Increased *de novo* lipogenesis: It is well demonstrated that elevated fasting lipogenesis occurs in HCV in humans[36] as a result of up-regulated genes mediating fatty acid *de novo* synthesis and uptake[37]. Coupled with impaired cholesterol synthesis, therefore, HCV has been reported to induce a paradoxical state in which, while cholesterol synthetic pathways are up-regulated, synthesis of the end product cholesterol is actually impaired as a result of diversion of the intermediate geranylpyrophospate, which is necessary to HCV life cycle[36,38,39].

Decreased oxidation of fatty substrates: The seminal study by Okuda *et al*[40] provided the first evidence for perturbed mitochondrial function as a direct result of HCV core protein. These Authors found that expression of HCV core protein uniformly increased ROS in 3 different cell lines and also increased lipid peroxidation

products in 2 out of the 3 *in vitro* systems. Interestingly, inhibition of mitochondrial electron transport prevented such an oxidative stress induced by core protein[40]. These findings were confirmed in HCV transgenic mice indicating that oxidative injury occurs as a direct result of HCV core protein expression both *in vitro* and *in vivo*[40].

Evidence for disruption of fatty acids metabolism in patients with chronic HCV infection has been reported by an elegant metabolomic profile analysis study by Roe *et al*[41] Moreover, Sato *et al*[42] reported that the rate of change in total ketone body concentration between 12 and 15 h after the start of fasting (an indirect index of decreased oxidation of fatty substrates) was significantly lower in chronic hepatitis C patients than in healthy volunteers and such a reduction was associated with increasing viral load and IR.

Decreased export of fatty substrates from the hepatocyte into the bloodstream: Using the HCV subgenomic replicon expression system, Domitrovich *et al*[25] have demonstrated that interaction with the nonstructural protein 5A and apoB 100 results in the inhibition of apolipoprotein B100 secretion.

In conclusion, in order to induce steatogenic pathways, HCV infection affects each and all the four chief classical steps involved in NAFLD steatogenesis[9,43].

HCV genotype diversity

This topic has recently been analyzed in depth by Roingeard *et al*[44]. Data suggest that, with the notable exception of phosphatase and tensin homologue (PTEN) down-regulation, rather than having specific "steatogenic strategies", HCV genotype 3 seemingly amplifies steatogenic mechanisms reported to occur in NAFLD[45]. Specific differences in the core protein aminoacid sequence have been reported to account for the more elevated steatogenic activity featured by HCV genotype 3[46]. Moreover, alcohol abuse might be another, spurious, mechanism accounting for the finding that HCV genotype 3 is more frequently associated with steatosis and that such steatosis is more severe[9,11]. HCV genotype 3, indeed, tends to be more common among intravenous drug addicts, a population typically featuring high alcohol consumption[47-49]. Finally, the theoretical possibility that genotype 3 HCV infection might exert a more pronounced steatogenic activity *via* more increased IR remains controversial[14,44].

Microsomal triglyceride transfer protein

Microsomal triglyceride transfer protein (MTP) is effective in stabilizing apoB lipidation, a key step in the assembly of VLDL particles and thus in the export capacity of triglycerides from the hepatocyte into the bloodstream[50]. Core and nonstructural HCV proteins promote steatosis by impairing MTP activity[25,51]. Such a pathogenic mechanism accounts for the acknowledged finding that both hypo-lipidemia and steatosis are reversed by HCV eradication[11,44]. Interestingly, compared to those infected with other HCV genotypes, patients with genotype 3 HCV infection have been reported to exhibit significantly reduced MTP activity[52]. Although not a definite evidence for a HCV genotype 3 direct inhibition of MTP, these data may circumstantially suggest such an effect[44], so accounting for the elective steatogenic potential of HCV genotype 3 even in the absence of worsened IR.

PPAR-alpha

In liver cells, the highly expressed nuclear receptor PPAR-alpha promotes oxidation of fatty acids by stimulating their entry into mitochondria, where they undergo oxidation. Lipid-lowering fibrates, are ligands of PPAR-alpha[53].

Conversely, decreased PPAR-alpha activity will result in increased fatty deposition mediated by impaired fatty acid uptake and decreased mitochondrial oxidation of lipidic substrates[44].

Interestingly, genotype 3 HCV displays PPAR-alpha mRNAs lower than those observed in patients with genotype 1 HCV infection[54] suggesting this to be one more pathogenic mechanism accounting specifically for genotype-3 steatogenesis[44]. Indirectly confirming that steatosis represents a selective advantage for HCV survival, a pilot study has suggested the potential usefulness of PPAR-alpha agonist bezafibrate in the treatment of CHC patients[55]. Unfortunately, long-term treatment of transgenic mice expressing HCV core protein with clofibrate, induced hepatocellular carcinoma (HCC) with mitochondrial abnormalities and hepatic steatosis[2,56].

Sterol regulatory element-binding protein

Sterol regulatory element-binding proteins (SREBPs), SREBP-1c being the hepatic isoform, are a family of transcription factors which, following activation, are translocated intranuclearly, eventually up-regulating the transcription of lipogenic genes, including fatty acid synthase (FAS)[57]. The relationships between SREBP-1c and HCV proteins are unclear. Of interest, FAS serum levels predict the development of steatosis in patients with viral hepatitis due to HCV[58]. It remains controversial whether HCV genotype 3 core proteins are capable of inducing FAS more efficiently than HCV genotype 1 proteins[59,60]. However, it is possible that HCV genotype 3 activates SREBPs more efficiently *via* increased oxidative stress (PI-3-K-Akt pathway and inactivation of PTEN)[61].

PTEN

Studies *in vitro* have reported intra-hepatic down-regulation of PTEN to be another, NAFLD-like[24], genotype 3-core protein specific steatogenic mechanism which does not occur in HCV genotype 1 infection[62].

In conclusion, Figure 3 recapitulates the principles of genotype-specific molecular mechanisms of steatogenesis which exploit the host's metabolic machinery[63-69].

FIBROGENESIS

Cross-sectional and prospective studies have shown that

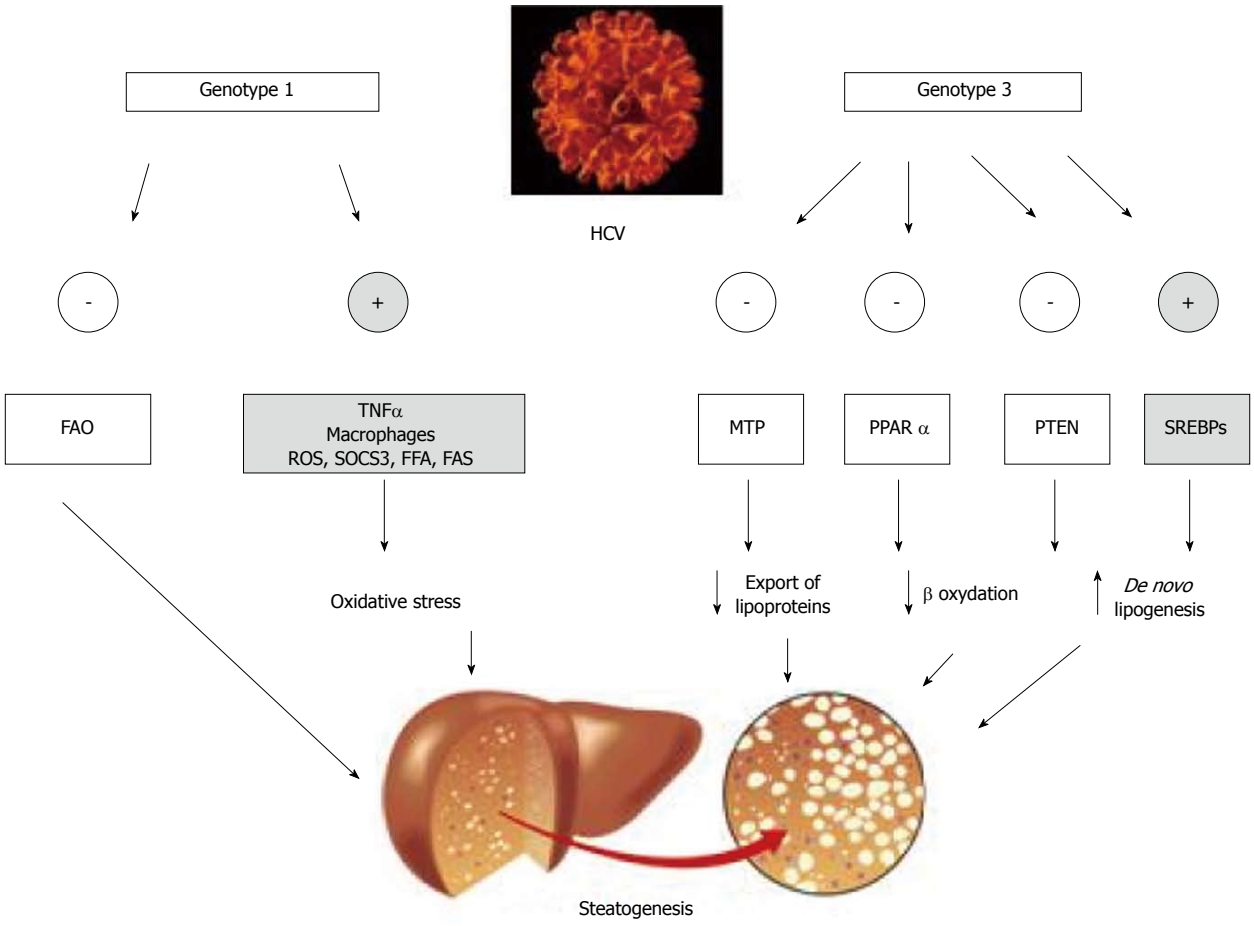

Figure 3 Genotype-specific steatogenic pathways associated with hepatitis C virus infection[63-69]. The negative sign (-) indicates inhibited and the positive sign (+) indicates stimulated pathway. FAO: Fatty acid oxidase; FAS: Fatty acid synthase; FFA: Free fatty acids; MTP: Microsomal triglyceride transfer protein; PPAR-alpha: Peroxisome proliferator-activated receptor alpha; PTEN: Phosphatase and tensin homologue; ROS: Reactive oxygen species; SOCS3: Suppressor of cytokine signaling; SREBPs: Sterol regulatory element-binding proteins; TNF alpha: Tumor necrosis factor alpha; HCV: Hepatitis C virus.

fibrosis progression risk is increased in the presence of HCV-steatosis (reviewed in[11]). Several published studies, summarized in Table 1[68,70-101], have evaluated the association of steatosis and fibrosis in either cross-sectional or prospective studies.

The vast majority of these reports have consistently provided robust evidence for steatosis being associated with fibrosis. Among such studies, probably the most single conclusive evidence that steatosis represents a risk factor for fibrosis progression comes from the study by Leandro *et al*[79]. These Authors conducted a meta-analysis on data belonging to 3068 CHC patients recruited at 10 centers in Europe, Australia, and north America. Steatosis and fibrosis were found in 50.9% and 87.6% of cases, respectively. At logistic regression analysis, fibrosis was independently associated with steatosis and other variables. Based on the finding that, in the subgroup analyses, the association between steatosis and fibrosis was invariably dependent upon a concurrent association of steatosis with liver inflammatory changes, the Authors reasonably speculated that the association of steatosis and fibrosis is likely mediated by hepatic inflammation.

A more restricted number of studies, however, have identified a genotype-dependency of the association of steatosis with fibrosis[68,95-97]. Finally, only in a minority of studies fibrosis and steatosis were not associated[91,98-101].

In conclusion, an impressive body of evidence supports the strong association of steatosis with fibrosis in chronic HCV infection. It is logical to hypothesize that inflammatory hepatic changes represent the candidate lesion mediating the progression, in the liver, from fatty to fibrotic changes.

IMPAIRED TREATMENT RESPONSE

Several studies, reported in Table 2, have identified steatosis as a predictor of poor treatment outcome in chronic HCV infection since the early 2000s[96,102-111].

These findings appear to be consistent across different HCV genotypes. It may be argued that steatosis, in patients with HCV infection, is closely associated with hepatic fibrosis and multiple metabolic derangements (HCADS). Therefore steatosis, further to directly impairing SVR *per se*, may also act as a marker of dysmetabolic milieu (HCADS) and that it is the latter that actually impairs SVR.

Table 1 Steatosis is associated with fibrosis

Method and findings	Conclusion	Ref.
98 CHC patients who had undergone repeat liver biopsies before antiviral treatment (median follow-up 5.8 yr)	In HCV patients with genotype 3 infection, steatosis was a risk factor for fibrosis progression	[68]
297 consecutive patients with HCV	Steatosis and inflammation scores were the only parameters independently predicting fibrosis	[70]
96 non-cirrhotic treatment-naive CHC patients	In untreated CHC patients fibrosis progression was strongly associated with worsening of steatosis	[71]
1428 CHC treatment-naïve patients included in a French therapeutic trial	The variables independently associated with steatosis were genotype 3 , higher age, triglycerides and body mass index (BMI) values and septal fibrosis	[72]
131 biopsy-proven CHC individuals	Hepatic steatosis was related to genotype, fibrosis degree, and serum leptin level	[73]
160 CHC patients	Irrespective of viral genotype, patients who had steatosis showed significantly more fibrosis than non-steatosic	[74]
Cross sectional study evaluating: 233 hepatic biopsies from 219 CHC patients and hepatectomy specimens from 65 patients transplanted for HCV-related cirrhosis. Longitudinal study: 41 patients with two biopsies and 10 patients with three biopsies performed over 2-8 yr	Steatosis was associated with fibrosis independently of necroinflammation, but declined in cirrhosis	[75]
Retrospective study conducted on 324 US patients with CHC from a university medical center and a regional VA medical center	Steatosis was independently associated with advanced fibrosis stage	[76]
135 treatment-naive CHC patients who had undergone repeat liver biopsies after a median interval of 61 months after the baseline biopsy	Irrespective of HCV genotype, steatosis was a chief contributor to fibrosis progression in mild CHC and the probability of such a fibrosis worsening is directly dependent on the proportion of steatotic hepatocytes	[77]
116 CHC patients undergoing liver biopsy	The MTHFR C677T polymorphism, responsible for hyperhomocysteinemia, contributed to increasing steatogenesis and steatosis which in its turn, hastened hepatic fibrosis progression	[78]
Meta-analysis on individual data from 3068 patients with biopsy- proven CHC recruited from 10 centers in Europe, Australia, and United States	Steatosis was significantly and independently associated with fibrosis in CHC. Hepatic inflammation may mediate fibrogenesis in patients with liver steatosis	[79]
180 patients infected with genotype 1b HCV	At multivariate analysis, fibrosis was significantly related to age, alanine transaminase, diabetes, hepatitis B core antibody, steatohepatitis and grading	[80]
Overall, 600 consecutive individuals: 500 with CHC; and 100 with CHB	IR, was associated with 1 and 4 HCV genotypes and high viral load. The association of significant fibrosis with IR occurs independent of steatosis	[81]
153 chronic hepatitis C patients enrolled in the Swiss hepatitis c cohort study and for whom a liver biopsy and plasma samples were available	By multiple regression analysis, CTGF levels were independently associated with steatosis, a past history of alcohol abuse, plasma leptin and HCV RNA levels; when only patients with genotypes non-3 were considered, CTGF levels were independently associated with a past history of alcohol abuse, plasma leptin levels and steatosis	[82]
107 consecutive CHC patients	Multiple regression analysis revealed that, HOMA-IR, fibrosis and oxidative stress were independently associated with steatosis, whereas steatosis was independently associated with oxidative stress and HOMA-IR. Steatosis and HAI were also independent predictors of fibrosis	[83]
143 AA and 157 CA adults with untreated chronic HCV genotype 1 infection	In 3-variable models including race and biopsy adequacy, the factors significantly associated with fibrosis progression were age when infected, steatosis, ALT level, and necroinflammatory score	[84]
228 HCV treatment-naive patients who met the inclusion/ exclusion criteria	Genotype 1 and presence of steatosis were found to be associated significantly with MS. After adjusting for confounding variables, MS remained independently associated with a lack of SVR	[85]
346 untreated, nondiabetic patients solely infected with either genotype 1 or 3	HOMA-IR rather than steatosis was independently associated with fibrosis for both HCV genotype 1 and genotype 3. Exclusion of cirrhotic subjects did not alter the findings with respect to the greater contribution of IR compared to hepatic steatosis, as a predictor of fibrosis	[86]
Retrospective study of 460 patients with CHC	Elevated AST, alpha fetoprotein, and presence of grade 2 and 3 steatosis are independent parameters associated with stage 3 and 4 fibrosis in patients with CHC	[87]
112 HCV RNA positive subjects who had two liver biopsies performed	On multivariate analysis, only baseline steatosis was significantly associated with fibrosis. Kaplan-Meier analysis demonstrated that steatosis impacted on time to progression to both significant fibrosis and cirrhosis	[88]
Of 253 HCV RNA-positive persons who underwent at least one liver biopsy	The presence of T2D, steatosis and duration of HCV infection were independent predictors of advanced fibrosis	[89]
Metanalysis of 12 published studies, including 1989 HIV/HCV co-infected patients	In co-infected patients, HS was associated with higher body mass index, diabetes mellitus, elevated alanine aminotransferase, necroinflammatory activity and fibrosis	[90]
Liver biopsy samples were collected from 59 patients with HCV without a sustained virologic response (SVR) or cirrhosis	There were no associations between fibrosis progression and histologic features including inflammation, fibrosis, or steatosis	[91]
170 genotype 1 CHC patients	At multivariate analysis Severe (F3-F4 fibrosis), , was independently associated with older age, IR, steatosis > 10%, and moderate-severe necroinflammatory activity in CHC patients	[92]

92 untreated consecutive adults with chronic HCV infection admitted for liver biopsy	In multivariate analyses, fibrosis was associated with high AST level, age ⩾ 40 yr, and steatosis	[93]
152 LT recipients with HCV were followed up with repeated liver biopsies for a median of 2.09 yr after index biopsy	In the multivariate analysis, steatosis at 1 yr was an independent predictor of subsequent F2 to F4 fibrosis. Steatosis was a stronger predictor of fibrosis in the setting of sirolimus use	[94]
755 consecutive chronic hepatitis C patients (178 with genotype 3), admitted to three referral hospitals in Switzerland	Fibrosis was associated with steatosis in genotype 3 infected individuals alone	[95]
574 CHC patients with chronic hepatitis C from a single United States center	In CHC owing to genotype 1 infection HCV, fibrosis was associated with steatosis severity	[96]
Clinical data and liver histology findings in 510 HCV patients were analysed	Age at liver biopsy, BMI and duration of HCV were independent risk factors for increased fibrosis in HCV patients. Steatosis as a risk factor for fibrosis is evident in HCV genotype-1	[97]
60 HCV patients compared to 41 NASH patients and 18 CHB individuals	Compared to patients who had mild steatosis, those CHC individuals with moderate steatosis exhibited higher fibrosis stages	[98]
251 CHC women	Severity of fibrosis was associated with a longer duration of infection, a higher BMI, advanced steatosis and the menopause	[99]
Ninety HCV infected patients undergoing liver biopsy	Steatosis was not found to be independently associated with fibrosis	[100]
Liver biopsies from 66 out of 306 HCV/HIV non-cirrhotic patients without cirrhosis at baseline who underwent a second biopsy were case-matched with a control group selected from a cohort of 233 HCV mono-infected patients	Progression of fibrosis was similar in HIV/HCV-co-infected compared to HCV mono-infected individuals and no clinical or laboratory predictor of worsening liver disease was identified	[101]

AA: African American; CA: Caucasian American; CHB: Chronic hepatitis B; CHC: Chronic hepatitis C; CTGF: Connective tissue growth factor; HAI: Hepatic activity index; HOMA-IR: Homeostasis model of insulin resistance; HS: Hepatic steatosis; LT: Liver transplantation; T2D: Type 2 diabete; HCV: Hepatitis C virus; HIV: Human immunodeficiency virus.

Table 2 Steatosis is associated with poor treatment outcome

Method and findings	Conclusion	Ref.
574 patients with chronic hepatitis C	Steatosis reduces the likelihood of achieving early and SVR in genotype 1 infected patients	[96]
HCV genotype 2 and 3 patients	Steatosis is the independent predictor of relapse	[102]
148 consecutive adults with HCV admitted for liver biopsy	Steatosis in chronic hepatitis C is not a negative prognostic factor of response to combined antiviral therapy	[103]
932 patients infected with HCV genotype 2 or 3	Steatosis was associated with significantly higher rates of relapse, irrespective of viral load, in patients infected with HCV genotype 3 who had an RVR	[104]
A total of 116 patients [HCV-G4 85 (73.3%); HCV-G1 31 (26.7%)] were included	The NAS steatosis score correlates with response to antiviral therapy	[105]
885 HCV patients	Steatosis did not influence the efficacy of treatment in our study population. Baseline viral load is a confounding factor, particularly in patients infected with genotype 3 and once baseline viral load was accounted for, the association between steatosis and SVR was not relevant	[106]
250 patients with genotype 4 chronic hepatitis C, treated with different regimens of combined interferon	Among genotype 4 chronic hepatitis C patients, severe fibrosis, severe steatosis, treatment with standard interferon and a high serum AFP level were all negatively associated with SVR	[107]
207 HCV patients	Features of the metabolic syndrome are associated with hepatic steatosis in most of these patients. Steatosis is significantly more common in genotype 3 compared with other genotypes, and in these patients, an SVR is associated with steatosis clearance	[108]
357 HCV-infected US veterans	Steatosis is independently associated with stage III-IV fibrosis. However, only HCV genotype, and not steatosis, obesity, or stage III-IV fibrosis, was associated with SVR to interferon alpha-2b and ribavirin treatment	[109]
80 Japanese patients with CHC. treated with IFN alpha-2b and ribavirin for 24 wk evaluated retrospectively	HS is an important predictor of poor response to therapy of IFN-alpha-2b and ribavirin in patients with CHC	[110]
Liver specimens with both CHC and significant steatosis (> 33%) or SH were categorized as group 1 (84 specimens). A control group (group 2) of 231 CHC patients without evidence of steatosis > 33% or SH	Overall SVR for patients with HCV and significant steatosis or SH is considerably lower than for HCV and steatosis less than 33% and no SH	[111]

CHC: Chronic hepatitis C; IFN: Interferon; NAS: Nonalcoholic fatty live disease activity score; RVR: Rapid virologic response; SH: Steatohepatitis; SVR: Sustained virologic response; AFP: Alpha fetoprotein; HCV: Hepatitis C virus; NASH: Non-alcoholic steatohepatitis.

HCC

Published studies on steatosis as a risk factor for HCC in those with HCV infection are summarized in Table 3[112-120]. With reference to such studies, it is of note that evidence linking steatosis and an increased risk of developing HCC in those with HCV infection is not limited to case-reports alone.

Data appear to be consistent and most studies support this conclusion while a single case-control study has provided negative results concerning such HCV-steatosis HCC association[119]. In this limited case-control study

Table 3 Steatosis is associated with increased hepatocellular carcinoma risk

Method and findings	Conclusion	Ref.
1818 patients with histologically proven CHC treated with IFN Cumulative incidence and HCC risk were analyzed over a mean follow-up period of 6.1 yr HCC developed in 179 study subjects	Severe steatosis, is an independent factor significantly associated with HCC	[112]
The 5-yr occurrence rate of HCC in 353 consecutive patients with histologically proven HCV cirrhosis and persistent viral replication prospectively followed and screened for HCC was 34% in genotype 3 and 17% in non-3 genotype group	For patients with HCV cirrhosis and ongoing infection, infection with genotype 3 (a potent steatogenic virus) is independently associated with an increased risk of HCC development	[113]
The steatohepatitis-HCC variant was found in 35.5% of 62 HCC cases In 14 of the 22 cases (63.6%) of SH-HCC, the non-neoplastic liver showed changes of NAFLD/NASH superimposed on otherwise typical features of HCV-C	This study suggests a possible NAFLD/NASH pathway leading to SH-HCC in the setting of HCV-C	[114]
This retrospective study investigated the features of 5 patients who developed HCC after 10 yr of achieving SVR	In 3 patients, liver tissues were obtained at the treatment of HCC. These tissues showed marked improvement in both activities and fibroses, but severe steatosis in 1 patient	[115]
Two-hundred and sixty-six patients, who achieved SVR, were enrolled in this retrospective study	Age, hepatic fibrosis, and hepatic steatosis at pre-interferon treatment might be risk factors for developing HCC after SVR	[116]
A retrospective study was conducted in 88 patients undergoing curative resection of HCV-associated HCC	Hepatic steatosis is a useful predictor of postoperative recurrence of HCV-related HCC	[117]
94 consecutive patients with cirrhosis due to HCV who underwent liver transplantation and had pathology available for review were retrospectively identified	In patients with HCV-related cirrhosis, the presence of hepatic steatosis is independently associated with the development of HCC	[118]
The histological severity of steatosis in the index liver biopsies of 25 patients with chronic hepatitis C who subsequently developed HCC was compared with matched controls who did not. As determined by percentage area of biopsy core occupied by steatosis on computer assisted morphometric evaluation, and graded semiquantitatively, steatosis was comparable among cases and controls	The odds of developing HCC among those with steatosis grades 1 and 2 did not differ significantly from those without steatosis. There was no association between increasing morphometric percentage area occupied by steatosis and the subsequent development of HCC. Neither steatosis grade or percent area of steatosis on biopsy were selected in multivariate regression analysis as independent predictors for the development of HCC	[119]
161 patients with chronic HCV infection	At multivariate analysis hepatic steatosis, (together with aging, cirrhosis, and no IFN treatment) was an independent, significant risk factor for HCC	[120]

CHC: Chronic hepatitis C; IFN: Interferon; SH-HCC: Steatohepatitis-hepatocellular carcinoma (HCC); SVR: Sustained virologic response; HCV: Hepatitis C virus.

patients with a SVR to antiviral therapy were excluded.

Based on data reported in Table 2, given that steatosis poses an additional risk for HCC, increased surveillance is necessary in HCV patients with steatosis. According to Nkontchou *et al*[113] such a caution needs to be particularly exercised in those with HCV genotype 3. Moreover, a possible NAFLD/NASH pathway leading to steatohepatitic-HCC in the setting of HCV-cirrhosis requires further investigation[114].

Hepatocarcinogenesis in those with HCV steatosis involves both IR and, particularly, deranged lipid metabolism. The molecular bases underlying the pathogenesis of HCV-HCC have been accurately detailed elsewhere[14,15,37,121,122].

HCV AND STEATOHEPATITIS

Steatohepatitis is the stereotypical histological hallmark of a wide spectrum of etiologically diverse liver conditions which invariably display concurrent steatosis and inflammatory-fibrotic changes occurring to a variable extent[123].

Compared to simple steatosis, less has been published concerning steatohepatitis occurring in the setting of HCV infection. Probably this occurs as a result of steatohepatitis being a much more uncommon finding which occurs in 4% to 10% of cases, averaging 6.13 % of 2316 published cases as shown in Table 4[70,124-127].

Acknowledged risks for the development of steatohepatitis in patients with chronic hepatitis C include BMI[128]; either trigliceride and HDL cholesterol (genotype 1) or AST (genotpe 3)[127].

Similar to steatosis, steatohepatitis has been associated with advanced fibrosis[124] and impaired SVR[111] suggesting that the biological significance of steatohepatitis closely overlaps with that of high-grade steatosis. However, whether steatohepatitis is an independent risk factor for HCC in HCV-infected individuals, remains to be shown.

ATHEROSCLEROSIS

In the past, individuals with chronic liver disease were deemed to be spared from atherosclerosis but this paradigm has been changed by an ever increasing body of literature concerning hepatitis C[129]. HCV infection is not a unique model of atherosclerosis and numerous reports document an association of atherosclerosis with infections due to a large variety of pathogens[130].

Data reviewed elsewhere[129] indicate that HCV is associated with excess cardiovascular mortality and that, possibly, achieving SVR might prevent at least a fraction

Table 4 Prevalence of steatohepatitis associated with hepatitis C virus infection *n* (%)

Series	Steatohepatitis	Ref.
297 patients	17 (6)	[70]
170 patients	17 (10)	[124]
1458 liver biopsies	80 (5.5)	[125]
95 patients	4 (4)	[126]
296 liver biopsies	24 (9)	[127]
2316 biopsies/patients	142 (6,13)	Cumulative

of such deaths. Studies evaluating the association of HCV sero-positivity (either HCV-Ab or HCV-RNA) with surrogate markers of atherosclerotic burden (Carotid Intima Media Thickening or plaques) have been reviewed recently elsewhere[129]. Data from our and other groups indicate that viral (HCV viral load) and host features (liver histology changes) are likely to contribute to increased vascular risk in these individuals[131,132]. Interestingly, Adinolfi *et al*[133] have recently reported that HCV infection is a risk factor for human ischemic stroke.

The mechanisms underlying excess cardiovascular risk include both HCV direct vessel colonization and indirect mechanism associated with HCV infection such as pro-atherogenic cardiometabolic derangement, *i.e.*, HCADS *per se*, increased expression of pro-inflammatory cytokines and adhesion molecules, hyperomocysteinemia and steatosis[129,134].

CONCLUSION

We have discussed data favoring the interpretation of steatosis as an example of successful viral strategy. HCV steatosis fulfills all requirements expected to occur in order to perpetuate HCV life cycle.

Indeed, for steatosis to develop, the host's metabolic pathways are engaged giving life to a variant (HCADS) of the commonly occurring metabolic syndrome. Such an HCADS will typically display, further to steatosis, the corollary of ordinary metabolic syndrome including IR, hyperuricemia and expanded visceral adipose tissue. However, given that intermediate products of the cholesterol biosynthetic pathway rather than cholesterol itself are necessary to HCV replication, HCV will perturb the distal biosynthetic pathway, eventually leading to hypocholesterolemia.

A better understanding of the physiology of HCADS will likely result in a more successful handling of disease with improved antiviral success rates.

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TOPIC HIGHLIGHT

WJG 20th Anniversary Special Issues (2): Hepatitis C virus

Production and pathogenicity of hepatitis C virus core gene products

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Abstract

Hepatitis C virus (HCV) is a major cause of chronic liver diseases, including steatosis, cirrhosis and hepatocellular carcinoma, and its infection is also associated with insulin resistance and type 2 diabetes mellitus. HCV, belonging to the *Flaviviridae* family, is a small enveloped virus whose positive-stranded RNA genome encoding a polyprotein. The HCV core protein is cleaved first at residue 191 by the host signal peptidase and further cleaved by the host signal peptide peptidase at about residue 177 to generate the mature core protein (a.a. 1-177) and the cleaved peptide (a.a. 178-191). Core protein could induce insulin resistance, steatosis and even hepatocellular carcinoma through various mechanisms. The peptide (a.a. 178-191) may play a role in the immune response. The polymorphism of this peptide is associated with the cellular lipid drop accumulation, contributing to steatosis development. In addition to the conventional open reading frame (ORF), in the +1 frame, an ORF overlaps with the core protein-coding sequence and encodes the alternative reading frame proteins (ARFP or core+1). ARFP/core+1/F protein could enhance hepatocyte growth and may regulate iron metabolism. In this review, we briefly summarized the current knowledge regarding the production of different core gene products and their roles in viral pathogenesis.

Key words: Hepatitis C virus; Core protein; Alternative reading frame/core+1 proteins; Insulin resistance; Steatosis; Hepatocellular carcinoma; Interferon

Core tip: In addition to the mature core protein (a.a. 1-177) and the cleaved peptide (a.a. 178-191), different alternative reading frame (ARF)/core+1 proteins could be expressed from the core+1 reading frame of hepatitis C virus (HCV) core gene. Core gene products play an important role in the HCV pathogenesis. Core protein could induce insulin resistance, steatosis, and even hepatocellular carcinoma. The peptide (a.a. 178-191) may play a role in the immune response and steatosis development. ARF proteins/core+1/F protein could enhance hepatocyte growth and may regulate iron metabolism. We summarized the current knowledge regarding the HCV core gene products and their pathogenicity in this article.

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INTRODUCTION

Hepatitis C virus (HCV) accounts for approximately

15%-20% cases of acute hepatitis. After acute infection, around 50% to 80% of HCV patients will develop chronic infection. HCV persistently infected individuals are at risk to develop liver inflammation, steatosis, fibrosis, cirrhosis and hepatocellular carcinoma (HCC)[1-4]. Epidemiological studies also indicate that HCV is associated with insulin resistance and type 2 diabetes mellitus[5,6].

HCV is a small enveloped RNA virus belonging to the family *Flaviviridae* and genus *hepacivirus*. The HCV genome is a single, positive-stranded RNA with a nucleotide length of about 9.6 kb. This genome encodes a polyprotein precursor of approximately 3000 amino acids, which is processed by host and viral proteases into at least 10 different proteins, which are arranged in the order of NH_2-C-E1-E2-p7-NS2-NS3-NS4A-NS4B-NS5A-NS5B-COOH. C, E1, and E2 are structural proteins while NS2-NS5B and perhaps also p7 are non-structural proteins. The release of C, E1, E2 and, p7 from the polyprotein is mediated by the cellular signal peptidase located in the endoplasmic reticulum, whereas the cleavages between NS2-NS5B are mediated by viral NS2/3 and NS3/4A proteases (for a review[7,8]).

Following the discovery of HCV, the presence of great nucleotide diversity among isolates was reported[1,9]. Due to the lack of proof-reading mechanism in the NS5B polymerase, a closely related but diverse population of viral variants known as quasispecies is produced at a rate of approximately one mutation per replication cycle within infected individuals[10]. Accumulation of nucleotide substitutions in the virus has resulted in diversification into subtypes and distinct genotypes. Therefore, the HCV RNA genome sequences are highly heterogeneous. At present, HCV is classified into seven major genotypes and numerous subtypes[11]. There is 30%-50% variation among viral genotypes and 15%-30% variation among different subtypes, while there is 1%-5% variation in HCV nucleotide sequence from a single infected patient[12,13]. Viral pathogenesis and response to anti-viral treatment are different among different HCV genotypes, *e.g.*, genotype 3 infection is associated with a high level of liver steatosis while genotypes 1 and 4 are more resistant to interferon (IFN) based therapies than genotypes 2 and 3[11].

The pathobiological changes caused by HCV infection have been attributed to both the host immune responses and the direct viral cytopathic effects[8]. Viral pathogenesis caused by direct viral cytopathic effects is the outcome of the interactions between the host cell and different HCV proteins. In this review, we will only focus on the pathogenicity of HCV core gene products.

PRODUCTION OF HCV CORE GENE PRODUCTS

HCV core protein and the cleaved peptide from the conventional open reading frame

The HCV core protein is cleaved at residue 191 by the host signal peptidase (SP) to release it from the precursor polyprotein. This immature core protein is further cleaved by the host SP peptidase (SPP) within the C-terminal transmembrane region to generate the mature core protein and the cleaved peptide. The cleavage of HCV core protein by SPP is essential for HCV assembly[14,15]. The cleavage between core and E1 proteins by SP facilitates further cleavage of core protein by SPP[16]. The exact C terminal amino acid residue of the mature, virion-associated core protein is not known. The studies of core protein expressed in insect cells have indicated that SPP cleaves after residue 177[17], 179 or 182[18], and the studies in human cells have supported that the mature protein terminates at residue 177[15]. Moreover, a recent report demonstrated that a core protein with amino acids 1 to 177 efficiently trans-complemented the viral assembly[19]. Therefore, although the exact C terminus of the core protein awaits further investigation, it is likely that the mature core protein contains 177 amino acid residues. And, the cleaved peptide generated by the sequential cleavages of SP and SPP is composed of amino acid 178 to 191 (Figure 1A).

Comparing with other HCV proteins, core protein is thought to be the most conserved one: results of nucleotide and deduced amino acid sequence analysis across diverse HCV strains reveal 81%-88% nucleotide and 96% amino acid sequence homology[20,21]. Assembly of the virion is initiated by the oligomerization of core protein. Small molecules directly binding to core protein could potentially be potent antiviral agents[22].

The mature core protein having 177 amino acids consists of two domains: positively charged domain 1 (a.a. 1-117) and hydrophobic domain 2 (a.a. 118-177) (Figure 1A). The domain 1 is rich in basic residues, and is implicated in RNA-binding and homo-oligomerization. The domain 2 is important for the membrane association activity of the core protein as well as for its folding and stability (for a review[23,24]). The amphipathic helices Ⅰ and Ⅱ in domain 2 spanning from residue 119 to 136 and residue 148 to 164, respectively, are involved in the association of HCV core protein with lipid droplets[25]. In addition, the region spanning from residue 112 to 152 is associated with membranes of the endoplasmic reticulum and mitochondria[26,27].

The core protein may also localize with nucleus[26,28] and bind to the nuclear proteasome activator PA28-γ/REGγ, resulting in PA28-γ-dependent degradation of the core protein[28].

The cleaved peptide (a.a. 178-191) is highly conserved with close to 100% identity among different HCV genotypes[19,29].

ARF/core+1 proteins from the +1 reading frame

In addition to the proteins translated from the conventional open reading frame (ORF), existence of a new antigen encoded in the -2/+1 alternative reading frame (named ARF) was first demonstrated through bioinfor-

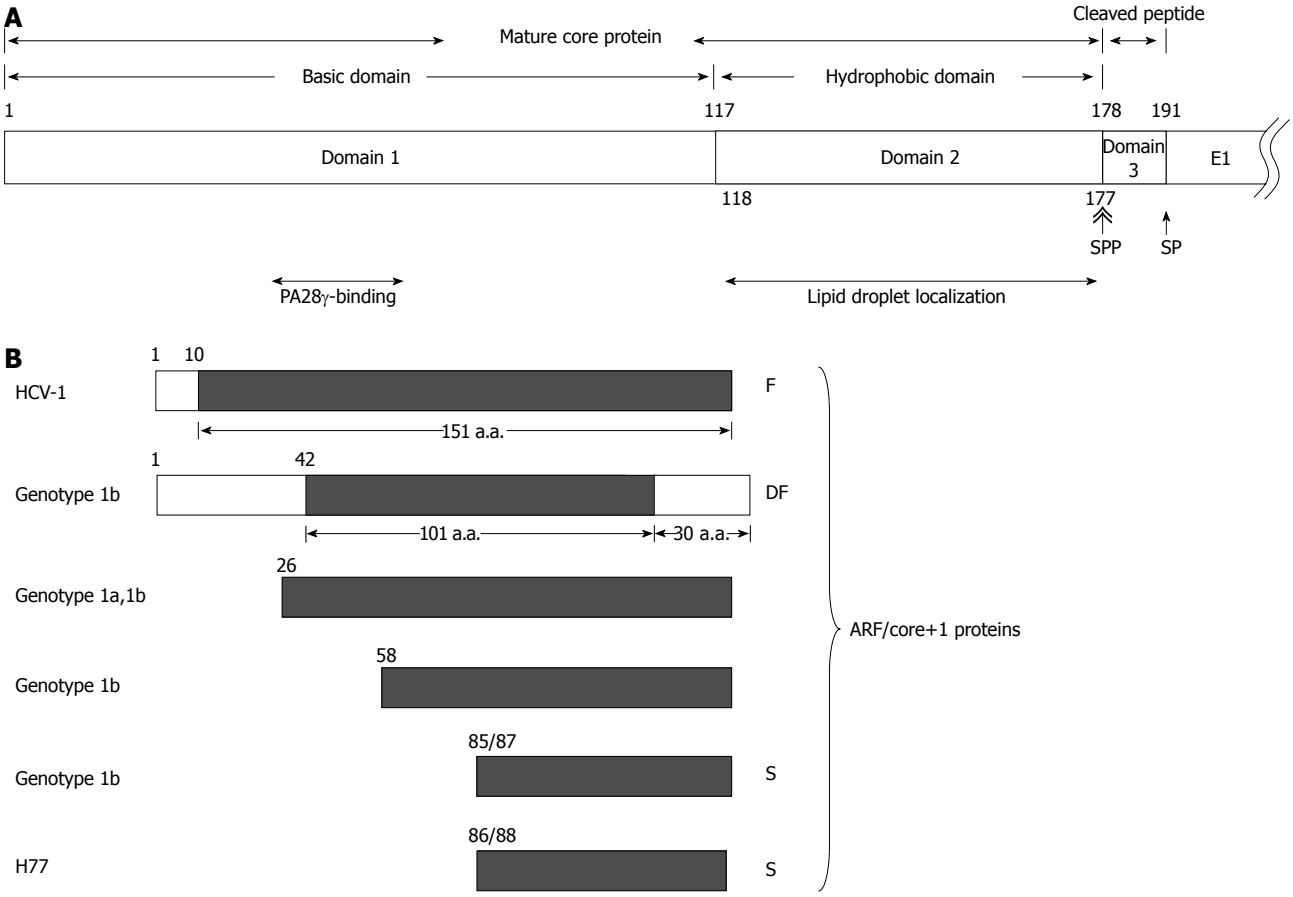

Figure 1 Various hepatitis C virus core gene products. A: The hepatitis C virus (HCV) polyprotein is cleaved at residues 191/192 by the host signal peptidase (SP) and further cleaved at residue 177/178 by signal peptide peptidase (SPP) to release the mature core protein (a.a. 1-177) and the cleaved peptide (a.a. 178-191) from the precursor polyprotein. The mature core protein consists of the positively charged domain 1 (a.a. 1-117) and the hydrophobic domain 2 (a.a. 118-177). The highly basic domain 1 is involved in RNA-binding and its oligomerization. The region containing residues 44-71 of domain 1 binds to PA28γ. Domain 2 is involved in the association of HCV core protein with lipid droplets; B: Different alternative reading frame (ARF)/core+1 proteins from different HCV isolates/genotypes. The polypeptides from the conventional open reading frame are marked by empty rectangles while those from the alternative reading frame (ARF/core+1) by filled rectangles. The termination codon of ARF/core+1 proteins from other isolates/genotypes may be different from those shown in this figure.

matics and patient-based research in 2001[30].

Besides the core protein, a smaller protein was first detected in 1994 when the HCV-1 isolate was *in vitro* translated[31]. In 2001, this smaller protein (named F) was found to be synthesized by the ribosomal frameshift into the -2/+1 reading frame, not from the conventional viral open reading frame. Results from sequence analysis of different HCV genotypes and from the reactivity of patients' sera also indicated the existence of a protein product encoded from this -2/+1 reading frame[32].

In 2002, translation of this -2/+1 reading frame (named core+1) was also verified by the fusion protein reporter assay and antibody response[33]. Therefore, proteins from this alternative reading frame was named ARFP/F/core+1 at first (for a review[34]).

In addition to F protein, proteins with different lengths were synthesized from this alternative reading frame through various translational mechanisms used in different HCV genotypes and/or strains. The ARFP/double-frameshift (DF) protein from genotype 1b is a chimera: N-terminal with 42 amino acids of the core protein, followed by 101 amino acids of ARFP in the middle, and end with the C-terminal 30 amino acids of the core protein[35]. Translation from the ARF could be started from non-AUG codon 26 (core+1), GUG or GCG, of genotype 1a or 1b, or initiated from codon 58 (GUG) of genotype 1b[36,37]. Actually, translation initiation from this ARF was detected most efficient at the internal AUG codon at position 85/87 of genotype 1b or 86/88 of H77 strain[37-39], named core+1/S (short form). Therefore, proteins translated from the +1 reading frame are composed of proteins with different lengths[34,40] (Figure 1B). All proteins containing amino acids from this ARF are called ARFP, core+1 proteins or ARF/core+1 proteins. Specific proteins from this ARF are designated after ARFPs or core+1, *e.g.*, F protein was called ARFP/F, core+1/F or ARFP/core+1/F[34,41].

Different ARF/core+1 proteins could have similar subcellular localization, *e.g.*, both F and core+1/S are cytoplasmic proteins, primarily associated with the endoplasmic reticulum[37,42]. Further immunoflurescence and subcellular fractionation analyses indicated that core+1/S and core+1/F are cytoplasmic proteins with partial endoplasmic reticulum distribution at interphase, whereas in

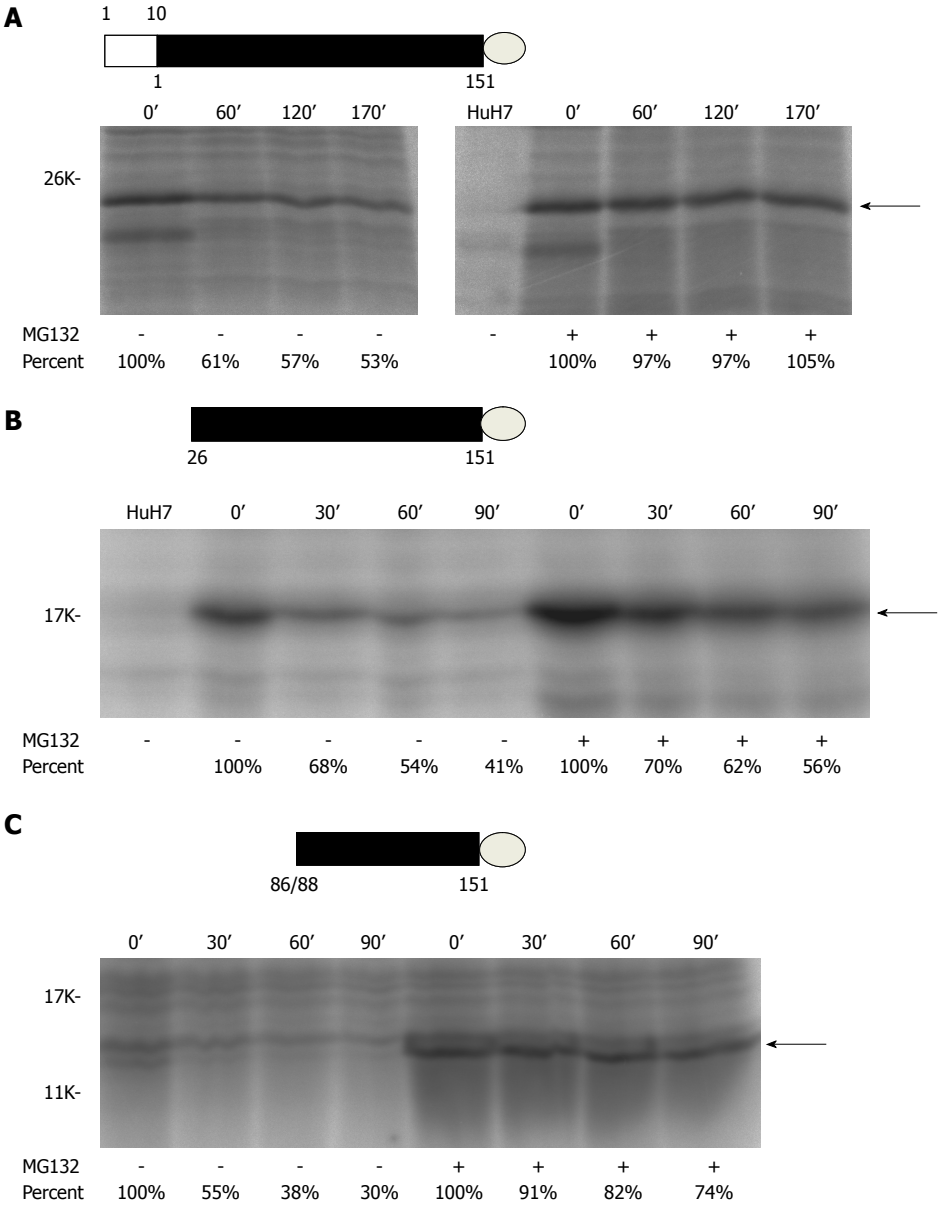

Figure 2 Pulse-chase experiments of different recombinant alternative reading frame/core+1 proteins (H77 sequence was used for this study). A: Authentic F[alternative reading frame (ARF)/core+1/F] protein (the empty rectangle marks the sequence overlapping with the core protein while the filled rectangle for the core+1 coding sequence) with a V5 tag (represented by a gray circle) at its C-terminus; B: The ARF/core+1 protein translated from AUG of amino acid 26 with a V5 tag at its C-terminus; C: The ARF/core+1 protein translated from AUG of amino acids 86/88 with a V5 tag at its C-terminus. HuH7 cells were mock-transfected or transfected with various constructs expressing different recombinant ARF/core+1 proteins as indicated. Forty-eight hours after transfection, cells were incubated in methionine-free medium for two hours and subsequently radiolabeled with ^{35}S-methionine in the same medium (160 mCi/mL) for two hours. Then, regular medium with or without MG132 treatment was used for further cultivation. At the indicated times, cells were disrupted and proteins were extracted to perform the immunoprecipitation assay using rabbit anti-F polyclonal antibody.

dividing cells they also localize to the microtubules of the mitotic spindle[41]. ARF/core+1 proteins seem to be labile in the cells. F protein was labile in the cells and its degradation is ubiquitin-independent[42,43]. Moreover, core+1/S is also very unstable[41]. In the cells, the half-lives of several ARF/core+1 proteins were around 30 to 120 min (Figure 2A-C). Biochemical properties of different ARF/core+1 proteins are largely unknown. Core+1/S, a highly basic polypeptide, was found to be highly disordered under native conditions, with a tendency for self-association[44].

PATHOGENICITY OF HCV CORE GENE PRODUCTS

Pathogenicity of HCV core protein

At present, the transgenic mouse model was used mostly to study the pathogenic roles of core protein in animals[45]. Core protein was shown to induce the ROS overproduction in the liver of transgenic mice[46]. In one study, core protein could induce steatosis only in the transgenic mice[47]. In another study, the transgenic mice expressing

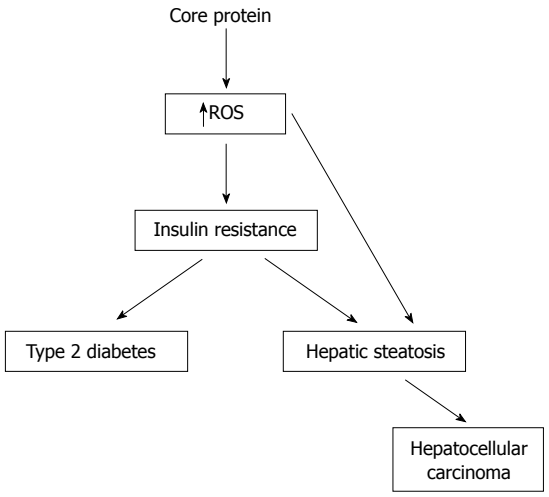

Figure 3 Pathogenecity of hepatitis C virus core protein in the transgenic mice. Some studies have showed that the transgenic mice with core protein developed steatosis only, or steatosis followed by hepatocellular carcinoma. In other studies, the transgenic mice with constitutive core protein expression developed insulin resistance, then leading to type 2 diabetes on a high-fat diet. Most of these mice would develop hepatic steatosis and some of them would even develop hepatocellular carcinoma.

core protein developed steatosis and HCC in the absence of inflammation[48]. On the other hand, the transgenic mice with constitutive core protein expression developed insulin resistance at 1 to 2 mo-old, then leading to type 2 diabetes on a high-fat diet. Most of these mice would develop hepatic steatosis at 6-mo-old and some of them would develop HCC at 16 to 23 mo-old[24,49-51] (Figure 3).

Transgenic mice with constitutive expression of core protein are usually lack of immune response to this protein. Therefore, transgenic mouse models suitable to study fibrosis and cirrhosis caused by core protein are not available yet. A Cre/loxP recombination system has been developed in transgenic mice to study the inflammation caused by the core protein[52]. This inducible system in transgenic mice may be suitable to study fibrosis and cirrhosis caused by core protein in the near future.

Molecular mechanisms regarding the pathogenic roles of core protein were studied extensively in the cell culture and transgenic mouse models.

Interaction of HCV core with cellular proteins

HCV proteins orchestrate a complex and dynamic interaction network with cellular proteins contributing to viral persistence and pathogenecity. Through high-throughput yeast two-hybrid screening assay and computation-based analysis, a virus-human protein interactome network has been constructed[53]. Cellular proteins related to four pathways are major targets by HCV proteins: insulin, Jak/STAT, TGF-β and focal adhesion pathways. Core protein appeared as a major perturbator of IJT network (insulin, Jak/STAT and TGF-β pathways) in this study. Seventy-six cellular proteins were found to interact with core protein in this yeast two-hybrid screening assay. By interacting with PLSCR1, connecting insulin and JAK/STAT, core protein could therefore interfere with both insulin and JAK/STAT pathways. Through interacting with Yin Yang 1, connecting IJT network, core protein could perturb these three pathways[53]. To explore the protein-protein interactions further, the yeast two-hybrid membrane protein system was performed. Eleven human proteins interacting with core protein were identified in this assay. A virus-human protein interactome network has also been constructed[54]. This network suggests that core protein may (1) interfere the host innate immune response through interacting with SLC25A5; (2) induce oxidative stress through interacting with NDUFS2 and ETFB; (3) affect focal adhesion pathway through SLC25A5 and ENO1; and (4) elevate hepatic iron level through its cellular partner FTL[54]. Therefore, core protein could potentially target insulin, Jak/STAT, TGF-β and focal adhesion pathways.

Through extensive literature review, more than 100 cellular proteins (including the proteins mentioned above) were found to interact with core protein and the interaction network of core protein was constructed. These cellular proteins are involved in the processes of signal transduction, transcription, nucleic acid binding, apoptosis, cell cycle, cytoskeleton and kinase activity[55]. Pathogenicity of core protein may be resulted from its interaction with these cellular proteins.

Modulation of cellular gene expression by core protein

HCV core protein could modulate cellular gene expression by directly interacting with transcription factors or indirectly through affecting the signal transduction pathways. Expression of numerous cellular genes is regulated by HCV core protein (for a review[56]). This review focuses only on the cellular genes modulated by core protein identified through global analysis, *i.e.*, microarray. Core protein could stimulate hepatocyte growth, which is at least partly mediated through up-regulation of Wnt-1 expression, both in Huh-7 cells and transgenic mice[57,58]. Stat3 signaling pathway was induced when primary human hepatocytes was immortalized by core protein[59]. Genes involved in lipid metabolism (*e.g.*, SREBP pathway) were affected by core protein in either cultured cells or transgenic mice[60,61]. Core protein could also induce interferon-inducible gene 27 in primary human hepatocytes[62]. Moreover, transgenic mice that conditionally express intermediate HCV core protein develop inflammation possibly through activation of complement 3[52]. On the other hand, core protein may mute the cellular inflammatory response *via* inhibition of cyclooxygenase 2 expression during HCV infection[63]. In B cells, core protein may impair antigen presentation by downregulation of MHC class Ⅱ molecules[64]. Therefore, gene expression profiles regulated by core protein are mainly involved in lipid metabolism, signal transduction, protease activity and immune responses[59,60,65-67]. It is important to notice that cellular gene expression profiles modulated by core proteins from different genotypes are not the same[60,67].

MicroRNAs (miRNAs) affect gene silencing *via* translational inhibition and/or mRNA degradation[68]. The miRNA dysfunction is believed to play important roles in human diseases, including viral infectious diseases, *e.g.*, HCV infection (for a review[56]). HCV core protein could down-regulate the expression of miRNA-122 and miRNA-124 in the cells[69,70]. On the other hand, core protein could down-regulate p21(Waf1/Cip1) expression by enhancing miRNA-345 expression in human hepatoma cells[71]. In monocytes, core protein could also increase the miRNA-155 expression, which in turn up-regulate the TNF-α production[72]. Recently, core protein was reported to induce steatosis through up-regulation of the miRNA-27 expression[73]. It is not surprising to know that differentially expressed microRNAs were detected in HuH-7 cells expressing core proteins from different genotypes[74].

Pathogenicity of core protein should be at least partially through modulating cellular gene expression (mRNA and/or miRNA production).

Effect of core protein on apoptosis

Apoptosis is important for a host to defend viral infections, to inhibit viral spread and persistence. Induction of apoptotic pathways in HCV-infected patients, primarily as a result of host immune responses, could lead to viral suppression and virus-mediated liver damage. In HCV-infected liver, however, despite enhanced hepatocyte apoptosis, viral persistence is observed. To date, it is not known whether the infectious virions act as pro- or anti-apoptotic agent *in vivo*. For virtually all HCV proteins, pro- and anti-apoptotic effects have been described. However, which HCV protein affecting apoptosis *in vivo* is still unknown (for a review[56,75,76]).

The data regarding the effect of HCV core protein on apoptosis is controversial. Core protein was reported to enhance Fas-mediated apoptosis[77]. However, Fas-mediated apoptosis was inhibited in the transgenic mice expressing core, E1, E2 and NS2 proteins[78]. Core protein could either enhance or inhibit TNFα-mediated apoptosis[79-81]. The discrepancy of these results is possibly due to that different virus strains and/or different experimental systems were used. Recently, core protein from genotype 3a was demonstrated to have a stronger effect on anti-apoptosis than the one from genotype 1a[82]. Moreover, it has been mentioned that other factors, possibly cell-type specific, might be involved in different pro- and anti-apoptotic effects of core protein in the cells[83]. Therefore, it is unclear whether core protein inhibits or induces apoptosis in hepatocytes. However, it is still believed that inhibition of apoptosis and enhanced cell proliferation are important in progression of HCC[76]. Recently, core protein was reported to have anti-apoptotic effect in B cells that were isolated from two individual donors[64].

Immunomodulatory role of core protein

Through escaping immune detection and/or suppressing the host immune responses, HCV is efficient to establish persistent infection[83]. HCV core protein could modulate immune response in many ways. It is well known that core protein would suppress interferon signaling (for a review[84]). Core protein is reported to block interferon signaling by interacting with the STAT1 protein[85,86]. Through inhibition of interferon regulatory factor-3 (IRF-3) dimerization, core protein suppressed interferon β expression[87]. By reducing the interaction with DEAD-box RNA helicase (DDX3), core protein with specific mutations also attenuated type I interferon response[88]. Core protein is also reported to abrogate DDX3 and interfere with DDX3-enhanced interferon signaling in two different cells[89]. In addition, core protein may alter NK cell function by inducing apoptosis in these cells[90]. Core protein was also reported to stimulate TLR2 pathway assisting the virus to evade from the innate immune system[91]. Therefore, core protein impairs the innate immunity through these activities.

In hepatocytes, HCV infection or core protein expression could up-regulate the CD55 expression, limiting excessive complement activation[92]. Moreover, hepatocytes infected with HCV or expressing core protein displayed significant repression of complement 9 expression[93]. On the contrary, in the transgenic mice with core protein, complement 3 was up-regulated in inflamed liver. Moreover, administration of CD55 reduced hepatic inflammation[52]. It is not known which factor(s) caused the discrepancy. However, it is possible that core protein may modulate the complement activity in different situations.

In the transgenic mice, core protein could suppress T cell response *via* enhancing Fas-mediated apoptosis in these cells[94]. In addition, through interaction with gClqR, core protein could up-regulate the expression of PD-1 and SOCS-1 in T cells and monocytes/macrophages[95,96], of Tim-3 in monocytes[97], and of STAT3 on human monocytes, macrophages, and dendritic cells[98], and in turn, suppress the functions of these cells. Core protein could also inhibit cathepsin S-mediated MHC class Ⅱ maturation, contributing to weak immunogenicity of viral antigens in chronically infected humans[99]. Therefore, core protein affected the innate and adaptive immunities.

Jurkat cells expressing core proteins suppressed $CD4^+$ and $CD8^+$ T-cell responses to anti-CD3 plus anti-CD28 stimulation by up-regulation of both FOXP3 and CTLA-4 expression[100]. Therefore, core protein also inhibited the functions of regulatory T cells.

In summary, HCV core protein could modulate immune responses through different mechanisms.

Role of core protein in interferon treatment

As mentioned earlier, core protein is known to inhibit interferon signaling[84-89]. Therefore, core protein sequence variation may be associated with interferon (IFN) therapy resistance. Indeed, substitutions of amino acid 70 and amino acid 91 in the core protein of genotype 1b were reported as independent factors associated with a non-virological response toward interferon treatment. Especial-

ly, substitutions of arginine by glutamine at amino acid 70, and/or leucine by methionine at amino acid 91 were significantly more common in non-virological responses toward interferon treatment[101]. After this finding, numerous reports confirmed this association (for a review[11,84]). Consistent with these clinical findings, an *in vitro* study has also demonstrated that cells with core mutants (R70Q, R70H, and L90M) were significantly more resistant to the interferon treatment than the cells with the wild-type core protein. Moreover, the interferon-resistance of the cells with these core mutants may be through IL-6-induced, SOCS3-mediated suppression of interferon signaling[102].

Role of core protein in oxidative stress

HCV infection is characterized by a systemic oxidative stress. The possible mechanisms of HCV-induced oxidative stress include (1) activation of NAD(P)H oxidase of Kupffer cells and PMN cells during inflammation; (2) iron overload and lipid peroxidation; (3) activation of NAD(P)H oxidase by NS3 protein; (4) increased production of mitochondrial ROS/RNS by the electron transport chain due to core and NS5A proteins; (5) decreased GSH output due to liver damage; (6) decreased antioxidants and antioxidant gene expression; (7) alcohol, drugs, and other chemicals; (8) increased cytokines that increase ROS; (9) increased expression/activity of COX-2; and (10) increased expression of CYP2E1 (for a review[103]). HCV core protein is reported to be associated with endoplasmic reticulum (ER) and interacted directly with mitochondria. Then, core protein would cause ER stress, inhibit mitochondrial electron transport and increase ROS production[104,105].

In the transgenic mice, core protein could induce overproduction of ROS in liver. At the same time, some genes of the antioxidant systems, including heme oxygenase-1 and NADH dehydrogenase quinone 1, were down-regulated in the liver with HCV infection[46]. Similarly, in cooperation with NS3 protein, core protein would impair the induction of cytoprotective Nrf2 target genes in the cells[106]. The expression of a variety of cytoprotective genes is regulated by short cis-acting elements in their promoters, called antioxidant response elements (AREs). A central regulator of ARE-mediated gene expression is Nrf2. Therefore, core protein could induce ROS production and impair cytoprotective response.

On the other hand, it was reported that core protein expression leads to intracellular oxidative stress, and that vital cellular functions are, in turn, protected by the up-regulation of cellular antioxidant defense mechanisms in cultured hepatoma cells[107]. Furthermore, core protein could activate the antioxidant defense Nrf2/ARE pathway in a ROS-independent manner[108]. Therefore, core protein could induce ROS production and, at the same time, activate the cytoprotective response.

In summary, core protein could induce ROS production. Meanwhile, core protein might up- or down-regulate Nrf2 target genes in different conditions.

Role of core protein in insulin resistance and diabetes

HCV infection is known to be associated with insulin resistance (IR)[109], leading to the development of type 2 diabetes[6,110]. IR in chronic hepatitis C is reported to associate with genotypes 1 and 4, the serum HCV RNA level, and liver fibrosis[111]. Therefore, IR could be promoted by HCV *via* a genotype-specific mechanism. HCV core protein is a pathogenic factor for the development of IR[84,112,113].

Core protein could cause ER stress, increase oxidative stresses[104,105], which can further exacerbate IR[114]. IR caused by core protein (genotype 1b) in transgenic mice was at least partially mediated by induction of TNF-α over-production, responsible for phosphorylation of serine residues of insulin receptor substrates (IRS-1 and IRS-2) and down-regulation of glucose transporter gene expression. Indeed, administration of antibodies against TNF-α to these mice could restore insulin levels to normal and return insulin sensitivity to normal[51]. Further analysis of this mouse model indicated that a PA28-γ-dependent pathway was required for core protein-mediated IR[49]. To impair the insulin signaling, core protein (genotype 1a) increases IRS-1 phosphorylation at Ser(312) by activating JNK in hepatocytes[115] (Figure 4).

Core proteins from different genotypes down-regulate IRS-1 through genotype-specific mechanisms: the core protein of genotype 3a promoted IRS-1 degradation through the down-regulation of PPAR-γ and by upregulating SOCS-7 while the core protein of genotype 1b activated mTOR[116]. Further study indicated that core protein of genotype 3a increases SOCS-7 expression through PPAR-γ in Huh-7 cells[117]. Indeed, deletion of SOCS-7 in the transgenic mice leads to enhanced insulin action[118]. In addition to SOCS-7, over-expression of SOCS-3 has also been linked to insulin resistance[119]. Indeed, core protein (genotype 1b) up-regulated SOCS-3 and caused ubiquitination of IRS1 and IRS2 in the transgenic mice. Furthermore, core protein-induced down-regulation of IRS1 and IRS2 was not seen in the SOCS3(-/-) mouse embryonic fibroblast cells[120]. Actually, activation of SOCS family members is a general mechanism associated with the core proteins from genotypes 1-4 except a rare genotype 1b variant failed to activate any of the SOCS tested. This leads to identifying the role of the amino acids 49 and 131 of core protein in mediating SOCS transactivation[121].

Sequence variations in core protein may affect IR development. Indeed, in Japanese patients without cirrhosis and diabetes mellitus, a.a. substitutions of the genotype 1b core protein [Glu70 (His70) and/or Met91] are the significant determinants of severe IR[122].

Role of core protein in steatosis

Steatosis or "fatty liver" is common in patients infected with HCV. Steatosis caused by HCV infection should be classified into two types: metabolic steatosis in patients infected with non-genotype 3, and viral steatosis

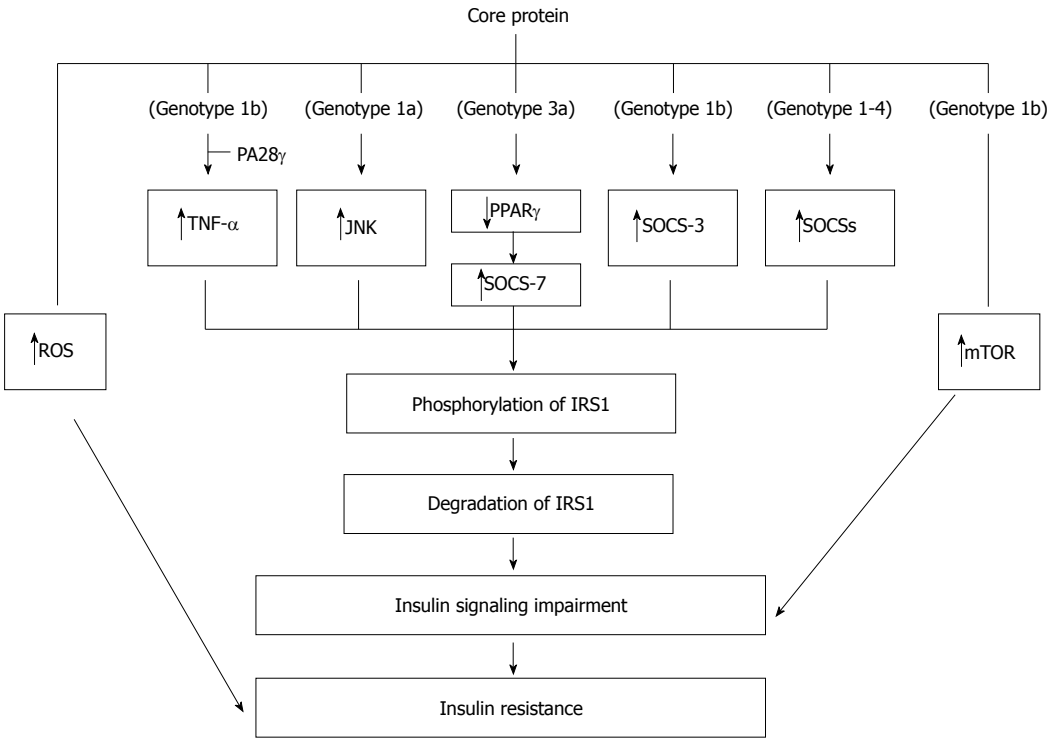

Figure 4 Molecular mechanisms regarding the insulin resistance induced by core protein. Core proteins from different isolates/genotypes may use common and/or distinct mechanisms to cause insulin resistance.

in patients infected with genotype 3. Metabolic steatosis occurs in the setting of obesity, hyperlipidemia, and IR, whereas viral steatosis is caused by HCV as a direct cytopathic effect[112,123-126]. Transgenic mice with core protein developed hepatic steatosis[47,127]. Therefore, core protein plays an important role in the development of steatosis[128]. Three mechanisms have been proposed regarding the induction of triglyceride accumulation in the liver cells by core protein: firstly by impaired lipoprotein secretion, secondly by increased lipogenesis, and thirdly by impaired fatty acid degradation[124-126,128] (Figure 5).

To impair lipoprotein secretion, core protein inhibits the activity of microsomal triglyceride transfer protein (MTTP), which plays a key, rate-limiting role in VLDL assembly/secretion. Thus, its inhibition results in the accumulation of triglycerides in the cells, which causes steatosis[129]. Furthermore, through interaction with mitochondria, core protein induces ROS production. The production of ROS results in peroxidation of membrane lipids and structural proteins, that are involved in the trafficking and secretion apparatus, blocking VLDL secretion.

To increase lipogenesis, core protein activates transcription factor SREBP-1c through up-regulation of liver X receptor alpha (LXRα) and retinoid X receptor alpha (RXRα), leading to enhanced activity of various enzymes involved in cellular lipid biosynthesis[61,130-133]. Interestingly, the genes related to fatty acid biosynthesis and srebp-1c promoter activity were up-regulated by core protein in cell line and mouse liver in a PA28γ-dependent manner[50]. Recently, a study reported that accumulation of triglycerides in HepG2 cells with core proteins was due to delta-9 desaturase, an enzyme involved in fatty acid biosynthesis (primarily the synthesis of oleic acid), activated by core protein. Moreover, polyunsaturated fatty acids could counteract the impact of core protein on lipid metabolism[134].

To impair fatty acid degradation, core protein is believed to down-regulate PPARα and MCPT-1, resulting in the reduction of fatty acid oxidation activity[135-138], though some contradictory results were also reported[139,140]. Recently, core protein is reported to induce the expression of miRNA 27 to repress PPARα expression[73]. Down-regulation of PPARα and MCPT-1 by core protein may be mediated by repressing the SIRT1-AMPK signaling pathway[141].

Recently, a bipartite model has been proposed as a novel mechanism for core protein-induced steatosis: core protein first requires DGAT1 to gain access to lipid droplets[142], and then lipid droplets-localized core protein interferes with triglyceride turnover, thus stabilizing lipid droplets and leading to steatosis[143].

In the transgenic mice, HCV core-induced nonobese hepatic steatosis is associated with down-regulation of the leptin gene in visceral fat and concurrent hypoadiponectinemia. Moreover, steatosis is ameliorated by adiponectin administration[144].

Collectively, the multiple activities of core protein may participate in the triglyceride accumulation in chronic HCV infection.

The higher prevalence and much more severity of liver steatosis are observed in patients infected with HCV

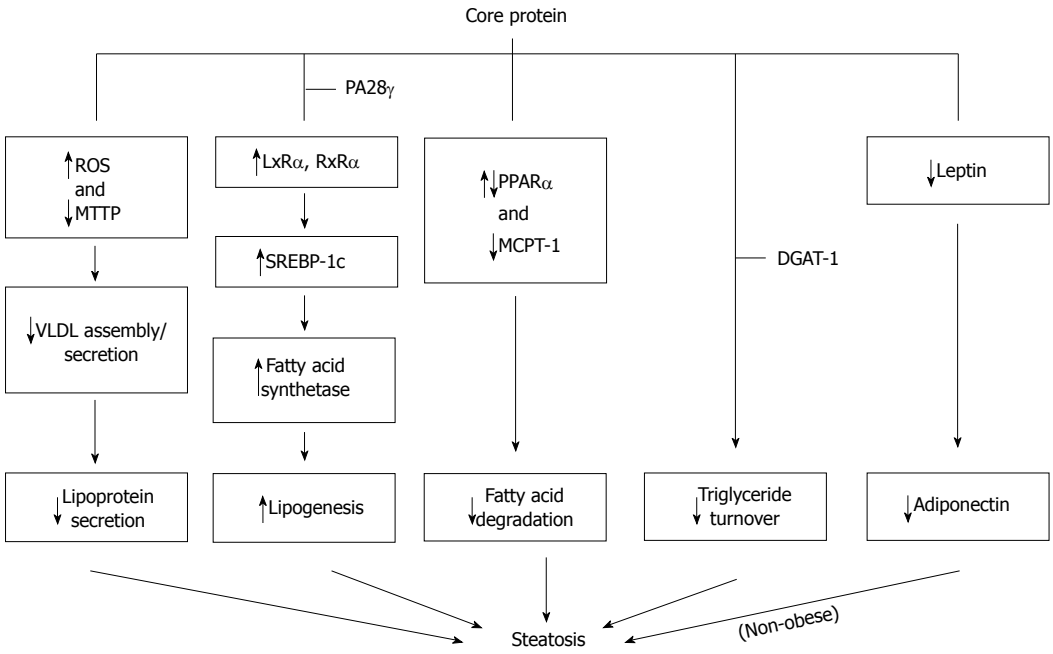

Figure 5 Molecular mechanisms proposed for the steatosis caused by core protein. Core protein from one genotype may use more than one mechanism to induce steatosis.

genotype 3 than in patients infected with other genotypes. Indeed, core protein from genotype 3a but not from genotype 1b could down-regulate PTEN in hepatocytes and trigger the formation of large lipid droplets[145]. Recent studies examining a possible mechanism of steatosis formation in genotype 3a isolates have focused on the a.a. 164 of core protein[146]. Core protein has Phe at 164 position up-regulated fatty acid synthetase promoter stronger[147].

Sequence variations in core protein may affect steatosis development. Indeed, substitutions at a.a. position 70 and/or 91 of the genotype 1b core gene are associated with the lipid accumulation that causes steatosis[148,149]. Moreover, polymorphisms at the a.a. position 182 and 186 of the genotype 3 core gene are correlated with the intrahepatic steatosis[150].

Role of core protein in fibrosis and cirrhosis

The molecular mechanism(s) of HCV-related fibrosis is unclear. Hepatic stellate cells (HSCs), one of the sinusoid constituent cells, play a critical role in liver fibrosis[151]. Oxidative stress and various cytokines are well known profibrogenic mediators[152]. HCV may induce fibrosis by the following mechanisms: (1) stimulating secretion of profibrogenic cytokines in hepatocytes; (2) interacting with sinusoidal endothelium; and (3) provoking fibrogenesis *via* HSCs. Transgenic mice with conditional core protein expression developed inflammation and fibrosis[52]. Therefore, core protein plays an important role in liver fibrosis.

Core protein could stimulate secretion of profibrogenic cytokines in hepatocytes. Indeed, core protein is known to up-regulate TGFβ1 expression in hepatoma cells[153]. Moreover, core protein in hepatoma cells promotes liver fibrogenesis *via* up-regulation of CTGF with TGFβ1 when co-cultured with HSCs[154]. Recently, TGF-β was also reported to be up-regulated in transgenic mice with core protein. Moreover, hepatoma cells expressing core protein could activate stellate cells in the co-culture system and this activation was TGF-β dependent[155]. In addition, core protein could increase the TNF-α production in monocytes[72].

Core protein could also provoke fibrogenesis *via* HSCs directly. Non-enveloped core protein could be secreted by infected cells[156,157]. These secreted core proteins could stimulate fibrosis in HSCs *via* either obese receptor[158] or toll-like receptor 2[159].

Little is known regarding the molecular mechanism(s) of HCV-related cirrhosis. Core protein could up-regulate and sustain HIF-1α expression under hypoxia, thereby contributing to increased VEGF expression, a key regulator in the hypoxic milieu of liver cirrhosis[160]. Therefore, core protein may play a role in liver cirrhosis through the up-regulation of VEGF expression.

Role of core protein in hepatocellular carcinoma

The major risk factor for the development of HCC in HCV-infected patients is pre-existing cirrhosis. Therefore, the main hypothesis for HCV carcinogenesis is that it occurs through the effects of chronic inflammation and hepatocellular injury. However, HCC can still develop in a small proportion of non-cirrhotic patients with chronic hepatitis C, suggesting that HCV may be directly involved in hepatocarcinogenesis. This was supported by the report that transgenic mice with constitutive expression of HCV structural and nonstructural proteins would develop HCC[161]. HCV core protein plays a very important role in the development of HCC. This was supported by

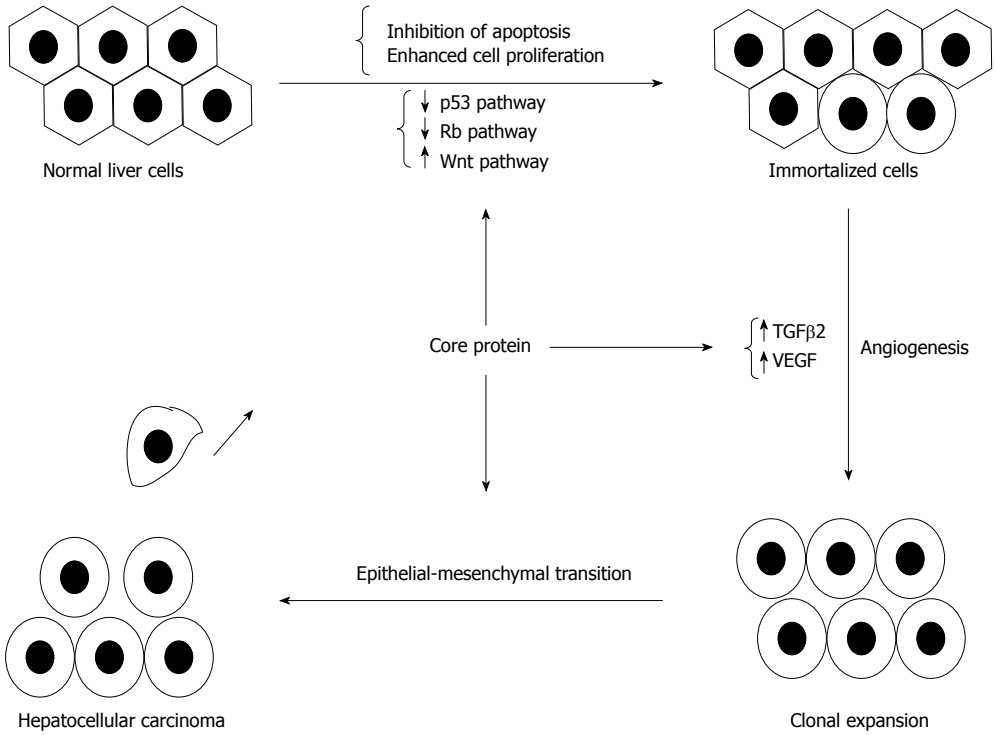

Figure 6 Involvement of core protein in the development of hepatocellular carcinoma. In addition to inducing immortalization of hepatocytes, core protein could enhance angiogenesis and promote the epithelial-mesenchymal transition to result in hepatocellular carcinoma.

the report that transgenic mice with constitutive HCV core protein expression would develop HCC[162].

Core protein may induce HCC development through its contribution to the onset of oxidative stress, steatosis and anti-apoptosis[48,50,76]. In addition to overcoming apoptosis, disruption of hepatocyte growth control is another key molecular event leading to the development of HCC. Actually, core protein could increase cell proliferation through the interaction with cellular proteins (*e.g.*, p53, p73 and pRb), or through the modulation of cellular gene expressions (*e.g.*, p21) and intracellular signal transductions, such as MAPK and Wnt/β-catenin pathways[56-58,163,164]. Moreover, core protein could stimulate primary human hepatocytes to escape from senescence and promote an immortalized phenotype[165,166] (Figure 6).

Furthermore, core protein is reported to trigger hepatic angiogenesis by induction of TGF-β2 and VEGF[167].

Core proteins derived from HCC were demonstrated to shift the TGF-β responses from tumor suppression to epithelial-mesenchymal transition (EMT)[168]. Recently, core protein is reported to epigenetically silence SFRP1 and enhance HCC aggressiveness by inducing EMT[169]. Another report also showed that core protein could promote the migration and invasion of hepatocyte *via* activating transcription of extracellular matrix metalloproteinase inducer[170].

Collectively, the multiple regulatory activities of core protein may result in the development of HCC.

HCV core protein-induced pathogenesis may be genotype-specific. Indeed, over expression of core gene from genotype 3a showed stronger effect in regulating expression of Cox-2 as compared to that from genotype 1a in Huh-7 cells[171]. Sequence variations within HCV core gene are reported in tumors and adjacent non-tumor tissues from the same patients[172-174]. Therefore, sequence variations of core protein within the same genotype may have different pathogenic effect. Indeed, amino acid substitutions in the core protein of genotype 1b are associated with HCC development[175-178], especially at amino acids 70 and 91[84].

Role of core protein in other cancers

In addition to HCC, HCV core protein may be also associated with the development of intrahepatic cholangiocarcinoma (ICC) and hilar cholangiocarcinoma[70,179]. However, more studies are needed to reveal the molecular mechanisms.

Role of HCV core protein in co-infection with HIV or HBV

When co-infection with HIV/HCV occurs, HCC is more likely to occur at a younger age and with a shorter duration of HCV infection compared to those with HCV mono-infection[180]. This indicates HIV could worsen the pathogenic effects caused by HCV. On the other hand, HCV core protein could interact with HIV-1 Nef protein to stimulate HIV-1 replication in macrophages[181]. Moreover, HCV core protein could induce neuroimmune activation and potentiate HIV-1 neurotoxicity[182].

Dual infection with HCV and HBV in cirrhotic patients has been linked to an increased risk of HCC[183], indicated the interactions between these two viruses. A

zebrafish model of ICC was established recently by dual expression of hepatitis B virus X protein (HBx) and HCV core protein in liver. Further studies in this model revealed that TGF-beta1 plays an important role in HBx- and HCV core protein-induced ICC development[184].

Pathogenicity of the HCV core cleaved peptide

Though the exact C terminus of core protein has not been determined yet, it is likely that the mature core protein contains 177 amino acid residues[15,19]. Therefore, the cleaved peptide generated by the cleavages of both SP and SPP is from amino acid 178 to 191 (Figure 1A). This peptide is the E1 signal peptide region that facilitates the proper cleavage at core-E1 junction. All signal peptide sequences contain a hydrophobic core region, but, despite of this, they show great variation in both overall length and amino acid sequence[185]. However, the cleaved peptide (a.a. 178-191) is highly conserved with close to 100% identity among different HCV genotypes[19,29]. Sequence conservation in this cleaved peptide suggests that it should play important roles during virus infection. However, no individual residue among these 14 amino acids of the cleaved peptide is absolutely required for infectious virus production, as individual substitutions resulted in wild-type titers and a core protein fragment comprising residues 1 to 177 efficiently complemented assembly in *trans*[19]. Signal peptides have been suggested to have additional functions[186]. For example, the signal peptide of the lymphocytic choriomeningitis virus glycoprotein is presented by major histocompatibility complex class I as an immunodominant epitope[187], and the liberated HIV-1 gp160 leader sequence is associated with calmodulin[185,186]. A previous report argued for an additional function for this cleaved peptide. The synthetic peptide containing HCV core protein a.a. 178-187, which shows sequence homology with CYP2A6 and CYP2A7, could induce primary CTL responses in peripheral blood mononuclear cells in an HLA-A*0201-restricted manner[188]. If the cleaved peptide (a.a. 178-191) was further processed into (a.a. 178-187), it will be interesting to know how this process occurs in the cells.

Amino acid substitutions at positions 182 and 186 of the HCV genotype 3a core protein have been identified to cause increased intracellular lipid accumulation in hepatic cells[150]. These amino acid substitutions did not affect the production of mature core protein[150], in agreement with the results of a previous report[19]. Therefore, polymorphisms in the cleaved peptide (a.a. 178-191) may contribute to steatosis development. Jhaveri *et al*[150]. speculated (1) that the cleaved peptide interacts with host proteins within the ER membrane that mediate lipid metabolism and trafficking; and (2) that this interaction may differ between genotypes. It will be interesting to find out which cellular proteins could interact with this cleaved peptide.

Pathogenicity of HCV ARF/core+1 proteins

Detection of the specific antibodies against ARF/core+1 proteins and the T-cell responses in HCV-infected patients provided strong evidence that ARF/core+1 protein is expressed *in vivo*[30,32,33,189]. However, abolishing the production of ARF/core+1 proteins had no effect on HCV replication in cultured cells or uPA-SCID mice, suggesting that ARF/core+1 proteins is probably not important for the HCV reproductive cycle[190]. On the other hand, the gene sequence conservation of this open reading frame argues that ARF/core+1 proteins should serve an important function[34].

The role of ARF/core+1 proteins in viral pathogenesis is largely unknown. It was shown that the F protein, unlike core protein, is not involved in NF-kappaB regulation[191]. Moreover, F protein, unlike core protein, could not up-regulate the expression of the fibrosis marker α-smooth muscle actin[58]. Actually, F protein does not share the major properties identified previously for the core protein, other than repressing p21 expression[192]. Down-regulation of p21 expression by F protein suggests that F protein may regulate cellular proliferation. Cellular MM-1 protein was found to interact with F protein. Further analysis indicated that F protein can enhance the gene trans-activation activity of c-Myc, apparently by antagonizing the inhibitory effect of MM-1[193]. The ability of F protein to enhance the activity of c-Myc also raises the possibility that F protein may enhance cellular proliferation. Indeed, F protein could induce hepatocyte proliferation in the transgenic mice possibly through β-catenin signaling pathway[58]. These results suggest that F protein may play a role in hepatocellular transformation in HCV patients. This hypothesis was supported by the finding that HCV sequences derived from HCC tissues could produce F protein more efficiently than those derived from non-HCC tissues[173,174,194]. Moreover, high occurrence of anti-core+1 antibodies was detected in the serum of HCC patients[195].

HCV core protein may regulate iron metabolism through interacting with FTL[54]. Recently, core+1/ARF protein was found to decrease hepcidin transcription through an AP1 binding site[196]. This indicates that core+1/ARF protein may also affect iron metabolism because hepcidin is the main regulator of iron metabolism. Moreover, HCV core and F proteins were shown to induce hepatocyte proliferation in the transgenic mice possibly through β-catenin signaling pathway[58]. Therefore, these two proteins seem to have redundant pathogenic roles. This explains the findings that HCV patients who do not produce normal anti-core antibodies have unusually high levels of anti-core+1/ARFP[197], and that the HCV-1b core+1 products are negatively regulated by core expression[38].

F protein was also found to interact with cellular prefoldin 2 protein. Moreover, expression of F protein resulted in aberrant organization of tubulin cytoskeleton[198], which suggests that F protein may affect cellular functions. On the other hand, it is possible that F protein may serve as a modulator to prevent high level of HCV replication and thus contributes to viral persistence in

chronic HCV infection since HCV replication requires intact microtubule and actin polymerization[198].

It is not known whether different ARF/core+1 proteins regulate cellular activities through similar pathways. Similar subcellular localization and short half-lives of F and core+1/S proteins[41,42] suggest that these two proteins may have similar regulatory activities. However, further investigations are needed to clarify this issue.

CONCLUSION

In addition to the mature core protein (a.a. 1-177) and the cleaved peptide (a.a. 178-191) encoded from the conventional open reading frame of HCV core gene, several ARF/core+1 proteins could be expressed from the core+1 reading frame.

Through interacting with cellular proteins, modulating cellular gene expression, inducing reactive oxygen species production, and modulating cellular apoptosis, core protein could induce insulin resistance, steatosis, and even hepatocellular carcinoma. The cleaved peptide (a.a. 178-191) may play a role in the immune response and steatosis development. Though labile, ARFP/core+1/F protein could interact with cellular proteins and enhance hepatocyte growth. The core+1/ARF protein may also affect iron metabolism.

The present-day knowledge about the pathogenic roles of core gene products discussed here is obtained from cell culture and transgenic mouse models. The transgenic mice with constitutive expression of core gene products are tolerant to these proteins, leading to an insufficient immune response. A Cre/loxP recombination system has been developed in transgenic mice to study the inflammation caused by core protein. This inducible system in transgenic mice may be suitable to study the fibrosis and cirrhosis caused by core protein in the near future.

Studies of the cellular mechanisms involved in the pathogenesis of core gene products should help in the design of therapeutic drugs.

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TOPIC HIGHLIGHT

WJG 20th Anniversary Special Issues (3): Inflammatory bowel disease

Inflammatory bowel diseases and human reproduction: A comprehensive evidence-based review

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Abstract

To evaluate the effects of inflammatory bowel diseases (IBDs) on human reproduction, we reviewed the current literature using a systematic search for published studies (articles and/or abstracts) without limits for English language. We searched on Medline (through PubMed), the Institute for Scientific Information, the Web of Science and the websites for the registration of controlled trials (http://controlled-trials.com/). Bibliographies of retrieved articles, books, expert opinion review articles and reviewed bibliographies from subject experts were manually searched. Titles and abstracts were screened initially, and potential relevant articles were identified and reviewed. Whenever possible, data were analyzed by comparing IBD patients *vs* healthy controls, and patients with active IBDs *vs* those with disease in remission. The effects of IBDs on female fertility, fertility in infertile couples, pregnancy and male infertility were examined separately. Patients with IBDs in remission have normal fertility. At the moment, there is no established guideline for the preservation of fertility in women with IBD undergoing surgery. Further data are needed regarding guidelines for the management of these patients. Data regarding IBDs and infertility are currently completely lacking. Considering the prevalence of intestinal pathology in young adults of childbearing age, this field is of great scientific and clinical interest, opening up important future perspectives. Another important and as yet unexplored point is the response to treatments for infertility in patients with IBDs. In particular, the question is whether the reproductive outcomes (clinical and biological) can be influenced by the IBD of one of the partners. The goals for successful reproductive outcomes in IBD population are correct counseling and disease remission. IBDs significantly affect several reproductive aspects of human (female, male, couple) reproduction. Further data are needed to develop guidelines for the clinical management of subjects of reproductive age with IBDs.

Key words: Inflammatory bowel diseases; Fertility; Infertility; Pregnancy

Core tip: The current comprehensive evidence-based review evaluated the most recent data regarding the

effects of inflammatory bowel diseases on human reproduction.

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INTRODUCTION

Inflammatory bowel diseases (IBDs) predominantly affect younger patients of reproductive age. To date, several reviews[1-4] have been published in the English literature whose aim was to evaluate and clarify the impact on obstetric outcomes of both the IBDs themselves and the drugs commonly used to treat them. However, very little is known on the overall effect of IBDs on human reproduction.

The study of human reproduction includes not only the effects of IBDs and their treatment on pregnancy but also their effects on the menstrual cycle and hormonal patterns, on the subfertile women scheduled for ovulation induction cycles or assisted reproductive techniques (ARTs), on future reproductive potential in younger and/or adolescent women, and on male fertility, including data on semen parameters and libido/hormonal patterns.

Based on these considerations, the current study was designed to provide an evidence-based overview on the effects of IBDs on all aspects of human reproduction, including both female and male fertility, and to provide a critical synthesis useful for clinical practice.

SEARCH STRATEGY

To obtain evidence-based data and to provide evidence-based recommendations, a systematic search for studies (articles and/or abstracts), without English language limitation, was performed.

It included the combination of the following medical subject headings or keywords: "assisted reproduction techniques", "ART", "Crohn's disease", "complication", "effectiveness", "efficacy", "embryo", "endometrium", "fertility", "fetal", "foetal", "infertility", "inflammatory bowel disease(s)", "IBD(s)", "intracytoplasmic insemination", "ICSI", "*in vitro* fertilization", "IVF", "libido", "management", "menstrual cycle", "menses", "neonatal", "obstetrics", "ovary", "pregnancy", "prevention", "safety", "semen analysis", "sex hormones", "sperm", "spermatozoa", "subfertility", "surgery", "therapy", "treatment", "UC", "ulcerative colitis".

We searched on Medline (through PubMed), the Institute for Scientific Information , the Web of Science, and the websites for the registration of controlled trials (http://controlled-trials.com/). Bibliographies of retrieved articles, books, expert opinion review articles, and reviewed bibliographies from subject experts were manually searched. Titles and abstracts were screened initially, and potentially relevant articles were identified and reviewed

For each issue, we analyzed mainly meta-analyses and/or randomized controlled trials (RCTs). When meta-analytic data or data from RCTs were lacking, prospective non-randomized and then cohort studies were included in the final analysis.

To construct a comprehensive review, data regarding both primary endpoints, as traditionally suggested by evidence-based medicine (pragmatic view), and intermediate endpoints, crucial to understanding the mechanisms of action (mechanistic view), were extrapolated and analyzed.

Whenever possible, data were analyzed by comparing IBD patients *vs* healthy controls, and patients with active IBDs *vs* those with disease in remission.

FEMALE FERTILITY

Women with inactive Crohn's disease (CD) or ulcerative colitis (UC) appear to have normal fertility. In remission, female fertility seems not to be diminished. A case-control study by Elbaz *et al*[5] showed that there was an increased need for fertility treatment of women with IBD; however, this association was no longer significant after controlling for maternal age (increasing maternal age is associated with subfertility).

In CD, fertility is normal or slightly reduced[6-8]; older referral center studies estimated infertility rates of 32%-42%[8-11], but community-based and population-based studies suggest infertility rates of 5%-14%, similar to that of the general population[7]. Women with UC have normal fertility until they undergo surgery[9,10].

Several reasons for the potentially reduced fertility in IBD women have been hypothesized. We have identified two main sources: psychological problems and surgery-related problems.

Psychological problems

Relatively few data are available regarding sexual dysfunctions in women with IBD. Moody *et al*[12] did not find any significant change in rates of dyspareunia and overall frequency of sexual intercourse between women with IBD and matched controls. On the other hand, a mismatch of perception and reality seems to significantly affect family planning decisions in women with IBD. A recent large study[13] was published whose aim was to evaluate whether, and to what extent, IBD patients' perceptions of risk influence their reproductive behavior and to describe IBD patients' specific concerns related to fertility and pregnancy. "Voluntary childlessness" was the main cause of the reduced fertility rate (number of live births per woman) reported in IBD patients[13]. This fear of infertility was most evident in women with CD and pre-

vious surgery[13]. In particular, IBD-related reproductive risks seemed to be overestimated by the examined subjects. The main reproductive concerns of IBD patients regarded pregnancy risks, drug-related teratogenicity or toxicity, long-term risks and IBD inheritance[13].

Surgery-related problems

Women with active CD have decreased fertility[6], perhaps related to the formation of adhesions caused by the disease itself and/or surgery, resulting in tubal infertility[14]. Fertility may normalize after induction of remission in women with CD[15]. Some surgical procedures, such as rectal excision and pouch formation, appear to have detrimental effects on male and female fertility.

In UC with ileal pouch anal anastomosis (IPAA), the ability to become pregnant is significantly reduced, probably because of the presence of post-surgical adhesions in the pelvis, secondary obstruction of the fallopian tubes or altering the normal tubo-ovarian relationship necessary for ovum capture and transport[16-18]. Two studies that evaluated the impact of proctocolectomy with pelvic pouch or end ileostomy on pelvic anatomy, showed that 50 percent of females had complete unilateral or bilateral obstruction of the fallopian tubes[19,20]. These studies suggested that pelvic dissection is the likely cause of adhesions and altered pelvic anatomy.

A systematic review showed that IPAA for UC results in decreased fertility[21]. The conception rate in women with UC was 40% before IPAA and only 29% after IPAA[21]. A meta-analysis estimated that the risk of infertility after IPAA increased by a factor of three[22]. The high use of fertility treatments after IPAA compared with other groups illustrates the difficulty these women have in getting pregnant after surgery. Olsen *et al*[10] found that after IPAA, 29% of children were born after *in vitro* fertilization compared with only 1% in the general population. Another study[10] of Danish and Swedish patients found that females had significantly decreased fecundity (probability of becoming pregnant per month of unprotected intercourse) after IPAA compared with before IPAA. The data regarding fertility after IPAA do not state the number of women who voluntarily chose not to become pregnant and the impact of age on female fertility, which could greatly influence the interpretation of the results.

In a Canadian study, Johnson *et al*[23] confirmed that the infertility rate was significantly higher in IPAA patients compared with patients managed non-operatively (38.6% *vs* 13.3%). In that study, the effect of age on the risk of becoming pregnant was investigated for all patients who attempted to become pregnant in both the IPAA and non-operative management groups. There was a negative association between advancing age and success of becoming pregnant: for each additional year of age there was a 12% decrease in the odds of becoming pregnant[23]. Increasing age was associated with decreased reproductive ability among all females included in that study[23].

A recent Finnish study showed that, although the probability of a women conceiving in any short time period seemed to be reduced to 47% of the average, the lifetime chance of having at least one live birth after IPAA was 80%[24]. Thus, women with IPAA mostly suffer a reduction in the probability of conception rather than complete infertility.

Strategies are needed to improve fertility after IPAA. One possibility would be to perform sub-total colectomy/end ileostomy and delay IPAA, but this would likely be unacceptable to most females. A second strategy would be to preserve tubal patency and normal tubo-ovarian relationship. Various materials are available to prevent post-surgical adhesions in the pelvis; however, as there are no data regarding their efficacy in improving fertility, further research is needed[25].

To date, there are no established guidelines for the preservation of fertility in women with IBD undergoing surgery. However, we believe that an effective strategy should be based on the following principles: (1) proper selection of patients with a specific clinical indication for surgery; (2) proper evaluation of the patient based on factors predictive of ovarian reserve [*i.e.*, age, anti-Müllerian hormone (AMH), antral follicle count]; and (3) surgery that is as minimally destructive of the radical pelvic anatomy as possible.

FERTILITY IN INFERTILE COUPLES

The purpose of this section is to evaluate the effects of the IBDs in the infertile couple. In particular, our attention focused on reproductive outcomes (*i.e.*, all intermediate steps and final results) of an infertile couple in which one or both partners are affected by IBDs. Both CD and UC mainly affect young adults of reproductive age. Whereas substantial data are available concerning pregnancy in young IBD women or fertility in IBD men, no study has been conducted on IBDs in infertile couples.

In the few last years, only two studies[26,27] investigated the ovarian reserve status in CD women, as reflected by serum AMH. The first study[26] showed that women with CD do not have severe ovarian reserve alterations compared with a control population. However, age $\geqslant$ 30 years and a colonic location of the disease could be associated with an accelerated loss of follicles. Another study[27] confirmed that serum AMH levels of reproductive-age women with CD were significantly lower compared with the controls, and the Crohn's Disease Activity Index (CDAI) and AMH were inversely correlated. Thus, these data could encourage gastroenterologists to inform CD women of the risk of delaying childbirth.

Finally, only one report[28] described the reproductive outcome following intracytoplasmic sperm injection (ICSI) for male factor infertility associated with CD and 6-mercaptopurine (6-MP) chemotherapy. The authors[28] reported the first successful birth after ICSI for severe oligozoospermia associated with CD.

PREGNANCY

Several recent data are available in literature on IBDs and pregnancy. However, the majority of published studies are reviews or retrospective analyses.

Effects of pregnancy on IBDs disease activity

Pregnancy seems to have a beneficial effect on IBD symptoms, especially when it occurs during disease remission. A small, but significant, decrease in the Harvey-Bradshaw index of disease activity during pregnancy, in comparison with the year preceding and following pregnancy, was observed in a retrospective analysis on women with CD[29]. Similar results were found in a large European prospective study, showing that 74% of CD and 67% of UC patients with active disease at conception achieved remission later during pregnancy[30].

Factors affecting remission or exacerbation of IBDs have been investigated extensively. In particular, smoking has a negative effect on the course of CD, and some authors showed that the reduced disease activity in pregnancy was partly the result of reduced tobacco smoking during pregnancy[31]. By contrast, exacerbation of disease, particularly in the first trimester of pregnancy, could result from discontinuation of maintenance therapy.

The state of the disease at conception is a factor influencing the course of pregnancy[32-34]. In fact, patients with active disease at conception often continue to have symptoms during pregnancy, whereas a normal course of pregnancy can be expected in patients who conceive when in remission. In a cohort study with a 10-year follow-up period, it was observed that if conception occurred during remission, the risk of a flare-up was comparable to that in non-pregnant patients with IBD. Instead, when conception occurred during an active disease period, two-thirds of patients relapsed during pregnancy and more than 60% of these patients experienced further deterioration[35].

The course of IBD in the postpartum period remains controversial. A small prospective study reported a decrease in relapse rate in CD, as well as in UC patients, 4 years after pregnancy, in comparison with the 3 years before pregnancy[36]. Similarly, in a large cohort study, the yearly flare-up rates decreased from 0.34 to 0.18 in UC and from 0.76 to 0.12 in CD. Two studies[37,38] also reported reduced stenosis and resection rates in women with IBD after pregnancy. Mechanisms potentially involved could be related to the hormone relaxin, the effect of pregnancy on the immune response, as well as feto-maternal HLA disparity[38,39]. The effects of breastfeeding on flare-up rates in IBD mothers are also controversial[40,41].

Effect of IBDs on pregnancy/perinatal outcomes

Current data indicate that quiescent disease has minimal impact on the course and outcome of pregnancy in IBD patients, whereas patients with active disease at conception have increased rates of spontaneous abortion[33] and a significantly increased risk of preterm delivery and low birth weight[32,42]. Overall pregnancy outcomes in women with IBDs (CD or UC) were similar to those of non-IBD pregnant patients[31].

A meta-analysis[43], including 3907 subjects, reported increased risks for preterm delivery (OR = 1.87, 95%CI: 1.52-2.31) in both CD and UC patients; low birth weight (OR = 2.82, 95%CI: 1.42-5.60) in CD but not in UC; and congenital abnormalities (OR = 3.88, 95%CI: 1.14-10.67) in UC but not in CD. A Swedish population study found 4.5% and 1.2% of children born to IBD patients had, respectively, low and very low birth weight, as compared to 2.9% and 0.6% in the overall Swedish population[44]. Moser *et al*[32] additionally found an increased incidence of poor maternal weight gain during pregnancy in CD patients with quiescent disease at conception.

In both CD and UC patients, pregnancies ended more frequently with caesarean section compared with the general population[45]. Furthermore, pregnant CD and UC patients who needed to be hospitalized had a higher risk of undergoing a caesarean section (OR = 1.72, 95%CI: 1.44-2.04 and OR = 1.29, 95%CI: 1.01-1.66, respectively) in comparison with patients without IBD[46].

Prevention and treatment of IBDs during pregnancy

Most of the drugs used to treat IBD are not associated with increased risk of congenital anomalies or adverse effects on the fetus (Table 1). The 2010 European Crohn´s and Colitis Organisation (ECCO) guidelines state "medical treatment for Crohn's disease (except methotrexate) should generally continue during pregnancy, because the benefits outweigh the risks of medication"[46]. Moreover, as complications and adverse pregnancy outcomes mainly occur in patients with active disease, the main concern should be to achieve remission before conception and maintain quiescent disease during pregnancy. Patients who received counseling regarding the benefits and risks of drug treatment before conception and during pregnancy were more likely to remain compliant[34].

It is generally regarded as safe to keep using aminosalicylates (ASA) during pregnancy [Food and Drugs Administration (FDA) category B drug], despite some reports noting a higher incidence of neural tube defects, oral cleft and cardiovascular defects[47]. In a recent meta-analysis of treatment of IBD patients with 5-ASA drugs, IBD did not significantly increase the risk of congenital abnormalities (OR = 1.16), stillbirth (OR = 2.38), spontaneous abortion (OR = 1.14), preterm delivery (OR = 1.35) or low birth weight (OR = 0.93)[48].

Several studies[49,50] reported that sulfasalazine assumption during pregnancy does not give rise to increased rates of birth defects in women with IBD. Sulfasalazine therapy should be accompanied by extra folate supplementation, as this medication halts folate synthesis by inhibiting dihydrofolate reductase. Folic acid supplementation decreases the augmented risk of oral clefts and cardiovascular anomalies associated with folate antagonist treatment during pregnancy[51]. Caution should be applied regarding the use of some mesalamine formulations (*e.g.*,

Table 1 Safety indications for drugs in pregnancy and breastfeeding

	UC	ECCO rating pregnancy	ECCO rating breastfeeding	Personal observations
5-ASA (except sulfasalazine)	B	Safe	Safe	Avoid high doses for long time, asacol preparations may be switched to another mesalamine
Sulfasalazine	B	Safe	Safe	Folate supplements are required
AZA/6-MP	D	Safe	Probably safe	Discuss breastfeeding with the patient. Avoid lactation in the four hours after intake of the drug
Cyclosporine A	C	Probably safe	Contraindicated	Use only in severe cases of UC to avoid urgent colectomy during pregnancy
Methotrexate	X	Contraindicated	Contraindicated	Discontinue at least 4 mo prior to conception
Corticosteroids	C	Safe	Safe	Very low risk of malformations. If possible, not use for long time
Infliximab	B	Probably safe	Safe	Can be used safely in the first two trimesters of pregnancy. If possible, avoid in the third trimester
Adalimumab	B	Probably safe	No data	Can be used safely in the first two trimesters of pregnancy. If possible, avoid in the third trimester
Metronidazole	B	Probably safe	Best avoided	Very slight increase of cleft lip. Use only if strictly necessary
Ciprofloxacin	C	Probably safe	Probably safe	Use only for short periods and avoid in the first trimester
Thalidomide	X	Contraindicated	Contraindicated	

5-ASA: 5-aminosalicylates; AZA: Azathioprine; 6-MP: 6-mercaptopurine; ECCO: European Crohn's Colitis Organisation; UC: Ulcerative colitis.

asacol) that contain dibutyl phthalate (DBP) as a coating agent. The use of DBP-coated medications produces measurable phthalate metabolite levels in urine. Prenatal exposure to DBP can cause congenital malformations in the male urogenital tract[52]. Finally, the sulfasalazine metabolite sulfapyridine is secreted into breast milk. ASA are generally considered safe during lactation, although a case of bloody diarrhea in an infant has been reported[53-55].

The use of antibiotics during pregnancy, *i.e.*, matronidaole and quinolones, should be considered with caution. Metronidazole is considered a low-risk drug during pregnancy (FDA class B). While several studies did not find an association between metronidazole treatment and birth defects[56], a large case-control study showed an increased incidence of cleft lip and/or cleft palate in infants of mothers exposed to metronidazole in the first trimester of pregnancy[57]. Thus, it should be limited to short-term use for the treatment of pouchitis. In addition, as metronidazole is excreted in breast milk, breastfeeding during its administration is not recommended[56].

Quinolone antibiotics are FDA category C drugs and should be avoided because they carry an increased risk of arthropathy because of their high affinity for bone and cartilage. ECCO recommends avoiding quinolone use in the first trimester of pregnancy[57]. Data on breastfeeding are limited, but quinolone use is probably compatible with breastfeeding[58,59].

The recent London position statement on biological therapy for IBD states that anti-tumor necrosis factor (TNF) therapy is considered low risk and can be used in the preconception period and during the first two trimesters of pregnancy[60]. The cytokine TNF-α not only plays a pivotal role in the inflammation process underlying IBD, but also plays physiological roles in host defense mechanisms and pregnancy. During pregnancy, TNF-αprobably plays a role in protecting the fetus against teratogenic stress[61]. Despite the role of TNF in pregnancy, treatment with anti-TNF antibodies can be considered safe in the preconception period and the first part of pregnancy, because IgG antibodies do not cross the placenta in the first pregnancy trimester and transplacental IgG transport mainly takes place during the late second and third trimester of pregnancy[62,63]. Maternal transfer of IgG during the last trimester of pregnancy provides the neonate with sufficient acquired immunity to defend itself while its own immune system is becoming fully functional.

The currently available TNF inhibitors (etanercept, infliximab, adalimumab, golimumab and certolizumab) are all classified as FDA category B drugs, indicating that no teratogenic effects of these drugs were observed in animal reproduction studies; however, adequate and controlled human safety data are still lacking. Transfer of anti-TNF antibodies to the fetus during the last part of pregnancy may mean exposure of the neonate in the first months after birth, raising potential concerns about infection and response to vaccines[60].

Infants exposed to immunosuppressive drugs during pregnancy probably should be considered to be immunocompromised, as are their mothers. ECCO guidelines state that live vaccinations (BCG, rotavirus, mumps-measles-rubella (MMR) and varicella zoster) are contraindicated until exposure to immunosuppressants has been discontinued for at least 3 mo[64]. IgA is the predominant immunoglobulin in human milk, so secretion of TNF inhibitors in milk is likely to be very limited and breastfeeding under anti-TNF treatment can be considered safe[65].

Natalizumab is an α-4 integrin inhibitor approved for treatment of CD in the US, but not in Europe. Experience with natalizumab in the context of IBD is still limited, even if this biological compound is widely used for treating multiple sclerosis. It has received an FDA category C label. Thus, at present insufficient data are available to reach a definite conclusion on the safety of natalizumab during pregnancy and lactation.

Corticosteroids are FDA category C drugs. They are

believed to be safe throughout pregnancy at doses up to 15 mg per day[60], whereas higher doses increase the risk of infection and premature delivery[66]. Systemic treatment with corticosteroids during the first trimester of pregnancy was found to slightly increase the risk of oral clefts (OR = 3.35, 95%CI: 1.97-5.69), while the overall risk of congenital malformations is not significantly increased (OR = 1.45, 95%CI: 0.80-2.60)[67].

Pregnant women are preferably treated with prednisone or prednisolone, as the bulk of these compounds are inactivated by placental 11β-hydroxy steroid dehydrogenase, the physiological mechanism in place to protect the fetus from elevated maternal cortisol levels during pregnancy. Treatment with corticosteroids is compatible with breastfeeding[68,69].

Cyclosporine crosses the placenta but is rapidly cleared in the neonate and has no known teratogenic effect. FDA categorizes cyclosporine in pregnancy category C for lack of controlled studies in humans; however, cyclosporine does not appear to be a major teratogen.

A meta-analysis[58] on the use of cyclosporine in pregnancy showed that cyclosporine use in pregnancy was not associated with major malformations, but slightly decreased birth weight and duration of gestation. In IBD patients, cyclosporine use in refractory UC during pregnancy has been shown to be safe and effective. Its main use in pregnant IBD patients is the prevention of urgent colectomy in fulminant UC[70,71].

Although a number of cases are reported where no overt adverse effects were observed in breastfed infants of mothers treated with cyclosporine, the use of this drug during lactation is generally not advised as cyclosporine is secreted in milk at high concentrations, leading to potential nephrotoxicity and immunosuppression in exposed infants[72].

ASA and 6-MP are still designated as FDA category D drugs, indicating that increased risk for the fetus exists, but the risk must be weighed against the possible benefits of the drug. A recent Danish cohort study showed an increased risk of preterm delivery and low birth weight in women exposed to azathioprine (AZA) or 6-MP during pregnancy, but no significant increase in congenital malformations[50].

The CESAME study[73], a cohort study comparing IBD patients exposed to thiopurine therapy during pregnancy with women receiving other treatments or women without any drug therapy, showed that thiopurine exposure was not associated with low birth weight prematurity and congenital abnormalities, but was associated with preterm birth. Exposure in men at the time of conception was not associated with congenital abnormalities[74]. Thiopurine treatment is generally considered a contraindication for breastfeeding.

Methotrexate has teratogenic properties and is contraindicated during pregnancy (FDA category X). Methotrexate metabolites have long tissue half-lives; therefore, its administration must be stopped 3 to 6 mo before conception[75]. Folic acid supplementation after methotrexate withdrawal is also recommended because methotrexate acts as a folate antagonist. Methotrexate is also contraindicated during lactation because of its potential accumulation in the child's tissues.

Thalidomide and its analog lenalidomide partly counteract the effects of TNF-α and have been used in patients with refractory CD, even if currently available systematic evidence does not clearly demonstrate the benefit of these drugs[76]. The teratogenicity of these drugs has been well documented, indicating increased risks for limb defects, central nervous system defects, and congenital abnormalities in the cardiovascular, respiratory, gastrointestinal, and genitourinary tracts. Thus, thalidomide is absolutely contraindicated in pregnancy (FDA category X) and patients taking thalidomide are advised to use two complementary contraceptive methods[77,78]. Although lenalidomide appears less teratogenic in animal studies, the lack of studies demonstrating its safety in humans leads to the absolute contraindication of this drug in pregnant patients or in patients wishing to become pregnant[77,78].

Management of delivery in women with IBDs

Pregnancy should not be considered high risk in all cases of IBDs. Delivery, therefore, can be carried out in clinics of the second or third level. Only patients with active IBD necessitating steroid and/or anti-TNF treatment, patients with ileostomies, and patients with ileoanal pouches should be referred to high-risk pregnancy clinics.

Active perianal disease at the time of delivery is an indication for caesarean section, whereas patients without history of perianal disease or inactive perianal disease do not require caesarean delivery[45]. In UC patients with IPAA, caesarean section rates of almost 50% have been reported, but the incidence of pouch-related complications was low and pouch function was found to be unrelated to the mode of delivery[79]. However, the most recent ECCO guidelines state that the presence of an ileoanal pouch in CD patients is an indication for caesarean section[80]. Moreover, patients with IPAA surgery in the past are always advised to have caesarean section, and we recommend that an abdominal surgeon should be present during the surgery.

MALE FERTILITY

Clinical data

Infertility in men with IBD has been relatively less studied than infertility in women with IBD. A recent European consensus states that both male and female fertility are not significantly affected in non-operated IBD patients when disease is quiescent, compared to the general population[81]. It is important to note that IBD patients remain voluntarily childless more frequently than non-IBD controls[82]. In a survey among 255 Australian IBD patients, fear concerning IBD heritability, side effects of the medication on the child, and medical advice given by physicians were the most important reasons for voluntary childlessness[13].

Table 2 Effects of inflammatory bowel disease medications on erectile dysfunction, male infertility and partner's pregnancy

	Erectile dysfunction	Infertility	Pregnancy complications	Recommendations
5-ASA (except sulfasalazine)	No	Single reversible case	No reports	No recommendations to discontinue prior to conception
Sulfasalazine	Single case	Yes, reversible, not dose dependent	One study	Switch to other 5-ASA preparations
AZA/6-MP	No reports	No	Controversial	No recommendations to discontinue prior to conception
Cyclosporine A	No reports	No	No reports	No recommendations to discontinue prior to conception
Methotrexate	Yes	Controversial	No reports	Discontinue 3-4 mo prior to conception
Steroids	No reports	No	No reports	Lack of data to discontinue
TNF-α inhibitors (infliximab, and only a case report for adalimumab)	No reports	Reduce sperm quality, but no infertility	No	No recommendations to discontinue prior to conception, but barrier methods during pregnancy

5-ASA: 5-aminosalicylates; AZA: Azathioprine; 6-MP: 6-mercaptopurine; TNF: Tumor necrosis factor.

A recent systematic review[83] on fertility in non-surgically treated IBD showed that in men with CD (a total of 493 men in two population-based and one referral center studies), there was an 18%-50% reduction in fertility compared with controls, although whether the cause was involuntary infertility or voluntary childlessness was not indicated. There was no evidence of reduced fertility in men with UC[83].

IBD and male fertility intermediate end-points

Active disease, IBD treatment, and psychological factors affect male reproductive and sexual function[84]. Most male IBD patients considered "maintaining remission" as important at conception[85] and in fact inflammation has a negative effect on male fertility[86]. Men who were in remission or who had only mild disease activity had rates of erectile dysfunction similar to those of healthy control subjects, whereas men with more severe IBD activity had higher rates[87].

IBD and semen parameters

There are no large studies that assess semen abnormalities in IBD patients on no medication at all. Some studies suggest that factors such as disease activity and nutritional status could affect semen[88-90]. Furthermore, there are reports of antisperm antibodies in both men and women with IBD, and these antibodies might contribute to infertility. Antisperm antibodies might be a result of the increased immunological response, caused by increased intestinal permeability, against antigens of gut microbiota possessing common antigenicity with spermatozoa[91]. Dimitrova *et al*[92] showed that antisperm antibody incidence in 50 patients with ulcerative colitis was statistically significant compared with 50 healthy blood donors[92]. A little study on 10 IBD patients (four women and six men) and reported the presence of antisperm antibodies in semen of male patients and cervical secretions of female patients at the time of ovulation[93].

Surgery and male fertility

The most common surgery in IBD patients is IPAA. Proctocolectomy with IPAA has been associated with sexual dysfunction in men. A large study found 1% and 2% of sexual dysfunction at 1 year and 12 years after surgery, respectively[94]. Furthermore, 3% of men reported experiencing either retrograde ejaculation or no ejaculation 10 years after surgery[94].

A meta-analysis of 43 observational studies that evaluated patients after IPAA found the pooled incidence of sexual dysfunction to be 3.6%[95]. On the other hand, a study of 122 men who had undergone IPAA evaluated male sexual function using a validated index based on erectile function, orgasmic function, sexual desire, intercourse satisfaction and overall satisfaction[96]. This study showed that, despite any negative effect on erectile function (in particular retrograde ejaculation), the other four features had a statistically significant improvement[96]. A Dutch study confirmed that despite an elevated rate of sexual dysfunction of up to 25% in 35 men after IPAA for UC, 90% of patients were satisfied with the operation[97].

A randomized, placebo-controlled trial of sildenafil for erectile dysfunction following rectal excision performed either for cancer or IBD in 32 men showed that post-operative sexual dysfunction can be treated successfully with sildenafil in most cases (79% of the sildenafil-treated group compared to only 17% of the group taking placebo)[98].

There are other types of surgery beyond IPAA, for example, total colectomy with end ileostomy, colectomy with ileo-rectal anastomosis, and proctocolectomy with ileo-anal anastomosis without a pouch. The reports on sexual function after these procedures are limited, but only ileo-rectal anastomosis avoids the extensive pelvic dissection of the other procedures, which produces adverse effects on sexual function.

Medical treatments and male fertility

Table 2 shows the effects of IBD medications on erectile dysfunction, infertility and pregnancy. In the general population, medications are responsible for erectile dysfunction in up to 25% of cases[99]. The medications used

to treat IBD rarely cause erectly dysfunction; however, antidepressants and anti-anxiety medications, frequently prescribed for patients with IBD, could cause erectile dysfunction because depressive mood negatively influences sexual function (patients with a first diagnosis of IBD had higher rates of depression than patients with diagnosis of colon cancer)[100].

On the other hand, many drugs have been reported to impair semen parameters; sulfasalazine, methotrexate, and infliximab seem to affect sperm quality[57]. Of note, confounding factors that could affect male fertility, such as smoking, alcohol consumption, disease status, and medication use, are not always reported in studies investigating the effects of medical treatments on male fertility**.** Men with IBD rarely have documented physician counseling regarding the potential effects of medication on fertility and pregnancy because gastroenterologists' lack of knowledge of the effects of medications on male fertility or a lack of documentation of their counselling practices[101].

Except for sulfasalzine, the risk of adverse fetal outcomes of other medications must be weighed against the benefit of maintaining good health of the father at the time of conception.

The only 5-ASA medication shown to cause male infertility is sulfasalazine[102,103]. Sulfasalazine causes reversible non-dose-dependent semen abnormalities (oligospermia, reduced motility and abnormal morphology)[104,105] and infertility in up to 60% of men[106]. Impaired sperm maturation and oxidative stress from the sulfapyridine constituent of the drug are thought to be the cause[107-110]. Male fertility is restored two months after sulfasalazine withdrawal or switching to other mesalazine preparations[111-115]. A single case of impotence was reported that was resolved after switching to olsalazine[116]. One study found an association between sulfasalazine use in men and a higher rate of congenital malformations in their children[106]. There is a single case report of mesalazine-induced oligospermia and infertility that reversed when the drug was stopped[117]. Given these data, no recommendations are made to discontinue 5-ASA (except sulfasalazine) for men planning to conceive.

A study of 18 men with IBD showed that AZA did not reduce semen quality, and thus male fertility, in IBD: no changes in semen parameters were noted after 11 ± 5 mo of AZA administration or during long-term treatment (49 ± 14 mo)[118]. In a survey of 164 male renal transplant patients, long-term therapy with cyclosporine, AZA and prednisone demonstrated no effects on fertility[119].

The teratogenic effect of AZA and its metabolite 6-MP remains controversial. In one retrospective study, the incidence of pregnancy-related complications was significantly increased when the fathers used 6-MP within 3 mo of conception[120]. Specifically, in men with IBD under 6-MP, congenital anomalies and spontaneous abortions were detected in 4% and 6% of cases, respectively. These data are substantially different from those observed in men with IBD who were not taking this drug (no congenital anomalies and 2% of spontaneous abortions)[120]. However, the rates of congenital abnormalities and spontaneous abortions were below those of the general population - 3% and 10%, respectively[121]. In a Danish population-based cohort study, the paternal use of AZA or 6-MP before conception was associated with an increased, but not statistically significant, risk of congenital abnormalities[122].

Two other studies did not observe any significant effect of preconception thiopurine exposure of the father on pregnancy outcomes. Francella *et al*[123] showed that there was no statistical difference in conception failures (defined as a spontaneous abortion), abortion secondary to a birth defect, major congenital malformations, neoplasia or increased infections among offspring of male patients taking 6-MP compared with controls. In 2010, Teruel *et al*[124] confirmed there were no significant differences in terms of unsuccessful pregnancies between the exposed group (MP 9, AZA 37) and the control group. Given these data, no recommendations are made to discontinue AZA/6-MP for men planning to conceive.

There are no data regarding the effects of cyclosporine on fertility of men with IBD. In animal models, cyclosporine A causes damage of testicular tissue and sperms[125], but antioxidants may protect from testicular toxicity[126,127]. In humans, the small studies conducted do not seem to suggest any association between cyclosporine and male infertility: a decrease in serum antisperm antibodies[128,129], normal semen analysis, and successful pregnancies are described[130]. There are no reports of adverse pregnancy among partners of men who were taking cyclosporine. No recommendations are made to discontinue cyclosporine A for men planning to conceive.

Methotrexate is the one most often associated with impotence[131-133]. However, few data are available on the effects of methotrexate on male reproductive capability. In animal models, it causes altered spermatogenesis and degeneration of spermatocytes, Sertoli cells, and Leydig cells[134-136]. Opinions differ in the literature on the effects of methotrexate on male fertility: the concurrent administration of other chemotherapeutic agents is a limitation[137].

There are small studies reporting on methotrexate use with no other agents. In patients affected by psoriasis, there are cases of documented reversible sterility when the methotrexate was stopped[138] and there are several case series that report no change in sperm quality[139-142]. There are no reports of adverse pregnancy outcomes among the partners of men exposed to methotrexate before conception[143]. However, the active metabolites of methotrexate can remain in cells or tissues for several months after discontinuation[144]. Given these data, it is recommended that the drug should be discontinued at least 3-4 mo before attempts at conception for men with IBD[145-147].

Few data are available on the effects of steroid therapy on male fertility[118,148]: it appears there is little

impact. Although steroids may inhibit apoptosis of damaged sperm cells, they may prevent pathological excessive apoptosis, which results in oligospermia[149]. No recommendations can be made to discontinue steroid use due to insufficient data.

There are few studies also on the effects of anti-tumor necrosis factor agents; the data do suggest, however, that these medications may impair male fertility. Infliximab may also serve to counter the negative effects of TNF-α on sperm quality[150]: supraphysiological levels of TNF-α were seen to cause chromatin and DNA damage, and reduce sperm motility[151]. Reports on male fertility in ankylosing spondylitis patients showed a decrease in sperm motility in patients on conventional treatment *vs* anti-TNF-α-treated patients[152].

In a study of 10 men, infliximab increased semen volume with a trend towards decreased sperm motility and morphology, but its impact on male fertility was not reported[153]. Villinger *et al*[154] showed sperm abnormalities that were more pronounced in patients with active spondyloarthritis, but the sperm quality of patients with inactive disease receiving long-term TNF inhibition is comparable to that in healthy controls. Another report on four patients with ankylosing spondylitis who fathered six healthy children during infliximab treatment may provide some reassurance for male patients treated with infliximab[155]. The data support continuation of anti-TNF treatment when IBD patients plan fatherhood[154].

The recent Austrian evidence-based consensus on the safe use of infliximab in inflammatory bowel disease[156] states that infliximab may lead to reduced quality by decreasing sperm motility and affecting sperm morphology, although these findings do not demonstrate that male fertility would be reduced[157].

Infliximab treatment of men prior to planned conception does not seem to cause embryo toxicity. For anti-TNF alpha, as it is unknown whether the fetus will be affected by exposure to anti-TNF alpha through semen, the ECCO consensus on reproduction in IBD advises barrier methods during pregnancy. Data on the impact of other biologicals on male fertility are currently lacking, although there is a case report of 35-year-old father of a healthy 4-year-old child who was successfully treated for 3 years with adalimumab for ankylosing spondylitis[158]. No recommendations are made to discontinue anti-TNF-α treatment for men planning to conceive.

CONCLUSION

IBDs significantly affect several reproductive aspects of human (female, male, couple) reproduction. Moreover, further data are needed in order to develop guidelines for the clinical management of subjects of reproductive age with IBDs. In fact, at the moment, there are no established guidelines for the preservation of fertility in women with IBD undergoing surgery. Further data are needed regarding the management of these patients.

Data regarding IBDs and infertility are currently completely lacking. Considering the prevalence of intestinal pathology in young adults of childbearing age, this field is of great scientific and clinical interest, opening up important future perspectives. Further studies should, in fact, be conducted to determine whether the treatments for infertility have an effect on the state of IBD (remission, flare-up, recurrence). Another important point as yet unexplored is the response to treatments for infertility in patients with IBDs. In particular, the question is whether the reproductive outcomes (clinical and biological) can be influenced by the IBD of one of the partners.

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TOPIC HIGHLIGHT

WJG 20th Anniversary Special Issues (5): Colorectal cancer

Emerging role of the β-catenin-PPARγ axis in the pathogenesis of colorectal cancer

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Abstract

Multiple lines of evidence indicate that Wnt/β-catenin signaling plays a fundamental role in colorectal cancer (CRC) initiation and progression. Recent genome-wide data have confirmed that in CRC this pathway is one of the most frequently modified by genetic or epigenetic alterations affecting almost 90% of Wnt/β-catenin gene members. A major challenge is thus learning how the corrupted coordination of this pathway is tied to other signalings to enhance cell growth. Peroxisome proliferator activated receptor γ (PPARγ) is emerging as a growth-limiting and differentiation-promoting factor. In tumorigenesis it exerts a tumor suppressor role and is potentially linked with the Wnt/β-catenin pathway. Based on these results, the identification of new selective PPARγ modulators with inhibitory effects on the Wnt/β-catenin pathway is becoming an interesting perspective. Should, in fact, these molecules display such properties, new research avenues would be opened aimed at developing new molecular targeted drugs. Herein, we review the basic principles and present new hypotheses underlying the crosstalk between Wnt/β-catenin and PPARγ signaling. Furthermore, we discuss the advances in our understanding as to how their altered regulation can culminate in colon cancer and the efforts aimed at designing novel PPARγ agonists endowed with Wnt/β-catenin inhibitory effects to be used as therapeutic and/or preventive agents.

Key words: Colorectal cancer; Wnt/β-catenin pathway; Peroxisome proliferator-activated receptor γ; Genomic instability; Peroxisome proliferator activated receptor γ ligands

Core tip: Genetic and epigenetic modifications of the Wnt/β-catenin pathway play a fundamental role in the initiation and progression of colorectal cancer (CRC). The nuclear receptor peroxisome proliferator activated receptor γ (PPARγ) acts as a differentiation-promoting transcription factor with a potential link with Wnt/β-catenin. In this review, we discuss the basic principles underlying the crosstalk between Wnt/β-catenin and PPARγ signaling, present the most recent progress in understanding as to how their alterations can culminate in CRC and, finally, suggest new hypotheses and perspectives on the identification of selective PPARγ modulators endowed with Wnt/β-catenin inhibitory effects to be used as molecular targeted drugs.

Sabatino L, Pancione M, Votino C, Colangelo T, Lupo A, Novellino E, Lavecchia A, Colantuoni V. Emerging role of the β-catenin-PPARγ axis in the pathogenesis of colorectal cancer. *World J Gastroenterol* 2014; 20(23): 7137-7151 Available from: URL: http://www.wjgnet.com/1007-9327/full/v20/i23/7137.htm DOI: http://dx.doi.org/10.3748/wjg.v20.i23.7137

INTRODUCTION

More than 1.2 million colorectal cancers (CRC) are diagnosed every year, accounting for approximately 10% of all cancers worldwide. Despite the progress made in surgical and therapeutic management, still 600000 deaths are caused every year by CRC representing over half of all gastrointestinal cancer deaths. CRC survival is highly dependent on the tumor stage at the time of diagnosis; over one-third of patients die within five years from the initial diagnosis and most of fatal outcomes result from liver metastases[1,2].

Colorectal cancer can have a hereditary (10%) or sporadic (90%) origin; in both cases, environmental factors contribute to its development. Interestingly, one third of patients have an increased risk of developing a CRC due to familial factors synthesized by yet unidentified genes. It is well established that CRC results from the cumulative effects of sequential genetic and epigenetic alterations, leading to a progressive and irreversible loss of cell growth and differentiation control[3]. In the last decades, a large number of investigations have identified several "driver genes" in CRC initiation and progression, including wingless-type (*WNT*) MMTV integration site family, *RAS*, mitogen-activated protein kinase (*MAPK*), phosphatidyl-inositol3-kinase (*PI3K*), trasforming growth factor β (*TGF-β*), tumor protein p53 (*TP53*) and DNA mismatch-repair genes. The various and sequential pathways in which these genes are involved support the theory that CRC is a heterogeneous, complex and multifactorial disease[3]. Indeed, at least three well-defined pathways, the traditional (adenoma-carcinoma sequence or Vogelstein's model[4,5]), the alternative and the serrated pathway underlie these malignancies, leading to genomic instability that perpetuates a widespread loss of DNA integrity. Thus, genomic instability is emerging as a hallmark of the carcinogenic process and at least three distinct types have been described: chromosomal instability (CIN); microsatellite instability (MSI) and CpG island methylator phenotype (CIMP). CIN is the most common type of genomic instability, occurs in 60%-80% of CRCs and results in an imbalance of the chromosome number "manifested as aneuploidy". MSI is an alternative pathway that accounts for 15%-20% of sporadic CRCs in which the characteristic signature is deletion of repetitive regions of DNA that in most cases generates frameshift mutations in the coding sequences of genes leading to their inactivation. CIMP is a novel instability pathway characterized by the widespread hypermethylation of CpG islands at several genomic loci[2,6,7]. These data suggest that CRC is a highly heterogeneous disease, *i.e.*, clinicopathologically similar tumors strikingly differ as distinct biological subtypes and, consequently, in their response to treatment and patient's survival. Extensive molecular profiling, genome-wide studies and the integrative analysis of genomic data support the notion that clinically distinct subtypes exist and provide insights into the pathways that are dysregulated in CRC[8,9]. Multiple lines of evidence indicate that Wnt signaling plays a fundamental role in CRC development as it is altered from the very early stages[10]. Consistently, almost 90% of CRCs present genetic or epigenetic alterations of Wnt players such as adenomatous polyposis coli (APC), AXIN, β-catenin, sex determining region Y (SRY)-box 9 (SOX9), regardless of the CIN or MSI signature, according to the cancer genome atlas[11]. These observations support the hypothesis that mutationally corrupted cancer (stem) cells, distributed among normal epithelial colonic cells, are the driving force of initiation and progression. Along with driver genes mutations implicated in tumor initiation, Wnt signaling alterations may influence the CRC course and prognosis and be instrumental in determining the optimal patients' treatment.

In this context, peroxisome proliferator-activated receptor gamma (PPARγ) signaling is drawing increasing attention because of its role in CRC pathogenesis and because novel compounds identified as selective ligands could be used as pharmaceutics to improve therapies' efficacy[12,13]. A number of studies have shown that PPARγ levels within primary tumors correlate with patients' prognosis[14-16]. Evidence has also been provided that PPARγ tumor suppressive activity can be altered at multiple levels through aberrant phosphorylation, DNA promoter hypermethylation and microRNAs modulation. Thus, modifications of PPARγ appropriate levels, subcellular localization and activity presumably play a key role in colorectal tumorigenesis[13-15].

In this review, we present recent evidence and new hypotheses that underscore the growing impact of dysregulated Wnt and PPARγ signaling in CRC initiation and progression. Furthermore, we discuss the advances in our understanding as to how these pathways crosstalk and impact colon cancer biology and response to the therapy. Finally, we discuss new therapeutic perspectives of molecular target drugs represented by selective PPARγ modulators endowed with Wnt/β-catenin inhibitory effects.

WNT/β-CATENIN PATHWAY AND CRC INITIATION AND PROGRESSION

The epithelial cells of the intestine have a relatively short life span compared to cells of other epithelial tissues. They, in fact, orchestrate a unique mechanism of constant cell migration starting from the bottom of the crypts and going upwards to the luminal surface. It is well established that long-lived multipotent intestinal

stem cells (ISCs) reside at the bottom of the intestinal crypts and give rise to transit amplifying progenitors that, upon constant upward migration, undergo cell cycle arrest and terminally differentiate into the diverse intestinal cell lineages. Once a differentiated cell has reached the villus tip or the colonic surface, it undergoes apoptosis and is shed into the lumen. The intestinal renewal system is tightly controlled and depends on the spatial organization of signals that emanate from supportive mesenchymal as well as from differentiated epithelial cells[17]. Until recently, ISCs were a rather elusive entity at the bottom of the intestinal crypt; only in the last few years, important efforts have been made in the stem cell research field to characterize their existence, position and function. In 2007 Van der Flier *et al*[18], through both a Wnt transcriptome analysis and in situ studies, identified a panel of 17 putative stem cell markers expressed at the crypt base[17]. Two genes displayed the strongest relationship with the Wnt signaling: Leucine rich repeat containing G protein coupled receptor 5 (Lrg5) and achaete-scute like2 (Ascl2). Lgr5 was subsequently localized at the crypt base of the mouse small intestine and positive cells were shown to be able to differentiate into all epithelial cell lineages. These studies suggest that Lgr5 and Ascl2 are intestinal-specific stem cell markers and emphasize a "crucial role" of the Wnt cascade not only during embryonic development but also in adult organs, particularly in tissue homeostasis, cell renewal and ISC maintenance[18-20]. Wnt is the "fusion of two terms" the segment polarity gene wingless (wg) discovered in Drosophila and the proto-oncogene integration-1 (int-1). The first direct connection of the Wnt pathway with CRC came out in the early 1990s. The APC gene was found to be involved in a hereditary cancer syndrome, termed familial adenomatous polyposis (FAP) but also in sporadic CRC[4,5]. Soon thereafter, the large "scaffold" cytoplasmic APC protein was found to interact with β-catenin providing the molecular basis of the seminal work by Fearon and Vogelstein: CRC develops as a stepwise accumulation of genetic hits in specific genes and pathways[4,5]. From that time, many additional components of the pathway and disease connections have been identified so that the list of new target genes, as well as new interacting pathways, constantly grows. Recent studies have disclosed that the interplay between Wnt and Hippo signaling pathways is indispensable to coordinate proliferation and differentiation during organ growth. Interaction of Wnt ligands with their receptor complexes triggers two major intracellular signaling cascades that are traditionally indicated according to the role played by β-catenin[21,22]. The Wnt "canonical" signaling is "activated" upon the binding of one of multiple Wnt factors, a family of soluble secreted proteins, to one of ten possible Frizzled receptors (Fzd) in the presence of a low density lipoprotein receptor related co-receptor (LRP5 or 6). This interaction generates a cascade of events involving the cytosolic adapter protein, Disheveled (Dvl), that promotes the dissociation of a multiprotein "destruction complex" resulting in the stabilization of β-catenin and its translocation to the nucleus. Upon displacement of the transcriptional repressor Groucho, β-catenin interacts with and activates members of the T cells factors and lymphoid enhancing factors (TCF/LEF) to promote the expression of target genes involved in cell differentiation and proliferation such as c-myc (MYC), cyclin D1 (CCND1), axin 2 (AXIN2), CD44 and Survivin (Figure 1A)[23]. When the Wnt pathway is "inactive", β-catenin binds to its destruction complex formed by the scaffold proteins, APC and axin, and is phosphorylated by the specific kinases glycogen synthase kinase 3β (GSK3β) and casein kinase 1 (CK1)[23,24]. This leads to its ubiquitination and subsequent targeting to the proteasome for degradation. β-catenin consists of three main domains: the N-terminal region of 141 aminoacids, a central core domain of 513 aminoacids, and a C-terminus of 107 aminoacids, which contains the transactivation domain. Phosphorylation of β-catenin by GSK3β and CK1 occurs at aminoacids S33, S37, T41 and S45, generating a recognition tag for ubiquitination and subsequent proteasomal degradation[25,26]. The central "core region" contains 12 imperfect armadillo repeats each formed by 42 aminoacids; the repeats consist of three α-helices and, together, the twelve repeats form a superhelix containing a long positively charged groove[27]. This structure appears to facilitate the binding to the negatively charged β-catenin binding domains (CBD) within TCF/LEF[28,29] or other interacting proteins such as APC, axin, and cadherins. Recent studies have identified two lysines, K312 and K435, defined "charged buttons", within the armadillo repeats 5 to 9 of β-catenin, that form salt bridges with negatively charged glutamate or aspartate in the CBD of the interacting proteins[28]. Mutations of one of the components of the pathway (APC, axin, β-catenin) or autocrine signaling due to constitutive Wnt production by tumor cells cause an "active" Wnt signaling. In most cases, *APC* loss-of-function mutations result in a truncated and inactive protein; in other cases, mutations in β-catenin phosphoacceptor sites turn into an active oncogene; for instance, S37A β-catenin is expressed at high levels in several human carcinomas[30]. β-catenin phosphorylation is also hampered through GSK3β sequestration into multivesicular compartments and/or other still unknown mechanisms.

The critical step in the Wnt canonical pathway appears then to be the ratio of cytosolic and/or membrane-associated β-catenin levels *vs* its nuclear counterpart[30]. Consistently, nuclear β-catenin is an indicator of an active Wnt signaling, likely operating in cancer initiating cells, and is a useful biomarker associated with CRC disease progression and poor prognosis; more recently, it has predominantly been observed at the invasive front of CRC tissues. According to these data, a recent meta-analysis suggests that increased cytoplasmic expression of β-catenin, not accompanied by nuclear accumulation, has no relationship with the prognosis[31]. Finally, growing evidence indicates that aberrant activation of the

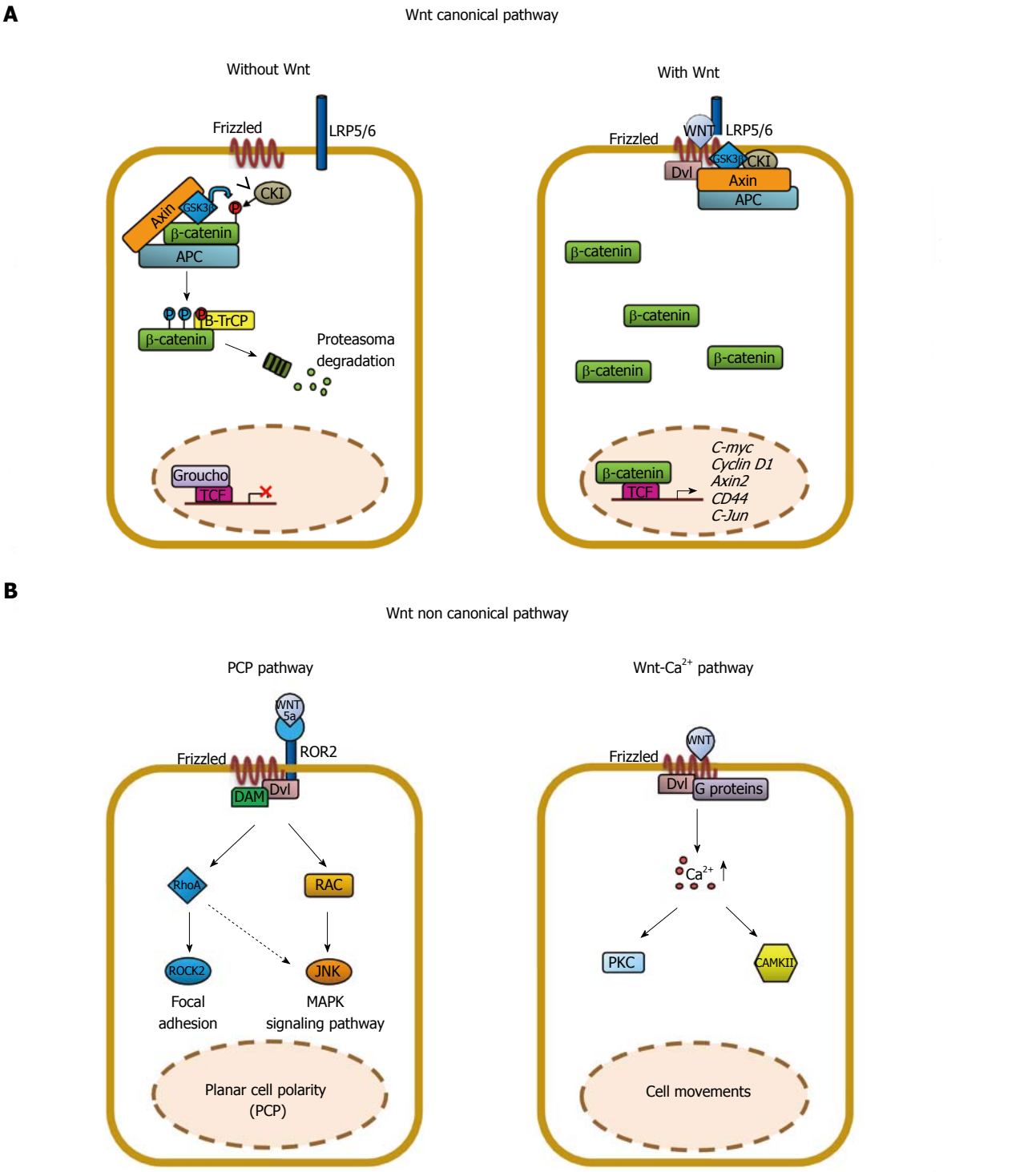

Figure 1 Schematic representation of the Wnt/β-catenin signaling in epithelial cells. The Wnt signaling pathway can be subdivided into a "canonical" or β-catenin-dependent and "non-canonical" or β-catenin-independent. A: In the absence of Wnt ligands, a multi-subunit destruction complex, composed by adenomatous polyposis coli (APC), Axin, GSK3β, CKI, binds and phosphorylates β-catenin tagging for ubiquitination and subsequent proteasomal degradation (βTrCP). The "canonical" Wnt signaling is initiated by the binding of one of 19 Wnt ligands to one of 10 Frizzled receptors (Fzd), in the presence of the co-receptor LRP5 or 6. This leads to recruitment of Disheveled and inhibition of the APC destruction complex. Accumulation of β-catenin in the cytoplasm leads to its translocation to the nucleus where it interacts with TCF/LEF to drive transcription of Wnt target genes including c-myc, cyclin D1, axin2 and others; B: The "non-canonical" Wnt signaling is initiated by the binding of Wnt5a to ROR2, alone or in combination, with a Frizzled receptor leading to the activation of the planar-cell polarity (PCP) pathway through Rock2, RhoA, Rac or JNK. Alternatively, Wnt11 can bind a Frizzled receptor alone and activate the Wnt/calcium pathway that involves the calcium/calmodulin dependent Kinase II (CamKII), protein-kinase-C (PKC) and nuclear factor of activated T cells (NFAT). Importantly, the "non-canonical" Wnt pathway inhibits the "canonical" one either impairing β-catenin accumulation in the cytoplasm or the β-catenin/TCF/LEF complex formation.

Wnt cascade leads to stem cell expansion, proliferation and disturbed tissue architecture (Figure 1A).

The so-called Wnt "non-canonical" signaling is independent of β-catenin function and is less characterized than the "canonical" one. It is initiated by the binding of Wnt5a to receptor tyrosine kinase-like orphan receptor 2 (ROR2), alone or in combination with a Frizzled receptor, leading to the activation of the planar-cell polarity (PCP) pathway through Rock2, RhoA, Rac or JNK. Alternatively, Wnt11 can bind a Frizzled receptor alone and activate the Wnt/calcium pathway that involves the calcium/calmodulin-dependent kinase Ⅱ (CamKⅡ), protein-kinase-C (PKC) and nuclear factor of activated T cells (NFAT) (Figure 1B)[32]. Importantly, the Wnt "non-canonical" pathway inhibits the canonical one either impairing β-catenin accumulation in the cytoplasm or the β-catenin/TCF/LEF complex formation. In this review, for space reasons, we will focus only on the Wnt "canonical", β-catenin-dependent signaling. In epithelial cells, membrane-bound β-catenin interacts with E-cadherin forming cell adhesion complexes that anchor the extracellular matrix to the cytoskeleton[33]. Upon β-catenin nuclear translocation, the interactions with E-cadherin are reduced, impairing cell-cell interactions and providing cells a migration and invasion potential into the neighbouring tissues and, eventually, into the circulation. These events are the basis of the epithelial mesenchymal transition (EMT), a process implicated in tumor progression and metastasis[34]. Both activation of the Wnt/β-catenin signaling and E-cadherin loss are important effectors of EMT in CRC. β-catenin nuclear accumulation at the invasive front of CRCs has been associated with migrating cancer stem cells (MCSCs), metastatic spreading and EMT. Conversely, the serrated pathway has been associated with a lower frequency of nuclear β-catenin localization or reduced membrane-bound β-catenin expression than the traditional one (adenoma-carcinoma sequence), suggesting that the Wnt "non-canonical" pathway may influence metastasis formation especially in right-sided tumors[6,14].

PPARγ SIGNALING PATHWAY IN CRC INITIATION AND PROGRESSION

PPARs are ligand-activated transcription factors belonging to the nuclear receptor superfamily. PPARs activate transcription by recognizing specific sequence motifs, defined PPRE (peroxisome proliferator response elements), located in the regulatory regions of target genes as heterodimers with the retinoid X receptors (RXR)[12,35-37]. In the absence of ligand, PPARs are complexed with corepressor proteins such as the nuclear receptor corepressor (NCoR) or silencing mediator of retinoid and thyroid receptors (SMRT) and act as transcriptional repressors. Ligand binding induces conformational changes that allow displacement of the co-repressor complexes and recruitment of transcriptional coactivators, including members of the steroid receptor coactivator (SRC) family and histone acetyltransferases, such as p300/CBP (Figure 2)[12,35-37]. A variety of endogenous and exogenous lipophilic molecules, such as polyunsaturated fatty acids, prostaglandines, leukotrienes and hypolipidemic drugs, have been identified as PPAR ligands. The structural heterogeneity of these compounds seems to reflect the conformation of the ligand binding domain (LBD), that forms a large Y-shaped hydrophobic pocket with relatively low ligand specificity (Figure 3A). Yet these compounds display selective binding for each of the three PPAR isotypes identified so far PPARα (NR1C1), PPARβ/δ (NR1C2) and PPARγ (NR1C3) that, in addition to their ligand specificity, display distinct tissue expression patterns. PPARγ, in particular, is expressed in adipose tissue, muscle, gastrointestinal tract, blood cells, macrophages and liver. PPARγ modulates cellular and whole-body glucose and lipid homeostasis, increases insulin sensitivity in adipose and muscle tissues following activation by the antidiabetic agents thiazolidinediones (TZDs)[12,35-37]. PPARγ has also been implicated in the modulation of immune and inflammatory processes, vascular homeostasis and cell differentiation both in normal and neoplastic tissues[13,37,38]. In line with this, PPARγ is expressed in a variety of tumors and its role in cancer initiation/progression has been debated for long time[13,37]. *In vitro* studies have shown that PPARγ activation induces growth arrest of epithelial-derived cancer cell lines, including those from thyroid, lung, prostate, breast, pituitary and colon[36]. Consistently, some PPARγ downstream targets, such as p18, p21, and p27 are induced, determining a cell cycle block[39,40]. PPARγ-dependent upregulation of the tumor suppressor gene phosphatase and tensin homolog (PTEN) inhibits PI3-kinase and AKT phosphorylation reducing cell migration and proliferation[41]. The anti-proliferative effects are reinforced by downregulation of the anti-apoptotic protein B-cell CLL/lymphoma 2 (Bcl-2)[42]. More recently, PPARγ has been endowed with anti-angiogenic activity through inhibition of VEGF and its receptors in various cells[43] and with anti-inflammatory activity through inhibition of NFκB-mediated gene transcription[44]. Finally, PPARγ hampers the EMT and thus metastasis formation[45]. All these data strongly support for PPARγ a role as a tumor suppressor; other studies, in contrast, support a role as tumor promoter[46,47]. A more recent work suggests for PPARγ a dual function as a tumor promoting factor in neuroblastoma cells and tumor suppressor in breast cancer cells[48]. Also *in vivo*, contentious results have been reported: administration of PPARγ ligands increases the incidence of colon tumors in Apc+/Min mice[49,50]. In contrast, PPARγ has no effects on tumor incidence in Apc/1638N and 1309 mice, using both genetic and pharmacological models[51,52]. Recently, exposure to pioglitazone, a TZD family member, suppresses colon tumor growth in Apc+/Min mice[52]. Data obtained by a *Pparg* tissue-specific biallelic knockout in ApcMin/+ mice have apparently solved these

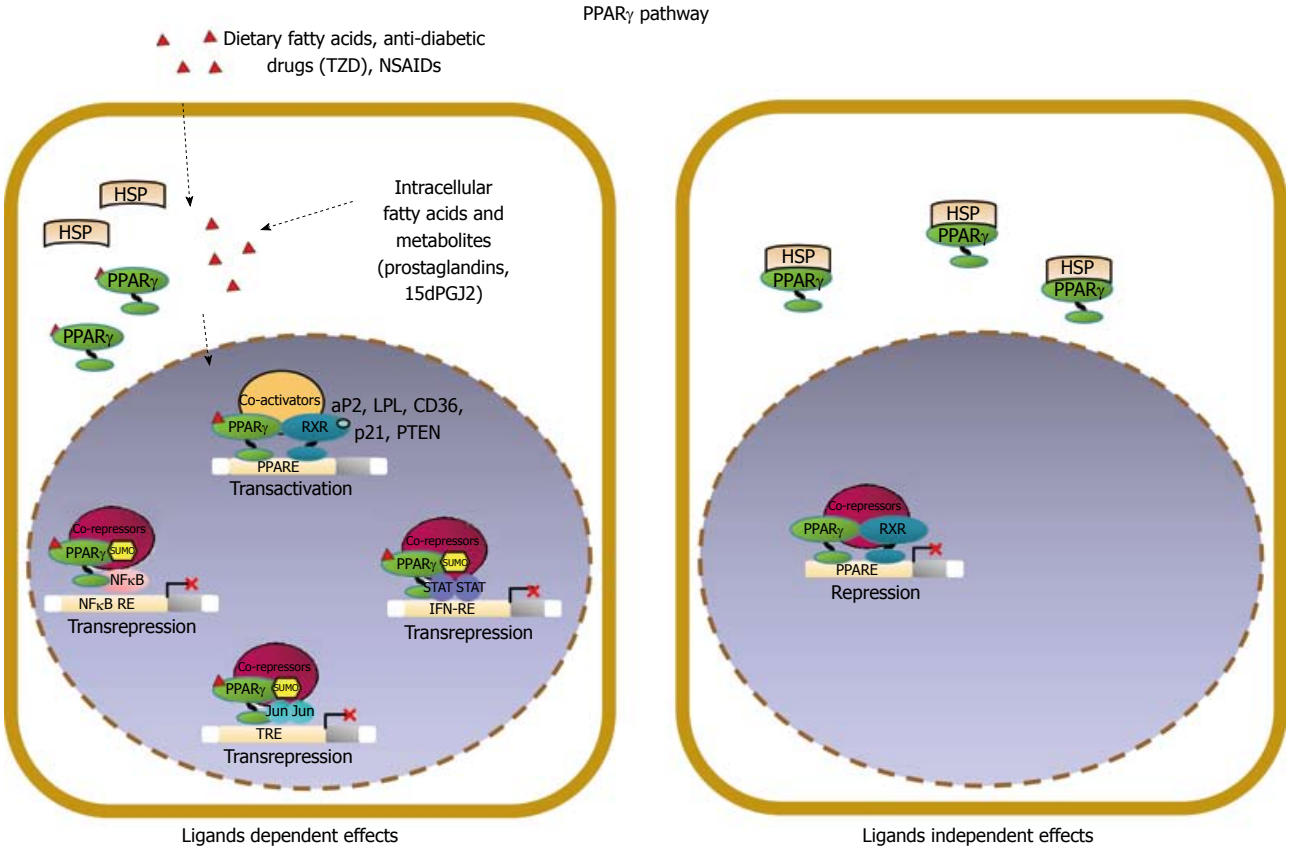

Figure 2 Schematic model of PPARγ signaling in epithelial cells. PPARγ acts as a pro-differentiating transcription factor in colonic epithelial cells where it is abundantly expressed. A variety of endogenous and exogenous compounds, including lipophilic molecules such as polyunsaturated fatty acids and prostaglandines, have been identified as PPARγ ligands; in particular, 15-deoxy-Δ12,14-PGJ2 (15dPGJ2), is considered a natural ligand for PPARγ. Two molecular mechanisms have been proposed to explain PPARγ effects in maintaining cellular differentiation and homeostasis referred to as PPARγ ligand-dependent or PPARγ ligand-independent effects. (1) in the ligand-dependent transactivation, PPARγ binds the cognate PPRE as heterodimer with RXR and activates target gene expression (PTEN, p21, CDH1) through the recruitment of coactivators; (2) an alternative mode of action is known as ligand-dependent trans-repression, in which the SUMOylated form of the receptor interacts with transcription factors such as NFκB, STAT or JUN and represses their target genes transcription. This is attained through the recruitment and stabilization of corepressor complexes at the promoter regions of proinflammatory or protumorigenic genes by a functionally distinct pool of PPARγ that is specifically SUMOylated at susceptible aminoacid residues in the presence of selected agonists.

contraddictory observations. In this mouse strain, an increased tumor incidence and tumor size is observed, consistent with the *in vitro* data: PPARγ ligands inhibit cell growth even in the presence of *APC* mutations[53]. In azoxymethane (AOM)-treated mice, the most widely used preclinical model of sporadic CRC in rodents, PPARγ inhibits colon carcinogenesis and TZDs act as potent suppressors of tumor formation[54]. Of note, some of the effects attributed to TZDs can be due to PPARγ-independent effects[55]. A direct role of PPARγ as tumor suppressor is confirmed by the observation that hemizygous *Pparg* colon-specific knockout mice display a significantly higher incidence of colon tumors following AOM treatment[56]. Epidemiological studies in humans have clearly established a link between chronic inflammatory conditions, such as inflammatory bowel diseases (IBD), and a higher risk of CRC[57]. In colitis-associated cancers (CAC), tumor promotion is mainly due to the presence of a leucocyte infiltration and to inflammatory mediators; moreover, administration of nonsteroidal anti-inflammatory drugs to IBD patients reduces the risk of CRC development[57,58]. In spite of the results obtained in murine models, evidence of a PPARγ involvement in human colon carcinogenesis is still circumstantial. PPARγ is expressed at high levels in about 60% of sporadic human CRCs and specific loss-of-function gene mutations have been reported in 8% of primary CRCs[59]. Increasing evidence suggests that PPARγ activity is attenuated during the transition from adenoma to carcinoma, likely explaining why PPARγ agonists can block the early stages of tumorigenesis, inhibiting the aberrant crypt focus (ACF) formation but with little or no effect on advanced tumor stages[36]. PPARγ phosphorylation operated by the mitogen activated kinases ERK1 and 2 and its ligand-independent SUMOylation negatively regulate its function[44,60]. Both loss-of-function mutations and the reduced activity due to posttranslational modifications, however, do not fully explain the low PPARγ levels found in 35%-40% of sporadic CRCs[61]. Interestingly, they are associated with a more aggressive course, EMT activation, and patients' worse prognosis, suggesting that PPARγ can be considered an independent prognostic factor[16,62]. *PPARG* has recently been shown to be post-transcriptionally modu-

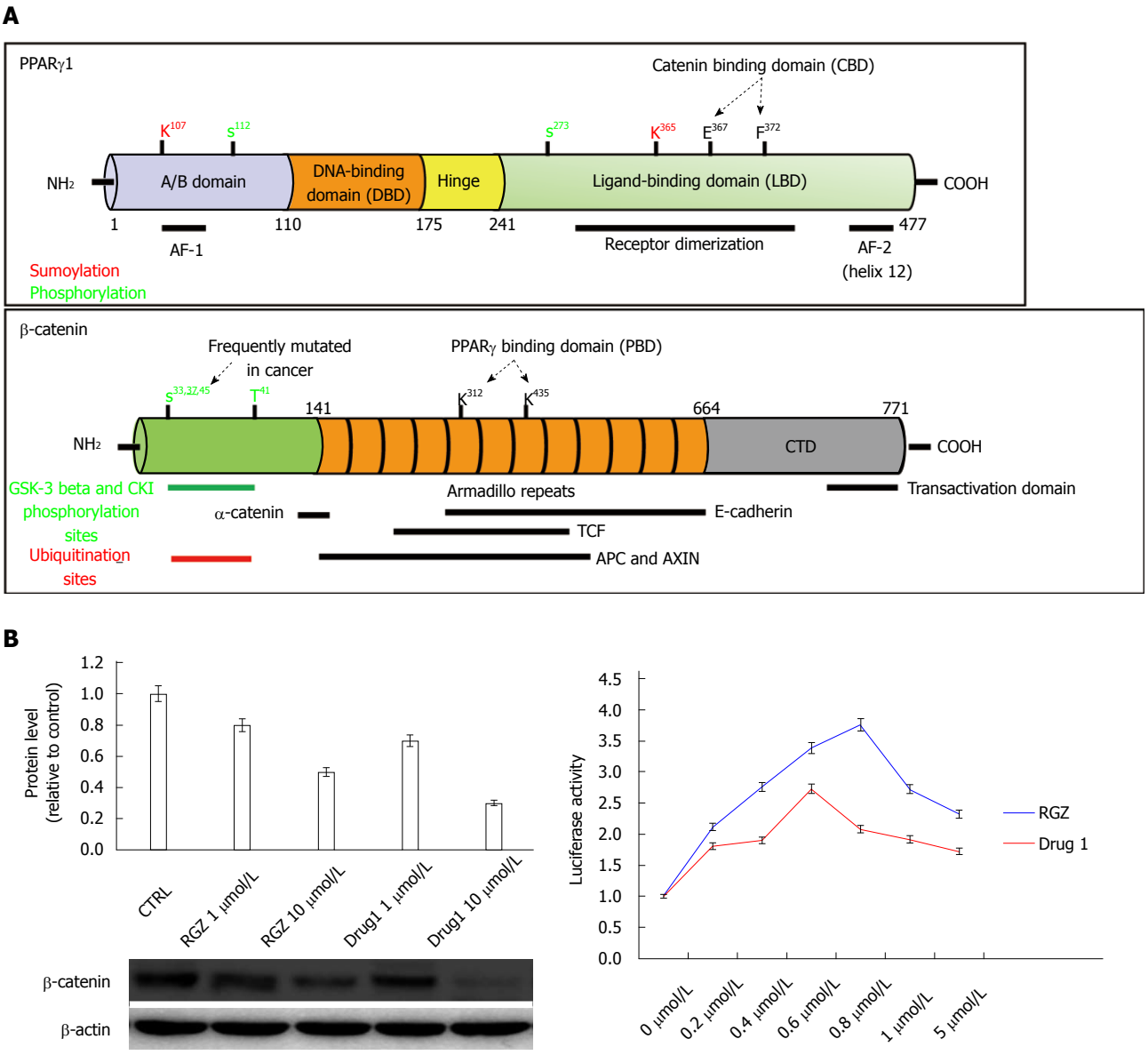

Figure 3 Structural and functional domains of PPARγ and β-catenin. A: The mature PPARγ protein consists of four structural/functional domains: (1) the variable A/B region at the N terminus contains the ligand-independent transactivation domain AF1 (residues 1–71 of PPARγ1); lysine 79 and serine 84 residues are targets of SUMOylation and phosphorylation events, respectively; (2) the C region is the DNA binding domain, characterized by two C4 Zinc-finger motifs, that interact with the major groove of the DNA; (3) the D or hinge region allows receptor dimerization and DNA binding; and (4) the E/F region is the ligand binding domain (LBD) constituted by 12 α-helices and 4 β-strands where the agonist accommodates. This region (helices 7 and 8) includes a β-catenin binding domain (CBD) essential for the interaction with β-catenin. The most important aminoacid residues implicated in PPARγ activity regulation are shown. The full length β-catenin is essentially composed by three domains: (1) the N-terminal domain involved in the ubiquitin-mediated degradation; (2) the arm repeat domain, containing 12 armadillo repeats that mediate the binding with cadherins, APC, TCF/LEF, CREB binding protein (CBP) and PPARγ; and (3) the carboxy terminal (CTD) or transactivating domain interacts with coactivators such as CBP or corepressors such as β-catenin inhibitor and TCF-4 (ICAT). The most important aminoacid residues implicated in β-catenin activity regulation are shown; B: Luciferase activity from HEK293T cells transfected with a PPRE-driven luciferase reporter gene and exposed to the compound indicated as Drug1 is lower than that obtained from cells exposed to rosiglitazone, indicating a reduced transactivation potential in line with the notion of a partial agonist. HT29 colon cancer cells treated with Drug1 exhibit inhibition of cell growth and a 40% higher ability to downregulate β-catenin than rosiglitazone, likely through a mechanism involving β-catenin nuclear export and proteasome-mediated degradation.

lated by miRNAs[48,63].

WNT/β-CATENIN AND PPARγ SIGNALING CROSSTALK IN CRC

Recent reports suggest that, in addition to members of the canonical Wnt signaling pathway, β-catenin can interact with a variety of coactivators and transcription factors, implying an even wider involvement in physiologic and pathologic processes[17,22]. Specific interactions of β-catenin with nuclear receptors signaling pathways seem to be fundamental in gut physiology. Such cross-regulation, in addition, provides a molecular platform to evaluate alterations in cell adhesion and transcription occurring during tumor progression[64]. All components of

the Wnt pathway can affect nuclear receptors functions by events including transcriptional activation or repression and protein phosphorylation. Conversely, nuclear receptors and their ligands confer a dynamic impact upon Wnt functions as shown by the effects on their target genes. In this context, liver receptor homologue 1 (LRH-1) is activated upon association with β-catenin, promotes cyclin D1 and cyclin E transcriptional activation and governs the self-renewal of intestinal crypt cells. Proliferation of epithelial cells is thus enhanced, contributing to CRC development[65]. In contrast, β-catenin activity is repressed by association with retinoic acid receptor, vitamin D receptor and androgen receptor. Retinoic acid and its synthetic derivatives, 1α, 25-dihydroxyvitamin D3, the active form of vitamin D and its synthetic derivatives, have shown chemopreventive effects in animal models of CRC and are employed in cancer therapy[64]. Although the mechanism by which these receptors inhibit the Wnt/β-catenin pathway is not fully understood, several hypotheses have been put forward. In the case of the androgen receptor, the complex with β-catenin represses β-catenin activity and tumor formation in some target tissues[66]. Molecular interactions between components of the Wnt/β-catenin and PPARγ signalings have been reported in several studies, suggesting the potential for cross-regulation at different levels (Figure 3A). PPARγ protein is generally elevated in human CRC specimens and altered in colon tissues from the APC/Min mice; these results are correlated with and attributed to high β-catenin levels and activation[25,56,67,68]. Girnun and collaborators provided the first evidence that PPARγ is capable of inhibiting colon carcinogenesis by suppressing β-catenin in cells that express a functional Wnt/β-catenin pathway[56]. Loss of one *Pparg* allele is sufficient to increase sensitivity to chemical carcinogenesis likely due to the higher β-catenin levels that may prime the colonic epithelium to respond more rapidly to a carcinogenic insult. Addition of PPARγ selective ligands can alter the balance between this nuclear receptor and β-catenin in preadipocytes by activating PPARγ and inducing β-catenin proteasomal degradation in a GSK3β-dependent manner[68]. Oncogenic β-catenin mutants in the phosphoacceptor sites at residues S33, S37, T41 and S45 of the N terminal region of the protein, escape phosphorylation by GSK3β and proteasomal degradation by several hypothesized molecular routes[25] (Figure 3A). The accumulated protein can translocate to the nucleus and suppress PPARγ activity as assessed by lack of transcription of selected target genes[25]. Interestingly, despite the reduced activity, the total amount of PPARγ in these cells is higher likely due to posttranscriptional and not transcriptional events[67]. The selective inhibition of target genes expression may alternatively be ascribed to the interaction of β-catenin with PPARγ-associated transcriptional complexes recruited on the DNA that results in transcription inhibition or squelching of critical PPARγ coactivators[25] (Figure 3A). Notably, β-catenin interaction with the PPARγ transcriptional complexes involves the same sequence motifs of the central region that are required for binding to TCF/LEF without affecting the transcriptional activation of the target genes of this latter complex[25]. TCF factors may form binary complexes with β-catenin or ternary complexes including PPARγ. Indeed, ternary complexes containing β-catenin/PPARγ/TCF4 have already been found in tumor tissues although their biological significance remains elusive[67]. In cells with an intact form of APC or β-catenin, a dominant model can be hypothesized whereby PPARγ suppresses tumorigenesis by activating transcription of its own target genes but also facilitating the GSK3β-dependent degradation of β-catenin. In CRC-derived HT29 cells, that harbor a mutated APC and a wild type β-catenin, selective ligands activate PPARγ stimulating not only target gene expression but also interaction with β-catenin leading to its proteasomal degradation (our unpublished data). Given the fact that the canonical Wnt signaling pathway may be altered at multiple levels, PPARγ could exert its tumor suppressive activity in a context-dependent manner. Data in the literature, in fact, suggest that the cellular response to extracellular effectors and intracellular signalings depends on the relative amounts of β-catenin and PPARγ present in a given cell and on changes of this ratio that can influence their crosstalk (Figure 4). Furthermore, the molecular mechanisms by which PPARγ ligands can elicit transcription of different target genes owing to the differential recruitment of co-activators have not been completely elucidated and the reciprocal effects of PPARγ activation on Wnt signaling pathway are only at the beginning to emerge. We and others have shown that the overall survival of CRC patients is markedly better when PPARγ expression in primary tumours is detectable[14,16,61,62]. Notably, reduced PPARγ expression is not correlated with "activated" β-catenin (*i.e.* nuclear β-catenin) suggesting that these pathways signal differently in cancer and that there may be subtle tissue specific differences in their regulation. Defining how PPARγ influences the Wnt/β-catenin activities will be important to modulate downstream effectors as possible treatment of intestinal diseases.

OLD AND NEW COMPOUNDS TARGETING BOTH β-CATENIN AND PPARγ PATHWAYS

In this section we discuss how natural and synthetic compounds can affect β-catenin and PPARγ activity. The rationale for targeting β-catenin stems from the important functions that this protein serves in cell-adhesion and in the Wnt signaling. Thus, drugs targeting aberrantly activated members of the pathway have the potential as cancer therapeutics. In addition to the known mutations, the Cancer Genome Atlas Network has identified mutations in other genes of the Wnt pathway such as transcription factor 7-like 2 (TCF7L2; previously known

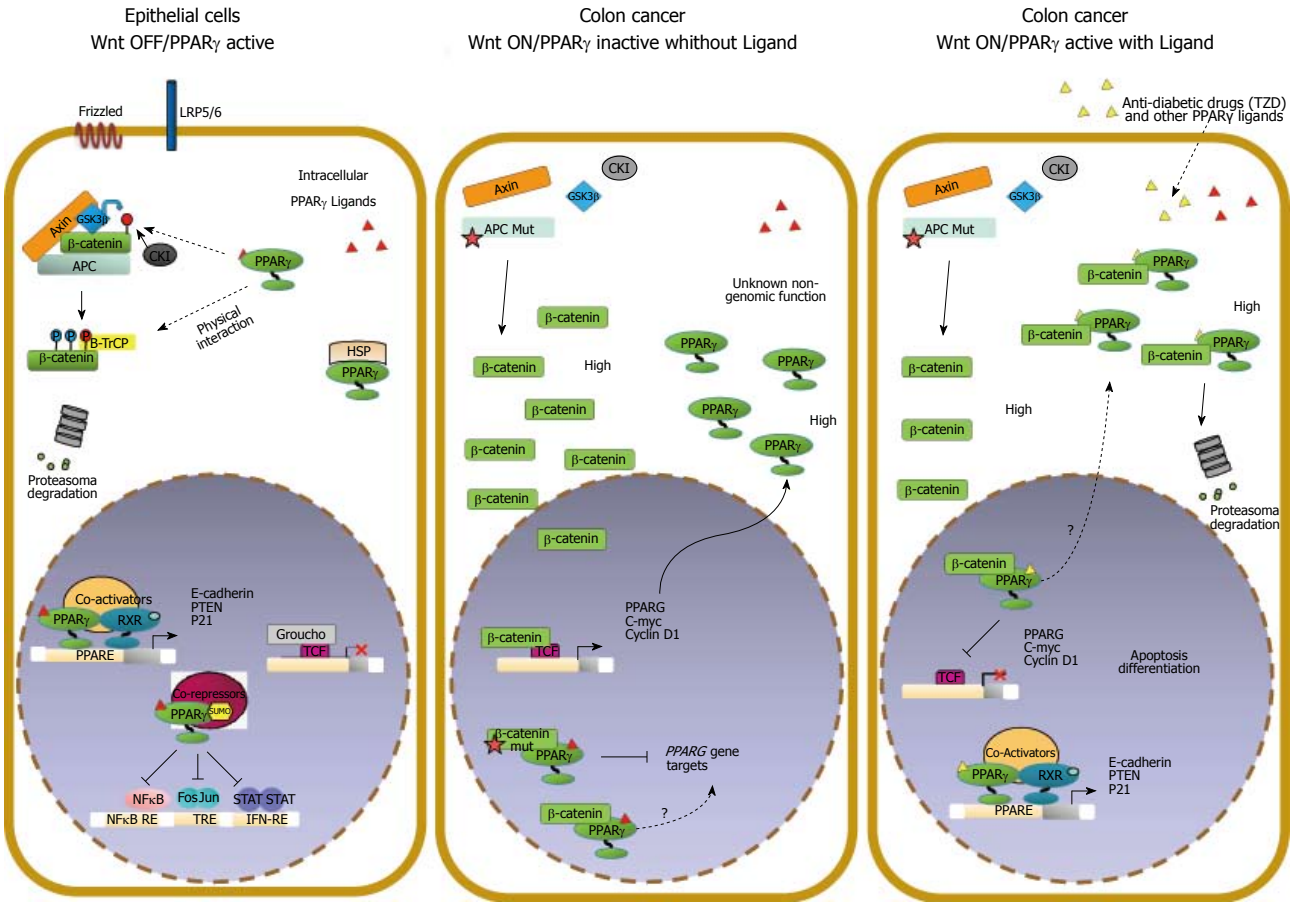

Figure 4 Molecular interactions between Wnt/β-catenin and PPARγ signaling in colorectal cancer cells. The Wnt/β-catenin and PPARγ signal transduction pathways likely act in a coordinated manner to ensure epithelial cells a balance between growth and differentiation. In this condition, β-catenin is targeted by PPARγ for phosphorylation and subsequent degradation. In CRC, the Wnt pathway is generally overactive and β-catenin is stabilized and translocates to the nucleus to activate Wnt target genes. In a "Wnt on" state, PPARγ protein is generally elevated likely due to high β-catenin levels. The selective inhibition of PPARγ target genes expression may be ascribed to different mechanisms: interaction of β-catenin with PPARγ-associated transcriptional complexes recruited on the DNA that results in transcription inhibition or in squelching of critical PPARγ coactivators through the alternative binding with β-catenin. Our data and those already published suggest a hypothetical model whereby a ligand-bound PPARγ suppresses Wnt/β-catenin signaling in a cancer-cell context dependent manner by: (1) activating transcription of its own target genes; (2) facilitating the GSK3β-dependent degradation of β-catenin; and (3) competing in the nucleus with transcription factors such as LEF/TCFs in blocking prosurvival β-catenin target genes also in cells harboring a mutated *APC*.

as TCF4), SOX9, CTNNB1 and Wilms tumour gene on the X chromosome (WTX; also known as FAM123B) known to activate the Wnt/β-catenin signaling[11]. Furthermore, many of the cell surface markers (including LGR5/GPR49, CD44, CD24 and Epcam) used to identify tumor stem cell populations are Wnt direct targets[17]. Similar considerations underlie PPARγ targeting. Given that PPARγ acts as a tumor suppressor in CRC, attempts have been made to identify and/or synthesize new molecules that can enhance this activity. The list of natural or synthetic compounds able to interfere with either Wnt/β-catenin and/or PPARγ signaling is long and constantly growing (Table 1). Herein we will describe the effects of only some of them.

A large number of natural compounds derived from dietary intake, plants and marine organisms, fungi and microrganisms, display chemopreventive and/or chemotherapeutic activity through modulation of the Wnt/β-catenin signaling[69-71]. Direct targeting of this pathway has been difficult, largely owing to the lack of pathway-specific targets and the potential redundancy of many components; moreover, inhibition of β-catenin signaling could cause side effects in normal, adult cells. To overcome these difficulties, it has been suggested to specifically target Wnt factors and Wnt receptors as an attractive cancer therapeutic strategy. Promising results have been obtained with Wnt3A or FZD7-neutralizing antibodies *in vivo*.

Among the small-molecule inhibitors, the non-steroidal anti-inflammatory drugs (NSAIDs) (indomethacin, sulindac, aspirin) and the selective COX-2 inhibitor (celecoxib) prevent β-catenin-dependent transcription in colorectal cells. Other existing drugs include molecular targeted agents such as the CBP/β-catenin antagonist ICG-001[71]. The common mechanism by which NSAIDs and their derivatives act is through inhibition of β-catenin/TCF pathway transcriptional activity and, consequently, down-regulation of target genes such as cyclin D1.

Indomethacin is a COX-1 and COX-2 inhibitor and

Table 1 Natural and synthetic compounds targeting Wnt/β-catenin and PPARγ pathways

	Subcategory	Drug	Pathways	Effects	Target tissues	Ref.
Natural ligands	Polyphenols	Quercetin EGCG Curcumin Resveratrol DIF	β-catenin/TCF WNT GSK-3β	β-catenin, TCF, cMyc, cyclin D1, survivin, conductin reduction	CRC, Adipose Tissue, Kidney	[81,91-101]
	Phytochemicals	Capsaicin Cladosporol Thymoquinone	β-catenin GSK-3β	β-catenin, cMyc reduction; GSK-3β, PPARγ activation	CRC	[89,102-105]
	Vitamins	Retinoids 1α25,-dihydroxy Vitamin D3	β-catenin Dab2	β-catenin reduction	CRC	[79,106-108]
Synthetic ligands	NSAIDs	Aspirin Sulindac Celecoxib Indometacin Diclofenac NS398	β-catenin, TCF, PPARγ	β-catenin, TCF cMyc reduction; PPARγ activation	CRC	[72-74,76,77,109-116]
	Small Molecules	PNU 74654 2,4-diamino-quinazoline ICG-001 FH535 Others	β-catenin/TCF CBP Dv1 Axin Tankyrase 1,2	Block Wnt Wnt/β-catenin suppression	CRC	[71-80]
	PPARγ ligands	TZDs Lutein	β-catenin/TCF	β-catenin degradation; PPARγ activation	CRC	[82-86,89]
	Partial PPARγ Agonists	Drug1	β-catenin/TCF	β-catenin degradation; PPARγ activation	CRC	[86-90]

NSAIDs: Non-steroidal anti-inflammatory drugs; CRC: Colorectal cancer.

exhibits anti-inflammatory and analgesic properties. In addition to the more general inhibition of the β-catenin/TCF pathway mentioned above, indomethacin impairs β-catenin gene expression itself at early times, as shown by the significant reduction of the corresponding mRNA. Furthermore, indomethacin stimulates β-catenin degradation in an APC/GSK3β and proteasome-independent manner (Wnt "non-canonical" pathway) even in cells bearing a mutated APC or β-catenin. These results support the potential chemotherapeutic activity of the molecule[71-73].

Sulindac inhibits β-catenin/TCF pathway and reduces β-catenin levels in human colon cancer cells. The antiproliferative effects of sulindac and its derivatives are confirmed in different mouse models of multiple intestinal adenomas and also in human colorectal adenomas. Like indomethacin, also sulindac causes β-catenin degradation mainly through an APC/GSK3β-independent mechanism, while the canonical pathway and, in turn, the proteasomal degradation are activated at late times, especially after induction of apoptosis[71,74]. Finally, a third alternative degradation mechanism in CRC cells is mediated by an increase of cGMP levels due to the cGMP phosphodiesterase (PDE) inhibition. High cGMP levels activate the cGMP-dependent kinase (PKG) that, in turn, stimulates β-catenin phosphorylation reducing its protein levels. It has been proposed that phosphorylation by PKG is an alternative way to induce proteasomal-mediated β-catenin degradation in cells with an inactive APC/GSK3β-destruction complex[75].

Aspirin also down-regulates the Wnt/β-catenin pathway in CRC cells leading to reduced transcription of target genes. Unlike other NSAIDs, this effect seems to be mediated by stabilization of β-catenin in its transcriptionally inactive form (*i.e.*, phosphorylated form), hampering its activity as transcription factor[76].

All NSAIDs, in addition to their effects on β-catenin and related pathway, act as PPARγ ligands[77]. As such, they stimulate PPARγ-dependent effects as cell cycle block, differentiation and apoptosis, adding to those reported on β-catenin and providing the basis of a double benefit in cancer therapy.

The active form of vitamin D, 1α, 25-dihydroxyvitamin D3, plays a relevant role in chemoprevention in animal models of colon cancer by targeting the Wnt/β-catenin pathway. Although the molecular mechanisms have not been elucidated yet, the vitamin D receptor has been proposed to specifically interact with β-catenin competing for TCFs binding. The resulting complex is no longer able to activate transcription of β-catenin target genes. Indeed ternary complexes have been reported and their putative functions suggested; in the case of the vitamin D receptor, the active complex is no longer formed, the novel is not functional thus explaining the negative results[78,79]. Also small molecules, such as PKF115-584, CGP049090, PKF222-815, derived from fungi, and PKF118-744, PKF222-310 from actinomycete strains, inhibit colon cancer cell proliferation by blocking β-catenin/TCF4 interaction and, subsequently, repressing their target genes[80].

Resveratrol, a polyphenol belonging to the stilbene phytochemical family, is found in dark grapes, red wine, peanuts and shown to block colon cancer cell proliferation through inhibition of the Wnt/β-catenin pathway. Specifically, low (subapoptotic) concentrations of resveratrol reduce the expression of Bcl9, Pygo I and II and interfere with β-catenin nuclear localization[81]. In addition, resveratrol displays a PPARγ agonist effect inducing cell growth arrest and apoptosis doubling its beneficial antitumor activity. The molecular mechanisms underlying these activities are currently under investigation.

Different classes of new molecules have been isolated and/or synthesized as putative PPARγ ligands. When administered to cells in culture they are assessed for PPARγ-dependent effects and compared with full agonists. Among all molecules tested, only TZDs can interfere with the Wnt/β-catenin pathway, indicating that they function not only as PPARγ transcriptional activators[82]. Therefore, it would be useful to examine whether other partial agonists could display such a

repressive effects on β-catenin signaling in order to establish a structure/function relationship. As mentioned, PPARγ displays a Y shaped ligand binding domain in which molecules with heterogeneous structures can accommodate. The aminoacid residues of the receptor involved in the interactions with full agonists have been identified as well as the conformational changes implicated[83-85]. The new partial agonists could accommodate in a distinct binding pocket, induce different conformational changes of the LBD that, in turn, result in the activation of only a subset of PPARγ target genes. Such an alternative mode of action has been reported for luteolin and appears to be carried out by an additional ligand whereby only genes involved in lipid and glucose metabolism are transcribed, through a differential recruitment of coactivators[86].

On the basis of these considerations, attempts are currently made to synthesize or isolate novel molecules able to act as PPARγ partial agonists[87,88]. These should only partially transactivate the receptor, promote transcription of genes involved in cell growth arrest, differentiation and/or apoptosis and, additionally, exhibit inhibition of β-catenin at different levels. In line with this, we have recently shown that cladosporol A, a secondary metabolite from the fungus *Cladosporium tenuissimum*, displays antiproliferative properties in human colorectal cancer cells through up-regulation of p21waf1/cip1 and down-regulation of cyclin D1, cyclin E, CDK2 and CDK4[89]. The effects observed are mediated through activation of PPARγ as a partial agonist. Interestingly, cladosporol A causes β-catenin nuclear export and its proteasomal-mediated degradation. Consistently, also cyclin D1 and c-Myc are reduced, indicating that the β-catenin/TCF pathway is inhibited, further strenghtening the antiproliferative properties of this drug (Zurlo *et al*[89] submitted for publication). We have also tested a new compound belonging to the class of chiral phenoxyacetic acids for its ability to act as a PPARγ ligand and activate downstream genes[90]. As shown in Figure 3B, Drug 1 acts as a partial agonist displaying a transactivation potential that is only 60% of that obtained with rosiglitazone. Interestingly, when tested for the ability to downregulate β-catenin, Drug 1 shows a 40% higher ability than rosiglitazone, likely through the proteasomal destruction machinery. These results suggest that it is possible to design novel PPARγ partial agonists with the ability to recruit selected coactivators and stimulate transcription of a subset of genes. The possibility to enhance β-catenin degradation and counteract an active and oncogenic Wnt signaling is an added value that should further stimulate the search for such compounds as novel drugs in cancer therapy.

CONCLUSION

CRC remains the fourth most common cause of cancer related death in western countries despite the discovery of a number of key genetic and epigenetic alterations involved in its initiation/development and the progress in the treatment. Therefore, an interdisciplinary approach that combines new techniques aimed at the identification of novel biomarkers with the treatment of advanced CRCs will aid in guiding future therapeutic interventions. Recent genome-wide studies and the integrative analysis of genomic data support the notion that clinically distinct CRC subtypes exist and provide insights into the pathways that are dysregulated in this malignancy. The Wnt/β-catenin signaling is one of the most frequently modified pathways in CRC, suggestig that drugs targeting aberrantly activated members have the potential as cancer therapeutics. PPARγ is emerging as a growth-limiting, differentiation-promoting signal with known potential link with Wnt/β-catenin signaling. Accumulating evidence suggests that PPARγ overexpression has prominent suppressive activities in CRC growth, acting as an independent biomarker of good prognosis. Naturally occurring and novel synthetic agonists capable of differently modulating PPARγ signaling and interfering with related pathways show great promise in animal models and in preclinical studies. Specifically, novel PPARγ agonists, endowed with Wnt/β-catenin pathway inhibitory activities, are currently designed and investigated to provide new molecular target therapies in CRC.

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TOPIC HIGHLIGHT

WJG 20th Anniversary Special Issues (9): Hepatitis B virus

Molecular identification of hepatitis B virus genotypes/subgenotypes: Revised classification hurdles and updated resolutions

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Abstract

The clinical course of infections with the hepatitis B virus (HBV) substantially varies between individuals, as a consequence of a complex interplay between viral, host, environmental and other factors. Due to the high genetic variability of HBV, the virus can be categorized into different HBV genotypes and subgenotypes, which considerably differ with respect to geographical distribution, transmission routes, disease progression, responses to antiviral therapy or vaccination, and clinical outcome measures such as cirrhosis or hepatocellular carcinoma. However, HBV (sub)genotyping has caused some controversies in the past due to misclassifications and incorrect interpretations of different genotyping methods. Thus, an accurate, holistic and dynamic classification system is essential. In this review article, we aimed at highlighting potential pitfalls in genetic and phylogenetic analyses of HBV and suggest novel terms for HBV classification. Analyzing full-length genome sequences when classifying genotypes and subgenotypes is the foremost prerequisite of this classification system. Careful attention must be paid to all aspects of phylogenetic analysis, such as bootstrapping values and meeting the necessary thresholds for (sub)genotyping. Quasi-subgenotype refers to subgenotypes that were incorrectly suggested to be novel. As many of these strains were misclassified due to genetic differences resulting from recombination, we propose the term "recombino-subgenotype". Moreover, immigration is an important confounding facet of global HBV distribution and substantially changes the geographic pattern of HBV (sub)genotypes. We therefore suggest the term "immigro-subgenotype" to distinguish exotic (sub)genotypes from native ones. We are strongly convinced that applying these two proposed terms in HBV classification will help harmonize this rapidly progressing field and allow for improved prophylaxis, diagnosis and treatment.

Key words: Hepatitis B virus; Hepatitis; Classification; Genotype; Subgenotype; Phylogenetic tree

Core tip: Hepatitis B virus (HBV) eradication could be achieved through three important points: (1) efficient universal vaccination; (2) accurate diagnostic assays; and (3) effective treatment of HBV carriers. Undoubtedly, these critical measures are not possible without fully understanding the genetics of the virus and be-

ing able to differentiate the isolates. In this review article we provide an update of HBV virology, focusing on classification and its impact on diagnosis, clinical outcomes, therapy, prophylaxis, evolution and epidemiology. Subsequently, the role of correct classification in describing HBV is highlighted, and misclassifications together with their causes are recounted. Finally, through the proposal of novel terms, HBV strains are reclassified.

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POSSIBLE ERADICATION OF HEPATITIS B VIRUS; MULTI-FACTORIAL COMPLICATIONS

Undoubtedly, the World Health Organization's (WHO) announcement in 1980 that smallpox virus was eradicated through vaccination represented one of the extraordinary human breakthroughs in the battle against infectious diseases[1,2]. Unfortunately, it seems that vaccination alone is not enough for hepatitis B virus (HBV) eradication.

HBV was discovered by Blumberg *et al*[3] (1925-2011) in 1965. Five years later, the first HBV vaccine and diagnostic blood test were developed[4]. The HBV vaccine is considered to be the first widely used vaccine against cancer and a chronic disease. In contrast to other vaccines, the clinical trial of this vaccine was short, and the vaccine was quickly approved by the United States Food and Drug Administration (FDA)[5]. Multiple studies have confirmed that the incidence of acute hepatitis, chronic liver disease as well as hepatocellular carcinoma (HCC) is decreased in the HBV-vaccinated population. Owing to the administration of more than one billion doses of HBV vaccines since 1982[6], the worldwide mortality rate of HBV has diminished significantly[7].

The WHO and other alliance organizations established the annual World Hepatitis Day in 2008. July 28th was selected for this particular day in honor of Prof. Blumberg's birth date. The experts and healthcare organizations put their efforts into raising global awareness of viral hepatitis, especially about HBV and hepatitis C virus (HCV). The experts educate people all around the world about prevention, transmission, diagnosis and treatment against viral hepatitis infection. Undoubtedly, like smallpox eradication and its global preventative program, the World Hepatitis Day also moves up the knowledge of the global strengthening of preventive and control measures against viral hepatitis. Consequently, it is anticipated that increasing HBV vaccination coverage worldwide will definitely have positive impact on HBV eradication in the near future.

Despite advances that have resulted in several generations of HBV vaccines, a series of viral screening assays and effective treatment options, HBV is still considered a dangerous, life-threatening illness and a serious public health problem. The WHO estimates that at least two billion people (one fourth to one third of the world's population) had been infected with the virus; 400 million people are infected chronically[8]. HBV-related diseases are currently ranked ninth on the global list for causes of mortality, and HBV is the fifth most important infectious agent, resulting in about one million deaths annually[9]. Due to the significant public health risk that HBV poses, it is important to compile comprehensive knowledge of both viral and host properties to enable elimination of HBV infection in the near future.

Host-related complications

The existence of a large reservoir of chronically infected HBV carrier patients hampers eradication of the virus[10]. The prevalence of chronic infection varies from region to region, such that different geographical parts of the world exhibit different sero-epidemiological patterns of HBV infection. The highest seroprevalence (8%) is found in Asia and the South Pacific region, which is considered a highly endemic region. In Sub-Saharan Africa, Alaska, the Mediterranean region and India, HBV seroprevalence is between 2% and 7%, which is considered an intermediate endemic range. In European countries and some parts of Central and South America, HBV seroprevalence is less than 2%, which is considered low[11].

Patients chronically infected with HBV have a greater than 100-fold chance of developing HCC compared to uninfected people[12]. From a global perspective, HBV is the leading cause of HCC and causes one million deaths annually[13]. Although the eradication of hepatitis B by means of universal vaccination seems technically achievable, this task is made difficult by the fact that hundreds of millions individuals are already chronically infected with HBV. The elimination of hepatitis B will only be successful when this group of chronically infected patients is cured naturally or through antiviral treatments. Only accurate and continuously improved diagnostic policies will identify the pool of carriers for appropriate treatment. To further complicate diagnosis, different types of chronicity like normal obvious infection (overt) or masked infection (occult) increase the complexity of the diagnostic algorithms[14,15]. Vaccinating patients in high-risk groups, which come in contact with infected people, would decrease the infectivity and risk of transmission to the healthy population and should therefore be considered a priority[4,6].

Virus related complexity

HBV is the prototypic species of a family of DNA viruses called *Hepadnaviridae*. The HBV genome is approximately 3200 bp long, circular, and consists of four genes

and seven open reading frames (ORFs). HBV itself evolves inter- and intra-genetically in reservoirs. Since the reverse transcriptase enzyme lacks proofreading activity, the nucleotide substitution rate for HBV is higher than that of other DNA viruses[16]. During persistent, long-term HBV infection and under different selective pressures, variants of HBV can emerge. Some variants are able to evade diagnostic, prophylactic and therapeutic measures. This extraordinary genomic diversity, together with a high replication capacity, allows HBV to adapt to different hosts[17]. Based on evolutionary analysis, HBV has eight confirmed genotypes (named A to H) and two tentative genotypes (called I and J), and almost forty subgenotypes (Figure 1A)[18,19]. Phylogenetic and evolutionary analyses of complete genome sequences have classified HBV into these eight distinct genotypes; each has an intergroup nucleotide divergence greater than 7.5%[20]. "Subgenotypes" are subgroups within the same genotype that meet two particular criteria. First, they have a nucleotide divergence between 4% and 7.5% over the full-length genome[21], and secondly, there is high phylogenetic bootstrap support. "Clades" further divide subgenotypes, and have less than 4% nucleotide diversity based on the complete genome sequences[20]. Prior to molecular analyses (genotype, subgenotype and clades), classification of HBV strains was based on the immunological heterogeneity of HBsAg, which led to the categorization into different HBV serotypes (subtypes). The development of DNA sequencing revealed that amino acid changes in the major hydrophilic region (MHR) region of the HBsAg are responsible for this classification. This serotype-based classification is still used, and epidemiological studies describe associations between serologic subtypes and genotypes[20,22].

HBV CLASSIFICATION METHODS

Phylogenetic analysis of the nucleotide sequences of the whole HBV genome represents the most conclusive method for HBV genotyping[23]. This method is considered the "gold standard" approach for genotyping and subgenotyping, though it is relatively expensive and time-consuming. Fortunately, sequencing is becoming cheaper and faster, so it may serve as a common molecular method in the very near future even for clinical routine purposes. Phylogenetic analysis can also be performed on individual genes instead of the complete genome, in particular on the HBV envelope (S) gene[24]. The results obtained from the partial sequence (HBV S gene) may be useful for determining the HBV genotype, but it will not be appropriate for determining the HBV subgenotype.

Online methods to genotype HBV

Bioinformatics is currently used in many fields of science, and numerous bioinformatics software tools are available online. Genotyping of microorganisms has also become readily available, particularly for viruses, since they carry small genomes compared to eukaryotic organisms. One of the most popular online systems is a sequence similarity algorithm, such as BLAST, which is available through the National Center for Biotechnology Information (NCBI). BLAST analysis can be used to identify the sequences that are most similar to the sample by comparing the sample sequence to sequences archived in GenBank. NCBI has generated additional online tools specifically to determine the sequence of certain genotypes and subgenotypes of viruses. Thus, HBV and several other viruses can currently be genotyped using the NCBI web-based HBV genotyping tool[25]. To further facilitate HBV genotype determination, several experts in the field have introduced different online HBV genotyping tools, such as the hepatitis B virus database (HBVdb)[26], HBV STAR[27], BioAfrica-Oxford HBV Automated Subtyping Tool[28], HepSEQ Genotyper[29,30] and the jumping profile Hidden Markov Model (jpHMM)[31].

French experts developed the HBVdb online genotyping system, allowing researchers not only to genotype HBV but also to create virus recombination and drug resistance profiles. A group from the United Kingdom designed the HBV STAR online tool to genotype HBV based on a statistically defined, position-specific scoring model. This tool is able to predict recombinant and non-human primate isolates. The HBV STAR tool also supports human immunodeficiency virus-1 (HIV-1) subtyping. Another group established a rapid high-throughput-genotyping system called BioAfrica-Oxford Automated Subtyping Tool, which is able to genotype and subtype several viruses, including HBV. The tool employs a phylogenetic approach to genotype viruses and uses bootscanning methods to detect recombination[30]. The HepSEQ Genotyper online tool is an international public health repository for hepatitis B developed by British scientists, which provides molecular, clinical and epidemiological information regarding HBV. HepSEQ Genotyper is capable of determining the HBV genotype and classifying clinically relevant mutations within the HBV genome such as vaccine escape, precore or antiviral-resistant mutations. This tool uses only HBV polymerase/surface genes for genotype computation and is therefore less accurate for detecting recombination. The jpHMM online tool was developed to subtype/genotype two viruses: HIV-1 and HBV. This tool uses a probabilistic approach to compare a sequence to a multiple alignment of a sequence family and can also be extended to detect recombination.

Molecular-based methods to genotype HBV

Several molecular approaches have been developed to characterize HBV genotypes, including polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP)[32,33], genotype-specific primers in a single or a multiplex-PCR set[34], oligonucleotide microarray chip (DNA Chip), restriction fragment mass polymorphism (RFMP), mass spectrometry (MS), PCR-invader assay, re-

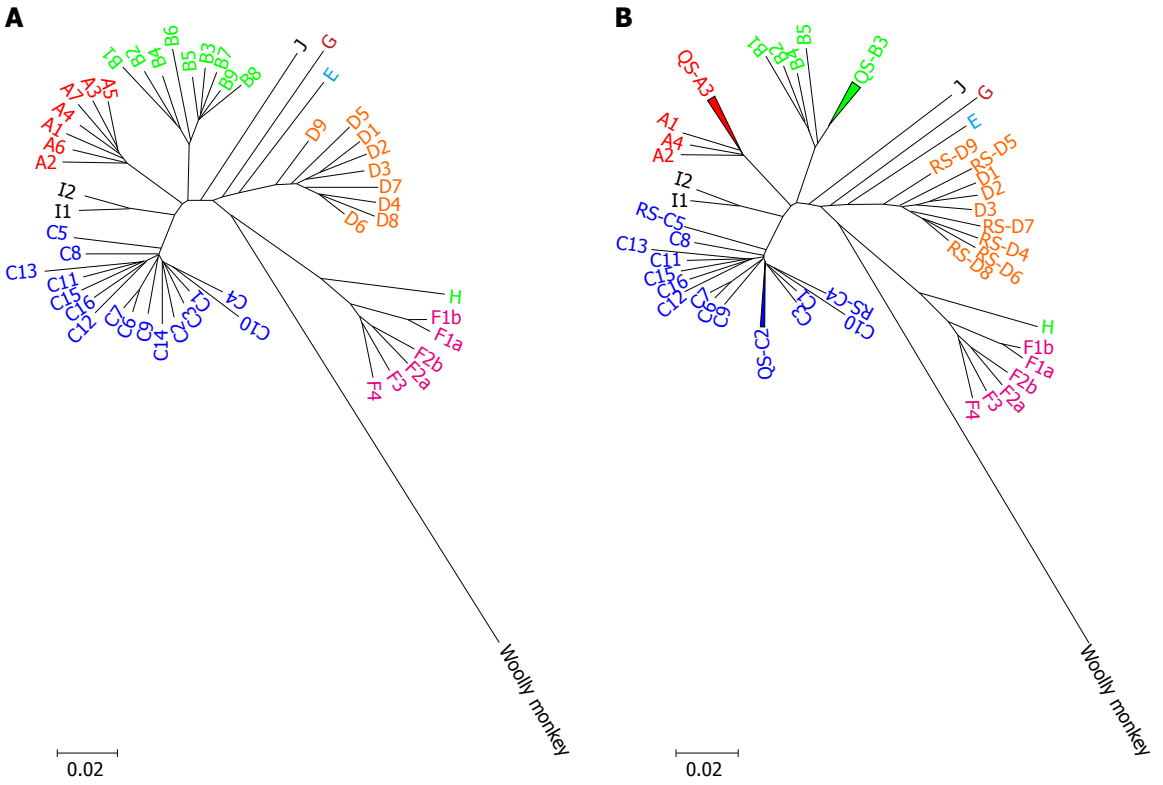

Figure 1 Neighbour-joining phylogenetic tree was conducted based on hepatitis B virus full-length genomes of all proposed genotypes and subgenotypes before (A) and after (B) reclassification. HBV genome sequence that used are listed below: JN182318: A1; HE576989: A2; AB194951: A3; AY934764: A4; FJ692613: A5; GQ331047: A6; FN545833: A7; AB642091: B1; FJ899779: B2; GQ924617: B3; GQ924626: B4; GQ924640: B5; JN792893: B6; GQ358137: B7; GQ358147: B8; GQ358149: B9; AB697490: C1; GQ358158: C2; DQ089801: C3; HM011493: C4; EU410080: C5; EU670263: C6; GU721029: C7; AP011106: C8; AP011108: C9; AB540583: C10; AB554019: C11; AB554025: C12; AB644280: C13; AB644284: C14; AB644286: C15; AB644287: C16; GU456636: D1; GQ477452: D2; EU594434: D3; GQ922003: D4; GQ205377: D5; KF170740: D6; FJ904442: D7; FN594770: D8; JN664942: D9; FN594748: E; FJ709464: F1b; DQ899146: F2b; AY090459: F1a; DQ899142: F2a; AB036920: F3; AF223965: F4; GU563556: G; AB516393: H; FJ023659: I1; FJ023664: I2 ; AB486012: J and AY226578: Woolly monkey as an out-group. HBV: Hepatitis B virus.

al-time PCR, hybridization strips as INNO-LiPA, reverse dot blot assay and sequencing[35].

Among them, PCR-RFLP is widely used to genotype HBV since it is simple and inexpensive. Nevertheless, there are many reports that indicate this technique is not always accurate and may often result in an 'untypable' genotype[36]. Genotype-specific primers in single and multiplex PCR sets are also utilized to distinguish different genotypes. This method is rapid and inexpensive and widely used for large population studies; however, any mutation in the genome may alter the result of the assay due to primer-DNA hybridization mismatching[37]. Recently, the DNA Chip method[38], invader assays[39], real-time PCR[40-42], hybridization and dot blot methods[43] have been employed for HBV genotype detection[38]. These techniques are highly sensitive; however, fidelity can be affected by any mutation within the HBV genome. Mass spectrometry[44] and RFMP[45] can also be used for genotyping of HBV. These methods can detect drug resistant variants, but fidelity is also affected by mutations, and the methods themselves are costly and require specialized equipment. Despite the emergence of many methods to genotype HBV, sequencing is still considered the "gold standard" method for genotyping, followed by phylogenetic and evolutionary analysis. This method is the most reliable, since it is able to determine the whole genome sequence of HBV.

Methods to determine HBV subtypes

Three decades ago, HBV subtyping was introduced as the first classification system for hepatitis B, in which categories were assigned based on the amino acid sequences at the surface antigen region[22,46]. This sero-molecular taxonomy based on the specific motifs of HBsAg led to 10 different sero-subtypes of HBV. Different HBV subtypes can be distinguished based on enzyme-linked immunosorbent assay (ELISA) or enzyme immunoassays (EIA) using specific monoclonal antibodies against pre-S2 and S regions[47]. Since sequencing of the HBV HBsAg region reveals subtype-specific motifs, serological subtyping (serotyping) of HBV can also be predicted by HBsAg amino acid sequence mapping[22].

The relationship between particular subgenotypes and subtypes has already been demonstrated[20,21]. Also, different impacts of HBV subtypes on results of serological assays for HBsAg have been demonstrated[48]. Little is known about the driving forces behind the divergence of HBV genotypes, subgenotypes and subtypes. We believe that they are the result of long-term adaptation to the host's genetic background of certain human

populations where they circulated. Subtypes distributed consistently with the pre-historic human migration[49]. Genotype, subgenotype and subtype-specific variability is stably transmitted within the host population and is considered to stay constant from the beginning of the infection in an individual[50].

Methods to determine HBV subgenotype

Although all aforementioned methods are able to detect HBV genotype, no molecular method has been introduced to determine HBV subgenotypes accurately. There are several studies that used PCR-based approaches to distinguish subgenotypes of HBV, however, they were designed to differentiate only limited subgenotypes. For instance, investigators introduced multiplex-PCR or semi-nested PCR assays to differentiate between HBV subgenotypes B1, B2, C1 and C2[51] or Ba and Bj[52]. Therefore, sequencing is still the only method that is able to determine subgenotypes of HBV accurately following careful phylogeny analysis. Based on our own experience in the field of HBV epidemiology, we would like to explain in more detail how the HBV subgenotype can be accurately determined.

Introducing "HBV subgenotyping guidelines"

To determine HBV subgenotype, several rules must be strictly applied: (1) Analyzing the full-length genome is the foremost prerequisite for determining the accurate subgenotype. This is necessary for the analysis of entire genes and ORFs at the nucleotide level. It also allows for accurate subgenotyping, even if mutations or recombinations occur in the particular strain of interest. In the past, using only the partial genome has led to several misclassifications of HBV subgenotypes. The preeminent example is the introduction of the subgenotypes A4 and A5 through partial genome analysis by Olinger *et al*[53] in 2006. Further analysis revealed that these strains should not be considered independent subgenotypes[54,55]. The partial genome is inadequate for HBV subgenotyping, because of the particular genomic structure in which four genes and seven proteins are arrayed in a concise genome. Furthermore, analysing partial genes instead of the full-length genome can sometimes lead to false epidemiological signals from distant geographical regions. For instance, Khedive *et al*[56] analysed 681 bp of isolated strains from HBV carriers in Iran and reported five strains with HBV subgenotypes D5 and D8, which is inconsistent with the known geographical distribution of subgenotypes. According to all epidemiological studies from Iran, D1 is the most frequent subgenotype of HBV, followed by a minor population of D2. Moreover, recent analysis showed that Iran is the most probable location of the common ancestor of subgenotype D1-D3[57]. Also, there is no accurate evidence of other subgenotypes of genotype D in Iran or its neighbouring countries. Thus, the reported identification of D5 and D8 is not geographically congruent with previous studies from Iran. Also, in other studies, like searching for the common source of HBV infection, full-length genome analysis provides strong evidence[10,58,59]; (2) Adherence to the ranges of intra-genotypic nucleotide divergence (more than 4.5% and less than 7.5%) that define distinct subgenotypes is the second most important factor for correctly identifying subgenotypes of HBV. Disregarding this rule in several cases, like with A5 and A7, has resulted in misclassification of strains[60,61]; (3) Bootstrap values greater than 75% are necessary to support the monophyletic tree to introduce a cluster as an independent subgenotype. There are several subgenotypes that have been introduced without considering this critical rule. Shi *et al*[62] misclassified the B3 subgenotype and Huy *et al*[63] misclassified C2 of HBV due to ignoring this criteria; (4) Recombinant strains should be excluded from any subgenotyping analysis as much as possible because they can disrupt the topology of a phylogenetic tree and falsely increase nucleotide divergence. For example, subgenotypes D8, D9, CD1 and CD2 have been misclassified[18,64-67]. The impact of recombination on HBV strain analysis will be discussed in more detail below; (5) To introduce novel subgenotypes, strains harbouring specific nucleotide and amino acid motifs should be identified. Some investigators have demonstrated this particular criteriaon for subgenotypes of A1, A3 and A6[55,68]; and (6) To avoid sampling bias, a minimum of three purported novel strains, together with all available subgenotype strains belonging to the same genotype, should be subjected to evolutionary and phylogeny analysis. Using random reference sequences, as opposed to selecting some particular reference sequence, is highly recommended for subgenotyping phylogeny analysis.

Since recombination is an inevitable part of HBV' s evolution, most subgenotypes that belong to a HBV genotype (such as genotype B) show a trace of recombination[69]. However, in several cases, the level of recombination is significant and the strain should not be classified as a subgenotype. This is discussed below in more detail. Table 1 presents examples of misclassified subgenotypes, their methodological errors, and the proposed correct subgenotype classification of HBV.

IMPORTANCE OF HBV CLASSIFICATION

Before passing away in 2011, the Nobel Prize winner Professor Blumberg emphasized the importance of eliminating HBV. He believed eradication could be achieved through three important points: (1) efficient universal vaccination; (2) accurate diagnostic assays; and (3) effective treatment of HBV carriers[4]. Undoubtedly, eradicating HBV is not possible without fully understanding the genetics of the virus and being able to differentiate the isolates. Thus, before delving into HBV classification and current methodological drawbacks, we first present a comprehensive overview of differences in HBV strains in terms of virological, epidemiological, clinical and evolutionary aspects.

Table 1 The misclassifications detected in hepatitis B virus subgenotyping

Genotypes	Subgenotypes old classification	Reasons (R) for misclassification				Suggested resolution	New proposed subgenotypes reclassification
A	A1, A2						A1, A2
	A3, A4, A5	R1	R2	R3	R5	Quasisubgenotype-A3	QS-A3
	A6						A4
	A7		R2	R3	R5	Quasisubgenotype-A3	QS-A3
B	B1, B2						B1, B2
	B3, B5, B7, B8, B9		R2	R3		Quasisubgenotype-B3	QS-B3
	B4						B4
	B6						B5
C	C1						C1
	C2			R3		Quasisubgenotype-C2	QS-C2
	C3						C3
	C4				R4	Trace recombination	RS-C4
	C5				R4	Trace recombination	RS-C5
	C6-C13						C6-C13
	C14		R2			Quasisubgenotype-C2	QS-C2
	C15, C16						C15, C16
	C/D1, C/D2				R4	Inter-genotypic recombinant	Not considered as subgenotype
D	D1, D2, D3		R2			Not decided yet	
	D4, D5, D6, D7, D8, D9				R4	Recombino-subgenotype	RS-D4, RS-D5, RS-D6, RS-D7, RS-D8, RS-D9

Number (R1-R5) indicates the reason of problems in classification. R1: Applying partial gene in introducing subgenotype; R2: < 4% nucleotide divergence; R3: Weak bootstrap value or no monophyletic cluster; R4: Recombination; R5: Bias in reference collection. QS: Quasi-subgenotype; RS: Recombino-subgenotype.

Genotypic virological differences

It has been proposed that genotypic virological differences have evolved and adapted through long-term evolution, as well as through sequence insertions and deletions in the HBV genome. The HBV prototype strains comprises 3215 nucleotide base-pairs (bp), which are found in HBV genotypes B, C and F and H. The length of HBV strains varies from 3182 bp in the shortest genotype (genotype D) to 3248 bp in the longest one (genotype G)[17]. Particular genetic characteristics are present in some genotypes. For example, because of the existence of two stop codons in the core region of genotype G of HBV, this strain does not have the ability to secrete hepatitis B e antigen (or HBeAg)[70]. Also, the G1896A precore (PC) mutation, which also ablates HBeAg expression due to a stop codon in the precore region, is rare in genotypes A, F and H, while this mutation has the highest prevalence in genotype D[16,71,72]. Some reports have demonstrated the high prevalence of the PC mutation in non-Japanese subgenotypes of genotype B (B2-B5) compared to subgenotype B1[73]. Also, A1762T and G1764A/T basal core promoter (BCP) mutation, which is significantly associated with advanced liver diseases, is more prevalent in genotypes A and H of HBV[74,75]. Moreover, nucleotide variation and deletion in the pre-S region are particularly prevalent in distinct genotypes, such as genotype C in comparison with genotype B[76]. Studies revealed that diversity within the HBV genome is directly associated with cirrhosis as well as HCC incidence and outcome[77]. Moreover, heterogeneity and substitution rates are dissimilar in different HBV genotypes. Based on intra-genotype genetic diversity, some genotypes like genotypes A, B, C, D, and F have been classified into different subgenotypes, while genotypes of E, G and H do not contain enough heterogeneity to be subdivided into subgenotypes[69].

Genotype epidemiological distinctions

In addition to virological aspects, epidemiological studies revealed that HBV genotypes are associated with differences in geographical distribution and route of transmission.

Geographical distribution: Different genotypes of HBV show distinct geographical distribution patterns. For example, genotype A is dominant in Northwest Europe and North America. Also, some strains of genotype A have been found in the Philippines, Hong Kong and in some parts of Africa and Asia. Genotypes B and C are mainly prevalent in Southeast Asia and can be also found in the Pacific islands[21]. Genotype D is the most globally distributed genotype, and it can be found from Southern Europe and Africa to India. It can be also detected among intravenous drug users on all continents[78]. Genotype E is mainly dominant in West Africa[79]. Genotype F is found in South and Central America[19]. Genotype G was first discovered in France and the United States[80], and it has recently been reported in Belgium[81]. Genotype H has been described in America and Japan[82]. Recently, two controversial genotypes (I and J) have been proposed in South Asia[37,83], which will be discussed comprehensively later in Section 6 (recombination).

Transmission route: Generally, HBV is transmitted through contaminated body fluids. Various routes of transmission include blood transfusions from infected patients, sexual intercourse, unsafe injections and mother-to-neonate transmission. Recently, tears, urine, saliva,

bites and broken skin have also become accepted as probable modes of transmission[7]. Since HBV is able to survive on surfaces for up to seven days, direct transmission through contaminated surfaces to persons that have frequent contact with HBV carriers has been also reported[84]. To complicate matters, the transmission routes largely depend on the regional prevalence of chronic carriers of HBV-infected individuals[84]. Differences in the natural history of infection with different genotypes have also impacted the modes of HBV transmission[85]. The local prevalence of HBV with its geographical distribution, regional and social cultures, as well as taboos can help to draw an informative transmission pattern for each genotype[10]. In highly endemic areas (prevalence rate > 8%), such as South Asia where genotypes B and C are dominant, perinatal (mother-to-child) transmission is most common. It has been proposed that genotype C is primarily transmitted perinatally in this region, since the seroconversion of HBeAg to anti-HBe took longer in these patients, which is consistent with the fact that it takes longer for genotype C than for other genotypes[86].

In European countries with a low prevalence of HBV (prevalence rate < 2%), where genotype A and D are dominant, sexual transmission and unsafe injection practices are the main modes of HBV transmission. Nosocomial transmission and unsafe injection practices are considered responsible for more than 60% of HBV infections in Central Europe[87]. Though genotype E is transmitted by heterosexual relations in Africa, genotype A and particularly genotype G were isolated from men who have sex with men in Europe and Canada[81,88]. In West Asia and in the Middle East (where genotype D is dominant), the route of transmission and HBV seroprevalence depend on the region. For instance, Iran has a low HBV endemicity (around 2%), and intravenous drug injections, tattooing and phlebotomy are considered the major potential risk factors and transmission routes of HBV infection in the country. Furthermore, socioeconomic status, life style, occupation, and cultural attitude in different ethnic groups greatly impact the route of HBV transmission[89,90].

Genotypes' impact on clinical outcomes

Increasing evidence suggests that HBV genotyping is important for determining HBV disease progression and designing appropriate antiviral treatment. Some genotypes are more associated with particular kinds of prognoses, such as acute forms of disease[88]. Several reports showed that genotype A evolves more rapidly in patients than genotype D does, which poses problems for treatment[16]. Also, patients infected with genotype C progressed to end stage liver disease earlier than those infected by genotype B[91]. Interestingly, it has been shown that patients infected with genotype F have higher mortality rates than those infected with genotype A or D[92]. In India, genotype D is associated with more severe liver complications than other genotypes[93]. In the United States, it has been shown that genotype D is an independent risk factor for fulminant hepatitis[94]. Interestingly, however, patients infected with genotype F have a higher rate of liver-related mortality than those infected with genotype D[95].

Genotype C and D generally tend to be related to more severe liver disease than genotype A and B and are more frequently associated with HCC[96]. In HBeAg-positive patients treated with standard interferon-alpha, the post-treatment HBeAg seroconversion and normalization of serum ALT levels are considerably better in those infected by genotype A and B than patients infected with genotypes C and D[97]. Furthermore, following pegylated interferon-alpha therapy, HBeAg seroconversion and a substantial decrease in serum titer of HBsAg was observed in genotypes A and B but not in patients infected with genotypes C and D[98-100]. In the case of antiviral therapy, it has been shown that genotype B is more frequently associated with lamivudine-resistant variants than genotype C. Likewise, in some studies, it has been observed that genotype A develops antiviral-resistant variants earlier than genotype D[74].

Genotypes' impact on prophylaxis measures

Current HBV vaccines are recombinant peptides that cover a "super antigenic" and highly conserved motif of HBsAg. Protective serum titers of anti-HBs (greater than 10 IU/L) develop in 95%-99% of healthy infants, children[101,102] and young adults[103]. Many studies have demonstrated that HBV vaccination has dramatically decreased the HBV chronicity rates and HBV-related complications[7]. The current vaccine is derived from genotypes A and D of HBV[104]. Despite its success in most cases, there are several reports of vaccination failure due to genotype complications[105]. In one case, a German patient who was vaccinated and showed production of formally protective anti-HBs antibodies developed acute hepatitis B following infection with genotype F of HBV[104]. In a similar report from Europe, an Irish man was vaccinated, but developed infection by genotype F[106]. Both patients received HBV vaccines produced using the S gene of HBV genotypes A and D, which are the most common genotypes in Europe. Furthermore, in both reports, the HBV isolates did not carry typical vaccine escape mutations. Several investigators have described S gene variations isolated from vaccine failure cases[107,108]. The frequency of these mutations varies in different genotypes. Of note, some of these mutations are considered wild type motifs in another genotype[109]. Such instances of failure serve as reminders that our current vaccination program is imperfect. Future efforts should be directed towards developing vaccines that protect against all genotypes of HBV and also account for vaccine escape mutations.

Evolutionary differences of genotypes

Phylogenetic analysis has demonstrated that genotype H is closely related to genotype F, though there is enough inter-nucleotide divergence between these two particu-

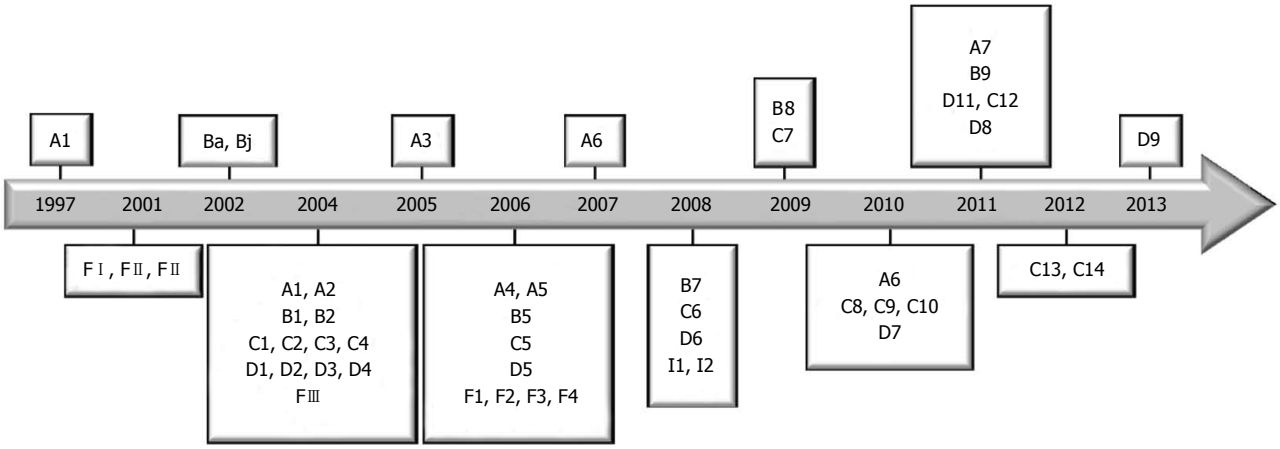

Figure 2 The time line of identification and designation of hepatitis B virus subgenotypes.

lar genotypes to distinguish them as distinct genotypes. Phylogenetic analysis also revealed that these two genotypes ("New World human genotypes") lie on the branch of the Woolly Monkey, which suggests cross-speciation between non-human and human genotypes of HBV[110]. This scenario was reiterated after evolutionary relationship analysis between genotypes D and E. Furthermore, they showed different evolutionary rates (number of substitution per site per year)[49].

SUBGENOTYPES ARE MORE DETAILED GENOTYPES

For some HBV genotypes, several subgroups can be easily defined as when the intra-genotypic nucleotide divergence stays between 4% and 7.5% over the full-length genome. According to conventions of identification, HBV subgenotypes are differentiated by numbers corresponding to the order of discovery; the numbers do not correspond to subgenotype evolution. For instance, D1-D4 were identified by Norder *et al*[21] earlier than D5[111]. However, D5 is the most ancient of all known subgenotypes for genotype D[112]. Also, it was noted that A6 (currently known as A4) is from a basal lineage that diverged earlier than the other African subgenotypes of genotype A[54,55]. Figure 2 illustrates the updated time line of HBV subgenotype identification. Uncovering the relationship between subgenotypes and subtypes of HBV has added significant value to molecular epidemiological studies of HBV[74].

It should be noted that because of inappropriately applied methods, some subgenotypes have been incorrectly classified in the past. One of the most common mistakes is applying phylogenetic analysis over a partial genome sequence instead of the full-length genome. Experts in the field have attempted to correct the errors in numbering and misclassification (Figure 1B, Table 1), but inaccurate subgenotyping of HBV is continuously being reported[54,58,62,69,113-118].

HBV subgenotypes and geographical distribution

Subgenotypes reflect properties and distributions of genotypes. Figure 3 shows the geographical distribution of genotypes and subgenotypes of HBV together with the prevalence of HBsAg in different areas around the world. For instance, subgenotype A2 is dominant in Europe, A1 is prevalent in Asia and most of Africa, A3 is found in Cameroon and Gambia, A6 (currently named A4) and quasi-subgenotype A3 (which includes previously named A4 from Mali, A5 from Nigeria and A7 from Cameroon) have been isolated from other regions[69]. Subgenotype B1 was isolated from Japan, while B2, B3, B4, B5, B6, B7, B8 and B9 were isolated from Taiwan, Indonesia, Vietnam, Philippines, the Arctic region, Nusa Tenggara (a region from Eastern Indonesia) and Indonesia, respectively[119]. Subgenotype C1 was isolated from Taiwan, C2-C16 were isolated from China, Oceania, Australian Aborigines, Philippines, Papua-Indonesia and Nusa Tenggara[120]. Two recombinant mixed strains C/D1 and C/D2 (combination of HBV genotype C and D) were specifically reported in Tibet[65,121]. Although the genotype D of HBV is distributed globally, its subgenotypes are locally confined to certain geographical regions. For example, subgenotype D1 is restricted to Iran and its neighboring countries[116,122-126]. Subgenotype D2 is derived from East Europe and Russia, D3 was detected in Serbia, South Africa and Alaska, D4 was found in Oceania and Somalia, and D5-D9 were reported from India, Indonesia Tunisia and Nigeria[67]. Genotype F is also widely distributed: F1a is dominant in Costa Rica and Salvador, F1b in Argentina, Chile and Alaska, F2a in Venezuela and Brazil, F2b in Venezuela, F3 in Panama, Venezuela and Colombia and F4 is circulating in Brazil, Argentina and Bolivia[127].

HBV subgenotypes and ethnic origin

It has been demonstrated that in some cases, such as for genotype B8, the distribution of HBV genotypes/subgenotypes is related to the ethnic origin[128], or B6 is considered confined to indigenous populations[129]. Inter-

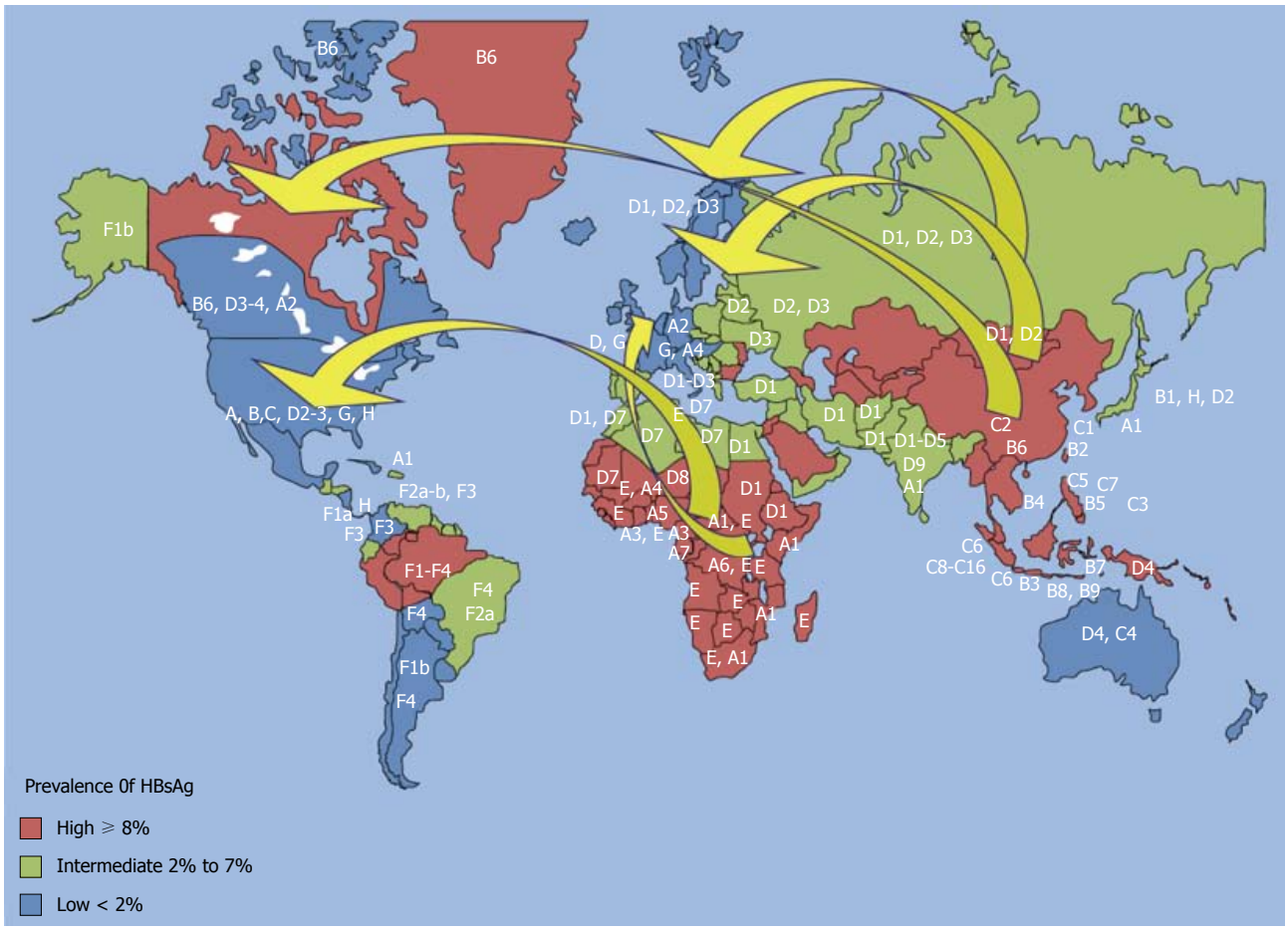

Figure 3 Geographical distribution of hepatitis B virus genotypes and subgenotypes in different regions of the world. Yellow arrows illustrate the directions of HBV subgenotype dispersal through immigration from highly and intermediate endemic countries to low HBV endemic areas (map of hepatitis B surface antigen (HBsAg) prevalence adapted from the website of the WHO). HBV: Hepatitis B virus; WHO: World Health Organization.

estingly, C4 can be detected in the Australian Aborigines but nowhere else, which suggests that C4 independently evolved from its ancestor in that region[130]. Strains isolated from indigenous populations, such as C3 and C5-C10 from Indonesia and B6 from the Canadian Arctic, proposed that HBV (sub)genotypes have evolved in different streams of human immigration[49].

HBV subgenotypes and clinical outcome

HBV subgenotypes also present differently in terms of clinical outcome. It is possible to uncover more details regarding the natural history of infection by comparing genotypes. For example, subgenotype A1, which is prevalent in West and South Africa, has a more severe clinical outcome compared to subgenotype A2. Patients infected with this subgenotype developed HCC at a young age in West and South Africa, whereas those who were infected with A2 and developed HCC in Europe were mainly older patients[131]. European patients infected with A2 had a mild clinical outcome and high chance of clearing HBsAg and losing HBV DNA[95]. Furthermore, in Europe more occult cases have been infected by A1 comparing with A2 or other (sub)genotypes[81]. Nevertheless, it is important to keep in mind that all these studies are hampered by the fact that it is difficult to adjust the studied patient cohorts for all potential confounding factors.

Subgenotype B6 is commonly associated with a mild clinical outcome in infected patients, while B1 can result in fulminant and acute hepatitis B infection. Patients infected with subgenotype B1 also developed advanced liver disease at an older age compared to patients infected with subgenotypes B2-B5[73,132]. In most studies in South Asia, genotypes B and C were compared. Though there is a paucity of data of comparing the clinical findings of different subgenotypes of genotype C, one study demonstrated an increased risk for HCC in patients infected by subgenotype C2 compared to C1 and genotype B subgenotypes[133]. Interestingly, precore mutations have not been observed in subgenotype F2, whereas subgenotype F1 frequently carries precore mutations[16]. In one large study, a higher percentage of patients who developed HCC had been infected by subgenotypes F2 or C2 compared to subgenotype A2, B6 or subgenotypes of genotype D[131].

CLASSIFICATION AND IMPORTANCE OF ACCURACY

In order to accurately investigate the impact of different

(sub)genotypes of HBV on different aspects of infection from prophylaxis, diagnosis and therapy, it is crucial to agree to a holistic classification. Numerous HBV strains have been described through PCR and sequencing, however, many disregarded well-established HBV (sub)genotype definitions, which has resulted in several misclassifications. Subsequently, experts in the field agreed that the classification of HBV should be mended and rectified[54,69,113-115]. There are several reviews or commentary articles regarding the misclassification of HBV that describe the history of identification of different (sub)genotypes. However, the reclassification and renovation of this system has been considered less often. We, along with others, believe that recombination is the main cause of misclassifications and major evolutionary characteristics of HBV should be investigated to help identify strains that require additional analysis for proper classification[115]. Besides this factor, massive but gradual conversion of geographical distribution of HBV should be concomitantly investigated.

RECOMBINATION AND ITS ETIOLOGY

Recombination in HBV is principally the result of the co-infection of a host with more than one strain from different (sub)genotypes. Different HBV strains can exchange their genetic material within the host cells. Recombination is favoured in particular geographical regions by three conditions: (1) two or more different HBV genotypes are circulating in the population; (2) the chronicity of HBV is high; and (3) public health level is low. Most recombinant strains have been reported from East Asia or Africa, where the prevalence of HBV infection is high and prophylaxis and control of infection is low. Due to the dense population, the chance of co-infection is boosted so risk of recombination is subsequently elevated. In contrast, HBV recombinant isolates have been reported rarely within typical European strains (such as subgenotypes A2, D2 and D3). This corroborates the necessity of efficient health control and prophylactic measures in order to decrease the risk of infection, co-infection and eventually recombination.

Currently, more than 30 recombinant strains have been described[126,134]. Sometimes recombination can occur between strains with high genetic homology, in which two different subgenotypes from a similar genotype are co-infected in a patient. Such recombination has been reported between HBV subgenotype B2 with B5 and between B1 with B6[128]. Also, there are some reports regarding recombination between two strains of different genotypes[65,66,121,135]. Although more than 60% of recombinant isolates have their breakpoint between nucleotides that span 1640-1900 (which encompasses the core region), recombinant strains with breakpoints in the S gene (350-500, 3150-100 or 650-830) have also been identified[134]. Markedly, the largest breakpoints have been detected among HBV isolates from Tibet, in which two different genotypes of C and D (subgenotypes C1 or C2 with D1 or D2) formed 50% of the recombination[135,136].

Recombination as a source of HBV misclassification

Recombination is an inevitable event in evolution and can cause errors in classification of HBV genotyping or subgenotyping[18,69,114,115]. In many cases, recombinant strains have been erroneously introduced as new genotypes or subgenotypes. For instance, Ghosh *et al*[137] introduced six HBV strains as a novel subgenotype of HBV named D9. In the phylogenetic tree, subgenotype D9 strains are grouped as a monophyletic and distinct cluster with a maximum bootstrap value. Not surprisingly, according to their analysis, these recombinant strains showed extraordinary nucleotide divergence from the other subgenotypes of genotype D (from minimum 5.2 ± 0.3 with D1 to 6.7 ± 0.4 with D7 and D8). This nucleotide divergence was odd, because it did not meet the threshold of genotype definition, yet it was much higher than normal nucleotide divergence within well-classified subgenotypes. More interestingly, there was a highly diverse pattern of recombination in these strains. Although all strains showed recombination with genotype E in the HBV core and pol regions, there was no identifiable recombination pattern among all strains. Indeed, different lengths of genotype D recombined with genotype E. Moreover, in some strains, some recombination with genotype C was observable. If PCR and sequencing had been perfectly carried out, subgenotype D9 strains should be classified as a D/E recombinant strain. Exactly the same scenario was suggested by another group for the D8 subgenotype[18]. Similar concerns have been raised regarding subgenotype D7, which was introduced as a novel subgenotype of genotype D. However, these strains harbored at least 900 nucleotides of genotype E in the backbone of this genotype D strain[138]. Likewise, subgenotype D4 may have recombined with D7 or D8, which were grouped in an exclusive cluster in the phylogeny tree[64]. The same concern was raised for other HBV subgenotypes, like genotype C, in which the recombinant strains C/D1 and C/D2 were suggested as new subgenotypes[65,66].

Proposing the term "recombino-subgenotype"

Owing to the availability of sequencing and free online phylogenetic software, novel subgenotypes of HBV are continually being suggested by researchers that disregard the fundamental definitions of HBV classification. To address the misclassification of HBV subgenotypes that lack enough nucleotide divergence and bootstrapping value to definitively be considered subgenotypes, we previously proposed the term of quasi-subgenotype[54]. This term has somewhat settled irregularities in subgenotyping of HBV and was respected by other studies[10,62,69,81,113,118,139-143]. To address the classification of recombinant strains, which are being introduced as independent subgenotypes, we propose to call the indefinitely recombinant strains as "recombino-subgenotype"

rather than an independent subgenotype. According to our proposed definition, the "recombino-subgenotype" is a lineage that shows strong evidence of recombination and its nucleotide divergence, together with supportive bootstrap value, fall within the range necessary to define subgenotypes. Although these strains are recombinant, in a comprehensive phylogenetic tree (only based on full-length genome), "recombino-subgenotypes" are clustered around intra-genotype clusters and could have supporting bootstrap value. While this new term cannot prohibit the introduction of HBV recombinants as novel subgenotypes, it can clearly differentiate the pure subgenotypes from recombinant strains. Therefore, we would like to offer the term "recombino-subgenotype" to differentiate recombinant from non-recombinant (sub)genotypes. We sincerely hope that this term will help to remind scientists about recombination as a potential pitfall in HBV classification as well as allow a more accurate description of novel HBV isolates derived by recombination in HBV taxonomy.

IMMIGRATION: INTRODUCING THE TERM "IMMIGRO-SUBGENOTYPE"

While recombination is an important virological aspect to be considered in classification, the epidemiological profile of HBV is another valuable scope to study. Although ancient dispersal of HBV alone has made the HBV pool a dynamic viral population, recent trends of globalization and increasing human mobility are significantly speeding up HBV distribution and recombination between viral strains. Immigration and direct human contact are the two main causes of changes in geographic dispersal of HBV genotypes and subgenotypes; however, their profound impact has yet to be studied in more detail. People typically emigrate from countries with high HBV endemicity, which alters the geographical distribution of HBV (sub)genotypes around the world, notably in low sero-prevalence regions including Europe and North America.

Recently, novel subgenotypes have been reported from immigrants who are not living in their original home areas. For instance, HBV subgenotype A6 (currently named A4) strains have been isolated from African immigrants currently residing in Europe and North America[54,55,81,113,135]. It has been predicted that within the next few decades, HBV epidemiological patterns will be completely modified by waves of immigration. Thus, such "immigro-subgenotypes" may replace native strains in regions with high immigration. Their integration might alter the local prevalence of carriers, routes of transmission, and will have a great impact on prophylactic, diagnostic and therapeutic measures. In a recent study, Mitchell *et al*[144] showed that roughly 53800 HBV chronic carriers settled in the United States each year between 2004 and 2008 from countries of intermediate or high HBV endemicity (2%-31%). In all states of the United States, genotypes A, B, C and D have been identified in immigrants, who were born in HBV-endemic areas[145]. This is one of the clearest pieces of evidence highlighting the direct impact of immigration on the introduction of exotic genotypes to areas with low endemicity. Therefore, we would like to propose the term "immigro-subgenotypes" to differentiate native strains from imported stains.

Effect of "immigro-subgenotype" on clinical outcome

The genotype-specific history of HBV strains should be considered when studying imported strains[133]. In the HBV-endemic area, the main route of transmission is usually perinatal. It has been estimated that over 21% of worldwide HBV-related mortality is associated with perinatal transmission[146]. The risk of perinatal transmission is 100%, when the mother is HBeAg-positive and does not take any antiviral medications or HBV immunoglobulins (HBIg). The risk of chronicity of the infant will be 90%, if prophylactic countermeasures are not administrated directly after birth[147]. When the patient is HBeAg-positive, viral load is usually high, which further increases the risk of transmission. As a genotype-related characteristic in HBV endemic regions, the HBeAg test of mothers is positive in the years of childbearing, and just after four decades of life seroconversion might happen. This shows the infectivity potential of HBV carriers infected by (sub)genotypes circulating in endemic regions like East Asia, Africa, Alaska and East Europe[133]. In a recent investigation in Italy, the immigrant population (mostly from Eastern European countries) showed a high prevalence of HBeAg-positivity with a mean age of 31 years[148]. In another study, Dervicevic *et al*[149] showed the integration of exotic genotypes of HBV with HBeAg-positivity and high viral load among antenatal women in the United Kingdom. Dervicevic *et al*[149] emphasized the trend of changing epidemiological patterns of HBV in the United Kingdom, where an influx of immigration brings almost 6000 HBV carriers annually to this country. In numerous studies in Belgium, exotic (sub)genotypes of HBV have been identified and found to have integrated into the native population[55,59,81,135,150]. In Bolivia, the exchange of native HBV subgenotype F4 and exotic ones (subgenotypes B2 and C2) between Bolivian and Japanese immigrants was clearly demonstrated by phylogenetic analysis[151]. Interestingly, the exotic strains have different mutational patterns in different ORFs of HBV[123], which would have a different impact on the course of infection, therapeutic, diagnostic and prophylactic measures[152,153].

Naturally occurring mutations associated with drug resistance have been reported in native populations in Asia and Europe[154]. Additionally, in a study conducted by Bottecchia *et al*[155], a primary drug resistance mutation (rtM204V) was found in the course of treatment-naïve immigrants infected by (sub)genotype E and A3. In a recent study from our group, we found that exotic (sub)genotypes (A6) carried clinically important mutations, which could help the virus to escape from diagnostic assays or prophylactic measures[81,135]. Finally, it

should be added that different disasters such as wars[156] or mass-casualties[157] can have direct and indirect impacts on the epidemiology of HBV. Since virological and clinical characteristics of HBV (sub)genotypes differ, it is crucial to monitor changes in epidemiological patterns of HBV infection as it relates to immigration[158].

CONCLUSION

In this review, we attempted to provide strong and up-to-date evidence about the impact of different (sub)genotypes on prophylaxis, diagnosis, clinical outcomes and treatment of HBV infection. Controlling HBV requires massive and unified efforts because modern human measures like vaccination and antiviral therapies have led to the rise of invasive strains, drug resistant, vaccine and diagnosis escape variants. Furthermore, immigration has changed the distribution of HBV and resulted in the emergence of exotic strains in destination territories. These strains, together with intra- and inter-(sub)genotypes recombination, complicate diagnosis, treatment and classification. Elimination of HBV infection requires concomitant vaccination, effective treatment and a vigorous diagnostic scheme. To organize all measures from prophylaxis to therapy, an accurate, holistic and dynamic classification system is essential. This system should be based on robust virological and epidemiological facts to cover all existing strains, and also have the capacity for newly identified strains in the future. Analyzing full-length genome sequences when classifying genotypes and subgenotypes is the foremost prerequisite of this classification system. Careful attention must be paid to all aspects of phylogenetic analysis, such as bootstrapping values and meeting the necessary thresholds for (sub)genotyping. Quasi-subgenotype refers to subgenotypes that were incorrectly suggested to be novel. As many of these strains were misclassified due to genetic differences resulting from recombination, we propose the term "recombino-subgenotype". We also suggest to introduce the term "immigro-subgenotype" to distinguish exotic (sub)genotypes from native ones; immigration demonstrates a confounding facet of global HBV distribution. We are strongly convinced that applying these two proposed terms in HBV classification will help harmonize this field and allow for improved prophylaxis, diagnosis and treatment.

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TOPIC HIGHLIGHT

WJG 20th Anniversary Special Issues (9): Hepatitis B virus

Treatment of chronic hepatitis B in clinical practice with entecavir or tenofovir

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Abstract

Results from phase Ⅲ clinical trials clearly demonstrate the efficacy and safety of entecavir and tenofovir in the controlled environment of randomized clinical studies. There are several studies with both drugs performed in clinical practice (also called "real life studies"). Despite the pros and cons, studies performed in real life conditions represent everyday practice and add important information about long term treatment effectiveness and safety in this clinical setting. This review shows that patients treated with first line nucleos(t)ide analogs at referral centres, with good clinical follow-up and adherence to international guidelines, can achieve high treatment response rates with a very low rate of adverse events.

Key words: Hepatitis B; Clinical practice; Entecavir; Tenofovir; Real life

Core tip: Patients treated with entecavir or tenofovir in routine clinical practice at referral centres, with good clinical follow-up and adherence to international guidelines, can achieve high treatment response rates with a very low rate of adverse events.

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INTRODUCTION

The hepatitis B virus (HBV) is estimated to have infected more than 2 billion people worldwide, of whom 400 million are chronically infected today and are at an increased risk of liver-related complications, including cirrhosis, liver failure, hepatocellular carcinoma (HCC) and death[1,2]. In most regions of America, HBV prevalence is relatively low, with hepatitis B surface antigen (HBsAg) positivity ranging from < 2% to 7% compared with Asia, Africa and the Middle East, where chronic hepatitis B (CHB) prevalence rates reach 5%-20% of the general population[2,3]. Indications for treatment have been established by several international guidelines[3,4]. Treatment end-points are complete viral suppression (undetectable levels of HBV DNA replication), hepatitis B e antigen (HBeAg) clearance and seroconversion in HBeAg-positive patients, and if possible HBsAg clearance and development of antiHBs antibody[3,4]. Patients achieving these serologic end-points may discontinue treatment after an additional 6-12 mo period of consolidation therapy according to the cited guidelines. The goal of HBV treatment is to improve survival by preventing disease progression to decompensated cirrhosis

and HCC[3,4].

Treatment induced suppression of HBV DNA to undetectable levels reduce the risk of disease progression improving liver fibrosis, and can result in fibrosis and cirrhosis regression[5,6]. Also, HBV DNA clearance is associated with increased rates of HBeAg and HBsAg seroconversion, the ultimate goal of HBV therapy. Since HBV DNA is integrated in the host genome, HBV persists in the covalently closed circular DNA form in the hepatocyte even if HBV DNA is not detectable in the serum. This HBV persistence may result in reactivation and hepatocarcinogenesis. Long-term treatment nucleos(t)ide analogs (NUC) is required in HBeAg negative and positive patients who cannot maintain off-treatment virologic suppression.

Pegylated interferon alpha (PEG-IFN alpha), entecavir (ETV) and tenofovir disoproxil fumarate (TDF) had been selected as the first-line therapy to initiate treatment in naïve CHB infected patients[3,4]. Other NUCs like lamivudine (LAM), adefovir (ADV) and telbivudine (LdT), are no longer recommended as first-line therapy since long-term therapy success with these drugs has been reduced with the emergence of drug-resistant mutants[3,4]. ETV and TDF were approved by different regulatory agencies in most countries between 2005 and 2009 on the basis of phase Ⅲ clinical trials results. Since their approval, observational studies have been performed in everyday clinical practice (also known as "real life studies"), with their long term use adding valuable information to the efficacy and safety profiles of these two drugs. The aim of this review is to analyze the currently available data of long term ETV and TDF use in clinical practice as first-line treatments for NUC naïve chronic HBeAg positive and negative HBV patients.

RESULTS FROM CLINICAL TRIALS

Results from phase Ⅲ clinical trials (CT) are critical for the approval of new drugs. Their main objective is to demonstrate the efficacy and safety of the drug being evaluated in comparison with the current standard of care, in a controlled setting. These trials are conducted on large patients groups under standardized conditions. "Ideal" young patients without comorbidities are included, and usually, patients with advanced liver disease are excluded. These strict inclusion/exclusion criteria are developed to facilitate analyzing the results and getting the new drug approved for its use in clinical practice. Also, patients treated within CT are strictly monitored and assist more frequently to clinical consultation and to laboratory monitoring than in routine clinical practice. Once approved, treating physicians use the same drug in "real life" patients, some of whom would have been excluded from these trials.

Entecavir

Entecavir is a potent inhibitor of HBV replication, which has been commercially available since 2005. In phase Ⅲ randomized clinical trials (RCT) ETV showed increased virologic, biochemical and histologic response rates when compared with LAM. ETV at a dose of 0.5 mg/d in treatment-naïve patients suppressed HBV DNA to undetectable levels by year 1 in 67% of HBeAg-positive and in 90% of HBeAg-negative patients compared with 36% and 72% in the LAM arms, respectively[7,8]. Recent reports showed that when administered for 2 to 5 years, ETV resulted in better HBV DNA suppression and higher HBeAg seroconversion rates[9-11]. ETV treatment for 3 years in HBeAg-negative and for 5 years in HBeAg-positive patients resulted in 95% and 94% HBV DNA undetectable levels, respectively[10,11]. In HBeAg-positive patients, treatment for 96 wk resulted in 31% HBeAg seroconversion rates[9]. In the ETV-901 study, continuing treatment in those patients who remained HBeAg positive at week 96 resulted in 23% HBeAg seroconversion rates and 1.4% HBsAg loss[11].

ETV has a high genetic barrier to resistance and a strong resistance profile, and has a very favorable safety profile. Recently reported results of more than 6 years of therapy showed that in NUC naïve patients the cumulative probability of genotypic resistance to entecavir was very low (1.2%) and that treatment was well tolerated[12,13]. Also, analysis of liver biopsies from the two phase Ⅲ entecavir studies (ETV-022 and ETV-027) and the open-label rollover study (ETV-901) have shown that ETV treatment can improve fibrosis of the liver and can cause fibrosis and cirrhosis regression[5]. Patients receiving treatment for at least 3 years had ⩾ 2 point decrease in the Knodell necroinflammatory score and no worsening of the Knodell fibrosis score in 96% of the cases, and ⩾ 1-point improvement in the Ishak fibrosis score in 88% of the cases[5]. Reversal of advanced fibrosis/biopsy-proven cirrhosis was demonstrated in nine of 10 patients with baseline Ishak fibrosis scores of 4-6 who underwent serial liver biopsies up to year 6.

Tenofovir

TDF is also a potent inhibitor of HBV replication, which has been commercially available since 2008. In phase Ⅲ RCT TDF showed increased virologic and biochemical response rates when compared with ADV. TDF at a dose of 300 mg/d in treatment-naive patients suppressed HBV DNA to undetectable levels by year 1 in 76% of HBeAg-positive and in 93% of HBeAg-negative patients compared with 13% and 63% in the ADV arms, respectively[14]. As previously shown with ETV, extending treatment with TDF is associated with increasing HBV DNA suppression and higher HBeAg seroconversion rates[15-17]. After 4 years of treatment, 96% of HBeAg positive and 99% of HBeAg negative patients achieved undetectable HBV DNA levels[15-17]. In HBeAg positive patients, HBeAg loss occurred in 41% of patients and HBeAg seroconversion in 29%; the cumulative probability of HBsAg loss was 11%[15,16]. Longer treatment with TDF is associated with higher HBV DNA negativization rates (98%-99%), and higher

HBeAg and HBsAg negativization and seroconversion rates[18].

As with ETV, long-term treatment with TDF has been associated with histologic improvement. Sustained viral suppression with TDF treatment over 5 years was associated with histological improvement in 87% of patients and 51% fibrosis regression; 74% of patients with cirrhosis (Ishak score 5 or 6) at baseline no longer had cirrhosis[6]. TDF was well tolerated over this treatment period[15-18], and no resistance with long term treatment has been reported to date[19,20].

RESULTS FROM CLINICAL PRACTICE STUDIES

Results from phase Ⅲ RCT clearly demonstrate the efficacy and safety of ETV and TDF in the controlled environment of randomized clinical studies. There are several studies with both drugs performed in clinical practice (also called "real life studies"). Some had been published in full text in peer review journals, and some had been only presented at the liver meetings organized by the American Association for the Study of Liver Diseases and the European Association for the Study of Liver. These studies contain a heterogeneous mixture of patients treated for different periods of time who are differentiated from those in clinical trials as based on a number of criteria and may, therefore, be more reflective of the treatment population and the real efficacy and safety of the drug (Table 1)[21]. Results from these studies are discussed in the following section and summarized in Table 2.

Entecavir

There are several studies of ETV treatment in clinical practice from different regions of the world. Most of them are from Europe and Asia, and a minority from America and Oceania. The Oriente study analyzed the results from 190 NUC-naïve patients treated for a year in 25 centres in Spain. The cohort was 73% male, 84% Caucasian, 30% HBeAg positive and 34% of the patients who underwent biopsy had advanced fibrosis/cirrhosis. At week 48, 83% of the patients (61% HBeAg-positive; 92% HBeAg negative) achieved a virological response, 26% of the HBeAg-positive patients lost HBeAg and 22% achieved seroconversion to antiHBe and 2% showed HBsAg clearance[22]. The European network of excellence for vigilance against viral resistance (VIRGIL) performed a multicentre cohort study at over 10 European referral centres between 2005 and 2010 including 243 NUC-naïve patients[23]. At week 144, 90% of HBeAg positive patients and 99% of HBeAg negative patients achieved a virologic response, and 34% of the HBeAg-positive patients lost HBeAg. In a single-centre cohort study from the King's College in the United Kingdom 3 treatment strategies were compared. One hundred and fifty four patients were treated with ETV monotherapy for a median of 28 mo: 76% of patients achieved HBV DNA undetectable levels, 8% of the HBeAg positive patients cleared HBeAg and 1% cleared HBsAg[24].

A retrospective/prospective, multicentre study was conducted at 19 Italian centres and included 418 consecutive NUC-naïve patients treated with ETV[25]. In their last evaluation, 100% of HBeAg positive patients and 99% of HBeAg negative patients achieved HBV DNA undetectable levels after 60 mo of treatment. In HBeAg positive patients, HBeAg seroconversion occurred in 31 patients (cumulative rate of 55%) and HBsAg loss in 15 patients (cumulative rate of 34%). One patient developed resistance to ETV (L180M, M204V, S202G) over the treatment period and was successfully treated with TDF[25].

A single centre study from Italy included 100 patients, 85 of whom were NUC-naïve treated with ETV for 36 mo. Overall, 94% of the patients achieved HBV DNA negativization, 33% of HBeAg positive patients cleared HBeAg and 15% cleared HBsAg[26]. Another multicentre study from Italy included 300 patients, 287 being NUC-naïve treated for 24 mo. At the end of follow up, cumulative rates of undetectable HBV DNA was 89%, 39 patients were HBeAg positive and 17 achieved negative HBeAg with antiHBe seroconversion in 15 cases (38.4%), and HBsAg loss was observed in 5 patients[27]. Unfortunately, both studies presented their overall results, including both NUC-naïve and NUC-experienced patients.

The results from a previously reported multicentre study performed in Argentina were recently updated[21,28]. One hundred and sixty nine consecutive patients were treated with ETV for a median 181 wk. Overall, 156 (92%) patients became HBV DNA undetectable, 92 (88%) of HBeAg positive and 64 (98%) of HBeAg negative patients. Cumulative clearance of HBV DNA by week 192 and 240 was 100% in both HBeAg positive and negative patients. Seventy four (71%) patients cleared HBeAg, 23 (14%) patients cleared HBsAg (19 HBeAg positive and 4 HBeAg negative, $P = 0.025$), and 22 (13%) patients developed protective titers of antiHBs. One patient developed virological breakthrough due to ETV resistance (M204V, S202G) over the treatment period[29]. In a follow up study, post-treatment outcomes of patient from this study were evaluated in clinical practice[30]. Thirty-five patients (20%) discontinued ETV treatment due to sustained virological response; 33 of these patients developed HBeAg seroconversion and 18 HBsAg seroconversion. Nine patients (26%), all HBeAg positive at baseline, developed virological relapse after a median 48 wk off treatment, 3 of them showed HBeAg reversion and 4 lost antiHBe. No patient with HBsAg seroconversion relapsed[30]. These results confirmed that ETV, after 12 mo consolidation therapy, can be discontinued in real life. Patients have to be followed since there is still a risk of virological relapse.

A single-centre prospectively followed cohort from Hong Kong analyzed 222 NUC-naïve patients receiv-

Table 1 Baseline characteristics of patients included in entecavir studies[21]

Characteristic, *n* (%)[1]	ETV-022	ETV-027	Oriente	Virgil	King's College Cohort	Italian cohort	Argentinean cohort	Hong Kong cohort	Japan cohort	China cohort	China cohort 2	Taiwan cohort	Taiwan cohort 2	United States cohort	United States cohort 2	Australia
Ref.	[7]	[8]	[22]	[23]	[24]	[25[	[29]	[32]	[33]	[34]	[35]	[36]	[37]	[38]	[39]	[40]
n	354	325	190	243	154	418	169	222	474	230	1768	98	248	169	136	163
Age, yr (mean ± SD or range)	35 ± 13[2]	44 ± 11[2]	44 (35-54)[3]	43 ± 14[2]	42[3]	58 (18-82)[4]	51 ± 13[2]	47 (21-77)[4]	47 (17-82)[4]	42 ± 12[2]	36 (16-70)[4]	48 ± 13[2]	39 (17-77)[4]	39 ± 12[2]	39 ± 12[2]	52 (24-86)[4]
Male	274 (77)	248 (76)	139 (73)	177 (73)	122 (79)	316 (76)	113 (77)	157 (71)	321 (67)	196 (85)	1414 (80)	67 (68)	172 (69)	100 (59)	83 (61)	113 (69)
Race					NR	NR		NR	NR	NR		NR				NR
White	140 (40)	193 (59)	60 (84)	114 (47)			143 (85)							7(4)	10 (7)	
Asian	204 (58)	122 (38)	18 (9)	70 (29)			26 (15)				1768 (100)			162 (96)	126 (93)	
Other	2 (< 1)	2 (< 1)	6 (3)	59 (24)												
Region	Europe 24%, North America 13%, South America 14%, Australia and Asia 49%	Europe 48%, North America 9%, South America 11%, Australia and Asia 33%	Europe	Europe	Europe	Europe	South America	Asia	Asia	Asia	Asia	Asia		North America	North America	Oceania
Genotype			NR		NR		NR	NR		NR	NR	NR				NR
A	94 (27)	33 (10)		40 (22)					12 (3)					0		
B	68 (19)	46 (14)		14 (8)					67 (16)					-61	-57	
C	111 (31)	57 (18)		25 (14)					336 (81)					-39	-41	
D	37 (10)	157 (48)		91 (50)		84 (90)			0 (0)					0		
HBeAg negative	6 (3)	322 (99)	133 (70)	157 (65)	106 (69)	347 (83)	65 (39)	132 (59)	252 (53)	117 (51)	602 (34)	57 (58)	0 (0)	0 (0)	0 (0)	10 (6.1)
HBV DNA, log10 IU/mL[1]	9.62 (2.01)[2,5]	7.6 (1.8)[2,5]	5.94 (4.64-7.39)[3]	6.2 ± 1.7[3]	4.6 (0.2)[6]	6.0 (1.5–9)[4]	6.88 (1.81)[2]	7.1 (4.0-> 8.8)[4]	6.7 (2.6-> 9.0)[4]	6.3 ± 1.4[3]	6.74 (1.04-9.69)[4]	6.0 ± 1.5[3]	7.6 (2.2-13.1)[4]	7.58 (3.77-9.70)[4]	7.48 (3.8-9.9)[4]	NR
ALT, IU/L[1]	140.5 (114.3)[2]	141 (114.7)[2]	71.5 (44-108)[3]	NR	NR	92 (11-2241)[4]	139 (231)[2]	92 (17-2168)[4]	70 (8-2121)[4]	68(3-2631)[4]	NR	138 (21-4190)[4]	201 (27-2415)[4]	62 (14-839)[4]	67 (14-1077)[4]	NR
Cirrhosis	26/329 (8)	15/303 (5)	0[7]	57 (24)	52 (34)	202 (49)	38 (23)	0	102 (22)	74 (32)	NR	13 (14)	39 (16)	NR	NR	26(16)

[1]Unless otherwise specified; [2]Mean ± SD; [3]Median (interquartile range); [4]Median (range); [5]log10 copies/mL; [6]Mean ± SE; [7]Advanced fibrosis in 34%. ALT: Alanine transaminase; NR: Not reported; NUC: Nucleos(t)ide analogue; VIRGIL: Vigilance against viral resistance; ETV: Entecavir.

ing ETV (0.5 mg daily) for up to 4 years[31,32]. The cumulative rate of patients achieving HBV DNA undetectable levels was 90% by year 4. HBeAg seroconversion occurred in 53% of HBeAg positive patients, and of HBsAg positive patients seroconversion developed only in one patient (0.5%). Only one case of resistance (rt180M, rt204V, and rt184S/C/G/A) was reported in this cohort, representing a 0.6% cumulative resistance rate up to year 4[32]. This low rate, by comparison with previously mentioned results, probably evidences the less frequent occurrence of HBsAg loss (mainly if not exclusively reported in HBeAg positive patients) in genotypes B and C (the most frequent in Asia) compared with genotypes A and D (the most frequent in Europe)[21].

A retrospective analysis from Japan included 474 patients who received ETV treatment for up to 4 years[33]. The cumulative rate of patients achieving HBV DNA undetectable levels was 96% by year 4. HBeAg seroconversion occurred in 42% of HBeAg positive patients, and of HBsAg patients seroconversion developed only in one patient (0.2%). In this cohort, 5 patients showed virological breakthrough during the treatment period, including 2 patients who developed ETV-resistant mutations[33]. Two hundred and thirty NUC-naïve patients treated for up to 5 years were retrospectively evaluated in China[34]. Incremental increases were observed in undetectable HBV DNA rates, with

Table 2 Summary of efficacy results from real-life studies of entecavir in nucleos(t)ide analogue-naïve patients *n* (%)[21]

Study	Median follow-up (range)	Patients (*n*)	Cut-off (assay limit) (IU/mL)	HBV DNA undetectable[1]	HBeAg seroconversion[1,2]	HBsAg loss[1]
ORIENTE[22]	52 wk (46-53 wk)	190	50	115 (82)	12 (21)	2 (1)
VIRGIL[23]	19 mo (3-45 mo)	243	80	126 (74)	13 (15)	3 (1)
King's College cohort[24]	28 mo (NR)	154	12	NR	NR	NR
Italian cohort[25]	58 mo (2-80 mo)	418	12	93 (99)	527 (31 patients)	337 (15 patients)
Argentinean cohort[29]	181 wk (108-248 wk)	169	6	34 (100)	71 (68)	23 (14)
Hong Kong cohort[32]	3 yr (12-60 mo)	222	12	51 (90)	16 (53)	1 (0.5)[3]
Japan cohort[33]	2.37 yr (0.5-7.2 yr)	473	12	70 (96)	93 (42)	1 (0.2)
China cohort[34]	27.5 mo (3-73 mo)	230	100	NR	17 (15)	1 (0.4)
China cohort 2[35]	191 wk (1-233 wk)	1768	50	1327 (83)	NR	NR
Taiwan cohort[36]	144 wk	98	NR	93 (95)	5 (12)	0
Taiwan cohort 2[37]	25.3 mo (12-69 mo)	248	6	33 (82)	64 (28)	NR
United States cohort[38]	25 mo (6-68 mo)	169	100	75 (44)	12 (8)	NR
United States cohort 2[39]	36 mo	136	100	115 (85)	41 (30)	0
Australia cohort[40]	26 mo (3-46)	163	12	134 (82)	66 (43)	1 (0.6)

[1]Unless otherwise specified; [2]Median (interquartile range); [3]Advanced fibrosis in 34%.

100% being undetectable at 5 years of treatment. Fifteen percent achieved HBeAg/antiHBe seroconversion, and only one patient cleared HBsAg (0.4%). Only one patient developed ETV resistance mutations (rtL180M + rtT184A + rtM204V), and was subsequently treated with ETV+ADV combination therapy[34].

In a sub-study of the randomized, observational study of entecavir to assess long-term [(REALM) outcomes associated with nucleoside/nucleotide monotherapy for patients with chronic HBV infection] trial, 1768 NUC-naïve patients were treated with ETV in a 'real-world' clinical practice setting in China[35]. The preliminary results of the virologic efficacy and limited safety data were recently presented. At week 144, 84% of ETV-treated patients had HBV DNA undetectable levels. Unfortunately HBeAg and HBsAg clearance rates were not reported. Importantly, in this large cohort of patients prospectively followed, ETV demonstrated to be very safe with no serious adverse events reported. In Taiwan 98 patients were treated with ETV, in a study comparing its efficacy with LdT[36]. Short term treatment, up to 48 wk, showed HBV DNA was undetectable in 95% of patients and the HBeAg seroconversion rate was 27%. None of the patients achieved HBsAg clearance. No resistance was reported. In the real-world study "Taiwan Retrospective study of Entecavir Treatment: a Multicenter E Antigen positive Treatment-Naïve Trial of Chronic Hepatitis B" (TREATMENTCHB), 248 HBeAg positive patients were treated with ETV[37]. Undetectable serum HBV DNA levels were achieved in 52% (111/213), 79% (101/128), and 82% (33/40) of patients at 1, 2, and 3 years of treatment, respectively. Of 248 patients, 99 (40%) achieved HBeAg loss at the time of data analysis. The rate of HBeAg seroconversion was 28% (64/231; 17 missing data of antiHBe antibody). The cumulative rates of HBeAg loss were 20%, 38%, and 49% at years 1, 2, and 3 of treatment, respectively. HBsAg loss rate were not reported[37].

A retrospective cohort study was performed including 333 consecutive treatment-naïve HBeAg positive patients treated with oral NUC monotherapy with LAM, ADV, ETV, or TDF for up to 12 mo at three gastroenterology clinics in the United States, where 96% of the cohort were Asians[38]. One hundred and sixty nine of them received treatment with ETV. At the time of evaluation 44% achieved HBV DNA undetectable levels and the HBeAg seroconversion rate was only 8%. In the entire cohort, a total of 118 patients switched therapy during the course of treatment: 38 switched to combination therapy and 80 switched to alternative monotherapy. The HBeAg seroconversion rates improved with time, being 21% at year 2, 28% at year 3, 38% at year 4, and 38% at year 5. There is no data about ETV patients treated outcome. A subgroup of this study, those receiving only ETV was reported[39]. One and hundred thirty six patients received treatment for up to 36 mo. Complete viral suppression rates at months 24 and 36 were 66% and 85%, respectively. The cumulative HBeAg seroconversion rates were 20% at month 24 and 30% at month 36. No patients achieved HBsAg loss or HBsAg seroconversion in this study. The results from this study suggest that, unlike the majority of the studies reported, achieving HBeAg seroconversion in real-life settings appears to be much more difficult than in registration trial settings. In this case, it might be related to lower ALT levels. Also, the low rate of HBsAg loss in this predominately Asian cohort may be associated with the predominance of HBV genotypes B and C as previously mentioned.

A study from Australia included 163 NUC-naïve patients treated with ETV for up to 36 mo[40]. It showed that 134 patients (82%) achieved complete virological suppression (HBV DNA levels < 12 IU/mL). Authors reported that the annual HBeAg positive to negative seroconversion rate was 14%; after 36 mo 66 patients (43%) achieved this serologic endpoint. In this cohort only one patient (HBeAg negative) cleared HBsAg. A recent review also from Australia showed similar results: 81%-89% HBV DNA suppression rates[41]. Unfortunately they do not report HBeAg and HBsAg clearance rates.

Results from these 13 studies, including 4434 pa-

Table 3 Baseline characteristics of patients included in tenofovir studies *n* (%)[21]

Characteristic[1]	Study 103	Study 102	King's College Cohort	United States cohort	European cohort	German cohort
Reference	[14]	14	[24]	[38]	[42]	[43]
n	176	250	60	28	302	184
Age, yr (mean ± SD or range)	34 ± 11[2]	44 ± 11[2]	403	36 ± 9[2]	55 (19-80)	44 ± 14[2]
Male	119 (68)	193 (77)	30 (50)	16 (58)	222 (74)	127 (69)
Race			NR		NR	
White	92 (52)	161 (64)		1 (4)		140 (76)
Asian	64 (36)	63 (25)		27 (96)		
Other	20 (11)	26 (10)				
Region	Europe 55%, North America 27%, Australia and Asia 18%	Europe 63%, North America 21%, Australia and Asia 16%[4]	Europe	North America	Europe	Europe
Genotype			NR		NR	NR
A	94 (27)	33 (10)				
B	68 (19)	46 (14)		18 (65)		
C	111 (31)	57 (18)		10 (35)		
D	55/173 (32)	156/243 (64)				
HBeAg negative	0 (0)	250 (100)	46 (77)	0 (0)	241 (80)	127 (69)
HBV DNA, log10 IU/mL[1]	8.64 (1.076)[2,5]	6.86 (1.31)[2,5]	4.2 (0.3)[6]	7.74 (3.34-8.66)[3]	5.9 (1.4 -> 9)[3]	6.9
ALT, IU/L[1]	142 (102.81)[2]	127.5 (101.21)[2]	NR	52.5 (8-468)[3]	88 (11-3733)[3]	
Cirrhosis	34/172 (20)	47/250 (19)	14 (23)	NR	105 (35)	20 (11)

[1]Unless otherwise specified; [2]mean ± SD; [3]median (range); [4]Australia or New Zealand; [5]log10 copies/Ml; [6]mean ± SE. ALT: Alanine transaminase; NR: Not reported; HBeAg: Hepatitis B e antigen.

Table 4 Summary of efficacy results from real-life studies of tenofovir in nucleos(t)ide analogue-naïve patients *n* (%)

Study	Median follow-up (range)	Patients (*n*)	Cut-off (assay limit) (IU/mL)	HBV DNA undetectable[1]	HBeAg seroconversion[1,2]	HBsAg loss[1]
King's College cohort[24]	12 mo	60	12	33 (76)	2 (7)	0
United States cohort[38]	12 mo (6-23 mo)	28	100	23 (82)	7 (5)	0
European cohort[42]	33 mo (0-66 mo)	302	12	91 (97)	18 (36)[3]	8 (13)
German cohort[43]	24 mo	184	69	170 (92)	NR	NR

[1]Unless stated otherwise; [2]among those hepatitis B e antigen (HBeAg) (+) at baseline; [3]Kaplan–Meier estimate. NR: Not reported; HBV: Hepatitis B virus; NR: Not reported; NUC: Nucleos(t)ide analogue; HBsAg: Hepatitis B surface antigen.

tients, showed that ETV is as effective in clinical practice as in clinical trials. Extending treatment duration is associated with increasing rates of HBV DNA complete suppression, HBeAg seroconversion and HBsAg loss. Different response rates between studies, mainly regarding serological response, may be associated with particular virological and host factors of each geographic region.

Tenofovir

There are fewer studies published with TDF than with ETV. Also, the population included in these studies tended to be heterogeneous, patients were treated for different periods of time (generally for shorter periods of time than with ETV) (Table 3), and may also be more reflective of the treatment population and the real efficacy and safety of the drug[22]. Results from these studies are discussed in the following section and summarized in Table 4.

The King's College Cohort from London (already discussed in the ETV section) included 60 patients receiving first-line TDF treatment[24]. Since TDF was approved after ETV, these patients received a shorter duration of treatment at the time of the analysis (9 mo compared with 28 mo). At 12 mo of treatment, 76% of TDF treated patients cleared HBV DNA, 7% achieved HBeAg seroconversion and no patient cleared HBsAg. In another previously mentioned study, 333 consecutive treatment-naïve CHB patients treated with oral NUC monotherapy with LAM, ADV, ETV, or TDF for up to 12 mo were evaluated at three gastroenterology clinics in the United States[38]. Twenty eight of them received treatment with TDF. At 12 mo of treatment, 82% of TDF treated patients cleared HBV DNA, 5% achieved HBeAg seroconversion and no patient cleared HBsAg.

Two large studies evaluating TDF use in clinical practice were recently reported[42,43]. A multicentre cohort study conducted at 19 European centres retrospectively and prospectively monitored 302 NUC-naïve patients followed for a median of 33 mo and the 3 years follow up study was presented[42]. Virological response rates increased over time from 84% at year 1 to 95% at year 3 in the overall population, from 66% to 86% in HBeAg positive patients and from 74% to 98% in HBeAg negative ones. The cumulative probability of HBeAg seroconversion steadily increased to 36% at year 3, with 8 patients

(13%) clearing HBsAg, and 5 of these stopping TDF successfully. Virologic breakthrough was reported in 2% of patients, with no potentially resistance-associated mutations identified to date. A prospective observational study including 400 TDF-naïve patients was performed in Germany and the 2 year data is available (GEMINIS study)[43,44]. Forty-six percent of the patients (n = 184) were NUC-naïve. At the time of evaluation, overall 92% of NUC-naïve patients achieved HBV DNA undetectable levels, 81% of HBeAg positive and 91% of HBeAg negative patients; 20% achieved HBeAg seroconversion, and there was 5% loss in HBsAg in HBeAg positive patients. No virologic breakthrough and no resistance have been reported to date.

SAFETY AND TOLERABILITY IN CLINICAL PRACTICE

Entecavir

ETV should be administered on an empty stomach (at least 2 h after a meal and 2 h before the next meal) and is generally well tolerated. The most commonly reported treatment related adverse events in phase Ⅲ clinical trials were headache, fatigue, dizziness, and nausea at comparable rates to LAM[7,8]. In the ETV-901 rollover study 1051 patients were enrolled from 10 prior Phase Ⅱ/Ⅲ studies and were treated with ETV for at least a 5 year period[13]. Most of the reported adverse events (AEs) were mild to moderate, 19% were grade 3-4 events, with only 4% of them possibly related to ETV. These grade 3-4 AEs were myalgias (5%), neuropathy (hypoparesthesia and hyperparesthesia, polyneuropathy) (4%), increased lipase (2%), increased serum creatinine (< 1%), increased serum lactate or decreased serum bicarbonate (< 1%), hypophosphatemia (< 1%), muscular weakness (< 1%), pancreatitis (< 1%) and creatinine phosphokinase elevation (< 1%)[45,46]. It was reported an overall discontinuation rate due to AEs was extremely low (< 1%).

Are these results from phase Ⅲ trials applicable to treatment in real life settings? After reviewing the experience from these studies (including 4434 patients), it seems that the ETV Safety profile in clinical practice is consistent with those of Phase Ⅲ studies, in that no major safety issues or serious side effects have been reported to date[21-25,29-40,44]. As a controlled trial, patients have been carefully selected in order to be able to be included. Excluded patients usually have advanced liver diseases or comorbidities. The latter commonly require administration of concomitant medications. The addition of different medications may have an important impact upon study drug pharmacokinetics, efficacy and safety. The strict inclusion criteria of these studies did not allow testing unexpected adverse events due to drug to drug interactions, nor potential toxicity in patients with advance liver disease. For this reason, studies reporting results in "real life" are necessary to add information to controlled clinical trials reports[44].

There are some safety concerns when using the newer NUCs in CHB cirrhotic patients. Lactic acidosis (LA) with ETV was first reported in 2009. Five of 16 HBV cirrhotic patients treated with EVT developed lactic acidosis. One of the patients died, and the other 4 recovered after treatment discontinuation. A significant correlation between the MELD score and the development of lactic acidosis was observed (P = 0.002). The single components of the MELD score - bilirubin, INR, and creatinine - also correlated with the development of lactic acidosis (P = 0.003, P = 0.003, and P = 0.008, respectively). LA developed in patients with more severe liver dysfunction (MELD score > 20)[45]. There were no cases of LA reported in the ETV 901 study or in the clinical practice studies, considering that 8% to 49% of patients included were cirrhotics[22-26,30-41]. A recent study using ETV and/or TDF in compensated or decompensated HBV cirrhotic patients in real-life clinical practice demonstrated that both drugs can be safely used in this subgroup of high risk patients[46]. Safety of ETV in decompensated HBV cirrhotic patients was confirmed in an open label study[47]. This data suggested that ETV can be used, but should be applied cautiously, in patients with severe decompensated liver disease. As per reported NUCs preclinical data, a usual concern with the long term administration is their potential carcinogenicity. After a 5 year period of ETV administration, only 3 cases of the novo non liver neoplasms were identified: two gastric and one pancreatic adenocarcinoma[13,44]. However, to date, there is no evidence for the occurrence of cancers as a result of ETV treatment in patients. A global phase Ⅳ study (the REALM study), preliminary results of which were discussed above[35], is continuing to address this safety concern in patients treated with ETV during a 10-year follow-up period.

Tenofovir

In phase Ⅲ trials the overall incidence of AEs was comparable in patients receiving TDF *vs* ADV[14]. The most common AEs in both studies included headache, nasopharyngitis, back pain, nausea, and fatigue. Nephrotoxicity may be a potential concern with TDF, based on evidence from post-marketing surveillance of patients receiving TDF for HIV infection, but so far the problem appears to be less evident in patients with HBV infection[22,43]. Results from the long term follow up of phase Ⅲ studies have been recently presented[20]. At year 6, less than 2% of patients discontinued TDF due to an adverse event, and less than 1.5% experienced a confirmed renal event (≥ 0.5 mg/dL increase in serum creatinine from baseline, phosphorus < 2 mg/dL, or CrCL < 50 mL/min)[21]. The use of tenofovir has been associated with greater loss of bone mineral density during the early months of therapy in HIV monoinfected patients, although no HBV monoinfected patient experienced bone fractures in these studies[16-20]. Bone mineral density (BMD) remained stable from year 4 through year 6, for both hip and lumbar spine[20]. Recent data suggests that in HBV monoinfected patients, bone mineral loss

might be related to vitamin D deficiency and no to TDF treatment[48]. Nevertheless, BMD should be periodically evaluated in HBV patients taking TDF[44]. Safety data collected from the European cohort study concerning TDF were generally consistent with the long term clinical study safety data[42].

Median serum creatinine, eGFR and phosphorus blood levels remained unchanged over time. Approximately 2% of the patients showed > 0.5 mg/dL increase of serum creatinine or < 2 mg/dL phosphorus or proteinuria. The proportion of patients with eGFR < 50 mL/min by MDRD increased from 3% at baseline to 6% at the end of the study. TmPO4/GFR ratio, a marker of urinary phosphate reabsorption, was reduced in nearly 20% of the patients at baseline and in nearly 30% during follow-up. TDF doses were reduced to 300 mg/48 h in 10 patients (3%, decline of eGFR in all) and discontinued in an additional 9 patients (3%, renal-related events in 2 cases). Overall, 5% stopped TDF (HBsAg loss in 5, adverse events in the remaining 11)[42]. In the GEMINIS study serum creatinine clearance and phosphorous levels remained stable. No frequent AEs were reported. Four renal events were detected, all in NUC-experienced patients (prior long-term LAM +/− ADV therapy) with comorbidities: diabetes (2 patients), renal insufficiency (2 patients), and cirrhosis (1 patient)[43].

A French multicentre prospective cohort (Vireal study) evaluated the tolerance of TDF treatment in a real life cohort, including elderly patients with comorbidities[49]. Unfortunately, 58% of the 441 HBV patients treated were NUC-experienced or resistant and were not included in the review of the virological response discussed above. The 2-year data reported no major safety issues. Forty-eight elderly patients were subsequently analyzed: mean age 71 ± 6 years, 73% male, 87% HBeAg-negative, 58% advanced fibrosis and 79% treatment experienced. Although 82% of elderly had prior GFR < 90 mL/min (estimated by CKD-EPI formula), GFR remained stable or improved in 91%. The mean GFR was 73, 69 and 70 mL/min at baseline, 1 and 2 years. This study showed that TDF safety and tolerance were similar in elderly and younger patients[49]. Also, TDF can be safely used in patients with mild renal impairment. A prospective, randomized, double-blind trial of TDF *vs* emtricitabine (FTC)/TDF combination in LAM-resistant patients compared mild renal impairment (MRI; CrCL 50 ⩽ 80 mL/min by Cockroft-Gault) patients (74/280; 26%) and normal renal function (NRF; CrCL ⩾ 80 mL/min) patients (206/280; 74%)[50]. No patients had a confirmed increase in serum creatinine of ⩾ 0.5 mg/dL, and 1% (2-NRF) had transient phosphorus < 2 mg/dL. Nine MRI patients had CrCL < 50 mL/min (pre-treatment range: 49-61 mL/min) that stabilized with dose adjustment. No differences were observed in percentage change in spine or hip BMD over 96 wk, and no clinically relevant bone loss was noted in either group. The safety of patients with MRI receiving TDF was similar to NRF patients; in MRI patients there was no evidence of increased risk for renal- or bone-related complications[50]. In TDF treated patients serum chemistries, including creatinine and phosphorus, should be monitored every 6 mo. Monitoring may be more frequent in patients with impaired baseline renal function or other medical conditions that increase the risk of renal failure[51].

TDF was also used in HBV patients with decompensated liver disease in a phase Ⅱ double-blind randomized study[52]. TDF, alone or in combination with FTC, was demonstrated to be safe in this population. As previously mentioned, TDF was safe when used in this group of patients in real-life clinical practice[46].

PREDICTORS OF RESPONSE IN CLINICAL PRACTICE

The factors that determine the likelihood of achieving a virological and/or serological response are called predictors of response. They can be classified as viral or host related, or as baseline or on-treatment depending on the time point of evaluation. Many viral and host factors affect treatment response, and not achieving the desired response might be related to a combination of them. Before initiating treatment, it is useful for patients and physicians to know the likelihood of achieving a response, so that they can decide whether treatment benefits outweigh its costs and its risks. Also, predictors may be helpful to guide the continuation of antiviral therapy[4].

In CHB therapy, some baseline and on-treatment predictors of subsequent response have been identified. These factors are stronger predictors of treatment outcomes and are more useful for IFN/PegIFN based than for NUCs based therapies[4]. Predictors of response for the existing NUCs at various time points vary for different agents. In HBeAg positive patients, baseline factors predictive of antiHBe seroconversion are low viral load (HBV DNA below 2×10^8 IU/mL), high serum ALT levels, and high activity scores on liver biopsy[4]. HBV genotype does not influence the virological response to any of the available NUCs[4,53]. Virological response (undetectable HBV DNA) at 24 wk during treatment with LAM or LdT and at 48 wk during treatment with ADV is associated with a lower incidence of resistance, *i.e.* an improved chance of maintained virological response in both HBeAg positive and HBeAg negative patients and with a higher chance of antiHBe seroconversion in HBeAg positive patients[4]. A decline of HBsAg, HBeAg and HBV DNA levels during NUC treatment in HBeAg positive patients may identify cases with subsequent HBeAg or HBsAg loss[4,54-56].

Evaluation of predictors of response tends to be difficult in real world studies since patients' characteristics are heterogeneous and treatment duration and parameters evaluated may vary between studies. But there are some data reported with ETV treatment in the studies cited above, and unfortunately none of the TDF studies reported predictors of response. In the

ORIENTE study, virological response in the HBeAg positive patients at week 12 correlated significantly with antiHBe seroconversion rate at week 48: odds ratio for this correlation at week 12 was 8 (95%CI: 1.17-54.5, $P < 0.05$). This correlation was also observed at weeks 24, 36 and 48 ($P = 0.003$, 0.002 and 0.017, respectively)[22]. In the single centre study from Italy, the presence of cirrhosis (OR = 1.730, 95%CI: 1.082-2.766, $P = 0.022$) and absence of HBeAg at baseline (OR = 0.479, 95%CI: 0.273-0.842, $P = 0.011$) were independent predictors of earlier clearance of serum HBV DNA[26].

In our study from Argentina, baseline HBV DNA $\geqslant$ 7 $\log_{10}$ IU/mL (HR = 9.40, 95%CI: 3.46-25.54, $P < 0.001$) and Metavir A score $\geqslant$ 2 (HR = 2.48, 95%CI: 1.39-4.40, $P = 0.002$) predicted HBeAg clearance in ETV treated patients[29]. Being HBeAg positive at baseline (HR = 11.1, 95%CI: 0.96-128, $P = 0.053$) and HBV DNA clearance before week 48 (HR = 7.76, 95%CI: 0.96-62.4, $P = 0.054$) tended to predict HBsAg seroclearance, but they were not statistically significant[29]. In the Hong Kong cohort baseline HBV DNA levels $\geqslant$ 8 $\log_{10}$ copies/mL and undetectable HBV DNA levels at week 24 were associated the higher possibilities of achieving undetectable HBV DNA at year 3 of ETV treatment[31]. In the Japan cohort, HBV DNA levels < 7.6 $\log_{10}$ copies/ml (OR = 15.8, 95%CI: 43.1-79.9, $P = 0.001$) predicted HBV DNA undetectable levels after 3 years of ETV treatment[33]. Serum albumin < 3.5 g/dL (RR = 2.0, 95%CI: 1.1-3.6, $P = 0.019$) was the only significant determinant of HBeAg seroconversion[33]. In the Chinese study, high baseline HBV DNA levels (OR = 0.532, 95%CI: 0.315-0.896, $P = 0.018$) and virological non-response at week 24 (OR = 6.093, 95%CI: 2.099-17.685, $P = 0.001$) to ETV monotherapy were the independent risk factors for a partial virologic response at 1 year[34]. In the TREATMENT CHB study form Taiwan, baseline ALT > 5-times ULN (HR = 1.810, 95%CI: 1.062-3.085, $P = 0.001$) and baseline HBV-DNA level (HR = 0.812, 95%CI: 0.700-0.942, $P = 0.014$) were independent factors associated with HBeAg loss in ETV treated patients[37]. In the Australian cohort, patients with baseline DNA levels < 10^8 $\log_{10}$ IU/mL *vs* > 10^8 $\log_{10}$ IU/mL ($P = 0.001$) and HBeAg negative patients ($P = 0.001$) achieved more rapidly complete virological suppression[40].

In summary, baseline HBV DNA levels and HBeAg status appeared to predict HBV DNA clearance and HBeAg clearance/seroconversion in clinical practice. There is little information about predictors of HBsAg clearance/seroconversion. There is no information about how HBsAg and/or HBeAg baseline and on-treatment levels impact on treatment response in real life.

CONCLUSION

Is ETV or TDF treatment effective in clinical practice? Can the results observed in CT be extrapolated to clinical practice? Efficacy is the ability of a drug or intervention to produce an effect under optimal conditions, whereas effectiveness is its usefulness in routine practice[57]. Clinical trials differ in many ways from clinical practice, and many patients treated in clinical practice would have been excluded from these trials. This is the main reason why studies performed in routine clinical practice provide useful information for the treating physician. This review shows that ETV and TDF used in clinical practice have similar response rates when compared with CT, with low rates of resistance and favorable safety profiles.

Studies performed in clinical practice have some limitations when compared with CT. They are, in most cases, retrospective; the treatment protocol is not standardized; adverse events may be under-reported since there is no strict register of safety parameters; and they include a variable number of patients. These treatments were conducted at referral centres by highly trained specialists with experience in the field who have participated in CT. This ensures treatment effectiveness, but tends to exclude less experienced investigation centres. Another concern is that patient compliance to these long term treatment regimens may be poorer and less controlled than compliance to short term strictly monitored treatments in CT. Most of the studies reviewed show a low rate of patients lost to follow up and a low rate of non-adherence[58]. Even if adherence is not strictly evaluated in these types of studies, it can be assumed that if there is a low rate of virological breakthroughs and resistance, adherence has to be good to maintain treatment responses.

Despite the pros and cons, studies performed in real life conditions represent everyday practice and add important information about long term treatment effectiveness and safety in this clinical setting. This review shows that patients treated with first line NUCs at referral centres, with good clinical follow-up and adherence to international guidelines, can achieve high treatment response rates with a very low rate of adverse events.

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TOPIC HIGHLIGHT

WJG 20th Anniversary Special Issues (9): Hepatitis B virus

Hepatitis B virus infection in Latin America: A genomic medicine approach

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Abstract

Hepatitis B virus (HBV) infection is the leading cause of severe chronic liver disease. This article provides a critical view of the importance of genomic medicine for the study of HBV infection and its clinical outcomes in Latin America. Three levels of evolutionary adaptation may correlate with the clinical outcomes of HBV infection. Infections in Latin America are predominantly of genotype H in Mexico and genotype F in Central and South America; these strains have historically circulated among the indigenous population. Both genotypes appear to be linked to a benign course of disease among the native and mestizo Mexicans and native South Americans. In contrast, genotypes F, A and D are common in acute and chronic infections among mestizos with Caucasian ancestry. Hepatocellular carcinoma is rare in Mexicans, but it has been associated with genotype F1b among Argentineans. This observation illustrates the significance of ascertaining the genetic and environmental factors involved in the development of HBV-related liver disease in Latin America, which contrast with those reported in other regions of the world.

Key words: Genomic medicine; Hepatitis B virus; Hepatitis B virus genotypes; Latin America; Mexico; Central America; South America

Core tip: We explore the influence of genetic and environmental factors that may participate in the clinical outcome of hepatitis B virus (HBV) infection among the Latin American population. Such features may be of interest to clinicians and scientists in the field of hepatology because this population differs importantly from others worldwide. A novel genomic medicine approach is required to implement new strategies for the prevention, management and treatment of HBV infection.

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INTRODUCTION

Hepatitis B virus (HBV) is a globally distributed human pathogen that can cause life-threatening liver disease such as chronic hepatitis, liver cirrhosis and hepatocellular cancer[1]. At least 350 million people worldwide contribute to the large reservoir of chronic carriers, especially in developing regions, which may now be facing epidemiological shifts due to immigration[1,2].

The study of the HBV reflects many of the chronological stages of scientific and technological development in the field of medical virology[3]. During the immunological phase, the discovery of the hepatitis B surface antigen protein (HBsAg)[4] supported worldwide serological testing for HBV infection; later, the use of DNA amplification and sequencing tools marked the era of molecular epidemiology to test for viral genomes and genotypes, respectively[3]. Most recently, the Bayesian coalescent and phylogeographic framework[5] coupled with bioinformatics and specialized software is rapidly contributing new data regarding the geographic origin and spread of HBV infection throughout different populations globally[6].

Significant progress has also been made in regards to understanding the epidemiology, virology, natural history and therapy of HBV infection[3]. Moreover, novel prevention, management and treatment strategies should now be studied with a genomic medicine approach. Herein, we consider that the interrelationship between genetic and environmental factors involved in the development of human diseases should be based on the features of each population[7,8]. Hence, HBV infection is a particularly suitable candidate to examine such an approach.

This paper provides a critical view of why the application of genomic medicine is required for the study of HBV among the Latin American (LA) population. This complex ethnic group, which arose from the mixture of Native American, Caucasian and African lineages, presents a combined distribution of HBV genotypes from the Old and New World, which may have an impact on the clinical outcomes of and treatment strategies for HBV infection.

WALKING WITH THE HEPATITIS B VIRUS

Origin and diversification of HBV

It is plausible that viruses have accompanied humans since their emergence on planet earth[9,10]. Within their genetic information and replication cycles may lie the history of key events in the diversification of all living creatures[11]. When did viruses first originate? It is an exciting question that still inspires researchers to develop new methods for genetic sequence analysis[12-14]. For example, the hepadnaviridae family is divided into two genera, orthohepadnavirus and avihepadnavirus, based on their genomic similarities and hosts[15]. Additionally, the Hepadnaviruses have the ability to integrate their genome into the host's genome[16,17]. This feature is useful for paleovirologists to identify ancient endogenous viral elements and estimate how long they have coexisted with their host[18-20]. Interestingly, rock and sediment fossils are crucial to date the age of any plant or animal species, while in some viruses, fossil information can be found in contemporary cells' genomes. The genus Avihepadnavirus primarily infects birds, and it is estimated that it emerged in the Mesozoic era, 65 million years ago[20], when mammals lived in the shadow of the dinosaurs[21]. In contrast, the genus *Orthohepadnavirus* affects mammals[15]. This genus includes the human hepatitis B virus, one of the most important etiological agents of viral hepatitis in the world[22]. The HBV genome is approximately 3.2 kb, made of partially doubled stranded DNA confined within a fenestrated nucleocapsid (core protein) enveloped in a membrane containing three surface antigen proteins (large, medium and small HBsAg)[15,23]. HBV genome replication is carried out by a viral DNA polymerase without DNA proof-reading capacity[23], which contributes to the high genetic diversity of HBV.

Based on their genomic divergence, HBV is classified into eight genotypes, designated A through H. New information has been gathered that suggests the existence of genotypes I and J[24]. However, the origin of HBV still remains unclear[25] due to the lack of consensus on the estimated evolutionary rates for HBV and the inconsistency between these data and archeological evidence[26]. Recently, Paraskevis *et al*[6] estimated that diversification among the different genotypes may have occurred 33000 years ago. Thus, by adapting these results to the hypothesis that HBV came from Africa[6,25,27], it is plausible that the most recent common ancestor (MRCA) of all HBV genotypes traveled together with humans from Africa to their arrival in Beringia. Archaeological and genetic evidence show that modern humans originated 200000 years ago[28]. In addition, it has been estimated that humans began to migrate out of Africa 100000 years ago, traveling through Israel, India, China, Australia, Europe and Russia before reaching the limits of the old continent[29]. Later, the expansion was detained by the glacier of the Beringia region for approximately 36000 years[30]. In this region, the pre-Amerindians faced an extremely hostile climate that reduced their population from 9000 to approximately 3200 people, who then crossed the strait to the Americas[30]. In the last 20000-13000 years, humans have dispersed to Alaska, the continental United States, Mexico and Central and South America[18,31-35]. The ancestral population of Greenland may have been the last to cross over to the new continent, roughly 5500

years ago[36].

From Africa to South America, man was exposed to diverse climates, geographic altitudes, foodstuffs and pathogens. Consequently, due to host-environment interactions, inhabitants may have undergone region-specific genotypic and phenotypic adaptations[37,38]. Examples include the anatomical structure of the Eskimos[39,40], the height and pigmentation of the African population[41], tolerance of hypoxic conditions in the Tibetans[42,43] and adaptations to varying diets[44] and infectious agents[45,46]; these adaptations allowed them to survive. Based on such features, it is likely that each new human settlement carried an HBV strain with its own MRCA, which may have developed specific adaptations to its host, allowing it to survive and spread efficiently through its autochthonous population. These changes are reflected at the genomic level, giving rise to what we now know as genotypes.

It is estimated that the first genotype to diversify was C, followed by B, D, A, F, E, H and G[6]. HBV genotypes A through E are predominant among Old World populations. Genotypes B and C are predominant in Asia; genotype D in Africa, Europe and India; and genotype A in sub-Saharan Africa, North Africa and Western Europe; genotype E is restricted to East Africa[47]. Furthermore, genotype G may be the most recent, with an estimated time of MRCA of 800 years[6]; however, because its genome contains fragments of other genotypes[48,49] and the number of complete sequences studied is limited, further research is required to determine its geographic origin[8]. Nonetheless, genotype G is restricted primarily to populations of men who have sex with men in America[8], suggesting that these patients play a key role in the spread of HBV to other parts of the world[50-53].

Genotype H is predominant in Mexico[7,8], while genotype F prevails in Central and South America[54,55]. Phylogeny tests performed by the Neighbor-Joining and Maximum Likelihood methods group these genotypes as "sisters", near the root of many phylogenetic trees[56-60] due to their strong genetic similarities. Thus, by this methodology, genotypes F and H belong to a monophyletic clade and can be considered direct descendants of the ancestor of all human hepatitis B genotypes. Because they share common epidemiological and transmission routes, such features may be of medical relevance to the clinical outcome and response to treatment among human populations in Mexico and Central and South America.

Human adaptations to HBV

Based on their historical background and types of infection[61], human populations have developed a wide spectrum of adaptations to HBV. From an evolutionary perspective, "adaptation" is defined as all changes that increase the success of the survival of an organism[62]. Applying this concept to HBV, we may consider three levels of the adaptation process: incomplete, semi-complete and complete; these classifications may be related to the clinical outcomes of HBV infection in humans.

Incomplete adaptation: Incomplete adaptation events may be exemplified in patients who develop fulminant hepatitis[63], wherein a hyperimmune response to viral antigens may lead to the deterioration of liver function, severely compromising the patient's life[64,65]. This is an inefficient state for the survival of HBV, which depends on the host to exist. Viruses exhibiting incomplete adaptation may be those that recently crossed the species barrier[66]. Alternatively, they may arise in the circulating population in the form of core and pre-core gene mutants, which enhance the encapsidation of virions and in some cases may cause fulminant hepatitis B[67-69]. In general, the frequency of fulminant hepatitis is approximately 0.1% to 1%[70,71], suggesting that the majority of HBV carriers have other forms of adaptation.

Another mechanism of incomplete adaptation, which is beneficial to the host, may occur when HBV DNA genomes are eliminated by an efficient and coordinated immune response[72]. In this type of infection, Th1 cytokines (IFN-γ, IL-2, TNF-α) quickly clear HBV DNA by means of an optimal polyclonal response against the viral antigens[73]. Then, T cell ($CD4^+$ and $CD8^+$) activity fights infection by cytolytic and non-cytolytic mechanisms[73,74]. This type of response may annihilate HBV; however, if all human populations responded in the same manner, today's HBV would not have achieved such a broad distribution.

Semi-complete adaptation: Semi-complete adaptation can be exemplified in patients with chronic hepatitis B infection. In this group, it is a common characteristic to detect HBV DNA and HBsAg after more than six months[75]. It is likely that an inadequate immune response and HBV evasion mechanisms are responsible for chronicity[74]. This type of infection does not immediately compromise the host's life, allowing hepatitis B virions to fulfill their life cycle and transmit to the susceptible population. Nevertheless, prolonged exposure to chronic infection is associated with the development of fibrosis and cirrhosis[22,75]. Additionally, the HBx protein has been related to the development of hepatocellular carcinoma by interacting with different signaling pathways[76], preventing DNA repair[77] and modulating the cell cycle[78]. There are 350 million people suffering from chronic HBV infection[71], many of whom are part of the Asian population, which harbors genotypes B and C and has a higher risk for hepatocellular carcinoma (HCC)[79]. This situation contrasts with the clinical presentation of HBV genotypes F and H[7,8] and may indicate that an important majority of the human population presents semi-complete adaptation; thus, in the long run, some HBV genotypes may be more aggressive than others.

Complete adaptation: Finally, complete adaptation comprises what is known as occult hepatitis B (OHB). In this scenario, infections are characterized by low viral loads (< 200 IU/mL) and the absence of HBsAg[80]. It is likely that the ability to integrate the HBV genome

into the host's cell[79], the stability of the cccDNA and immune tolerance[81] achieve a balance between the rate of viral replication and tissue damage, conferring a homeostatic state between the human host and the specific HBV genotype. Under this hypothesis, complete adaptation may allow patients to be asymptomatic for many years[82]. However, other viral infections (*i.e.*, HIV or HCV)[83], intravenous drug use, immunosuppression, antiviral therapy and even massive vaccination may break the equilibrium state, triggering the onset of symptoms. Additionally, these environmental "stress" factors may provoke genetic diversification, thus enhancing viral fitness and survival.

Finally, HBV could have accompanied humans from their origin to their arrival in South America, developing variable degrees of mutual adaptation depending on the degree of endemicity, HBV genotype, extent of exposure and genetic background of the human population. Thus, the clinical manifestations and outcomes of HBV infection may reflect the degree of adaptation between the human host and their specific viral genotype companion.

HBV IN LATIN AMERICA

The Latin American and Caribbean region encloses the Spanish, Portuguese and French-speaking countries of the American continent and covers almost 22000000 km^2. It includes Mexico, the islands of the Caribbean and Central and South America, which possess a rich cultural and natural heritage[84]. In this region, HBV endemicity ranges from low to high with at least 7 to 12 million people infected[85,86]. In the following section, we offer an overview of the relationship between the genetic backgrounds of LA hosts and HBV genotypes, both endemic and foreign, with an eye to the clinical outcomes of HBV infection.

Human population

The peopling of the Americas, and consequently the origin of the present-day LA population, is an active topic of research. In a recent population genetics study, Reich *et al*[87] proposed that Native Americans descend from at least three streams of Asian gene flow. One stream of ancestry comprises the Native American descendants that came from the homogenous "First Americans", who crossed the Bering Strait more than 15000 years ago (range, approximately 40000-15000 years). Two additional Asian lineages were detected among the Eskimo-Aleut speakers and Na-Dene-speaking Chipewyan, with a subsequent admixture between First Americans and the following streams of aforementioned Asian migrations[87]. The nomadic lifestyle of these people and the climatic changes allowed for their widespread expansion throughout the continent in a southward direction[87].

The establishment of human populations in continental Latin America began mainly in the northern part of Mexico. This dry land region, known as Aridoamerica, was inhabited by small and isolated semi-nomadic groups[88], while Mesoamerica, in central-south and southeast Mexico, with its extraordinary natural resources, especially in the Mexican Basin, invited the nomadic hunter-gatherers to become sedentary societies[87,88]. Anthropological evidence dates the earliest settlers in Mexico as far back as 30000 years, during the Lithic Period[89]. The climatic conditions and environment of Mesoamerica allowed the domestication and earliest cultivation of plants (7000-5500 BC), which resulted in the eventual emergence of agriculture systems. The growth of sedentary societies during the Pre-classical period (2200 BC) flourished in the Classical (150-900 AC) and Post-classical (900-1519 AC) period with the subsequent increase in population density[89].

The size of the most developed population, in Tenochtitlan, the Aztec capital city, at the time of the Spanish conquest was 25000000, which declined drastically to 5000000[90] due to warfare, overwork and the presence of epidemic diseases, thereby allowing the wide expansion and settlement of Europeans that gave rise to the initial genetic and cultural admixture[89,90]. The Spaniards later brought slaves from several regions of Africa and further admixture occurred[88]. These historical processes, in conjunction with successive colonization and industrial development from the 16th to the 18th century, brought other foreign settlers; in recent years, ongoing migration has come to shape the present-day gene pool of the Mexican population.

Several genome-wide analyses[87,88,91,92] have demonstrated that the Mexican population still retains its Native American ancestry, with the degree of Amerindian ancestry increasing from north to south (38%-76%) and a proportional decrease in European ancestry (50%-8.5%); African ancestry remains relatively low throughout the population (9%-18%)[92,93]. Likewise, most of the population of Central and South America underwent the same pattern of socio-demographic transformation as the Amerindian ancestry, which led to the heterogeneous distribution of the admixture background of LA. For example, the population of Argentina displays predominantly European ancestry (78%), followed by Amerindian (19%) and African ancestry (3%), although the precise proportions may differ according to the studied subpopulations[94]. Brazil and Colombia show similar proportions of European (71%) followed by the Amerindian ancestry (18%-19%), and the African ancestry (10%-11%)[95].

Epidemiology of HBsAg seroprevalence

HBV infection in Latin America has a heterogeneous distribution when estimated by the HBsAg serological marker. According to the World Health Organization (WHO), the majority of LA countries are considered low seroprevalence (< 2%) regions, including Mexico, Honduras, Nicaragua, Costa Rica, Panama, Cuba, Paraguay, Uruguay, Chile, Argentina, Peru and north Colombia. Regions with intermediate seroprevalence of HBV

infection in Central America are Guatemala, Belize, El Salvador, Honduras, Haiti the Dominican Republic and Puerto Rico (> 2.0%-< 8.0%). In South America, some countries or regions are classified as having intermediate endemicity, such as Ecuador, Venezuela, Guyana, Surinam, French Guyana and the south of Brazil; whereas Peru, south Colombia, northern Bolivia and northern Brazil are known for their high seroprevalence (HBsAg > 8%)[2,22,96,97]. However, WHO reports are primarily estimates that require updated feedback obtained by national, large-scale epidemiological studies, which are not commonly carried out in developing countries, including those of Latin America. For example, the introduction of HBV vaccination campaigns among members of the WHO has diminished its prevalence in several regions of Latin America[86].

Interestingly, countries that have a pattern of low endemicity for HBsAg may have a higher prevalence of anti-HBc, a marker of past or ongoing infection[98,99], suggesting that exposure to HBV infection may be higher than previously estimated by HBsAg alone. Additionally, it is unlikely that a given prevalence reported by the WHO is applicable to all regions or risk groups in a given country (*i.e.*, rural and urban areas, or native and mixed race populations). Furthermore, despite advancements in the sensitivity thresholds for HBsAg testing, many commercial kits have their limitations; thus, the surface antigen protein may be undetected[98,99]. Furthermore, among special populations, precautions should be taken in patients with comorbidities related to overt immunosuppression, such as cancer or HCV/HIV coinfections, as well as in cases of obesity and alcoholism, which may indeed mask HBV infection in the form of OHB[7,8]. Thus, given that Latin America is a diverse region and that the aforementioned situations are likely to be encountered, it may be advisable to proceed with further HBV testing by using the anti-HBc marker, as well as nucleic acid testing, because HBsAg provides only one view of the status of infection.

Molecular epidemiology and phylogeography

As previously mentioned, diagnostic tests based on molecular techniques, either manual or automated, to ascertain HBV genomes have revolutionized the clinical management of HBV infection worldwide. It is now a standard practice to use them to confirm serological testing, to decide who and when to treat, and during the follow-up of antiviral treatment. These guidelines are recommended by several liver associations worldwide. Paralleling these advancements is the gradual appearance of research in the field of molecular epidemiology[100,101] and phylogeography[58,102-104] of HBV genotypes in Latin America.

At the beginning of the molecular epidemiology era in Latin America, it was clear that the HBV genotypes F and H were the indigenous genotypes[54,57,105], while the incidence of the HBV genotypes A and D within this region was the result of admixture with European and African populations. Specifically, genotype F has been detected as the predominant genotype in Central America and occurs at a high frequency among the HBV-infected Amerindians in all countries of South America (*i.e.*, Venezuela, Colombia, Peru, Bolivia, Argentina, and Brazil), as well as in Native Alaskan populations[106]. In general, its prevalence depends on the degree of admixture of the population with Amerindians[57] (see section Clinical Outcomes for further discussion of this topic). Moreover, F sequences isolated from sporadic cases among non-Amerindian populations appear as nested clades within the Amerindian genotype F radiation[6]. On the contrary, HBV genotype H has been predominantly isolated from both Amerindians and mestizos in Mexico with a frequency that ranges from 60% to 100% depending upon the ethnicity and geographic location of the sample population[7,107].

Genotypes F and H display a close phylogenetic relationship (Figure 1, section A), which suggests an introduction of the F/H ancestral strains to the Americas before the recent European colonization[54]. It has recently been proposed that the estimated time of the MRCA for genotypes F and H within the New World was approximately 8900 years ago[6]. It appears that both the ancestral and distinct F/H lineages (*i.e.*, clusters or subgenotypes) emerged under appropriate conditions for human settlement, development of agricultural systems and an increase in population size throughout the American continent[103]. However, genotype F presents greater genetic diversity than genotype H. Phylogenetic tree topology and genetic distances built using maximum likelihood and maximum parsimony methods show the deep clusters and geographical structure typical of genotype F[103]. Notably, the phylogeography of genotype F denotes the presence of subgenotypes designated F1-F4; some are further classified in subdivisions "a" or "b" suggesting a high level of isolation of the Amerindian populations carrying HBV[6] (Figure 1, section B). The estimated times of the MRCAs for the F1-F4 subgenotypes are, in chronological order (*i.e.*, oldest to newest), 6.4 ky, 3.3 ky, 3.2 ky and 2.4 ky, respectively[6]. Additionally, characteristic amino acid positions of complete genomes of HBV genotype F confirm the existence of the four subgenotypes[58,104].

Within genotype H, at least four nested sub-clusters (Figure 1, section C) are noticeable, though they are not yet definite clades or subgenotypes. So far, these correspond to the geographic region of origin of the isolates[7]. Accordingly, genotype H isolates depict an intragenotypic divergence of 0.032%-3.82% (data not shown). In Table 1, the genetic distance by pair-wise analysis among the four F subgenotypes and the H_{MEX}, H_{USA}, H_{SA} and $H_{NON\text{-}HISPANIC}$ subsets range from 7.17% to 10.40% on the basis of complete genomes. Interestingly, the highest divergence is within the H subsets and F1 genotypes, followed by F2 and F4, while F3 had the lowest (Table 1). These data are consistent with the fact that F3 and H share amino acid positions[58,104] and with the

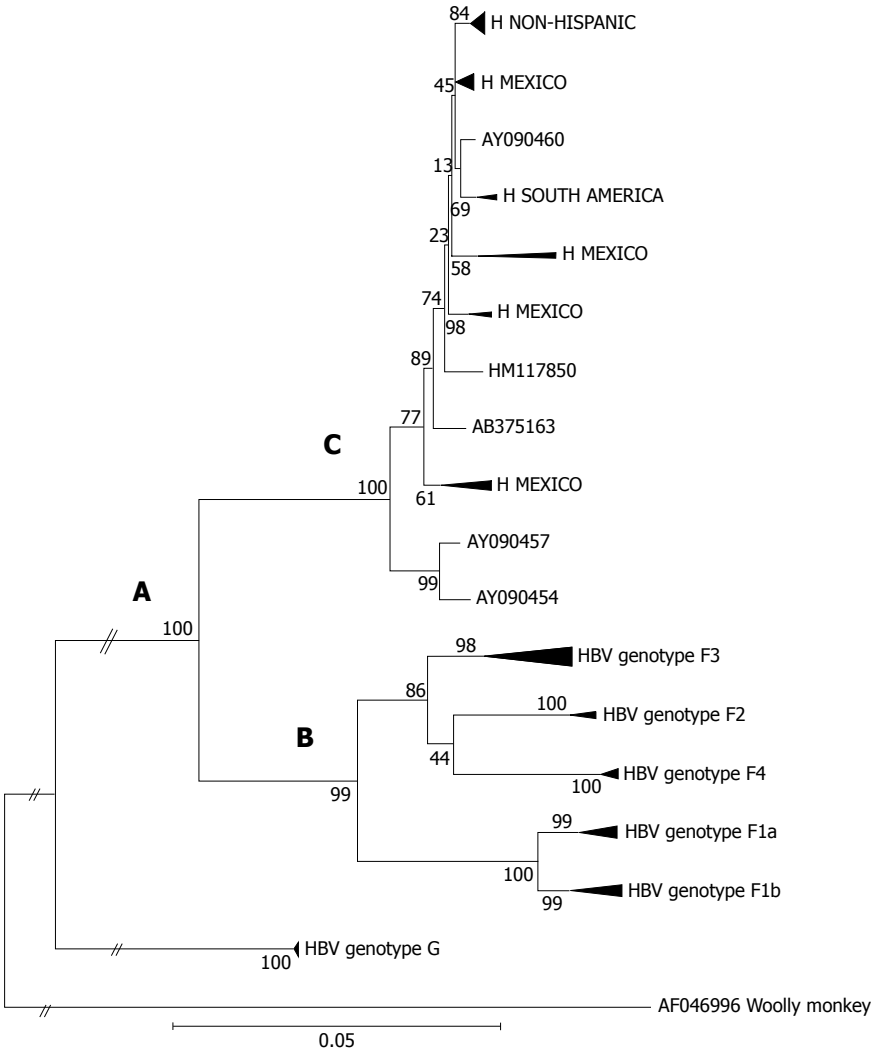

Figure 1 Maximum-Likelihood phylogenetic tree of the F/H genotype family (1000 bootstrap replicates, Mega 5.0). An illustration of the genetic relationship between genotype F and H and their subdivisions. Section A: Common ancestry; Section B: Hepatitis B virus (HBV) genotype F lineages; Section C: HBV genotype H subsets.

Table 1 Nucleotide divergence over the complete genome among hepatitis B virus genotypes F and H

Cluster	H_{MEX}	H_{USA}	H_{SA}	$H_{non\text{-}Hispanic}$
F1a	8.63 (8.11-9.9)	8.89 (8.16-9.5)	8.70 (8.5-8.8)	8.51 (8.22-8.91)
F1b	8.63 (8.22-10.1)	8.88 (8.04-9.86)	8.59 (8.19-9.39)	8.49 (8.04-9.31)
F2	8.07 (7.59-9.66)	8.83 (8.04-9.86)	8.77 (8.09-8.24)	7.9 (7.66-8.48)
F3	7.84 (7.17-9.91)	7.76 (7.28-8.94)	7.94 (7.24-8.97)	7.69 (7.24-8.82)
F4	8.36 (7.77-10.40)	8.66 (7.84-9.98)	8.50 (8.06-9.34)	8.29 (7.91-9.33)

Evolutionary distances between nucleotide sequences of 61 hepatitis B virus (HBV) genes calculated in accordance with the Gen Bank database. Values correspond to mean percentage of genetic distances of pair-wise analysis. Values in parentheses indicate ranges (bootstrap value 1000; Kimura two parameter method). H_{MEX}, H_{USA}, H_{SA}, $H_{non\text{-}Hispanic}$, refer to isolates of HBV genotype H from Mexico, United States, South America and Asian subjects, respectively in compliance with Figure 1. MEX: Mexico; SA: South America.

similarity in the estimated times of MRCA, 3.2 ky and 4.1 ky, respectively[6]. Furthermore, recent studies based on coalescence and phylogeographic methods have provided new insights into the introduction and spread of F genotypes (F1-F4) among the LA populations through human migration, especially in Colombia[58], Brazil[102] and Argentina[103] (Figure 2).

In regards to genotypes D and A, they have maximum frequencies of 35% and 5%, respectively, among urban populations in Guadalajara, Jalisco, Mexico[7], while in various cities in Argentina in South America, they occur at frequencies of 22% and 45%, respectively (see references cited in Table 2). Both cases are in accordance with the ethnic demographic shift that took place during the European colonization. Likewise, genotypes B and C are dispersed among the LA populations due to Asian immigrants (Tables 2, 3).

HBV genotype G is a minor strain that exists throughout the Americas in special populations with blood-

Figure 2 Pathway of hepatitis B virus in Latin America. A composite map of the theoretical pathway of hepatitis B virus (HBV) through Latin America based on molecular epidemiology and phylogeographic evidence that relate past and current human migration. (Adapted from references: 6, 55, 57, 58, 101, 103, 104).

borne infectious diseases, however due to the high frequency of this genotype in Mexico and the common practice of sexual relationships among native men, it may be associated with an Amerindian ancestry[7,8,98]. Finally, an uncommon genotype E was reported in Colombia[108], as well as an F3/A1 recombinant among an Afro-Colombian community[109].

Thus, with the advancement of molecular epidemiology and phylogeography, future progress can allow a better understanding of the relationship between the evolutionary history of the HBV genotypes and its impact on the clinical outcomes of HBV infection among the LA populations.

Clinical outcomes of HBV infection

Mexico: HBV endemicity and clinical outcomes among the native or mestizo Mexican population are associated with the predominance of genotype H, followed by genotypes A, D and G[7,8,50,98,107] (Table 3). Two outstanding features of HBV infection are OHB and low viral load regardless of the degree of endemicity[7]. HBV genotype A is most likely to be detected in acute infections and associated with mixed infections and high viral loads; in contrast, genotype D manifests at very low or undetectable viral loads or in mixed infections. The progression to chronic infection occurs primarily among mestizo adults through horizontal transmission and, to a lesser degree, in children by vertical transmission[7,99,110]. OHB is a key feature in native populations with high rates of endemic HBV infection, although surprisingly few clinical manifestations occur. Recently, in an analysis of native Mexican groups, we reported differences in cytokine levels in the serum that can distinguish OHB genotype H-infected patients from patients that resolved HBV infection. This result suggests that cytokine expression, along with specific immune responses, can influence the severity of OHB disease. Differences in immune response may be responsible for viral transcription repression, which in turn results in both HBsAg and minimal detectable levels of HBV DNA[111].

Moreover, it is plausible that the course of liver disease and immune response in native populations in Mexico may be different from those described in other areas of the world. In general, liver cirrhosis and HCC

Table 2 Hepatitis B virus genotypes, clinical and serological characteristics and type of liver damage in South America *n* (%)

Ref.	Country	Study population	*n*	HBsAg Positive	Anti-HBc Positive	HBV genotypes[1]							Diagnosis
						A	B	C	D	F	G	H	
Cortes-Mancera *et al*[130], 2011	Colombia	Patients with end-stage liver disease	131	14 (11)	6 (5)	-	-	-	-	7 (100) F1a, F3	-	-	Cirrhosis in 71% HCC in 12%; Cirrhosis and HCC in 17%
Devesa *et al*[131], 2008		HBV-infected patients	100	NA	NA	2 (2)	-	2 (2)	8 (8)	86 (86) F3	2 (2)	-	NA
Alvarado Mora *et al*[58], 2011		Blood donors	143	143 (100)	NA	8 (15)	-	-	-	40 (77)	8 (15)	-	NA
Nakano *et al*[134], 2001	Venezuela	Amerindian	12	12 (100)	NA	-	-	-	-	12 (100)	-	-	CH in 33%
Quintero *et al*[132], 2002		Afro-Venezuelan	222	8 (4)	NA	3 (50)	-	-	-	3 (50)	-	-	CH in 100%
Gutierrez *et al*[138], 2004		Blood donors	258	2 (0.8)	258 (100)	1 (9)	-	-	7 (64)	3 (27)	-	-	OHB in 4.3%; Rare severe liver disease
Kato *et al*[123], 2005		HBV-infected patients	2	NA	NA	-	-	-	-	2 (100) F1	-	-	AH in 100%
Devesa *et al*[131], 2008		Amerindian	89	NA	NA	3 (3)	-	-	5 (6)	81 (91) F1; F2a; F2b; F3	-	-	NA
Cardona *et al*[135], 2011		Amerindian	70	2 (3)	25 (36)	-	-	-	-	25 (100) F3	-	-	OHB in 34%;
Palumbo *et al*[133], 2007	Ecuador	Immigrants in Italy	2	2 (100)	0 (0)	2 (100)	-	-	-	-	-	-	CH in 100%
Casey *et al*[139], 1996	Peru	Military personnel	84	77 (88)	84 (95)	-	-	-	-	15 (100)	-	-	CH in 68%
Von Meltzer *et al*[140], 2008		HBV-infected patients	9	9 (100)	NA	-	-	-	-	9 (9) F1b	-	-	CH in 100%
Sitnik *et al*[143], 2004	Brazil	HBV-infected patients	103	103 (100)	NA	51 (49)	3 (3)	14 (14)	25 (24)	10 (10)	-	-	CH in 100%
Palumbo *et al*[133], 2007		Immigrants in Italy	12	12 (100)	0 (0)	3 (25)	-	-	9 (75)	-	-	-	CH in 100%
Bertolini *et al*[144], 2012		Blood donors	228	228 (100)	NA	32 (14) A1, A2	-	3 (1)	189 (83)	3 (1) F2a, F4	-	1 (0.4)	NA
Mello *et al*[102], 2013		HBV-infected patients	12	NA	NA	-	-	-	-	12 (100) F2a, F1b, F4	-	-	NA
Eloy *et al*[145], 2013		HBV-infected patients	119	80 (100)	80 (100)	74 (92)	-	4 (5)	1 (1)	1 (1)	-	-	Asymp in 70; AH in 2%; CH in 28%
Araujo *et al*[146], 2013		HBV/HIV-coinfected patients	1	1 (100)	1 (100)	-	-	-	-	1 (100) F4	1 -100	-	CH in 100%
Khan *et al*[147], 2008	Bolivia	Japanese immigrants	287	10 (8)	NA	-	1 (1) Ba	5 (50) C2	-	4 (4)	-	-	NA
Khan *et al*[147], 2008		Native population	200	12 (6)	NA	-	1 (8)	3 (25)	-	8 (68); F4	-	-	NA
Di Lello *et al*[141], 2009,	Chile	HBV-infected patients	40	NA	NA	3 (7)	2 (5)	3 (7)	-	27 (67)	-	-	NA
Venegas *et al*[142],2011		HBV-infected patients	21	21 (100)	21 (100)	-	-	-	-	21 (100) F1b	-	-	CH in 100%
Solari *et al*[149], 2009	Argentina	HBV-infected patients	21	21 (100)	-	8 (38)	3 (14)	-	3 (14)	7 (33)	-	-	CH in 100%
Pezzano *et al*[150], 2011		HBV-infected patients	139	128 (100)	128 (100)	22 (28)	1 (0.8)	3 (2)	28 (22)	60 (47)	-	-	AH in 37%; CH in 63%
Trinks *et al*[148], 2012		HBV-infected patients	33	33 (100)	33 (100)	15 (45) A1, A2	-	-	2 (6) D1	13 (39) F1b, F4	-	-	CH in 100%
Barbini *et al*[101], 2013		HBV-infected patients	29	29 (100)	29 (100)	4 (14) A2	-	-	2 (7) D2, D3	20 (62) F1b, F4	2 (7)	-	CH in 100%
Araujo *et al*[146], 2013		HBV/HIV-coinfected patients	1	1 (100)	1 (100)	-	-	-	-	1 (100); F1b	1 -100	-	CH in 100%

[1]The percentage of hepatitis B virus (HBV) genotypes is according to the number of samples that were sequenced. Subgenotypes are reported. NA: Not available; Asymp: Asymptomatic; AH: Acute hepatitis; CH: Chronic hepatitis; HCC: Hepatocellular carcinoma; OHB: Occult hepatitis B.

Table 3 Hepatitis B virus genotypes, clinical and serological characteristics and type of liver damage in Mexico and Central America *n* (%)

Ref.	Country	Study population	*n*	HBsAg Positive	Anti-HBc Positive	HBV genotypes[1]								Diagnosis
						A	B	C	D	E	F	G	H	
Sanchez *et al*[107], 2002	Mexico	HBV-infected adults	15	13 (87)	6 (40)	3 (20)	-	-	1 (7)	-	10 (67)	1 (7)	-	AH in 30%; CH in 27%; cirrhosis in 7%.
Sanchez *et al*[50], 2007		HBV-infected patients	67	67 (100)	NA	10 (15)	-	-	4 (6)			4 (6)	44 (66)	AH in 15%; CH in 48%
Ruiz-Tachiquin *et al*[151], 2007		Blood donors and HBV-infected patients	33	33 (100)	NA	-	-	4 (12)			1 (3)	-	26 (79)	Asymp in 64%; CH in 36%
Roman *et al*[98], 2010		Native adults	306	17 (5.6)	100 (32.7)	6 (24)	1 (4)	2 (8)	4 (16)		-	1 (4)	11 (44)	OHB in 14.2%; asymp in 100%
Arauz-Ruiz *et al*[115], 1997	Central America: Costa Rica, Nicaragua, Honduras, El Salvador, Guatemala	HBV-infected patients, blood donors and pregnant woman	330	330 (100)	71 (21)	13 (14)	-	1 (1)	5 (6)		71 (79) F1a; F1b; F2	-		CH in 21%; AH in 16%
Leon *et al*[122], 2005	Costa Rica	HBV-infected patients	50	32 (64)	18 (36)	-	-	-	2 (4)	-	48 (96)	-		CH in 47%; HCC in 16%
Kato *et al*[123], 2005	Panama	HBV-infected patients	2	NA	NA	-	-	-	-		2 (100) F1, F3	-	-	NA
Martinez *et al*[124], 2013		Chinese residents	320	42 (13)		-	11 (61) B2	7 (39) C1	-	-	-	-	-	No evident liver disease
Andernach *et al*[125], 2009	Haití	Pregnant woman	320	320 (100)		128 (71) A1, A2, A5	-	-	40 (22) D3, D4	11 (6)	-	-	-	NA
Couto *et al*[126], 2013		Haitian's in South Florida	27	NA	NA	-	-	-	19 (79)	-	-	-	-	NA

[1]The percentage of hepatitis B virus (HBV) genotypes is according to the number of samples that were sequenced. Subgenotypes are reported. NA: Not available; Asymp: Asymptomatic; AH: Acute hepatitis; CH: Chronic hepatitis; HCC: Hepatocellular carcinoma; OHB: Occult hepatitis B.

associated with HBV infection do not occur frequently in native populations in Mexico[112,113], even in comparison with the rest of Latin America[113]. However, it is noteworthy that Mexico ranks first in mortality due to alcoholic liver disease[114] and ranks lowest for mortality due to HCC in general[112]. This epidemiological profile contrasts with what occurs in Asia, where countries such as China are exposed to HBV genotypes B and C at earlier ages through vertical transmission and have a higher prevalence of HCC and different responses to antiviral therapy, suggesting that genetic and environmental factors may modulate the degree of adaptation to HBV infection, as previously mentioned.

Central America: In Central America, data regarding the HBV genotype distribution in countries such as Costa Rica, Nicaragua, Honduras, El Salvador and Guatemala were reported in the late nineties (Table 3). HBV genotype F (specifically, F1a, F1b and F2) in patients with acute and chronic infection was associated with seropositivity for HBsAg and anti-HBc, as well as pre-core stop mutations[115].

In Guatemala, HBsAg prevalence was reported to be as low as 0.5% in a group of 77 pregnant women[116] and 1.3% (*i.e.*, 6 cases) among 484 female sex workers from the Mexican-Guatemalan border[117]; in both populations, the infected individuals were asymptomatic carriers of HBsAg. However, among a group of Guatemalan refugees near the same border, the percentage of asymptomatic carriers of HBsAg increased to 17%[118]. In this country, the HBV genotype F1a was found, and the infection with this subgenotype apparently does not produce important hepatic inflammation. Likewise, in Belize, 35% of a studied population with acute hepatitis infection was indigenous[119], and chronic hepatitis B has not been reported in this region.

In Honduras and Nicaragua, HBsAg and anti-HBc seroprevalence have been reported in high-risk groups,

such as multi-transfused adult patients[120] and children with cancer[121]. In addition, genotype F was reported in 96% of HBV cases in Costa Rica, followed by genotype D in 4%[122]; in contrast, genotype F (specifically, F1 and F3) was found in two patients in Panama[123]. All of these cases were in HBV-infected patients who presented with chronic hepatitis B and HCC associated with HBx gene mutations in genotype F strains. Therefore, the genetic characteristics of mutant HBV may increase the rate of HBV-related liver damage and HCC in Central America. On the other hand, the presence of HBV genotypes B and C among Chinese residents living in Panama without liver disease[124] provides the opportunity to study the natural history of these genotypes in a new environment.

Central America is an important gateway for immigration in which further serological and molecular epidemiological studies may provide new evidence concerning shifts in the genotype distribution and its effect on the clinical manifestations of HBV infection.

The Caribbean region: In general, HBV genotypes have rarely been reported in the Caribbean, but it seems that the HBV genotype distribution and progression of liver damage could be different from those observed in Central America. HBV genotype A (specifically, A1, A2 and A5) was the dominant strain reported in Haiti, followed by genotype D (specifically, D3 and D4) among 320 pregnant women without liver inflammation[125]. It is noteworthy that despite the fact that this country has significant African ancestry, HBV genotype E has rarely been found, suggesting that this genotype emerged after the slave trade in the Americas[125]. Additionally, in another study in Haitians living in Florida, United States, genotype D was found with spontaneous pre-core region mutations[126], whereas OHB was reported in Cuba in HIV-infected patients[127] in whom these HBV genotypes were unknown. In a Jamaican study, the HBsAg prevalence was reported at 3.2% among patients with sexually transmitted diseases, but liver damage was not studied[128]. Further, HCC was found to be associated with HBV infection in 5.3% of 114 veterans in Puerto Rico[129] (Table 3).

South America: Countries in this region, including Colombia, Venezuela, Ecuador, Peru, Brazil, Bolivia, Chile and Argentina, have defined the predominant circulating HBV genotypes, although the numbers of HBV strains in some regions are limited. Thus, in Colombia, a connecting country between Central and South America, HBV genotype F (specifically, F1a and F3) is more frequent (77% and 86%, respectively) than genotypes A and G (15% and 2%, respectively)[58,130,131]. In these studies, HBV-infected patients were at end-stage disease due to cirrhosis and HCC[130], whereas in other studies, the type of liver damage in HBV-infected patients and blood donors was not reported. Various studies report HBV genotype F as the most frequent in Venezuela, Peru and Chile with differences in the frequencies of the subgenotypes (specifically, F1a, F2a, F2b and F3) among the Afro-Venezuelan[132], Amerindian[133-137] and mestizo[138-142] populations. In these groups, chronic hepatitis B is common and OHB has been found in blood donors and Amerindians[139-142] (Table 2).

Although genotype F (specifically, F4) has been reported in Brazil, genotype A (specifically, A1 and A2) has been found to be dominant, followed by genotype D, in patients with chronic hepatitis B; coinfection with genotypes F and G was reported in one HIV-infected patient[102,132,143,144-146]. Interestingly, genotype F4 was reported in Bolivia[147] and Argentina[101], which have populations of mixed ethnic backgrounds, and the characteristics of the HBV genotypes could have changed over time in these regions. In fact, this population had chronic liver disease, HCC was associated with HBV infection, HBsAg was positive in all cases, and the viral load was high. On the other hand, HBV genotypes B and C (specifically, Ba and C2) were found in Japanese immigrants living in Bolivia[147], which reflects immigration events into South America. Regarding HBV genotypes A and D in patients in Argentina[148-151], a chronic progression of the infection with these genotypes was reported, contrasting with OHB and the asymptomatic clinical outcome reported in Mexico[151] (Tables 2 and 3).

Therefore, research on HBV strains in LA should be clinically associated with the natural evolution of liver disease, hepatic complications and the response to different treatments. These issues may have a strong impact on the prevention and control of HBV transmission because they may influence the prognosis and response to the hepatitis B vaccine.

CONCLUSION

This overview has illustrated the influence of genetic and environmental risk factors in the onset and development of HBV-related liver disease. The people of Latin America share a similar genetic ancestry with varying degrees of admixture from three distinct lineages. The closely related HBV genotypes F and H have been in contact with the native population of the Americas and tend to cause mild liver disease with no further complications. In contrast, the mestizo population of South America presents acute and chronic liver disease with a tendency toward HCC. Based on these features, personalized medicine strategies provide a novel framework for the prevention, management and treatment of HBV-related liver disease in Latin America. Future genomic medicine research will have an important impact on the clinical approach to liver diseases. An integrated approach requires that genetic and environmental factors be taken into account, as both are involved in the endemicity and clinical outcome of HBV infection. Among the human host factors, the susceptibility to liver damage and the response to antiviral treatment, which in turn are modulated by the immune system, are linked to genetic

polymorphisms that are associated with specific ethnic backgrounds. Genes involved in various metabolic pathways are influenced by changes in environmental lifestyle factors, such as nutrition, physical activity and emotional stress. Other comorbidities that enter the picture are the worldwide obesity epidemic and increased consumption of alcohol, which impose a greater burden on liver health.

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TOPIC HIGHLIGHT

WJG 20th Anniversary Special Issues (9): Hepatitis B virus

Regulation of microRNA by hepatitis B virus infection and their possible association with control of innate immunity

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Abstract

Hepatitis B virus (HBV) chronically infects more than 350 million people worldwide. HBV causes acute and chronic hepatitis, and is one of the major causes of cirrhosis and hepatocellular carcinoma. There exist complex interactions between HBV and the immune system including adaptive and innate immunity. Toll-like receptors (TLRs) and TLR-signaling pathways are important parts of the innate immune response in HBV infections. It is well known that TLR-ligands could suppress HBV replication and that TLRs play important roles in anti-viral defense. Previous immunological studies demonstrated that HBV e antigen (HBeAg) is more efficient at eliciting T-cell tolerance, including production of specific cytokines IL-2 and interferon gamma, than HBV core antigen. HBeAg downregulates cytokine production in hepatocytes by the inhibition of MAPK or NF-κB activation through the interaction with receptor-interacting serine/threonine protein kinase. MicroRNAs (miRNAs) are also able to regulate various biological processes such as the innate immune response. When the expressions of approximately 1000 miRNAs were compared between human hepatoma cells HepG2 and HepG2.2.15, which could produce HBV virion that infects chimpanzees, using real-time RT-PCR, we observed several different expression levels in miRNAs related to TLRs. Although we and others have shown that HBV modulates the host immune response, several of the miRNAs seem to be involved in the TLR signaling pathways. The possibility that alteration of these miRNAs during HBV infection might play a critical role in innate immunity against HBV infection should be considered. This article is intended to comprehensively review the association between HBV and innate immunity, and to discuss the role of miRNAs in the innate immune response to HBV infection.

Key words: Hepatitis B virus; HepG2.2.15; Innate immunity; MicroRNA; Persistent infection; Toll-like receptor

Core tip: Hepatitis B virus (HBV) is the leading cause of chronic hepatitis, cirrhosis, and hepatocellular carcinoma in the world. HBV could interact with the host's innate and adaptive immune responses to establish chronic infection. HBV also interacts with Toll-like receptors (TLRs) and TLR signaling pathways, and regulates host immune responses through the regulation of microRNAs (miRNAs) to some extent. This article fo-

cuses on the involvement of miRNA in the association between HBV and TLR signaling pathways and reviews the miRNAs involved in HBV infection.

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INTRODUCTION

Hepatitis B virus (HBV), a member of hepadona viridae, has partially circular double-stranded DNA genome, 3.2 kb in length[1]. It contains four overlapping open reading frames that encode seven proteins: the precore protein, also known serologically as HBe antigen (HBeAg), the core protein (HBcAg), viral polymerase, three forms of the envelope protein known as S antigen (HBsAg) and X (HBx) protein[1,2]. HBV as well as hepatitis C virus (HCV) causes acute and chronic hepatitis, cirrhosis and hepatocellular carcinoma (HCC)[3]. Hepatic cirrhosis and HCC are the most common causes of death in patients with chronic liver disease[4].

The outcome of HBV infection is the result of complex interactions between HBV and the immune system including adaptive and innate immunity[5,6]. Toll-like receptors (TLRs) are important parts of the innate immune response in hepatitis virus infections[7]. There are several reports about the important role of TLRs and TLR-mediated signaling in the pathogenesis and outcome of HBV infection[2,5-11].

MicroRNA (miRNA) is one of the endogenous noncoding small RNAs, approximately 18-22 nucleotides in size, a post-transcriptional regulator that binds to the 3'-untranslated region (UTR) of the target gene messenger RNA, usually resulting in cleavage or inhibiting translation of the target gene mRNA[12,13]. It is estimated that the human genome may encode over 2000 miRNAs, which may control about 60% of the human genome[14,15]. Physiologically, miRNAs are able to regulate various biological processes such as cell proliferation, differentiation and apoptosis, neuroprocesses, carcinogenesis and immune response[16-18]. This article is intended to comprehensively review the association between HBV and innate immunity, and to discuss the role of miRNAs in the innate immune response to HBV infection.

INNATE IMMUNITY IS IMPORTANT FOR THE ERADICATION OF HBV

Interferons (IFNs) play an important role in the innate immune response to virus infection. IFN-α and IFN-β (type I IFNs) are secreted by almost all virus-infected cells including hepatocytes and by specialized blood lymphocytes. In contrast, the production of IFN-γ (type Ⅱ IFN) is restricted to cells of the immune system, such as natural killer (NK) cells, macrophages, and T cells. On the other hand, tumor necrosis factor alpha (TNF-α) primarily initiates innate immune response and triggers acquired immune responses[19]. TNF-α-induced apoptosis is important for clearance of hepatocytes infected with HBV and HCV, and IFN-γ accelerates the killing of theses hepatocytes[19,20]. The previous studies demonstrated that TNF-α and IFN-γ downregulate HBV gene expression in the liver of HBV transgenic mice by post-transcriptionally destabilizing the viral mRNA[21-23]. It has been widely believed that the cytotoxic T lymphocyte response clears viral infections by killing infected cells. However, Chisari's group[21-24] reported that noncytopathic clearance of HBV from hepatocytes by cytokines, which abolish viral replication and HBV gene expression, is another important mechanism. Isogawa *et al*[24] reported that TLR3, TLR4, TLR5, TLR7 and TLR9 ligands could induce antiviral cytokines and inhibit HBV replication in HBV transgenic mice, thereby indicating TLR activation as a powerful strategy for the treatment of chronic HBV infection. HBV replication can be controlled by innate immune response, involving TLRs, if it is activated in hepatocytes[24]. Together, these facts indicate that innate immunity including TLR signaling plays an important role in the pathogenesis of HBV infection.

TOLL-LIKE RECEPTORS AND ANTI-VIRAL DEFENSES

TLRs, germline-encoded pattern recognition receptors (PRRs), can play a central role in host cell recognition and response to various pathogens such as viruses[25]. TLR1, TLR2, TLR4, TLR5, and TLR6 are expressed on the cell surface while TLR3, TLR7, TLR8 and TLR9 are expressed within intracellular vesicles. TLR3, TLR7/8 and TLR9 are involved in the recognition of viral nucleotides such as double-stranded RNA, single-stranded RNA and DNA, respectively[26]. Other than TLRs, membrane-bound C-type lectin receptors (CLRs), cytosolic proteins such as NOD-like receptors (NLRs) and RIG-I-like receptors (RLRs), which include retinoic acid-inducible gene I (RIG- I), melanoma differentiation antigen 5 (MDA5), and lipophosphoglycan biosynthetic protein 2 (LPG2), and unidentified proteins that mediate sensing of cytosolic DNA or retrovirus infection, are also involved in the recognition of pathogen-associated molecular patterns (PAMPs)[25].

TLRs play a crucial role in defending against pathogenic infection through the induction of inflammatory cytokines and type I IFNs by myeloid differentiation primary response 88 (MYD88)-dependent and MYD88-independent pathway. In the MYD88-dependent pathway, MYD88 recruits a set of signal cascades such as MAPK and NF-κB through receptor-interacting serine/

threonine protein kinase (RIPK/RIP). In the MYD88-independent pathway, TLR3 activates NF-κB and MAPKs through RIPK. TLR3 also activates IFN regulatory factor 3 (IRF3) and IRF7 *via* TRIF/TICAM-1, inducing the production of type I IFN. The activated NF-κB and IRFs are translocated to the nucleus. NF-κB and MAPKs initiate the transcription of inflammatory cytokine genes, whereas IRFs initiate the transcription of type I IFN[2]. RIG- I and MDA5 pathways can also activate IRF3 to produce type I IFNs. RNA helicases RIG- I and MDA5, specific receptors for double-stranded RNA, and the downstream mitochondrial effector known as CARDIF/MAVS/VISA/IPS-1, are also major pathways for type I IFN induction.

ASSOCIATION BETWEEN HBV AND TOLL-LIKE RECEPTORS

TLRs have been recognized as playing an important role in the pathogenesis of chronic hepatitis B[8]. NF-κB is activated by three TLR adaptors, MYD88, Toll/interleukin (IL)-1 receptor (TIR)-domain-containing adaptor-inducing IFNβ (TRIF), and IFN promoter stimulator 1 (IPS-1), to elicit anti-HBV response in both HepG2 and Huh7 cells[27]. Down-regulations of TLR7 and TLR9 mRNA were observed in peripheral blood mononuclear cells (PBMC) of HBV-infected patients[28]. Chen *et al*[29] reported that TLR1, TLR2, TLR4 and TLR6 transcripts were also downregulated in PBMC of chronic hepatitis B patients. After being challenged by TLR2 and TLR4 ligands, cytokine production was impaired in PBMC of chronic hepatitis B patients on the basis of the levels of plasma HBsAg[29]. Xie *et al*[30] reported that HBV infection results in reduced frequency of circulating plasmacytoid dendritic cells (pDCs) and their functional impairment *via* inhibiting TLR9 expression. HBV replication suppresses the TLR-stimulated expression of pro-inflammatory cytokines (TNF, IL6) and the activation of IRF3[31]. It has also been reported that HBV could target RIG- I signaling by HBx-mediated IPS-1 down-regulation, thereby attenuating the antiviral response of the innate immune system[32].

HBV E ANTIGEN DOWNREGULATES CYTOKINE PRODUCTION

The HBV precore/core region of HBV genome also encodes HBeAg as well as the HBV core. The precore stop codon prevents the formation of precore protein and HBeAg[2,33]. The existence of HBeAg in serum is known to be a marker of a high degree of viral infectivity. In Japan, the major HBV genotypes are B and C, but our previous study[34] revealed that the precore mutation A1896 and the core promoter mutations at nt1762 and 1764 were found more frequently in acute liver failure than in acute hepatitis, and HBV genotype B was predominant in acute liver failure. It has also been shown that acute liver failure occasionally occurs in persons who are negative for HBeAg[35,36]. It is well known that perinatal transmission of HBV occurs in about 10%-20% of HBeAg-negative mothers without prevention of perinatal HBV transmission by combined passive and active immunoprophylaxis, and the babies are at risk of developing fulminant hepatitis[37]. Chronic hepatitis B with high HBV DNA and ant-HBe is associated with a severe and evolutive liver disease[38]. These clinical findings could be assumed to have immune tolerance for HBeAg, although the function of HBV precore or HBeAg is unknown. Previous immunological studies[39-41] demonstrated that HBeAg is more efficient at eliciting T-cell tolerance, including production of its specific cytokines IL-2 and IFN-γ, than HBV core antigen. We also demonstrated that HBeAg expression inhibits IFN and cytokine production[2] and that HBeAg physically associates with RIPK2 and regulates IL-6 gene expression[6]. Visvanathan *et al*[42] reported that the expression of TLR2 on hepatocytes, Kupffer cells, and peripheral monocytes was significantly reduced in HBeAg-positive chronic hepatitis B patients. Thus, HBV seems to have evolved strategies that block the effector mechanisms induced through IFN and/or cytokine signaling pathways, similar to other viruses[19].

MIRNAS WERE DIFFERENTIALLY EXPRESSED IN HEPG2.2.15 AND HEPG2

HepG2.2.15 cells assemble and secrete HBV virion that infects chimpanzees[43,44]. We examined the expression of approximately 1000 miRNAs in the human hepatoma cells HepG2.2.15 and HepG2 using real-time RT-PCR, the most sensitive technique for mRNA detection and quantification[45,46].

First, 1008 miRNAs were examined in the hepatoma cells HepG2.2.15 and HepG2, using quantitative real-time RT-PCR with specific primers (Qiagen, Hilden, Germany). SNORD61, SNORD68, SNORD72, SNORD95, SNORD96A and RNU6-2 were used as endogenous controls to normalize expression to determine the fold-change in miRNA expression between the test sample (HepG2.2.15) and control sample (HepG2) by 2-ddCT (comparative cycle threshold) method[21]. MiRNAs were annotated by Entrez Gene (NCBI, Bethesda, MD, United States), accessed on 2/27/2013. Data were analyzed with miRNA PCR array data analysis software (http://www.sabiosciences.com/mirnaArrayDataAnalysis.php). Scatter plot analysis is shown in Figure 1A. There were differences in expression between HepG2 and HepG2.2.15 (Figure 1B).

We then excluded 599 miRNAs according to the following criteria: (1) average threshold cycle was relatively high (> 30) in either HepG2 or HepG2.2.15, and was reasonably low in the other samples (< 30); (2) average threshold cycle was relatively high (> 30), meaning that its relative expression level was low, in both HepG2 and

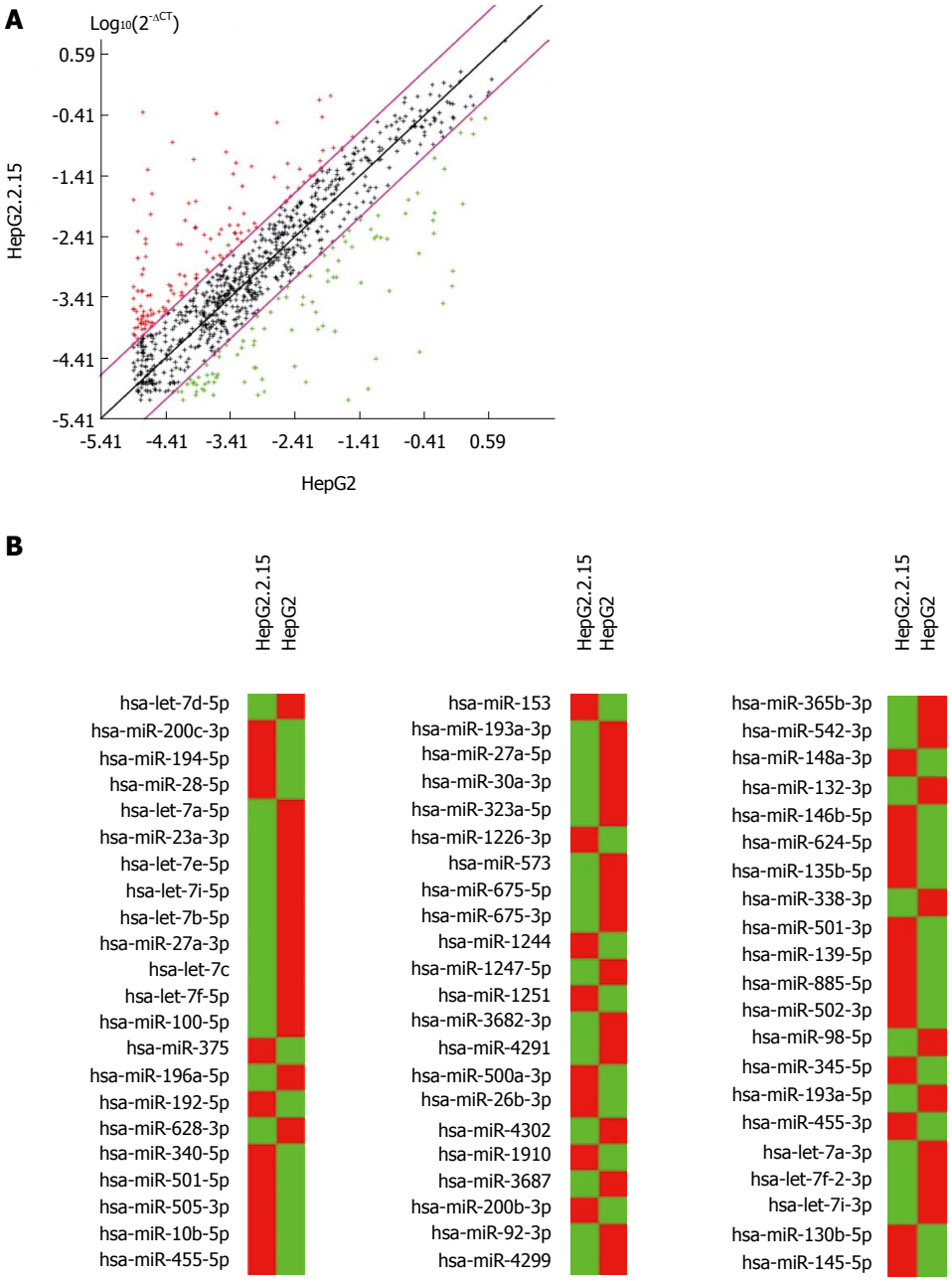

Figure 1 MicroRNAs expression in hepatoma cells HepG2.2.15 and HepG2. A: Scatter plots of 1008 miRNAs indicate 2^{-dCT} numerical values in HepG2 cells (x-axis) and HepG2.2.15 cells (y-axis). The black line indicates fold changes of 1. The pink lines indicate 5-fold change in miRNA expression threshold, comparing HepG2.2.15 with HepG2. Red + indicates miRNA expressed at least 5-fold higher in HepG2.2.15 than in HepG2 cells. Green + indicates miRNA expressed at least 5-fold lower in HepG2.2.15 than in HepG2 cells. Black + indicates that the difference of miRNA between the two cells was within 5-fold; B: Comparison of miRNAs expression between HepG2 and HepG2.2.15 cells. Red color indicates miRNA expressed at least 5-fold higher in HepG2.2.15 than in HepG2 cells. Green color indicates miRNA expressed at least 5-fold lower in HepG2.2.15 than in HepG2 cells. miRNAs: MicroRNAs.

HepG2.2.15; and (3) average threshold cycle was either not determined or was greater than the defined cut-off value (default 35) in both samples, meaning that its expression was undetected, making this fold-change result erroneous and uninterpretable.

Out of 409 miRNAs examined, 30 (7.3%) were upregulated by 5-fold or greater in HepG2.2.15 compared to HepG2. Twelve miRNAs (miR-200b-3p, miR-505-3p, miR-148a-3p, miR-145-5p, miR-194-5p, miR-885-5p, miR-192-5p, miR-146b-5p, miR-340-5p, miR-375, miR-139-5p and miR-200c-3p) were upregulated 10-fold or more in HepG2.2.15 cells. MiRNAs upregulated 5-fold or more are shown in Figures 1B and 2A. On the other hand, out of 409 miRNAs, 35 (8.6%) were downregulated 5-fold or more in HepG2.2.15 compared to HepG2. Twenty-two miRNAs (let-7c, miR-573, let-7b-5p, miR-338-3p, miR-100-5p, miR-92b-3p, miR-542-3p, miR-4302, miR-4291, miR-193a-5p, miR-98-5p, miR-4299, miR-132-3p, let-7f-2-3p, let-7f-5p, let-7i-5p, let-7d-5p, miR-193a-3p, let-7a-5p, let-7i-3p, miR-196a-p and let-7a-3p) were downregulated 10-fold or more in HepG2.2.15 cells. MiRNAs downregulated 5-fold or

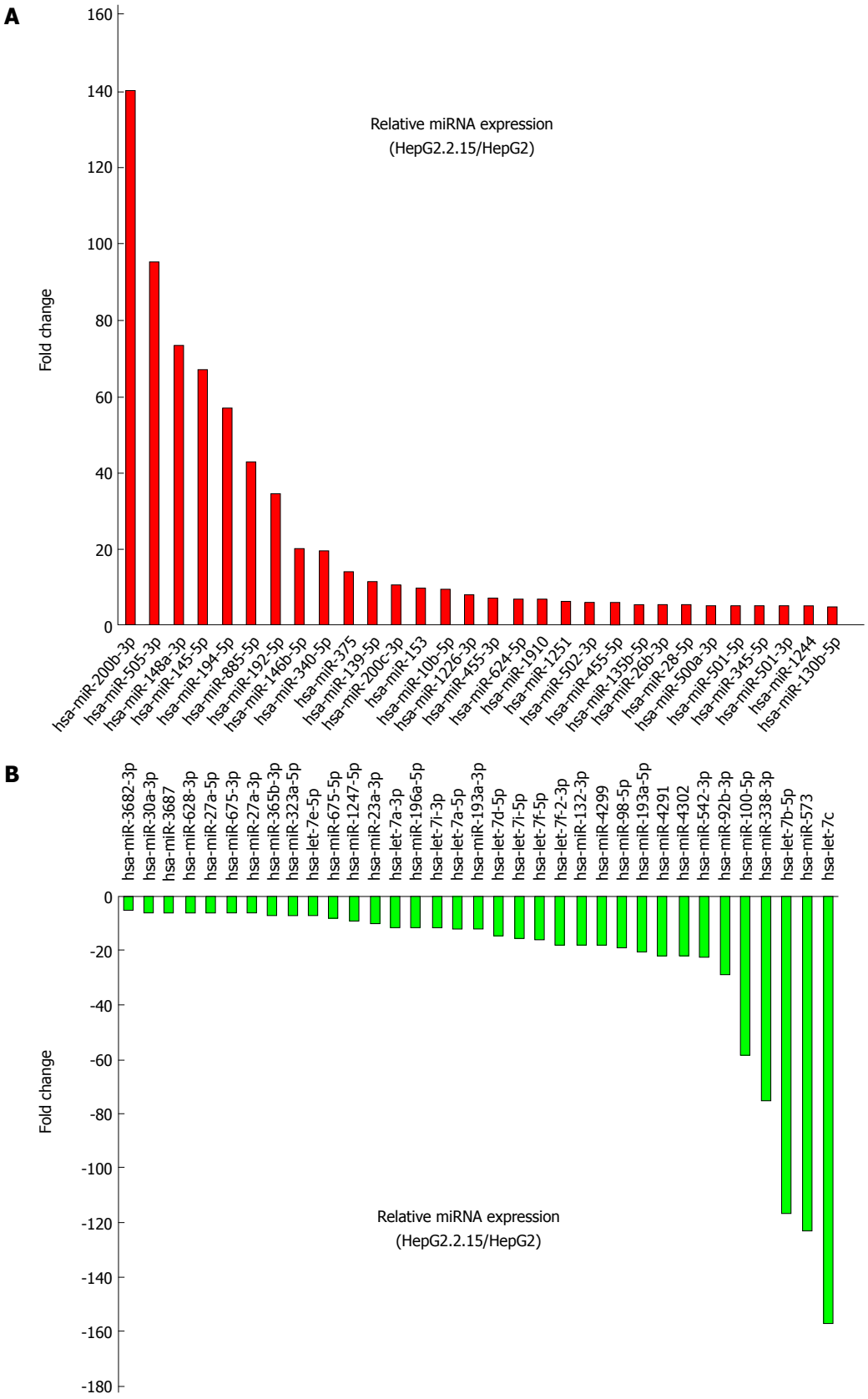

Figure 2 MicroRNAs expressed at more than 5-fold difference between hepatoma cells. HepG2.2.15 and HepG2 cells. A: MiRNA expressed at least 5-fold higher in HepG2.2.15 than in HepG2 cells; B: MiRNA expressed at least 5-fold lower in HepG2.2.15 than in HepG2 cells. miRNAs: MicroRNAs.

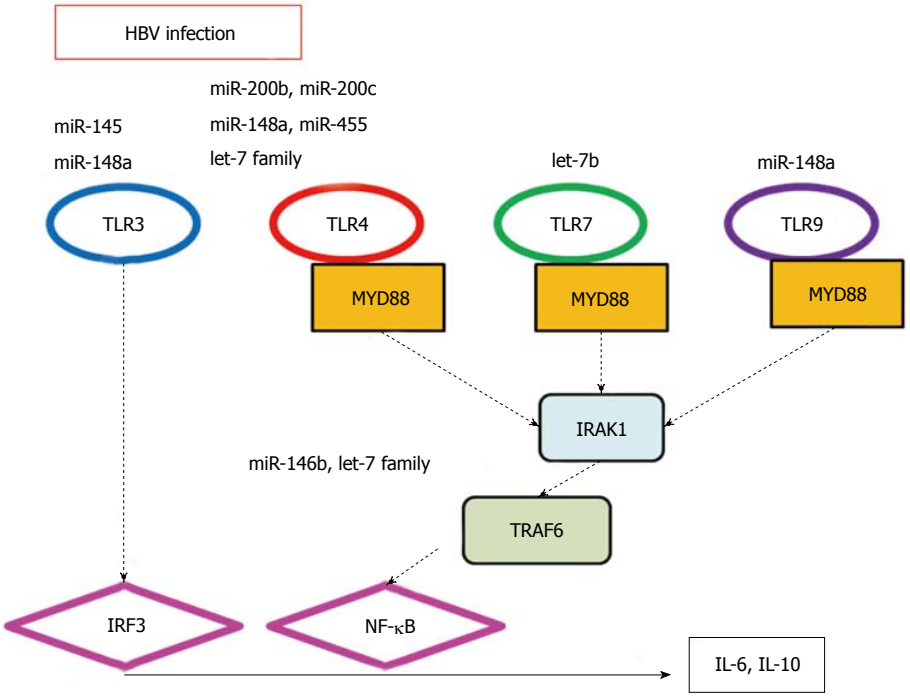

Figure 3 MicroRNAs and Toll-like receptor signaling pathway in hepatitis B virus infection. IRAK: Interleukin (IL)-1 receptor-associated kinase 1; IRF: Interferon regulator factor; miRNA: MicroRNA; MYD88: Myeloid differentiation factor 88; NF-κB: Nuclear factor-κB; TLR: Toll-like receptor; TRAF: Tumor necrosis factor receptor-associated factor.

Table 1 MicroRNAs associated with Toll-like receptor signaling pathways, upregulated by 5-fold or greater in HepG2.2.15 than in HepG2

MicroRNAs	Genomic location	Fold changes	Description of target molecules/pathways	Ref.
miR-200b-3p	1p36.33	140.15	TLR4 signaling through MyD88-dependent pathway	[47]
miR-148a-3p	7p15.2	73.36	TLR3, TLR4 and TLR9 agonists upregulated miR-148/152 expression	[48]
miR-145-5p	5q32	66.97	miR-145 promoted interferon-β induction by SOCS7	[49]
miR-146b-5p	10q24.32	20.05	TNF receptor-associated factor 6 and IL-1 receptor-associated kinase 1	[50]
miR-200c-3p	12p13.31	10.75	TLR4 signaling through MyD88-dependent pathway	[47]
miR-455-3p	9q32	7.36	miR-455 was involved in TLR4 signaling pathway through E2F1 transcription factor	[51]
miR-455-5p	9q32	5.76	miR-455 was involved in TLR4 signaling pathway through E2F1 transcription factor	[51]

Genomic location was analyzed using GeneCards (http://gene4.weizmann.ac.il/). TLR: Toll-like receptor.

more are shown in Figures 1B and 2B.

MIRNAS RELATED TO TLR PATHWAY UPREGULATED IN HEPG2.2.15 CELL LINES

Innate immunity represents the first line of defense against HBV, and we and others have reported its importance in the persistence of HBV infection[2,5-11]. So, we focused on miRNAs related to the TLR pathway. Among miRNAs upregulated 5-fold or more in HepG2.2.15 cells, 7 miRNAs (miR-200b-3p, miR-148a-3p, miR-145-5p, miR-146b-5p, miR-200c-3p, miR-455-3p and miR-455-5p) were reported to be related to TLR pathways (Table 1). MiRNAs miR-200b and miR-200c are the factors that modify the efficiency of TLR4 signaling through MYD88 in HEK293 cells[47]. TLR3, TLR4 and TLR9 agonists upregulated miR-148/152 expression and downregulated calcium/calmodulin-dependent protein kinase Ⅱ (CaMK Ⅱ) in dendritic cells (DCs) on maturation[48]. Thus miR-148/152 can act as fine-tuners in regulating the innate response and antigen-presenting capacity of DCs[48]. Exogenous miR-145 promoted IFN-β induction by targeting the suppressor of cytokine signaling 7 (SOCS7), through the nuclear translocation of signal transducer and activator of transcription 3 (STAT3) and SOCS7-silencing enhanced IFN-γ induction by stimulation with TLR3 ligand, poly(I-C)[49]. MiR-146 plays a role in the control of TLR and cytokine signaling through a negative feedback regulation loop involving down-regulation of interleukin (IL)-1 receptor-associated kinase 1 (IRAK1) and tumor necrosis factor (TNF) receptor-associated factor 6 (TRAF6) protein levels[50]. MiR-455 was involved in the TLR4 signaling pathway through E2F1 transcription factor[51].

Table 2 MicroRNAs associated with Toll-like receptor signaling pathways, downregulated by 5-fold or greater in HepG2.2.15 than in HepG2

MicroRNAs	Genomic location	Fold changes	Description of target molecules/pathways	Ref.
let-7e-5p	19q13.33	-7.29	Akt1 activated by TLR4-ligand LPS, positively regulated let-7e	[52]
let-7a-3p	9q22.32 11q24.1 22q13.31	-11.44	Repression of let-7 family relieves IL-6 and IL-10 mRNAs from negative post-transcriptional control in TLR4 signaling pathway	[53]
let-7i-3p	12q14.1	-11.57	let-7i regulates Toll-like receptor 4 expression	[54]
let-7a-5p	9q22.32 11q24.1 22q13.31	-11.96	Repression of let-7 family relieves IL-6 and IL-10 mRNAs from negative post-transcriptional control in TLR4 signaling pathway	[53]
let-7d-5p	9q22.32	-14.03	Repression of let-7 family relieves IL-6 and IL-10 mRNAs from negative post-transcriptional control in TLR4 signaling pathway	[53]
let-7i-5p	12q14.1	-15.10	let-7i regulates Toll-like receptor 4 expression	[54,55]
miR-132-3p	17p13.3	-18.18	TNF receptor-associated factor 6 and IL-1 receptor-associated kinase 1	[56]
let-7b-5p	22q13.31	-116.31	let-7b activates TLR 7	[56]

Genomic location was analyzed using GeneCards (http://gene4.weizmann.ac.il/). TLR: Toll-like receptor.

MIRNAS RELATED TO TLR PATHWAY DOWNREGULATED IN HEPG2.2.15 CELL LINES

Among miRNAs downregulated 5-fold or more in HepG2.2.15 cells, 8 miRNAs (let-7e-5p, let-7a-3p, let-7i-3p, let-7a-5p, let-7d-5p, let-7i-5p, miR-132-3p and let-7b-5p) were reported to be related to TLR pathways (Table 2). Protein kinase Akt1, which is activated by the TLR4-ligand lipopolysaccharide (LPS), positively regulated let-7e and miR-181c but negatively regulated miR-155 and miR-125b[52]. Repression of the let-7 family relieves IL-6 and IL-10 mRNAs from negative post-transcriptional control in the TLR4 signaling pathway[53], and the miRNAs let-7i and let-7b activate TLR4 and TLR7, respectively[54-56].

ROLE OF MIRNAS IN REGULATION OF INNATE IMMUNE RESPONSE IN HBV INFECTION

In the present study, 30 and 35 miRNAs were upregulated and downregulated, respectively, by 5-fold or greater in HepG2.2.15 compared to its parental cell line HepG2. These results indicate that miRNAs could play an important role in chronic persistent HBV infection. Su *et al*[57] reported that miR-155 enhances innate antiviral immunity through promoting the JAK/STAT signaling pathway by targeting SOCS1, inhibiting HBV replication. The possibility cannot be ruled out that HBV persistently infects hepatocytes through the regulation of miRNAs.

We also speculated that several of the miRNAs involved in the TLR signaling pathway play a critical role in innate immunity against HBV infection[5,24] (Figure 3). It has been reported that miR-21[58], miR-22[59,60], miR-122[58], miR-194[61] and miR-219-1[62] are associated with chronic persistent HBV infection as well as its clearance. In the present study, miR-194 was upregulated 10-fold or more in HepG2.2.15 cells.

CONCLUSION

MicroRNAs miR-122 and miR-130a play an important role in chronic hepatitis C[63,64]. Regulation of miRNAs also plays an important role in HIV infection[65]. In HCV infection, a set of miRNAs that regulate host immune response are modulated[66]. We and others have demonstrated that HBV modulates the host immune response. It might be possible that HBV as well as HCV regulates host immune response through the regulation of miRNAs in some steps toward chronic infection. MiRNAs and their regulation play a critical role in HBV infection, and HBV may regulate the TLR signaling pathway through the regulation of miRNAs.

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TOPIC HIGHLIGHT

WJG 20th Anniversary Special Issues (9): Hepatitis B virus

When to stop nucleos(t)ide analogues treatment for chronic hepatitis B? Durability of antiviral response

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Abstract

Introduction of nucleos(t)ide analogues (NAs) for oral antiviral therapy has dramatically improved the clinical outcome in patients with chronic hepatitis B (CHB). Although current international guidelines for the management of CHB provide information regarding when to begin the antiviral therapy with NAs, there is no clear consensus on when to stop the treatment, especially for those who respond to the therapy. Hepatitis B surface antigen loss has been regarded as an ideal endpoint of oral antiviral therapy with NAs, however since this is rarely achieved, practical endpoints have been suggested by the international guidelines. Despite the stopping rules recommended by the international guidelines, whether oral antiviral therapy with NAs can be safely discontinued is of major concern. While attention has been drawn to whether antiviral treatment with NAs can be a finite therapy, there is lack of sufficient data on off-treatment durability of highly potent NAs. Based on the available evidences, current guidelines for stopping NA therapy seems to be inadequate in terms of off-treatment durability, with relapse rates of more than 40% for both hepatitis Be antigen (HBeAg)-positive and HBeAg-negative patients. Therefore, further studies are required to accumulate data on off-treatment durability of highly potent NAs, and future studies are warranted to identify adequate predictive markers that could provide supplementary information to guide the timing of stopping NA therapy.

Key words: Chronic hepatitis B; Antiviral therapy; Nucleos(t)ide analogue; Durability; Cessation

Core tip: Introduction of nucleos(t)ide analogues (NAs) for oral antiviral therapy has dramatically improved the clinical outcome in patients with chronic hepatitis B (CHB). While attention has been drawn to whether antiviral treatment with NAs can be a finite therapy in patients with CHB, current guidelines for stopping NA therapy seems to be inadequate in terms of off-treatment durability in both hepatitis Be antigen (HBeAg)-positive and HBeAg-negative patients. In the present work, we discussed the validity of current stopping rules of NA therapy and addressed areas of uncertainty in deciding the best timing to stop NA treatment.

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INTRODUCTION

Hepatitis B virus (HBV) infects more than 350 million

people worldwide and is a major cause of chronic liver disease, which may eventually evolve to cirrhosis and hepatocellular carcinoma (HCC)[1,2]. There are several host and viral factors which affect the natural course of HBV infection, and active HBV replication has been described as the key driving force for the subsequent HBV-related immune clearance that determine liver injury and progression of liver disease, implicating the importance of sustained viral suppression, or ideally, elimination of the virus[3].

In recent years, introduction of nucleos(t)ide analogues (NAs) for oral antiviral therapy has dramatically improved the clinical outcome in patients with chronic hepatitis B (CHB). Although current international guidelines for the management of CHB provide information regarding when to begin the antiviral therapy with NAs, there is no clear consensus on when to stop the treatment, especially for those who respond to the therapy.

In this article, we discuss the validity of current stopping rules of NA therapy recommended by the international guidelines by assessing the durability of antiviral response after cessation of NAs based on currently available literature. We also address areas of uncertainty in deciding the best timing to stop NA therapy.

TREATMENT GOALS AND STOPPING RULES

Seroclearance of hepatitis B surface antigen (HBsAg) is a condition that is most similar to complete and definitive remission status of chronic HBV infection. In this view, the international guidelines have suggested HBsAg loss as an ideal endpoint of oral antiviral therapy with NAs. However, HBsAg loss is almost negligible even after a long-term therapy[4-7]. Therefore it is anticipated that most patients with CHB probably require lifelong oral antiviral treatment with NAs. The primary goals of antiviral therapy are sustained suppression of HBV replication and hepatic inflammation thereby improving long-term outcomes and prolonging patient survival by preventing the development of progressive fibrosis, cirrhosis and/or HCC[8-10]. Response to treatment is assessed based on biochemical, virological, serological, as well as histological parameters. Treatment with NAs can effectively suppress HBV replication and prevent the progression of disease.

On the other hand, long-term treatment with NAs is associated with significant problems in the management of CHB. Generally, adherence to long-term therapy is an important issue in patient management, and safety of long-term NA treatment is of concern since they are primarily eliminated by the kidney[11]. Financial burden of long-term NA treatment represent another important issue in the management of CHB. In this regard, international guidelines have suggested the timing of stopping antiviral NAs based on the studies demonstrating off-therapy durability of responses to NA therapy in patients with CHB (Table 1). A finite therapy would not only offer reduced financial burden and increased treatment safety but also encourage the patients to stay adherent to the treatment.

Table 1 Criteria for stopping nucleos(t)ide analogue therapy in chronic hepatitis B patients

	HBeAg-positive chronic hepatitis B	HBeAg-negative chronic hepatitis B
APASL 2012	HBeAg seroconversion with undetectable HBV DNA for at least 12 mo	HBsAg seroclearance or NA therapy > 2 yr and undetectable HBV DNA on three separate occasions, 6 mo apart
EASL 2012	HBsAg seroclearance or HBeAg seroconversion with undetectable HBV DNA and 12 mo of consolidation therapy	HBsAg seroclearance
AASLD 2009	HBeAg seroconversion with undetectable HBV DNA and > 6 mo of consolidation therapy	HBsAg seroclearance

HBeAg: Hepatitis Be antigen; APASL: Asian Pacific Association for the Study of the Liver; EASL: European Association for the Study of Liver; AASLD: American Association of the Study of Liver Disease.

For patients with hepatitis B e antigen (HBeAg)-positive CHB, the European Association for the Study of Liver (EASL) guideline recommends HBsAg loss with or without seroconversion as an ideal finite goal of NA therapy[9]. However, NA therapy-induced HBsAg seroclearance or seroconversion is only achievable in a minority of patients. Hence, the EASL guideline also provides a reasonable option that is comparable to the recommendations of the asian pacific association for the study of the liver (APASL) and american association of the study of liver disease (AASLD) guidelines. The APASL guideline suggests that treatment can be stopped after HBeAg seroconversion with undetectable HBV DNA by a sensitive polymerase chain reaction assay for at least 12 mo[8]. Likewise, the EASL and AASLD guidelines recommend that treatment can be stopped after HBeAg seroconversion with undetectable HBV DNA and an additional 6-12 mo of consolidation therapy[9,10]

For patients with HBeAg-negative CHB, however, currently there is no clear consensus on the optimal duration of oral antiviral therapy with NAs. Both the EASL and AASLD guidelines recommend long-term NA therapy until HBsAg seroclearance has been achieved[9,10]. On the contrary, the APASL guideline suggests that unless HBsAg seroclearance has been achieved, cessation of NA therapy can be considered after at least 2 years of treatment if HBV DNA remains undetectable on three separate occasions 6 mo apart[8].

Despite the stopping rules recommended by the international guidelines, whether oral antiviral therapy with NAs can be safely discontinued is of major concern. Accordingly, many studies have been conducted to evaluate the durability of antiviral response after cessation of NAs in patients with CHB (Table 2).

Table 2 Off-therapy durability of response to nucleos(t)ide analogue therapy in chronic hepatitis B patients

Ref.	NA	*n*	Treatment duration	Cumulative relapse rate
HBeAg-positive CHB				
Song *et al*[13]	LMV	98	10.3 ± 3.1 mo	1 yr, 37.5%; 2 yr 49.2%
Chien *et al*[14]	LMV	82	16 (3-55) mo	48%[1]
Dienstag *et al*[12]	LMV	39	36.6 (4.8-45.6) mo	77%[1]
Ryu *et al*[18]	LMV	61		6 mo, 15%; 1 yr, 21%; 2 yr, 31%
van Nunen *et al*[15]	LMV	59		3 yr, 54%
Byun *et al*[16]	LMV	132	14 ± 7 mo	6 mo, 58%; 1 yr, 66%
Yoon *et al*[17]	LMV	95	26 mo	1 yr, 52%; 2 yr, 55.7%
Fung *et al*[44]	LMV	22	23 (5-91) mo	44%[1]
Lee *et al*[19]	LMV	178	26 (12-77) mo	1 yr, 15.9%; 5 yr, 30.2%
Reijnders *et al*[21]	Various	132	26 (16-43) mo	67%[1]
Wang *et al*[20]	LMV	82	24 (12-54) mo	1 yr, 23.4%; 2 yr, 25.0%; 4 yr, 29.4%
Chaung *et al*[22]	Various	39		90%[1]
HBeAg-negative CHB				
Santantonio *et al*[23]	LMV	15	52 wk	74%[1]
Fung *et al*[24]	LMV	50	2 yr	6 mo, 30%; 12 mo, 50%; 18 mo, 50%
Chien *et al*[45]	LMV	85	7.4 (6-12) mo	61%[1]
Chan *et al*[46]	LMV	139	24 mo	90%[1]
Shouval *et al*[47]	ETV	257	48 wk	3%[1]
	LMV	201	48 wk	5%[1]
Paik *et al*[25]	LMV	50	24 mo	1 yr, 20.9%; 2 yr, 36.0%; 3 yr, 43.1%
Liu *et al*[26]	LMV	61	⩾ 24 mo	6 mo, 26.2%; 1 yr, 43.6%; 5 yr, 56.1%
Hadziyannis *et al*[27]	ADV	33	⩾ 4 yr	45%[1]
Jeng *et al*[28]	ETV	95	721 (395-1762) d	1 yr, 45.3%
Kim *et al*[48]	Various	45		6 mo, 48.9%; 1 yr, 73.3%

[1]Overall relapse. NA: Nucleos(t)ide analogue; CHB: Chronic hepatitis B; LMV: Lamivudine; ETV: Entecavir; ADV: Adefovir dipivoxil.

OFF-THERAPY DURABILITY OF ANTIVIRAL RESPONSE

HBeAg-positive patients

Initial studies with lamivudine showed disappointing results with its antiviral durability after cessation of therapy. Although a study of 39 patients reported lamivudine-induced HBeAg seroconversion to be durable in 77% of patients[12], a subsequent study of 34 HBeAg-positive CHB patients in whom lamivudine was stopped after HBeAg seroconversion showed that the cumulative relapse rate was 37.5% after 1 year and increased to 49.2% after 2 years of discontinuation[13]. Similarly, Chien *et al*[14] also reported that 48% of the patients relapsed after discontinuing lamivudine for 12 mo. In parallel with these results, several studies from different groups showed that the off-treatment durability of lamivudine-induced HBeAg seroconversion was not sustained in a large proportion of patients, with cumulative relapse rate of more than 50% after 1 year[15-17].

On the other hand, some studies have reported a higher antiviral durability after cessation of therapy. In a study of 61 patients, Ryu *et al*[18] reported that the cumulative relapse rate was 15% at 6 mo and 31% at 2 years after stopping lamivudine therapy. A more recent study by Lee *et al*[19] also showed a durable off-treatment response in 178 patients with lamivudine-induced complete response. In this study, the cumulative relapse rate was 15.9% at 1 year and 30.2% at 5 years. These results were supported by a more recent prospective study with 82 patients demonstrating a cumulative relapse rate of 23.4% at 6 mo and 29.4% at 4 years after discontinuation of lamivudine[20].

However, in a series of recent studies with various antiviral NAs the off-treatment response was shown to be not durable despite consolidation therapy after HBeAg seroconversion. A study of 132 patients who achieved HBeAg seroconversion by various NAs demonstrated an overall relapse rate of 67% despite consolidation therapy after HBeAg seroconversion[21]. Moreover, a more recent study of 39 patients treated with various NAs reported that almost all (90%) patients who stopped NA therapy after achieving HBeAg seroconversion and clinical response experienced recurrent viremia despite consolidation therapy prior to discontinuation of NAs[22].

To summarize, the results from these studies imply that the current guidelines of stopping NAs after achieving HBeAg seroconversion with undetectable HBV DNA do not seem to result in a durable off-treatment antiviral response in the majority of HBeAg-positive CHB patients.

HBeAg-negative patients

Earlier studies showed consistent results that the antiviral response was not durable in patients who stopped NA therapy even if the guideline recommendations were followed. In a small study of 15 HBeAg-negative patients who stopped lamivudine after a year of therapy, 86% of the patients developed virological and/or biochemical relapse[23]. Succeeding studies with more stringent cessation criteria showed some improvements in the durability of NA-induced antiviral response, yet the results were

still disappointing. In a retrospective study of 50 HBeAg-negative patients, the cumulative relapse rates at 6 mo was 30% and at 18 mo was 50% despite successful lamivudine treatment for 2 years followed by withdrawal[24]. Moreover, several prospective studies also demonstrated that off-treatment response rates were below satisfaction. In a long-term prospective study of 50 HBeAg-negative patients who stopped lamivudine after treatment for 24 mo, the cumulative relapse rate was 43.1% at 3 years after withdrawal[25]. Similarly, Liu *et al*[26] have also reported a cumulative relapse rate of 56.1% at 5 years after stopping lamivudine treatment.

In a series of recent studies with antiviral NAs other than lamivudine, the durability of off-treatment response was shown to be comparable to that of lamivudine treatment. A prospective cohort study of 33 HBeAg-negative patients who had been treated with adefovir for 4-5 years and monitored for 5.5 years after cessation of treatment showed an overall relapse rate of 45% during the follow-up period[27]. Interestingly, among 18 of 33 patients who achieved sustained response, 13 (72%) patients showed HBsAg clearance. Of note, serum HBsAg levels at the end of treatment showed significant association with HBsAg clearance, suggesting a possible role of HBsAg quantitation for predicting the antiviral response to NA therapy.

A more recent study by Jeng *et al*[28] demonstrated off-treatment durability of entecavir therapy in 95 HBeAg-negative patients using the stopping rule recommended by the APASL guideline. In this observational study, patients were treated with entecavir for a median of 721 d, and followed up for at least 12 mo. The cumulative relapse rate after 1 year of stopping entecavir treatment was 45.3%.

For the patients with HBeAg-negative CHB, current data show that about half of the patients attain durable antiviral response after discontinuation of NA therapy. This is somewhat disappointing as the off-treatment durability is not satisfactory despite following the stopping rule recommended by the guideline. Therefore, further studies would be required to find adequate factors that could predict the best timing of stopping the NA therapy.

HBSAG QUANTITATION AS A PREDICTIVE MARKER

Current guidelines for stopping NA therapy seems to be inadequate in terms of off-treatment durability, with relapse rates of more than 40% for both HBeAg-positive and HBeAg-negative patients. As a result, further studies are warranted to identify adequate predictive markers that could provide supplementary information to guide the timing of stopping NA therapy.

One of the recently proposed predictive factors for HBsAg loss is serum HBsAg quantitation. Since serum HBsAg level seems to correlate with the amount of covalently closed circular DNA (cccDNA) within the infected hepatocytes[29-31], serial monitoring of serum HBsAg levels during the course of antiviral therapy may provide useful information regarding off-treatment response. Clinically, low serum levels of HBsAg and HBV DNA were reported to be predictive of spontaneous HBsAg seroclearance in treatment-naïve patients[32]. For HBeAg-positive patients on NA therapy, older age, high baseline alanine aminotransferase and HBeAg loss were reported as the predictive factors for HBsAg loss[33]. Based on these findings, it has been suggested that monitoring of serum HBsAg levels along with serum HBV DNA levels may guide the timing of stopping NA therapy[34]. However, the clinical significance of serum HBsAg quantitation for predicting HBsAg loss during antiviral therapy with NAs has been challenged by several studies. In a study investigating the effect of long-term entecavir or tenofovir treatment on serum HBsAg levels in CHB patients, Zoutendijk *et al*[33]. reported that the predicted median time to HBsAg loss was 36 years for HBeAg-positive and 39 years for HBeAg-negative patients Moreover, another study from a French group, based on their mathematical modeling, suggested that more than 50 years of NA therapy would be required for the clearance of HBsAg[35], implying that cessation of NA therapy would be almost impossible. Nevertheless, there is an increasing attention in the clinical utility of serum HBsAg quantitation, and further studies are needed to validate its role for monitoring the antiviral response and predicting the off-treatment durability.

CONCLUSION

For the minority who have achieved the ultimate goal of NA therapy, *i.e.*, HBsAg loss, the treatment may be discontinued. However, loss of HBsAg does not necessarily indicate complete eradication of the virus as cccDNA persists within the infected hepatocytes, and thus there is still a risk of developing HCC[36,37]. Therefore, long-term surveillance for HCC would be mandatory albeit successful achievement of HBsAg loss and discontinuation of NA therapy.

In addition, for patients with liver cirrhosis, it would be beneficial to maintain than to discontinue NA therapy. This is supported by an increasing data that long-term viral suppression with NA therapy not only decreases hepatic inflammation but also induces regression of cirrhosis which may translate to improved clinical outcome[38-43].

In conclusion, while attention has been drawn to whether antiviral treatment with NAs can be a finite therapy, there is lack of sufficient data on off-treatment durability of highly potent NAs, particularly tenofovir, and based on the available evidences, current guidelines for stopping NA therapy seems to be inadequate in terms of off-treatment durability. Therefore, further studies are required to accumulate data on off-treatment durability of highly potent NAs. Furthermore, search for adequate predictive markers that could provide supplementary information to guide the timing of stopping NA therapy is warranted. Clinical studies addressing this issue seems to be highly desirable in the future.

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TOPIC HIGHLIGHT

WJG 20th Anniversary Special Issues (9): Hepatitis B virus

Association between metabolic factors and chronic hepatitis B virus infection

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Abstract

There are limited data regarding the relationship between chronic hepatitis B virus (HBV) infection and metabolic factors. This article aims to highlight the link of metabolic factors with hepatitis B surface antigen (HBsAg) serostatus, HBV load, and HBV-related hepatocellular carcinoma (HCC). Although HBsAg-positive serostatus was positively correlated with a high risk of metabolic syndrome in students, chronic HBV-infected individuals have high serum adiponectin levels. The androgen pathway in HBV carriers with a low body mass index is more triggered which leads to enhanced HBV replication. High HBV load was inversely associated with obesity in hepatitis B e antigen (HBeAg)-seropositive HBV carriers; while in HBeAg-seronegative HBV carriers, high HBV load was inversely related to hypertriglyceridemia rather than obesity. For overweight and obese HBV-infected patients, high HBV load was positively associated with serum adiponectin levels. Several large cohort studies have revealed a positive link of diabetes with incidence of HBV-related HCC. However, the association between incidence of HCC and metabolic factors other than diabetes is still inconclusive. More long-term prospective studies should elucidate the association of chronic HBV infection and its outcomes with metabolic factors in clinical practice.

Key words: Hepatitis B surface antigen; Hepatitis B viral load; Hepatocellular carcinoma; Diabetes; Obesity; Adiponectin

Core tip: Facing the increasing burden of metabolic syndrome and chronic hepatitis B worldwide, this review tries to highlight the association of metabolic factors with chronic hepatitis B. Intriguingly, hepatitis B virus carriers are reported to have higher serum adiponectin levels, previously linked with individuals with low body mass index. Obesity and hypertriglyceridemia (metabolically bad factors) are inversely associated with high hepatitis B viral load; a crucial predictor for primary liver cancer. In contrast, serum adiponectin levels (a metabolically good factor) are positively related to high hepatitis B viral load in individuals with high body mass index.

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INTRODUCTION

Chronic hepatitis B virus (HBV) infection is well-known as a major risk factor for hepatocellular carci-

noma (HCC)[1-4]. The burden of obesity and metabolic syndrome has been increasing in recent decades[5]. Subsequently, there is growing concern regarding the association between metabolic factors and chronic HBV infection. This review article tries to highlight the association of metabolic factors with hepatitis B surface antigen (HBsAg) serostatus, HBV load, and HBV-related HCC.

METABOLIC FACTORS AND HBsAg SEROSTATUS

Chronic-HBV-infected university freshers (4475 men and 3751 women) had a higher risk of metabolic syndrome (OR = 1.58, 95%CI: 1.04-2.47) compared to individuals with seroprotective titers after HBV vaccination[6], after controlling for age, sex, body mass index (BMI), uric acid quartiles, smoking, alcohol consumption, and physical activity. However, another population-based cross-sectional study (53528 participants) showed that the likelihood of developing metabolic syndrome was lower in HBsAg-positive (n = 5995, 12.6%) than HBsAg-negative (adjusted OR = 0.84, 95%CI: 0.76-0.93) adults after controlling for age and sex[7]. High triglyceride level (≥ 150 mg/dL) (adjusted OR = 0.65, 95%CI: 0.60-0.69) and high blood pressure (adjusted OR = 0.89, 95%CI: 0.83-0.94) were inversely associated with being HBsAg-positive. One of the probable explanations of the inconsistency between the above student- and population-based studies is the different age compositions (freshers *vs* 30-79 years) and comparison groups (individuals with seroprotective titers after HBV vaccination *vs* being HBsAg positive). It is worth mentioning that being positive for hepatitis C virus (HCV) antibody was positively associated with reduced high-density lipoprotein (adjusted OR = 1.61, 95%CI: 1.37-1.88), while inversely associated with high triglyceride level (adjusted OR = 0.63, 95%CI: 0.55-0.71) according to the population-based study[7]. Thus, the likelihood of developing metabolic syndrome in HCV carriers (n = 1792, 3.8%) was similar to that in non-HCV carriers.

There have been controversial results. A hospital-based cross-sectional study (243 men and 264 women; mean age: 46.6 years) showed no significant relationship between chronic HBV infection and insulin resistance or ultrasonographic hepatic steatosis[8]. Another cross-sectional population study reported that HBV-infected Hong Kong Chinese (n = 91) had lower intrahepatic triglyceride content measured by proton-magnetic resonance spectroscopy (P < 0.001), lower serum triglycerides (P < 0.001), lower metabolic syndrome (11.0% *vs* 20.2%, P = 0.034), and a lower risk of fatty liver (adjusted OR = 0.42, 95%CI: 0.20-0.88) than controls (n = 922)[9]. The association of HBV with selected adipokines is also under investigation. For example, adiponectin possesses anti-inflammatory effects and is inversely associated with BMI, type 2 diabetes and several metabolic disorders[10]. Recently, we demonstrated that HBV-infected individuals, though heavier than healthy controls, had higher serum adiponectin levels (P < 0.0001) and a higher proportion of adiponectin levels over the 75th percentile (adjusted OR = 4.25, 95%CI: 2.36-7.66) after controlling age, sex, BMI, and insulin resistance index[11]. The link between HBsAg serostatus and metabolic factors should be further clarified from the perspective of HBV load.

METABOLIC FACTORS AND HEPATITIS B VIRAL LOAD

Some animal models considered HBV a "metabolovirus" because the gene expression of HBV and key metabolic genes in hepatocytes was shown to be similarly regulated[12]. The androgen production in HBV carriers with a low BMI (< 23 kg/m^2) was more triggered and up-regulated HBV replication, as shown in a transcriptional animal model and a campus-based study[13,14]. A Taiwanese community-based study including 3587 HBV-infected participants revealed that high HBV load was inversely associated with extreme obesity (adjusted OR = 0.17, 95%CI: 0.05-0.63) and central obesity (adjusted OR = 0.44; 95%CI: 0.25-0.78) in HBeAg-seropositive patients; while high HBV load was inversely associated with hypertriglyceridemia (adjusted OR = 0.74, 95%CI: 0.61-0.89) in HBeAg-seronegative patients[15]. Liver steatosis was neither associated with HBV load in HBeAg-seropositive patients (adjusted OR = 1.46, 95%CI: 0.90-2.36) nor in HBeAg-seronegative patients (adjusted OR = 0.88, 95%CI: 0.72-1.08). The above findings altogether implicate that metabolically bad factors (obesity and hypertriglyceridemia) may cause liver damage through hepatic steatosis and oxidative stress, independently of HBV replication.

Although adipokines were observed to contribute to histological liver injury of chronic HBV-infected patients hospitalized for liver biopsy[16], an experimental animal model demonstrated that HBV replication boosted the increase in circulating adiponectin levels through activation of peroxisome proliferator-activated receptorγ (*PPARγ*) gene expression. Reciprocally, adiponectin and *PPARγ* agonist treatment triggered HBV replication[17]. Consistently, we also revealed that the logarithmic transformation of HBV load was positively associated with serum adiponectin levels, but only in patients with a higher BMI (BMI ≥ 23 kg/m^2) (P = 0.018) adjusted for age, sex, BMI, HBeAg serostatus, liver function, and homeostasis model assessment of insulin resistance[11]. In patients with a lower BMI, HBV load tended to be up-regulated by the activated androgen production more than the adiponectin pathway[14]. More elucidation of adiponectin pathways in HBV carriers may help develop adjuvant treatments of HBV infection in the future.

METABOLIC FACTORS AND HBV-RELATED HCC

The potential link between diabetes mellitus and metabolic factors with HBV-related HCC has aroused increasing concern[18-22], not necessarily related to serum HBV

load, a well-known risk factor of HCC[23,24]. For example, a long-term community-based cohort revealed that HBV-related HCC risk was associated with diabetes (adjusted OR = 2.27, 95%CI: 1.10-4.66) rather than extreme obesity (adjusted OR = 1.36, 95%CI: 0.64-2.89)[18]. However, the study performed no adjustment of hepatitis B viral load or HBeAg serostatus.

The relationship between HCC and metabolic factors other than diabetes, however, is more inconclusive. A large European cohort study of 289273 men has reported an inverse link between cancer occurrence of the liver and intrahepatic ducts and serum total cholesterol[25]. Tsan *et al*[26,27] analyzed a National Health Insurance claims database and found protective effects of statins on HBV- and HCV-related HCC incidence. Notably, secondary data analyses using claims database in Taiwan usually lack important confounding information including BMI, blood pressure, liver function, cigarette or alcohol habits, and medication adherence. Besides, clinicians may decide to withhold or withdraw statins for patients with abnormal liver function, though some human trials of statins were shown to improve hepatic steatosis and hepatic fibrosis[28]. This concern in real practice might confound the true protective role of statins in HBV- and HCV-related HCC incidence.

High triglyceride levels (≥ 150 mg/dL) were inversely associated with subsequent HBV-related HCC incidence (adjusted OR = 0.60, 95%CI: 0.40-0.90)[18]. This finding is consistent with the inverse association between serum triglycerides and HBV load in HBeAg-seronegative patients[15]. HBV X protein could inhibit the secretion of apolipoprotein B, located on the surface of every triglyceride-rich very-low-density lipoprotein particle[29]. Once HBV actively replicates, HBV X protein increases rapidly and impairs the production of very-low-density lipoprotein and circulating triglycerides. However, an animal study reported fibrate-induced anti-proliferative effects in cultured human HCC cells[30]. The investigators demonstrated that the protective effects were independent of the PPARα pathway. There are still no prospective human studies prospectively exploring fibrate use and HCC occurrence in HBV-infected individuals.

CONCLUSION

The controversy regarding the association between the presence of HBsAg and metabolic factors should be further understood from the perspective of HBV load. High HBV load was inversely associated with obesity in HBeAg-seropositive HBV carriers; while in HBeAg-seronegative individuals, high HBV load was inversely related to hypertriglyceridemia. HBV replication did not interact with obesity or hypertriglyceridemia to cause liver damage. The activation of *PPARγ* gene expression at a high BMI and androgen pathway at a low BMI might be associated with high HBV load. Among metabolic factors, diabetes has been the best known risk factor of HBV-related HCC. More better-designed long-term prospective research should focus on elucidating association of metabolic factors with chronic HBV infection and its relevant outcomes.

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TOPIC HIGHLIGHT

WJG 20th Anniversary Special Issues (11): Cirrhosis

Influence of unrecorded alcohol consumption on liver cirrhosis mortality

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Abstract

Unrecorded alcohol includes illegally distributed alcohol as well as homemade or surrogate alcohol which is unintended for consumption by humans (*e.g.*, cosmetics containing alcohol). The highest unrecorded alcohol consumption occurs in Eastern Europe and some of these countries have an over proportional liver cirrhosis mortality. Compounds besides ethanol have been hypothesized as being responsible for this observation. On the other hand, chemical investigations were unable to prove that unrecorded alcohol regularly contains contaminants above toxicological thresholds. However, illegally produced spirits regularly contain higher percentages of alcohol (above 45% by volume), but for considerably less costs compared with licit beverages, potentially causing more problematic patterns of drinking. In this review, it is investigated whether patterns of drinking rather than product composition can explain the liver cirrhosis mortality rates. Statistical examination of World Health Organization country data shows that the originally detected correlation of the percentage of unrecorded alcohol consumption and liver cirrhosis mortality rates disappears when the data is adjusted for the prevalence of heavy episodic drinking. It may be concluded that there is currently a lack of data to demonstrate causality between the composition of illicit spirits (*e.g.*, higher levels of certain contaminants in home-produced products) and liver toxicity on a population scale. Exceptions may be cases of poisoning with antiseptic liquids containing compounds such as polyhexamethyleneguanidine, which were reported to be consumed as surrogate alcohol in Russia, leading to an outbreak of acute cholestatic liver injury, histologically different from conventional alcoholic liver disease.

Key words: Epidemiology; Liver cirrhosis; Alcoholic beverages; Unrecorded alcohol; Risk assessment

Core tip: Various constituents and contaminants of unrecorded alcohol (*i.e.*, illicitly or informally produced alcohol) were implicated as over proportionally causing liver disease. Quantitative risk assessments were not able to corroborate these claims by identifying such contaminants above toxicological levels, however. The higher rates of liver disease can be alternatively explained by more detrimental patterns of drinking in regions with a high prevalence of unrecorded alcohol consumption.

Lachenmeier DW, Monakhova YB, Rehm J. Influence of unrecorded alcohol consumption on liver cirrhosis mortality. *World J*

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INTRODUCTION

In Central and Eastern Europe, large discrepancies can be found between recorded alcoholic beverage consumption and alcohol-related mortality[1]. An example is Hungary, a country in which liver disease mortality is circa four times that of countries with comparable per capita consumption of alcohol (*e.g.*, Refs.[2,3]). An explanation for this finding might be the particularly high unrecorded alcohol consumption[2].

Despite the high levels of unrecorded alcohol consumption in some countries (up to 40% of total consumption), there is an absence of data about chronic long-term health consequences that may specifically be influenced by unrecorded alcohol consumption. The proof of causality is particularly difficult because people may co-consume both forms of alcohol (*i.e.*, recorded and unrecorded alcohol)[4]. Rehm *et al*[4] have provided an example of a Russian person having died of alcoholic liver cirrhosis, and for whom unrecorded alcohol products were the main form of alcohol consumption in his final years. Nevertheless, he would have been expected to exclusively consuming recorded alcohol for some years before switching out of economic reasons to unrecorded alcohol consumption (typically to medicinal, cosmetic or other surrogate alcohol) when his patterns of drinking became more and more detrimental[5]. With this change and the following exposure to "other" forms of alcohol, there are 3 potential effects according to Rehm *et al*[4]: "(1) The alcoholic liver cirrhosis would have taken exactly the same course; (2) The alcoholic liver cirrhosis would have taken a different course, for example, later onset, longer duration, or no fatal outcome, if this person had consumed recorded alcohol only; and (3) The alcoholic liver cirrhosis would not have occurred with consumption of recorded alcohol".

The determination of causality of the link of unrecorded alcohol with chronic disease is, therefore, a challenging task[6,7]. As a result, few studies have researched the question if unrecorded alcohol consumption may have detrimental health effects that are not found in recorded consumption, *e.g.*, because unrecorded alcohol may contain some compounds that are not present in recorded alcohol. An exception is a study from India[8], which found an association between unrecorded alcohol consumption (country liquor) and an increased risk of alcoholic liver disease, particularly alcoholic liver cirrhosis. It is notable that the country liquor contained lower alcoholic strengths than the local types of recorded alcohol. However, the study may have been confounded by social status and other factors that were not well controlled[4].

The connection between unrecorded alcohol consumption levels and liver cirrhosis mortality rates can be inferred from Figure 1. Hungary, Moldova and Romania show very high levels of unrecorded adult (15+) per capita alcohol consumption [4.0 L (Hungary, Romania) or 10 L (Moldova) of pure alcohol per year]. For these countries, liver cirrhosis mortality rates (47, 43 and 119 per 100000 adult population for Hungary, Romania and Moldova, respectively) were much higher than for other European countries. In contrast, comparably low unrecorded alcohol consumption in France, Spain, and Switzerland (0.34, 1.4, and 0.50 L of pure alcohol per year) were associated with low liver cirrhosis mortality rates in these countries (less than 12 deaths per 100000 adult population from alcoholic liver disease).

INVESTIGATION INTO THE INFLUENCE OF UNRECORDED ALCOHOL CONSUMPTION ON LIVER CIRRHOSIS MORTALITY

To provide further and more systematic insight into the connection between unrecorded alcohol consumption and liver cirrhosis, the following data was taken from the Global Information System on Alcohol and Health (GISAH) of the World Health Organization (WHO)[9]: (1) The liver cirrhosis age-standardized mortality rate is "the number of individuals in a given population (100000 people) that died from alcoholic liver disease during a calendar year (2005)". Only adults (population above 15 years) were taken into account; (2) The levels of unrecorded and total consumption (in liters of pure alcohol) for the year 2005 and for the population older than 15 years; and (3) Heavy episodic drinkers, defined as "the percentage of adults (aged 15+) who have drunk at least 60 g (approximately 6 standard alcoholic drinks) or more of pure alcohol on at least one occasion weekly".

The following definitions are quoted verbatim from the WHO GISAH website[9] for reasons of clarity: "recorded alcohol consumption refers to official statistics (production, import, export, and sales or taxation data), while unrecorded alcohol consumption refers to alcohol which is not taxed and is outside the usual system of governmental control, such as home- or informally-produced alcohol (legal or illegal), smuggled alcohol, surrogate alcohol (which is alcohol not intended for human consumption), or alcohol obtained through cross-border shopping (which is recorded in a different jurisdiction). Recorded adult per capita consumption of pure alcohol is calculated as the sum of beverage-specific alcohol consumption of pure alcohol (beer, wine, spirits, other) from different sources. The first priority in the decision tree is given to government statistics; second are country-specific alcohol industry statistics in the public domain (Canadean, IWSR-International Wine and Spirit Research, OIV-International Organisation of Vine and Wine, Wine Institute, historically World Drink Trends); and third is the Food and Agriculture Organization of the United Nations' statistical database (FAOSTAT). The method of

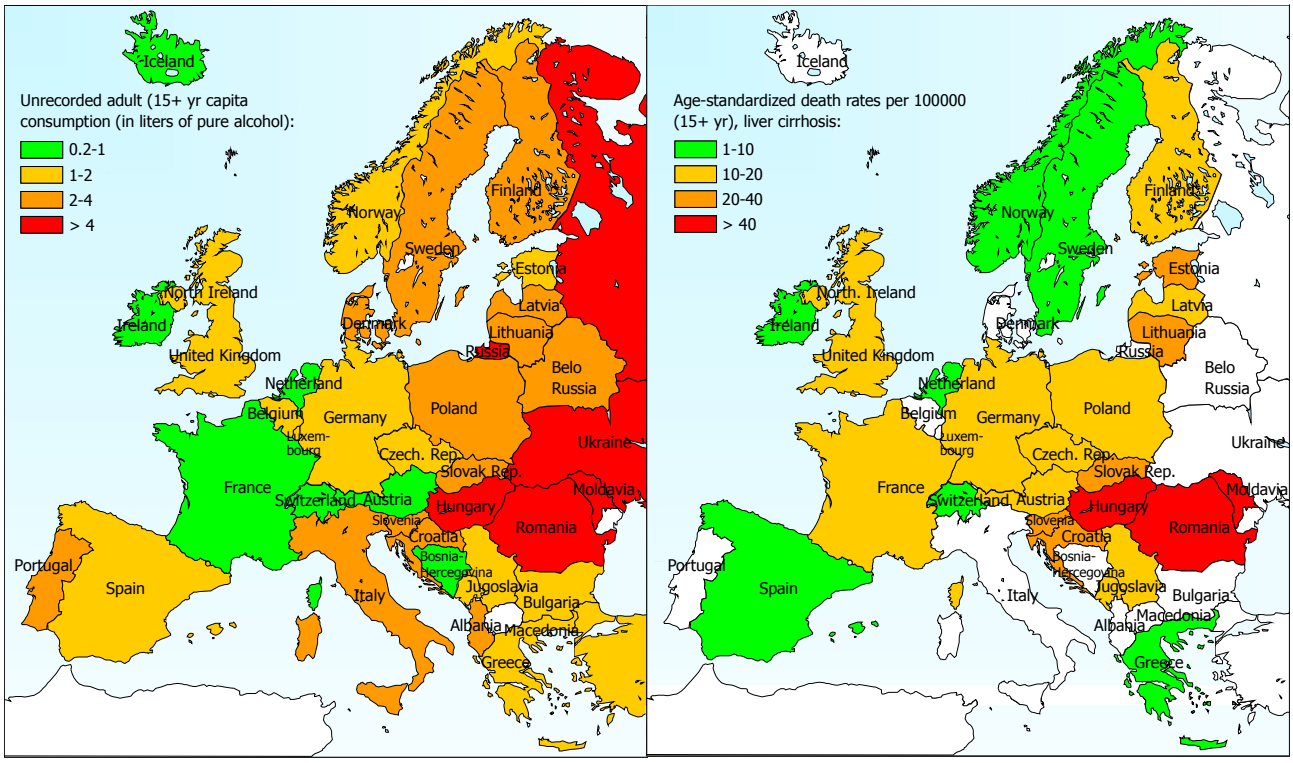

Figure 1 Comparison of levels of unrecorded alcohol consumption and liver cirrhosis mortality rates in Europe for 2005. Data from World Health Organization[9]; no data available for uncolored countries.

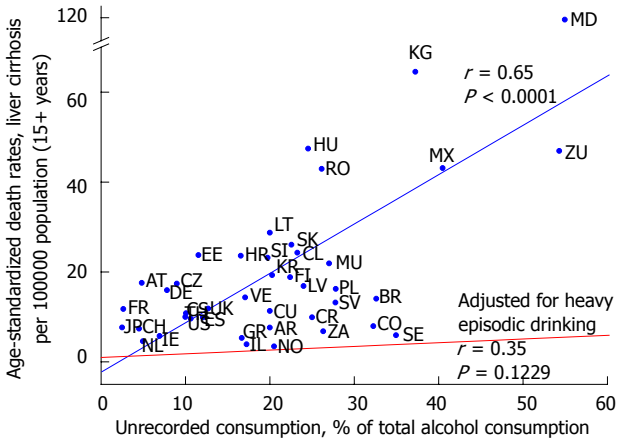

Figure 2 Correlation between unrecorded alcohol consumption and liver cirrhosis mortality for countries worldwide. Own calculation based on World Health Organization data[9]; the lower curve shows the result after controlling for heavy episodic drinking.

measurement of unrecorded alcohol consumption gives the first priority in the decision tree to nationally representative empirical data; these are often general population surveys in countries where alcohol is legal. Second are specific other empirical investigations, and third is expert opinion. Survey questions on consumption of unrecorded alcohol are converted into estimates per year of unrecorded adult per capita consumption. Usually surveys underestimate consumption. However, in countries where survey based estimates exceeded the recorded consumption, unrecorded was calculated as total consumption estimated from survey minus recorded adult per capita consumption. In some countries, unrecorded is estimated based on confiscated alcohol confiscated by customs or police. Total consumption is the sum of recorded and unrecorded consumption".

The full methodology is available on the WHO GISAH webpages[9,10]. The percentage of unrecorded alcohol consumption for each country was then calculated from these data.

Data analysis was conducted with Origin V.7.5 (Originlab, Northampton, United States). Mathematical correlation between the investigated parameters was evaluated using linear regression for all countries for which data was available. The 0.05 probability level was used to define statistical significance. The results are shown in Figure 2.

There appears to be a statistically significant correlation between mortality from liver cirrhosis and the amount of unrecorded alcohol consumption ($r = 0.65$, $P < 0.0001$). This trend upholds even when the data is controlled for per capita consumption ($r = 0.35$, $P = 0.04$; for calculation see Ref.[11]), but becomes non-significant after controlling for heavy episodic drinking ($r = 0.35$, $P = 0.12$). However, five countries (Hungary, Estonia, Slovakia, Mauritius, and Ecuador) still showed over proportional liver cirrhosis mortality after the adjustment for heavy episodic drinking. However, as consumption of alcohol *per se* may also cause liver cirrhosis, it remains unclear what the specific contribution of unrecorded al-

Table 1 Compounds potentially associated with toxicity in unrecorded alcohol

Compounds in unrecorded alcohol	Occurrence	Toxic effect	Risk assessment
Ethanol	Main components of every alcoholic beverage; often found in higher strength in unrecorded alcohols	Several acute and chronic health effects including liver cirrhosis	Major risk factor of unrecorded alcohol on a population scale
Methanol	Minor constituent in all alcoholic beverages, higher in some fruit spirits; acute toxic concentrations due to adulteration	Poisoning at high concentrations	Extremely high risk for consumers of highly contaminated products; poisoning outbreaks are uncommon but cause high morbidity/mortality
Higher alcohols (*e.g.*, propanol, butanol)	Minor constituents in all alcoholic beverages, higher concentrations in certain fruit spirits	Similar to ethanol	Lack of causality with effects of unrecorded alcohol (exposure below thresholds)
Acetaldehyde	Minor constituents in all alcoholic beverages, higher concentrations in certain fruit spirits	Carcinogenicity	Could constitute higher chronic risk if contained at higher levels, but epidemiological evidence is missing
Ethyl carbamate	High levels in stone fruit spirits	No human data. Hepatocellular tumors in rodents	See acetaldehyde
Diethyl phthalate	Denatured alcohols	Developmental effects, hepatotoxicity in animals	See acetaldehyde
Coumarin	Flavoring in cosmetic alcohol	Hepatotoxicity in animals	See acetaldehyde
Polyhexamethyleneguanidine	Antiseptic liquids in Russia	Potentially causing cholestatic hepatitis in humans	Unclear causality, but observational studies suggest a plausible risk

cohol may be[11,12].

MECHANISMS POTENTIALLY LEADING TO OVER PROPORTIONAL LIVER CIRRHOSIS MORTALITY ASSOCIATED WITH UNRECORDED ALCOHOL CONSUMPTION

The large variance of cirrhosis mortality rates between the countries Hungary and Romania and the rest of Europe, mentioned above, were suggested to having been caused by some specific compounds in unrecorded alcoholic beverages[2] but not by differences in drinking behavior between recorded and unrecorded alcohol (*e.g.*, regarding the volume or patterns of consumption)[13,14].

Some studies (see summary in Refs.[12,15]) have used various chemical methods to systematically analyze the composition of unrecorded alcoholic beverages with the focus on potential harmful components (Table 1). If we examine the potential long-term health consequences of unrecorded alcohol consumption such as liver cirrhosis, a single consistent finding was regularly described: the concentration of ethanol is higher in unrecorded alcohols (considerably above 40% vol) than in the recorded alcoholic beverages[4]. These higher contents of ethanol alone may cause detrimental effects, for example, regarding injuries and ethanol intoxication. The alcoholic strength of unrecorded alcohol is normally not labeled, so that the consumer is unaware that some high strength types should be diluted with water before consumption. Therefore, the original high-strength beverages could be consumed directly[12].

Other components besides ethanol analyzed in unrecorded alcohols were below toxicological thresholds in most of the samples[15-17]. For example, some hepatotoxic contaminants such as copper or ethyl carbamate were found in some samples of unrecorded alcohol, however, the intake in alcohol consumers was below 1% of the threshold doses in rodents[16]. In conclusion, there are considerable research needs regarding unrecorded alcohol[18]. In light of the current state of research, the authors believe that the two indicators "volume of alcohol consumption" and "drinking patterns" are the major contributors that cause the observed differences in liver cirrhosis mortality[16]. Both indicators may be influenced by unrecorded alcohol because it typically contains higher concentrations of ethanol and its lower costs may additionally increase the drinking amounts[16]. Unrecorded alcohol consumption is also inversely associated with socioeconomic status (SES) and education, both of which are also factors connected to alcohol-related death, disease and injury[19]. Further confounding factors in the population consuming unrecorded alcohol may be drug use, viral hepatitis or HIV, which could potentially contribute to multifactorial liver disease[12]. However, there is currently an absence of quantitative epidemiological research on these risk factors in connection with unrecorded alcohol consumption.

METHANOL AND PHMG: EXCEPTIONS TO THE RULE

While unrecorded alcohol seldom contains substances more toxic than ethanol itself, the exception to the rule may be isolated outbreaks of methanol poisoning[20] as well as the occurrence of polyhexamethyleneguanidine hydrochloride (PHMG). PHMG is a substance that was linked to widespread acute cholestatic liver injury in Russia connected to the consumption of surrogate

alcohol[21]. In that case, the surrogate that was ingested was an antiseptic fluid, which consisted of ethanol (93%), diethyl phthalate (DEP) (0.08%-0.15%) and PHMG (0.10%-0.14%). While PHMG is the disinfecting ingredient[22], DEP is used to denature the alcohol[23]. Several other Russian studies detected PHMG together with DEP in solutions consumed as surrogate alcohol connected to intoxications[6,22,24]. Ostapenko *et al*[21] concluded from clinical and laboratory findings in 579 cases that the cholestatic hepatitis histologically different from conventional alcoholic liver disease was connected to PHMG exposure. A history of hepatitis and cirrhosis caused by long-term alcohol consumption may have contributed to a more severe course of the intoxication. Besides PHMG, multifactorial liver damage may have been caused by further factors such as DEP or chronic viral hepatitis. Nevertheless, the causality in these poisoning cases in Russia remains questionable because the exact composition of the surrogate alcohol that has been consumed is often unknown, and the studies were not controlled for confounding factors such as volume and patterns of drinking[12,17,25].

CONCLUSION

Comprehensive literature reviews proof that concerns about the health effects of unrecorded alcohol were typically overstated[4,15]. To provide only one example, the first quantitative risk assessments of compounds in alcohol have provided evidence that the effect of alcohol itself regarding harm to the liver is more than 5000 times greater than the one of ethyl carbamate[26,27]. Liver cirrhosis mortality rates connected to unrecorded alcohol consumption may be rather explained by the higher ethanol contents, detrimental patterns of drinking, lower SES and poor health status and the interaction between these indicators than by reference to alcohol quality[4].

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TOPIC HIGHLIGHT

WJG 20th Anniversary Special Issues (11): Cirrhosis

Comorbidity in cirrhosis

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Abstract

Cirrhosis patients' comorbidities are their other diseases than cirrhosis. Comorbidities are neither causes nor consequences of cirrhosis, but they can increase mortality and are therefore clinically important. They are also an important source of confounding in epidemiologic studies. Comorbidity scoring systems have been developed as tools to measure the cirrhosis patient's total burden of comorbidity, and they are useful in the clinic and for epidemiologic studies. The recently developed CirCom score is the only comorbidity scoring system developed specifically for cirrhosis patients, and it may be preferred over the older, generic, and more complex Charlson comorbidity index. Studies of individual comorbid diseases can provide insight into the interactions between cirrhosis and other diseases and thus into the pathophysiology of cirrhosis. This article reviews the literature on comorbidity in cirrhosis.

Key words: Liver cirrhosis; Comorbidity; Prognosis; Epidemiology

Core tip: Cirrhosis patients' comorbidities are their other diseases than cirrhosis. They can increase mortality and are therefore clinically important. They are also an important source of confounding in epidemiologic studies. Comorbidity scoring systems have been developed as tools to measure the cirrhosis patient's total burden of comorbidity, and they are useful in the clinic and for epidemiologic studies. Studies of individual comorbid diseases can provide insight into the interactions between cirrhosis and other diseases and thus into the pathophysiology of cirrhosis. This article reviews the literature on comorbidity in cirrhosis.

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INTRODUCTION

Cirrhosis patients' comorbidities are their other diseases than cirrhosis[1,2]. Comorbidities increase mortality and are therefore clinically relevant[3,4]. The presence of comorbidity may also be an important source of confounding and should be accounted for in epidemiologic studies of cirrhosis patients.

Comorbidities must be distinguished from complications such as ascites, variceal bleeding, and hepatic encephalopathy. Complications are at least to some extent a consequence of the portal hypertension and loss of liver function resulting from cirrhosis, whereas comorbidities are neither causes nor consequences of cirrhosis[1]. Sometimes the distinction is difficult: for example, is hepatocellular carcinoma a complication or comorbidity to cirrhosis? Cirrhosis develops in response to a repeated injury to the hepatocytes, and hepatocellular carcinoma in a cirrhosis patient likely develops in response to the same injury[5]. Therefore it is reasonable to interpret hepatocel-

lular carcinoma as a complication to cirrhosis although it can also develop in patients without cirrhosis. There are many diseases whose causal relationship with cirrhosis is unclear, and the categorization of a disease as a complication or comorbidity may change as our understanding of cirrhosis pathophysiology evolves.

The aim of this article is to review the evidence regarding comorbidities' impact on the mortality of cirrhosis patients. The cirrhosis patient's total burden of comorbidity may be assessed by a comorbidity scoring system, and such a system may be helpful for clinical decision-making and for confounder control in epidemiologic studies. The prognostic impact of individual comorbidities, on the other hand, may point to areas where cirrhosis and comorbid diseases interact. Studies of individual comorbidities may therefore improve our understanding of the pathophysiology of cirrhosis. This article reviews studies of comorbidity scoring systems and studies of the impact of individual comorbidities on the clinical course of cirrhosis.

COMORBIDITY SCORING SYSTEMS

The purpose of a comorbidity scoring system is to express a patient's total burden of comorbidity as a single number rather than a list of diagnoses: Comorbidity scores make it easier to communicate a patient's comorbidity burden, and they also facilitate epidemiologic studies because inclusion or exclusion criteria can be based on a comorbidity score, analyses can be stratified according to the comorbidity score, and the comorbidity score may serve as a confounding factor that can be adjusted for in the statistical analysis.

A comorbidity scoring system should reflect the combined effects of all a patient's comorbidities. This might be complex, but for purposes of mortality prediction it appears that there is no need to consider more than two diseases for each patient[3]. It is possible to develop comorbidity scores for other outcomes than mortality, *e.g.*, surgical risk or variceal bleeding, but existing scoring systems have been developed to predict mortality. Two comorbidity scores have been validated as predictors of mortality among cirrhosis patients: The Charlson comorbidity index and the CirCom score[3,4]. The Charlson comorbidity index and a modified version thereof, the CCI-OLT, have also been shown to predict mortality among liver transplant recipients[6,7].

Charlson comorbidity index

The Charlson comorbidity index assigns a numeric score ranging from one to six to 17 diseases according to their effect on mortality (Table 1). The sum of a patient's scores is a measure of the total burden of comorbidity[8]. In studies of cirrhosis patients, liver disease must be excluded from the Charlson index because liver diseases cannot be considered co-morbidities.

The Charlson index was developed to predict mortality among hospitalized patients, but it was not developed for cirrhosis patients or for patients with any other particular index disease[8]. There are other reasons why it is probably suboptimal for use among cirrhosis patients: First, it was developed based on only 559 patients[8], so rare but severe diseases may not have been included. Second, psychiatric diseases were not considered for inclusion, but eight percent of Danish cirrhosis patients have been diagnosed with a psychiatric disease other than substance abuse[3]. Third, it does not consider the duration between the occurrence of the comorbidity and the development of cirrhosis; but the impact of, *e.g.*, a peptic ulcer or an acute myocardial infarction decreases over time[9,10], whereas the opposite is true for cancer and diabetes[8]. Fourth, the prognostic impact of many diseases has changed since the Charlson index was developed in 1984[11-13]. Despite these shortcomings, the Charlson index has been shown to be strongly associated with mortality among cirrhosis patients in Denmark and the United Kingdom[4,14]. Moreover, it was not only associated with the risk of death from any cause, it was also associated with the risk of death from cirrhosis[4].

CirCom score

Our group recently developed a cirrhosis-specific comorbidity scoring system using data from healthcare registries on 12976 Danish cirrhosis patients, most of whom had alcoholic cirrhosis[3]. We defined 34 comorbidities on the basis of hospital discharge diagnosis codes[3]. Fifty-five percent of patients had at least one of these comorbidities at the time of cirrhosis diagnosis. The final comorbidity scoring system-the CirCom score-included nine diseases (Table 1). The prevalence of any of these nine diseases was 24.2% at the time of cirrhosis diagnosis, with the highest prevalence for chronic obstructive lung disease (7.3%), cancer (6.7%), and heart failure (5.2%).

The CirCom score is based on nine diseases of which at most two count towards a patient's CirCom score (Figure 1). Although simpler than the Charlson index, the CirCom score was slightly better at predicting mortality: In the full cohort of 12976 cirrhosis patients the C statistic for the CirCom score was 0.6% points (95%CI: 0.3%-0.8%) higher than the C statistic for the Charlson index. The Net Reclassification Index, a newer measure of predictive ability, was 3.6% (95%CI: 2.3%-5.0%) higher for the CirCom score. In the two validation cohorts of 419 patients with alcoholic cirrhosis and 4656 patients with chronic hepatitis C infection, the CirCom score remained superior, although not by a statistically significant amount[3].

CCI-OLT

The Charlson comorbidity index has been evaluated in a study of 221 Italian liver transplant recipients. The prevalence of comorbidity was 57%, and patients with a comorbidity score > 1 had higher risks of graft loss and death than patients with a score of 0 or 1[7]. Thus the Charlson index predicted both death and graft loss, but

Table 1 Comorbidity scoring systems for patients with cirrhosis

Target population	Charlson comorbidity index	CirCom	CCI-OLT
	Patients with any disease	Patients with cirrhosis	Orthotopic liver transplant recipients
HIV/AIDS	6		
Cancer (metastatic)	6	3[1]	
Cancer (non-metastatic or hematologic)	2	1[1]	
Liver disease (mild)	1		
Liver disease (severe)	3		
Diabetes (no complications)	1		1
Diabetes (with complications)	2		1
Kidney disease	2	3	2
Hemiplegia	2		
Peptic ulcer	1		
Connective tissue disease	1		2
Chronic obstructive lung disease	1	1	3
Dementia	1		
Epilepsy		1	
Cerebrovascular disease	1		
Peripheral vascular disease	1	1	
Congestive heart failure	1	1	
Acute myocardial infarction	1	1[1]	2
Substance abuse other than alcoholism		1	

[1]Add two points if the comorbid disease is active. The numbers indicate the comorbid diseases' weight. HIV: Human immunodeficiency virus; AIDS: Acquired immunodeficiency syndrome; CCI-OLT: Charlson comorbidity index for orthotopic liver transplantation.

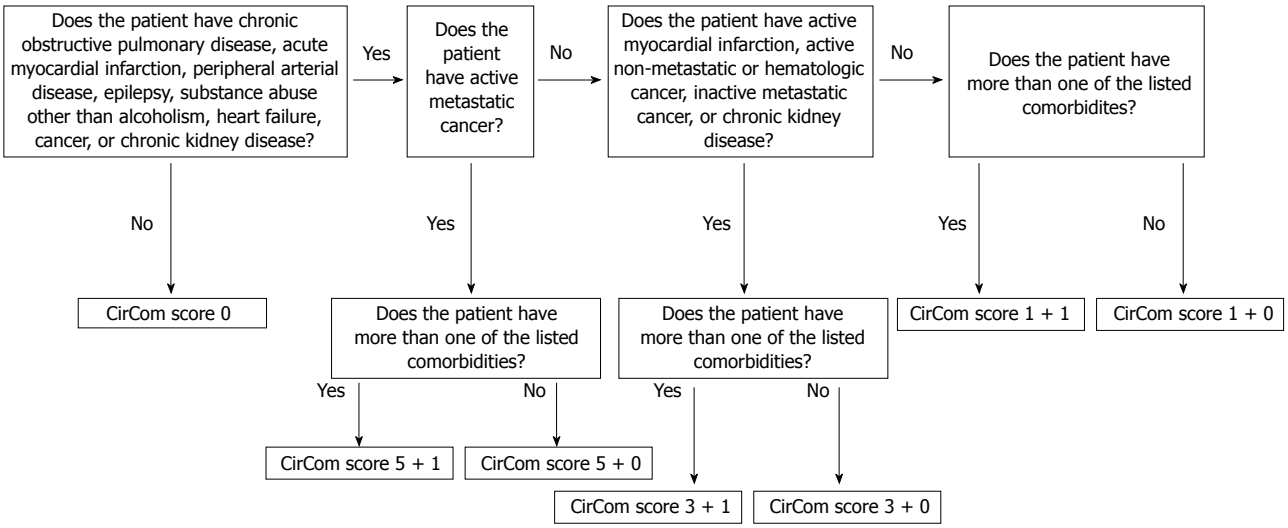

Figure 1 Algorithm for computing of CirCom scores[3]. Reprinted from reference 3, with permission from Elsevier.

none of the individual comorbid diseases in the Charlson index was a statistically significant predictor of mortality, and only chronic obstructive lung disease was a statistically significant predictor of graft loss (HR = 4.71, 95%CI: 1.07-20.83).

The Charlson comorbidity index has been modified for analyses of kidney transplant recipients[15], and the same modified index with only nine comorbidities has been evaluated in two studies of orthotopic liver transplant recipients followed from transplantation. In the two studies, 30% and 40% of patients had one or more of these nine comorbidities[6,16]. The first study followed 169 patients for one month after transplantation. It showed that the prevalence of the nine comorbidities was similar for those who lived or died, hence comorbidity did not predict mortality[16]. The second study followed 624 patients for up to twelve years after transplantation and found that a simplified index with five comorbidities predicted survival. This comorbidity scoring system was named CCI-OLT (Table 1).

Which comorbidity scoring system should be preferred?

For the cirrhosis patient, the choice between the CirCom score and the Charlson comorbidity index is not obvious. The CirCom score was developed in a cohort dominated by patients with alcoholic cirrhosis, and it has not been validated using data from other countries than Denmark or data obtained in the clinic[17]. The Charlson index is more extensively validated[4,14], but has limitations, as described above. Based on the available evidence, clinicians

Table 2 Effects of comorbid diseases on the mortality of patients with liver cirrhosis in the CirCom cohort of 12976 Danish cirrhosis patients. Hazard ratios are adjusted for gender and age differences[3]

Comorbidity	Hazard ratio (95%CI)
Diabetes with complications	1.16 (1.10-1.22)
Diabetes without complications	1.03 (0.98-1.08)
Acute myocardial infarction	1.59 (1.40-1.79)
Peripheral arterial disease	1.29 (1.18-1.40)
Heart failure	1.27 (1.20-1.34)
Valvular heart disease	1.17 (1.04-1.30)
Cardiomyopathy	1.16 (1.03-1.30)
Arterial hypertension with complications	1.16 (0.98-1.37)
Cardiac arrhythmia	1.14 (1.08-1.20)
Mesenteric vascular disease	1.13 (0.85-1.49)
Ischemic heart disease without myocardial infarction	1.13 (1.06-1.21)
Mesenteric vascular disease	1.13 (0.85-1.49)
Cerebrovascular disease	1.09 (1.02-1.16)
Venous thromboembolism	1.20 (1.08-1.33)
Chronic obstructive lung disease	1.24 (1.18-1.30)
Peptic ulcer with bleeding or perforation	1.17 (1.11-1.23)
Chronic pancreatitis	1.09 (1.03-1.16)
Chronic inflammatory bowel disease	1.08 (0.95-1.22)
Peptic ulcer without complications	1.03 (0.97-1.10)
Acute pancreatitis	1.01 (0.92-1.12)
Chronic kidney disease	1.59 (1.37-1.83)
Psoriasis	1.05 (0.92-1.21)
Connective tissue disease	0.99 (0.91-1.08)
Epilepsy	1.22 (1.11-1.35)
Schizophrenia	1.15 (1.00-1.32)
Bipolar disorder	0.98 (0.76-1.26)
Depression	1.00 (0.91-1.09)
Dementia	1.04 (0.95-1.15)
Substance abuse other than alcoholism	1.25 (1.14-1.37)
Metastatic cancer	1.72 (1.53-1.94)
Non-metastatic solid cancer	1.35 (1.27-1.43)
Hematologic cancer	1.30 (1.10-1.53)
Human immunodeficiency virus infection	0.79 (0.49-1.26)
Osteoporosis	1.03 (0.93-1.15)
Obesity	1.02 (0.92-1.12)

and researchers may prefer the simpler, yet slightly better cirrhosis-specific CirCom score, but more comparative studies are necessary to determine which comorbidity scoring system is better.

For the liver transplant recipient, the CCI-OLT should be preferred because it assigns transplantation-specific weights to the comorbid diseases from the Charlson index. Liver transplant recipients are a highly selected group that excludes patients with severe comorbidities, and the post-transplant immunosuppression may affect the prognosis of the comorbidities. Therefore it makes good sense to have a comorbidity index specifically for liver transplant recipients. The greater detrimental effect of chronic obstructive lung disease than of cancer highlights the importance of having a transplant-specific comorbidity index[6,7].

INDIVIDUAL COMORBIDITIES

Studies of individual comorbidities' effect on the clinical course of cirrhosis can provide insight into the pathophysiology of cirrhosis. Unfortunately, only few such studies have been conducted[18], and all have focused on the prognosis with respect to death. This section presents the available evidence.

Diabetes

Diabetes is the best studied comorbidity to cirrhosis, but studies have reached different conclusions. Among the 12976 Danish cirrhosis patients included in the CirCom study, diabetes without complications was unassociated with mortality whereas diabetes with complications did increase mortality (Table 2)[3]. A study from the Netherlands including 226 patients diagnosed with cirrhosis in 2001-2011 found that diabetes was unassociated with all-cause and liver-related mortality[19], and a smaller Mexican study found that the reduced survival for cirrhosis patients with diabetes was due to confounding by cirrhosis severity and renal impairment[20]. Earlier studies have been reviewed by Garcia-Compean *et al*[21] who concluded that diabetes mellitus does increase mortality in cirrhosis, and that the excess mortality in diabetes patients is due to hepatocellular failure, not to diabetes[22]. More detailed studies are needed to clarify the interactions between cirrhosis and diabetes.

Cardiovascular disease

The hyperdynamic circulation in cirrhosis provides some protection against atherosclerosis, ischemic events, and overt heart failure[23,24], but acute myocardial infarction, peripheral arterial disease, and heart failure were all strong predictors of mortality in the CirCom study. Other cardiovascular diseases were weaker predictors (Table 2)[3]. Coronary disease, defined by acute myocardial infarction or coronary disease on angiography, was also a predictor of mortality among liver transplant recipients[6]. The reasons for these associations are unclear.

Venous thromboembolism

In the CirCom cohort, venous thromboembolism in the form of deep venous thrombosis or pulmonary embolism increased mortality 1.20-fold (95%CI: 1.08-1.33) after adjustment for gender and age (Table 2)[3]. By contrast, both manifestations were unassociated with mortality in a study of United States veterans with cirrhosis after adjustment for gender, age, race, Charlson comorbidity index, insurance type, and presence of cirrhosis complications (HR = 1.01, 95% CI: 0.83-1.23)[25]. This could indicate that the association in the Danish cohort is due to uncontrolled confounding by cirrhosis complications, with greater risk of thromboembolism for cirrhosis patients with complications. Coagulation in liver disease is complex[26], and it remains unclear whether venous thromboembolism is a marker of severe liver function loss.

Lung disease

In the CirCom cohort, chronic obstructive lung disease increased cirrhosis patients' mortality (Table 2)[3], and it

was also the strongest predictor of mortality in the studies of liver transplant recipients[6,7]. Chronic obstructive lung disease is a relative contraindication for the non-selective beta blockers that reduce the risk of variceal bleeding[27], but endoscopic ligation is a satisfactory alternative treatment[28]. The mechanisms behind the adverse effect of chronic obstructive lung disease are therefore unclear. Smoking has also been identified as an adverse prognostic factor in patients with cirrhosis[29], but this association is unexplained, too[30].

Gastrointestinal disease

Alcohol is the dominant cirrhosis etiology in Denmark, yet the prevalence of chronic pancreatitis in the CirCom cohort was only 4.5%[3], a prevalence similar to that seen among alcohol abusers with or without cirrhosis[31]. This observation is consistent with the hypothesis that cirrhosis and chronic pancreatitis develop along different pathogenetic lines[31,32]. The same hypothesis might explain why chronic pancreatitis increased mortality only 1.09-fold (95%CI: 1.03-1.16) (Table 2)[3], despite the generally increased cancer risk and mortality in patients with chronic pancreatitis[33]. Another possible explanation to this unexpectedly weak association is that patients with chronic pancreatitis are immediately screened for cirrhosis and therefore have their cirrhosis diagnosed in an earlier stage than other cirrhosis patients. No other studies have examined chronic pancreatitis in cirrhosis.

Acute pancreatitis, chronic inflammatory bowel disease, and uncomplicated peptic ulcer had no clinically or statistically significant effect on cirrhosis patients' mortality in the CirCom cohort (Table 2), but patients with a history of complicated peptic ulcer had a 1.17-fold (95%CI: 1.11-1.23) increased mortality (Table 2). The reasons are unclear, but cirrhosis patients' high risk of rebleeding from peptic ulcer-26% within five years after first bleeding-is likely to have contributed[34].

Chronic kidney disease

Chronic kidney disease increases morbidity and mortality among the general population[35] and among liver transplant recipients[6]. It was also a strong predictor of mortality in the CirCom cohort (Table 2)[3]. The circulatory dysfunction that ultimately leads to the hepatorenal syndrome may worsen an existing kidney dysfunction[36], and the International Ascites Club has been involved in defining and studying the complex interplay between kidney disease and cirrhosis[37,38].

Connective tissue disease

Connective tissue disease is included in the Charlson comorbidity index and also a predictor of mortality among liver transplant recipients (HR = 2.32, 95%CI: 1.02-5.25)[6]. It was, however, unassociated with mortality in the CirCom cohort (Table 2)[3]. One possible explanation is that better treatment methods introduced after the Charlson index's development in 1984 have improved the prognosis of connective tissue diseases, but at least in rheumatoid arthritis there seems to have been no improvements in overall survival[39]. This explanation is therefore questionable. An alternative explanation is that connective tissue diseases have a smaller impact on the mortality of cirrhosis patients than on other patients, possibly because cirrhosis patients do not survive long enough to suffer the long-term consequences of these diseases. Further studies are needed to substantiate this speculation.

Epilepsy

Idiopathic epilepsy-*i.e.*, epilepsy not due to brain tumor, vascular disease, alcoholism, or metabolic disease-has been found to increase mortality 1.6-fold in the general population[40]. Even so, we had not expected epilepsy to increase mortality as much as it did in the CirCom cohort, the hazard ratio being 1.22 (95%CI: 1.11-1.35), on par with chronic obstructive lung disease (Table 2)[3]. The reasons for this strong association are unclear. It is possible that hepatic encephalopathy may manifest as status epilepticus[41], or that some patients given a diagnosis of non-convulsive epilepsy did in fact have hepatic encephalopathy. In both instances a diagnosis of epilepsy would be a marker of cirrhosis with hepatic encephalopathy, and this complication has a very poor prognosis[42]. St Germaine-Smith *et al*[43] previously constructed an epilepsy-specific comorbidity index. It included cirrhosis without complications in the "mild liver disease" category which was unassociated with mortality, whereas cirrhosis with complications was in the "severe liver disease" category which was associated with a three-fold increase in mortality. These findings suggest that cirrhosis and epilepsy do not always interact to cause a poor prognosis, supporting the hypothesis that epilepsy is a marker of severe cirrhosis, not a cause. It is also possible that epilepsy promotes the development of hepatic encephalopathy or vice versa; that treatments for epilepsy are detrimental to cirrhosis patients; or that the explanation lies in alcohol which is a cause of status epilepticus[44] and also an adverse prognostic factor in cirrhosis. Further research is clearly needed.

Psychiatric disease

In developing the CirCom score we had expected psychiatric disease to be a strong predictor of mortality in cirrhosis patients due to its association with substance abuse and suicide risk[45]. Schizophrenia was indeed an adverse prognostic factor, whereas bipolar disorder and depression were unassociated with mortality (Table 2). No other studies have examined the prognostic impact of psychiatric diseases in cirrhosis.

Substance abuse

Alcohol abuse is highly prevalent among cirrhosis patients in the Western world, but since it is a cause of liver disease it should not be considered a comorbid condition. Substance abuse other than alcoholism increased mortality in the CirCom cohort (Table 2)[3], possibly because it

is a marker of adverse lifestyle factors, malnutrition, and low socioeconomic status[46].

Non-hepatic cancer

Cirrhosis patients have an increased risk of non-hepatic cancer[47,48]. The mechanisms are unclear, but lifestyle factors associated with both cirrhosis and cancer development-primarily alcohol consumption and tobacco-are clearly important. Unsurprisingly, non-hepatic cancer increased the mortality of the CirCom cohort (Table 2)[3]. Some cancer forms may aggravate ascites formation and portal hypertension, *e.g.*, by causing portal vein thrombosis, and patients with advanced cirrhosis may not tolerate chemotherapy[49]. These two mechanisms indicate that cirrhosis and non-hepatic cancer may worsen each other's prognosis. Reuken *et al*[50] have previously reported that cancer increases mortality among cirrhosis patients with urinary tract infections, and Gundling *et al*[51] have shown that, among cirrhosis patients in general, metastatic cancer was a stronger predictor of mortality than was non-metastatic cancer.

Miscellaneous comorbid diseases

In the CirCom cohort, human immunodeficiency virus (HIV) infection did not increase mortality (Table 2), whereas in the Charlson index HIV infection has the same weight as metastatic cancer (Table 1). This discrepancy is likely the result of the considerable progress made in the clinical management of HIV infection[52]. Finally, in the CirCom cohort osteoporosis and obesity did not affect mortality (Table 2).

CONCLUSION

Comorbidity affects the prognosis of cirrhosis patients. Measures of a patient's total burden of comorbidity are important for epidemiologic studies and for clinical use. The CirCom score may be the preferred comorbidity scoring system because it is simpler yet slightly better than the Charlson comorbidity index, but more comparative studies are needed. Studies aiming to update the CirCom score to other cirrhosis populations will also improve its clinical value and credibility[53].

The available evidence of interactions between cirrhosis and individual comorbid diseases is sparse. A better understanding of such interactions will improve our understanding of cirrhosis pathophysiology, and clinical epidemiologic studies may help by answering questions like these: (1) does the comorbid disease increase the risk of developing cirrhosis complications? That would provide evidence that the comorbid disease affects portal pressure or liver function loss, or that it reduces the efficacy of cirrhosis treatments; (2) is there prognostic synergy between cirrhosis and the comorbid disease? That would provide evidence that the comorbid disease is more detrimental to cirrhosis patients than to others due to a pathophysiological interaction with cirrhosis; (3) is cirrhosis a risk factor for developing the comorbid disease? That would providence evidence that cirrhosis may affect the pathogenesis of the comorbid disease; or (4) are cirrhosis patients with decompensated as opposed to compensated cirrhosis at greater risk of developing the comorbid disease? That would provide evidence that portal hypertension and/or loss of liver function facilitates the development of the comorbid disease.

A better understanding of the interactions between cirrhosis and individual comorbid diseases is the first step towards clinical advances, *e.g.*, the possibility to tailor cirrhosis treatments to specific comorbidity patterns. Currently, our understanding of the impact of comorbidities in cirrhosis is in its infancy.

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TOPIC HIGHLIGHT

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Noninvasive diagnosis of cirrhosis: A review of different imaging modalities

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Abstract

Progressive hepatic fibrosis can lead to cirrhosis, so its early detection is fundamental. Staging fibrosis is also critical for prognosis and management. The gold standard for these aims is liver biopsy, but it has several drawbacks, as it is invasive, expensive, has poor acceptance, is prone to inter observer variability and sampling errors, has poor repeatability, and has a risk of complications and mortality. Therefore, non-invasive imaging tests have been developed. This review mainly focuses on the role of transient elastography, acoustic radiation force impulse imaging, and magnetic resonance-based methods for the noninvasive diagnosis of cirrhosis.

Key words: Liver; Cirrhosis; Elastography; Acoustic radiation force impulse imaging; Magnetic resonance elastography

Core tip: In order to overcome the well-known drawbacks of liver biopsy, different non-invasive imaging tests have been developed for diagnosing and staging liver fibrosis. At present, transient elastography and acoustic radiation force impulse imaging are the most widely used. Reviewing literature, it seems that acoustic radiation force impulse imaging, having the advantage of being included in ultrasound equipments, could provide higher reproducibility and successful measurements rate, with a more precise examination than transient elastography. Magnetic resonance-based methods, especially hepatospecific contrast medium uptake/excretion measurements and elastography, are promising but still not universally available tools.

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INTRODUCTION

Fibrosis is the common result of several chronic hepatic diseases that, if progressive, can lead to cirrhosis, which is developed by 20%-30% of patients. Since fibrosis can be reversible, its early detection is fundamental[1,2]. Staging is also needed, because it is critical for prognosis and management, especially for chronic viral hepatitis: antiviral therapy is recommended in chronic hepatitis B (CHB) with cirrhosis, while in chronic hepatitis C (CHC) the treatment may not be indicated with minimal or absent fibrosis[3,4]. Furthermore, the assessment of residual

fibrosis in CHC patients who achieved a sustained virological response to interferon is of strategic importance, for prognostication and to define a cost-effective surveillance, especially for patients with eradicated infection but ongoing complications[5]. Moreover, staging is needed for the treatment of CHC with protease inhibitors, which are effective but expensive[6]. Finally, staging is important in human immunodeficiency virus/hepatitis C virus co-infection, because of the more rapid progression and to the diminished response to therapy[7]. The gold standard for diagnosing and staging fibrosis is liver biopsy (LB), which gives information on presence and extent of fibrosis but also on other concomitant processes. Fibrosis is mainly staged with the METAVIR system, which comprises five stages: F0 (no fibrosis), F1 (portal fibrosis without septa: minimal fibrosis), F2 (portal fibrosis with few septa: moderate fibrosis or clinically significant fibrosis), F3 (septal fibrosis with many septa but no cirrhosis: severe fibrosis) and F4 (cirrhosis). LB has several drawbacks, as it is invasive, expensive, has poor acceptance, is prone to interobserver variability and sampling errors, has poor repeatability, and has a risk of severe complications of 0.57%, and a mortality rate of 0.009%-0.12%[8-10]. Therefore, non-invasive imaging tests for evaluation of liver fibrosis have been developed. This review focuses on the most widely used imaging methods and on possible future developments for the non-invasive diagnosis of cirrhosis, with particular emphasis on transient elastography, acoustic radiation force impulse imaging, and magnetic resonance (MR) elastography.

ELASTOGRAPHIC TECHNIQUES

This group comprises imaging techniques that observe tissue deformation after applying a force, that can be so slow that is considered "quasi-static" [strain elastography (SE) and strain-rate imaging (SRI)] or dynamic [acoustic radiation force impulse (ARFI), transient elastography (TE), point shear-wave elastography (pSWE), shear wave elastography (SWE)].

TE

Technical aspects

TE is a dynamic quantitative technique, which uses acoustic waves ("thumps"-50 Hz), generated by an external driver. Liver stiffness (LS) measurement is performed in the right lobe, with patient in dorsal decubitus, with the right arm above the head. A portion of parenchyma free of large vessels, > 6 cm thick, must be chosen; LS is measured at depth of 25-65 mm, in a 1 cm × 4 cm area. At least 10 valid measurements must be obtained, with a success rate, defined as the number of valid acquisitions divided by the attempts, > 60%, and a ratio of the interquartile range to the median of 10 measurements ⩽ 0.3. Liver elasticity is expressed in kilopascals (kPa).

Clinical applications, normal and pathological values

LS ranges from 2.5 kPa to 75 kPa; mean LS in normal adults is 5.81 ± 1.54 and 5.23 ± 1.59 kPa, respectively for men and women[11]. The main published meta-analyses have proved the reliability of TE and its usefulness (Table 1). Different cut-off values for different etiologies have been proposed for the diagnosis of cirrhosis: 12.5 kPa in CHC, 13.4 kPa in CHB, 10.3 kPa in non-alcoholic fatty liver disease (NAFLD), 22.4 kPa in alcoholic steatohepatitis (ASH), 17.3 kPa in primary biliary cirrhosis and primary sclerosant cirrhosis[12-16]. Liver biopsy is often not recommended in the NAFLD patients, because of its cost, the potential risk of complications and the absence of consensus regarding the histopathological criteria that firmly differentiate between the NAFLD entities; because of the remarkable increase in the prevalence of NAFLD, which represents the most common chronic liver disease in the general population and is expected to increase in future as a result of an ageing population, and the concomitant efforts in developing novel therapies, a non-invasive, simple and reproducible technique as TE is needed in the clinical practice[17]. TE does not always provide a perfectly corresponding estimation of fibrosis stage; one of the known reasons for this is that LS is affected by other histological findings, as edema, steatosis, inflammation or necrosis. Acute or chronic inflammation, in fact, can produce higher LS, indicating the presence of falsely higher fibrosis stages[18]. Several studies have reported the usefulness of TE for longitudinal monitoring of antiviral treatment, mainly reporting a decrease in LS, which could indicate a regression of fibrosis. Particularly, Martinez *et al*[19] performed TE at baseline and at weeks 24, 48, and 72 in patients with CHC: LS significantly decreased in treated patients and remained stable in untreated patients. These results are not universally accepted; it must be kept in mind that both reduction in fibrosis and necroinflammation might contribute to the decrease of LS. For example, Wong *et al*[20] reported that the absolute change in LS poorly correlated with the modifications of fibrosis stage, and resolution of advanced fibrosis could only be assumed with significantly decreased liver stiffness to 5.0 kPa or less after treatment.

Pros and cons

TE is rapid and easy to perform and can be repeated over time. TE can assess a sample area about 100 times bigger than a biopsy sample; therefore it should be more representative. Despite this, with TE is impossible to be sure that the chosen area is free of parenchymal inhomogeneities, which could affect the measurement. The success rate of TE is dependent on operator expertise, as well as on other factors (age, width of the intercostal space, ascites, BMI, visceral fat). Sporea *et al*[21] reported a rate of reliable measurements of 81.6%, which is in line with Castera *et al*[22]. The obesity problem has been partially solved by the development of XL probe, increasing the success rate in obese patients from 45%-50% to about 75%[23,24]. As above mentioned, necroinflammation influences LS. D'Ambrosio *et al*[25] reported that 30% of patients with persistent F4 had LS values suggestive of a less severe disease, and this was explained by the

Table 1 Diagnostic performance of transient elastography for the diagnosis of cirrhosis in different chronic liver disease from different etiology, data from meta-analysis

Meta-analysis	No. of studies	Etiology	Cut-off (kPa)	Sensitivity	Specificity	AUROC
Bota *et al*[53]	13	Various	-	89%	87%	-
Shaheen *et al*[107]	4	HCV	12.5	86%	93%	0.95
Talwalkar *et al*[108]	9	Various	-	87%	91%	-
Friedrich-Rust *et al*[109]	50	Various	-	-	-	0.94
Tsochatzis *et al*[110]	40	HCV	15 ± 4.1	83%	89%	-
Adebajo *et al*[111]	5	HCV	-	98%	84%	-
Stebbing *et al*[112]	22	Various	15.08	84.45%	94.69%	-
Abd El Rihim *et al*[113]	23	Various	-	83.40%	92.40%	-
Chon *et al*[114]	18	HBV	11.7	84.60%	81.50%	0.929

AUROC: Area under receiver operating characteristic; HCV: Hepatitis C virus; HBV: Hepatitis B virus.

onset of liver remodelling and necroinflammation. Park *et al*[26] reported that 3 mo after acute exacerbation ALT levels decreased and stabilised, while LS required 3 more months for stabilisation: LS should be postponed for > 3 mo after stabilisation of ALT levels to restore the reliability. As patients with higher ALT levels tend to have higher LS, several authors suggested to use different cut-offs to adjust the inflammation-induced overestimation. In particular, Chan *et al*[27] proposed that for patients with normal ALT, a LS value > 12 kPa would indicate F4, while for patients with ALT 1-5-times > ULN, this value should be > 13.4 kPa. Kim *et al*[28] reported that the cut-off value for F4 was 10.1 kPa in patients with normal ALT, whereas it was 15.5 kPa in those with elevated ALT.

ARFI

Technical aspects

ARFI is a dynamic technique that uses ultrasound-induced radiation force impulses to obtain both qualitative measure of displacement, both quantitative measure of shear waves speed (ARFI quantification). It involves targeting a region of interest (ROI) of 5 mm × 10 mm, at maximum depth of 8 cm, chosen while performing B-mode imaging. The ultrasound probe produces short-duration acoustic "push" pulses (262 ms), with a transmission frequency of 2.67 MHz, which generate shear waves, propagating perpendicularly, tracked using ultrasound, thus obtaining the shear wave speed quantification in m/s. Patients should be supine, with the right arm in maximum abduction. Probe is placed parallel to the intercostal space. 5-10 measurements are performed in the right lobe, with the patient holding breath gently. Several technical aspects must be taken into account. The influence of deep inspiration on measurement is still debated, as Karlas *et al*[29] reported that it could increases values by an average of 13%, while Horster *et al*[30] and Goertz *et al*[31] did not report differences. Eiler *et al*[32] evaluated 132 healthy children, reporting a shear waves speed (SWS) of 1.16 ± 0.14 m/s, stating that neither age or depth had influence on results. This is in contrast with the study by Lee *et al*[33], who evaluated the age-related modifications in 202 healthy children, founding a mean SWS of 1.14 ± 0.020 < 5 years and of 1.08 ± 0.023 > 10 years. Eiler's *et al*[32] study is in contrast also with other two studies: Sporea *et al*[34], which found a poor correlation between subcapsularly-measured values and fibrosis; and Chang *et al*[35], which found that the measurement depth with lower variability was 4 cm. Moreover, also D'Onofrio *et al*[36] reported that higher values could be obtained in the superficial right lobe: the absence of this aspect in children is probably due to a lower age-related fibrosis in the superficial parenchyma. In the study of Eilers *et al*[32], an interlobar difference was found, with lower values in the right lobe. This difference has been reported also by other authors, reporting both higher both lower values in the left lobe[33-39]. Rifai *et al*[40], instead, reported that ARFI values of both lobes were comparable; in addiction, Goertz *et al*[41] reported a lower number of faulty measurements in right posterior segments. There is no definite explanation for this: probably the presence of heart-beating artifacts in the left lobe and the direct compression with the probe during the examination can be issues; these aspects, however, should not to be considered as a limitation of ARFI, since they can also reflect real interlobar differences and heterogeneity in disease progression. Regarding this, it was demonstrated that biopsies taken in both lobes during laparoscopy presented differences in fibrosis stage in up to 33% of cases[42]. However, since the reference standard for the assessment of fibrosis is LB of the right lobe, it is recommend to measure LS in this lobe[43]; an approach with bilateral multiple measurements is worthy of further investigation, as it may lead to interesting diagnostic results. ARFI must be performed in fasting conditions: Popescu *et al*[44] reported that mean LS increased significantly 1 h after food intake, but 3 h after the meal the difference was no longer significant.

Clinical applications, normal and pathological values

It is still difficult to definitely determine the real value of ARFI for the early diagnosis of fibrosis; it is also difficult to compare the large amount of published papers on this issue. It must be noted that the newest release of the Siemens ARFI system is based on two acoustic pulses, and the maximum depth nowadays achievable is 8 cm, so the more recent published data should be more indicative of what can be obtainable. Moreover, high variability in normal values has been reported; for example, in both

Table 2 Diagnostic performance of acoustic radiation force impulse for the diagnosis of cirrhosis in different chronic liver disease from different etiology, data from main published meta-analysis

Meta-analysis	No. of studies	Etiology	Cut-off (m/s)	Sensitivity	Specificity	AUROC
Bota *et al*[53]	13	Various	-	87%	87%	-
Friedrich-Rust *et al*[57]	9	Various	1.80	-	-	0.93
Nierhoff *et al*[115]	36	Various	1.87	-	-	0.91

AUROC: Area under receiver operating characteristic.

the preliminary studies from the Verona group, D'Onofrio *et al*[36] and Gallotti *et al*[45] reported a mean value of about 1.5 m/s: these results should be considered a outliers, if compared to other studies, but however possible, as it has been also reported by other authors[46]. The main published meta-analyses suggest that ARFI is a reliable method for the diagnosis of cirrhosis (Table 2). Almost all published studies report an increase in SWS with the increase of fibrosis, despite there is a wide overlap between consecutive stages; moreover, mean values indicating cirrhosis have a wide range, while cut-offs have a narrower range. For these reasons, what it seems most important, more than the accurate staging, is to give the correct task to this new technique, as previously stated by D'Onofrio *et al*[47]: the correct use of ARFI must be based on the possibility of this technique to detect significant modifications of LS, related to the development of a significant amount of fibrosis. In fact, as for TE, it seems unreal that this technique could identify very small and localized amount of fibrosis, as it happens in F1, or to differentiate between early stages; it seems to be more "real", technically feasible and clinically useful the differentiation between the two extremes of the grading scale, i.e. the distinction between non-cirrhotics and cirrhotics. For example, in the study by Fierbinteanu-Braticevici *et al*[48] there is a wide overlap between F0-F1 and F2 stages, and the increase in SWS is more significant between F2 and F3 than between F1 and F2, and this is consistent with the more important increase in fibrosis deposit between stages F2 and F3 than between F1 and F2. In the international multicentric study by Sporea *et al*[49], the chosen cut-offs were really strict: F = 1 > 1.19 m/s; F = 2 > 1.33 m/s; F = 3 > 1.43 m/s; F = 4 > 1.55 m/s. The difference between non-cirrhotics and cirrhotics was just 0.12 m/s. In order to make ARFI a useful tool, the chosen cut-off values must not be too strict, but they should be adapted in relation to clinical aspects, imaging findings and technical settings, in order to avoid an overestimation of pathology and to identify inconsistent diseases. This is a further evidence of the necessity of placing ARFI in the right setting, in a protocol that includes an ultrasound (US) evaluation of the liver and a clinical evaluation of the patient, rather than use its results alone.

As above mentioned, liver biopsy is often not recommended in patients with non-alcoholic fatty liver disease (NAFLD), because of its cost, the potential risk of complications and the absence of consensus regarding the histopathological criteria that firmly differentiate between the NAFLD entities. ARFI can represent a useful tool in diagnosing the onset of fibrosis in NAFLD and non-alcoholic steatohepatitis (NASH), in which B-mode evaluation can be inaccurate; Fierbinteanu-Braticevici *et al*[50] reported a high diagnostic performance in predicting cirrhosis in these patients (AUROC = 0.984). Most studies report at least equivalence between TE and ARFI: Friedrich-Rust *et al*[51] (AUROCs of 0.91 and 0.91 for cirrhosis), Piscaglia *et al*[37] (high correlation, $r = 0.891$), Vermehren *et al*[52] ($r = 0.75$, $P < 0.001$), Bota *et al*[53] (mean difference in rDOR = 0.12), Cassinotto *et al*[54] (no significant difference). Some other studies reported a superiority of ARFI: in the 2013 multicentric study by Friedrich-Rust *et al*[55], the diagnostic accuracy for cirrhosis of ARFI and TE was 0.97 and 0.93; similarly, Rizzo has shown a superiority of ARFI *vs* TE regardless of fibrosis stage[56]. Other studies reported a slightly lower diagnostic accuracy of ARFI: the Friedrich-Rust *et al*[57] pooled meta-analysis reported a comparable accuracy of ARFI and TE for the diagnosis of significant and severe fibrosis in 2012, with a trend to be inferior for the diagnosis of cirrhosis; also in the 2012 international multicentric study by Sporea *et al*[49] TE was better than ARFI for predicting cirrhosis.

Pros and cons

A first advantage of ARFI is its integration into conventional US equipment, as opposed to TE: this enables the preliminary evaluation of the whole liver, seeking for signs of cirrhosis and for focal lesions. Then, ARFI is US-guided, so it should be more reliable than TE, for the possibility to position the ROI in an area free of vessels, lesions, biliary ducts or other inhomogeneities. Moreover, ARFI is easy, rapid, and painless; results are immediately available; intra-operator and inter-operator correlation is good[58]. Several studies reported higher rates of valid measurements in comparison to TE: Crespo *et al*[59] reported that ARFI was successfully performed in its whole cohort, while TE failed in 11% of patients; Rifai *et al*[40] reported that ARFI was feasible in all patients, while TE gave invalid results in 34% of patients. Then, ARFI can be performed in patients with ascites or in obese patients. Some limits of ARFI are that the elasticity measurement cannot be performed a posteriori; the ROI has a predetermined and not-changeable size. The influence of necroinflammation on measurements is a debated issue, as it initially appeared poorly relevant. However, a multicentric study[60] showed that, for the same fibrosis degree, the threshold was slightly lower for patients with normal ALT and higher for those with altered ALT; this

study concluded that necroinflammation partially affects ARFI, but with lower extent than TE. The influence of steatosis is another debated issue: Guzman-Aroca *et al*[61] reported that ARFI was not influenced by the severity of steatosis; Marginean *et al*[39] found that SWS in patients with steatosis was statistically higher compared to healthy controls. Righi *et al*[62] reported the influence of chronic autoimmune diseases (primary biliary cirrhosis, autoimmune hepatitis, primary sclerosing cholangitis, overlap syndromes) on ARFI: SWS was significantly higher.

SWE

SWE is a dynamic technique, which does not require manual compression, similar to ARFI; it provides a quantitative measure of SWS using ultrasound-induced radiation forces to create a Mach cone. SWS is calculated as a colorimetric elastographic map, showing quantitative tissue stiffness, expressed as kilopascal. Few and controversial papers focus on the application of SWE in chronic liver diseases. In particular, Leung *et al*[63] reported that the AUROC of SWE and TE was respectively 0.98 and 0.92 for F4; SWE had significantly higher accuracy than TE in all stages and a higher successful rate. Poynard *et al*[64] reported that the performance of SWE for staging was lower than those of TE. Ferraioli *et al*[65] reported an AUROC of 0.98 for SWE and 0.96 for TE when comparing F0-F3 *vs* F4.

REAL-TIME STRAIN ELASTOGRAPHY

It is based both on strains and on shear waves; the stress is manually induced or by internal body movements. Qualitative maps of the strain are produced, in which colors range from red for soft components to blue for hard components. LS evaluation can be either qualitative or semi-quantitative, by analyzing strain histograms and distribution pattern of the pixels in the ROIs[66]. Different quantitative assessment methods as elastic ratio or liver fibrosis index were proposed by Koizumi *et al*[67] and Tomeno *et al*[68]. Real-time strain elastography (RTE) has several advantages over TE, as it allows the evaluation of LS while performing the US exam. RTE does not seem to suffer from breathing artifacts, nor from ascites, steatosis, BMI, or skin thickness[69]. Some studies reported the utility of RTE to evaluate liver stiffness, but with controversial results[67-72].

CONTRAST-ENHANCED ULTRASOUND

Very few studies have tested contrast-enhanced ultrasound (CEUS) for fibrosis assessment. Orlacchio *et al*[73] used time intensity curve analysis and found an AUROC of 0.88 for the distinction between F0-2 *vs* F3-4. Sugimoto *et al*[74] proposed a subjective assessment of CEUS images to identify morphologic changes of portal vein branches, reporting AUROCs of 0.96 for F1 *vs* F2-4 distinction, 0.97 for F1-2 *vs* F3-4, and 0.91 for F1-3 *vs* F4.

PERFUSION COMPUTED TOMOGRAPHY

Few studies have been performed on perfusion computed tomography applied to fibrosis evaluation; Ronot *et al*[75] found that mean transit time could differentiate F1 from F2-3 with a sensitivity of 0.71 and a specificity of 0.65. Motosugi *et al*[76] reported that portal venous perfusion in cirrhotics was significantly lower than in patients without cirrhosis. Kanda *et al*[77] reported that mean hepatic arterial perfusion and arterial perfusion fraction were significantly higher in cirrhotics than in healthy controls.

MR

Several MR-based techniques can be used to evaluate cirrhosis. Regarding unenhanced MR, Banerjee *et al*[78] reported that T1 mapping strongly correlated with fibrosis degree, with AUROC of 0.94; Hshiao *et al*[79] reported that standard deviation, mean, and entropy of pixel intensity in selected ROIs of dynamically grey-level scaled T2-weighted images were significantly smaller in patients with cirrhosis. Balassy *et al*[80] studied the modifications induced by fibrosis in susceptibility-weighted images and found that liver-to-muscle signal intensity (SI) ratio decreased in parallel with the increase of fibrosis and performed well in grading fibrosis (AUROC = 0.93 for F4). Regarding contrast-enhanced MR, especially with gadolinium-EOB-DTPA, a reduced SI in patients with cirrhosis is mostly reported. Particularly, Feier *et al*[81] found that relative enhancement values correlated strongly with fibrosis stage, with an AUC of 0.83 for > F4; Norén *et al*[82] found that liver-to-spleen contrast ratios at 10 and at 20 min and contrast uptake rate had AUROCs values of respectively 0.80, 0.78, and 0.71 with regard to severe *vs* mild fibrosis; Verloh *et al*[83] found that the mean relative enhancement in patients with Child-Pugh Score A cirrhosis had significant increase between arterial, late arterial, portal and hepato-biliary phases, while for Child-Pugh B+C cirrhosis, relative enhancement increased until portal phase and was significantly reduced in C cirrhosis during hepatobiliary phase; Nojiri *et al*[84] found that SI at 25 min could discriminate F = 0-3 *vs* F = 4, with AUROC of 0.87; Goshima *et al*[85] reported that sensitivity, specificity, and AUROC demonstrated by linear regression formula generated by volumetric ratio and contrast enhancement index in predicting fibrous scores were 91%, 100% and 97% for F4. Kim *et al*[86] reported that the relative enhancement [(hepatocyte phase SI - precontrast SI)/pre-contrast SI] of patients with Child-Pugh cirrhosis was significantly higher than that of patients with Child-Pugh B or C cirrhosis. Few studies have been performed on perfusion MRI. Nilsson *et al*[87] quantitatively assessed hepatic uptake of gadolinium in the whole liver as well as on a segmental level, finding a larger parenchymal liver volume, lower hepatocyte function and more inhomogeneous distribution of function in cirrhotics. Hagiwara *et al*[88] reported that the most discriminating perfusion parameter to differentiate F0-2 *vs* F 3-4 was

distribution volume (AUROC = 0.82, sensitivity = 0.77, specificity = 0.79). Diffusion-weighted imaging (DWI) uses the diffusion properties of water molecules in biological tissues; the microscopic movement of water molecules in biological tissues can be measured by apparent diffusion coefficient (ADC) values derived from DWI. Fibrosis should modify this movement, and this has been proved by several studies: Cece *et al*[89] found a significant difference between patients and controls and between different METAVIR stages in respect of liver mean ADC values. DWI images analysis could be also performed directly evaluating the SI of DWI images: Tosun *et al*[90] reported that the SI of cirrhotic liver in b = 1000 images was significantly higher than those of the normal volunteers. Despite these encouraging results, the correlation between ADC values of different diffusion b values and the influence of necroinflammation have not been definitely determined; for example, Onur *et al*[91] found that mean ADC values of CHC patients were significantly lower than mean ADC values of the control group at b = 100 and b = 600 gradients, while no significant difference was found at b = 1000 gradient; moreover, no significant correlation was found between ADC values and histopathologic scores of CHC; Bulow *et al*[92] stated that ADC values can be confounded by fat and iron. Finally, the Wang *et al*[93] reported that MRE outperformed DWI: the AUROC for DWI was 0.86 for F0 *vs* F1-4, 0.83 for F0-1 *vs* F2-4, and 0.86 for F0-2 *vs* F3-4, all significantly lower than the equivalent AUROCs for MRE. Diffusion tensor imaging (DTI) is an evolution of DWI, which uses additional gradients to detect the degree of diffusion in multiple dimensions. Tosun *et al*[90] reported that ADCs reconstructed from conventional DWI and DTI of the patients were significantly lower than those of the normal volunteers; despite this, DWI performed better than DTI for the diagnosis of fibrosis and inflammation. MR spectroscopy has been poorly used in the assessment of fibrosis. Some authors found that 31P-MR spectroscopy measurements correlate with the fibrosis stage whereas others found no correlation[94-99].

MR ELASTOGRAPHY

Technical aspects

MR elastography (MRE) provides a qualitative and quantitative imaging of LS by measuring acoustic shear waves progression. It uses vibrations produced by an external driver; the shear modulus of tissues can be then assessed using a specific MRI sequence. The resulting data are processed to generate quantitative maps (elastograms), displaying LS. The external device is triggered and synchronized with the MR pulse sequence. Different driving mechanisms have been developed, as electromechanical drivers, piezoelectric stack drivers, focused-ultrasound-based radiation force systems. Different pulse sequences can be used.

Clinical applications, normal and pathologic values

Although not as widely available as TE or ARFI, many studies confirm the usefulness of MRE in fibrosis detection. Yin *et al*[100] reported that a cut-off of 2.93 kPa is optimal for distinguishing healthy livers from fibrotic ones (sensitivity = 98%, specificity = 99%); Kim *et al*[101] reported that the best cut-off for advanced fibrosis was 4.15 kPa (AUROC = 0.954, sensitivity = 0.85, specificity = 0.929). Ichikawa *et al*[102] found that mean stiffness value increased with increasing stages of fibrosis: F0 = 2.10 ± 0.10 kPa; F1 = 2.42 ± 0.29 kPa; F2 = 3.16 ± 0.32 kPa; F3 = 4.21 ± 0.78 kPa; and F4 = 6.20 ± 1.08 kPa; the mean AUROC values for discriminating fibrosis stages were F1 = 0.984; F2 = 0.986; F3 = 0.973; and F4 = 0.976. Wang *et al*[93] reported an overall sensitivity, specificity, and AUROC of 0.83, 0.99, and 0.95 for the distinction between F0 and F1-4. Venkatesh *et al*[103] found that MRE was significantly more accurate than serum fibrosis markers for the detection of significant fibrosis (AUROC 0.99 *vs* 0.55-0.73) and cirrhosis (AUROC 0.98 *vs* 0.53-0.77); sensitivity, specificity, positive predictive and negative predictive values for MRE for significant fibrosis and cirrhosis were 97.4%, 100%, 100% and 96%, and 100%, 95.2%, 91.3% and 100%, respectively. Choi *et al*[104] found that LS values measured on MRE were more strongly correlated with fibrosis stage than with the contrast enhancement index (SIpost/SIpre, where SIpost and SIpre are, respectively, the liver-to-muscle signal intensity ratio on hepatobiliary phase images and on unenhanced images): MRE showed higher sensitivity and specificity for predicting F1 (91% and 87%), F2 (87% and 91%), F3 (80% and 89%), and F4 (81% and 85%) compared with contrast enhancement index.

Regarding NAFLD, Kim *et al*[101] reported that the best cutoff for advanced fibrosis was 4.15 kPa (AUROC = 0.954, sensitivity = 0.85, specificity = 0.929), concluding that MR elastography can be a useful diagnostic tool for detecting advanced fibrosis in NAFLD. Chen *et al*[105] reported that the mean hepatic stiffness for patients with simple steatosis (2.51 kPa) was lower than that for patients with inflammation but no fibrosis (3.24 kPa). The mean hepatic stiffness for patients with inflammation but no fibrosis was lower than that for patients with hepatic fibrosis (4.16 kPa). Liver stiffness had high accuracy (AUROC = 0.93) for discriminating patients with NASH from those with simple steatosis, with a sensitivity of 94% and a specificity 73% by using a threshold of 2.74 kPa; the author concluded that in patients with NAFLD, hepatic stiffness measurements with MR elastography can help identify individuals with steatohepatitis, even before the onset of fibrosis; NAFLD patients with inflammation but no fibrosis have greater liver stiffness than those with simple steatosis and lower mean stiffness than those with fibrosis.

Pros and cons

The main advantage of MRE is that the acquisition time is relatively short, so it could be included in standard protocols, providing a comprehensive evaluation of the liver. Second, it provides quantitative maps of tissue stiffness over large regions, so it is much less operator dependent

than ultrasound-based techniques, and can accurately reflect the distribution of fibrosis in the whole liver. Finally, MRE has been reported to be more accurate than any non-invasive alternative, with a success rate higher than TE; moreover, it can be suitable for patients with obesity or ascites[106]. Despite these pros, MRE remains poorly available, more expensive and not suitable for patients with contraindications to MR.

CONCLUSION

At present, TE and ARFI are the most widely used non-invasive methods for the diagnosis of cirrhosis. ARFI has the great advantage of being included in standard US equipment, so it can be used as a complement to the conventional B-mode whole-liver evaluation, with higher reproducibility and success rate, providing also a more precise examination than TE. MR-based methods, especially hepatospecific contrast medium uptake/excretion measurement and MRE, are promising tools; in the future, a wider availability of these techniques should be expected, in order to add these measurements to standard MRI protocols, to obtain a better comprehensive liver assessment.

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TOPIC HIGHLIGHT

WJG 20th Anniversary Special Issues (11): Cirrhosis

Nanotechnology applications for the therapy of liver fibrosis

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Abstract

Chronic liver diseases represent a major global health problem both for their high prevalence worldwide and, in the more advanced stages, for the limited available curative treatment options. In fact, when lesions of different etiologies chronically affect the liver, triggering the fibrogenesis mechanisms, damage has already occurred and the progression of fibrosis will have a major clinical impact entailing severe complications, expensive treatments and death in end-stage liver disease. Despite significant advances in the understanding of the mechanisms of liver fibrinogenesis, the drugs used in liver fibrosis treatment still have a limited therapeutic effect. Many drugs showing potent antifibrotic activities *in vitro* often exhibit only minor effects *in vivo* because insufficient concentrations accumulate around the target cell and adverse effects result as other non-target cells are affected. Hepatic stellate cells play a critical role in liver fibrogenesis , thus they are the target cells of antifibrotic therapy. The application of nanoparticles has emerged as a rapidly evolving area for the safe delivery of various therapeutic agents (including drugs and nucleic acid) in the treatment of various pathologies, including liver disease. In this review, we give an overview of the various nanotechnology approaches used in the treatment of liver fibrosis.

Key words: Liver fibrosis; Nanotechnology; Nanoparticles; Hepatic stellate cells; Antifibrotic drugs, Cirrhosis

Core tip: New drugs or new drug delivery strategies to cure liver fibrosis are needed to find effective therapeutic options for this pathologic condition. Therapies based on nanotechnologies have emerged as an innovative and promising alternative to conventional therapies. This work aims to review the most recent literature about the use of nanotechnology approaches to reduce liver fibrosis.

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INTRODUCTION

Chronic liver diseases (CLD) are disorders that chronically affect the liver, undermining its capacity to regenerate after injury and triggering a wound-healing response that involves a range of cell types and mediators which try to limit the injury and set in motion the fibrogenesis

mechanisms. The sustained signals associated with CLD of whatever origin (infections, drugs, metabolic disorders, autoimmunity, *etc.*) are required for significant fibrosis to accumulate, which predisposes to the development of cirrhosis and its complications.

Liver fibrogenesis in response to a chronic infection associated with hepatitis viruses (HBV and HCV), chronic alcohol consumption, genetic abnormalities, steatohepatitis, autoimmunity, *etc.* is the consequence at the cellular and molecular levels of the activation of hepatic stellate cells (HSCs) and their transformation into myofibroblasts which overproduce extracellular matrix, mainly type Ⅰ and Ⅲ collagens. Liver fibrosis may regress following specific therapeutic interventions, but no anti-fibrotic drugs are currently available in clinical practice other than those which eliminate the risk factors. Indeed, several clinical trials testing potential anti-fibrotic drugs [such as angiotensin Ⅱ antagonists, interferon gamma, peroxisomal proliferator activated receptor (PPAR) gamma ligands, pirfenidone, colchicine, silymarin, polyenylphosphatidylcholine, ursodeoxycholic acid and Interleukin-10], have failed to observe either a halt in the progression of liver fibrosis or its reversal[1].

An important disadvantage of the standard therapy is that it is unable to provide a sufficient concentration of the therapeutic agent to treat liver disease and/or it leads to side effects.

Recently, therapies based on nanotechnologies have emerged as an innovative and promising alternative to conventional therapy. Nanotechnology is a rapidly growing branch of science focused on the development, manipulation and application of materials ranging in size from 10-500 nm either by scaling up from single groups of atoms or by refining or reducing bulk materials into nanoparticles (NPs). Currently, nanoparticles are being constructed with biocompatible materials and they possess great potential in delivering drugs in a more specific manner: either passively by optimizing the physicochemical properties of the drug nanocarriers (such as the size and surface properties) or actively by using tissue/cell-specific homing devices which allow the targeting of the disease site, while minimizing side-effects. Therefore, NPs can be engineered as nanoplatforms for the effective and targeted delivery of drugs, also thanks to their ability to overcome many biological, biophysical, and biomedical barriers.

In recent years, nanomedicine-based approaches have been explored for liver disease treatment. In this review, we will describe the most common NP types employed in the treatment of fibrotic liver diseases.

NANOTECHNOLOGIES IN HUMAN DISEASES

Therapeutic NPs are generally defined as nanostructures constituted by therapeutic drugs, peptides, proteins or nucleic acids loaded in carriers with at least one length in the nanometer range. Drugs and imaging labels which cannot achieve an effective and targeted delivery due to biological, biophysical, and biomedical barriers can be engineered as NPs. The possibility to incorporate drugs and genes into NPs through the conjugation or coating of ligands specifically binding to target cells or tissues opens a new era for delivering drugs and genes selectively to the disease site. There are several advantages to using NP delivery systems: (1) protection of the therapeutic agent, especially nucleic acid, against inactivation until it reaches the site of action; (2) feasibility of incorporation of both hydrophilic and hydrophobic agents; (3) optimization of pharmacological effectiveness (increased bioavailability of drugs); (4) reduction of toxicity and side effects of the drug; (5) reduction of drug blood level fluctuations (lower risk of ineffective or toxic concentration); (6) potential broad spectrum of administration routes (external, ophthalmic, oral and parenteral); (7) controlled drug release; and (8) active targeting due to the possibility of obtaining a greater affinity of the nanoparticle system (functionalized nanoparticle) for certain tissues. Following systemic administration, conventional NPs are opsonized by plasma proteins, recognized as foreign bodies and rapidly captured by the reticuloendothelial system (RES). The liver and the spleen are the major organs of accumulation of NPs[2,3] due to their rich blood supply and the abundance of tissue-resident phagocytic cells, therefore liver targeting by NPs may be favorable for treating liver diseases[2,3].

The uptake and distribution of NPs depend on their size: (1) NPs with a mean diameter > 400 nm are quickly captured by the RES; and (2) NPs with a diameter < 200 nm show prolonged blood circulation and a relatively low rate of RES uptake[4]. On the other hand, to reduce opsonization by blood proteins and to prolong bloodstream circulation by limiting RES uptake and reducing immunogenicity/antigenicity, biologically inert hydrophilic polymers, such as poly(ethylene glycol) (PEG), have been covalently linked to the nanocarrier surface[5,6]. These types of NPs are commonly called "stealth" NPs[5,6].

Nanomedicine is referred to as the field of medicine that deals with the application of nanotechnology to address medical problems[7-9], and recently nanomedicine-based approaches have been explored for liver disease treatment.

COMMON NP TYPES TO BE EMPLOYED FOR THE TREATMENT OF LIVER DISEASE

The variety of materials that can be used to create NPs is enormous and the number of NPs used in biomedical research and drug delivery is rapidly increasing. They can be classified into two major types: inorganic and organic NPs. Here, we briefly summarize only the structure, properties and characteristics of some of the most commonly-used NPs for the treatment of liver fibrosis.

Inorganic NPs have received great attention because

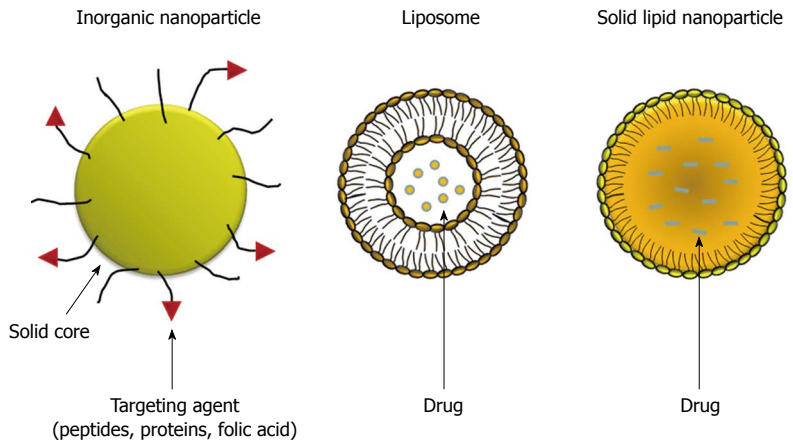

Figure 1 Major types of nanoparticles including: inorganic nanoparticles, liposomes and solid lipid nanoparticles.

of their outstanding properties. Generally, inorganic nanoparticles can be defined as particles with a metal oxide (iron oxide, titanium oxide, *etc.*) or metal (gold and silver) central core and with a protective organic layer on the surface. The organic outer layer both protects the core from degradation and also allows the conjugation of biomolecules with reactive groups (amines and thiols) to link peptides, proteins and folic acid (Figure 1). In recent years inorganic NPs have gained significant attention due to their unique material- and size-dependent physicochemical properties, which are not possible with organic NPs. Their unique optical, magnetic and electronic properties can be tailored by controlling the composition, size, shape, and structure. In some cases, inorganic nanoparticles are attractive alternatives to organic NPs for drug delivery and imaging a specific tissue because of their physical features, such as optical and magnetic properties, in addition to their inertness, stability and easy functionalization[10-13].

Polymeric NPs, liposomes, solid lipid nanoparticles (SLN) and nanostructured lipid carriers (NLC) form a large and well-established group of organic nanoparticles (Figure 1). These biodegradable, biocompatible polymeric NPs have attracted significant attention as potential drug delivery systems since they can be applied in drug targeting to particular organs/tissues and as carriers of DNA in gene therapy, and are also able to deliver proteins and peptides via oral administration.

Biodegradable natural polymers include chitosan, albumin, rosin, sodium alginate and gelatin, while synthetic polymers include polylactic acid (PLA), polylactic-glycolic acid (PLGA), polycaprolactones (PCL), polycyanoacrylates and polyaminoacid conjugates)[14-17]. PLA and PLGA biodegradable polymeric nanoparticles have recently been approved by the US Food and Drug Administration for human use.

Liposomes are spherical artificial vesicles consisting in one or more phospholipid bilayers enclosing an aqueous compartment. Liposomes can encapsulate a wide variety of lipophilic (hydrophobic) and hydrophilic drugs within their dual compartment structure. Hydrophobic drugs can be incorporated into the bilayer, while hydrophilic drugs can be contained within the inner aqueous core formed by the lipid membrane. NPs made with liposomes are the simplest form of NPs and have several advantages, such as easy preparation, good biocompatibility, reduced systemic toxicity and increased uptake[11,18]. Conventional liposomes, termed "non-stealth" liposomes, are rapidly removed from the blood circulation because of their high affinity for the RES. However, this phenomenon has been avoided by coating them with hydrophilic molecules (such as PEG derivatives) linked to the liposomal formulation by a lipid anchor. This modification significantly prolongs liposome circulation over time[19], and therefore improves pharmacological potency, reduces the dose and widens the range of indications.

In the early 1990s a new class of colloidal drug carriers, SLNs, were developed[20]. SLNs have been reported to be an alternative carrier system to emulsions, liposomes and polymeric nanoparticles. SLNs are particles measuring above the submicron range (from about 50 to 500 nm). SLNs are produced by substituting the liquid lipid (oil) with a solid lipid, *i.e.*, the lipid is solid at both room and human body temperatures. SLN are mainly composed of physiological solid lipid dispersed in water or, if necessary, in aqueous surfactant solution. The solid lipid core may contain triglycerides, glyceride mixtures, fatty acids, steroids or waxes. SLNs offer the advantages of the traditional systems but avoid some of their major disadvantages. SLNs are relatively easy to produce without the use of organic solvents, and may be produced on a large scale at low cost[21]. They do not cause toxicity or biodegradability problems, being obtained from physiological lipids and, since the mobility of a drug in solid lipid is lower compared to that in liquid lipid, they can control drug release[22-27].

NLCs were developed at the end of the 1990s to overcome some limitations related to older generation SLNs[25,27,28]. NLCs are produced using blends of solid lipids and liquid lipids (oils), the blends being solid at room and body temperatures. Both NLCs and SLNs are made of physiological, biodegradable, and biocompat-

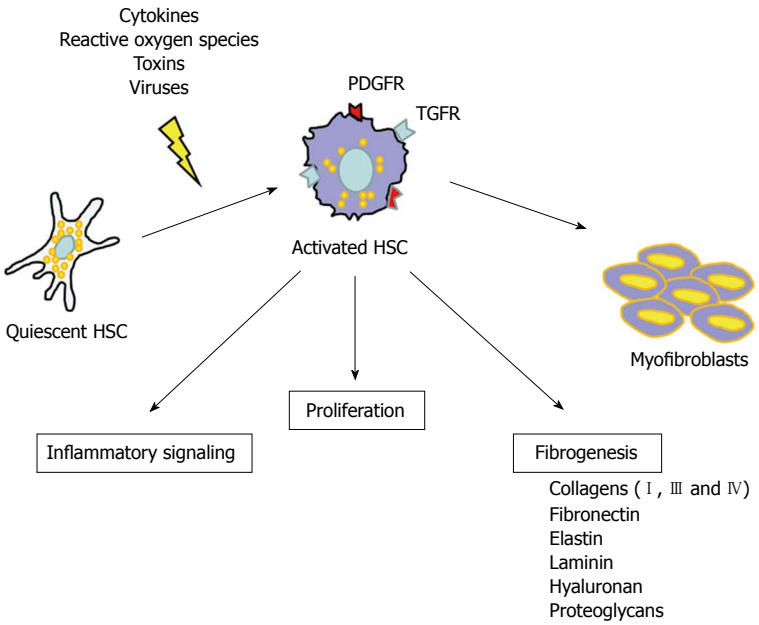

Figure 2 Quiescent stellate cell activation is initiated by different types of stimulus (cytokines, reactive oxygen species, toxins, viruses). The activated cell is transformed into myofibroblasts which contain contractile filaments. Activation of hepatic stellate cells is associated with a gradual replacement of the extracellular matrix (ECM) by the collagen rich fibers and the production of fibrous bands. In advanced stages of fibrosis, the liver contains more ECM components than normal, including collagens (Ⅰ, Ⅲ and Ⅳ), fibronectin, elastin, laminin, hyaluronan, and proteoglycans. PDGFRs: Platelet-derived growth factor receptors; TGFR: Transforming growth factor receptor.

ible lipids and surfactants. NLCs, similar to SLNs, are colloidal particles ranging in size from 100 to 500 nm. Compared to SLNs, NLCs possess a higher drug loading capacity, lower water content, reduced drug expulsion during storage and longer physical stability[22,23-31].

TREATMENT OF LIVER FIBROSIS BY NANOTECHNOLOGY APPROACHES

Liver fibrosis is an abnormal liver condition in which there is a scarring of the liver. It is the consequence of a chronic liver injury and a continual wound-healing process mainly triggered by hepatitis viruses (HBV and HCV) chronic infection, alcohol consumption, genetic abnormalities, steatohepatitis, autoimmune damage, *etc*. Key players in the fibrotic process, which takes place at the cellular and molecular levels, are the HSCs. Their activation and transformation into myofibroblasts, initiated by different types of stimuli, such as cytokines[32], reactive oxygen species[33], toxins[34] and viruses[35], determine an overproduction of extracellular matrix (mainly type I and Ⅲ collagens) which greatly contributes to intrahepatic connective tissue expansion during fibrogenesis (Figure 2). Moreover, activated HSCs secrete pro-fibrotic and pro-inflammatory mediators which perpetuate their activated state, and due to their contractile features they also play a pivotal role in the portal hypertension setting, the major cause of clinical complications of liver cirrhosis[36,37].

In the clinical setting, the conventional anti-fibrotic treatments are still limited, often due to non-specific drug disposition. Thus, the aim of efficient antifibrotic drug delivery using nanotechnology approaches is to achieve liver-specificity with subsequent targeting of the fibrotic region. In this context, HSCs have been the major target for delivering drugs to fibrosis using nanotechnology approaches (Figure 3).

The strongest experimental evidence on possible new approaches for the treatment of liver fibrosis derives from the use of different HSC-selective nanoparticle carriers, most of which are based on the conjugation of targeting ligands directed against receptors expressed by activated HSCs at the surface of various types of NPs. In fact, activated HSC cells express or over-express various receptors, such as mannose-6-phosphate/insulin-like growth factor Ⅱ (M6P/IGF Ⅱ) receptor, PPARs, integrins, platelet-derived growth factor receptors (PDGFRs), retinol binding protein (RBP) receptor and galactosyl receptor, which could be the target of NPs.

M6P/IGF Ⅱ receptor

M6P/IGF Ⅱ receptor is involved in the activation of latent transforming growth factor β (L-TGFβ). M6P/IGF Ⅱ receptor is highly and specifically up-regulated on activated HSC during liver fibrosis[38,39]. TGFβ is a fibrogenic cytokine with many functions, including collagen production and inhibition of its degradation[40]. TGFβ binds to the TGFβ type-Ⅱ receptor on the cell surface, which then heterotetramerizes with a type-I receptor, in most cases activin-like kinase 5. Several preclinical results using different animal models of liver fibrosis suggest that selective localization of a drug to HSC can be possible by targeting the M6P/IGF Ⅱ receptor. First, Beljaars *et al*[41] demonstrated that in rats with liver fibrosis M6P-human serum albumin (albumin chemically modified with 28 M6P groups, M6P-HSA) could be taken up and selectively accumulated in activated HSC. The binding of M6P-HSA to HSC was specific and mediated by binding to M6P/IGF-Ⅱ receptor. This first evidence therefore suggested that M6P-HSA is a suitable carrier for the selective delivery of antifibrotic drugs to activated HSC. Based on these findings, Adrian *et al*[42,43] coupled M6P-HSA to the surface of liposomes and injected them via the penile vein in rats with liver fibrosis induced by bile

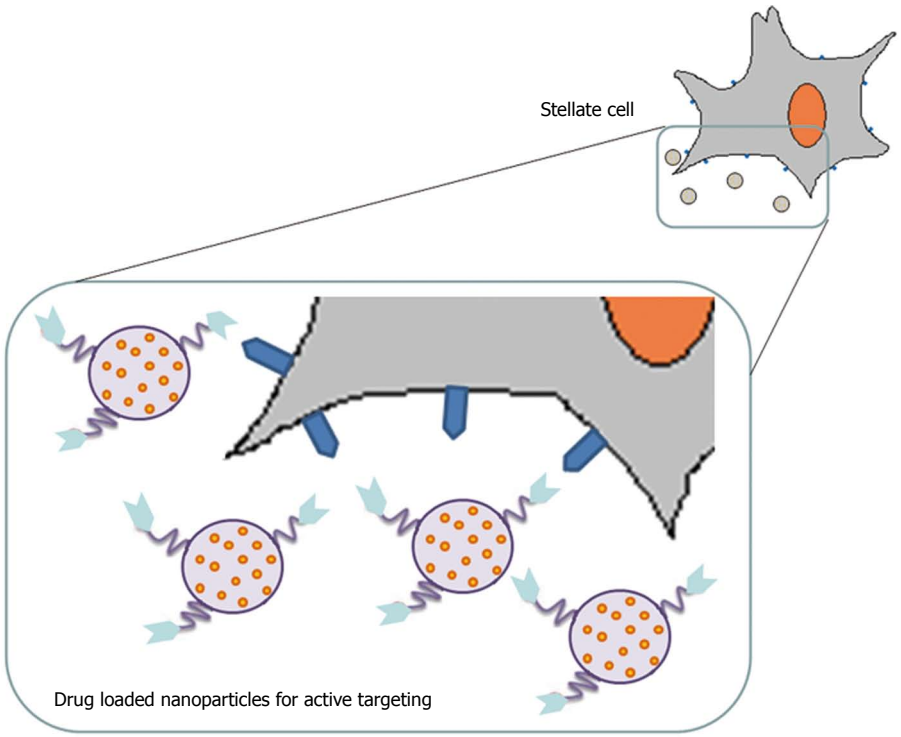

Figure 3 Nanoparticles are loaded with drugs to actively target the disease site by using cell-specific homing devices.

duct ligation. M6P-HSA liposomes were rapidly cleared from the blood circulation in the diseased rats and mainly accumulated in the liver. These studies demonstrated that liposomes coupled with M6P-HSA are potentially effective drug carriers and therefore open up new possibilities for pharmacological interference with a disease as complex as liver fibrosis. Subsequently, to explore new potential therapeutic interventions based on a genetic approach for the treatment of liver fibrosis, inactivated hemagglutinating virus of Japan (HVJ, also known as Sendai virus) containing plasmid DNA was fused with M6P-HSA liposomes to yield HVJ liposomes that selectively target HSCs[43]. Adrian *et al*[44], showed that following *iv* injection into mice with liver fibrosis, M6P-HSA-HVJ-liposomes efficiently associated with HSC. This approach therefore offers new possibilities for treating liver fibrosis.

PPARs

PPARs, which belong to the superfamily of nuclear hormone receptors with transcriptional activity controlling multiple processes, have been implicated in liver fibrogenesis[45]. To date, four isoforms of PPARs have been identified, namely PPARδ, β, γ and δ. Growing evidence shows that activated HSCs express PPARδ and its expression exerts important effects on fibrogenesis in animal models[46], and that treatment with PPARδ ligands, Wy-14643 (WY) or fenofibrate, dramatically reduces hepatic fibrosis[47]. PPARβ and PPARδ are also highly expressed in HSCs, and their activation increases hepatic fibrosis[48]. There is clear evidence that activation of HSCs and their transdifferentiation into myofibroblasts is accompanied by significantly decreased PPARγ expression, and that treatment with PPAR-γ agonist rosiglitazone inhibits HSC activation[49]. Therefore, PPARs are considered a promising drug target for antifibrotic therapy[50-52]. M6P-HSA-conjugated liposomes have also been used to deliver ligands for PPAR to activated HSCs. Recently, Patel *et al*[53] reported a significant enhancement of liver uptake, improvement in histopathological morphology and decreased fibrosis grade when the PPARγ ligand rosiglitazone was loaded in M6P-HSA-conjugated liposomes and administrated intravenously in rats with liver fibrosis.

Integrins

Activated HSCs express increasing amounts of integrins. Integrins are a large family of heterodimeric cell surface receptors which mediate the interaction between cells and extracellular matrix molecules (such as collagens and fibronectin) and recognize a common motif in their ligands, among which the best studied is the RGD sequence (arginine-glycine-aspartic acid)[54-56]. Collagen VI, abnormally produced in the liver by activated HSCs and deposited during fibrogenesis, is recognized by cell-surface integrins, mainly integrin δ1β1, through the specific interaction of the receptor with the RGD sequence present in the matrix molecule. Therefore, the RGD sequence has been used as a homing device to target integrins and hence HSCs in fibrotic liver. In 2000 a carrier which showed binding and internalization to HSC was successfully used for the first time[57], suggesting the possibility to deliver anti-fibrotic agents directly to HSC. Subsequently, Du *et al*[58] developed cyclic RGD-labeled sterically-stabilized liposomes (SSLs) to deliver IFNδ-1b to HSC. They demonstrated that cyclic RGD peptide-

labeled liposomes were selectively taken up by activated HSCs in a liver fibrosis rat model, and that liposome encapsulated IFNδ-1b displayed an improved efficiency in blocking fibrogenesis. In another study, Li *et al*[59] also used SSLs labeled with cyclic RGD peptide to encapsulate hepatocyte growth factor (HGF), in order to prevent its degradation and therefore to reverse fibrogenesis processes[60]. When HGF was encapsulated in SSL labeled with cyclic RGD peptide (RGD-SSL-HGF) it was more effective than SSL-HGF in promoting liver fibrosis regression in cirrhotic rats, indicating that HGF loaded in RGD-SSL enhanced its effect on activated HSCs[60].

PDGFRs

PDGF is the most potent proliferative factor in liver fibrosis. Its receptor (PDGFR) has two forms, PDGFRδ and PDGFRβ. PGFRs are cell surface tyrosine kinase receptors for members of the PDGF family. In particular, PDGF-β receptor is highly upregulated on activated HSCs[61]. Recent studies in rats with hepatic fibrosis showed that pPB-SSL-IFN-γ, a targeted SSL modified by a cyclic peptide (pPB) with affinity for the PDGF-β receptor to deliver IFN-γ (pPB-SSL-IFN-γ) to HSCs, improves the anti-fibrotic effects of IFN-γ and reduces its side effects to some extent[62,63].

RBP receptor

RBP receptor expressed by HSCs, is involved in the uptake and storage of vitamin A. Sato *et al*[64] assessed the anti-fibrotic properties of vitamin A-coupled liposomes containing small interfering RNA (siRNA) against gp46, the rat homolog of human heat shock protein 47 and involved in the inhibition of collagen secretion, in three experimental models of liver fibrosis induced by dimethylnitrosamine, carbon tetrachloride (CCl_4), and bile duct ligation. They showed that the treatment decreased collagen deposition, induced apoptosis of HSCs, improved liver function tests and prolonged survival in the treated rats.

Galactosyl receptor

Hepatic fibrosis is also the result of oxidative damage to the liver due to exposure to environmental metalloid toxicants. Therefore, a promising strategy has been developed to deliver antioxidants to damaged liver by nanocarriers. The galactosyl receptor expressed on the hepatocytes mediates the internalization of molecular asialoglycoproteins and small particles. With this in mind, liposomes decorated with p-aminophenyl δ-*D*-galactopyranoside, which binds to galactosyl receptor, have been used as carriers for targeted *iv* delivery of the antioxidant flavonoid quercetin (QC) in animals with liver fibrosis[65,66]. It has been shown that the administration of QC loaded in galactosylated liposomes in rats results in the maximum prevention of arsenic deposition (a contaminant responsible for oxidative damage present in drinking water, particularly in developing countries such as India and Bangladesh) and protects the liver from sodium arsenite ($NaAsO_2$)-induced collagen deposition and fibrosis initiation. However, whereas free QC does not protects rats from oxidative damage, galoctosylated liposomes QC might be therapeutically useful to prevent $NaAsO_2$-induced acute liver toxicity.

Apoptosis-inducing agents

Oxymatrine (OM) is an alkaloid extracted from the medicinal plant *Sophora alopecuroides L.* which, among its multiple pharmacological functions, can induce apoptotic cell death in different cell types[67,68] and exerts antiviral effects, inhibiting HBV and HCV replication[69,70]. In addition, OM has also been demonstrated to have anti-fibrotic effects, being effective in reducing collagen production and deposition in CCl_4-induced liver fibrosis in rats[71]. Based on these findings, Chai *et al*[72] used OM-RGD liposomes in both *in vitro* and *in vivo* experiments and demonstrated that delivery of OM to HSCs with this formulation attenuates hepatic fibrosis by inhibiting viability and inducing HSC apoptosis, thus highlighting its possible application in the treatment of hepatic fibrosis. The influence of OM on the fibrotic process has also been evaluated in a bile duct ligation rat model of liver fibrosis using self-assembled polymeric vesicles based on biodegradable poly(ethylene glycol)-b-poly(e-caprolactone) (PEG-b-PCL), referred to as polymersomes (PM), and modified with RGD peptide to obtain RGD-PM-OM[73]. Yang *et al*[73], demonstrated that intravenous injection of RGD-PM-OM and PM-OM formulations showed significant benefits in ameliorating the degree of liver injury and fibrosis as shown by lower levels of fibrosis markers in the serum compared to free OM. This novel approach therefore appears to be more effective than conventional treatment with OM.

Curcumin or diferuloylmethane, a yellow polyphenol extracted from the rhizome of turmeric *Curcuma longa*, has been extensively studied for its therapeutic effects in a variety of disorders because of its antineoplastic, antioxidant and anti-inflammatory effects[74-76]. Several studies have shown its potential anti-fibrotic activity[77-84] but the compound has poor aqueous solubility, which results in low bioavailability and low concentrations at the target site[85,86]. Consequently, nanotechnology approaches have been developed to deliver curcumin to targets. For example Bisht *et al*[87] developed a polymeric nanoparticle formulation of curcumin (NanoCurc™). Nanocurcumin was synthesized utilizing the micellar aggregates of cross-linked and random copolymers of *N*-isopropylacrylamide, with *N*-vinyl-2-pyrrolidone and poly(ethylene glycol) monoacrylate (PEG-A). Nanocurcumin showed to be readily dispersed in aqueous media with a comparable *in vitro* therapeutic efficacy to free curcumin against a panel of human cancer cell lines[87]. The same authors subsequently performed *in vivo* studies using NanoCurc™ to treat animals with hepatic injury and fibrosis induced by CCl_4 administration[88]. Results following intraperitoneal injection of NanoCurc™ were extremely promising, as NanoCurc™ enhanced the bio-

availability of intrahepatic curcumin concentrations compared to control void NPs, attenuated hepatocellular injury and levels of pro-inflammatory cytokines, inhibited CCl_4-induced liver injury, prevented hepatic fibrosis and induced HSC apoptosis. The exact mechanism by which curcumin induces a protective hepatocellular environment is not clear. Curcumin might work through multiple mechanisms. As reported by Bisht *et al*[88] NanoCurc™ accumulates in hepatocytes and in the non-parenchymal cell compartment, which contains pro-fibrotic stellate cells and myofibroblasts. Authors have demonstrated that NanoCurc™ inhibits pro-fibrogenic transcripts associated with activated myofibroblasts and directly induces HSC apoptosis. However, another possibility is that NanoCurc™ might also affect hepatic progenitor cells or bile duct cells and thus ameliorate the effects of CCl4-induced liver injury by influencing these cells.

CONCLUSION

The extremely wide diffusion of CLD worldwide and the relatively ineffective therapeutic options especially for advanced liver fibrosis demand new drugs or new drug delivery strategies to cure liver fibrosis. Therapies based on nanotechnologies have emerged as an innovative and promising alternative to conventional therapies. The number of NPs used in biomedical research and drug delivery is rapidly increasing. Several reports in the literature, most of all in animal models, have shown that different HSC-selective NP carriers, based on the conjugation of targeting ligands directed against several receptors expressed by activated HSCs at the surface, can reduce liver fibrosis. These data, if confirmed in humans, could open up a new era in the treatment of liver fibrosis. In the next few years the clinical validation of CLD therapies based on nanotechnologies will hopefully be demonstrated.

However, much more needs to be done, particularly because the use of nanoparticles also creates unique environmental and societal challenges[89]. Toxicity associated with nanomaterials should be considered before NPs are widely utilized as drug delivery systems, especially for inorganic nanoparticles[90,91]. In this respect, the risk associated with organic NPs seems to be less problematic because this type of nanoparticles are very often typically either made from, or covered with natural or highly biocompatible polymers (such as PEG)[92].

Finally, it is necessary to develop a regulatory framework based on objective scientific research which will ensure that human exposure to unwanted engineered nanomaterials in the environment will be limited to safe levels. However, the therapeutic use of nanomaterials in medicine requires a different framework in which the therapeutic benefits will be balanced against the potentially harmful risks.

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TOPIC HIGHLIGHT

WJG 20th Anniversary Special Issues (11): Cirrhosis

New determinants of prognosis in bacterial infections in cirrhosis

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Abstract

Despite major advances in the knowledge and management of liver diseases achieved in recent decades, decompensation of cirrhosis still carries a high burden of morbidity and mortality. Bacterial infections are one of the main causes of decompensation. It is very important for clinical management to be aware of the population with the highest risk of poor outcome. This review deals with the new determinants of prognosis in patients with cirrhosis and bacterial infections reported recently. Emergence of multiresistant bacteria has led to an increasing failure rate of the standard empirical antibiotic therapy recommended by international guidelines. Moreover, it has been recently reported that endothelial dysfunction is associated with the degree of liver dysfunction and, in infected patients, with the degree of sepsis. It has also been reported that relative adrenal insufficiency is frequent in the non-critically ill cirrhotic population and it is associated with a higher risk of developing infection, severe sepsis, hepatorenal syndrome and death. We advise a change in the standard empirical antibiotic therapy in patients with high risk for multiresistant infections and also to take into account endothelial and adrenal dysfunction in prognostic models in hospitalized patients with decompensated cirrhosis.

Key words: Bacterial infections; Liver cirrhosis; Drug resistance; Bacterial; Endothelial dysfunction; Relative adrenal insufficiency

Core tip: Despite major advances in the management of cirrhosis, it still carries high morbidity and mortality. Bacterial infection is one of the major causes of decompensation. This review deals with the new determinants of prognosis in patients with cirrhosis and bacterial infection reported recently. It summarizes the existing evidence for emergence of multiresistant bacteria, endothelial dysfunction, and relative adrenal insufficiency; and resultant changes in medical practice are given.

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INTRODUCTION

Decompensation of liver cirrhosis carries a huge burden of morbidity and mortality in society. In the past 30 years there has been major progress in the knowledge and management of liver disease; despite this, there are approximately 29 million people in the European Union who still suffer from a chronic liver condition. Available data suggest that about 0.1% of the European popula-

tion is affected by cirrhosis, corresponding to 14-26 new cases per 100000 inhabitants per year or an estimated 170000 deaths per year[1,2].

Bacterial infection is a major cause of decompensation. Patients with cirrhosis are at an increased risk of developing bacterial infections, sepsis, severe sepsis and death[3]. Thus, infection is present at admission or develops during hospitalization in about 25%-30% of patients[4,5]. Bacterial infection is not only more frequent but also more severe in cirrhosis, causing a four-fold increase in the probability of death, reaching 38% at 1 mo[6]. Infection can accentuate circulatory dysfunction leading to the development of hepatorenal syndrome (HRS) and can also induce an excessive pro-inflammatory response that could contribute to the development of sepsis-related organ failure (acute-on-chronic liver failure) and septic shock[7]. Therefore, it is important to identify determinants of poor prognosis in patients with bacterial infections and cirrhosis in order to be alert to the group of patients with highest risk of death and, if possible, reverse the deleterious effect of these determinants by modifying the standard clinical practice performed in this major disease.

This review aims to summarize the recently reported data regarding recent changes in the epidemiology of bacterial infections in cirrhosis, endothelial dysfunction, and relative adrenal insufficiency; all of which are the new determinants of prognosis in patients with cirrhosis and bacterial infections reported recently.

EMERGENCE OF MULTIRESISTANT BACTERIA IN CIRRHOSIS

The discovery of antibiotics in the early to mid-20th century remains one of the most significant achievements to date, but inherent with its use is the development of antimicrobial resistance. In consequence, epidemiology of bacterial infections is continuously changing and the emergence of multiresistant (MR) bacteria in the general and cirrhotic population has risen as a new determinant of prognosis.

In the 1980s, epidemiological surveillance showed that most infections were community acquired and approximately 70% to 80% of the isolated organisms were gram-negative bacilli (GNB)[4]. Since the 1990s, practice in hepatology has involved invasive procedures (*i.e.*, variceal ligation, transjugular intrahepatic portosystemic shunt, and arterial chemoembolization or percutaneous ablation of hepatocellular carcinoma) and also severely ill patients have been treated in Intensive Care Units. Consequently, in the 2000s some important changes were reported: 39% of infections were of nosocomial origin and gram positive cocci (GPC) was the most frequently isolated bacteria in the nosocomial setting. GPC were also isolated more frequently in the admissions which required invasive procedures or treatment in the Intensive Care Unit. Another important change observed was the emergence of SBP caused by quinolone-resistant GNB in patients under long-term norfloxacin prophylaxis. At that time, only 1.2% of infections caused by *Enterobacteriaceae* were resistant to third generation cephalosporines (TGC)[4]. On that account, international clinical guidelines recommend the use of TGC to treat the most common infections in cirrhosis as they are active against *Enterobacteriaceae* and streptococci, but not against enterococci, and they also have a good security profile[8-10].

Types of multiresistance patterns around the world

The employment of TGC for two decades has led to the emergence of MR bacteria, as evidenced by various reports from very different geographical areas[5,11-22]. MR bacteria are resistant to at least three of the main antibiotic families including β-lactamics[23]. The most common MR bacteria are extended-spectrum β-lactamase-producing *Enterobacteriaceae* (ESBL), non-fermentable GNB such as *Pseudomonas aeruginosa*, *Stenotrophomonas maltophilia* or *Acinetobacter baumanii*, methicillin-resistant *Staphylococcus aureus* (MRSA), vancomycin-susceptible *Enterococcus* (VSE) and vancomycin-resistant *Enterococcus* (VRE).

The emergence of MR bacteria is present worldwide, but in a heterogeneous manner. Thus, different geographical areas have different epidemiological patterns of multiresistance; for example, ESBL-producing *Enterobacteriaceae* are predominant in South Europe and Asia[5,11-19], while MRSA and VRE are frequently isolated in centers of the United States[22]. Moreover, Carbapenemase-producing *K. pneumoniae* has only been reported in some centers in Italy[24]. Table 1 summarizes the prevalence and risk factors for the development of MR bacteria across the world.

Risk factors and clinical impact of multiresistance bacterial infections

The main risk factors for the development of infections caused by MR bacteria are: nosocomial origin of infection [hazard ratio (HR): 4.43], long-term norfloxacin prophylaxis (HR: 2.69), recent infection by an MR bacteria (HR: 2.45), and recent use of β-lactams (HR: 2.39)[5]. Other reports suggest that infections that develop in the setting of recent contact with the health-care environment (health-care associated), like dialysis centers, are also at risk of developing MR bacteria infections[16].

The emergence of MR bacteria has a major impact on the clinical evolution of infected patients with cirrhosis, through impairment of the efficacy of standard empirical antibiotic therapy. In an in-depth prospective study regarding this topic, the final resolution of infection was still high in community-acquired infections (83%), but low in healthcare associated infections (73%), and extremely low in nosocomial infections (40%)[5]. Final resolution was significantly lower in infections caused by multiresistant strains (70% *vs* 92%, $P < 0.0001$), particularly in SBP and pneumonia (50% and 55%, respectively). In consequence, septic shock was more frequently observed in MR infections (26% *vs* 10%, $P < 0.0001$) and hospital mortality in MR infections duplicate that observed in infections caused by susceptible bacteria (25% *vs* 12%, $P =$

Table 1 Prevalence, clinical impact and risk factors of multiresistant bacterial infections around the world

Ref.	Infections	Prevalence of MR bacteria	Risk factors	Clinical impact
Song *et al*[14], 2006 South Korea	SBP	29% ESBL-producing *Enterobacteriaceae*: 14% in community-acquired, 67% in nosocomial episodes	No data	No impact
Angeloni *et al*[61], 2008 Italy	SBP	8% ESBL-producing *Enterobacteriaceae*	Healthcare-associated infections	Higher initial treatment failure No impact on mortality
Umgelter *et al*[20], 2009 Germany	SBP	10% VSE, 1% *Pseudomonas aeruginosa*	No data	Higher initial treatment failure Higher hospital mortality
Piroth *et al*[19], 2009 France	SBP and bacterioascites	8% MRSA 5% VSE 4% ESBL-producing *Enterobacteriaceae*	No data	No data
Cheong *et al*[13], 2009 South Korea	SBP	15% ESBL-producing *Enterobacteriaceae*	Previous exposition to β-lactams Nosocomial infection	Independent predictor of 30-d mortality
Song *et al*[12], 2009 South Korea	SBP	4%-7.5% ESBL-producing *Enterobacteriaceae*	Recent hospital stay Previous SBP Antibiotic treatment in the last month	Higher initial treatment failure Higher hospital and 30-d mortality
Merli *et al*[16], 2010 Italy	All	20% ESBL-producing *Enterobacteriaceae* 7% MRSA	Antibiotic treatment in the last month HCA infection	Higher hospital mortality
Ariza *et al*[17], 2012 Spain	SBP	6% ESBL-producing *Enterobacteriaceae* 2% *Pseudomonas aeruginosa* 2% *Acinetobacter baumannii* 1% VSE	Nosocomial infection Previous exposition to β-lactams Diabetes mellitus Upper gastrointestinal bleeding	Independent predictor of mortality at 30 d
Fernández *et al*[5], 2012 Spain	All	8%-9% ESBL-producing *Enterobacteriaceae* 3% *Pseudomonas aeruginosa* 3%-4% MRSA 3%-7% VSE	Nosocomial infection Long-term norfloxacin prophylaxis Treatment with β-lactams in the last 3 mo MR bacteria in the last 6 mo	Lower infection resolution Higher risk of septic shock Higher hospital mortality
Novovic *et al*[21], 2012 Denmark	SBP	1% ESBL-producing *Enterobacteriaceae* 12% VSE-VRE	No data	Higher hospital mortality
Tandon *et al*[22], 2012 United States	All	9% VRE 6.5% ESBL-producing *Enterobacteriaceae* 5% MRSA	Systemic antibiotics in the past 30 d Nosocomial infection	No data

ESBL: Extended-spectrum β-lactamase-producing *Enterobacteriaceae* (bacteria with chromosomal β-lactamases are also included); MRSA: Methicillin-resistant *Staphylococcus aureus*; VSE: Vancomycin-susceptible enterococci; VRE: Vancomycin-resistant enterococci; SBP: Spontaneous bacterial peritonitis.

0.001)[5].

New recommendations of empirical antibiotic therapy

According to this new epidemiological data it has been recommended to change the empirical antibiotic therapy (*i.e.*, third generation cephalosporins) employed in nosocomial infections. Marked epidemiological differences observed among countries and centers suggest that local epidemiology should be evaluated regularly and new guidelines should be tailored according to the specific local epidemiological pattern of multiresistance[25].

In general, our recommendation for nosocomial infections is that in areas with a high prevalence of ESBL-producing *Enterobacteriaceae*, carbapenems should be used. It is also important to tailor the antibiotics according to the severity of infection: in severe sepsis and septic shock it is important to cover all possible bacteria, therefore glycopeptides should be added[5]. In areas with high prevalence of VSE and MRSA, a glycopeptide should be used. In the United States and other regions with a high rate of infections caused by VRE, glycopeptides should be replaced by linezolid or daptomycin. In areas with low prevalence of MR bacteria but high prevalence of *Enterococcus faecalis*, piperacillin-tazobactam should be used.

In healthcare associated infections our recommendation is to treat as nosocomial infections those patients under long-term norfloxacin prophylaxis or those with an MR bacteria infection in the previous six months because these two factors identify a subgroup of patients with high risk of MR bacteria infection (Figure 1).

In summary, recent data demonstrate that currently recommended empirical antibiotic therapy is not appropriate for the treatment of nosocomial and some healthcare associated infections in cirrhosis because of the high prevalence of MR bacteria in these settings. New antibiotic strategies for these infections should be tailored according to the local epidemiological patterns of multiresistance and early de-escalation of antibiotics according to the microbiological results is also mandatory to slow down the development of new resistances.

CIRCULATORY AND ENDOTHELIAL DYSFUNCTION

It is well known that a clinically important characteristic of cirrhotic patients is systemic circulatory dysfunction which is characterized by arterial splanchnic vasodilation which progresses in parallel with the degree of liver impairment and portal hypertension. This situation is due to a local release of vasoactive substances, especially nitric oxide[26-28] but also prostaglandines, P substance, carbon monoxide, calcitonin gene-related peptide and endocannabinoids[29-33]. In the initial phase of the disease, the

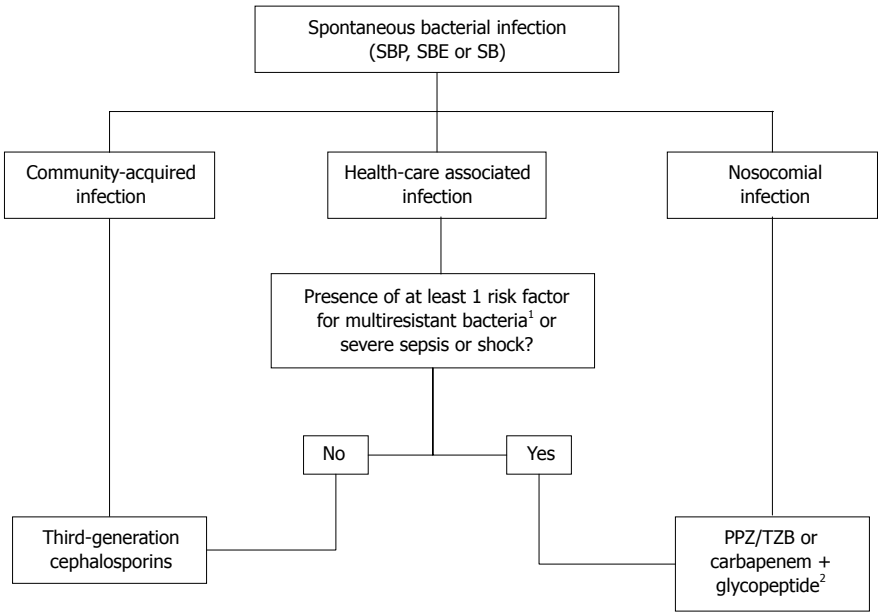

Figure 1 Proposed algorithm for the empirical treatment of infections in cirrhosis. [1]Risk factors for multiresistant bacteria in Health care associated infections are long-term norphloxacin prophylaxis or previous infection by multiresistant (MR) bacteria within 6 mo; [2]Piperaziline/tazobactam in areas of low MR bacteria but high *Enterococcus faecalis* prevalence. Meropenem and glycopeptides in areas with high prevalence of extended-spectrum β-lactamase-producing *Enterobacteriaceae* and methicillin-resistant *Staphylococcus aureus*. SBP: Spontaneous bacterial peritonitis; SBE: Spontaneous bacterial empyema; SB: Spontaneous bacteremia; PPZ/TZB: Piperaziline/tazobactam.

reduction of effective arterial volume is compensated for by an increase in cardiac output. Nevertheless, while liver dysfunction progresses patients develop cirrhotic cardiomyopathy characterized by diastolic dysfunction which can affect inotropic function during stress[34]. Therefore, patients develop arterial hypotension and compensating activity of vasoactive systems (sympathetic and renin-angiotensin-aldosterone) with sodium and water retention and ascites production[26]. When circulatory dysfunction continues to progress, vasopressin is activated which leads to dilutional hyponatremia and hepatorenal syndrome[35].

Bacterial infection is a main cause of circulatory dysfunction. Infection in cirrhosis is characterized by a more intense inflammatory response than that observed in non-cirrhotic population. Accordingly, cirrhotic patients with SBP present very high levels of cytokines[36] which exacerbate circulatory dysfunction[37]. Thus, infection in a patient with baseline circulatory dysfunction can have devastating effects. Up to 30% of patients develop progressive circulatory dysfunction with acute renal failure, cardiac impairment, hepatic encephalopathy, type-1 hepatorenal syndrome and death[38]. On that account, expansion of effective arterial volume with intravenous albumin reduces renal failure and improves survival in SBP[39].

Moreover, advanced cirrhosis is characterized by an increased intestinal permeability and bacterial translocation which results in severe infections like spontaneous bacterial peritonitis, spontaneous bacteremia, and spontaneous empyema[40,41]. Increased intestinal permeability is caused by many factors including structural changes in intestinal mucosa due to circulatory dysfunction[42,43], hypomotility secondary to sympathetic nervous system hyperactivity, and oxidative damage produced by high levels of nitric oxide and proinflammatory cytokines[44,45]. Translocation of not only viable bacteria, but also bacterial products like DNA, have been associated with a higher inflammatory response and worse prognosis, due to the development of acute-on-chronic liver failure[46,47].

Endothelial dysfunction

There are well recognized markers of endothelial dysfunction; these involve mainly the von Willebrand factor (vWF), but also P-selectin and isoprostanes. It has been reported that serum levels of vWF increase according to the degree of liver dysfunction and portal hypertension. Endothelial dysfunction, and higher levels of vWF have been associated with a higher incidence of decompensations related to portal hypertension and mortality[48,49]. In one study it has been suggested that vWF is released in the hepatosplanchnic vascular bed[49]. Furthermore, regarding infection in cirrhosis, it has been reported that the degree of endothelial dysfunction increases according to the degree of sepsis, vWF serum levels increased progressively among non infected patients, infected patients without sepsis, infected patients with sepsis and, showing the highest levels of vWF, patients with septic shock[50] (Figure 2).

The high mortality associated with endothelial dysfunction could be explained beyond its association with circulatory dysfunction. The increasing levels of vWF in parallel with increasing degrees of sepsis would reflect increasing endothelial activation produced by increasing levels of cytokines. Moreover, cytokines and inflammation activate coagulation cascade and lead to hemostatic abnormalities leading to poor organ perfusion reaching, in some cases, the extreme degree of disseminated intra-

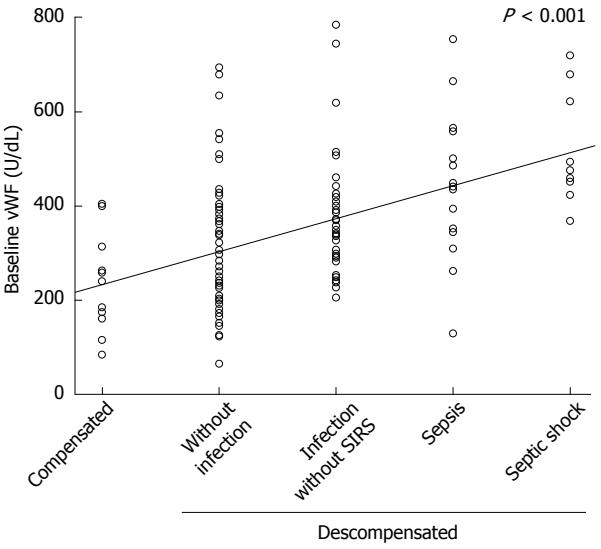

Figure 2 Correlation between von Willebrand factor and degree of sepsis. SIRS: Systemic inflammatory response syndrome. (thanks *Hepatology* journal for permission to reproduce the figure).

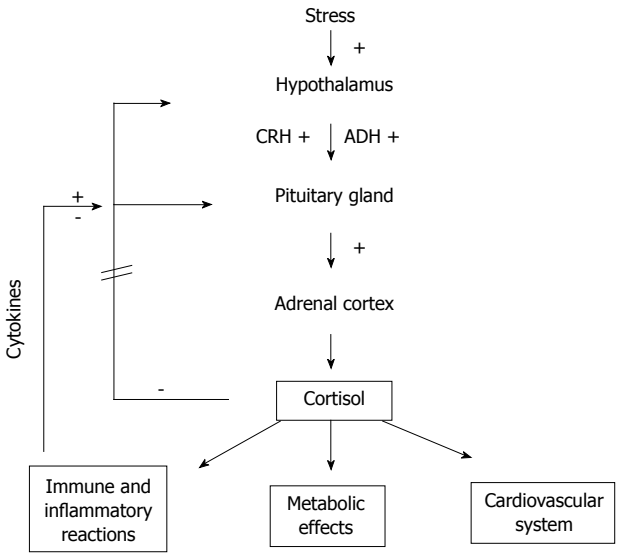

Figure 3 Hypothalamic-pituitary-adrenal axis in the critical illness. CRH: Corticotrophin releasing hormone; ADH: Antidiuretic hormone; ACTH: Adrenocorticotropic hormone. Original work of the authors.

vascular coagulation and multiple organ failure.

In summary, endothelial dysfunction is clearly associated with poor prognosis and vWF should be taken into account in predictive models in hospitalized patients.

ADRENAL DYSFUNCTION

A normal adrenal function is essential to surviving critical illness. Cortisol maintains vascular tone, endothelial integrity, vascular permeability and total corporal water distribution[51]. In consequence, an inappropriate adrenal response, *i.e.*, relative adrenal insufficiency (RAI), during a critical illness like severe sepsis or septic shock has important clinical consequences. These patients secrete cortisol and corticotrophin at the initial stage of the disease, but less than needed to overcome stress. Activation of the axis is triggered by cytokines and other factors that promote the release of corticotrophin releasing hormone (CRH) and vasopressin in the hypothalamus[51,52]. These hormones stimulate pituitary secretion of corticotrophin (ACTH) which induces adrenal production of cortisol. In addition, the levels of cortisol binding protein decrease fast, leading to higher levels of free cortisol, which is the active component of cortisol[53]. Furthermore, negative feedback of cortisol upon CRH and ACTH is inactive, thus maintained activation on the hypothalamic-pituitary-adrenal axis can be exerted[51]. Finally, there is an increase in the number and sensibility of cortisol receptors[51,52]. In this sense, during critical illness there is an integrated multilevel response that optimizes the cortisol effect in peripheral tissues, and cytokines and bacterial products are also able to modify the response of hypothalamic-pituitary-adrenal axis at each level[51] (Figure 3).

There is a high prevalence of RAI in patients with cirrhosis and septic shock and it is associated with liver and renal failure, refractory septic shock and hospital mortality[54-56]. Two recent studies confirmed a high prevalence of RAI in septic shock[57] and in digestive bleeding[58]. However, there are contradictory results on the beneficial effects on outcome produced by the administration of steroids at stress doses[55-59]; large-scale randomized controlled trials are required to clarify this point.

It has recently been reported that RAI is not only common in critically ill patients with cirrhosis but also in non-critically ill patients hospitalized by decompensation of cirrhosis. RAI prevalence in this setting is 26%, and it is associated with a higher degree of circulatory dysfunction evidenced through lower mean arterial pressure (76 ± 12 mmHg *vs* 83 ± 14 mmHg, $P = 0.009$), higher serum levels of noradrenaline (544 ± 334 pg/mL *vs* 402 ± 316 pg/mL, $P = 0.02$), plasma renin activity (7.1 ± 9.9 ng/mLh *vs* 3.4 ± 5.6 ng/mLh, $P = 0.03$), and lower serum sodium levels (131 ± 7 mEq/L *vs* 135 ± 5 mEq/L, $P = 0.007$). Furthermore, patients with RAI presented a tendency to a higher inflammatory state with a higher prevalence of systemic inflammatory response syndrome (SIRS) (60% *vs* 41%, $P = 0.08$) and higher plasmatic levels of tumoral necrosis factor alpha (54 ± 115 pg/mL *vs* 27 ± 24 pg/mL) and interleukine-6 (916 ± 2532 pg/mL *vs* 244 ± 439 pg/mL). Patients with RAI showed a higher probability of developing infections (41% *vs* 21%, $P = 0.008$), severe sepsis (27% *vs* 9%, $P = 0.003$), type-1 hepatorenal syndrome (16% *vs* 3%, $P = 0.002$), and death (22% *vs* 7%, $P = 0.01$) (Figures 4 and 5)[60].

The higher incidence of infections observed in patients with RAI is explained by the presence of an important circulatory dysfunction which leads to bacterial translocation. The higher degree of inflammation would contribute to mucosal barrier damage and bacterial translocation and also to the development of renal failure and hepatorenal syndrome by worsening circulatory dysfunction, as already described. The addition of a baseline low vascular tone and a functional deficit of cortisol which leads to a further decrease in vascular tone would con-

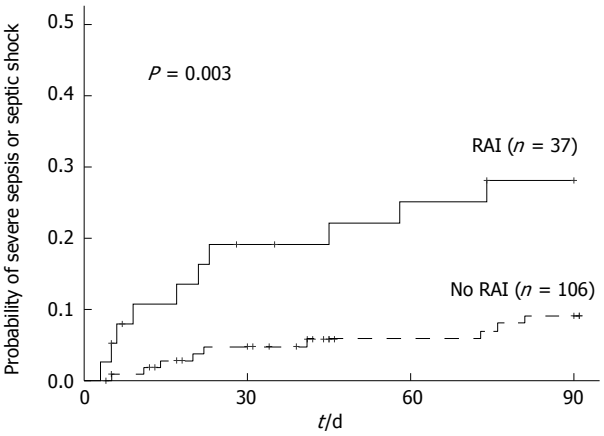

Figure 4 Probability of development of severe sepsis and septic shock in patients with and without relative adrenal insufficiency. Probability of developing new episodes of severe sepsis or septic shock in patients with relative adrenal insufficiency (RAI) (continuous line) or with normal adrenal function (doted line) during 3 mo follow-up. Probability was significantly higher in patients with RAI. (thanks *Hepatology* journal for permission to reproduce the figure).

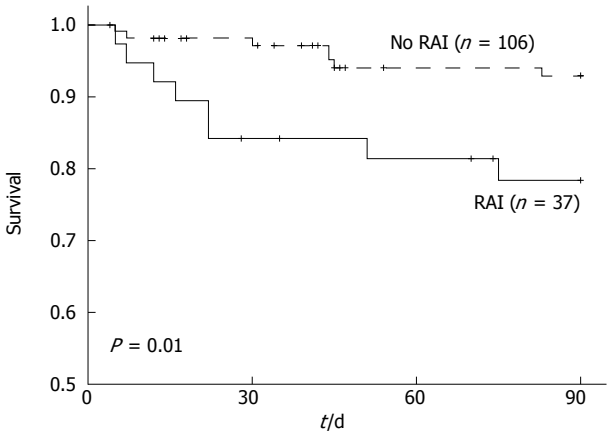

Figure 5 Probability of survival at 3 mo in patients with and without relative adrenal insufficiency. Probability of survival at 3 mo in patients with relative adrenal insufficiency (RAI) (continuous line) or with normal adrenal function (doted line). Probability was significantly higher in patients with RAI. (thanks *Hepatology* journal for permission to reproduce the figure).

tribute to the development of severe sepsis and septic shock[60].

Circulatory dysfunction and inflammation are causes of RAI through vasodilation and reduction in adrenal blood flow which diminishes adrenal function; high levels of proinflammatory cytokines also directly inhibit cortisol synthesis by the adrenal glands.

To sum up, RAI has a negative impact on prognosis in critically-ill and non critically-ill cirrhotic patients and large-scale randomized controlled trials should be performed, aimed at evaluating cortisol supplementation during critical illness and antibiotic prophylaxis during admission in the non-critically ill population.

CONCLUSION

New determinants of prognosis in patients with liver cirrhosis and bacterial infections have been identified in recent years. Recent changes in epidemiology and new findings in pathophysiology have been reported. On one hand, it has been reported worldwide emergence of multiresistant bacteria which leads to changes in the current recommended empirical antibiotic therapy in those patients with risk factors for MR bacteria infection, with the warning of tailoring it according to local patterns of multiresistance and de-escalating as soon as possible to diminish the impact of wide-spectrum antibiotics on the appearance of new resistant strains.

On the other hand, it has been reported an association between endothelial dysfunction and higher portal pressure and more episodes of decompensation of cirrhosis. Moreover, it is well known that relative adrenal insufficiency is related with refractory shock and mortality in critically-ill cirrhotic patients, and, in addition, it has been recently reported that relative adrenal insufficiency is related with a high risk of developing infections, septic shock and mortality in the non critically-ill cirrhotic population. Thus, endothelial dysfunction and relative adrenal insufficiency are clearly associated with poor prognosis and should be taken into account in prognostic models in hospitalized patients with decompensation of cirrhosis. Trials focused on whether steroid administration in patients with septic shock and RAI improves survival, and evaluation of antibiotic prophylaxis in the non critically-ill population with RAI would be interesting fields of research.

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TOPIC HIGHLIGHT

WJG 20th Anniversary Special Issues (11): Cirrhosis

Cellular and molecular mechanisms in the pathogenesis of liver fibrosis: An update

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Abstract

There have been considerable recent advances towards a better understanding of the complex cellular and molecular network underlying liver fibrogenesis. Recent data indicate that the termination of fibrogenic processes and the restoration of deficient fibrolytic pathways may allow the reversal of advanced fibrosis and even cirrhosis. Therefore, efforts have been made to better clarify the cellular and molecular mechanisms that are involved in liver fibrosis. Activation of hepatic stellate cells (HSCs) remains a central event in fibrosis, complemented by other sources of matrix-producing cells, including portal fibroblasts, fibrocytes and bone marrow-derived myofibroblasts. These cells converge in a complex interaction with neighboring cells to provoke scarring in response to persistent injury. Defining the interaction of different cell types, revealing the effects of cytokines on these cells and characterizing the regulatory mechanisms that control gene expression in activated HSCs will enable the discovery of new therapeutic targets. Moreover, the characterization of different pathways associated with different etiologies aid in the development of disease-specific therapies. This article outlines recent advances regarding the cellular and molecular mechanisms involved in liver fibrosis that may be translated into future therapies. The pathogenesis of liver fibrosis associated with alcoholic liver disease, non-alcoholic fatty liver disease and viral hepatitis are also discussed to emphasize the various mechanisms involved in liver fibrosis.

Key words: Liver; Liver fibrosis; Cirrhosis; Fibrogenesis; Hepatic stellate cells; Myofibroblast; Extracellular matrix

Core tip: Liver fibrosis is a dynamic process that results from an imbalance between the production and dissolution of the extracellular matrix. Development of liver fibrosis is orchestrated by many cell types, including hepatic stellate cells (HSCs). The activation of HCSs is a complex process, leading to multiple potential sites for therapeutic interventions. Additionally, the differences between the pathogenesis of liver fibrosis associated with different etiologies may provide the determination of new therapeutic approaches. This review summarizes the most significant data that has contributed to the understanding of the cellular and molecular pathogenesis of liver fibrosis, which may be translated into future therapeutic strategies.

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INTRODUCTION

Liver fibrosis is a common pathological consequence of a variety of chronic stimuli, including viral, autoimmune, drug induced, cholestatic and metabolic diseases[1-4]. Liver fibrosis can be defined as a result of the progressive accumulation and decreased remodeling of the extracellular matrix (ECM), which disrupts the normal architecture of

the liver[2]. If left untreated, fibrosis can progress to liver cirrhosis, ultimately leading to organ failure and death. The characterization of the underlying mechanisms of liver fibrogenesis has indicated that fibrosis is driven by a dynamic process involving the increased synthesis of matrix components and a failure of physiological mechanisms of matrix turnover. Moreover, the capacity of the liver to undergo fibrosis regression following cessation of the liver insult has been highlighted[4-6]. These findings have provided progressed the understanding of the pathogenesis of chronic liver diseases and have presented opportunities for novel therapeutic approaches for the management of liver fibrosis.

This review presents key advances in the new insights into the cellular and molecular mechanisms that regulate liver fibrosis, which may represent future therapeutic targets.

ECM IN LIVER FIBROSIS

During chronic liver injury, an increase of fibril-forming collagen and the replacement of the low density, basement membrane-like interstitial matrix occurs[4,6,7]. There is also an accumulation of other matrix proteins, including elastin, hyaluronan, proteoglycans and fibronectin. This type of matrix has the capacity to activate quiescent HSCs, leading to the loss of hepatocyte microvilli and the disappearance of endothelial fenestrations (Figure 1)[4,7,8]. This architectural change of endothelial cells also impairs the transport of solutes from the sinusoid to the hepatocytes, further contributing to hepatocyte dysfunction[7]. Moreover, the accumulation of ECM itself provokes positive feedback pathways that further amplify fibrosis[8]. The alteration of ECM proteins influences cellular behavior *via* cell membrane receptors. The most potent proteins are integrins that permit communication between the ECM and the cytoskeleton[9-11]. Patsenker *et al*[11] demonstrated that the inhibition of integrin alpha-V-beta slows the progression of biliary fibrosis and suggested that this inhibition could have potential therapeutic utility.

ECM remodeling is critical in the preservation of homeostasis during liver injury. This homeostasis depends on the fine balance between matrix metalloproteinases (MMPs) and their inhibitors, tissue inhibitors of matrix metalloproteinases (TIMPs). While the excessive increase in the ECM is controlled by MMPs (especially MMP-1, 2, 8 and 13), progressive fibrosis is correlated with the marked increase of TIMPs (TIMP-1 and TIMP-2)[12,13]. Moreover, because TIMP-1 has also anti-apoptotic effects on HSCs, it induces fibrogenesis by promoting fibrogenic cell survival. Several studies have reported that the regulation of TIMPs in HSCs may accelerate the elimination of fibrotic liver tissue and the reversal of fibrosis[14,15]. Enhancing the degradation of excess ECM by increasing the activity of MMPs or decreasing that of TIMPs is an additional approach in the development of antifibrotic drugs.

Angiogenesis is another response to chronic liver injury that leads to sinusoidal remodeling and pericyte amplification[16-18]. Consequently, many potent angiogenic mediators are involved in the exaggerated wound healing response to chronic liver injury, leading to an excessive accumulation of ECM[17,18]. The ECM can also affect cell function indirectly by releasing cytokines. These include transforming growth factor β (TGF-β), platelet derived growth factor (PDGF), hepatocyte growth factor (HGF), connective tissue growth factor (CTGF), tumor necrosis factor-α (TNF-α), basic fibroblast growth factor (bFGF) and vascular endothelial growth factor (VEGF)[19].

CELL TYPES INVOLVED IN THE PATHOGENESIS OF LIVER FIBROSIS

Although the cellular source of ECM components in fibrotic liver has been a matter of controversy for many years, recent investigations have revealed that ECM accumulation during chronic liver injury is driven by a heterogeneous population of cells. Currently, it is accepted that liver fibrogenic cells (myofibroblasts) play a central role during liver fibrosis. Their origin has been extensively studied, and several sources of myofibroblasts (MFs) have been identified[3,20-27]. Because HSCs are the main ECM-producing cells in the injured liver[20] they are currently considered to be the major source of MFs[3,20-22]. Hepatic MFs may also originate from portal fibroblasts and bone marrow derived mesenchymal cells[24,28]. Two other minor contributors of fibrogenic cells are the epithelial-mesenchymal transition (EMT)[29,30] and endothelial to mesenchymal transition (Figure 2)[31,32].

HSCs

Activation of HSCs is recognized as a central event during liver fibrosis, and the molecular mechanisms of this cellular alteration continue to attract increasing attention, creating many new findings[33,34]. However, there is limited knowledge about HSC activation from the viewpoint of cell fate or lineage regulation[35-37]. Recently, many studies have shown that HSCs are derived from mesodermal-derived multipotent mesenchymal progenitor cells (MMPC), which also give rise to neural cells and other mesenchymal cells[38,39]. Supporting these findings, HSCs also express neural and mesenchymal lineage markers. Because cell types derived from MMPC may undergo transdifferentiation within their lineages, the notion that HSC transdifferentiation may reside in these mesenchymal lineages is reasonable[39]. In normal liver tissue, HSCs exist in a quiescent state, storing retinoids and synthesizing glial fibrillary acidic protein (GFAP)[40-43]. Following liver injury, HSCs are activated with a gradual loss of retinoids and GFAP, leading to a reduction in the expression of adipogenic/lipogenic factors. Meanwhile, a complex network of autocrine/paracrine fibrogenic signals promotes the transdifferentiation of HSCs to a myofibroblastic phenotype.

Portal fibroblasts

Portal fibroblasts are spindle shaped cells of mesenchy-

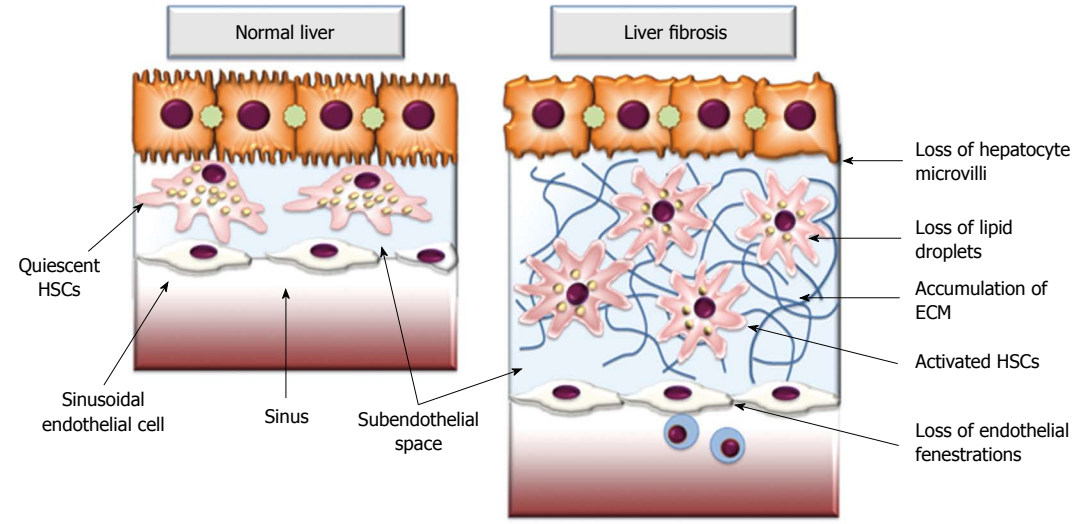

Figure 1 Extracellular matrix accumulation in subendothelial space activates quiescent hepatic stellate cells leading to the loss of hepatocyte microvilli and disappearance of endothelial fenestrations. These architectural changes impair transport of solutes from the sinusoid to the hepatocytes, further contributing to the hepatocyte damage. ECM: Extracellular matrix; HSCs: Hepatic stellate cells.

mal origin that undergo myofibroblastic differentiation, primarily in biliary and cholestatic liver injuries[44-46]. Although they possess biological similarities with activated HSCs, portal fibroblasts have different genetic profiles and signaling responses[45,46]. The latter could enable the development of disease specific antifibrotic therapies targeting these cells.

Fibrocytes

Fibrocytes originate from hematopoietic stem cells and have the ability to differentiate into MFs. In cases of tissue damage, fibrocytes proliferate and migrate to the injured organ and secrete growth factors that promote deposition of the ECM[47-49]. Several studies have suggested that the extent of fibrocyte differentiation into MFs depends on the organ and the type of injury[48,49]. Other studies have demonstrated that liver injury induces migration of fibrocytes to lymphoid organs[49], suggesting that the function of these cells may not be limited to ECM deposition.

Bone marrow-derived MFs

A fraction of hepatic MFs can also arise from bone marrow-derived mesenchymal stem cells (MSCs), which are defined as multipotent progenitor cells with the capacity to differentiate into lineage-specific cells[44,48,49]. Currently, it is not clear whether circulating MSCs significantly contribute to ECM deposition in the course of liver fibrosis or not, but they most likely represent a population that is distinct from hematopoietic-derived fibrocytes[49].

EMT

EMT is a process during which fully differentiated epithelial cells undergo phenotypic transition to fully differentiated mesenchymal cells. Liver cell culture studies have shown that hepatocytes and cholangiocytes may undergo EMT and acquire mesenchymal features, including FSP-1 expression[50-52]. However, more recent reports provide strong evidence against EMT in the liver as a source of MFs, convincingly arguing for an epithelial origin of ECM-producing cells[52,53].

HSCS IN LIVER FIROSIS

During liver fibrogenesis, parenchymal injury and the resulting inflammatory reaction generate a large panel of signals that stimulate the induction of specific transcription factors and morphogens in quiescent HSCs, thereby initiating the activation and the acquisition of fibrogenic and proinflammatory properties. Sustained activation leads to discrete changes in hepatic stellate cell (HSC) behavior, including proliferation, chemotaxis, fibrogenesis, contractility, retinoid loss and WBC chemoattractant/cytokine release[1]. In these phases there is a release of proinflammatory, profibrogenic and promitogenic stimuli acting in an autocrine and paracrine manner (Figure 3).

ACTIVATION OF HSCS

Activation of HSCs by neighboring cells

In the early stage of injury, all neighboring cell types can contribute to the paracrine stimulation of HSC activation.

Hepatocytes

Hepatocyte apoptosis is a common feature in liver injury. This process is mediated partially by Fas and may also involve TNF-related-apoptosis-inducing ligand (TRAIL)[54-56]. Recent data have shown that the engulfment of the apoptotic bodies of hepatocytes by HSC lines results in a profibrogenic response and activates Kupffer cells[57,58]. A similar profibrogenic response can be observed following disruption of Bcl-xl (an anti-apoptotic mediator) that leads to hepatocyte apoptosis[59,60].

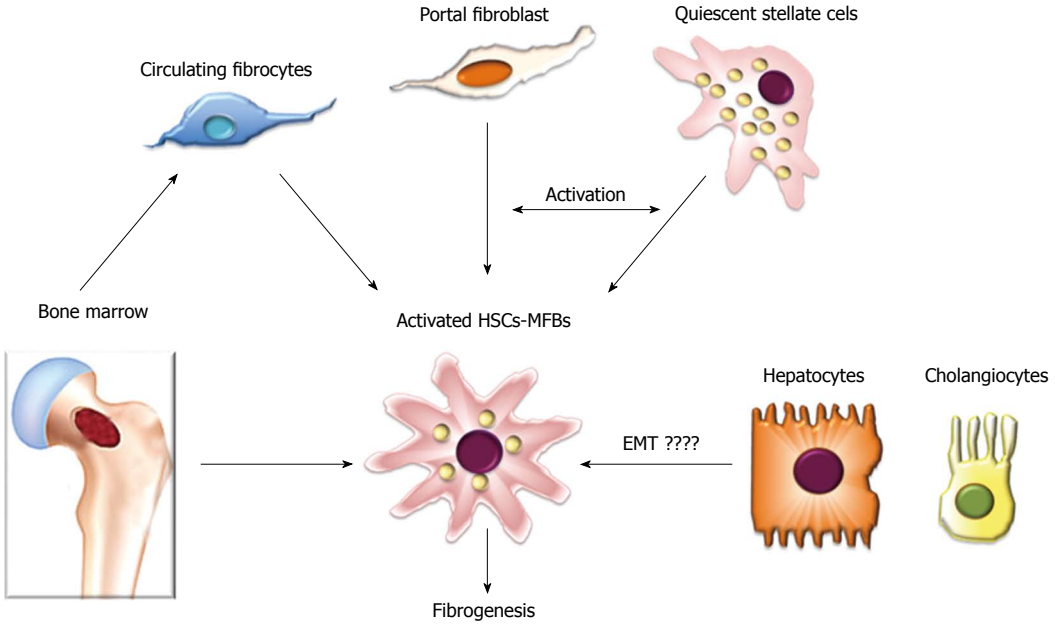

Figure 2 Hepatic myofibroblasts are a heterogenous population of fibrogenic cells. Hepatic stellate cells are considered to be a major source of liver fibrogenic cells followed by portal fibroblasts that play an important role in the fibrogenic process during cholestatic liver diseases. Other sources of hepatic myofibroblasts include circulating fibrocytes and bone marrow-derived cells that constitute a minor proportion of liver fibrogenic cells. The epithelial origin of liver fibrogenic cells is unlikely. EMT: Epithelial mesenchymal transition; MFBs: Myofibroblasts; HSCs: Hepatic stellate cells.

HSC activation by hepatocyte-derived apoptotic bodies is partially mediated by the interaction of hepatocyte DNA with Toll-like receptor 9 (TLR9) expressed in HSCs[61]. Hepatocytes also produce fibrogenic lipid peroxides[62]. Experimental studies have demonstrated that either blockage of hepatocyte apoptosis or selective stimulation of apoptosis in HSCs could be a therapeutic strategy for the prevention of fibrosis[63-66]. However, this approach has not been successful in clinical trials[26].

Liver sinusoidal endothelial cells

In response to injury, sinusoidal endothelial cells contribute to HSC activation, owing to their capacity to produce fibronectin, TGF-β1 and PDGF[67]. Conversely, recent data indicate that restoration of liver sinusoidal endothelial cell differentiation may contribute to fibrosis regression by promoting HSC quiescence[68-70]. It has been proposed that a loss of endothelial fenestration following injury leads to changes in liver sinusoidal endothelial cell differentiation and, consequently, HSC activation[3].

Kupffer cells

Kupffer cells and infiltrating monocytes express a number of chemokine receptors that influence fibrosis progression and resolution[71-74]. Indeed, different macrophage subsets have been described in experimental models; however, their molecular profile is incomplete and additional studies are warranted[74-78]. To date, profibrogenic macrophages have been shown to have high Gr1 (Ly6c) expression and to activate HSCs[74,78]. Additionally, another subset of monocytes (Gr1Io) is vital for fibrosis regression[79,80].

Lymphocytes

Lymphocytes, especially CD4 T-helper lymphocytes, may activate HSCs *via* cytokine production. Previous experimental models imply that during liver injury Th2 lymphocytes, a subset of T-helper lymphocytes, are more fibrogenic as compared to the Th1 lymphocytes subset[81,82].

Natural killer cells

Recent findings indicate that natural killer (NK) cells inhibit liver fibrosis by directly killing activated HSCs[83-86]. In cases of liver injury, NK cells induce apoptosis of HSCs by IFN-γ. Moreover, IFN-γ not only inhibits HSC activation directly but also amplifies NK cell cytotoxicity against HSCs *via* upregulation of NKG2D (best defined natural cytotoxicity receptor) and TRAIL expression on NK cells[87-90]. It has been shown that HSCs in the early stages of activation are more prone to be killed by NK cells than quiescent or fully activated HSCs, because they still produce retinoic acid that is important in the induction of NK cell-activating ligands (MICA in humans)[91]. Thus, activation of NK cells could be a novel, therapeutic target to treat liver fibrosis[91,92]. It should be noted that another T cell subset, NKT cells, has diverse effects on liver fibrosis depending on the stage of the disease[91-93].

Leukocytes recruited to the liver during injury produce compounds that modulate HSC behavior. Neutrophils are an important source of reactive oxygen species (ROS) that also produce nitric oxide (NO), which may counteract the effect of superoxide on collagen production[94,95].

Platelets that produce TGF-β1, PDGF and epidermal growth factor (EGF) are also an important source of

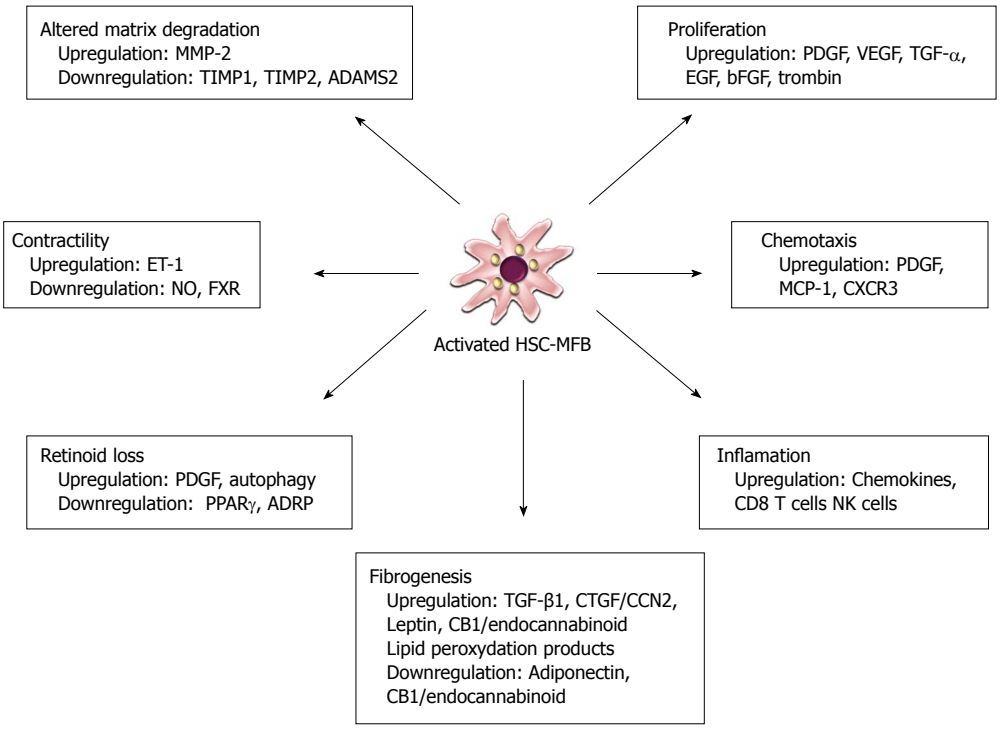

Figure 3 Hepatic myofibroblasts myofibroblasts have multiple functions during liver fibrogenesis. In the activated form, hepatic stellate cells show *de novo* properties, including increased proliferation, fibrogenesis, contractility, chemotaxis, matrix degradation, retinoid loss and secretion of chemokines. Each of these properties is controlled by the release of many cytokines acting in an autocrine and paracrine manner offering many potential sites for therapeutic intervention. MMP: Matrix metalloproteinase; TIMP: Tissue inhibitor of matrix metalloproteinase; ADAMS2: A disintegrin and metalloproteinase 2; PDGF: Platelet derived growth factor; VEGF: Vascular endothelial growth factor; TGF-α: Transforming growth factor-α; EGF: Epidermal growth factor; bFGF: Basic fibroblast growth factor; TGF-β1: Transforming growth factor-β1; CTGF/CCN2: Connective tissue growth factor; ET-1: Endothelin 1; NO: Nitric oxide; FXR: Farnesoid X receptor; PPARγ: Peroxisome proliferators activated nuclear receptorsγ; ADRP: Adipose differentiation related protein.

paracrine stimuli in HSC activation and fibrogenesis[96-98].

Molecular activation of HSCs

ROS: ROS that are generated through lipid peroxidation have the ability to activate HSCs and stimulate the progression of fibrosis[99,100] They can originate from hepatocytes, macrophages, cholangiocytes and inflammatory cells[99,100]. Moreover, ROS can also be produced by HSCs in response to several fibrogenic mediators, such as PDGF, TGF-β leptin and Angiotensin Ⅱ[101-104]. Although it has been suggested that the loss of antioxidant capacity in activated HSCs amplifies the effects of lipid peroxidation products, more recent studies have indicated that activated HSCs have an increased ROS-detoxifying capacity compared to quiescent HSCs[62,105-107]. It has also been demonstrated that increased glutathione levels and hydrogen peroxide detoxifying enzymes protect HSCs from ROS-induced necrosis and apoptotic cell death, respectively[107]. Because ROS can activate signal transduction pathways and transcription factors, including JNK and NFκB, they also upregulate the expression of fibrosis-associated genes, including COL1A1, COL1A2, MCP1 and TIMP1 in HSCs[102-104]. At the cellular level, ROS are generated *via* mitochondrial damage, mitochondrial transport chain or *via* activation of cytochrome P450 (especially cytochrome P450 2E1), xanthine oxidase and NADPH oxidase[108]. It has been demonstrated that, through the induction of oxidative stress, homologs of NADPH oxidase (NOX) might contribute not only to HSC activation but also to the activation of Kupffer cells and macrophages[109]. More recently it has been shown that the phagocytic NADPH oxidase NOX2 is expressed in HSCs and its activation leads to the induction of fibrogenic cascades[110,111]. Angiotensin Ⅱ-mediated induction of NOX1 was also described as profibrogenic[111,112]. In a recent study, Jiang *et al*[113] demonstrated that NOX4 plays an important role in ROS production and HSC activation. They proposed that inhibition of NOX4 might be a promising new strategy for translational trials in liver fibrosis. The cytochrome P450 2E1 (CYP2E1) may also contribute to activation of HSCs *via* the generation of ROS. In the presence of cells that express CYP2E1 (E47 cells), the production of collagen by HSCs is increased[114,115]. Conversely, in the presence of antioxidants or a CYP2E1 inhibitor the increase in collagen production is blocked, suggesting that the CYP2E1 derived ROS are responsible for the increased collagen production[115].

Because ROS constitute a heterogeneous group of species with widely varying chemical reactivity and biological properties, the blockade of oxidative stress as a therapeutic target is still under investigation. Early results demonstrated that the use of an antioxidant mitoquinone might decrease liver inflammation possibly through the induction of the antioxidant transcription factor Nrf2[116].

In a more recent study, chloride channels that are involved in HSC activation by superoxide anion radicals were proposed as a potential target for new anti-fibrotic drugs[117].

Toll-like receptors: Toll-like receptors (TLRs), receptors for microbial products, are present in HSCs and Kupffer cells, introducing a role of immunity in HSC activation and hepatic fibrosis. In chronic liver diseases, increased intestinal permeability results in an enhanced portal inflow of gut-derived microbial products, lipopolysaccharides (LPS), bacterial DNA, peptidoglycan and viral and fungal components[118]. The impact of intestinal decontamination on liver fibrogenesis has been reported. Parallel to this data, mice with a knockout of TLR4 (the LPS receptor), TLR2 and TLR9 were shown to be protected from liver fibrosis[118]. The stimulation of HSCs by LPS or bacterial products through TLR4, TLR9 and TLR2 has been shown to induce a proinflammatory response[118,119]. The activation of HSCs in response to LPS and its receptor TLR4 may elicit a fibrogenic response by down-regulating a transmembrane suppressor of TGF-β-1, BAMBI[119-121]. By contrast, it has been indicated that in addition to LPS (exogenous ligand) TLR4 signaling may also be activated by endogenous ligands from cellular compartments that are released and/or increased during tissue injury, including high mobility group box 1 protein (HMGB1)[122,123]. This chromatin-associated, highly conserved nuclear protein has been shown to be upregulated during liver fibrosis. *In vitro* studies have demonstrated that HMGB1 activates TLR4 signaling in HSCs to enhance their inflammatory phenotype, indicating that TLR4 signaling need not rely solely on gut-derived LPS for activation during liver injury[123]. HMGB1 also has a synergistic effect with TGF-β1 to stimulate fibrogenic protein expression, which is likely to be TLR4-dependent[123]. It has been suggested that inhibition of HMGB1 and TLR4 signaling activity may therefore be important targets of antifibrotic therapy, warranting further investigation by *in vitro* and *in vivo* studies[122,123].

Gene regulations in activated HSCs

There are countless changes in gene transcription that may take place after HSC activation. Among the many target genes of transcription factors described in HSCs include: Type 1 collagen, α-SMA, TGF-β-1, TGF-β receptors, MMP-2, TIMPs 1 and 2[124-126]. The transcription factors that activate these downstream targets are Ets-1, Mef2, CREB, Egr-1, Vitamin D receptor, Foxf1, JunD and C/EBPβ[127].

HSCs also express many nuclear receptors, such as the retinoid responsive RxR and RAR, the farnesoid X receptor (FXR), the pregane X receptor (PXR) and peroxisome proliferators-activated nuclear receptorsγ (PPARγ)[128-130]. While RXR an FXP suppress collagen production, PXR is activated by steroids and antibiotics, dimerizes RXR to induce cytochrome p450 and thereby induces fibrosis[128]. By contrast, PPARγ down-regulates HSC activation and reduces collagen production[128-130].

MicroRNAs: Micro RNAs (mi-Rs) regulate posttranscriptional gene repression by decreasing target mRNA levels. Many mi-Rs are expressed in HSCs and control fibrosis progression[131] including mi-R29, mi-R19b and miR 221/222, among others[132-134]. Based on gene array analysis, mi-R29, which is a physiological inhibitor of various ECM proteins, including collagens, is down regulated by TGF-β and LPS in cultured HSCs[132,133]. MiR-19b is an inhibitor of TGF-β signaling and its expression is decreased in patients with advanced fibrosis, while its overexpression in HSCs blocks activation[132]. In contrast, miR-221/222 is upregulated in human livers in parallel with progression of liver fibrosis. Its expression also increases during HSC activation, and its contribution to HSC proliferation has been proposed[134].

DNA methylation and histone modifications: DNA methylation of genes expressed in quiescent HSCs contributes to the maintenance of the quiescent phenotype. During activation, HSCs express DNA-methyl binding proteins (MeCP2). These proteins promote the silencing of antifibrogenic genes and increase the expression of histone methyl transferases, leading to enhanced transcription of collagen, TIMP-1 and TGF-β[135-137].

It is noteworthy that epigenetic changes can also modulate fibrosis susceptibility[136]. In a recent study, offspring from the progeny of male fibrotic rat ancestors are found to be more resistant to liver fibrosis than their counterparts with no previous history of fibrosis[137]. In experimental models, DNA methylation and histone acetylation in the sperm of rats with fibrosis may also take place in the resistance to the wound healing process, leading to hypomethylation of the PPARγ gene, resulting in elevated hepatic expression of this antifibrogenic transcription factor in adult offspring[137].

PROLIFERATION OF HSCS

The most potent mitogen in HSCs is PDGF. Other mitogens that stimulate HSC proliferation are VEGF, thrombin and its receptors, EGF, TGFα and bFGF[3,104]. Downstream pathways in HSCs include PI3 kinase and ERK/MAP kinase, among others[104,138]. PDGF signaling at the cell membrane of HSCs can also be enhanced by a co-receptor, neuropilin-1[139]. In addition to its mitogenic effect, PDGF also stimulates Na^+/H^+ exchange, providing a potential site for therapeutic intervention by blocking ion transport[140]. Signaling pathways for these mitogens have been clearly identified in HSCs, offering many potential sites for therapeutic intervention[141,142].

CHEMOTAXIS OF HSCS

HSCs can migrate towards many chemokines, including VEGF, PDGF, MCP-1, CXCR4 and CXCR3[3]. For example, CCR5 and its ligand RANTES stimulate the mi-

gration of HSCs[143]. Hypoxia is another activator of HSC migration. In hypoxic conditions the motility of HSCs is not only induced by ROS but also by VEGF in an autocrine manner because prolonged hypoxia induces HSCs to produce and secrete VEGF in an HIF-1α-dependent manner[144].

The role of ECM in migratory behavior of HSCs has been previously described. Additionally, cellular fibronectin containing an alternatively spliced domain A (EⅡA) has been shown to induce motility of HSCs, supporting the role of ECM in HSC behavior[145].

Interestingly, while adenosine blunts chemotaxis and fixes cells at sites of injury *via* the loss of actin fibers, enhanced adenosine signaling may also stimulate HSC fibrogenesis[146,147]. Therefore, understanding the dual role of adenosine will be important in the development of antifibrotic agents. Recent epidemiologic studies demonstrated that caffeine exerts its protective effect by inhibiting adenosine signaling in HSCs[148,149].

HSCS IN FIBROGENESIS

Production of the ECM, in particular collagen type Ⅰ, is a major characteristic of HSCs. The expression of collagen type Ⅰ in HSCs is regulated posttranscriptionally by multiple stimuli and pathways. Prominent among these is TGF-β, the most profibrogenic cytokine in the liver[150,151]. TGF-β is produced by Kupffer cells, liver sinusoidal endothelial cells, hepatocytes and HSCs and has paracrine/autocrine effects on HSCs[150,151]. It has three major isoforms: TGF-β1, TGF-β2 and TGF-β3. In addition to its role in the stimulation of collagen type Ⅰ, TGF-β also stimulates the production of other matrix components, including cellular fibronectin and proteoglycans[150,151]. Although none appears to be as potent as TGF-β, a variety of other factors have profibrogenic effects on HSCs, including retinoids and angiotensin Ⅱ[103,152]. TGF-β1 is stored as an inactivated protein and, when activated, signals *via* its receptors to Smad proteins, which enhance the transcription of target genes, such as procollagens Ⅰ and Ⅲ[150,151]. The response of SMADs in HSCs differs between acute and chronic injury to further favor matrix production[151]. Because TGF-β1 may also contribute to liver homeostasis during regeneration, therapeutic antagonization of TGF-β1 is challenging[153].

Connective tissue growth factor (CTGF/CCN2) is a growth factor protein that is upregulated by hyperglycemia, hyperinsulinemia and alcohol-induced cellular injury[154,155]. While the stimulation of CTGF/CCN2 in hepatocytes is TGF-β dependent, this stimulation in HSCs is independent of TGF-β, highlighting the fact that, in exception to the general rule, cytokine signaling in HSCs is not always autocrine[156].

Adipokines are polypeptides mainly secreted in adipose tissue and, to lesser extent, by stromal cells. In the liver, they not only contribute to the hepatic manifestation of obesity but are increasingly recognized as key mediators of liver fibrogenesis. Leptin, adiponectin and ghrelin are the main adipokines that contribute to liver injury[157-161]. Leptin is an adipogenic hormone that promotes HSC fibrogenesis and activates Kupffer cells, macrophages and endothelial cells to produce TGF-β1[162]. It modulates the HSC phenotype through the leptin receptor (OB-R), which leads to stimulation of the Janus kinase 2 (JAK 2) and signal transducer and activator of transcription 3 (STAT 3) pathways[157]. Leptin also partially suppresses PPARγ, which can reverse HSC activation and maintain senescence[163]. Recently, it has been demonstrated that leptin deficiency may reduce the activity of norepinephrine, thereby reducing fibrogenesis[164]. Reduced activity of norepinephrine leads to decreased activity of NK cells and attenuates the release of profibrogenic cytokines and reduces ECM production[164]. Adiponectin, a counter-regulatory hormone of leptin, inhibits hepatic fibrogenesis both *in vivo* and *in vitro*[160,162]. Ghrelin also appears to attenuate hepatocellular damage and fibrosis in experimental studies[161].

Neurochemical and neurotrophic factors also contribute to the fibrogenic function of HSCs. Following liver injury, activated HSCs express specific receptors (CB1 and CB2) that are components of the endocannabinoid system that regulates the fibrogenic cascade[165-168]. Two receptors exert opposing effects; while CB1 stimulation induces fibrogenesis, the stimulation of the CB2 receptor is anti-fibrotic and hepatoprotective[165-167]. The overexpression of these receptors is observed both in experimental models of liver fibrosis and in the livers of patients with chronic liver disease[165,167]. Therefore, efforts for therapeutic strategies are being directed to either antagonize CB1 or agonize CB2. Non-brain penetrant CB1 antagonists have shown promising results in experimental models[168]. Similarly, opioids that contribute to fibrogenesis by stimulating HSCs can be antagonized by naltrexone[169,170]. Serotonin and thyroid hormones are also involved in fibrogenesis, with agonists or antagonists for these mediators already in existence[41,171].

CONTRACTILITY OF HSCS

Activation of HSCs is accompanied by an increase in expression of proteins characteristic of contractile cells[172]. In the process of becoming contractile, HSCs develop an increased expression of the cytoskeletal protein α-smooth muscle actin (α-SMA)[172]. It has also been reported that HSC contraction is mediated by both Ca^{2+} dependent and Ca^{2+} independent mechanisms[173,174]. Contractility of HSCs has a multitude of effects in the injured liver, including perisinusoidal constriction and portal hypertension, leading to an increase in portal resistance during liver fibrosis[174]. Contractile HSCs impede portal blood flow by constricting sinusoids and by contracting the cirrhotic liver[174-177]. This contractility is likely associated with multiple different systems, including endothelin-1. Endothelin-1 receptors are expressed in both quiescent and activated HSCs[176]. Nuclear receptor FXR antagonizes endothelin 1[176]. There is a shift in the

predominant type of endothelin receptor and increased sensitivity to endothelin-1 after activation of HSCs[178]. The effect of endothelin-1 may also be reversed by locally produced vasodilator substances; particularly, nitric oxide (NO) may counteract the constrictive effects of endothelin-1[179]. Similarly, carbon monoxide also mediates sinusoidal dilatation[179].

RETINOID LOSS OF HSCS

Retinoid is stored as retinyl esters in the form of perinuclear droplets in the cytoplasm of quiescent HSCs. Activation of HSCs is accompanied by the loss of these characteristic droplets. The form of retinoid released outside the cell during activation is retinol, suggesting that there is intracellular hydrolysis of esters prior to export[127]. Several nuclear retinoid receptors have been identified in HSCs. Lecithin retinol acetyl transferase (LRAT) catalyzes the esterification of retinol into retinyl ester in liver[180]. In liver injury models, LRAT-deficient animals exhibit increased fibrogenesis in the liver[181]. In contrast, treatment with retinoid acid decrease activation of HSCs by inhibiting TGF-β[182].

PPARs regulate glucose and lipid metabolism[129]. Their expression decreases with the activation of HSCs[128,130]. In contrast, forced expression of PPARγ in activated HSCs inhibits collagen expression, blocks TGF-β1 signaling and increases cytoplasmic lipid droplets[129].

Adipose differentiation related protein (ADRP), an intracellular lipid storage protein, is present in quiescent HSCs and its expression is reduced during HSC activation. ADRP is induced by retinoid exposure, suggesting that ADRP may have a regulatory role between lipid content and cellular activation through an unknown mechanism[183,184].

Because energy homeostasis is maintained through autophagic digestion of lipid droplets in many cells, it has been hypothesized that autophagy drives HSC activation by digesting lipid droplets, thereby providing energy required for the activation process[185,186]. Recent studies have demonstrated that inhibition of autophagy downregulates the fibrogenic properties of HSCs, revealing HSC autophagy as a therapeutic target[185-187].

HSCS IN INFLAMMATION AND WBC CHEMOATTRACTION

HSCs may produce chemokines that amplify inflammatory responses by inducing migration of inflammatory cells[141,188]. Additionally, cell surface expression of chemokines by HSCs promotes ICAM-1- and VCAM-1-dependent adhesion and migration of lymphocytes[189]. Therefore, some of these chemokines are attractive therapeutic targets[188]. The interaction of HSCs with immune cells (especially with T cells) promotes or inhibits their maturation[190]. The results from a recent proteomics analysis supports the immunosuppressive role of activated HSCs[191]. It has been suggested that HSCs also have the capacity to interact with bacterial LPS because they express TLRs[94,118,119].

PATHOGENESIS OF FIBROSIS ASSOCIATED WITH VARIOUS ETIOLOGIES

Alcoholic liver disease

The pathogenesis of liver fibrosis in alcoholic liver disease (ALD) is complex and may be cell specific and controlled through feedback mechanisms and cross-talk between neighboring and distant cells. The development of liver fibrosis in alcoholics has been linked to the oxidation of ethanol to the highly reactive compound acetaldehyde. After alcohol consumption, acetaldehyde stimulates type I collagen synthesis and gene transcription in cultured rat and human HSCs through the activation of protein kinase C (PKC)[192]. Acetaldehyde was also shown to increase NFκB (p65) and its binding to the α2(I) collagen promoter as well as to enhance NFκB by a mechanism dependent on H_2O_2 accumulation[90,193-195]. The activity of cytochrome P450 isoform 2E1 (CYP2E1) is an important source of ROS in alcohol-induced injury. It has been reported that the inhibition of CYP2E1 activity prevented the induction of collagen I gene expression in rat stellate cells overexpressing CYP2E1[196]. Oxidative stress also activates c-Jun N-terminal kinase (JNK), a protein that regulates the secretion of proinflammatory cytokines in cultured HSCs[144]. The results of a recent study indicated that butein inhibited ethanol- and acetaldehyde-induced activation of HSCs at different levels, acting as an antioxidant and inhibitor of ethanol-induced MAPK, TGF-β and NFκB/IκB transduction signaling; therefore, butein is a promising agent for antifibrotic therapies[197].

Alcohol inhibits the anti-fibrogenic effects of NK cells by stimulating TGF-β production by HSCs, inducing suppressors of cytokine signaling (SOCS-1) and ROS in hepatocytes, thereby sustaining HSC activation and reducing HSC apoptosis[90,198]. Recently, it has been suggested that alcohol increases the binding of the early growth response-1 (Egr-1) transcription factor to the TNF-α promoter and enhances macrophage sensitivity to LPS in the progression of liver injury to fibrosis[199]. Recent discoveries have revealed that alcohol inhibits PPARα, suppressing sterol-regulatory element binding protein-1 (SREBP-1), which is involved in fatty acid synthesis, leading to the activation of HSCs and ultimately fibrosis[90,200]. Other recently identified novel molecules and physiological/cell signaling pathways include hedgehog (Hh) signaling, fibrinolysis and involvement of novel cytokines such as osteopontin. Alcohol increases liver progenitor cell accumulation by providing an increase of Hh and Hh ligands in an autocrine manner[201]. Osteopontin (OPN), which is secreted by several cell types in the presence of alcohol, activates NFκB and activator protein 1 (AP-1) as well as several other genes, including urokinase plasmino-

gen activator (uPA), MMPs and TGF-β[202,203]. Moreover, the profibrogenic plasminogen activator inhibitor (PAI-1) was increased in liver cells after alcohol consumption, leading to the inhibition of uPA, plasmin and fibrinolysis, thereby tipping the balance in favor of fibrosis[204].

Non-alcoholic fatty liver diseases and non-alcoholic steatohepatitis

Although the role of HSC activation in non-alcoholic fatty liver disease (NAFLD) has not been completely clarified, several studies have reported increased HSC activation in non-alcoholic steatohepatitis (NASH)[205]. Although the TGF-β signaling pathway plays a major role in the activation of HSCs in liver fibrosis, many other signaling pathways are implicated in liver fibrosis in NAFLD, including the hedgehog (Hh), PI3K/AKT and JAK/STAT signaling pathways[206].

Several studies have demonstrated that insulin resistance is associated with advanced stages of fibrosis in NAFLD[206,207]. Because insulin promotes HSC activation and insulin sensitizers can attenuate hepatic fibrosis in NASH, it has been suggested that insulin resistance plays an important role in NASH-related fibrogenesis[208,209].

It is understood that oxidative stress induces the activation of HSCs in NASH[108]. The role of oxidative stress in fibrogenesis is supported by the finding that antioxidants, such as vitamin E and astaxanthin, can decrease NASH-related fibrogenesis[210].

Recently reported data also indicate that adipokines affect not only lipid metabolism but also inflammatory and fibrotic processes in NAFLD[157] (the adipokines are described in more detail in the section "HSCs in fibrogenesis"). Recent data related to the newly described adipokines visfatin, chemerin and vaspin in NASH fibrogenesis is limited, warranting further studies to better understand their importance in the pathogenesis of NASH[211,212].

It has been hypothesized that various factors might contribute to the development of liver fibrosis in NAFLD, including LPS-derived from gut bacteria. Because LPS presents its effects by binding TLRs and because a recent finding in a murine NAFLD model demonstrated that TLR9 knockout mice demonstrate less steatohepatitis and liver fibrosis than controls, a role for TLRs in the progression of fibrosis of NASH have been proposed[213]. Recently, it has also been suggested that NK cells may play a pivotal role in NAFLD-related liver fibrogenesis. Although the population of hepatic NK cells in NAFLD patients is controversial, it has been shown that activation of the Hh pathway lead to hepatic accumulation of NK cells, resulting in progression of liver fibrosis in NASH[214].

In experimental studies as well as studies in patients with NASH, PPARγ agonists and especially pioglitazone have been shown to diminish liver fibrosis[209,215]. These data support the key role of PPARs in fibrosis in NASH. Among the other nuclear receptor family, liver X receptors (LXRs) play important roles in the regulation of cholesterol absorption, efflux, transport and excretion. In a more recent experimental study LXR ligands were found to suppress the activation of HSCs and the expression of fibrosis related genes[216].

Chronic viral hepatitis

During liver fibrogenesis, hepatotrophic viruses can induce HSC activation through several mechanisms. Immune cell types, especially NK cells, are engaged in the hepatitis B virus (HBV)-related acceleration of fibrosis[217]. It has been demonstrated that hepatitis B virus X protein (HBx) expression in hepatocytes leads to paracrine activation and proliferation of HSCs[218]. Moreover, in patients with chronic HBV, superinfection of hepatitis delta virus (HDV) accelerates the progression of fibrosis. The large isoform of hepatitis delta antigen (LHDAg) can induce liver fibrosis through the regulation of TGF-β-mediated signal transduction. LHDAg synergistically activates HBx protein-mediated TGF-β and AP-1 signaling, enhancing the level of TGF-β-induced PAI-1[219].

It has been found that the biology of activated HSCs is modulated by hepatitis C virus (HCV)-derived proteins in a profibrogenic manner[220]. Recent findings indicate that both oxidative stress and mitochondrial dysfunction are related to HCV pathogenesis. The blockade of oxidative stress as a therapeutic target in patients with HCV hepatitis remains under investigation[221,222]. Recent studies have indicated that hepatic iron accumulation is also correlated with histologic disease severity and with HSC numbers in patients with HCV infection, supporting the assumption that hepatic iron concentration may also influence fibrogenesis[223]. Huang *et al*[224] demonstrated that specific single nucleotide polymorphisms of TLR4 are related to the rate of progression of fibrosis in patients with HCV hepatitis. It has been suggested that this finding presents a link between a genetic marker and disease pathogenesis.

Although a correlation between HCV viral load and the progression of fibrosis has not been demonstrated in HCV hepatitis, HIV RNA levels predict the fibrogenic progression of chronic hepatitis in HCV/HIV-co-infected individuals[225,226]. In contrast, patients infected with HIV alone do not show significant liver fibrosis, indicating that HIV infection is not profibrogenic per se but rather accelerates the fibrogenic process in the presence of hepatic damage induced by hepatotropic viruses[225-227]. A recent, elegant study by Bruno *et al*[228] demonstrated that HIV gp120 modulates HSC behavior, including directional cell movement and expression of proinflammatory cytokines. They concluded that these results identify a direct pathway that most likely links HIV infection with liver fibrosis *via* envelope proteins, presenting new prospective strategies for the management of liver diseases in HCV/HIV-co-infected patients.

CONCLUSION

In conclusion, there have been considerable advances in the understanding of the mechanisms that underlie he-

patic fibrogenesis. A critical event in liver fibrogenesis is that the ECM is a dynamic structure, and even advanced fibrosis may be reversible. Multiple interactions between the ECM, HSCs, endothelial cells and immune cells have been identified. The central event in fibrogenesis appears to be the activation of HSCs, which is a complex process, leading to multiple potential sites for therapeutic interventions. Although specific, effective and safe antifibrotic therapies are not currently available for the identification of potential new therapeutic agents, once available, they will mediate the progression of hepatic fibrogenesis.

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TOPIC HIGHLIGHT

WJG 20th Anniversary Special Issues (11): Cirrhosis

Gallstones in patients with liver cirrhosis: Incidence, etiology, clinical and therapeutical aspects

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Abstract

Gallstones occur in about one third of the patients having liver cirrhosis. Pigment gallstones are the most frequent type, while cholesterol stones represent about 15% of all stones in cirrhotics. Increased secretion of unconjugated bilirubin, increased hydrolysis of conjugated bilirubin in the bile, reduced secretion of bile acids and phospholipds in bile favor pigment lithogenesis in cirrhotics. Gallbladder hypomotility also contributes to lithogenesis. The most recent data regarding risk factors for gallstones are presented. Gallstone prevalence increases with age, with a ratio male/female higher than in the general population. Chronic alcoholism, viral C cirrhosis, and non-alcoholic fatty liver disease are the underlying liver diseases most often associated with gallstones. Gallstones are often asymptomatic, and discovered incidentally. If asymptomatic, expectant management is recommended, as for asymptomatic gallstones in the general population. However, a closer follow-up of these patients is necessary in order to earlier treat symptoms or complications. For symptomatic stones, laparoscopic cholecystectomy has become the therapy of choice. Child-Pugh class and MELD score are the best predictors of outcome after cholecystectomy. Patients with severe liver disease are at highest surgical risk, therefore gallstone complications should be treated using noninvasive or minimally invasive procedures, until stabilization of the patient condition.

Key words: Liver cirrhosis; Pigment gallstones; Cholesterol gallstones; Lithogenesis; Risk factors; Asymptomatic gallstones; Laparoscopic cholecystectomy

Core tip: Gallstones often occur in patients with liver cirrhosis. Their prevalence increases with age and with disease severity. In most cases, stones are of pigment type; in about 15% of cases, they are cholesterol stones. This review presents new data on pathogenesis and risk factors for gallstones in patients with liver cirrhosis. An evidence-based approach to gallstones in these patients is described. Patients with liver cirrhosis and asymptomatic gallstones should be followed-up closely and offered laparoscopic cholecystectomy once symptoms develop. In patients with advanced liver disease, noninvasive or mini-invasive procedures should be used to treat the complications of gallstones.

Acalovschi M. Gallstones in patients with liver cirrhosis: Incidence, etiology, clinical and therapeutical aspects. *World J Gastroenterol* 2014; 20(23): 7277-7285 Available from: URL: http://www.wjgnet.com/1007-9327/full/v20/i23/7277.htm DOI: http://dx.doi.org/10.3748/wjg.v20.i23.7277

INTRODUCTION

Gallstone disease (GD) is a common disease in many parts of the world: gallstones are present in 10%-15% of the population in developed countries. There are two main types of gallstones with regard to the chemical composition: cholesterol and pigment type. Cholesterol gallstones represent the major type of gallstones in developed countries. In many cases, gallstones are mixed,

with the predominance of one or the other component. Pigment gallstones might be black stones (metabolic), in patients with hemolytic conditions, or brown stones (infectious), in patients with biliary infections/infestations.

Liver cirrhosis develops as the end-stage of chronic liver diseases. It is a quite common disease, with a rising prevalence in Western countries[1,2]. This is due to the growing epidemics of obesity and metabolic syndrome, having fatty liver as hepatic expression, and also to the fact that the spread of hepatitis C virus (HCV) infection in the United States and Europe occurred after the 1970s and a long duration of infection is necessary for cirrhosis to develop.

Among the many liver disorders that can lead to cirrhosis, some progress rapidly (years) and others more slowly (decades). Gallstones usually develop after a longer duration of cirrhosis. Gallstone prevalence in patients with liver cirrhosis ranges between 25% and 30%, being at least twice that in the general population.

In this paper we have reviewed the current literature in order to present the mechanisms responsible for the development of GD in patients with liver cirrhosis, as well as the clinical and therapeutical aspects of gallstones formed in this setting.

PREVALENCE AND INCIDENCE

The first data indicating a higher prevalence of gallstones in cirrhotics were derived from necroptic studies[3-7]. Prospective ultrasound studies have later confirmed the higher prevalence[8-13] and incidence[9,14-17] of gallstones in cirrhotic patients. The global cumulative incidence of gallstones was first evaluated in 72 patients followed-up for a mean of 2 years: 12 patients (16.6%) developed gallstones. The cumulative incidence was 5.5 cases/100 cirrhotics/year, and it was higher in advanced (decompensated) cirrhosis, irrespective of etiology[15]. Conte *et al*[17] followed-up 618 cirrhotic patients for almost 4 years, and found that 141 (22.8%) developed gallstones in this period, with an estimated cumulative probability of 6.5%, 18.6%, 28.2%, and 40.9% at 2, 4, 6, and 8 years, respectively. The multivariate analysis confirmed that advanced cirrhosis (Child class B and C) was associated with a greater risk for gallstones in these patients.

Although a number of risk factors for lithogenesis in liver cirrhosis have been identified, there are still aspects insufficiently elucidated. This is why cohort and case-control studies continue to be published[16,18-22], trying to better define the risk factors and the pathogenesis of gallstones in the cirrhotic patients.

GALLSTONE PATHOGENESIS

In most patients with liver cirrhosis, gallstones are of black pigment type[21,23-25]. Liver cirrhosis is considered as the major risk factor for pigment lithogenesis in adults. Only a small proportion of cirrhotic patients harbour cholesterol stones.

The major abnormalities leading to gallstone formation are the changes in bile composition (supersaturation of the bile in calcium bilirubinate for pigment stones, or supersaturation in cholesterol for cholesterol stones), enhanced crystal nucleation in the presence of mucin and its congeners, and gallbladder hypomotility (stasis) that allows crystals to grow into gallstones.

Changes in bile composition

Pathogenesis of black pigment stones: Pigment gallstones invariably contain a mucin glycoprotein matrix ("scaffolding")[26]. Black pigment stones develop in the sterile bile supersaturated in calcium bilirubinate. Supersaturation occurs in the presence of an increased concentration of unconjugated bilirubin or of an increased concentration of free ionized Ca^{2+} in the bile[27-30]. The unconjugated bilirubin fraction represents in physiological conditions less than 1% of the total amount of bilirubin in bile. It increases significantly in case of: (1) increased excretion of unconjugated bilirubin due to defective conjugation or hemolysis; (2) increased hydrolysis of conjugated bilirubin in bile due to enhanced beta-glucuronidase activity; (3) defective acidification of the bile due to mucin hypersecretion, resulting in increased ionization of unconjugated bilirubin and precipitation of Ca^{2+}; (4) decreased solubilization of bilirubinate anions in the presence of reduced bile salt concentration; and (5) induced enterohepatic cycling of unconjugated bilirubin.

Increased hemolysis and/or hydrolysis of conjugated bilirubin in bile lead to a shift in the ratio of bilirubin conjugates in the bile of cirrhotic patients in favour of bilirubin monoconjugates, especially monoglucuronides[24]. Bilirubin monoglucuronide is more easily deconjugated in bile by the β-glucuronidase secreted by hepatic parenchymal[31] or biliary epithelial cells, or is deconjugated through non-enzymatic hydrolysis.

Most mechanisms involved in pigment lithogenesis are also present in liver cirrhosis. A higher prevalence of hypersplenism and hemolysis was found in cirrhotics with gallstones than in those without gallstones[10,25]. Hemolysis could be promoted in advanced liver disease by hypersplenism, Kupffer cell destruction and altered membrane lipid composition.

The very low bile salt/unconjugated bilirubin molar ratio found in cirrhotic patients as compared to controls is an independent physico-chemical factor predisposing to pigment gallstone formation[24]. The reduction of the global bile acid pool size in cirrhotic patients is due to the impaired bile acid synthesis in the liver. Solubilization of the unconjugated bilirubin in bile, which is dependent on its interaction with bile salts, is reduced in liver cirrhosis. Vlahcevic *et al*[32] found a decreased cholic acid, but relatively preserved chenodeoxycholic acid synthesis in cirrhotic patients. They explained the fact that cirrhotic patients form rather pigment than cholesterol stones by demonstrating a reduced secretion of phospholipids and especially of cholesterol in their bile[33].

The decreased biliary secretion of phosphatidylcho-

line and cholesterol diminishes the detergent effect on membranes of the bile salts, potentially leading to bile salt-induced injury of the gallbladder mucosa. This favors pigment lithogenesis not only by production of mucosal β-glucuronidase from the biliary epithelial cells, but also by mucin glycoprotein hypersecretion and possibly by reactive oxygen species (ROS) production[34].

An induced enterohepatic cycling of unconjugated bilirubin, favored by alcohol abuse and low-protein diets[35] might contribute to gallstone formation in liver cirrhosis.

Pathogenesis of cholesterol stones in liver cirrhosis: Cholesterol gallstones occur more rarely in cirrhotic patients. Coelho *et al*[21] in their study on 369 transplant recipients with liver cirrhosis and gallstones observed on direct examination of the explanted livers that 318 patients (86.2%) had pigment stones, and 51 patients (13.8%) had cholesterol stones. The type of gallstones was not evaluated in relation to the etiology of liver cirrhosis. Viral C infection was in 132 patients (33% of the transplanted patients) the cause of liver cirrhosis[21].

Cholesterol gallstones occur in liver cirrhosis mainly in patients with viral C and non-alcoholic fatty liver disease (NAFLD) cirrhosis, and are due to the cholesterol supersaturated bile. Chronic HCV infection seems to be a risk factor for GD in patients with liver cirrhosis: gallstones are more frequent in patients with viral C as compared with viral B or alcoholic cirrhosis[20,36]. An increased incidence of gallstones in subjects with chronic HCV infection was found not only in cirrhosis, but also in the stage of chronic viral C hepatitis[37].

Non-alcoholic fatty liver diseases is associated with an increased prevalence of gallstones[38-40] due to obesity and increased insulin resistance. The risk of GD increases with the severity of liver disease; the highest prevalence of gallstones was found in the more advanced stages of fibrosis (cirrhosis) in NAFLD patients[39].

Enhanced nucleation in bile

Both cholesterol and pigment stones form on a matrix of mixed mucin glycoproteins secreted by the epithelial cells lining the biliary tree. Mucin hypersecretion, favored by gallbladder wall inflammation, accounts for the enhanced nucleation in cirrhotic patients.

Advanced liver disease is associated with a reduced apolipoprotein (apo) A1 and apoAII secretion in alcoholic patients with liver disease[41,42] and also in cirrhosis of other etiology. This might contribute to the enhanced crystal nucleation in cirrhotics' bile, as apo A-I and A-II act as antinucleating factors.

Gallbladder hypomotility

Unlike its contribution to the formation of cholesterol gallstones, the role of gallbladder hypomotility in pigment lithogenesis has longtime been controversial. However, larger fasting gallbladder volumes in patients with liver cirrhosis have been unanimously found[43-45]. Some earlier studies revealed a normal gallbladder emptying in patients with liver cirrhosis[43,44], but later most ultrasonographic[45-49] and scintigraphic[50] studies documented the reduced gallbladder contractility in these patients. The contradictory findings were mainly due to the different test meals used in these studies for evaluating gallbladder emptying.

Changes in the neurohormonal control of gallbladder motility and the structural changes of the gallbladder wall (edema caused by hypoalbuminemia and venous congestion in the context of portal hypertension) might account for the impaired gallbladder emptying in cirrhotics.

The level of circulating CCK is higher in cirrhotic patients than in controls[44,51,52]. This was explained by the impaired hepatic degradation in cirrhosis, as CCK-8 is normally metabolized on its first passage through the liver[53]. In spite of the higher levels of circulating CCK, gallbladder motility is diminished in liver cirrhosis, possibly due to a higher resistance of the gallbladder at the receptor site. Increased plasma concentrations of intestinal peptide hormones that have an inhibitory influence on gallbladder smooth muscle, such as VIP, somatostatin[54], glucagon[55] and pancreatic polypeptide were also detected in liver cirrhosis, as a consequence of their impaired degradation in the liver. The increased levels of relaxing peptides might explain the earlier cessation of the humoral stimulation of gallbladder emptying in cirrhotics: refilling of the gallbladder is more important and starts before complete emptying of the stomach in cirrhotics as compared to controls[45].

Hypocontractility of the gallbladder in patients with liver cirrhosis is proportional with the severity of liver disease, and is more important in cirrhotics with gallstones than in those without gallstones[56]. This suggests that gallbladder stasis might be a contributor to gallstone formation in the advanced stages of cirrhosis.

RISK FACTORS FOR LITHOGENESIS

Age and gender

Diehl *et al*[25], in a clinical study on 551 patients undergoing cholecystectomy, found that patients with pigment stones were older than those with cholesterol stones: most subjects older than 70 years had pigment stones ($P < 0.00001$), and cirrhosis was strongly associated with pigment gallstones. Other studies also found that gallstone prevalence increased with age in cirrhotics[5,10,17,20]. Advanced age was shown to represent also an independent risk factor for gallstone symptom development in patients with liver cirrhosis[57].

Necroptic studies, as well as clinical and ultrasound surveys resulted in contradictory data regarding the gender influence on gallstone formation in cirrhosis. Some studies indicated a higher prevalence in men[3,4,12-14,19], up to a 1/1 female/male ratio. The increased estrogen level in cirrhotic males was suggested to favor gallstone formation[12], but no correlation between presence of clinical signs of hyperestrogenemia in men and the incidence of

gallstones could be demonstrated[3]. In other studies, the female/male ratio was comparable with that of gallstone carriers in the general population[9,10,57]. Even if the prevalence in these studies was higher in cirrhotic females, gallstones were considerably more frequent in the cirrhotic males when compared with control males. Regarding development of symptoms, males seem to have a lower risk (OR = 0.20, P = 0.0049) than female patients with cirrhosis[57].

Family history of GD

GD is a complex disease, resulting from the interaction between environmental factors and numerous genetic influences. The familial aggregation of gallstones supporting the genetic influence on gallstone formation has been known since decades. A large family study, comprising 358 families with 1038 subjects having symptomatic gallstones, suggested that the genetic factors account for at least 30% of the etiology of symptomatic GD[58].

The first human susceptibility genes for cholesterol gallstone formation were recently identified. A genome-wide association study (GWAS) detected a highly significant association of GD with the DH19 polymorphism in the *ABCG8* gene, the gene controlling the cholesterol transporter in the bile[59]. This variant of the *ABCG8* gene was found to be associated with GD in a linkage and association study in siblings with gallstones[60], and it was later confirmed in many populations. An update inventory of human gallstone genes can be found in two recent reviews[61,62].

The Gilbert syndrome variant rs6742078 in the promoter of the *UGT1A1* gene, the gene encoding uridine 5'-diphosphate (UDP)-glucuronyltransferase 1A1 (UGT1A1), that is responsible for bilirubin conjugation, was identified as a candidate gene for GD in a genome-wide association study of Sardinian subjects having increased serum bilirubin levels[63]. This variant promotes formation of pigment gallstones. It was confirmed as a candidate gene for pigment stones in German and Chilean patients[64], especially in men, and was shown to increase the risk not only for pigment gallstones, but for all types of gallstones. The association of the variant of *UGT1A1* not only with the stone bilirubin content but also with the global risk for gallstones confirms the presence of common factors in the pathogenesis of cholesterol and pigment gallstones[64]. An increased gallstone risk was later found in Swedish twins carriers of this variant[65], supporting also the nucleation in the bilirubin supersaturated bile as an initial step in cholelithogenesis. Buch *et al*[64] calculated that the population-attributable fraction of the common *ABCG8* and *UGT1A1* variants in men was 21.2%. If estimated for all European gallstone carriers, this fraction was between 15% and 20%[62].

It would be of interest to evaluate the presence of these variants in patients with liver cirrhosis and gallstones. A genetic susceptibility might represent an independent risk factor for the occurrence of gallstones in cirrhotic patients. It has been already demonstrated that a positive family history of GD increases the risk of developing symptoms in cirrhotic gallstone carriers[57].

Etiology of liver cirrhosis

All prevalence studies agree that the lithogenetic risk in patients with liver cirrhosis is related to the cirrhotic change of the liver, which develops as the end stage of chronic liver diseases of any etiology. However, for some liver diseases, the lithogenetic risk was demonstrated to be higher.

Chronic alcoholism: Friedman *et al*[66] did not find an association between chronic alcoholism and lithogenesis. Trotman and Soloway[67] observed that a history of alcoholism in cholecystectomized patients did not influence gallstone type, cholesterol or pigment. Some clinical studies found a protective effect of alcohol in moderate consumers (39 g/d), suggesting a reduced bile lithogenicity as responsible for this effect[68]. Other studies noted an association between pigment stones and chronic alcoholism without cirrhosis[69], which was explained by the direct effect of chronic alcohol consumption on the liver, bile and red blood cells (macrocytosis) leading to an increased proportion and decreased solubilization of unconjugated bilirubin in the bile. Alcohol consumption was shown in one study to reduce the risk of symptoms in noncirrhotic women with gallstones[70]. In summary, epidemiological studies have been contradictory regarding the protective/favoring effect of alcohol for gallstone formation in patients without liver cirrhosis.

But all studies agree that alcohol-related liver cirrhosis is associated with an increased prevalence of gallstones. And previous alcohol abuse was found to be an independent risk factor for gallstone formation in a prospective follow-up of cirrhotic patients of all etiologies[16]. Regarding the risk for gallstone symptoms, this is significantly lower in patients with alcoholic versus viral cirrhosis (OR = 0.23, P = 0.0116)[57].

Chronic HCV infection: HCV infection represents a major cause of liver cirrhosis in the developed countries, where its prevalence is rising[1]. Some prospective[71] and retrospective[19,72] studies evidenced a higher gallstone risk in patients with chronic HCV infection in the stage of liver cirrhosis. A study derived from a populational survey in the United States (NHANES Ⅲ) found that anti-HCV positive men had a higher prevalence of gallstones than the HCV-negative, and that gallstone prevalence was higher in those with severe disease[35]. Stroffolini *et al*[20] noted a significantly higher prevalence of GD in viral C versus viral B or alcohol-related cirrhosis.

A study performed on 453 patients with chronic HCV-infection (cirrhotics excluded) demonstrated that HCV infection represented an independent risk factor for gallstone formation[37]. Prevalence of GD was higher in patients than in controls, gallstones occurred at a younger age and central obesity and fatty liver (steatosis) were the significant risk factors for their occurrence. A causal

link between chronic HCV infection and GD was thus proved, at least for the subgroup of obese subjects with liver steatosis.

The increased insulin resistance, commonly present in obese subjects and in those with fatty liver disease, could represent the link between chronic HCV infection and cholesterol GD. Increased insulin resistance favors cholesterol lithogenesis *via* increased biliary saturation in cholesterol. Although gallstone composition has not been evaluated in these patients, one can presume that cholesterol stones are the prevalent type in patients with viral C liver disease.

NAFLD: NAFLD is characterized by the fat accumulation in the liver in the absence of alcohol abuse and includes a large spectrum of liver changes, from simple fatty liver to non-alcoholic steatohepatitis (NASH) and liver cirrhosis. Cholesterol GD and NAFLD share many common risk factors, such as insulin resistance, type 2 diabetes mellitus, central obesity, hypertriglyceridemia and metabolic syndrome. These common factors account for the higher prevalence of cholesterol GD in patients with NAFLD[38-40]. Given this frequent association, it was even suggested that routine liver biopsy for diagnosing and staging NAFLD might be justified during cholecystectomy[73].

A recent study by Fracanzani *et al*[39] showed a progressive increase of gallstone prevalence with the severity of fibrosis in NAFLD (P for trend = 0.0001): from a gallstone prevalence of 15% in fibrosis stages 0-2, to 29% in stage 3 and 56% in stage 4 (cirrhosis). Female gender, prediabetes/diabetes, central obesity, older age and metabolic syndrome were significantly more frequent in NAFLD patients with gallstones than in NAFLD patients without gallstones.

Obesity and type 2 diabetes mellitus

Abdominal (central) obesity, type 2 diabetes mellitus and hypertriglyceridemia are risk factors for cholesterol gallstones in the general population, and have also been found to be independent risk factors for GD in patients with liver cirrhosis[13,17,22]. Increased insulin resistance represents the link between these disorders, being also responsible for NAFLD development. The increased gallstone risk in cirrhotics with obesity and type 2 diabetes mellitus might thus mainly refer to patients with NAFLD-induced liver cirrhosis, but to date, no study has evaluated this aspect.

Duration/severity of liver disease

The main determinant for gallstone formation in patients with cirrhosis of the liver appears to be the severity of liver disease. Advanced liver cirrhosis indicates a long duration of the disease. Most authors have shown that prevalence of gallstones was higher in the advanced stages of the disease: in decompensated versus compensated, or in Child C *vs* Child A patients, respectively[4,9,11,12,14,15]. This was confirmed in patients with NAFLD, in whom gallstone prevalence significantly correlated with the severity of liver fibrosis[39].

CLINICAL ASPECTS

Asymptomatic (silent) stones

In most patients, gallstones remain asymptomatic (silent) during the entire life. Gallstones are asymptomatic even if accompanied by dyspeptic symptoms, provided biliary pain is absent. They are often discovered incidentally at abdominal ultrasonography performed for various indications. Epidemiological studies suggest that about 70%-80% of gallstones in the general population are/remain asymptomatic and about 20% will eventually develop symptoms and complications within 5 and 20 years after diagnosis[74,75]. However, a recent large epidemiological study[76] found that in the general population, a significant proportion of cholecystectomies (41.3%) are still performed in asymptomatic patients.

In patients with liver cirrhosis, gallstones are also usually asymptomatic and have more chances to be detected at the periodical check-ups of liver disease by ultrasonography. A higher percentage of cholecystectomies used to be found in cirrhotic patients than in the normal population in the same area[8,18]. In his retrospective study, Maggi *et al*[18] found that only 62% of the cholecystectomized cirrhotics had a history of biliary pain. This could be due either to the detection of unknown latent cirrhosis during cholecystectomy, or because cholecystectomy is more readily recommended in case of altered liver function tests in these patients, presumed erroneously to indicate symptomatic or complicated lithiasis.

The risk of developing symptoms and complications is also low for patients with liver cirrhosis, but it was evaluated only in a few studies.

Symptomatic stones

Gallstones are symptomatic when pain occurs: pain is either colicky or continuous, steady. The simplest definition of biliary pain is that of pain located in the right hypocondrium or epigastrium, which irradiates to the back, occurs (usually, but not always) postprandially, is intense and lasts more than 15-30 min.

In the era before the introduction of laparoscopic cholecystectomy (LC), a small study found that out of 64 patients hospitalized with the diagnosis of liver cirrhosis and having gallstones, 14 (22%) developed biliary complications necessitating cholecystectomy[77]. Fifty patients (78%) remained asymptomatic at a 2-year follow-up. It was concluded that complications of gallstones do not occur more frequently than in gallstone carriers in the general population. In those patients with asymptomatic gallstones who were later submitted to elective surgical treatment for porto-systemic shunt, morbidity and mortality of the associated cholecystectomy did not differ from the rates observed in a group of 170 patients who underwent only portal surgery during the same period. But if complications occurred, emergency operation car-

ried a higher risk in cirrhotic patients. According to this study, the proportion of symptomatic versus asymptomatic gallstones seems to be similar in cirrhotic patients with that in the general population.

In a case-control study of 140 patients with liver cirrhosis and gallstones, both symptomatic and asymptomatic, the univariate analysis showed that advanced age, female gender, positive family history of gallstones, viral etiology of cirrhosis and duration of cirrhosis were significantly associated with the symptomatic stones[57]. In the multivariate analysis, only family history of gallstones and advanced age were independent risk factors for symptom development. Male gender and alcoholic etiology of cirrhosis negatively correlated with symptom presence, suggesting their protective effect on symptom development.

Recognizing the risk factors for symptoms is a very important issue for the medical decision. Once symptoms appear, patients are at risk for pain recurrence and complications. When symptoms do occur, morbidity and mortality are higher than in noncirrhotic patients. Early cholecystectomy in patients with surgical risk, as soon as the first symptoms occur, could avoid emergency surgery for complications in a more advance stage of liver disease.

TREATMENT

Expectant management for asymptomatic stones

Expectant management (observation alone) is the appropriate recommendation for patients with asymptomatic gallstones in the general population, due to the low risk to develop symptoms and/or complications. Cholecystectomy is not only an expensive procedure, but it carries a risk, even low, of morbidity and mortality in patients who might otherwise never develop symptoms or complications.

The same recommendation should be made for the cirrhotic patients with silent gallstones. Asymptomatic gallstones in cirrhotic patients are best managed conservatively, with close monitoring and surgery if symptoms or complications occur. Given the higher risk for surgery in the presence of advanced liver disease, the management options should be discussed with the patients when gallstones are diagnosed, and they should be actively involved in the process of therapeutic decision.

Prophylactic cholecystectomy

For the time being, there are no published randomized trials to evaluate the better approach for patients with silent gallstones: cholecystectomy or expectant management[78]. Prophylactic cholecystectomy is generally not recommended for gallstone carriers in the general population, except for some special situations. It should be also not recommended in cirrhotic patients given their higher surgical risk as compared to patients without liver disease. The management of asymptomatic gallstones found incidentally in these patients during abdominal surgery for another indication is controversial. Concomitant cholecystectomy might be a reasonable option for patients with well compensated cirrhosis undergoing elective abdominal surgery for other conditions[77]. However, in a small study on 34 patients with liver cirrhosis, all patients, even those in Child A or B class, who underwent additional cholecystectomy during the non-shunting operation for esophageal varices required blood transfusion[79].

Laparoscopic or open conventional cholecystectomy

Before the introduction of laparoscopic cholecystectomy (LC), the postoperative mortality in patients with cirrhosis undergoing conventional open cholecystectomy (OC) was between 7.5% and 25.5%[77,80-82]. As expected, patients with the most severe liver disease were at the highest risk. And although at the beginning of the 1990s it was already documented that LC had important advantages over OC when considering hospital stay and morbidity of gallstone patients, a consensus statement on LC in 1992 stipulated that patients with end-stage cirrhosis of the liver and with portal hypertension were not candidates for LC.

One year later, a first study was published regarding the outcome of LC in cirrhotic patients[83]. Lacy *et al*[84] reported a 9% conversion rate, no morbidity and an average hospital stay of patients of less than 2 d. Case series, case-control studies[85] and meta-analyses[86-90] were thereafter published. The first meta-analysis published in 2003 by Puggioni *et al*[86] showed that LC offered the advantages of less blood loss, shorter operative time, and shorter length of hospitalization in patients with cirrhosis. Further meta-analyses and randomized controlled trials (RCTs) confirmed that shorter operative time, reduced hospital stay, rapid recovery, reduced complications rate[86,89,90] and earlier resumption of a normal diet[90] were the most important advantages of LC in patients appropriately selected. The Child-Pugh classes and especially the MELD score were established as the best predictors of a better outcome after LC[88,91].

Patients with advanced cirrhosis (Child-Pugh class C) remain at significantly higher risk of complications and mortality for cholecystectomy. Progress in the surgical equipment, better therapy for liver failure, and multiplication of surgical options, including laparoscopic cholecystostomy and percutaneous transhepatic cholecystostomy have increased the safety of the intervention also for these patients. A recent systematic review and meta-analysis comparing LC and OC, which included all published RCTs and a total of 2005 cirrhotic patients undergoing cholecystectomy, showed that the mortality rates reported for both LC and OC were "substantially lower than those reported for OC in the 1980s"[89], acknowledging the progress in the management of these patients in the last two decades. Laparoscopic cholecystectomy *per se* did not entirely account for the lower mortality in cirrhotics. A rigorous selection of patients for surgery, based on imaging techniques with higher diagnostic accuracy and

a better control of the underlying liver disease, as well as the availability of less invasive techniques for temporary management-surgical or endoscopic-in those with advanced liver cirrhosis have substantially contributed to the improved outcome of these patients.

CONCLUSION

Gallstones occur often in patients with liver cirrhosis. Their prevalence increases with age and with the severity (*i.e.*, duration) of disease. They are in most patients pigment stones, while about 15% of cirrhotics have cholesterol stones. Lithogenesis is induced in these patients by the metabolic changes in the liver, involving bilirubin and biliary lipid secretion, and is favored by gallbladder hypomotility. Gallstones develop in cirrhosis of all etiologies, but more frequently in alcoholic, viral C and fatty liver disease. Asymptomatic gallstones should not be operated in cirrhotic patients, but patients should be followed-up closely and offered LC once symptoms develop. In patients with advanced liver disease, noninvasive or minimally-invasive procedures should be used to treat the complications of gallstones.

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TOPIC HIGHLIGHT

WJG 20th Anniversary Special Issues (11): Cirrhosis

Nutrition and exercise in the management of liver cirrhosis

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Abstract

Liver cirrhosis (LC) patients often have protein-energy malnutrition (PEM) and decreased physical activity. These conditions often lead to sarcopenia, which is the loss of skeletal muscle volume and increased muscle weakness. Recent studies have demonstrated that PEM and sarcopenia are predictors for poor survival in LC patients. Nutrition and exercise management can improve PEM and sarcopenia in those patients. Nutrition management includes sufficient dietary intake and improved nutrient metabolism. With the current high prevalence of obesity, the number of obese LC patients has increased, and restriction of excessive caloric intake without the exacerbation of impaired nutrient metabolism is required for such patients. Branched chain amino acids are good candidates for supplemental nutrients for both obese and non-obese LC patients. Exercise management can increase skeletal muscle volume and strength and improve insulin resistance; however, nutritional status and LC complications should be assessed before an exercise management regimen is implemented in LC patients. The establishment of optimal exercise regimens for LC patients is currently required. In this review, we describe nutritional status and its clinical impact on the outcomes of LC patients and discuss general nutrition and exercise management in LC patients.

Key words: Liver cirrhosis; Protein-energy malnutrition; Sarcopenia; Obesity; Exercise

Core tip: Recent studies have shown that sarcopenia is a predictor of poor survival in liver cirrhosis (LC) patients. LC-associated sarcopenia develops based on impaired nutrient metabolism and decreased physical activity. To improve this condition, nutrition and exercise management is imperative. Energy intake with branched chain amino acid supplementation is a promising method for nutrition management. Exercise can increase skeletal muscle volume and strength; however, nutritional status and LC complications should be assessed before exercise management begins. Obesity is another health issue for LC patients; improvement of insulin resistance is a key component in nutrition and exercise management for obese LC patients.

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INTRODUCTION

Liver cirrhosis (LC) is a critical stage of chronic liver disease with poor outcomes. Substantial data have indicated that poor liver function and the occurrence of hepatocellular carcinoma (HCC) are responsible for the shortened survival of LC patients[1-4]. Accumulating data have also demonstrated that LC patients often develop protein-energy malnutrition (PEM) at a rate of 25.1%-65.5%[5-8] and that PEM plays a crucial role in their poor survival[6,9-11].

LC-associated PEM occurs in combination with poor dietary intake, malabsorption, increased intestinal protein loss, decreased hepatic protein synthesis, abnormal substrate utilization, and hypermetabolism[12,13]. Individuals with PEM typically suffer from a loss of skeletal muscle volume and from muscle weakness; this condition is classified as sarcopenia[14]. Aging-related sarcopenia is defined as primary sarcopenia, while LC is a cause of secondary sarcopenia[15]. Recent studies have demonstrated that sarcopenia is an independent predictor of poor survival in LC patients with or without HCC[16,17]. However, overnutrition is increasingly affecting humans worldwide[18], and thus, overweight/obesity are frequently observed in LC patients. For example, 72.4% of patients had excess caloric intake in a study of compensated hepatitis C virus (HCV)-related LC[19], and 61% of compensated HCV-related LC patients have a body mass index (BMI) $\geqslant$ 25 kg/m^2[20]. Both chronic HCV infection[21] and overweight/obesity can cause insulin resistance, which raises the risk of liver fibrosis progression[22] and HCC occurrence[23] in HCV-related LC. Thus, clinicians are now confronted with problems related to malnutrition and overnutrition in the management of LC. In this review, we describe nutritional status and its clinical impact on the outcomes of LC patients and discuss nutrition and exercise management strategies for LC patients.

ENERGY METABOLISM ASSOCIATED WITH PEM IN LC PATIENTS

Metabolic activity

Metabolic activity can be assessed by comparing a measured resting energy expenditure (REE) and a predicted REE[24]. There are notable differences in metabolic activity among LC patients; previous studies have reported that 15%-33.8% of LC patients exhibited hypermetabolism, while 8%-31% were hypometabolic[7,8,25,26]. Earlier studies with LC patients demonstrated that a hypermetabolic state is strongly associated with decreased muscle volume[27]. Increased beta-adrenergic activity may explain, at least in part, hypermetabolism[26]. In a multicenter prospective study, a detailed analysis of metabolic activity and energy balance in LC patients was conducted. The results showed that PEM significantly correlated with Child-Pugh grade, that hypermetabolic and hypometabolic patients showed a significant decrease in kg of free fat mass, and that hypermetabolic patients had a positive energy balance due to decreased physical activity, while hypometabolic patients had a negative energy balance due to a reduced caloric intake[7].

The relationship between metabolic activity and outcomes in LC patients has been investigated. A study found that survival rate is significantly higher in normal metabolic LC patients than in hypometabolic or hypermetabolic LC patients[10]. Furthermore, some results have suggested that LC-related hypermetabolism is a factor associated with both transplant-free[25,28] and post-transplantation survival[29]. Hypermetabolic LC patients have decreased transplant-free survival compared with non-hypermetabolic LC patients (9.7 mo *vs* 31.8 mo, $P = 0.05$)[28]. Moreover, in a study of patients with end-stage liver disease, pre-transplantation hypermetabolism was associated with decreased post-transplantation survival[29].

Carbohydrate and lipid metabolism

The liver plays a critical role in carbohydrate and lipid metabolism. Ingested carbohydrates are taken up by the liver and converted into and stored as glycogen. In the fasting state, glucose is generated in the liver *via* glycogenolysis and gluconeogenesis; thus, blood glucose levels are maintained[30]. Because LC patients have decreased gluconeogenesis ability and glycogen stores capacity[31], they are prone to entering into a starvation state after a relatively short fasting period (*e.g.*, overnight)[32]. In this situation, lipid metabolism is enhanced; energy metabolism shifts from a carbohydrate preference to lipid oxidation preference[33-35]. Accordingly, free fatty acid (FFA) levels are elevated in LC patients. A previous study found that impaired re-esterification rather than accelerated lipolysis elevates FFA in LC patients[36].

Protein metabolism

Because albumin synthesis is decreased in LC patients, serum albumin levels inversely correlate with the grade of liver dysfunction[37]. Furthermore, in a study of compensated LC patients with alanine aminotransferase levels > 50 IU/L, a positive correlation between serum albumin levels and skeletal muscle volume was observed[38]. LC-associated PEM accelerates protein catabolism, which is the overall breakdown of cellular proteins, mainly in skeletal muscles, and which provides amino acids, especially branched chain amino acids (BCAAs), for protein synthesis and energy supply[39-41]. BCAAs consist of leucine, isoleucine, and valine. In a study with LC patients, energy efficacy (increased energy expenditure/energy equivalent of the supplemented nutrient) was significantly higher in BCAAs (96% ± 16%) than in glucose (96% ± 16% *vs* 41% ± 8%, $P < 0.01$) and fatty acids (96% ± 16% *vs* 27% ± 13%, $P < 0.05$)[42]. Moreover, BCAAs are consumed for ammonia detoxification in LC patients in whom hepatic detoxification to urea is impaired. Skeletal muscles and, to a lesser extent, the brain clear blood ammonia by incorporating ammonia into the process of glutamine production from glutamate. During the process, BCAAs are required for glutamate synthesis[40]. Thus, there is a frequent lack of BCAAs in LC patients, resulting in decreased albumin synthesis. In contrast to decreased BCAA levels, aromatic amino acid (AAA) levels are typically increased in LC patients[43,44], although underlying mechanisms for the altered AAA metabolism in LC are not fully understood. A decrease in the BCAA to AAA ratio (Fischer ratio; BCAA to tyrosine ratio, BTR) is thought to play a causal role in hepatic encephalopathy by enhanced brain AAA uptake and subsequent neurotransmission disturbance[45]. Recent studies have suggested that this amino acid imbalance occurs in the early stages of LC[46].

IMPACT OF SARCOPENIA ON LC PATIENT OUTCOMES

Sarcopenia

As described above, protein breakdown from skeletal muscles is an important pathologic mechanism for sarcopenia in LC patients. Recently, some analyses have indicated that hyperammonemia can cause sarcopenia. The results of an animal experiment demonstrated that skeletal muscle autophagy is induced by hyperammonemia and may contribute to sarcopenia in cases of LC[47]. Another study showed that skeletal muscle from LC patients had increased expression of myostatin, a known inhibitor of skeletal muscle accretion and growth. That study found that myostatin expression is induced by hyperammonemia in murine myotubes, suggesting a mechanism by which sarcopenia develops in LC patients[48].

Recent studies have examined outcomes in LC patients with sarcopenia[16,17]. In a study of LC patients in which sarcopenia was observed in 40% of the patients, sarcopenia, Child-Pugh scores, and model for end-stage liver disease (MELD) scores were each found to be independent factors for mortality, with the mortality risk more than 2-fold higher in sarcopenic than nonsarcopenic patients[16]. Interestingly, the study also revealed a strong relationship between sarcopenia and sepsis-related death, which may reflect the impaired immunity found in LC patients. In line with those findings, a prospective study of LC patients demonstrated that PEM is an independent predictor of bacterial infection[49]. Furthermore, sarcopenia has been shown to correlate with poor survival after liver transplantation[50,51].

Sarcopenic obesity

The current global obesity epidemic has created a new condition: the combination of sarcopenia and obesity, described as sarcopenic obesity[52]. Because LC patients occasionally have sarcopenia (40%)[16] and obesity (30%-31%)[53,54], it can be deduced that a considerable number of them may have sarcopenic obesity. Furthermore, obesity is frequently accompanied by nonalcoholic fatty liver disease (NAFLD), and the prevalence of this liver disease is increasing in industrialized countries[55-57]. NAFLD can progress to nonalcoholic steatohepatitis and LC. Given this global trend, sarcopenic obesity will likely be a major condition in LC patients in the future.

Obesity typically occurs in tandem with decreased physical activity[58,59], which may create a vicious cycle of sarcopenia progression. Obesity also induces insulin resistance and systemic inflammation, both of which prompt hypercatabolism and impair the anabolic effect of muscles, resulting in protein breakdown stimulation and muscle synthesis suppression[59-61]. Moreover, a recent study revealed that sarcopenic obesity is more closely associated with insulin resistance than sarcopenia or obesity alone[62]. Taken together, this new condition appears to accelerate sarcopenia progression.

Although sarcopenia has been reported to be predictive of poor survival in LC patients[16,17], the impact of sarcopenic obesity on LC patient outcomes remains unknown. However, it has been suggested that obesity is an independent predictor of hepatic decompensation in LC patients[53]. Furthermore, obesity has been shown to be a risk factor for LC-related death or hospitalization[63,64]. A study of cancer patients revealed that sarcopenic obesity is associated with a poorer functional status compared with obesity without sarcopenia and is an independent predictor of survival[65]. These findings provide the rationale for further studies to clarify whether sarcopenic obesity worsens LC patient outcomes.

ASSESSMENT METHODS FOR PEM IN LC PATIENTS

Table 1 lists the methods used to assess PEM and sarcopenia.

Indirect calorimetry

Indirect calorimetry can measure oxygen consumption per minute (V_{O_2}) and carbon dioxide production per minute (V_{CO_2}), thus calculating energy expenditure and non-protein respiratory quotient (npRQ). npRQ is considered to be a good marker for PEM assessment. In LC patients, npRQ is lower than in normal controls due to a shift of preferred energy metabolism from carbohydrate to lipid oxidation. A recent study of LC patients has revealed that the survival rate is significantly lower in patients with low npRQ (< 0.85) than in patients with scores above 0.85 ($P < 0.01$)[10]. Although the utility of indirect calorimetry in assessing energy metabolism has been proven, the high cost constrains its clinical application.

Anthropometric measurement

Because skeletal muscle volume reflects nutritional status, anthropometric measurement has been conducted to assess PEM in LC patients[66,67]. PEM indices include triceps skinfold thickness (TSF), arm muscle circumference (AMC), and arm circumference (AC). A study with LC patients reported that decreased AMC and TSF correlate with malnutrition and decreased liver functional reserve[67]. Accumulated data found a significant association between nutritional status estimated by anthropometric measurement and outcomes in LC patients. A previous study suggested that AMC may improve the prognostic capacity of Child-Pugh scores in LC patients[68]. Another study demonstrated that AMC and TSF may be useful in predicting survival of LC patients. In addition, the prognostic power of AMC was found to be higher than that of TSF[9]. A more recent study examined whether the anthropometric indices are alternatives to npRQ. When the measured values were expressed as percentages of normal values, percent of AMC and percent of AC were found to significantly correlate with npRQ, and a formula using %AC and Child-Pugh scores could represent npRQ[69]. External validation is needed to verify the relationship between the measurement values and

Table 1 Methods to assess protein-energy malnutrition and sarcopenia in liver cirrhosis patients

Method	Ability	Advantage	Disadvantage
PEM			
Indirect calorimetry	To calculate energy expenditure and npRQ npRQ being a marker for survival	Non-invasive and accurate	Expensive
Anthropometric measurements	To estimate nutritional status and liver function AMC and TSF serve as markers for survival %AMC and %AC serve as alternatives to npRQ	Simple and inexpensive	Possible errors related to the measurements
Bioimpedance analysis	To estimate body cell mass PA serves as a measure to estimate nutritional status and as a marker for survival	Convenient and inexpensive Comparable with the DXA and MRI methods in the assessment of skeletal muscle volume	Limitations in patients with ascites
Sarcopenia			
Imaging method	To assess skeletal muscle volume		
CT and MRI		Accurate	Radiation-exposed (CT)
DXA		Comparable with the CT and MRI methods Less radiation exposure and lower cost than the CT method	
Handgrip strength	To measure muscle strength A marker for nutritional status A predictor of hepatic decompensation	Simple and inexpensive	Possible errors related to measurements

PEM: Protein-energy malnutrition; LC: Liver cirrhosis; npRQ: Non-protein respiratory quotient; AMC: Arm muscle circumference; TSF: Triceps skinfold thickness; AC: Arm circumference; PA: Phase angle; DXA: Dual energy X-ray absorptiometry; MRI: Magnetic resonance imaging; CT: Computed tomography.

npRQ. Although anthropometric measurements are simply and inexpensively performed, the interpretation of the measured values should be performed carefully. For example, a study suggested that AMC may be affected by edema[70], a symptom frequently observed in LC patients. Furthermore, possible errors related to anthropometric measurements should be noted: repeated measurements providing different values (unreliability, imprecision, undependability) and measurements departing from true values (inaccuracy, bias)[71].

Bioimpedance analysis

Bioimpedance analysis (BIA) is another measure to assess PEM. This method is based on the measurement of tissue conductivity[72]. Skeletal muscle is a major body component with low resistance and is therefore a dominant conductor[73]. A study with LC patients has demonstrated that BIA is a reliable bedside tool for the estimation of body cell mass, although it is limited in the case of LC with ascites[74]. The phase angle (PA) is a derived measure calculated from two parameters of BIA: PA = arctangent reactance/resistance × 180°/π[75]. Several studies have demonstrated that PA is useful in the assessment of the nutritional status in hemodialysis[76] or preoperative[77] patients. Another study has suggested that PA can serve as a prognostic indicator in cancer patients[78]. With regard to LC, a recent study indicated that PA is a promising parameter for the assessment of patient nutritional status[79]. Furthermore, a study suggested that PA is more predictive of survival than commonly used body composition information: a low PA is associated with shorter survival time[80]. Several studies have revealed that the estimated values of skeletal muscle mass obtained by BIA are not significantly different from those obtained by magnetic resonance imaging (MRI)[73] or dual energy X-ray absorptiometry (DXA)[81] (see below). Because of its convenience and low cost, BIA is a potential alternative to these imaging methods[14].

Methods for sarcopenia assessment

Imaging methods: There are several methods for sarcopenia assessment. Computed tomography (CT) is an imaging method that permits the precise measurement of skeletal muscle volume. CT technology enables specific tissue demarcation according to a CT measure of the tissue, thereby permitting calculation of its area. Human muscle tissue has a CT number in the range of -29 to +150 hounsfield units (HU). Muscles at the third lumbar (L3) vertebra encompass the psoas, erector spinae, quadratus lumborum, transversus abdominis, external and internal obliques, and rectus abdominis. A recent analysis revealed that the calculated L3 muscle area accurately represents the whole-body skeletal muscle volume (r = 0.86-0.94, $P < 0.001$)[82]. Based on that finding, the L3 muscle area normalized for stature (cm^2/m^2) can be used as an index of skeletal muscle volume (the L3 skeletal muscle index, L3 SMI)[65]. Although cutoff values for diagnosing sarcopenia have not been established, a recent study used cutoff values of 38.5 cm^2/m^2 for women and 52.4 cm^2/m^2 for men[65]. MRI has also been used for the assessment of skeletal muscle volume and sarcopenia[73,83,84].

DXA is another imaging method used in sarcopenia assessment. This method allows for the measurement of bone, fat, and lean-tissue content. Appendicular skeletal muscle mass (ASM) accounts for more than 75% of the total body skeletal muscle mass and can thus serve as a marker for sarcopenia[59,85]. ASM divided by height

squared (ASM/Ht^2; kg/m^2)[86] and ASM as a percentage of body weight (ASM/Wt)[87] have been proposed as indices for sarcopenia. Sarcopenia has been defined as an ASM < 1 SD[62] or < 2 SD[59] below the sex-specific mean for a young reference group. The accuracy of the DXA method has been shown to be comparable to that of the CT or MRI method[84,88], and the DXA method requires less radiation exposure and costs than the CT method[88].

Handgrip strength: Decreased muscle strength reflects a decreased volume of skeletal muscle. The European Working Group on Sarcopenia in Older People (EWGSOP) recommends handgrip strength as a practical measure of muscle strength[14]. Handgrip strength has been shown to be a useful marker for the assessment of nutritional status in LC patients[89]. Moreover, a previous analysis has revealed that handgrip strength can be a useful predictor of hepatic decompensation in LC patients[6]. However, it should be noted that considerable variation in the measurement methods has the potential to introduce measurement errors[90].

NUTRITION MANAGEMENT FOR LC PATIENTS

Management for PEM in LC patients

Dietary management: Poor dietary intake is an important cause of PEM in LC patients. In a study of nutritional status in LC patients, decrease in daily caloric intake paralleled worsening of progressive liver failure: 48% and 34% of Child A patients, 51.7% and 35.8% of Child B patients, and 80.3% and 62.9% of Child C patients at admission had a caloric intake below 30 kcal/kg of body weight and protein intakes below 1 g/kg of body weight, respectively ($P < 0.001$). Furthermore, poor dietary intake was found to be an independent predictor for in-hospital mortality[67]. Some studies have aimed to clarify whether efforts to increase dietary intake can improve the outcome of LC patients, and short-term follow-up has suggested an improvement of nutritional status[91,92]. A study of alcoholic LC patients demonstrated that an increase in dietary intake altered the energy metabolism of Child C patients from preferred lipid oxidation to preferred carbohydrate metabolism. However, the dietary management appeared to be limited in improving nutritional status in end-stage LC patients, such as those with refractory ascites[92]. The European Society for Clinical Nutrition and Metabolism (ESPEN) guidelines recommend that energy and protein intake should be 35-40 kcal/kg of body weight per day and 1.2-1.5 g/kg of body weight per day, respectively[93].

The timing of dietary intake can influence energy metabolism. Because LC patients are prone to entering a starvation state after a relatively short fasting period, a large number of small meals ("nibbling" pattern) rather than a small number of large meals ("gorging" pattern) is considered preferable to maintain optimal energy metabolism[94,95]. Several studies of LC patients found that late nocturnal energy supplementation altered energy metabolism from preferred lipid oxidation to preferred carbohydrate metabolism[96,97]. More recently, a randomized controlled trial with LC patients suggested that nocturnal energy supplementation may be superior to daytime energy supplementation for protein accretion[98].

BCAA supplementation: As previously discussed, a lack of BCAAs in LC patients can accelerate muscular protein catabolism, decreased albumin synthesis, and hyperammonemia and associated hepatic encephalopathy. A loss of skeletal muscle volume (*i.e.*, sarcopenia)[16], low serum albumin levels[99-104], and hepatic encephalopathy[105] have been found to be predictors of poor survival in LC patients. These findings lead to the notion that BCAA supplementation may restore impaired protein metabolism and thereby improve outcomes of LC patients. Indeed, previous studies have revealed that BCAA administration stimulates albumin synthesis[40] and protein synthesis in skeletal muscles[106]. Of the BCAAs, leucine[106-108] is considered to play a central role in the synthesis process, of which, the mammalian target of rapamycin (mTOR)[106,107] appears to be a key component in controlling its signaling pathway.

BCAA administration can be conducted either orally or intravenously. A BCAA-enriched amino acid solution has been used in the treatment of acute hepatic encephalopathy for several decades, and its utility has been demonstrated[109]. Oral BCAA-enriched formulas, BCAA granules and BCAA and carbohydrate mixtures, have been used in the effort to achieve preferred nutritional status and improved outcomes of decompensated LC patients[110]. Studies with LC patients have demonstrated that serum albumin levels and npRQ increased with oral BCAA supplementation[111,112]. In a study of HCV-related LC, the intake of BCAA and carbohydrate mixtures as late evening snacks was more effective in increasing serum albumin levels and improving energy metabolism than ordinary food intake[111]. Long-term follow-up studies of BCAA supplementation for LC patients showed positive results. In a randomized clinical trial with decompensated LC patients, supplementation with BCAA granules contributed to preventing progressive liver failure[113]. A similar randomized controlled trial found that supplementation with BCAA granules increased serum albumin levels and contributed to decreased liver failure and mortality[114].

Thus, BCAA supplementation is an effective therapeutic strategy for improving energy metabolism and overall outcomes in LC patients. This nutritional treatment is recommended in several guidelines[93,115]. The optimal timing of BCAA administration during the course of LC remains to be determined, although one randomized controlled trial suggested that patients with a BTR of < 4 should begin BCAA treatment even in cases of compensated LC[116]. Given the close relationship between BCAAs and protein synthesis in skeletal muscles, future studies focusing on the benefits of BCAA supple-

mentation on sarcopenia in LC are necessary. In addition, some evidence suggests that BCAAs are essential for lymphocyte responsiveness and are necessary to support other immune cell functions[117]. Whether BCAA treatment can improve immunity in LC patients with sarcopenia and decrease the incidence of severe infection requires investigation.

Nutrition management of obese LC patients

With the increasing prevalence of obesity worldwide, the prevalence of obese LC patients is increasing[54]. Given that obesity accompanied by LC can accelerate hepatic decompensation[53], enhance hepatocarcinogenesis[118,119], and result in poor patient survival[63,64], nutrition management is imperative for obese LC patients. The restriction of excessive caloric intake without exacerbation of impaired nutrient metabolism is necessary for successful LC management. Furthermore, obesity is closely linked to insulin resistance; this metabolic problem increases the risk of disease progression, hepatocarcinogenesis, and mortality in LC patients[120]. Considering that obesity can exacerbate sarcopenia-associated insulin resistance[62,121], nutrition strategies for insulin resistance appear to be important, particularly in LC patients with sarcopenic obesity. Recent studies have suggested that BCAA supplementation is effective in improving insulin resistance[122,123]. Of the BCAAs, leucine appears to play a critical role in controlling carbohydrate metabolism; the amino acid regulates the oxidative use of glucose by skeletal muscle through the stimulation of glucose recycling *via* the glucose-alanine cycle[122]. Further trials are required to establish dietary regimens, such as dietary nutrient balance, for obese LC patients.

EXERCISE MANAGEMENT FOR LC PATIENTS

Physical activity and exercise capacity in LC patients

A recent survey of LC patients reported that physical activity levels were lower in LC patients than in healthy controls[124]. The survey results also suggested that low levels of physical activity were inversely associated with insulin resistance. In a study of compensated LC, low levels of physical activity and poor caloric intake were closely linked to sarcopenia[125]. These findings indicate that increased physical activity may prevent and improve sarcopenia in LC patients. Indeed, in studies of the elderly[126] or patients with certain types of chronic diseases[127], exercise management has been shown to be effective in preventing and improving sarcopenia.

Exercise capacity is described as the ability to use oxygen during exercise. The commonly used measure of exercise capacity is maximal oxygen consumption (VO_{2max})[128]. Studies with LC patients have shown decreased exercise capacity as evaluated by VO_{2max}[129,130] and an inverse relationship between exercise capacity and the severity of liver disease[130-132]. Recent research has demonstrated that a decrease in exercise capacity is not only associated with LC severity but also predictive of mortality after liver transplantation[133,134]. Earlier studies on exercise management demonstrated that physical training programs as short as approximately one month were useful in increasing VO_{2max} or peak oxygen consumption (VO_{2peak}) in LC patients[131,135].

Given these findings, exercise management is a key component in the management of LC patients because it can lead to increases in physical activity, skeletal muscle volume and strength, and exercise capacity, ultimately improving the quality of life and survival.

Assessment of nutritional status and complications for exercise management

The current guidelines for physical activity and health in older adults (men and women aged $\geqslant$ 65 years and adults aged 50-64 years with clinically significant chronic conditions and/or functional limitations) recommend that moderate-intensity aerobic physical activity should be performed for a minimum of 30 min five days each week in addition to two sessions of resistance training and flexibility exercises each week[136]. The applicability of these recommendations depends on the severity of the chronic conditions and complications. With regard to LC, inappropriate exercise may cause undesirable outcomes due to the impaired energy metabolism and/or complications associated with LC, including ascites[137], hepatic encephalopathy[138], portal hypertension[139], and hepatopulmonary syndrome[140]. For example, in patients with LC, portal pressure and portal hypertension reportedly increased with moderate exercise (30% of the maximum), suggesting that such physical load poses a risk for variceal bleeding[139]. Moreover, exercise under insufficient nutrient intake can promote protein catabolism and thereby a loss of skeletal muscle mass in LC patients[141,142]. The assessment of nutritional status and complications is therefore mandatory before any exercise management of LC patients.

Exercise regimens for LC patients

The optimal exercise regimens for LC patients remain uncertain. However, there are some preliminary data with regard to efficacious exercise management for LC patients. Recently, based on a survey of compensated LC patients, researchers recommended the following exercise regimen: walking 5000 or more steps per day with a total caloric intake of approximately 30 kcal/ideal body weight[125]. The authors claimed that the regimen has the potential to maintain and increase skeletal muscle volume in LC patients. Most recently, a randomized pilot study with LC patients, in which most participants had Child-Pugh grade A LC, examined whether an exercise program combined with leucine supplementation (10 g/d) can improve patient outcome. The program included three sessions per week of a 1-h treadmill and cycle ergometry exercise at an intensity of 60%-70% of the maximum heart rate, over a period of 12 wk. The intervention group had improved exercise capacity, as shown by the 6-min walk test (from

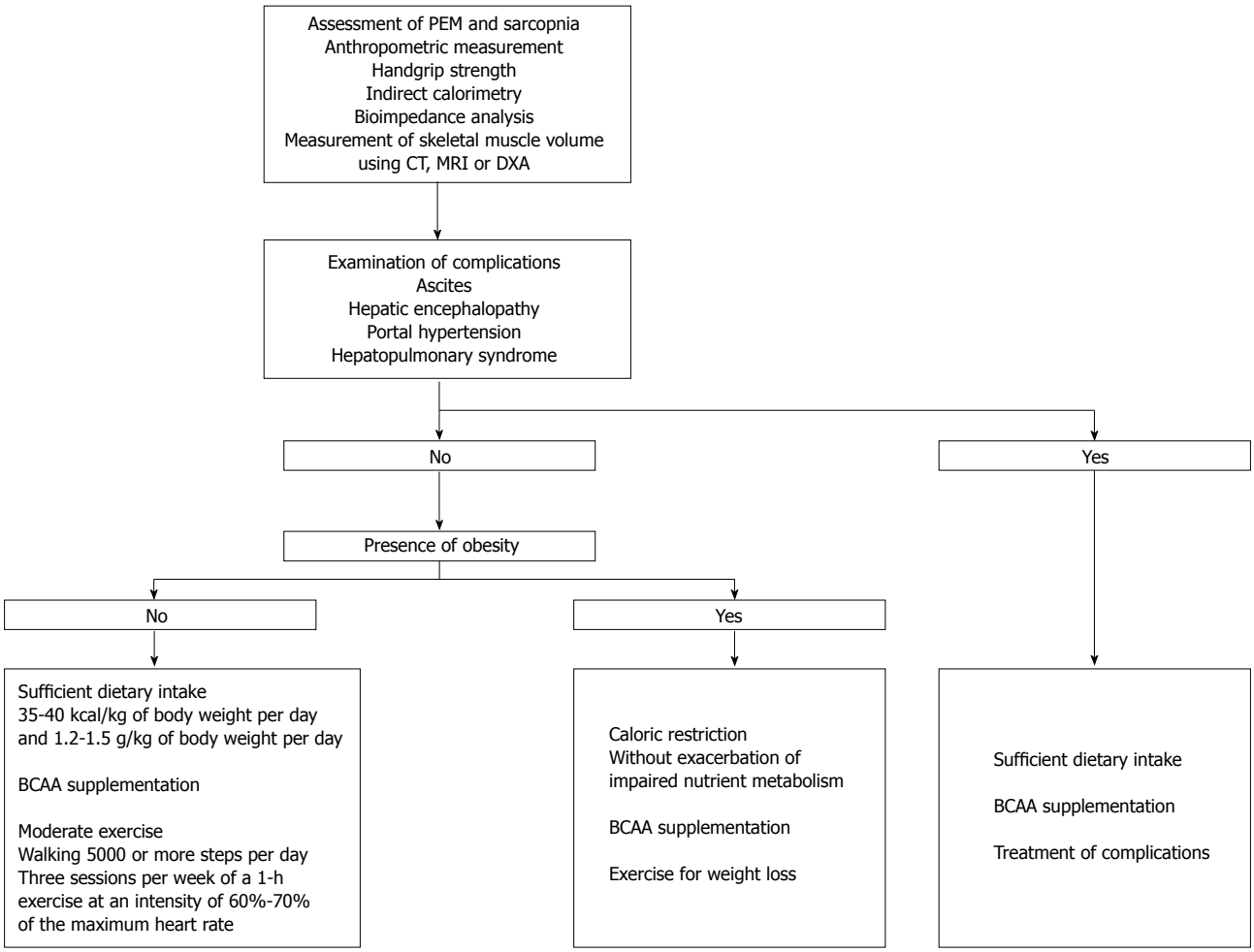

Figure 1 A practical approach for managing liver cirrhosis patients with sarcopenia or sarcopenic obesity. LC: Liver cirrhosis; PEM: Protein-energy malnutrition; CT: Computed tomography; MRI: Magnetic resonance imaging; DXA: Dual energy X-ray absorptiometry; BCAA: Branched chain amino acid.

median 365 m to median 445 m) and the 2-min step test (from median 100 steps to median 150 steps), increased lower thigh circumference, and improved health-related quality of life; the control group had no significant changes[143]. During the study, no adverse events due to the implementation of the exercise program were observed. These studies suggest the possibility that moderate exercise combined with LC-specific nutritional support can increase skeletal muscle volume and improve the outcomes of LC patients. Other studies have indicated that aerobic exercise can be expected to improve insulin resistance in patients with chronic liver disease[144,145]. This favorable effect of exercise on insulin sensitivity is particularly important for obese patients[144,146]. Future intensive studies are required to establish efficacious and safe exercise regimens for LC patients.

CONCLUSION

Substantial data exist clearly demonstrating that PEM confers a risk of poor survival in LC patients. PEM in LC patients is highly associated with sarcopenia and a decrease in serum albumin levels. These conditions have also been reported to be predictors of poor patient survival. Nutrition and exercise management can improve PEM and sarcopenia in LC patients. Nutrition management includes sufficient dietary intake and an improvement of impaired nutrient metabolism. In contrast, the current rise in obesity prevalence has increased the number of obese LC patients. Restriction of excessive caloric intake without exacerbation of impaired nutrient metabolism is necessary for those patients. BCAAs are good candidates for supplemental nutrients for both obese and non-obese LC patients. Exercise management can increase skeletal muscle volume and strength and can improve insulin resistance; however, assessment of nutritional status and LC complications is mandatory before the implementation of an exercise program for LC patients. The establishment of optimal exercise regimens for LC patients is required. Figure 1 shows a tentative practical approach for managing LC patients with sarcopenia or sarcopenic obesity. The further development of methods for nutrition and exercise management will improve the overall health outcomes of LC patients.

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TOPIC HIGHLIGHT

WJG 20th Anniversary Special Issues (11): Cirrhosis

Impairment of innate immune responses in cirrhotic patients and treatment by branched-chain amino acids

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Abstract

It has been reported that host defense responses, such as phagocytic function of neutrophils and natural killer (NK) cell activity of lymphocytes, are impaired in cirrhotic patients. This review will concentrate on the impairment of innate immune responses in decompensated cirrhotic patients and the effect of the treatment by branched-chain amino acids (BCAA) on innate immune responses. We already reported that phagocytic function of neutrophils was significantly improved by 3-mo BCAA supplementation. In addition, the changes of NK activity were also significant at 3 mo of supplementation compared with before supplementation. Also, Fisher's ratios were reported to be significantly increased at 3 mo of BCAA supplementation compared with those before oral supplementation. Therefore, administration of BCAA could reduce the risk of bacterial and viral infection in patients with decompensated cirrhosis by restoring impaired innate immune responses of the host. In addition, it was also revealed that BCAA oral supplementation could reduce the risk of development of hepatocellular carcinoma in cirrhotic patients. The mechanisms of the effects will also be discussed in this review article.

Key words: Branched-chain amino acids; Liver cirrhosis; Innate immunity; Natural killer cell activity of lymphocytes; Phagocytic function of neutrophils

Core tip: This review will discuss the recent research on impairment of innate immune responses in cirrhotic patients and the treatment by branched-chain amino acids (BCAA). It was revealed that BCAA oral supplementation could improve not only nutrition status but phagocytic function of neutrophils and natural killer activity of lymphocytes in cirrhotic patients. Therefore, BCAA supplementation might reduce the risk of bacterial and viral infection in patients with decompensated cirrhosis. Additionally, it was also revealed that BCAA oral supplementation could reduce the risk of development of hepatocellular carcinoma in cirrhotic patients. The mechanisms of the effects of BCAA described above will also be discussed.

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INTRODUCTION

Innate immunity is the first defense mechanism of host against pathogens such as bacteria and viruses. Patients with insufficient innate immunity may have increased

risk of bacterial and viral infection. Patients with liver cirrhosis, especially those with decompensated cirrhosis, are liable to suffer from bacterial infection such as bacteremia and spontaneous bacterial peritonitis (SBP), which could be lethal to the patients. The incidence of bacterial infections in cirrhotic patients is almost 4-7 times greater than that of general hospital population[1,2]. SBP occurs in 10%-25% of hospitalized cirrhotic patients and its mortality rate is 17%-50%[3]. The high incidence of bacterial infections in patients with cirrhosis has prompted an assessment of defects in their immune defense against microorganisms. The functional studies of peripheral blood neutrophils have shown defective phagocytic activity and intracellular killing activity in patients with cirrhosis[4-9]. In addition, natural killer (NK) cells also constitute the first line of host defense against invading pathogens. They usually become activated in an early phase of viral infection. Effective clearance of an acute viral infection requires the coordinated function of multiple arms of the immune systems, including innate immune systems mediated by NK cells and cytokines such as interferon, as well as adaptive immune responses. It was reported that not only phagocytic function of neutrophils but NK activity of lymphocytes were impaired in cirrhotic patients[10-14].

So far, there are few therapies for restoration of activity of immune responses of innate immunity in patients with liver cirrhosis. Branched-chain amino acid (BCAA) supplementation has been previously shown to improve serum albumin levels and Fisher's ratios in those patients[15-17]. In addition, BCAA was also shown to improve impaired glucose tolerance of the cirrhotic patients with creatinine height index (CHI) greater than 80 by Urata *et al*[18]. It was reported that BCAA oral supplementation might reduce the incidence of events comprising the primary endpoint (which was a composite of death by any cause, development of liver cancer, rupture of esophageal varices, or progress of hepatic failure[19]. In addition, recent studies have revealed that BCAA promotes albumin synthesis in rat hepatocyte through activation of mammalian target of rapamycin (mTOR) signal transduction system[20]. Much is still unknown on the effects of BCAA supplementation on reactions of innate immunity. This review will concentrate on the impairment of innate immune responses in decompensated cirrhotic patients and the effect of the treatment by BCAA on innate immune responses.

IMMUNE RESPONSES IN CIRRHOTIC PATIENTS

Increased risk of infection and death in cirrhotic patients

It was reported that alterations of the immune system are common in patients with end-stage liver disease and associated with an increased risk of infection and death[1-3,21-24]. Bacterial infection involving such as urinary tract, ascites, blood, respiratory tract is a severe complication of decompensated cirrhosis. Additionally, it might induce longer hospital stay and increased mortality[21]. It was previously reported that infections in patients with cirrhosis increase mortality 4-fold; 30% of patients die within 1 mo after infection and another 30% die by 1 year[22]. In patients with cirrhosis and severe sepsis, high production of proinflammatory cytokines seems to play a role in the worsening of liver function and the development of organ failures such as shock, renal failure, acute lung injury or acute respiratory distress syndrome, coagulopathy, or hepatic encephalopathy[23].

Impairment of innate immune response in cirrhotic patients

Functional abnormalities of neutrophils and macrophages[4-9], NK cells[10-14], and the complement system[25] contribute to impaired innate immune responses. On Neutrophils, it was reported that there was a defect of neutrophil phagocytosis and a defect of intracellular killing of bacteria in cirrhotic patients. In addition, it was revealed that these neutrophil defects are caused by both reduced production of superoxide and defects of degranulation[4]. In addition, the function of macrophage Fc gamma receptors was reported to be impaired in patients with cirrhosis, and this impairment probably contributes to the high incidence of bacterial infections among such patients[6]. Neutrophil migration and phagocytosis were reported to be decreased in cirrhotic patients with previous episodes of bacterial infection compared with non-infected patients. In addition, expression of complement receptor type Ⅲ (CR3) in circulating neutrophils was significantly higher in cirrhotic patients. These data suggest that deficient neutrophil recruitment to the infection site and impaired phagocytic activity may contribute to bacterial infections in cirrhotic patients with advanced liver disease[5]. Polymorphonuclear cells (PMNs) obtained from cirrhotic patients were reported to be less effective than those from controls in producing O_2- after stimulation with opsonized zymosan, while they were more effective in producing NO, NO synthase activity was higher in leukocytes from cirrhotic patients than in controls[8]. It was also reported that the plasma of cirrhotic patients induced neutrophil phagocytic dysfunction and the degree of the impairment was greater in cirrhotic patients with more severe disease[9]. The study also clarified that dysfunction of phagocytic function of neutrophils was associated with increased expression of toll-like receptors 2 and 4. Stable cirrhosis is characterized by neutrophil phagocytic dysfunction which may be subtle and only revealed in inflamed peripheral tissues where excessive inflammatory mediators continue to be released (Table 1).

NK cell activity was revealed to be significantly decreased in cirrhotic patients compared with normal controls and that in patients with other, non-malignant diseases, supporting the notion that immune-surveillance mechanisms may be affected in these patients[10-12]. Chuang *et al*[12] reported that cirrhotic patients with Child Pugh's C grade of severity of liver disease had lower NK cell activity. The depression of NK cell activity in cirrhotic patients was inversely correlated with prothrombin time ratios.

Table 1 Impairment of innate immune responses in cirrhotic patients

Impairment of phagocytic activity of neutrophils
Impairment of intracellular killing activity of neutrophils
Impairment of migration of neutrophils
Impairment of natural killer cell activity
Impairment of function of macrophage Fc gamma receptors
Impairment of opsonisation activity
Impairment of hemolytic complement function

Table 2 Impairment of adaptive immune responses in cirrhotic patients

Down regulation of major histocompatibility complex class II on monocyte/macrophages
Impairment of maturation and function of myeloid dendritic cells
Inhibition of T cell proliferation and T cell-mediated cytokine production
Inhibition of T-cell co stimulatory pathways
Impairment of tumor necrosis factor-alpha production of $CD4^+$ and $CD8^+$ cells
Increased fraction of $CD4^+$ $CD25^+$ cells
Increase in circulating anti-inflammatory cytokines

And NK cell activity in cirrhotic patients with hepatic encephalopathy was lower than that in patients without hepatic encephalopathy. Thus, the diminished NK cell activity in cirrhotic patients might be related to the severity of liver damage[12]. Reduction of NK cell activity might occur partially due to lower frequency of NK cell in peripheral blood[13]. However, the mechanisms of diminished NK cell activity was not clarified so far.

Also, acquired deficiencies of certain complement proteins and impaired opsonisation activity was reported to be implicated in the pathogenesis of the increased susceptibility to infections of patients with cirrhosis. Low serum C3 concentrations and decreased haemolytic complement function predispose to infection and increased mortality in patients with cirrhosis[25].

Impairment of adaptive immune response in cirrhotic patients

On the adaptive immune response in cirrhotic patients, there was evidence to suggest that the response was defective[26,27]. Generally speaking, impairment of adaptive immune responses can be due to either impairment of antigen presentation of professional cells or to reduction of T cell responses. T cell such as cytotoxic T lymphocyte (CTL) and helper T cell (Th) recognize a peptide-MHC complex on antigen presenting cell (Table 2). In cirrhotic patients, levels of interleukin-10 (IL-10) in blood became high due to increase of endotoxin and tumor necrosis factor-alpha (TNF-α)[28-30]. IL-10 could reduce cytokine responses of T cells, reduction of MHC class Ⅱ expression on antigen-presenting cells and suppression of co-stimulatory signals[31-34].

In addition, there was a report on association of T cell responses *in vitro* and markers of bacterial translocation, serum IL-10, monocyte HLA-DR expression and T cell subsets in cirrhotic patients[24]. Advanced liver disease predisposes to bacterial translocation and endotoxaemia which can contribute to elevated circulating levels of IL-10 and down-regulation of MHC class Ⅱ on antigen-presenting cells. Peter *et al*[24] evaluated antigen-specific T-cell responses toward common viral antigens in order to investigate defects in cellular immunity in cirrhosis. Compared to healthy controls, patients with cirrhosis had higher circulating levels of LBP and IL-10, an expansion of peripheral blood $CD14^+$ monocytes with low HLA-DR expression and an increased fraction of CD25-positive $CD4^+$ and $CD8^+$ T cells. These findings were reported to be most pronounced in cirrhotic patients with systemic inflammation. Furthermore, TNF-α production in responding T cells was attenuated in patients with a high frequency of $CD14^+$ HLA-DR- monocytes. The results of the study suggested that bacterial translocation, endotoxaemia, inflammation and T cell activation in cirrhosis are accompanied by an increase in circulating anti-inflammatory cytokines, reduced monocytic MHC class Ⅱ expression and attenuated cytokine production in T cells.

TREATMENT BY BCAA AND THE EFFECT ON INNATE IMMUNE RESPONSES IN CIRRHOTIC PATIENT

BCAA and mTOR signaling pathway

BCAA comprise three essential amino acids: leucine (Leu), isoleucine (Ile), and valine (Val). And chemical formulas of Leu, Ile, and Val are $HO_2CCH(NH_2)CH_2CH(CH_3)_2$, $HO_2CCH(NH_2)CH(CH_3)CH_2CH_3$, and $HO_2CCH(NH_2)CH(CH_3)_2$ respectively.

They are often used as supplemental therapy to improve protein malnutrition in patients with liver cirrhosis. Long-term oral supplementation with BCAA granules to cirrhotic patients improved their nutrition status (*i.e.*, hypoalbuminemia)[19,35]. In addition to the role of acting as nutrient substrates, recent studies have demonstrated that BCAA also serve as physiologically active substances. BCAA have been shown to have pharmacological effects, such as induction of protein synthesis[36] and promotion of glucose uptake in skeletal muscle[37]. In rat primary hepatocytes, albumin synthesis is significantly increased by BCAA administration, which is dependent on activation of the mammalian target of rapamycin (mTOR), mainly induced by leucine (Figure 1)[20].

mTOR integrates the input from upstream pathways, including insulin, growth factors (such as IGF-1 and IGF-2), and amino acids[38]. In addition, mTOR also senses cellular nutrient, oxygen, and energy levels[39]. The mTOR pathway is dysregulated in human diseases, such as diabetes, obesity, depression, and certain cancers[40]. mTOR is the catalytic subunit of two molecular complexes: mTORC1 and mTORC2[41]. mTOR complex 1 (mTORC1) is composed of mTOR, regulatory-associated protein of mTOR (Raptor), mammalian lethal

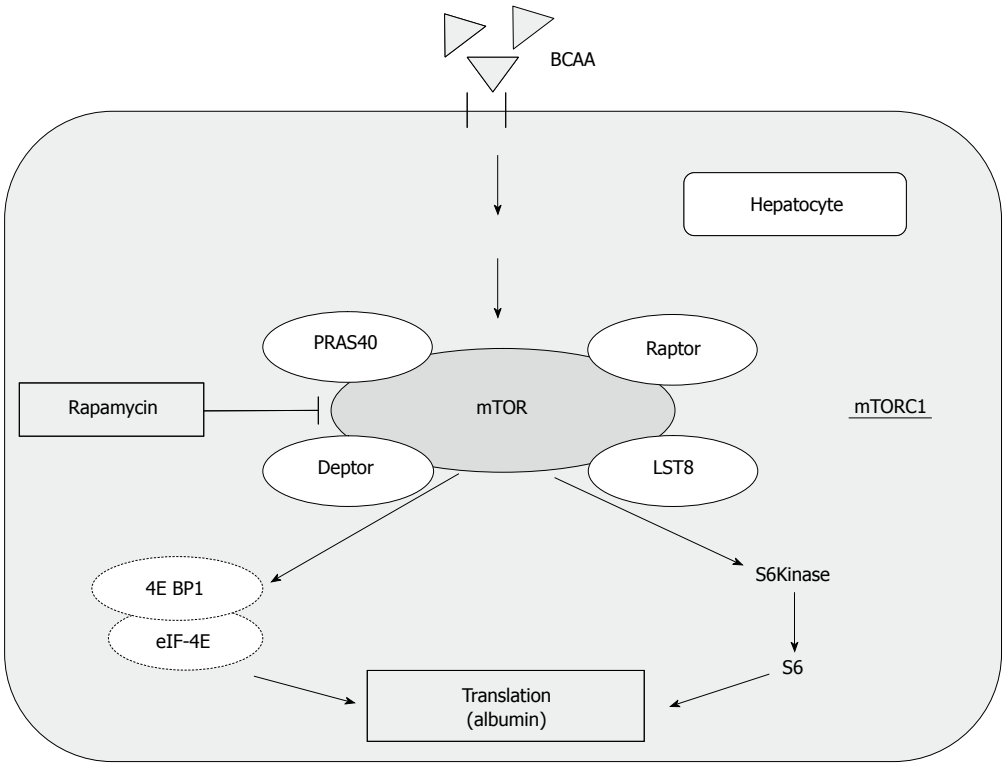

Figure 1 Mammalian target of rapamycin signaling pathway. Branched-chain amino acids (BCAA) promotes albumin synthesis through activation of the mammalian target of rapamycin (mTOR) signal transduction system. mTOR complex 1 (mTORC1) is composed of mTOR, Raptor, LST8, PRAS40 and Deptor. Raptor: Regulatory-associated protein of mTOR; LST8: Mammalian lethal with SEC13 protein 8; PRAS40: Protein-rich Akt subsrtrate of 40 kDa; Deptor; DEP domain-containing mTOR-interacting protein; eIF-4E: Eukaryotic translation initiation factor 4E; 4E BP1: Eukaryotic translation initiation factor 4E binding protein 1.

with SEC13 protein 8 (LST8) and the recently identified partners PRAS40 and DEPTOR (Figure 1)[42,43]. This complex is characterized by the classic features of mTOR by functioning as a nutrient/energy/redox sensor and controlling protein synthesis[38,42]. The activity of this complex is stimulated by insulin, growth factors, serum, phosphatidic acid, amino acids (particularly leucine), and oxidative stress[42,44].

In this paragraph, several reports on promotion of albumin synthesis by BCAA through activation of the mammalian target of rapamycin (mTOR) signal transduction system were introduced (Figure 1)[20].

Restoration of innate immune responses by oral supplementation of BCAA in cirrhotic patients

We reported on restoration of innate immune responses such as phagocytic function of neutrophils and NK cell activity[45,46]. In the reports, patients with decompensated cirrhosis received 12 g BCAA daily for 3 mo. Phagocytic function of neutrophils and NK cell activity of lymphocytes as well as Fisher's ratio were determined before and at 1 and 3 mo of BCAA supplementation. For quantification of phagocytic function, fluorescent intensities of cells in the neutrophil region in the cytogram were determined by flow cytometry after incubation of whole blood with fluorescent microspheres. NK cell activity was estimated by ^{51}Cr release assay using K-562 cell line as target cells. Fisher's ratio was reported to be significantly increased at 1 mo of BCAA supplementation compared with that before oral supplementation and also at 3 mo of BCAA supplementation compared with that before oral supplementation. In addition, the phagocytic function of neutrophils was reported to be significantly increased at 3 mo of supplementation compared with that before BCAA supplementation. It was also reported on the effects of BCAA supplementation on NK activity of lymphocytes. NK cell activity were also significantly improved at 3 mo of supplementation compared with that before BCAA supplementation.

In the chapter of discussion in our report, the several points were discussed as follows[46]. There were few therapies to restore the reactions of innate immunity such as phagocytic function of neutrophils and NK activity of lymphocytes. The mechanisms of the improvement by BCAA supplementation were not completely elucidated. It has been reported that BCAA supplementation promotes albumin synthesis through activation of the mTOR signal transduction system[20]. In addition, not only synthesis of albumin but HGF synthesis was enhanced by BCAA supplementation. Therefore, production of a factor which could stimulate neutrophil function of phagocytosis such as tuftsin[47,48] might be increased by BCAA by mTOR signal transduction system. Alternatively, improvement of poor nutritional status in cirrhotic patient by BCAA might restore phagocytic function of neutrophils and NK activity of lymphocytes by indirect mechanisms. In the study, phagocytic function of neutrophils and NK activity of lymphocytes were

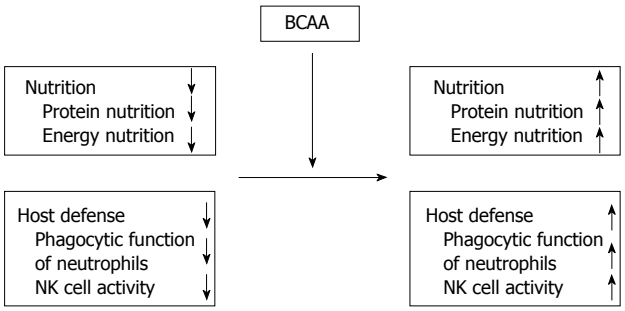

Figure 2 Restoration of nutrition state and host immune responses by branched-chain amino acids in decompensated cirrhotic patients. Branched-chain amino acids (BCAA) oral supplementation could not only improve nutrition state (protein nutrition, energy nutrition) but restore host defense mechanisms [phagocytic function of neutrophils and natural killer (NK) cell activity] in cirrhotic patients.

significantly restored before serum albumin level became statistically improved. That might imply a clue to clarify the mechanisms of effect on improvement of reactions of innate immunity by BCAA oral supplementation. To estimate the effects of BCAA supplementation on the innate host responses in cirrhotic patients, further studies on intrahepatic neutrophils, Kupffer cells and pit cells might be valuable.

Reduction of the risk of hepatocellular carcinoma development by BCAA in cirrhotic patients

A multicenter, randomized, and nutrient intake-controlled trial on the comparative effects of BCAA, conducted in 646 patients with decompensated cirrhosis, showed that the incidence of events comprising the primary endpoint (which was a composite of death by any cause, development of liver cancer, rupture of esophageal varices, or progress of hepatic failure) significantly decreased in the BCAA supplementation group as compared with the control group[19]. Marchesini *et al*[35] also reported that long-term oral supplementation of BCAA granules to cirrhotic patients were reported to improve not only their nutrition status (*i.e.*, hypoalbuminemia) but their event-free survival. In addition, Muto *et al*[49] reported that close association exists between insulin resistance due to hyperinsulinemia and BCAA, and that this association contributes to the progression of hepatocellular carcinoma (HCC) in cirrhotic patients. It was also reported that the risk for liver cancer was significantly reduced by oral BCAA supplementation in the patients with a BMI of 25 or higher. Oral supplemental treatment with BCAA might reduce the risk of liver cancer in cirrhotic patients[49]. Recent studies have revealed that BCAA supplemental therapy to patients with liver cirrhosis improves their insulin resistance and hyperinsulinemia[50,51], which can account for the reduced risk of HCC. BCAA is supposed to prevent insulin resistance through improving glucose tolerance by promoting insulin-independent glucose uptake by skeletal muscle[52].

There have been only a few reports to date regarding the suppression of liver cancer progression by BCAA. Murata *et al*[53] showed that isoleucine prevents tumor growth in a mouse liver metastatic model of colon cancer through inhibition of vascular endothelial growth factor (VEGF). Yoshiji *et al*[54] reported that BCAA exerts a chemopreventive effect against HCC, which is associated with the suppression of VEGF expression and hepatic neovascularization in obese diabetic rats. Both of these reports suggest an anti-angiogenesis activity of BCAA or Isoleucine through suppression of VEGF expression.

In the study by Miuma *et al*[55], they analyzed the expression of vascular VEGF in HepG2 cells under high-insulin culture conditions, and examined the effect of BCAA on VEGF expression. VEGF secretion was significantly increased by 200 nmol/L of insulin under BCAA deficient conditions, but it was repressed by the addition of BCAA. BCAA activated the mTOR pathway and increase HIF-1a expression under high-insulin culture conditions, however quantitative PCR analysis showed that insulin-induced expression of VEGF mRNAs decreased 2 h after the addition of BCAA. The half-lives of VEGF mRNAs were shortened in the presence of BCAA compared to the absence of BCAA. Therefore, the results of the study suggested that BCAA regulate VEGF expression mainly at the post-transcriptional level in patients who have hyperinsulinemia and are in the process of developing HCC. They also examined which of the Valine, Leucine, and Isoleucine components of BCAA were essential for VEGF mRNA degradation. All three BCAA components were revealed to be required for acceleration of insulin-induced VEGF mRNA degradation.

Recently, another study on the mechanisms of reduction of the risk of HCC development by BCAA was reported[56]. Hagiwara *et al*[56] reported the result of the study to investigate the effects of BCAA on insulin-induced proliferation of hepatic tumor cells and determine the underlying mechanisms. BCAA was reported to suppress insulin-induced cell proliferation of H4 II E, HepG2 cells. They demonstrated that BCAA inhibited PI3K/Akt pathway not only by promoting negative feedback loop from mammalian target of rapamycin complex 1 (mTORC1)/S6K1 to PI3K/Akt pathway, but also by suppressing mTORC2 kinase activity toward Akt. Their findings suggested that BCAA supplementation may be useful to suppress liver cancer progression by inhibiting insulin-induced PI3K/Akt and subsequent anti-apoptotic pathway, indicating the importance of BCAA supplementation to the obese patients with advanced liver disease.

Furthermore, NK cells were previously revealed to play important roles not only in the defense against viral infection but in tumor surveillance[57,58]. Therefore, the restoration of NK cell activity by BCAA supplementation might partly contribute to the reduction of the risk of HCC in cirrhotic patients.

CONCLUSION

BCAA oral supplementation could improve not only nutrition status (both protein nutrition and energy nutrition

) but phagocytic function of neutrophils and NK activity of lymphocytes in cirrhotic patients (Figure 2). BCAA supplementation might reduce the risk of bacterial and viral infection in patients with decompensated cirrhosis. In addition, administration of BCAA could improve glucose intolerance and hyperinsulinemia in cirrhotic patients. It has also been reported that the risk of developing HCC could be significantly reduced following long-term administration of BCAA in obese cirrhotic patients with diabetes mellitus.

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TOPIC HIGHLIGHT

WJG 20th Anniversary Special Issues (11): Cirrhosis

Does antiviral therapy reduce complications of cirrhosis?

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Abstract

Chronic hepatitis B infection is associated with the development of cirrhosis, hepatocellular carcinoma, and finally liver-related mortality. Each year, approximately, 2%-5% of patients with hepatitis B virus (HBV)-related compensated cirrhosis develop decompensation, with additional clinical manifestations, such as ascites, jaundice, hepatic encephalopathy, and gastrointestinal bleeding. The outcome of decompensated HBV-related cirrhosis is poor, with a 5-year survival of 14%-35% compared to 84% in patients with compensated cirrhosis. Because the risk of disease progression is closely linked to a patient's serum HBV DNA level, antiviral therapy may suppress viral replication, stabilize liver function and improve survival. This article briefly reviews the role that antiviral therapy plays in cirrhosis complications, particularly, in decompensation and acute-on-chronic liver failure.

Key words: Antiviral therapy; Cirrhosis; Complication; Hepatitis B; Decompensation

Core tip: The goals of antiviral therapy in hepatitis B virus-related cirrhosis would be to improve the hepatic disease severity, improve the clinical symptoms and quality of life, and prolong patient's survival. Despite the limitations, antiviral therapy with nucleos(t)ide in patients with HBV-related cirrhosis can prevent the development of complications from cirrhosis, particularly, decompensation and acute-on-chronic liver failure (ACLF). Early antiviral treatment is important for patients with severe decompensated cirrhosis and ACLF. Thus, physicians could treat these patients using lamivudine with careful monitoring for the development of resistance or using the most potent antiviral agent, such as entecavir or tenofovir.

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INTRODUCTION

Chronic hepatitis B (CHB) infection is associated with the development of cirrhosis, hepatocellular carcinoma (HCC), and finally, liver-related mortality[1,2]. According to studies of the natural course of cirrhosis, every year, 2%-5% patients with hepatitis B virus (HBV)-related compensated cirrhosis develop decompensation (*i.e.*, ascites, jaundice, hepatic encephalopathy, and gastrointestinal bleeding)[3,4]. The prognosis of patients with decompensated HBV-related cirrhosis is poor, with a 5-year survival rate of 14%-35% compared to 84% in patients with compensated cirrhosis[4,5].

Abundant evidence indicates that the risk of disease progression is closely linked to a patient's serum HBV DNA level[6-9]. A population-based prospective cohort study in Taiwan showed that the progression to cirrhosis in HBV-infected patients was strongly associated with the

serum level of the circulating virus. The risk of cirrhosis significantly increased with an elevated hepatitis B viral load and was independent of the hepatitis B e antigen (HBeAg) status and the serum alanine aminotransferase (ALT) level[2].

Currently, six drugs are approved by the US Food and Drug Administration to manage CHB: interferon (IFN) or its pegylated version, lamivudine (LAM), adefovir (ADV), entecavir (ETV), telbivudine (TBV), and tenofovir disoproxil fumarate (TDF). This article briefly reviews the effects of antiviral therapy on the complications of cirrhosis, particularly, in decompensation and acute-on-chronic liver failure (ACLF).

ANTIVIRAL THERAPY IN PATIENTS WITH COMPENSATED CIRRHOSIS

A cohort study of the natural history of compensated cirrhosis has shown that the risk of the development of a decompensation episode (*i.e.*, ascites, jaundice, hepatic encephalopathy or variceal bleeding) is higher in HBV-DNA positive patients compared to HBV-DNA negative patients (RR = 4.05, 95%CI: 1.09-15.1)[4]. A randomized study using LAM in patients with HBV-related compensated cirrhosis found that the Child-Turcotte-Pugh (CTP) scores increased in 3.9% of the patients in the LAM group compared to 7.4% in a placebo group ($P = 0.047$), and LAM significantly reduced the rate of disease progression, which was defined as an increase of at least 2 points in the CTP score, 7.8% in the LAM group *vs* 18% in the placebo group ($P = 0.001$)[10]. A study evaluated the effect of LAM on portal pressure using hepatic venous pressure gradient (HVPG), which precisely reflects portal pressure[11]. Among the 19 patients with HVPG > 10 mmHg, HVPG significantly decreased at 12 mo of LAM therapy (14.4 mmHg *vs* 12.4 mmHg, $P = 0.007$). These data suggest that long-term LAM treatment can prevent complications from chronic HBV infection.

Another study evaluated the long-term results in 69 patients treated with ETV using liver biopsy after a median of 6 years[12]. Overall, 88% had a reduction in Ishak fibrosis score by ≥ 1 points including all 10 patients with advanced fibrosis or cirrhosis at baseline (Ishak score ≥ 4). A randomized double blind comparison of TDF with ADF has reported the long-term effect of TDF in patients with CHB, including patients with advanced fibrosis. Among the 641 patients enrolled the trial, 96 (28%) had cirrhosis at baseline with Ishak fibrosis scores ≥ 5, 71 (74%) of the patients with cirrhosis initially had reduced fibrosis (≥ 1 unit score decrease) at year 5 and were no longer cirrhotic[13]. These findings suggest the role of antiviral therapy in cirrhosis regression.

ANTIVIRAL THERAPY IN PATIENTS WITH DECOMPENSATED CIRRHOSIS

Until now, liver transplantation has been the ultimate therapeutic option for decompensated cirrhosis. However, because of the shortage of donor organs, transplantation has not been an option for many patients. Thus, the management goal for decompensated cirrhosis is to reduce disease-related complications and the liver-related mortality rate.

Patients with decompensated HBV-related cirrhosis tend to have low or undetectable HBV DNA levels. However, some patients have high rates of HBV replication with high serum HBV DNA levels. The natural history of decompensated HBV-related cirrhosis is influenced by the levels of HBV replication, and sustained viral suppression may result in reduced hepatic necroinflammation and fibrosis progression, thereby preventing decompensation in patients with cirrhosis[14].

Among the antiviral therapy options, IFN has been associated with serious complications, including life-threatening hepatitis flares and infectious complications in decompensated HBV-related cirrhosis[15,16]. In contrast, oral nucleos(t)ide analogues (NAs) are well-tolerated in patients with decompensated HBV-related cirrhosis. Most clinical guidelines strongly recommend using oral NAs for patients with decompensated HBV-related cirrhosis independent of the HBV DNA levels[17,18].

LAM is a nucleoside analogue that inhibits HBV DNA synthesis. Yao *et al*[19]. showed a dramatic decline in the CTP scores (≥ 3 points) of 69% of the severely decompensated cirrhosis patients with LAM treatment. In 38% of patients, the CTP scores decreased to < 7, and their statuses on the United Network of Organ Sharing waiting list changed to inactive. A randomized controlled trial in Asia demonstrated less liver-related morbidity in the LAM-treated patients with HBV-associated advanced compensated cirrhosis compared to the untreated controls because of the reduced incidence of hepatic decompensation and lower risk of HCC. Increased CTP scores were noted in 3.4% of the patients in the LAM group compared to 8.8% of the patients in the placebo group ($P = 0.02$). Variceal bleeding occurred in 2 patients in the LAM group *vs* 3 patients in the placebo group. Spontaneous bacterial peritonitis and liver-related death did not occur in either group[10]. LAM generally improved the liver functions and prognosis of the patients with HBV-related cirrhosis. However, some of the patients died or underwent liver transplantation. Another study evaluated patients with decompensated HBV-related cirrhosis treated with LAM and found that most deaths occurred within the first 6 mo because of hepatic failure complications. Elevated pretreatment serum bilirubin, creatinine and HBV DNA levels were significantly associated with 6-mo mortality rates[20]. This finding suggests that early treatment with antiviral agents might be important.

During long-term LAM therapy, a substantial proportion of cirrhotic patients exhibited viral resistance to LAM and virologic response loss[21,22]. LAM resistance develops in up to 70% of patients after 5 years of continuous therapy, with an annual incidence of up to 20%

in antiviral-naïve patients[18]. On the contrary, the resistance rate to ETV at 4 years of treatment is ≤ 0.5% in antiviral-naïve patients[23]. Thus, LAM treatment is no longer considered to be the first-line therapy in CHB or cirrhosis patients because of its lower genetic barrier and higher resistance rate compared to ETV or TDV[24].

ADV is an acyclic nucleotide analogue of adenosine monophosphate. A total of 128 patients with LAM-resistant HBV-related decompensated cirrhosis were treated with 10 mg/d of ADV and achieved undetectable serum HBV DNA levels (< 400 copies/mL) in 81% of cases; the CTP scores improved in ≥ 90% of the patients at 48 wk of treatment[25]. In a long-term follow-up study of up to 240 wk, 73% of the patients showed improved fibrosis compared to the baseline. However, the cumulative probability of subsequent genotypic resistance to ADV was 20%, and renal toxicity was confirmed in 3% of patients[26]. Although ADV has a better genetic resistance profile than LAM, a lower antiviral potency and the potential risk of nephrotoxicity remain a concern for routine use as a first-line treatment in patients with HBV-related decompensated cirrhosis.

ETV is a cyclopentyl guanosine analogue that shows potent inhibition of the priming, DNA-dependent synthesis and reverse transcription of the HBV polymerase. ETV has a more potent activity against wild type HBV compared with LAM or ADV[27,28]. Several studies have used ETV in cirrhotic patients. A retrospective study from Korea evaluated the effect of ETV in 70 CHB patients with decompensated cirrhosis (CTP scores ≥ 7)[29]. Compared to the baseline, the 55 patients treated with ETV for 12 mo showed improved CTP (8.1 *vs* 6.6, respectively, $P < 0.05$) and Model for End-Stage Liver Disease (MELD) scores (11.1 *vs* 8.8, respectively, $P < 0.05$). The 2-year cumulative incidence of HCC was 6.9%, and the cumulative death rate was 17%. Theses findings suggest that ETV monotherapy improves hepatic function and provides overall benefits that are comparable to antiviral therapy in patients with HBV-related decompensated cirrhosis.

A randomized, open-label study compared the efficacy of ETV 1.0 mg ($n = 100$) or ADV 10 mg ($n = 94$) daily for up to 96 wk in subjects with decompensated cirrhosis (CTP scores ≥ 7)[30]. ETV showed more profound reductions in the HBV DNA levels than ADV (treatment difference, -1.74 log copies/mL, $P < 0.0001$). The ETV group showed a greater reduction of the HBV DNA levels at all time points through week 48 and a greater proportion of subjects who achieved HBV DNA < 300 copies/mL at weeks 24 (ETV 49% *vs* ADV 16%, $P < 0.0001$) and 48 (ETV 57% *vs* ADV 20%, $P < 0.0001$). In both groups, two-thirds of the subjects showed improvement or stabilization in CTP and MELD score. The cumulative HCC and death rates were 12% and 23% for ETV, respectively, and 20% and 33% for ADV, respectively. However, mean time to HCC and HCC-free survival did not differ significantly between the groups. These findings suggest that although clinical benefits were demonstrated in both groups, ETV is superior to ADV in its virologic efficacy through week 48.

A recent study investigated the efficacy of ETV on the clinical outcomes of two cohorts: the ETV cohort (subjects administered ETV 0.5 mg/d) and the historical control cohort (subjects who underwent routine clinical care). In the patients with cirrhosis (482 ETV-treated patients, 69 treatment-naïve patients), the ETV-treated patients had reduced risks for all clinical outcomes compared to the treatment-naïve patients after adjusting for the MELD score: hepatic events (HR = 0.51, 95%CI: 0.34-0.78, $P = 0.002$); HCC (HR = 0.55, 95%CI: 0.31-0.99, $P = 0.049$); liver-related mortality (HR = 0.26, 95%CI: 0.13-0.55, $P < 0.001$); and all-cause mortality (HR = 0.34, 95%CI: 0.18-0.62, $P < 0.001$). However, the risk for hepatic events in the ETV-treated cirrhotic patients who failed to achieve undetectable HBV DNA levels was comparable to that in the untreated patients.

TBV is a synthetic thymidine nucleoside analogue with potent antiviral activity against HBV. A double-blind randomized trial using TBV ($n = 114$) and LAM ($n = 114$) for 104 wk has shown that TBV was an independent predictive factor for HBV DNA < 300 copies/mL and ALT normalization ($P = 0.037$) in patients with HBV-related decompensated cirrhosis (CTP score ≥ 7)[31]. The changes in the CTP and MELD scores were comparable between both the groups. Cumulatively, 27% of the TBV patients and 36% of the LAM patients developed genotypic resistance during the 2-year period. These results suggest that because of the significant rate of virologic breakthrough, TBV has limitations as a first-line therapy in patients HBV-related decompensated cirrhosis.

TDF is an ancyclic nucleotide analogue and a potent inhibitor of HBV polymerase/reverse transcriptase. A multicenter study in Turkey determined the long-term effects of LAM, ETV and TDF. The mean CTP score change was comparable in 3 groups. A minimum 1-point decrease in the CTP score occurred in 29.6% of patients in the TDF group, 37.7% of patients in the ETV group and 21.9% of patients in the LAM group ($P = 0.35$). The MELD score (per year) decreased more in the TDF group than in the ETV group ($P = 0.04$). Regarding the complications from cirrhosis, variceal bleeding-free time and encephalopathy free-time were longer in the ETV group than in the TDF group, but theses differences were not statistically significant ($P = 0.38$ and $P = 0.87$, respectively)[32]. Until now, TDF has been the most potent suppressor of HBV replication, and no-resistance has been reported. Considering both the potency and resistance profiles, TDF and ETV are superior to LAM, ADV and TBV and can be considered to be a first-line therapy in HBV-related decompensated cirrhosis.

ANTIVIRAL THERAPY IN PATIENTS WITH ACUTE SEVERE EXACERBATION OR ACUTE ON CHRONIC LIVER FAILURE

The definition of reactivation of hepatitis B is the reap-

pearance of necroinflammatory activity of the liver in a person known to be in an inactive HBsAg carrier state or with resolved hepatitis B[33]. The definition of flare or exacerbation is the intermittent elevation of aminotransferase activity to > 10 times the upper limit of normal and more than twice the baseline value[34]. Severe acute exacerbations characterized by high ALT level, jaundice and hepatic decompensation may progress to ACLF with sepsis-like immunological changes[35]. ACLF is defined as an acute hepatic insult with manifestations of jaundice and coagulopathy (INR > 1.5) that are complicated within 4 wk by ascites and/or encephalopathy in a patient with previously diagnosed or undiagnosed compensated chronic liver disease[36]. ACLF is accompanied by the development of multi-organ failure, thereby leading to a high mortality rate. The prognosis of ACLF in CHB is poor, with 3-mo mortality rates > 50% without liver transplantation. Liver transplantation remains the only definite therapeutic option for these patients. In recent years, data have emerged regarding the efficacy of NA.

IFN-based therapy may aggravate the hepatic decompensation in the severe exacerbation of CHB. Previous studies using LAM for patients with severe acute exacerbation have not shown any survival benefit from antiviral therapy[37,38]. However, there is a study that suggests the beneficial effects of antiviral treatment on short-term survival. Among the patients with severe acute exacerbation of CHB, more patients in the LAM treatment group with baseline bilirubin levels < 20 mg/dL survived compared to patients in the control group (P = 0.013). However, the mortality rates in the two groups did not differ among the patients with baseline bilirubin level ⩾ 20 mg/dL[39]. Another prospective study from Hong Kong used ETV (n = 36) and LAM (n = 117) to evaluate the overall mortality rate at week 48 in patients with severe acute CHB exacerbation. By week 48, the patients in the ETV group had a higher liver-related mortality rate (P = 0.044) and more liver-related complications than the LAM group despite their better virological responses (P = 0.007). However, the cause of the increased mortality in the ETV group is not completely understood[40]. Lactic acidosis might be one possible cause[41], but this finding must be confirmed by other centers. This study has a limitation of small number of patients in the ETV group.

A matched retrospective cohort study from China using patients with ACLF showed that the 3-mo mortality rate in the LAM group (n = 130) was lower than that of the control group (n = 130), with a MELD score of 20-30 (50.7% *vs* 75.7%, P = 0.0021)[42]. A retrospective study compared ETV 0.5 mg (n = 33), LAM 100 mg (n = 34) and no-NA (n = 37) in patients with HBV-associated ACLF. The HBV DNA levels and the ACLF recurrence rate were lower in both treatment groups. However, no significant difference in the 3-mo mortality rate was found (51.5% for ETV, 50% for LAM and 59.5% for no-NA)[43]. A recent study from China compared the short-term and long-term efficacies of ETV, LAM or no-NA in patients with HBV-related ACLF. The ETV and LAM groups showed similar cumulative mortality rates in the first 3 mo of treatment (P = 0.374). The no-NA group had a significantly higher mortality rate compared with the ETV group (P = 0.007) and the LAM group (P = 0.006). The recurrence of ACLF was found in 33.3% of patients in the no-NA group, 11.1% from the LAM group and 0% from the ETV group (P = 0.003)[44].

A prospective randomized study from India using TDF (n = 14) and placebo (n = 13) in patients with ACLF from the spontaneous reactivation of CHB showed that TDF significantly reduced the HBV DNA levels and improved the CTP and MELD scores and the 3-mo survival rate compared to a placebo [8/14 (57%) *vs* 2/13 (15%), P = 0.03]. A > 2 log reduction in the HBV DNA levels at 2 wk was found to be an independent predictor of survival[45]. However, the limitations of this study include its small sample size and the unavailability of liver transplantation.

Although most patients with acute exacerbation of CHB resolve spontaneously, a certain proportion of patients may progress to liver failure and death. Antiviral therapy has no obvious benefit for short-term survival. However, antiviral treatment may prevent future exacerbation and ongoing hepatic injury. Thus, an antiviral agent should be administered as early as possible and liver transplantation should be considered for patients with severe disease.

CONCLUSION

The goals of antiviral therapy in HBV-related cirrhosis would be to stabilize or improve the hepatic disease severity, improve the clinical symptoms and quality of life, and extend patient survival. Despite the limitations of the existing data, it is likely that antiviral therapy with nucleos(t)ide in patients with HBV-related cirrhosis can prevent the development of complications from cirrhosis, particularly decompensation and ACLF. In addition, early antiviral treatment is important for patients with severe decompensated cirrhosis and ACLF. Thus, hepatologists could treat these patients using LAM with careful monitoring for the development of resistance or using the most potent antiviral agent such as ETV or TDF.

However, there are several unmet needs with regard to antiviral agents. First, the designs of published studies have been heterogeneous and the sample sizes have been small and until now, the antiviral agent could not reverse advanced cirrhosis. Second, because the NAs are less effective against cccDNA formation in the hepatocyte, antiviral therapy should be maintained for life. Third, the long-term safety of NAs has not been confirmed in cirrhosis patients. The carcinogenicity of ETV has been reported in rodents after exposure to doses > 35-fold the dose administered in humans. Thus, the cumulative risk of human will require post-marketing surveillance. Regarding TDF, patients with pre-existing renal impairment may be at risk of nephrotoxicity from TDF and decreases in bone mineral density have been rarely reported in

HIV-positive patients treated with TDF. Fourth, patients with drug-resistance are limited in their choices of new antiviral agents.

Several innovative antiviral approaches have been evaluated *in vitro* and in animal models, such as selectively targeting antiviral agents to the liver or using antisense approaches, RNA interference, and HBV-specific immunomodulatory therapy (*i.e.*, S and pre-S antigen vaccines, DNA vaccination, T-cell vaccines and adoptive immunity transfer), alpha-glucosidase inhibitor derivatives, and monoclonal antibodies). Further studies are needed to examine patient outcomes after using newer antiviral therapy to prevent the complications of cirrhosis.

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TOPIC HIGHLIGHT

WJG 20th Anniversary Special Issues (11): Cirrhosis

Pathogenesis of liver cirrhosis

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Abstract

Liver cirrhosis is the final pathological result of various chronic liver diseases, and fibrosis is the precursor of cirrhosis. Many types of cells, cytokines and miRNAs are involved in the initiation and progression of liver fibrosis and cirrhosis. Activation of hepatic stellate cells (HSCs) is a pivotal event in fibrosis. Defenestration and capillarization of liver sinusoidal endothelial cells are major contributing factors to hepatic dysfunction in liver cirrhosis. Activated Kupffer cells destroy hepatocytes and stimulate the activation of HSCs. Repeated cycles of apoptosis and regeneration of hepatocytes contribute to pathogenesis of cirrhosis. At the molecular level, many cytokines are involved in mediation of signaling pathways that regulate activation of HSCs and fibrogenesis. Recently, miRNAs as a post-transcriptional regulator have been found to play a key role in fibrosis and cirrhosis. Robust animal models of liver fibrosis and cirrhosis, as well as the recently identified critical cellular and molecular factors involved in the development of liver fibrosis and cirrhosis will facilitate the development of more effective therapeutic approaches for these conditions.

Key words: Cirrhosis; Pathogenesis; Hepatic stellate cells; Cytokine; miRNA; Animal model; Therapy

Core tip: Cirrhosis is the end-stage condition of many types of chronic liver diseases but the underlying mechanisms are far from being clarified. Multiple cellular and molecular factors might be involved in the initiation and progression of cirrhosis. Activation of hepatic stellate cells is a pivotal event in the development of cirrhosis. Animal models are crucial for understanding the pathogenesis and the development of more efficient therapeutic strategies for cirrhosis, with which cirrhosis may become a treatable or even a reversible disease.

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INTRODUCTION

Liver cirrhosis is the final common pathological pathway of liver damage arising from a wide variety of chronic liver diseases[1-3]. The etiology of cirrhosis varies geographically, with alcoholism, chronic hepatitis C virus infection, and nonalcoholic fatty lives disease (NAFLD) being the most common causes in western countries[4-6], whereas chronic hepatitis B is the primary cause of liver cirrhosis in the Asia-Pacific region[7-9]. Liver cirrhosis has many other causes, include inherited diseases such as hemochromatosis and Wilson's disease[10-14], primary biliary cirrhosis, primary sclerosing cholangitis[15-18], and autoimmune hepatitis[14,19]. Some cases are idiopathic or crypto-

genic. In recent decades, NAFLD has become a leading cause of chronic liver disease in Western countries such as the United States, with a prevalence of as high as 30% in the general population[20]. Thus, NAFLD has attracted extensive attention as an important cause of chronic liver diseases[21-23].

Although the causes of liver cirrhosis are multifactorial, there are some pathological characteristics that are common to all cases of liver cirrhosis, including degeneration and necrosis of hepatocytes, and replacement of liver parenchyma by fibrotic tissues and regenerative nodules, and loss of liver function[24-27]. Fibrosis as a precursor of cirrhosis is a pivotal pathological process in the evolution of all chronic liver diseases to cirrhosis[2,28]. At present, effective strategies to treat liver cirrhosis are still lacking, partially because of a poor understanding of the molecular mechanisms leading to cirrhosis. Thus, a better understanding of the pathogenesis of liver cirrhosis would facilitate the development of more effective treatment options.

In this review, we aim to summarize the recent advance in the molecular pathogenesis, animal models, and therapeutic strategies for liver cirrhosis.

MULTIPLE CELL TYPES CONTRIBUTE TO PATHOGENESIS OF LIVER CIRRHOSIS

The liver is formed by parenchymal cells (*i.e.*, hepatocytes) and other cells commonly known as nonparenchymal cells. The walls of hepatic sinusoids are lined by three different nonparenchymal cells: liver sinusoidal endothelial cells (LSECs), Kupffer cells (KCs), and hepatic stellate cells (HSCs). Both hepatic parenchymal and nonparenchymal cells are involved in the initiation and progression of liver fibrosis and cirrhosis.

HSCs

HSCs, formerly known as fat-storing cells, Ito cells, lipocytes, perisinusoidal cells, or vitamin A-rich cells, reside in the space of Disse in the normal liver and their main function is storage of vitamin A and other retinoids[27,29]. Following multiple injurious insults and/or exposure to inflammatory cytokines such as platelet-derived growth factor (PDGF), transforming growth factor (TGF)-β, tumor necrosis factor (TNF)-α, and interleukin (IL)-1, HSCs undergo the transition from a quiescent to activated state. HSC activation is a pivotal event in initiation and progression of hepatic fibrosis and a major contributor to collagen deposition[30,31]. Activation of HSCs is characterized by cell proliferation and migration, contraction after transforming into myofibroblasts, generation of a large amount of collagen and other extracellular matrix (ECM), ultimately leading to liver fibrosis[32-34].

LSECs

LSECs constitute the sinusoidal wall, also called the endothelium, or endothelial lining. The structural characteristic of LSECs is the fenestrae on the surface of the endothelium[28,35,36] The endothelial fenestrae measure 150-175 nm in diameter, and act as a dynamic filter facilitating the exchange of fluids, solutes and particles between sinusoidal blood and the parenchymal cells[37-39]. LSECs have high endocytotic capacity[28,40]. Chronic alcohol abuse could result in defenestration, and a decrease in the number of fenestrae[37,41]. In cirrhotic liver, defenestration of sinusoidal endothelium and the presence of a subendothelial basement membrane are frequently present[35,42]. It is known that retinol deficiency can activate and transform HSCs into myofibroblasts with enhanced ECM production, resulting in perisinusoidal fibrosis and ultimately in cirrhosis[24,35]. Defenestration and capillarization of the hepatic endothelium are believed to be important in the initiation of perisinusoidal fibrosis by altering retinol metabolism. Studies in animals and humans have revealed that LSECs can secrete the cytokine IL-33 to activate HSCs and promote fibrosis[43]. Defenestration and capillarization of LSECs lead to impaired substrate exchange and are considered major contributing factors for hepatic dysfunction in liver cirrhosis[39]. On the contrary, differentiated LSECs can promote reversion of activated HSCs to quiescence and thereby accelerate regression and prevent progression of fibrosis through vascular endothelial growth factor (VEGF)-stimulated NO production[44,45].

KCs

KCs, also known as Browicz-Kupffer cells and stellate macrophages, are specialized macrophages located in the lining walls of the sinusoids of the liver that form part of the reticuloendothelial system (RES)[46]. Studies in animal models have shown that KCs are implicated in the pathogenesis of various liver diseases[47,48]. KCs can be activated by many injurious factors such as viral infection, alcohol, high-fat diet, and iron deposition. Activated KCs destroy hepatocytes by producing harmful soluble mediators and serving as antigen-presenting cells during viral infection[47]. KC-mediated hepatic inflammation is considered to aggravate liver injury and fibrosis[49,50]. KCs are involved in the activation of HSCs and formation of fibrosis. *In vitro* studies have shown that KC-conditioned medium can promote activation of cultured rat HSCs with enhanced matrix synthesis and cell proliferation by eliciting expression of PDGF receptor in HSCs[51]. KC-derived TGF-β1 stimulates proliferation and collagen formation of HSCs derived from rats fed with high-fat diet and ethanol[52,53]. Alcohol can induce the circulating level of Gram-negative bacterial lipopolysaccharide (LPS), which is a strong activator of KCs[54]. In genetic hemochromatosis, iron overload in KCs could induce the expression of intercellular adhesion molecule (ICAM)-1 on hepatocytes, therefore facilitating activation of HSCs and collagen deposition in the hepatic tissues[55]. Gelatinase secreted by activated KCs triggers the phenotypic change in HSCs by degrading collagen type Ⅳ[56]. KCs engulf apoptotic bodies and produce death ligands, in-

cluding Fas ligand and TNF-α, thereby promotes inflammation and fibrogenesis[57]. In addition, KCs activated by β-glucans increase portal pressure through the release of thromboxane A2 in normal and fibrotic livers[58].

Hepatocytes

Hepatocytes are the primary liver parenchymal cells, and play complicated roles in fibrosis and cirrhosis. Hepatocytes are targets for most hepatotoxic agents, including hepatitis viruses, alcohol metabolites, and bile acids[59]. Chronic liver diseases either promotes apoptosis or trigger compensatory regeneration of hepatocytes[60]. Damaged hepatocytes release reactive oxygen species (ROS) and fibrogenic mediators, induce activation of HSCs, and stimulate the fibrogenic actions of myofibroblasts[59]. Apoptosis of hepatocytes is a common event in liver injury and contributes to tissue inflammation, fibrogenesis, and development of cirrhosis. Steatohepatitis enhances Fas-mediated hepatocyte apoptosis, which correlates with active nuclear factor (NF)-κB and disease severity[61]. Both HCV infection and ethanol consumption induce hepatocyte apoptosis in animal models and humans, and induction may be related to downregulation of Bcl-2 signaling[62]. Chronic HCV infection can induce hepatocyte G1 arrest and impair hepatocellular function and limit hepatic regeneration[63,64]. In CCl_4-induced liver injury, hepatocyte apoptosis is induced at the early phase, which is followed by constant proliferation and if it persists, liver cirrhosis ensues at a later stage[65]. Hepatocytes are the major sources of matrix metalloproteinases (MMP-2, MMP-3 and MMP-13) and tissue inhibitors of matrix metalloproteinases (TIMP-1 and TIMP-2); all of which are involved in the pathogenesis of liver cirrhosis in CCl_4-induced liver cirrhosis in rats[66]. In the last fibrotic stage or cirrhosis, hypoxic hepatocytes become a predominant source of TGF-β1, further exacerbating hepatic fibrogenesis[67]. Recently, it has been shown that hepatocyte telomere shortening and senescence can result in fibrotic scarring at the cirrhosis stage, presenting a novel explanation for the pathophysiology of cirrhosis[68].

ROLE OF CYTOKINES IN LIVER FIBROSIS AND CIRRHOSIS

Liver cirrhosis is orchestrated by a complex network of cytokine-mediated signaling pathways regulating the activation of HSCs and fibrogenesis.

PDGF

PDGF is the strongest mitogen to HSCs among all polypeptide growth factors. PDGF family has four members, PDGF-A, -B, -C and -D[69]. PDGF and its receptors are markedly overexpressed in fibrous tissues, and its activity increases with the degree of liver fibrosis[70-72]. A variety of factors such as viruses, chemicals, or mechanical damage to hepatocytes can induce KCs to synthesize and release PDGF[73]. Upon binding to its specific receptor on the membrane of HSCs, PDGF activates corresponding signal molecules and transcription factors, leading to the activation of its downstream target genes and activation of HSCs[74,75]. PDGF has been shown to upregulate the expression of MMP-2, MMP-9 and TIMP-1, and inhibit the activity of collagenase, thereby reducing ECM degradation[69,75]. PDGF-B and PDGF-D are potent PDGF isoforms in PDGF receptor (PDGFR)β signaling within HSCs, as evidenced by PDGFRβ autophosphorylation and activation of extracellular signal-regulated kinase (ERK)1/2, C-Jun N-terminal kinase (JNK), p38 mitogen-activated protein kinase (MAPK), and protein kinase (PK)B/Akt pathways[75-77]. PDGF-D can activate HSCs and exerts mitogenic and fibrogenic effects, and therefore plays an important role in matrix remodeling in liver fibrosis[72].

TGF-β

TGF-β is the strongest known inducer of fibrogenesis in hepatic fibrosis[78,79]. TGF-β is mainly synthesized by HSCs/myofibroblasts, KCs, LSECs, and hepatocytes in the liver. The TGF-β1 family is composed of six members, and among them, TGF-β1 has been shown to play a key role in the initiation and maintenance of liver fibrosis[78-82]. The expression level of TGF-β1 is increased in fibrotic liver and reaches a maximum at cirrhosis[67]. The pro-fibrogenesis effect of TGF-β1 is complicated, involving multiple aspects: the primary effect of TGF-β1 is to stimulate activation of HSCs, and the TGF-β1 autocrine loop in activated HSCs is an important positive feedback to the progression of liver fibrosis[80,81]. TGF-β1 induces expression of the matrix-producing genes and inhibits degradation of ECM by downregulating expression of MMPs and promoting TIMP, leading to excessive deposition of collagenous fibers and promoting the development of liver fibrosis[82,83]. In addition, TGF-β1 has been shown to inhibit DNA synthesis and induces apoptosis of hepatocytes. TGF-β1-induced apoptosis is thought to be responsible for tissue loss and decrease in liver size seen in cirrhosis[78]. Given the critical role of TGF-β1 in the pathogenesis of liver cirrhosis, specific blockade of TGF-β1/Smad3 signaling has shown some therapeutic value for liver fibrosis[82].

TNF-α

TNF-α is mainly produced by monocyte, macrophage, HSCs, and KCs. It has proinflammatory activities and cytotoxic effects in these cells. In the process of liver fibrosis, TNF-α plays an important role in the activation of HSCs and synthesis of ECM[84,85]. TNF-α can reduce the spontaneous apoptosis of activated rat HSCs by upregulating the antiapoptotic factors NF-κB, Bcl-XL and $p21^{WAF1}$, as well as downregulating the proapoptotic factor p53[86]. However, the effects of TNF-α on HSCs and fibrosis are complicated and even paradoxical, as demonstrated by studies showing that TNF-α could induce apoptosis in HSCs[87]. TNF-α has also been shown to exert antifibrogenic effect in rat HSCs by reducing

glutathione and inhibiting pro-collagen α1 expression[88]. In a rat model of nonalcoholic steatohepatitis (NASH), TNF-α antibody was shown to reduce the inflammation, necrosis and fibrosis in liver[89]. TNF-α signaling through activation of KCs plays an essential role in the pathogenesis of liver fibrosis in animal models of NASH[90].

Interferon

Interferon (IFN) is a family of soluble extracellular signaling molecules. Leukocytes synthesize IFN-α and IFN-β in response to virus infection, and T cells secrete IFN-γ upon stimulation with various antigens and mitogens. IFNs possess antiviral activity and is well-recognized for their antiviral effects[91]. Patients treated with IFNs exhibit a regression of liver fibrosis even if viral eradication is not achieved, indicating that IFN itself has antifibrotic activity via triggering the apoptosis of HSCs[92]. IFN-β could inactivate HSCs and decrease their production of α-smooth muscle actin (SMA) and collagen through inhibition of the TGF-β and PDGF pathways[93]. Similarly, IFN-γ has been demonstrated to reduce ECM deposition *in vivo* by inhibiting HSC activation via TGFβ1/Smad3 signaling pathways[94,95]. Treatment of rats with fibrosis by IFN-γ led to a reduced production and deposition of collagen, laminin, fibronectin, and pro-collagen type I in liver[95]. However, the effect of IFNs on fibrosis is not consistent, as demonstrated by a recent study showing that IFN-α and IFN-γ may exert opposite effects on apoptosis in HSCs. IFN-α was shown to elicit an antiapoptotic effect on activated HSCs, whereas IFN-γ was found to exert pro-apoptotic effect on HSCs by downregulating heat-shock protein 70[96].

ILs

ILs are a group of cytokines initially found to be expressed by leukocytes, but later on were shown to be produced by a wide variety of cells, such as CD4 T lymphocytes, monocytes, macrophages, and endothelial cells. ILs have a complicated role in immune response, inflammation, and liver fibrogenesis.

Pro-fibrogenic ILs: KCs and SECs can rapidly produce ILs in response to liver tissue damage. IL-1 can directly activate HSCs and stimulate them to produce MMP-9, MMP-13 and TIMP-1, resulting in liver fibrogenesis. In contrast, IL-1-receptor-deficient mice are less likely to sustain liver damage and exhibit reduced susceptibility to develop fibrosis[97]. Deficiency of IL-1α or IL-1β also makes the mice less susceptible to develop liver fibrosis in animal models of steatohepatitis[98]. Similarly, IL-1 receptor antagonists were found to protect rats from developing liver fibrosis in response to dimethylnitrosamine[99], and blocking IL-1 signaling could markedly attenuate alcohol-induced liver inflammation and steatosis. IL-1β was reported to increase the inflammatory and pro-steatotic chemokine monocyte chemoattractant protein-1 in hepatocytes, and augment Toll-like receptor (TLR4)-dependent upregulation of inflammatory signaling in macrophages[100].

Another profibrotic cytokine is IL-17, whose expression level increases with degree of liver fibrosis[101,102], indicating that IL-17 may be involved in disease progression and chronicity[101]. Studies in mice have shown that IL-17 induces liver fibrosis through multiple mechanisms, including upregulation of TNF-α, TGF-β1, and collagen 1α, which is dependent on signal transducer and activator of transcription (STAT)3 signaling pathway, and promotion of myofibroblast-like change of HSCs[102,103].

Antifibrogenic ILs: IL-10 is a cytokine that downregulates the proinflammatory response and has a modulatory effect on hepatic fibrogenesis[104,105]. IL-10 may have therapeutic potential for patients with HCV-related liver fibrosis who do not respond to IFN-based therapy[105]. IL-10 has been shown to exert antifibrotic effects through inhibiting HSC activity[106], and this was demonstrated in a rat model in which exogenous IL-10 was shown to reverse CCl_4-induced hepatic fibrosis by inhibiting the expression of TGF-β1, MMP-2 and TIMP-1[104,106,107].

IL-22 is known to play a key role in promoting antimicrobial immunity, inflammation, and tissue repair at barrier surfaces. IL-22 has been shown to induce HSC senescence, restrict liver fibrosis, and accelerate the resolution of liver fibrosis during recovery in a mouse model[108].

IL-6 is a pleiotropic cytokine involved in inflammatory pathways, hematopoiesis and immune regulation. IL-6 can attenuate apoptosis and promote regeneration of hepatocytes through NF-κB signaling and the Ras-MAPK pathway[109]. IL-6 reduces CCl_4-induced acute and chronic liver injury and fibrosis[110]. Pretreatment of fibrotic liver with IL-6 improves hepatic microenvironment and primes it for mesenchymal stem cell transplantation, leading to improvement in liver injury after fibrosis[111]. Meanwhile, increased blood level of IL-6 has been found in patients with NAFLD, and IL-6 could induce insulin resistance and inflammation in the liver[112,113], suggesting that IL-6 may play a role in the development of NAFLD.

miRNAS AND CIRRHOSIS

miRNAs represent a family of small noncoding RNAs controlling translation and transcription of many genes, which have recently emerged as post-transcriptional regulators. miRNAs play a key role in various hepatic pathologies, including hepatitis, cirrhosis and hepatoma[34,114]. miRNAs may play pro- and antifibrogenic roles, depending on cellular context and the nature of the stimuli.

Profibrogenic miRNA

miR-21 has an important role in the pathogenesis and progression of hepatic fibrosis. miR-21 can downregulate TGF-β expression and suppress HSC activation[115]. TGF-β1 induces expression of miR-181a and miR-181b,

and the latter can promote HSC proliferation by regulating p27 and the cell cycle. Elevation of serum level of miR-181b is suggested as a potential diagnostic biomarker for patients with cirrhosis[116].

miR-214-5p can increase expression of fibrosis-related genes (such as MMP-2, MMP-9, α-SMA, and TGF-β1) in LX-2 cells, and therefore, it may play crucial roles in HSC activation and progression of liver fibrosis[117].

miR-221 and miR222 are upregulated in human liver in a fibrosis progression-dependent manner and in mouse models of liver fibrosis. TGF-α or TNF-α induce expression of miR-222, which can bind to the CDKN1B (p27) 3'-untranslated region (UTR) and regulate expression of the corresponding protein[118].

Other fibrosis-associated miRNAs have been identified. For example, miR-199a, miR-199a*, miR-200a, and miR-200b were positively and significantly correlated with progression of liver fibrosis in both mouse and human studies. Overexpression of these miRNAs significantly increases the expression of fibrosis-related genes in HSCs[119]. miR-571 is upregulated in human hepatocytes and HSCs in response to TGF-β[120].

Antifibrogenic miRNAs

miRNA-150 and miRNA-194 are reduced in HSCs isolated from experimental rats with liver fibrosis. It has been demonstrated that these two miRNAs inhibit HSC activation and ECM production, at least in part, via inhibition of c-myb and rac1 expression[121]. In contrast, several miRNAs such as miR-29, miR 19b, miR-146a, and miR-133a are markedly downregulated in HSCs isolated from experimental animals with liver fibrosis, and restoration of these miRNAs attenuates hepatic fibrogenesis[30,122,123].

It is now thought that miRNAs can serve as biomarkers for HSC activation and liver fibrosis progression, and may represent therapeutic targets for hepatic fibrosis and cirrhosis.

ANIMAL MODELS OF LIVER FIBROSIS AND CIRRHOSIS

Animal models are crucial to understanding the pathogenesis and development of therapeutic strategies for liver fibrosis and cirrhosis. So far, many types of animal model have been developed in mice, rats, rabbits, and pigs to mimic the complicated process of fibrosis and cirrhosis. Animal models of liver fibrosis and cirrhosis can be induced by one of the following approaches: (1) Fibrosis induced by chemical compounds and toxins. These agents cause direct injury to hepatocytes and trigger secondary inflammatory reaction in the liver, which in turn activate HSCs and result in fibrosis. Commonly used chemical agents include CCl_4[124-126], thioacetamide[127,128] dimethylnitrosamine[129,130], dioxin[131], sodium arsenate[132], and ethanol[126,133,134]. These agents can be administered to experimental animals alone or in combination; (2) Special diet, such as choline-deficient, *L*-amino acid-defined, methionine-deficient diet[89,135,136], and high-fat diet[134,137,138]. Animals develop NAFLD and cirrhosis when they are fed these diets alone or in combination with other chemical agents; (3) Physical methods. Bile duct ligation creates obstruction of the extrahepatic bile duct[139,140], leading to cholestasis and subsequent injury to biliary epithelial cells and hepatocytes, infiltration of inflammatory cells in the portal area, fibrous tissue proliferation, and formation of liver fibrosis; (4) Fibrosis induced by immune reaction. Antigen-antibody complexes can provoke type Ⅲ hypersensitive reactions. Deposition of immune complexes in the portal area and around the central vein area causes allergic reaction and inflammation, stimulating HSCs to secrete collagen, and fibrosis formation. Common immunogens include plant protein concanavalin A[141] and xenogenic serum[142,143], such as serum from pigs, cattle, humans, and schistosoma. It was reported that 85.5% of rats immunized with subcutaneous injection of human serum albumin develop liver fibrosis and cirrhosis[144]. Similarly, injection of the excretory-secretory (ES) antigens of *Ascaris suum* into golden hamsters also successfully induces hepatic fibrosis[144]; and (5) Genetic modification. Forced overexpression of critical profibrotic genes and/or silencing of antifibrotic genes has been shown to induce cirrhosis in animals. For example, high-speed intravenous injection of naked plasmid DNA of TGF-β1 can induce transient and reversible liver fibrosis in mice[145]. Mice with liver-specific deletion of CYLD exon7/8 [CYLD(FF)xAlbCre] exhibit a prominent biliary phenotype with ductular reaction and biliary-type fibrosis[146].

THERAPY OF LIVER FIBROSIS AND CIRRHOSIS

Recent developments in our understanding of the process of hepatic fibrogenesis have revealed that the process is dynamic and reversible. Animal and clinical evidence has confirmed that any degree of fibrosis and even cirrhosis are potentially reversible by reasonable therapeutic strategies[147-149]. At present, the therapeutic strategies for liver fibrosis include the following.

Therapies to eliminate the etiological factors

Removing the etiological factors is the most direct and perhaps most effective method of treating liver fibrosis. As such, treatments against HBV and HCV infections[150,151], abstinence from alcohol abuse, weight and blood lipid control, chelation of overloaded iron and copper[152] are considered potentially effective therapies for a large proportion of liver fibrosis cases. In particular, the commonly used antiviral agents such as IFN-α, ribavirin, lamivudine, adefovir, entecavir, and especially pegylated IFN-α have been shown to exert antifibrotic effects[91,151,153-156].

Anti-inflammatory and immunosuppressive therapies

Intrahepatic inflammation and immune response are direct causes of injury to hepatocytes and activation of HSCs. Therefore, anti-inflammatory and immunosup-

pressive therapies are important measures to inhibit fibrogenesis, especially for fibrosis and cirrhosis resulting from viral hepatitis, autoimmune hepatitis, and primary sclerosing cholangitis. The anti-inflammatory drug celecoxib[157] and antioxidative agents taurine and vitamin E[158,159] all show some degree of antifibrotic effect. Likewise, glucocorticoids, azathioprine[160], colchicines[161] and rapamycin[162,163] appear to exert anti-inflammatory, antifibrotic and immunomodulatory effects, and therefore may potentially be useful in the treatment of liver fibrosis.

Suppressing activation and promoting apoptosis of HSCs

HSCs play a critical role in hepatic fibrogenesis, and therefore are potential target cells of antifibrotic therapy[164]. As such, inhibition of HSC activation is an attractive therapeutic approach for liver fibrosis. Inactivation of HSCs can be achieved by inhibiting the TGF-β1 signaling pathway and PDGF-B[165-167], and activated HSCs can be removed by inducing these cells to undergo apoptosis[27,31,164]. Some cytokines and growth factors such as insulin-like growth factor-1, IFN-α and IFN-γ have been found to induce apoptosis of HSCs[90,96,168]. Inhibitors of IκB kinase has also been shown to promote apoptosis of HSCs and exert antifibrotic effectd[31]. Other pharmacological agents such as gliotoxin, sulfasalazine, benzodiazepine ligands, curcumin and tanshinone I have been explored for their effects in inducing HSC apoptosis[27].

Protect liver function and promote hepatocyte regeneration

The hepatoprotective agent silymarin has been widely used in the management of chronic liver diseases and cirrhosis[129,169]. Ursodeoxycholic acid and tauroursodeoxycholic acid have shown protective effects against hepatocyte organelle injury, and have been confirmed as effective agents for the treatment of primary sclerosing cholangitis[170,171]. Calcium channel blockers (*e.g.*, verapamil) also show hepatoprotective and antifibrotic effects by stabilizing the hepatic cellular membrane[172] and lowering the portal vein pressure.

Hepatocyte apoptosis is a common event in liver injury and contributes to fibrogenesis and development of cirrhosis. Hence, preventing the hepatocytes from undergoing apoptosis and promoting hepatocytes regeneration can be useful therapeutic strategies for liver fibrosis and cirrhosis. Hepatocyte growth factor (HGF), an antifibrotic growth factor that induces apoptosis in HSCs and stimulates hepatocyte regeneration[173,174], has been attempted as a therapeutic agent for liver cirrhosis. In this respect, infusion of bone-marrow-derived cells and mesenchymal cells have been reported as a potentially effective method for the treatment of liver cirrhosis[175-177], because these cells can differentiate into hepatocyte-like cells in the liver and stimulate proliferation of hepatocytes by secreting some growth factors such as HGF. Furthermore, HGF-overexpressing human umbilical-cord-blood-derived mesenchymal stem cells have shown promising therapeutic effects on liver fibrosis[178].

Gene therapy and targeted therapy

Several critical genes implicated in the pathogenesis of liver cirrhosis such as TGF-β, PDGF-β, CTGF, and TIMP have been investigated as therapeutic targets for liver cirrhosis[179]. Antisense oligonucleotides[167,180,181] and siRNAs[182-184] against these genes have been tested *in vitro* and *in vivo*. Recently, miRNA has been found to play a regulatory role in the pathogenesis of liver fibrosis and cirrhosis through regulating the expression of profibrotic or antifibrotic genes, and influencing the proliferation and activation of HSCs. As such, miRNA-based therapy can potentially be useful for the treatment of liver fibrosis[185]. Furthermore, in order to target more directly the fibrogenic cells, attempts have been made to target the receptors of the profibrogenic proteins expressed on HSCs[184,186,187].

Complementary and alternative medicine

Evidence indicates that some traditional Chinese herbal medicines are effective in the treatment of liver fibrosis and cirrhosis, and have thus gained popularity worldwide[188,189]. These herbal medicines include the following categories: pure compounds (*e.g.*, salvianolic acid B[190] and oxymatrine[143]). The mechanisms by which tetrandrine[191], glycyrrhetinic acid[192] and curcumin[193]), single agents (*e.g.*, *Salvia miltiorrhiza*[194] and *Ganoderma lucidum*[195]), and composite formulae (*e.g.*, Fuzheng Huayu Capsule[196], Biejiajian[197], Yi-Gan-Kang granule[198] and Qinggan Huoxuefang[199]). Chinese herbal medicines exert antifibrotic effect are far from clear but may include antiviral and anti-inflammatory effects, immune regulation, inhibition of HSC activity, and promotion of collagen degradation. Further randomized controlled clinical trials are needed and the possible adverse effects should be carefully evaluated.

CONCLUSION

In summary, the etiology of cirrhosis is multifactorial and the mechanisms underlying pathogenesis of cirrhosis are far from being clarified. Further studies, particularly with appropriate animal models, to unveil the molecular mechanisms leading to liver fibrosis and cirrhosis are essential for the development of effective therapeutic approaches.

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TOPIC HIGHLIGHT

WJG 20th Anniversary Special Issues (12): Nonalcoholic fatty liver disease

Role of autophagy in the pathophysiology of nonalcoholic fatty liver disease: A controversial issue

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Abstract

Autophagy is a mechanism involved in cellular homeostasis under basal and stressed conditions delivering cytoplasmic content to the lysosomes for degradation to macronutrients. The potential role of autophagy in disease is increasingly recognised and investigated in the last decade. Nowadays it is commonly accepted that autophagy plays a role in the hepatic lipid metabolism. Hence, dysfunction of autophagy may be an underlying cause of non-alcoholic fatty liver disease. However, controversy of the exact role of autophagy in the lipid metabolism exists: some publications report a lipolytic function of autophagy, whereas others claim a lipogenic function. This review aims to give an update of the present knowledge on autophagy in the hepatic lipid metabolism, hepatic insulin resistance, steatohepatitis and hepatic fibrogenesis.

Key words: Non-alcoholic fatty liver disease; Non-alcoholic fatty liver disease; Steatohepatitis; Non-alcoholic steatohepatitis; Autophagy; Lipophagy; Lipid metabolism

Core tip: Autophagy is a mechanism involved in cellular homeostasis. In this review the current knowledge on the role of autophagy in non-alcoholic fatty liver disease (NAFLD) is summarised, with emphasis on the current controversy on the lipolytic *vs* lipogenic function in hepatic lipid metabolism. Furthermore the role of autophagy in the pathophysiology of insulin resistance, hepatocellular injury and fibrogenesis is reviewed to better understand its importance in NAFLD.

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INTRODUCTION

The term autophagy has been introduced by De Duve *et al*[1] over forty years ago to define a process of vacuolisation for the transport of intracellular material to the lysosomes for degradation. The knowledge and number of autophagy-related publications increased exponentially in the last decade, as the importance of autophagy

in (patho)physiology became recognised. Indeed, autophagy is progressively acknowledged as an important regulator of intracellular homeostasis. Dysfunction of this process has been linked with cardiovascular, respiratory, neurodegenerative and metabolic diseases and with cancer[2,3].

Non-alcoholic fatty liver disease (NAFLD) is characterised by macrovesicular fat accumulation in more than 5% of the hepatocytes in the absence of known causes of secondary steatosis[4,5]. This accumulation ranges from scarce to panacinar steatosis and usually starts in Rapaport's zone 3[6]. It is important to distinguish non-alcoholic fatty liver (NAFL, also known as simple steatosis) from non-alcoholic steatohepatitis (NASH), which is diagnosed when macrovesicular steatosis is accompanied by both hepatocyte ballooning degeneration and lobular inflammation[5,6]. Simple steatosis has a low risk for the development of advanced disease, while NASH is associated with an increased risk of hepatic and non-hepatic co-morbidities and mortality. NAFLD is epidemiologically linked with obesity and diabetes, and is currently considered as the hepatic manifestation of the metabolic syndrome. Given that the prevalence of these metabolic disorders rises, the prevalence of NAFLD and hence its clinical impact, is rising too[4,7].

A growing body of evidence indicates that autophagy and lipid metabolism are correlated. Dysfunctional autophagy may therefore contribute to the pathogenesis of NAFLD. However, controversies still exist and the exact role of autophagy in the hepatic lipid metabolism is not entirely elucidated yet. This review aims at summarising current knowledge on autophagy in NAFLD.

AUTOPHAGY

Autophagy is derived from the Greek "auto" and "phagos" and literally means "self-eating". Basal autophagy serves as a housekeeper in the continuous turnover of cellular contents, thereby removing damaged or dysfunctional cellular contents and supplying substrates for energy production. Autophagy can be induced in response to oxidative or metabolic stress[8,9]. Starvation is commonly used to induce autophagy in research settings. Moreover, liver tissue and hepatocytes are frequently used in research and as such were involved in many major discoveries[9].

There are three types of autophagy identified in mammalian cells: macroautophagy, chaperone-mediated autophagy (CMA) and microautophagy[2,10,11]. In macroautophagy, cytoplasmic material (*e.g.*, organelles or protein aggregates) is sequestrated in a double membrane structure, the autophagosome (Figure 1). This process initiates the formation of a phagophore (also known as isolation membrane), which subsequently lengthens to create an autophagosome. The autophagosome fuses with a lysosome to form an autolysosome where its content will be degraded. When a small portion of cytoplasm is engulfed directly by the lysosome, the term microautophagy is used. In CMA, proteins that contain a special targeting motif, recognised by heat shock cognate protein 70 (HSC70) and co-chaperones, will be selectively delivered to lysosomes where they are internalised *via* a lysosomal-associated membrane protein 2A (LAMP2A).

Among the three types of autophagy, macroautophagy (hereafter called autophagy) is considered to play the most important role in pathophysiology and is well studied in recent years. Even though autophagy was initially believed to be a non-selective bulky degradation pathway, selective forms such as "mitophagy" (selective autophagy of mitochondria), "peroxiphagy" (peroxisomes), "ribophagy" (ribosomes) or "xenophagy" (invading microbes) are also acknowledged[2,3,8,12].

The formation of autophagosomes is a dynamic and highly regulated process (Figure 1). It is regulated at the molecular level by autophagy related (*Atg*) genes. These genes were originally identified in yeast, but many orthologues in higher eukaryotes have been found[2]. A central regulator in autophagy is the mammalian target of rapamycin (mTOR)[13]. This protein complex inhibits the initiation of autophagosome formation by phosphorylating UNC51-like kinase 1 (ULK1). The class I phosphatidyli nositol 3-kinase (PI3K)/AKT pathway stimulates mTOR in response to growth factors, such as insulin. However, under conditions of low energy status the AMP/ATP ratio increases, leading to adenosine 5'-monophosphate-activated protein kinase (AMPK) activation and mTOR inhibition, thereby activating autophagy[2,13]. The beclin-1/VSP34 (a class Ⅲ PI3K)-interacting complex mediates nucleation of the phagophore[2,13]. Two ubiquitin-like conjugated complexes take care of elongation of the formed phagophore into an autophagosome: the ATG5-ATG12-ATG16L1 complex and light chain 3-Ⅱ (LC3-Ⅱ). An E1-like protein, ATG7, is a necessary mediator of both conjugation processes, hence an interesting target for the study of autophagy[14]. LC3 (also known as MAP1LC3) is the major mammalian orthologue of ATG8 and also one of the key regulators in autophagosome formation[3]. The active conjugated form of LC3, LC3-Ⅱ, is frequently used as a marker for autophagy in studies[15]. For further extensive review of autophagy regulation, see references[11,16].

AUTOPHAGY IN LIPID METABOLISM

Singh *et al*[17] were the first to convincingly correlate autophagy with the lipid metabolism. They considered it as a novel selective pathway in lipid breakdown and called it 'lipophagy', even though the first clues pointing towards a potential role of autophagy in lipid metabolism were already seen a couple of decades earlier[18]. In contrast, Shibata *et al*[19] claimed that autophagy was necessary for the genesis of lipid droplets (LDs) rather than being involved in the breakdown of LDs. Currently, several papers with supporting evidence for both theories have been published. In this section, some common findings will be discussed, followed by reviewing both the oppos-

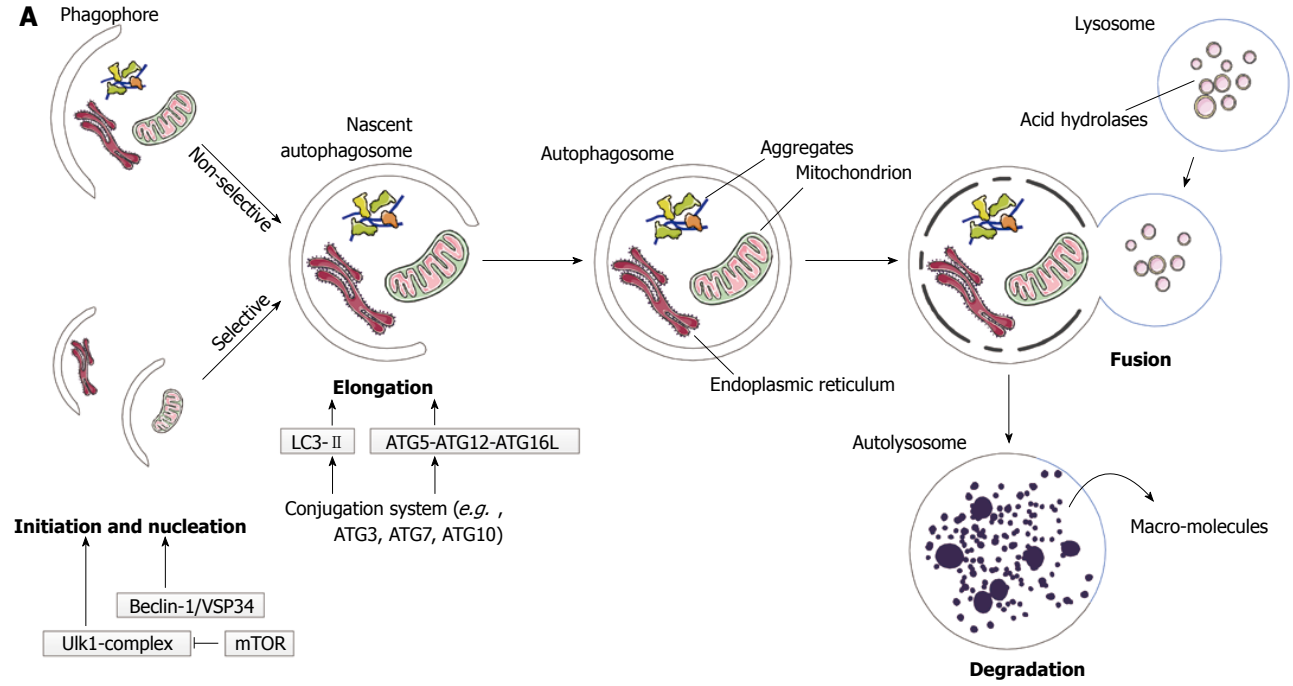

Figure 1 Macroautophagy. A: Schematic overview of macroautophagy. Macroautophagy starts with the formation of a double-layered membrane, the phagophore (isolation membrane). Phagophore formation is regulated by the ULK1 complex (initiation), which is under control of the mammalian target of rapamycin (mTOR) complex, and the beclin-1/VSP34 interacting complex (nucleation). Two major ubiquitine-like conjugated complexes take care of the elongation of the double membrane: light chain 3 (LC3)-Ⅱ and ATG5-ATG12-ATG16L1. ATG7 is one of the proteins necessary for formation of both elongation complexes. When an autophagosome is formed, it will fuse with a lysosome. The inner membrane of the autophagosome and the sequestered cytoplasm will be degraded and macromolecules can subsequently be (re-)used. Macroautophagy can be non-selective (random uptake of intracellular material) or selective [uptake of specific cargo, *e.g.*, mitochondria, endoplasmic reticulum (ER)]; B: Transmission electron microsocopy (TEM)-image of a normal mouse liver fasted overnight. The arrows indicate autophagosomes, the arrowheads indicate autolysosomes. N: Nucleus.

ing theories and the contextual alterations of autophagy in the lipid metabolism.

Common findings in autophagy and lipid metabolism

Despite the contradictory results in the recent literature, some common findings supporting the relationship between autophagy and the lipid metabolism in the liver deserve to be mentioned.

First, a close relationship between LDs and LC3 has been demonstrated. A notable portion of LC3-positive structures, as demonstrated by immunofluorescence microscopy, co-localised with markers for LDs in liver tis-

sue[19] and in cell lines[20-22]. Likewise, immunohistochemistry revealed positive LC3B dots localised on the surface of LDs[23]. Co-localisation could also be confirmed by LC3 immunogold staining transmission electron microscopy (TEM) and suggests a LD-regulating function of autophagy[17,19,20]. This co-localisation was not influenced by inhibition of autophagosome-lysosome fusion or knockout of autophagy, suggesting that conjugation to the active form of LC3 (LC3-Ⅱ) occurs on the surface of LDs and not only on autophagosomes[17].

Secondly, steatosis is most of the time present in acinar zone 3[6]. In parallel with this histological finding, immunohistochemical staining for autophagy (staining of LC3) is more localised around the central veins[23,24]. Zonal distribution of autophagy is also postulated from a theoretical point of view, based on findings in glutamine metabolism[25]. It is assumed that low rates of autophagy occur in periportal areas and constitutive high levels of autophagy pericentrally in well-nourished conditions. This assumption serves as a potential explanation for the pattern of steatosis in the liver.

Thirdly, LDs have shown to be associated with lysosomes. Immunofluorescence microscopy reveals increased co-localisation of LDs with lysosomal markers such as LAMP1[17] or lysotracker[22] in fat-loaden cells. Co-localisation decreases after inhibiting autophagosome formation pharmacologically or by knockdown techniques[17].

Autophagy as a lipolytic mechanism

Considering autophagy as a lipolytic mechanism is an attractive theory, because it helps explaining the ability of the liver to mobilise free fatty acids (FFA) rapidly, if needed, taking into account that hepatocytes have relatively low concentrations of cytosolic lipases[26].

Pharmacological inhibition, silencing or knockdown of autophagy (by targeting ATG5) in hepatocytes results in an increased hepatocyte triglyceride (TG) level and accumulation of LDs when cultured in the presence of an exogenous or endogenous lipid stimulus[17]. It was shown that this increase was due to impaired lipolysis (to fuel β-oxidation) and not to increased TG synthesis. Additionally, the opposite happened after pharmacological induction of autophagy: lipid stores in hepatocytes decreased. The development of hepatocyte-specific autophagy-deficient mice (by targeting *Atg7*) could confirm the *in vitro* results. Indeed, these mice develop hepatomegaly with increased TG and cholesterol content, compared to their wild type countermates[17]. Autophagy-deficient and -competent mice that were starved for 24 h showed an increased presence of TG in their livers (the so-called fasting-induced steatosis). In wild type mice this accumulation was less pronounced. Moreover, TEM demonstrated an increase of lysosomes and lipid-containing autophago(lyso)somes, supporting lipolysis. These findings were even more pronounced with prolonged fasting.

Obese mice (either genetically or dietary) show reduced ATG7 protein levels (although the mRNA expression is comparable) as well as decreased levels of autophagy[27]. Autophagy induction *via* liver-specific overexpression of *Atg7* in *ob/ob* mice improves the metabolic state and reduces the steatosis significantly. These findings further support a lipolytic function of autophagy. Unfortunately, the effects of induced autophagy on lipid metabolism were only reported in *ob/ob* mice and not in high fat diet (HFD)-fed animals.

When blocking autophagy pharmacologically, a decrease in the oxidation of FFA and in the VLDL production was observed, whilst stimulation of autophagy resulted in opposite effects[28,29]. *In vivo* results also showed a change in the distribution of lysosomal lipases (LAL) towards the autophagosome fraction after starvation, suggesting an increase in the autophagy-mediated lysosomal lipolysis[28].

Defects in forkhead box class O (FOXO) transcription factors are linked to steatosis and dyslipidaemia[30]. Mice with a liver-specific triple knockout of FOXO1/3/4 (LTKO) demonstrate steatosis and hypertriglyceridemia[31]. Reduced autophagy in these mice confirm FOXO1 mediated regulation of the key autophagy genes[32]. Of note, *Atg14* is regulated by the FOXO transcription factors 1 and 3. Knockdown of hepatic ATG14 increases hepatic and serum TG, whereas overexpression in HFD-fed animals decreases steatosis. In LTKO mice, overexpression of *Atg14* was able to counteract lipid disturbances including steatosis[31]. In contrast with these experiments, an increase rather than a decrease of FOXO1 levels was described in a small cohort of patients with NASH[33].

One of the latest contributions in the knowledge of autophagy regulation is the discovery of transcription factor EB (TFEB), which seems to be a master regulator of autophagy[34]. TFEB is involved in the lipid metabolism as its overexpression inhibits and its suppression induces steatosis, respectively[35]. The effects on the lipid metabolism were mediated by the stimulation of the peroxisome proliferator-activated receptor γ coactivator 1 α-peroxisome proliferator-activated receptor α (pgc-1α-PPARα) pathway as well as by autophagy. Overexpression of TFEB could not counteract steatosis caused by the disruption of autophagy, implying dependency of TFEB function on the autophagy mechanism[35].

It is generally known that antiretroviral therapy can induce steatosis. In an *in vitro* study, it was shown that the thymidine analogues zidovudine and stavudine inhibit the autophagic flux of hepatocytes in a dose-dependent manner, thereby inducing the accumulation of lipids and mitochondrial dysfunction[36]. Though not yet used for the treatment of steatosis, glucagon like peptide-1 (GLP-1) analogues were able to reduce endoplasmic reticulum (ER) stress and fat accumulation *in vitro* and *in vivo* by the activation of autophagy[37]. Carbamazepine and rapamycin induce autophagy and show to be effective in reducing steatosis in models of alcoholic and non-alcoholic fatty liver disease[38]. Very recently,

caffeine was shown to induce autophagy with increased lipid clearance[22]. Other possible mechanisms parallel to changes in autophagy that might explain the observed effects are formally not excluded, however, are less likely.

Data in humans are scarce. Studies in human liver tissue are limited partly by experimental restrictions, *e.g.*, the impossibility to use specific pharmacologic interventions or to perform consecutive biopsies. Some currently used markers, such as LC3, do not allow good identification of autophagosomal structures unless the target protein is overexpressed[23]. Moreover, immunohistochemical markers are a snapshot of a dynamic state and are not able to discern between increased autophagy versus decreased degradation of autophagosomes[15,23]. Nonetheless, a small immunohistochemical study on post-mortem liver tissue demonstrated decreased LC3 and increased p62 staining with an increased degree of steatosis, suggesting decreased autophagy in more severe steatosis[24]. Likewise, patients with proven NAFLD demonstrate increased numbers of autophagic vesicles and p62 accumulation on their liver biopsy[39].

Finally, two clinical observations in patients with NAFLD deserve attention. First, hypothyroidism was found to be more frequent in patients with NAFLD[40-42]. Thyroid hormone (T_3) is a known regulator of the basal metabolism and acts on different mechanisms. Recently, T_3 was shown to be a powerful inducer of autophagy *in vitro* and *in vivo*, and autophagy accounted for a crucial portion of T_3 stimulated β-oxidation[21]. As a result, autophagy may provide the explanation for this association.

Secondly, patients with NAFL have an increased prevalence of hypovitaminosis D[43]. Vitamin D prevents the development of steatosis, whereas knockout of its receptor (VDR) promotes steatosis[44]. Others have shown that vitamin D acts as a potent inducer of autophagy[13]. Indirectly, these two facts together are in line with lipophagy, but hitherto the hypothesis that these effects are directly mediated *via* autophagy has not been investigated.

Autophagy as a lipogenic mechanism

In fasting conditions, the body's energy supply is maintained by adaptive mechanisms. As insulin levels decrease, lipolysis in the adipose tissue (AT) is no longer inhibited and FFA are released in the serum. These FFA are captured by the liver and either used for formation of ketone bodies, or temporary stored as TG in LDs[19,45]. In rodents this mechanism may cause substantial accumulation of TG in the liver, known as fasting-induced steatosis. C57Bl/6 mice showed to be very prone to develop steatosis in fasting conditions[45]. Also in humans, the liver fat content increases on imaging (with ^{1}H-magnetic resonance spectroscopy) after 36h fasting[46].

Mice with a hepatocyte-specific autophagy deficiency do not show fasting-induced steatosis as compared to wild type animals. The remaining LDs are much smaller in number and size and the total TG content in the liver is lower. This lack of fasting-induced steatosis was first seen in very young mice (twenty-two days old), but was confirmed in eight to twelve week old mice[19,23,47]. Autophagy is therefore implicated in LD formation and growth. The co-localisation of LC3 (necessary for autophagosome formation) with LDs in starved wild type mice further supports these findings[19].

Subsequent *in vitro* research confirmed these findings in different cell lines (including hepatocytes)[20]. Cells subjected to knockdown of LC3 form less LDs and have less TG content compared to their controls. Neither FFA uptake, nor TG synthesis or TG breakdown are influenced in LC3 knockdown cells, suggesting an impaired ability to preserve synthesised TG within these cells[20].

Very recently, an improved metabolic profile was observed in both hepatocyte- or skeletal muscle-specific autophagy-deficient mice[48]. Adult mice with a deficiency in hepatic autophagy[47,48] and fed a control diet show reduced fasting-induced steatosis. Moreover, lipid accumulation did not develop[48] or increase[47] after feeding a HFD. Gene expression of proteins involved in fatty acid and TG synthesis was lower compared to control littermates. On the other hand, gene expression of proteins involved in β-oxidation and TG secretion was also reduced[47,48]. It is not clear whether these findings are epiphenomena or directly involved in preventing steatosis. The "mitokine" fibroblast growth factor 21 (FGF21), induced by mitochondrial stress, was held responsible as a central mediator of the metabolic alterations[48].

Decreased autophagy has been reported in dietary and genetic models of obesity, whereas overexpression of *Atg7* had beneficial metabolic effects[27] as discussed above. Nevertheless, suppressing ATG7 expression in lean mice increases hepatic glycogen content, but fails to alter lipid accumulation in the liver (as well as TG or FFA in serum)[27]. This study is therefore non-conclusive about a lipolytic or lipogenic function of autophagy.

Contextual variability of autophagy in lipid metabolism

Besides the duality in autophagy as a lipolytic or lipogenic process, there are also differences described depending on its context. In most cases these differences were found in experiments supporting autophagy as a lipolytic mechanism.

Based on *in vitro* experiments, basal autophagy is supposed to be a more important pathway for lipid metabolism than induced autophagy[17]. Hepatocytes in culture did not demonstrate signs of induced autophagy in response to lipid stimuli. Moreover, in contrast with endogenous lipid load, these hepatocytes were unable to adjust the autophagic flow to a sudden increase in the external lipid load. In line with this *in vitro* finding, the external lipid load by prolonged HFD decreases the efficiency of autophagy[17,27,29,37]. Intriguingly, a biphasic time course is observed with an increase in the autophagic flux and mRNA of autophagic markers after two weeks

HFD and a decrease afterwards (ten weeks HFD)[29]. Recent data (only presented as an abstract) suggest that autophagy decreases on short term HFD (three days) and normalises after long term HFD (ten weeks)[49]. Instead of a decrease, eight weeks of a diet high in fat load generates an increase in autophagy[50]. Paradoxically, a further increase of autophagy by a compound found in garlic decreases the lipid content, possibly by autophagy-independent mechanisms that have to be further elucidated. All together, it seems that alterations of autophagy are dynamic in states of overnutrition.

The term lipotoxicity covers all detrimental effects of fatty acids on the cellular integrity[51,52]. Hence, it is not surprising that lipids as such can influence autophagy. Short chain fatty acids are able to induce autophagy *in vitro*[53]. Unsaturated fatty acids (*e.g.*, oleic acid) stimulate autophagy and protect against apoptosis, whilst saturated fatty acids (*e.g.*, palmitic acid) inhibit autophagy and promote apoptosis[37,54,55].

Not only fatty acids, but also the lipid composition of membranes or vesicular compartments can influence autophagic behaviour. A long exposure to high lipid concentrations alters the membrane composition and diminishes the fusion capacity of autophagosomes and lysosomes[56]. This may explain the altered autophagy after prolonged fatty diets. Attenuation of CMA was also observed after lipid challenge[10]. However, some authors did not observe an attenuated fusion capacity in *ob/ob* mice Instead they report a decrease in clearance of autophagosomes due to a disturbed acidification of lysosomal compartments[57] and/or down-regulated cathepsin expression[39,57].

Variation of autophagic behaviour has also been reported with respect to tissue type. For example, autophagy is indispensable for adipogenesis and transdifferentiation of white AT[58,59], contradicting a potential lipolytic function in AT. These findings are in contrast with findings of autophagy in the liver, which mainly claim a lipolytic function of autophagy (as discussed above). In addition, an increased level of autophagy in AT of patients with metabolic syndrome or type 2 diabetes mellitus is observed[60-62].

Discrepancies and hypotheses

A clear-cut explanation for the aforementioned discrepancies in autophagy and lipid metabolism is currently lacking. Several hypotheses, however, have been put forward.

Because autophagy declines with age[63], some concerns were raised about the age of the laboratory animals[64]. Sometimes, juvenile rather than adult mice were used in experiments[19], which may be less dependent on autophagy. However, additional experiments with younger and older mice provide similar results[19,23,47,48], suggesting that the observed differences cannot be explained solely by age.

Secondly, small variances in the mouse strains might be responsible for different outcomes in *in vivo* experiments[48]. This explanation, however, is even so not likely to offer a solution. Most of the experiments were performed on a C57Bl/6 background, which is an inbred strain. Moreover, tracing back the cited resources of the hepatocyte-specific *Atg7* knockout mice that were used, leads to the same origin of the mice: *Atg7* flox mice (used by[17,23,65-67]) were created by Komatsu *et al*[14] and albumin-Cre mice (used by[17,23,48,66]) were created by the group of Magnuson[68]. Furthermore, *in vitro* experiments on hepatocyte cell lines also showed conflicting results[17,20,36,37].

Thirdly, many different methods can be used to examine lipid accumulation and autophagy. Liver steatosis can be induced by fasting, by genetic and/or by dietary alterations. Different genetic modifications or pharmacological interferences can also alter autophagy. Whereas papers claiming a lipolytic role use a wide range of methods (*e.g.*, *in vitro* methods, pharmacological interference, genetic modifications) (see 3.2), articles claiming the opposite mainly use *in vivo* knockout models and fasting-induced steatosis (see 3.3). However, one of the caveats in knockout models is the potential of influencing developmental stages, *e.g.*, transdifferentiation of white adipocytes requires autophagy[58,59]. This implies that the observed differences in autophagic lipid handling might be due to the method of inducing fat accumulation or an altered maturation of hepatocytes.

Fourthly, distinction is made between basal and induced autophagy. Basal autophagy is supposed to be the most important type of autophagy in the pathogenesis of NAFLD[17,69]. However, most studies are performed after total blockage of autophagy, making it difficult to discern between the two. A *Bcl-2* knock-in model is able to selectively block stimulus-induced (*i.e.*, by exercise or starvation) autophagy[70]. These mice have an impaired glucose-uptake during exercise and an impairment of the exercise-induced protection against glucose-intolerance and increased serum lipid levels caused by HFD. The liver and pancreas morphology did not alter after HFD, supporting the importance of basal autophagy in the lipid metabolism. Further elucidating the role of basal *vs* induced autophagy, including the relationship towards the lipid source, may provide a possible explanation for the divergent findings in the lipid metabolism.

Moreover, when studying autophagy, it is sometimes difficult to distinguish whether the observed effects are a secondary/adaptive process or directly casused by autophagy[59,71]. Furthermore, autophagy not only influences lipid metabolism, other organelles and cellular systems are also influenced by dysfunctional autophagy and may partly explain observed differences in the liver metabolism (*e.g.*, ER stress and dysfunctional mitochondria[44,72,73]). Disturbance of very low density lipoprotein (VLDL) production might also be implicated, as autophagy can degrade apoB, a necessary protein for the VLDL formation[71]. There even may be non-autophagic (and autophagy-independent) functions of ATG-proteins[26,27,74] in the lipid metabolism.

Inversely, the lipid metabolism is not solely depen-

dent on autophagy alone. Cytosolic lipases still account for a substantial part of the lipolysis[51]. For example, after total blockage of lipolysis by diethylumbelliferyl phosphate (DEUP), the cellular TG content increases more than after blocking autophagy alone[17]. Additionaly, if LD-formation is autophagy-dependent, small LD-like bodies are observed on TEM in autophagy-deficient cells, suggesting that the lumenally-sorted LD production (*i.e.*, LDs formed out of the ER) is not affected[19].

Finally, while the resolution limit of microscopical techniques does not allow to visualise the smallest LDs in living cells[75], it is possible that the observed effects of autophagy only reflect LD modulation after the formation of LDs. Autophagy might be a dynamically active process which controls LD size and the amount of lipotoxic FFA in the cytoplasm. The behaviour of autophagy will be context-dependent and drives the main outcome of autophagy in the lipid metabolism[51]. In this view, lipolysis and lipogenesis are no longer mutually exclusive and in fact co-exist.

AUTOPHAGY AND INSULIN RESISTANCE

The liver plays a central role in the glucose metabolism and an impaired insulin signalling is an important feature of NAFLD[4]. Autophagy substantially contributes to maintain the glucose homeostasis and is strictly regulated by insulin[76]. Similarly to the lipid metabolism, the exact interactions between the action of insulin and autophagy are not entirely clarified yet. An overview of the current knowledge is given in Figure 2.

Insulin stimulates mTOR *via* the class I PI3K /AKT pathway, and thus inhibits autophagy[2,16]. In case of a normal insulin sensitivity of the liver, insulin-dependent stimulation of this pathway is a possible mechanism of reduced hepatocellular autophagy in hyperinsulinemic states. Furthermore, an alternative pathway was found, which could explain the reduced autophagy in case of insulin resistance (IR)[32]. In HFD-induced IR, a down-regulation of autophagy was noticed due to a reduced FOXO1-mediated expression of key autophagy genes. The authors suggested that IR was a consequence of the reduced clearance of dysfunctional/aged mitochondria *via* mitophagy, as oxidative stress and altered mitochondrial integrity (and mass) are related to IR[32]. The impact of other dysfunctional organelles on IR was not investigated, but could also contribute to these findings.

Reduced autophagy was also linked to IR in a study with HFD and *ob*/*ob* mice[27]. In contrast to the aforementioned study, IR was not the cause but the result of decreased autophagy. Knockdown of autophagy in lean mice induced severe IR, while overexpression of *Atg7* in obese mice improved the insulin sensitivity and glucose tolerance, decreased the hepatic glucose production and decreased steatosis. An increased level of the calcium-dependent protease calpain 2, which can cleave several autophagy-related proteins, was observed and was held responsible for the decreased protein level of ATG7. The subsequent increase of cellular stress, with emphasis on ER stress, might be the mechanism behind IR[27].

Hepatic IR appears to be the result of ER stress-mediated processes[72], therefore ER stress secondary to decreased autophagy might be a plausible underlying mechanism for IR. Intracellular saturated fatty acids can also contribute to IR by an increase in ER stress, but also *via* an ER-stress independent mechanism[72]. On the other hand, ER stress stimulates autophagy[16,77], thus autophagy potentially acts as an escape mechanism to prevent cell injury and IR in particular.

Recently, Kim *et al*[48] described a novel endocrine and metabolic function of autophagy. Defective mitophagy causes cellular stress, inducing a stress response regulated by activating transcription factor 4 (ATF4), which promotes the expression of FGF21. FGF21 has several beneficial metabolic effects in lean and HFD-fed animals including an improved glucose tolerance and insulin sensitivity[48,78]. In human subjects, however, a positive correlation was observed between plasma FGF21 levels, IR[79] and steatosis[80]. This apparent paradox might be explained by the resistance to FGF21 (as with increasing levels of FGF21 less IR is actually expected, based on experimental data) or can either be explained by an adaptive increase of FGF21 after establishment of IR. In this study, mitochondrial dysfunction is no longer seen as detrimental, but rather as beneficial by reducing the fasting-induced steatosis and improving the glucose metabolism.

Finally, inhibiting and stimulating effects of protein kinase C (PKC), an important effector enzyme in several signal transduction cascades, on autophagy have been described[81,82]. Several isoforms of PKC are known and many of them can be activated by diacylglycerol (DG). Insulin resistance is also linked to PKC[83]. As DG is an intermediate as well as a product of lipolysis, these findings may indicate an additional crosslink between IR and autophagy. Not all DG is able to activate PKC due to differences in stereoisomers. DG produced by lipolysis does not show bioactivity, and therefore potential cross-talk is less likely[51,83]. On the other hand, active stereoisomers of DG (*i.e.*, 1,2-diacyl-glycerol) can be generated in lipid synthesis and interference with insulin signalling can still occur.

AUTOPHAGY AND HEPATOCELLULAR INJURY

In some patients, steatosis leads to cellular injury and inflammation (NASH) with a subsequent risk for progression to cirrhosis and eventually for hepatocellular carcinoma (HCC) in a subset of patients[4]. Because damaged organelles are removed by autophagy, dysfunction of autophagy likely will result in cellular injury. In line with this hypothesis, stimulation of autophagy could indeed reduce liver injury in animal models of ethanol-induced steatohepatitis[38,84]. Carbamazepine-induced autophagy also demonstrated a tendency to reduce cell injury in a

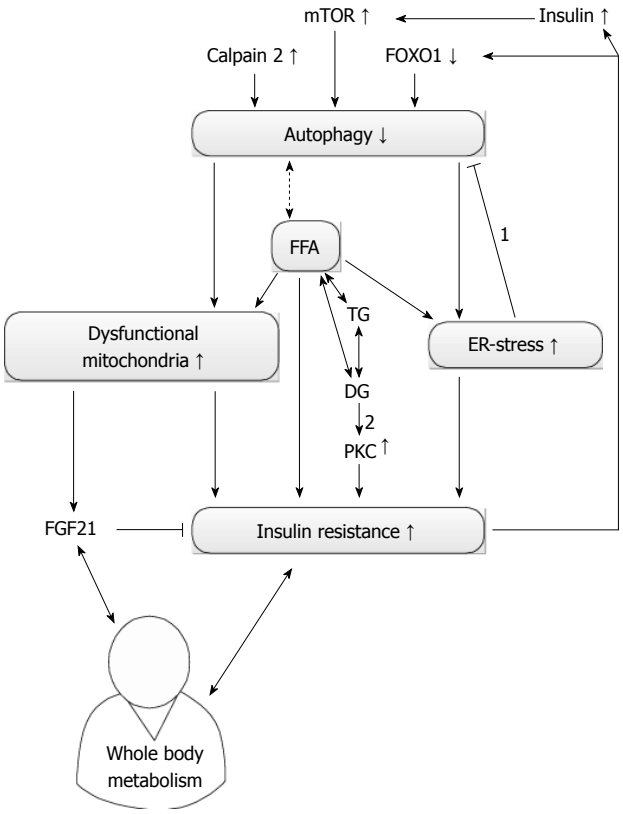

Figure 2 Current knowledge of autophagy and insulin resistance. Autophagy and insulin resistance (IR) seem to influence each other reciprocally. On one hand, an increased level of calpain 2 reduces autophagy and increases IR by increasing endoplasmic reticulum (ER) stress. The amount of dysfunctional mitochondria will also increase and contribute to IR. On the other hand, secondary hyperinsulinism due to IR can decrease autophagy if the insulin sensitivity remains present. Furthermore, autophagy can also be suppressed *via* an IR-mediated reduction in forkhead box class O 1 (FOXO1). Free fatty acids (FFA) can induce IR directly or by increasing ER stress. Correct stereoisomers of diacylglycerol (DG) can induce protein kinase c (PKC) dependent IR. Controversy exists on how autophagy influences the level of FFA and if subsequent correct stereoisomers of DG can be formed. Dysfunctional mitochondria can increase the level of fibroblast growth factor 21 (FGF21), which is able to reduce IR. FGF21 and IR interact with whole body metabolism. Arrows indicate a consequence of a certain alteration, bar headed arrows denote an inhibition. Double-headed arrows present a reciprocal influence. The dashed and double-headed arrow denotes the uncertain relation between FFA and autophagy. 1: ER stress actually increases autophagy; 2: Only right stereoisomers induce PKC. mTOR: Mammalian target of rapamycin; TG: Triglycerides.

mouse model of NAFLD[38].

A potential role exists for mitophagy since mitochondrial dysfunction leads to the production of reactive oxygen species (ROS), causes oxidative stress and is involved in the pathogenesis of NASH[73]. Many of the aforementioned studies do report damaged mitochondria when autophagy is defective[14,23,36,48,54,66]. In one study defective autophagy was accompanied by an increased production of ROS[36]. Knockdown of autophagy makes hepatocytes more susceptible to cell death caused by menadione-induced oxidative stress[69]. This type of cell death is caspase-dependent and activated *via* the mitochondrial death pathway due to c-JUN N-terminal kinase (JNK)/c-JUN overactivation. CMA is upregulated as a compensatory mechanism, but fails to overcome the induced oxidative stress. Furthermore, CMA as such also provides protection to menadione-induced cell death, but through a different mechanism[69].

Comparable to menadione, TNF-induced hepatic injury also causes increased cell death, JNK/c-JUN overactivation and activation of the mitochondrial death pathway in hepatocyte-specific autophagy-deficient mice. However, primary mitochondrial dysfunction followed by oxidative stress or impaired energy homeostasis is not responsible for cell injury in this model[47,65].

The protein p62/SQSTM1 selectively guides proteinaceous aggregates to autophagosomes and accumulates in autophagy-deficient cells. Presence of this protein contributes significantly to hepatocellular injury caused by autophagy deficiency, as double knockouts of autophagy (*Atg7*) and p62 (DKO) diminish hepatocyte injury compared to autophagy knockout (*Atg7*) only[67]. Nevertheless, p62 is believed to be a beneficial adaptive response to promote formation of relatively harmless aggregates. Toxic intermediates are formed during the aggregate formation and are considered responsible for cell injury. Furthermore, p62 also aids nuclear translocation of the beneficial transcription factor NF-E2 related factor 2 (NRF2), which induces transcription of various detoxifying enzymes[67]. Of note, despite the alleviation of cellular injury by DKO, a complete abolishment of the cellular injury comparable to control levels cannot be achieved as turnover of disturbed organelles is still not corrected.

Autophagy is linked to the inflammatory cytokines in adipocytes. When p62 is knocked out in adipocytes, an increased invasion of macrophages and production of pro-inflammatory cytokines in AT is observed[85]. Moreover, knockout of p62 in AT also causes obesity and glucose intolerance, whereas knockout of p62 in hepatocytes does not. Whether these inflammatory changes also occurred in liver tissue was not investigated. A direct inhibition of autophagy in human or mice adipocytes increases the production of pro-inflammatory cytokines[62] as was described in other tissues as well[86].

The role of autophagy in tumourigenesis is dual and depends on the stage of tumour development. Autophagy acts as a tumour suppressor in normal tissue and prevents the development of malignant neoplasia. Once a tumour is developed, autophagy drives survival of tumour cells by supplying nutrients[2]. An extensive discussion of the role(s) of autophagy in liver tumour development can be found in ref.[9].

AUTOPHAGY IN LIVER FIBROSIS

The knowledge on autophagy in fibrogenesis is scarce, but available evidence suggests an elementary role in different fibrogenic cells[87]. Autophagy seems to provide nutrients to fuel the processes involved in the activation of these cells.

Hepatic stellate cells (HSCs) are considered major fibrogenic cells in the liver. A central observation in the transdifferentiation from a quiescent state to active myo-

fibroblasts is the depletion of lipid stores, in HSC typically mainly composed of vitamin A[88].

Autophagy in HSC activation was first evidenced in 2011[89]. In this study, an increased autophagic flux was observed during HSC activation. Pharmacological inhibition of autophagy inhibited the activation of HSCs. Autophagy also interfered with the LD metabolism after stimulation by platelet derived growth factor BB (PDGF-BB), a known mitogen of HSCs, as shown by co-localisation of LC3B with LDs. Interestingly, autophagy seems to affect only larger LDs and co-localisation became absent once HSCs were activated.

The importance of autophagy in HSC activation was confirmed in another study using pharmacological and genetic tools to inhibit autophagy *in vivo* and *in vitro*[90]. The results showed an energy-supplying role (*via* delivery of FFA out of LDs for β-oxidation) of autophagy needed for the transdifferentiation of HSCs. Administration of oleic acid in autophagy-deficient stellate cells could partly restore the activation, but did not augment fibrogenesis in autophagy-competent cells.

In line with the aforementioned results, one may expect an increase in fibrosis when inducing autophagy. Paradoxically, reduced hepatic fibrosis is observed after administration of rapamycin, a known potent inducer of autophagy. The involvement of autophagy was, however, not specifically tested in these studies[91-93]. Rapamycin is known to have a complex mode of action. Hence, studies with other autophagy-inducers may clarify whether the rapamycin-induced reduction in hepatic fibrosis is due to autophagy-related mechanisms.

FUTURE PERSPECTIVES

Further research is clearly needed to elucidate the exact role of autophagy in NAFLD. Fortunately, the increasing research interest in NAFLD and the growing awareness of autophagy as a pathophysiological mechanism will most likely result in new discoveries in the next decade.

The major issue to be resolved is the apparent paradoxical behaviour of autophagy in the lipid metabolism as discussed in this review. At this moment, evidence towards a pro-lipolytic function in the liver is more solid and outnumbers the evidence against this theory. However, the evidence for a lipogenic role cannot be ignored. It has to be addressed whether the two roles might co-exist or whether one role dominates in the hepatocyte, and how their balance and/or function is regulated exactly.

Contextual differences were noted, but not fully understood. Detailed knowledge of basal and induced autophagy may resolve a part of this puzzle. It would be very interesting if future research can more specifically and separately investigate the role of basal and induced autophagy in liver metabolism. Additionally, the relationship with other cellular observations such as dysfunctional mitochondria should be clarified.

Most studies perform experiments on one specific cell type (*i.e.*, hepatocytes), although the liver is composed of many different cell types (*e.g.*, endothelial cells, HSCs and Kupffer cells[94]). More extensive research on whole liver tissue may be useful as several cell types are also involved in the development of steatohepatitis[4,88] or may influence each other. Moreover, potential zonal differences of autophagy in liver tissue should be investigated and correlated with the already known different functions of the acinar liver zones[25].

The liver is not a "cavalier seul" in the metabolism, but one of the central players of the whole body metabolism. The role of autophagy is site-specific. For example, in AT stronger evidence exists for a lipogenic action of autophagy[58,59] contrary to the results in hepatocytes. In order to understand the complex role of autophagy, other tissues and other metabolic pathways, including the glucose and protein metabolism, must be incorporated in the research projects.

Ultimately, if knowledge on autophagy in NAFLD increases, therapeutic interventions can be developed and tested. Systemic therapy will potentially be hampered if context- and tissue-dependent behaviour of autophagy appears to play an important role in the pathogenesis of the metabolic syndrome. Several clinical trials are already ongoing, almost exclusively in oncological settings (www.clinicaltrials.gov). Investigators should be stimulated to include repetitive evaluation of the metabolic parameters in their study as secondary objectives.

CONCLUSION

Autophagy is an important factor in the lipid metabolism, but its exact role has not yet been fully clarified and appears to be context- and tissue-specific. Increasing knowledge on its exact role in the complex pathophysiology of metabolic disturbances and NAFLD might make autophagy a target for treatment of the metabolic syndrome or NAFLD. We should, however, always keep in mind that altering a key cellular process such as autophagy might lead to a better metabolic state, but that this not automatically equals a better general health.

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TOPIC HIGHLIGHT

WJG 20th Anniversary Special Issues (12): Nonalcoholic fatty liver disease

Mediterranean diet and non-alcoholic fatty liver disease: New therapeutic option around the corner?

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Abstract

Non-alcoholic fatty liver disease (NAFLD) represents the most common chronic liver disease in Western countries, being considered as the hepatic manifestation of metabolic syndrome. NAFLD has a common pathogenic background to that of metabolic syndrome, and shares many risk factors such as obesity, hypertension, insulin resistance and dyslipidemia. Although there is no currently available evidence-based established treatment for NAFLD, all the recommendations from the medical associations indicate that the most effective treatment is to reduce weight through lifestyle modifications. Diet, indeed, plays a key role in the management of NAFLD patients, as both the quantity and quality of the diet have been reported to have a beneficial role in the onset and severity of the liver disease. Among all the diets that have been proposed, a Mediterranean diet was the most effective dietary option for inducing weight loss together with beneficial effects on all the risk factors associated with metabolic syndrome and NAFLD. Over the last few years, research has demonstrated a beneficial effect of a Mediterranean diet in NAFLD. In this review, we will examine all the available data on the association between diet, nutrients and the Mediterranean diet in association with onset and severity of NAFLD.

Key words: Mediterranean diet; Diet; Prevention; Metabolic syndrome; Non-alcoholic fatty liver disease

Core tip: In this review, we examine all the available data on the association between diet, nutrients and the Mediterranean diet in association with onset and severity of non-alcoholic fatty liver disease.

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INTRODUCTION

Non-alcoholic fatty liver disease (NAFLD) is the most common chronic liver disease in industrialized countries, and is characterized by increased hepatic fat accumulation in individuals not consuming excessive alcoholic beverages (typically a threshold of < 20 g/d for women and < 30 g/d for men is adopted)[1]. NAFLD is emerging as one of the most common causes of liver disease worldwide[2], particularly in Western countries such as the United States where approximately 30% of the population now has NAFLD[3]. It refers to a wide spectrum of liver disorders ranging from simple steatosis (more than 5% hepatocytes showing fat accumulation), to non-alcoholic steatohepatitis (NASH), which increases the risk of end-stage liver disease, namely liver cirrhosis and hepatocellular carcinoma[4].

The true prevalence of NAFLD is unknown because only a liver biopsy can distinguish simple steatosis from NASH, but it is unethical to perform liver biopsies on unselected asymptomatic patients from the general population. However, various strategies including unexplained alanine aminotransferase (ALT) elevation or fatty liver on ultrasound in nondrinking but otherwise unselected patients gives an estimated prevalence of NAFLD in the adult population of around 15%-30%[5]. Moreover, the problem is not limited to adults only because nearly 10% of obese children may have NAFLD[6]. Actually, NAFLD is considered as the hepatic manifestation of metabolic syndrome, sharing a common pathogenic background and with similar disease states such as obesity, type 2 diabetes mellitus, insulin resistance and dyslipidemia[1-4].

Several studies have recently shown the association of NAFLD with cardiovascular disease (CVD)[7]. In a study of biopsy-proven NAFLD with a follow-up of about 21 years, the main causes of death in patients with NAFLD were CVD and malignancy[8]. In addition, several studies showed that NAFLD might itself contribute to the increased CVD risk. The possible explanation for such a relationship between CVD and NAFLD includes the occurrence of many risk factors including insulin resistance, obesity, dyslipidemia, and an altered inflammatory state in both pathologies[7].

Cornerstones of NAFLD therapy are lifestyle interventions, and especially diet. These interventions are effective not only for improving NAFLD itself, but also associated conditions such as metabolic syndrome, type 2 diabetes, and the related risk of CVD[9].

In this review, we report data supporting the role of dietary approaches, including a Mediterranean diet, on the management of patients with NAFLD.

DIET AND NON-ALCOHOLIC FATTY LIVER DISEASE

Dietary habits are significantly linked with health state. A correct dietary intake, associated with a healthy lifestyle, may in fact contribute to the maintenance of a healthy status. Conversely, poor dietary habits may favor the occurrence of various disease states such as cancer, obesity, diabetes, dyslipidemia and hypertension, the combination of which may lead to metabolic syndrome that in turn increases the risk of death from cardiovascular and other chronic degenerative diseases.

Diet and lifestyle can significantly affect the clinical picture of NAFLD since most patients with NAFLD have excess body weight and other cardiometabolic risk factors, such as hypertension, dyslipidemia and diabetes[10,11]. Although promising pharmacological treatments are emerging, only a significant and sustained weight loss is the basis of any treatment plan for patients suffering from NAFLD[9].

Some intervention studies reported that weight loss induced by diet is able to reduce liver enzymes and hepatic steatosis. One of the most relevant studies has been conducted in NASH patients, where a dietary intervention study demonstrated that weight loss led to an improvement in liver histology and enzymes[12]. Patients were randomized into two groups, a combined lifestyle intervention group and a control group. At the end of the intervention period, patients who followed the lifestyle recommendations had an average weight loss of about 10% compared with only 0.2% in the control group. Interestingly, patients who lost more than 7% of their body weight had significant improvements in terms of activity score and inflammation, suggesting that the reduction in body weight resulting from lifestyle changes was beneficial in reducing the severity of NAFLD. The rapidity of weight loss is also extremely important in NAFLD treatment. It has been demonstrated that only modest weight loss (about 1 kg per week) is significantly associated with a decreased incidence of metabolic syndrome and improvement in the histological features of NAFLD, whereas fast weight loss, as seen in bariatric surgical procedures, often worsen the clinical features of NAFLD[13].

Quality modifications of dietary composition can also directly influence the clinical course of NAFLD beyond the "simple" caloric restriction. Indeed, diet composition, with modulation of either macro or micronutrients, can significantly affect most of the risk factors associated with fatty liver such as hypertension, serum lipids and insulin[10,11].

Carbohydrates and non-alcoholic fatty liver disease

As regarding carbohydrates, data from the literature report that a diet high in carbohydrates might worsen the clinical conditions of patients with NAFLD[14]. Actually, hepatic triglycerides showed a greater decrease among individuals treated with a carbohydrate-restricted diet than with an energy-restricted one, whereas a diet rich in carbohydrates may lead to increased levels of insulin, contributing to high levels of triglycerides and blood glucose. In an intervention study, Ryan *et al*[15] randomized NAFLD patients to hypocaloric diets containing different proportions of carbohydrates. After 16 wk, patients receiving a low percentage of calories from carbohydrates (40%) showed lower levels of liver enzymes compared to those receiving a high-carbohydrate diet, despite equal weight loss. Similarly, another intervention study using two energy-restricted diets with equal energies, but different proportions of carbohydrates, showed similar weight loss but a greater decrease in triglycerides in the group with a lower proportion of carbohydrates[16]. The type and quality of carbohydrates appear to be relevant for the occurrence of NAFLD, because the glycemic index seems to play a relevant role in the pathogenesis of NAFLD. Thus current recommendations of many scientific associations indicate a carbohydrate intake > 50% of the total energy, and choosing whole grain and low-

glycemic index foods[9].

Dietary fats and non-alcoholic fatty liver disease

Patients with NAFLD often have a high-fat diet that may be an independent risk factor for the development of the disease[17]. In a study by Yamamoto *et al*[18] the reduction of fat consumption for 6 mo induced a decrease in liver enzymes. Moreover, an increased fat intake in the diet has been linked to many components of metabolic syndrome and NAFLD. In particular, saturated fatty acids (SFA) have been reported to have deleterious effects on both lipid and glucose metabolism. A randomized, double-blind, crossover study examined the effects of three different diets in 86 healthy men: a control diet (14% SFA), the National Cholesterol Education Program Step I diet (9% SFA), and the National Cholesterol Education Program Step II diet (6% SFA). Although all reduced-fat diets decreased low-density lipoprotein (LDL)-cholesterol, they also decreased high-density lipoprotein (HDL)-cholesterol and increased triglycerides after 6 wk compared with the control diet. Furthermore, in response to the 6%-SFA diet, subjects with insulin resistance and higher body fat showed smaller reductions in LDL-cholesterol, larger reductions in HDL-cholesterol and increases in triglycerides as compared to subjects with normal insulin sensitivity[19].

The quality of dietary fats plays a key role in the pathogenesis of NAFLD as shown by the beneficial effect of monounsaturated fatty acids (MUFA). The replacement of carbohydrate and SFA with MUFA leads to reductions in glucose and blood pressure and to an increase in HDL-cholesterol in patients with diabetes. A MUFA-rich diet (40% of energy as fat) has also been demonstrated to decrease VLDL-cholesterol and triglycerides, and to be more acceptable to patients with diabetes than a high-carbohydrate diet (28% of energy as fat). Therefore, an increase in the intake of MUFA, particularly as a replacement for SFA and as a higher proportion in the diet in lieu of carbohydrates, may be beneficial for NAFLD patients[20].

Accordingly, polyunsaturated fatty acids (PUFA) may also have beneficial effects in patients with NAFLD. In a recent human dietary intervention study, we showed positive effects of n-3 PUFA administration in NAFLD patients[21]. Moreover, a meta-analysis demonstrated that n-3 PUFA have substantial beneficial effects in ameliorating liver steatosis and its related components[22]. More recently, we reported a significant amelioration in lipid variables, insulin and adiponectin levels as well as in the severity of fatty liver by introducing a n-3 PUFA-enriched olive oil into the diet of patients with NAFLD, thus indicating that PUFA might represent a therapeutic option for treating NAFLD[23].

In line with such evidence, recommendations in the recent guidelines in terms of dietary fats suggest a total fat contribution < 30%-35% of the total daily energy intake, with preference for MUFA and PUFA rather than SFA (recommended to be < 7% of total energy)[9].

MEDITERRANEAN DIET AND NON-ALCOHOLIC FATTY LIVER-RELATED CONDITIONS

Over the past decades, several dietary models have been proposed as the ideal diet for preventing NAFLD and metabolic-related conditions. However, among all the diets that have been proposed, only the Mediterranean diet demonstrated a beneficial effect according to scientific data.

Mediterranean diet was firstly discovered in the early 1960s by Ancel Keys who invented this term after the results of an epidemiological study, the "Seven Countries' Study" which demonstrated that the populations bordering the Mediterranean Sea (Italy and Greece) had a reduced incidence of cardiovascular disease and cancer in comparison to the other populations. In this study, the dietary profile of 12763 subjects aged between 40 and 59 years living in different countries of the world were analyzed: the United States of America, Finland, the Netherlands, Italy, Greece, the former Yugoslavia and Japan. The results of this study were that countries bordering the Mediterranean basin had the lowest rates of mortality and incidence of cardiovascular disease in comparison with the other countries. After these first results, other studies have confirmed these findings and the Mediterranean diet were recognized all over the world as a healthy diet, effective in reducing the risk of cardiovascular disease and cancer[24].

Mediterranean diet and cardiovascular disease

Much of the available data supporting the beneficial effect of Mediterranean diet on health derive from studies linking diet with CVD, the leading cause of mortality and morbidity all over the world and closely related to NAFLD. With regard to the primary prevention, the most relevant study is the EPIC-Elderly Prospective Cohort Study, a multicenter study that analyzed subjects living in 10 different European countries. In a paper published in the New England Journal of Medicine by Trichopoulou *et al*[25] a score of adherence that takes into account the main dietary variables, divided into food groups, typical of the Mediterranean diet was computed. This adherence score, based on food groups typically present in the Mediterranean diet (bread, pasta, fruit, vegetables, fish, legumes, moderate red wine consumption, and olive oil), gives a positive score to people consuming more than the median of the overall population in term of "Mediterranean" typical foods, and a negative score to those who consume a higher amount of foods which are not typical of the Mediterranean diet. A 2-points increase of this score was significantly associated with a 33%-reduced risk of mortality from cardiovascular causes (RR = 0.67, 95%CI: 0.47-0.94)[25].

Recently, a large intervention study conducted in Spain confirmed the beneficial effects of a Mediterra-

nean diet on the risk of CVD in primary prevention[26]. In a sample of 7747 adults (age range: 55-80 years) at high risk of cardiovascular disease but without a manifest disease who were followed for an average of 4.8 years, three different dietary interventions were tested. The participants were randomly assigned to a Mediterranean diet supplemented with extra-virgin olive oil, a Mediterranean diet supplemented with nuts or a control diet with a low contribution from fats. At the end of the follow-up, subjects who had followed the Mediterranean diet with either supplementation of extra-virgin olive oil or mixed nuts demonstrated a reduced risk of CVD. The hazard ratios were 0.70 (95%CI: 0.54-0.92) and 0.72 (95%CI: 0.54-0.96) for the group assigned to a Mediterranean diet with extra-virgin olive and the group assigned to a Mediterranean diet with nuts, respectively, *versus* the control group.

Alongside, Mediterranean diet has been also found protective versus cardiovascular disease in non-Mediterranean populations, such as in the United States In two large United States cohort studies, Mediterranean diet was found to ameliorate the cardiovascular risk profile[27,28]. The first, published by Mitrou *et al*[27], analyzed over than 200000 people aged between 50 and 71 years for a median follow-up period of 10 years; a greater adherence to the Mediterranean diet demonstrated a 22%-reduced risk of cardiovascular mortality in men (RR = 0.78; 95%CI: 0.69-0.87) and 29% in women (RR = 0.81; 95%CI: 0.68-0.97). The second, conducted in a population of over than 74000 women enrolled in the Nurses' Health Study confirmed the significant protective role of the Mediterranean diet, since women ith a higher adherence to the Mediterranean diet had a reduced risk of coronary heart disease by 29% (RR = 0.71, 95%CI: 0.62-0.82), and of overall mortality from CVD by 39% (RR = 0.61, 95%CI: 0.49-0.76)[28].

Our group conducted two systematic reviews and meta-analyses of prospective epidemiological studies that evaluated the adherence to the Mediterranean diet through the adherence score score in relation to different health outcomes[29,30]. In the updated analysis a 2-points increase of the adherence score to the Mediterranean diet resulted in a 10% reduction in the incidence and/or mortality from CVD (RR = 0.90, 95%CI: 0.87-0.93, $P < 0.0001$)[30].

Mediterranean diet and diabetes

A Mediterranean diet has also been proposed as a valid and effective non-pharmaceutical option for diabetes mellitus treatment. In a Spanish cohort study, that comprised a large number of participants analyzed for a period of 4 years, the possible association between adherence to Mediterranean diet and diabetes has been investigated[31]. This study demonstrated that patients with greater adherence to the Mediterranean diet had a concomitant reduction of developing diabetes by 83%. Furthermore, it was reported that a 2-points increase of the score was also related to a 35%-reduced risk (RR = 0.65, 95%CI: 0.44-0.95). These data were confirmed also in some intervention studies. In the first, Esposito *et al*[32] studies 215 patients with a new diagnosis of diabetes. Patients were randomized to a Mediterranean diet and a low-fat diet. It was reported that the Mediterranean diet ameliorates the glycemic status of these subjects, helps controlling the cardiovascular risk factors, and posticipates the need for hypoglycemic therapy when compared with a diet low in fat and low in carbohydrates. Another study, conducted by Estruch *et al*[33] reported similar results since 772 patients asymptomatic for CVD were randomized to a low-fat diet or to a Mediterranean diet. After only 3 mo of follow-up, subjects who followed the Mediterranean diet reported a significant reduction of blood glucose.

Moreover, the datum was also reported in a large case-control study, comprising about 340000 subjects enrolled in the EPIC study. The authors were able to demonstrate that a higher adherence to the Mediterranean diet was associated with a 12%-reduction of diabetes (OR = 0.88, 95%CI: 0.79-0.97)[34].

Mediterranean diet and obesity

The role of the Mediterranean diet in the development and/or modification of overweight-obesity has always been of great interest in clinical research. Recently, a sub-analysis of the EPIC study analyzed a cohort of 497308 people, by showing that a higher adherence to the Mediterranean diet was associated with a significantly lower body mass index and waist circumference within 3 years[35]. These results were confirmed by other studies in different populations. Indeed, a Spanish study conducted on over than 3000 men and women showed a significant inverse relationship between Mediterranean diet and obesity[36]. Likewise, the SUN study demonstrated that subjects reporting low adherence to the Mediterranean diet had a higher weight gain during follow-up than those who had followed the principles of the Mediterranean diet more strictly. Notably, a greater adherence to the Mediterranean diet was associated with a reduced risk of obesity (OR = 0.76, 95%CI: 0.64-0.90).

Finally, similar results were also obtained from a further study in a large Spanish population[37].

MEDITERRANEAN DIET AND NON-ALCOHOLIC FATTY LIVER DISEASE

Recently, the interest of investigators on the possible association between a Mediterranean diet and fatty liver disease has increased (Table 1). Although there is limited data linking a Mediterranean diet to NAFLD risk and severity, there is strong evidence from clinical studies supporting the hypothesis that such a diet might be beneficial for NAFLD-related disease states such as metabolic syndrome, CVD, and their risk factors[10,11].

In 2008, a *post hoc* analysis of an open label, quasi-randomized, controlled trial evaluated the possible influ-

Table 1 Studies investigating a Mediterranean diet and non-alcoholic fatty liver disease

Study	Country	Study design	Patients	*n*	Outcome	Intervention	Follow-up	Results
Fraser *et al*[38], 2008	Israel	Post hoc analysis of a quasi-randomized trial	Obese with diabetes	259	Reduction of liver enzymes through diets	3 diets: ADA diet Low-GI diet Modified MD	12 mo	MD determined the greatest reduction of liver enzymes at 6 and 12 mo
Tzima *et al*[39], 2009	Greece	Cross-sectional study (The ATTICA Study)	Healthy subejcts	1514 M 1528 F	Association of MD with liver enzymes and MS	None	-	Greater adherence to MD determines a moderate association between liver enzymes and MS
Pérez-Guisado *et al*[42], 2011	Spain	Prospective study	Obese with NAFLD	14	Effect of SKMD on NAFLD	SKMD	12 wk	SKMD determines reduction of liver enzymes and severity of steatosis
Ryan *et al*[40], 2013	Australia	Randomised cross-over dietary intervention	Non-diabetic NAFLD	12	Improvement of liver steatosis	MD Low-fat/ High-carnbohydrate diet	6 wk	MD reduces liver steatosis and improves insulin sensitivity
Kontogianni *et al*[41], 2013	Greece	Cross-sectional study	NAFLD	73	Adherence to MD and severity of NAFLD	None	None	Greater adherence to MD is associated with less severity of NAFLD and lower degree of insulin resistance

ADA: American Diabetes Association; GI: Glycemic index; MD: Mediterranean diet; MS: Metabolic syndrome; SKMD: Spanish ketogenic Mediterranean diet; NAFLD: Non-alcoholic fatty liver disease.

ence of three different diets on liver enzymes of 259 obese patients with type 2 diabetes[38]. Patients were randomized to a diet recommended by the American Diabetes Association, a low glycemic index diet and a modified Mediterranean diet. The three dietary profiles had a similar proportion of total fat but different proportions of carbohydrates and MUFA, as the Mediterranean diet had a higher intake of unsaturated fat and a lower percentage of energy coming from carbohydrates than the other two diets. In addition, the Mediterranean diet was modified in order to obtain all low-glycemic index foods. At 6 and 12 mo of follow-up, ALT levels decreased more significantly in the modified Mediterranean diet arm than in the other two dietary profiles, with mean values reduced from 19.8 to 14.4 U/L. Moreover, after adjustment for some traditional risk factors, including change in body mass, triglycerides, HOMA-IR and waist-to-hip index from baseline, ALT values were confirmed to be significantly reduced in the Mediterranean diet group with respect to the other groups. Although there were limitations related to the sample size and to clinical measurement that was limited to liver enzymes, this trial suggested for the first time that a Mediterranean diet could have a beneficial effect on ALT levels, and that this was not mediated by weight loss or decreases in other circulating biomarkers.

Later, the beneficial effect of a Mediterranean diet on liver enzymes was confirmed by the ATTICA study, that evaluated the prevalence of metabolic syndrome among over 3000 Greek adults[39]. The authors analyzed adherence to the Mediterranean diet through a scoring system named "MedDietScore", and found a slight but significant positive correlation between the AST/ALT ratio and the MedDietScore itself ($R = 0.17$).

Further evidence of the beneficial role of a Mediterranean diet on fatty liver came from the recent study by Ryan *et al*[40] The authors carried out a randomized, crossover dietary intervention study in 12 diabetic subjects with NAFLD. All patients had biopsy-proven NAFLD and were randomized to either a Mediterranean diet or control diet for a duration of 6 wk in random order, interposed by a wash-out period. At the end of the intervention period, mean weight loss was similar between the two groups of patients but a significant reduction in liver fat content was found with magnetic resonance imaging only after the Mediterranean diet phase with respect to the control phase. Moreover, patients improved their insulin sensitivity, measured via a hyperinsulinemic-euglycemic clamp, and their circulating levels of insulin only after the Mediterranean diet phase and not during the control phase. Of interest, no significant differences in AST and ALT values were observed. This intervention study is of extreme importance for the management of NAFLD, despite the low number of patients considered.

Very recently, adherence to a Mediterranean diet was investigated in association with the severity of NAFLD in a group of 73 patients[41]. Adherence to a Mediterranean diet was estimated through the MedDietScore and severity of NAFLD was measured through transient elastography in 58 out of 73 patients and through liver biopsies in 34 patients. In addition, 58 healthy controls were compared with the study population. A significant negative correlation between the MedDietScore and ALT, insulin levels, stage of fibrosis, and severity of steatosis was evidenced in the group of patients with NAFLD. In addition, logistic regression analysis showed that one unit increase in the MedDietScore was associ-

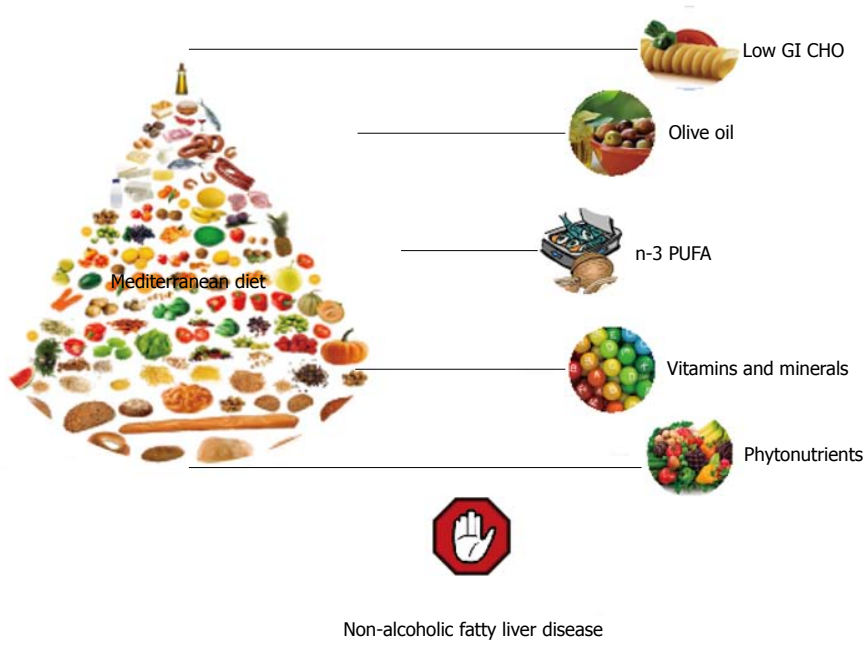

Figure 1 Beneficial properties of a Mediterranean diet on non-alcoholic fatty liver disease. PUFA: Polyunsaturated fatty acids.

ated with a 36% lower likelihood of having NASH after adjustment for confounders.

The Mediterranean-style diet is not a specific diet, but rather a collection of eating habits traditionally followed by people in the different countries bordering the Mediterranean Sea. It refers to a dietary profile commonly present in the Mediterranean regions in the last century and is characterized by a high consumption of fruit, vegetables, legumes, and complex carbohydrates, with a moderate consumption of fish and the use of olive oil as the main source of fats, and a low-to-moderate amount of red wine during meals. This eating pattern has been promoted worldwide as a model for healthy eating and has been reported to contribute to a favorable health status and to a better quality of life, as well as allowing an optimal intake of antioxidant vitamins, polyunsaturated fats and other beneficial nutrients for the prevention of chronic degenerative diseases[24]. In terms of NAFLD prevention, the beneficial effects of such dietary habits can be explained through several mechanisms that can vary from an effective dietary approach for weight loss, to a model diet that is plentiful in some beneficial nutrients such as MUFA and vitamins, to the presence of olive oil as the main contributor of fats (Figure 1). Indeed, olive oil has been demonstrated to have several different beneficial effects on metabolic syndrome and NAFLD, by improving glucose and lipid metabolism and preventing atherogenesis[20]. All these factors likely contribute, as a whole, in determining the preventive and therapeutic role of a Mediterranean diet on fatty liver disease.

CONCLUSION

In conclusion, a Mediterranean diet has recently been promoted as a healthy eating pattern for many conditions including metabolic syndrome, cardiovascular and neoplastic diseases. Over the last few years, an interesting inverse association with NAFLD has also been reported in some studies, indicating the Mediterranean dietary pattern as a new therapeutic option "right around the corner". To date, few studies have been conducted with the aim of investigating adherence to a Mediterranean diet in relation to the occurrence of NAFLD. Further studies are warranted to confirm these preliminary data and to suggest a reliable and easy-to-use tool for determining the adherence to the Mediterranean dietary pattern in a large population of patients affected by fatty liver disease.

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TOPIC HIGHLIGHT

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Pediatric fatty liver disease: Role of ethnicity and genetics

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Abstract

Non-alcoholic fatty liver disease (NAFLD) comprehends a wide range of conditions, encompassing from fatty liver or steatohepatitis with or without fibrosis, to cirrhosis and its complications. NAFLD has become the most common form of liver disease in childhood as its prevalence has more than doubled over the past 20 years, paralleling the increased prevalence of childhood obesity. It currently affects between 3% and 11% of the pediatric population reaching the rate of 46% among overweight and obese children and adolescents. The prevalence of hepatic steatosis varies among different ethnic groups. The ethnic group with the highest prevalence is the Hispanic one followed by the Caucasian and the African-American. This evidence suggests that there is a strong genetic background in the predisposition to fatty liver. In fact, since 2008 several common gene variants have been implicated in the pathogenesis of fatty liver disease. The most important is probably the patatin like phospholipase containing domain 3 gene (*PNPLA3*) discovered by the Hobbs' group in 2008. This article reviews the current knowledge regarding the role of ethnicity and genetics in pathogenesis of pediatric fatty liver.

Key words: Non alcoholic fatty liver disease; Ethnicity; Patatin like phospholipase containing domain 3 gene; Obesity; Insulin resistance; Glucokinase regulatory protein; Apolipoprotein C3 gene; Farnesyl-diphosphate farnesyltransferase 1

Core tip: The prevalence of hepatic steatosis varies among different ethnic groups. Ethnicity with the greatest prevalence of non-alcoholic fatty liver disease (NAFLD) is the Hispanic one followed by Caucasian and then African-Americans. NAFLD exhibits tight links with insulin resistance and metabolic syndrome. Several gene variants have been so far identified by Genome Wide Association Studies or by a candidate gene approach as associated with fatty liver disease. The *PNPLA3* rs738409 and the *GCKR* rs1260326 are the strongest variants associated with fatty liver in paediatrics.

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INTRODUCTION

Non-alcoholic fatty liver disease (NAFLD) comprehends

a wide range of conditions, encompassing from fatty liver or steatohepatitis (NASH) with or without fibrosis, to cirrhosis and its complications (*e.g.*, hepatocellular carcinoma and portal hypertension)[1,2]. The NAFLD diagnostic criteria are similar in adults and children: infiltration of more than 5% of hepatocytes, as confirmed by liver histology, in patients with no or low daily alcohol utilization and in absence of either viral, autoimmune or drug-induced liver disease[3-5]. NAFLD has become the most relevant form of liver disease in childhood[6] and its prevalence has highly increased over the past 20 years because of the increased obesity prevalence in children. It currently affects between 3% and 11% of the pediatric population[7,8] reaching the rate of 46% among overweight and obese children and adolescents[6]. Therefore, the screening for NAFLD should be recommended to overweight and obese children[9-11]. The liver histology is the gold standard for NAFLD diagnosis, but to perform biopsies isn't possible in all the cases. Liver enzymes values [aspartate aminotransferase (AST), and alanine aminotransferase (ALT)] are usually slightly elevated in children with steatosis without other causes of fatty liver[12]. Therefore, high serum AST and ALT levels, although they frequently do not well represent the grade of intrahepatic damage, are used as a non-invasive screening for pediatric NAFLD[13] along with liver ultrasound (US), that can detect the disease when steatosis involves > 20% of hepatocytes[14]. Although it does not represent the imaging gold standard, performing liver ultrasound has different advantages as screening : (1) relative cheapness; (2) massive expansion among pediatricians; and (3) practicability in the pediatric population[15]. Furthermore, very recently, in a large prospective pediatric cohort, it has been shown a good correlation between ultrasonographic steatosis score and the grade of fatty liver assessed by hepatic histology[14]. Computed tomography (CT) scan is not recommended in pediatric population because of the unjustifiable radiation related to the process. Magnetic resonance spectroscopy (MRS) and magnetic resonance Imaging (MRI) have been demonstrated to be the best methods to assess and quantify the amount of lipids present into the liver[16].

We reviewed the literature concerning the role of ethnicity and genetics in the pathogenesis of pediatric fatty liver disease.

PREVALENCE OF NAFLD AMONG ETHNIC GROUPS

Considering the global population in United States, Browning *et al*[17] described that the prevalence of NAFLD is the highest in the American Hispanic population (45%) and the lowest among African Americans (24%), with the Caucasians showing a midway prevalence (33%). The fatty liver prevalence in Europe, Australia, and Middle East encompasses from 20% to 30%[18]. On the basis of recent studies the NAFLD prevalence in Japan and China, such as Latin America, is comparable to the European prevalence (20%-30% in Japan and 15%-30% in China, respectively)[18]. In India, the fatty liver prevalence in urban populations encompasses from 16% to 32%; but in rural India, where there are traditional diets and lifestyles, the prevalence is lower (about 9%)[18]. About the prevalence of NAFLD in Africa there are few data. A Nigerian study estimated the prevalence to be about 9%[18]. This evidence suggests that a sedentary lifestyle and globalization of Western diet could be associated with an increase in the fatty liver prevalence in developing nations.

RISK FACTORS

The principal risk factor for fatty liver in childhood is the obesity. In fact, the pediatric prevalence of NAFLD is particularly high in those countries where childhood obesity is widespread[6,19]. Pediatric NAFLD is also highly correlated with insulin resistance and type 2 diabetes mellitus[20,21]. A high percentage (from 20% to 80%) of children with NAFLD may show associated hypertriglyceridemia and high LDL levels[22]. The prevalence of NAFLD increases in pre-diabetic children, and the subjects affected by NASH have an higher grade of insulin resistance than the individuals with simple fatty liver[8]. The NAFLD can also affect very young children, but its prevalence is higher in adolescents[23]. In fact, sex hormones and insulin resistance in puberty[24,25] and, moreover, the increased propensity for unhealthy food choices and sedentary lifestyle typical of the adolescents[26] can justify the higher rate of NAFLD in adolescents. In all the ethnicity, NAFLD is more prevalent in boys than in girls[22] with a male to female ratio of 2:1. This may be explained by the liver-protective role of estrogens, as well as by the potentially negative role of androgens in aggravating NAFLD[27,28]. Another risk factor that can promote the development of fatty liver is the excessive fructose consumption, in particular the fructose contained in the most common soda[29]. Substantial links have been demonstrated between increased fructose consumption and obesity, dyslipidemia, and insulin resistance (IR). The link between fructose ingestion and NAFLD is mainly explained by an increased hepatic *de novo* lipogenesis[30].

ROLE OF ETHNICITY IN DETERMINING HEPATIC STEATOSIS

The prevalence of hepatic steatosis varies among different ethnic groups. As previously shown, ethnicity with the greater prevalence is the Hispanic one followed by Caucasians and African-Americans[17]. NAFLD exhibits tight links with insulin resistance and metabolic syndrome (MS). It is, therefore, surprising that African-Americans, despite showing a similar or even higher degree of IR than Caucasians and Hispanics, have a lower prevalence of NAFLD[31] and a lower propensity for development of NASH[32]. The dissociation between fatty

liver and insulin resistance in African-Americans suggests that this group is protected from hepatic fat accumulation even in presence of IR. Browning *et al*[17] demonstrated that the ethnic differences in the prevalence of hepatic steatosis were not due to differences in the presence of risk factors for NAFLD such as increased body mass index (BMI), reduced insulin sensivity or ethanol ingestion. On the other, although there are no differences in the prevalence of risk factors among ethnicities, there are evident differences in body fat distribution especially concerning the three major fat depots (intraperitoneal, abdominal subcutaneous, and lower extremity)[33]. In fact, African Americans tend to accumulate less intravisceral fat, but more subcutaneous and mainly more gluteal fat than the other ethnic groups. Different is the association between regional adiposity and hepatic fat content. In fact, intra-peritoneal and lower extremities adiposity are strongly correlated with intra-hepatic fat, regardless of ethnicity[33].

While subjects with African ancestries have a low propensity to develop fatty liver, there are other ethnicities/races with a higher propensity to liver fat accumulation such as the subjects with Japanese descents. In fact, in a recent study, Azuma *et al*[34] considered ethnic difference in liver fat content among Japanese American in Hawaii, Japanese in Japan, and non-Hispanic whites in United States. Despite of a very similar BMI, compared with non Hispanic whites, Japanese-Americans had higher liver fat content which tended to become more significant with increasing BMI[34]. On the other hand, compared with Japanese, Japanese-Americans had a lower liver fat content, regardless of BMI[34].

What determines ethnic differences in hepatic steatosis is actually unknown.

In conclusion, insulin resistance and metabolic syndrome play a pivotal role in determining hepatic steatosis but they cannot explain such diversity among ethnic groups. In fact, as previously reported[17], the different ethnic predisposition to accumulate regional fat could partially explain the different ethnic prevalence of hepatic steatosis, since African-Americans have lower intraperitoneal fat accumulation than Hispanics and Caucasians. Therefore, the crucial issue to be resolved is why there is no association, in African-Americans, between the degree of IR and the degree of intra-hepatic fat. Is it possible that the hepatic steatosis does not play such an important role as we believe in developing IR? Why African-Americans despite high degree of IR have lower prevalence of NAFLD than other ethnic groups? Are there gene polymorphisms that could explain these paradoxes?

ROLE OF GENETICS IN DETERMINING HEPATIC STEATOSIS

PNPLA3 rs738409

The most important gene involved in determining hepatic steatosis is the patatin like phospholipase containing domain 3 gene (*PNPLA3*). The *PNPLA3* rs738409 single nucleotide polymorphism (SNP) is a non-synonymous variant, represented by a cytosine to guanosine substitution which encodes an isoleucine to methionine substitution at the amino acid position 148 (I148M) and was showed associated with NAFLD in a multiethnic cohort of adults[35] and children[36,37]. The *PNPLA3* encodes for the adiponutrin an enzyme present in the liver and adipose tissue showing both a lipogenic and lipolytic activity *in vitro*. The prevalence of the *PNPLA3* rs738409 minor allele (G) is 0.460 in Hispanics, 0.305 in Caucasians and 0.186 in African Americans[35].

It has been shown that this variant interacts with environmental factors (*i.e.*, obesity[37,38] and alcohol consumption[39]) that can themselves promote steatosis. In fact, these stressors seem to reveal the association between the rs738409 minor allele (G) and hepatic damage in populations in whom it is otherwise hidden[40]. It is interesting to underline that the same interaction appears with some nutrients. Indeed, the total carbohydrate and high omega (n)-6 to n-3 polyunsaturated fatty acids (PUFA) ratio can enhance the association between steatosis and *PNPLA3* variant[41].

Since the association between the *PNPLA3* rs738409 and fatty liver has been shown[35], a few researches tried to demonstrate this association physiopathology. Probably, this variant may lead to a gain of function of the protein, which could act as a lipogenic factor[42]. In fact, there is evidence that, administrating the mutated *PNPLA3* to knock-out mice for *PNPLA3* through viral vectors[43,44], the knock-out mice obtain a higher susceptibility to fatty liver[42]. Consistently, it was also shown that sterol regulatory element binding transcription factor 1 (*SREBP-1c*), activated by carbohydrate feeding, transcriptionally activates *PNPLA3* and other genes which encodes enzymes implicated in the fatty acid biosynthetic pathway[45]. Other researches demonstrating an interaction between the carbohydrates intake and the *PNPLA3* rs738409 in developing of NAFLD appear to support this mechanism[41]. However, the lack of association of the *PNPLA3* variant with increased plasma triglycerides is in contrast with this hypothesis[35,36].

Probably more interesting are the data deriving by evidence on the hydrolytic action of the *PNPLA3* given that the *PNPLA3* along with the acylglycerol transacetylase activity also has a triacylglycerol hydrolase function[46]. In fact, it has been recently showed that the rare allele could cause a lack of the *PNPLA3* hydrolytic function[46] reducing the protein capacity in hydrolyzing the n-9 of about 15%[46]. The n-9 are the most common fatty acids deriving from the diet (meat, olive oil, sesame oil, almonds, and avocados) but they can derive also being synthesized from essential polyunsaturated fatty acids such as the n-6[47]. In addition, Perttila *et al*[48] demonstrated that the *PNPLA3* 148M allele significantly slows down the triglycerides hydrolysis and then increases the cellular accumulation of triglycerides in presence of an excess free fatty acids (FFA). This might also explain the association between the PNPLA3 variant and the

Table 1 Gene variants associated with fatty liver disease identified by Genome Wide Association Studies

Ref.	Gene	Polymorphisms	Chromosome	Number of subjects studied
Romeo *et al*[35]	*PNPLA3*	rs738409	22	3383
Speliotes *et al*[71]	*GCKR*	rs1260326	2	7176
Petersen *et al*[60]	*APOC3*	rs2854116	11	258
		rs2854117		
Speliotes *et al*[71]	*NCAN*	rs2228603	19	7176
Speliotes *et al*[71]	*LYPLAL1*	rs12137855	1	7176
Speliotes *et al*[71]	*PPP1R3B*	rs4240624	8	7176
Adams *et al*[73]	*GC*	rs222054	4	928
Adams *et al*[73]	*LCP1*	rs7324845	13	928
Adams *et al*[73]	*SLC38A8*	rs11864146	16	928
Adams *et al*[73]	*LPPR4*	rs12743824	1	928
Kitamoto *et al*[72]	*SAMM50*	rs2143571	22	1326
Kitamoto *et al*[72]	*PARVB*	rs6006473	22	1326
		rs5764455		
		rs6006611		
Chalasani *et al*[66]	*FDFT1*	rs2645424	8	236

dietary lipids in modulating liver injury[49]. More recently, Li *et al*[50], generating mutant *PNPLA3* mice for I148M polymorphism, showed that the *PNPLA3* variant is associated with an increased formation of fatty acids and triacylglycerol and relative depletion of triacylglycerol long-chain polyunsaturated fatty acids. Metabolic studies in the transgenic mice showed that high level expression of *PNPLA3* I148M only in the liver and not in adipose tissue, affected both hepatic triacylglycerol (TAG) synthesis and catabolism. Also, it is interesting to note that *PNPLA3* I148M transgenic mice develop steatosis on a sucrose diet but not on a high-fat diet[51].

Interestingly, Speliotes *et al*[52] showed, among patient selected for NAFLD, that the G allele of the *PNPLA3* rs738409 polymorphism is associated with a favourable metabolic profile including decrease triglyceride levels, BMI, waist circumference and weight. These results argue strongly against rs738409 *PNPLA3* polymorphism increasing risk of NAFLD indirectly through an effect of these components of metabolic syndrome[52]. Despite this evidence, the effect of this polymorphism on liver damage is driven by the amount of visceral fat[37], and it has been demonstrated that weight loss reduce the effect of this polymorphism in obese children[53]. For this reason, the possibility exists that the association between the *PNPLA3* rs738409 SNP and NAFLD is modulated by the degree and the distribution of adiposity; this might explain the differences observed in the rs738409 *PNPLA3* phenotype.

Therefore, the *PNPLA3* I148M polymorphism increases the risk of NAFLD without a strong effect on metabolic syndrome components[52] but the abdominal fat, strictly correlated to metabolic syndrome components, can drive the effect of this polymorphism on liver damage. The weight loss, in fact, reduces the effect of this polymorphism[53].

GCKR rs1260326

Another gene that acts together with *PNPLA3* in determining hepatic steatosis is the Glucokinase Regulatory Protein (*GCKR*) gene[54] which encodes for the glucokinase regulatory protein (GCKRP). The GCKRP inhibits the glucokinase (GCK) activity competing with the glucose, substrate of GCK[55-57]. The rs1260326 in the *GCKR* gene is a functionally relevant SNP consisting of a C to T substitution encoding for a proline-to-leucine substitution at position 446 (P446L). It has been demonstrated that the GCKRP L466 variant encodes for a protein that has decreased regulation by physiological concentration of fructose 6 phosphate. This results indirectly in a constant increased GCK activity[58]. The increase in GCK hepatic activity leads to enhancement of the glycolytic flux, and then promotes hepatic glucose metabolism and raises the concentrations of malonyl coenzyme A, a substrate for *de novo* lipogenesis (DNL). DNL may contribute for about 20% in liver fat accumulation[59]. The *GCKR* SNP rs1260326 minor allele (T) frequency was 0.466 in Caucasians, 0.129 in African Americans, and 0.355 in the Hispanics ($P < 0.0001$)[54]. This allele is associated with higher fat content in the liver among all ethnic groups[54]. Therefore, the possibility exists that the lowest prevalence of *PNPLA3* rs738409 minor allele and *GCKR* rs1260326 minor allele (T) in African Americans could explain the lowest prevalence of hepatic steatosis in this ethnic group regardless of the unfavourable metabolic profile.

APOC3 rs2854116 and rs2854117

Apolipoprotein C3 gene (*APOC3*) variants are also involved, probably, in determining NAFLD. Petersen *et al*[60] showed that two SNPs in *APOC3* (rs2854117 and rs2854116 codifying for C-482T and T-455C respectively) are associated with NAFLD and marked insulin resistance in 95 healthy, non-obese, Asian Indian men. The rare-allele carriers and wild-type homozygotes had a NAFLD prevalence of 38% and 0% respectively ($P < 0.001$). The subjects with NAFLD had marked insulin resistance[60]. The mechanism suggested was that the above mentioned *APOC3* variants lead to higher plasma concentrations of apolipoprotein C3. The apo-

Table 2 Proposed mechanism of action of each genetic variant associated with fatty liver disease

Gene	Proposed mechanism of action
PNPLA3	The *PNPLA3* encodes for the adiponutrin, an enzyme expressed in the liver and adipose tissue showing both a lipogenic and lipolytic activity. This variant could cause both a gain of function of the enzyme (which could have a lipogenic activity in the liver) and a loss of function (that could predispose to steatosis by decreasing triglyceride hydrolysis in hepatocytes)
GCKR	The gene product is a regulatory protein that inhibits glucokinase in liver and pancreatic islet cells. The polymorphism could lead to increased hepatic glucokinase activity. This enhance the glycolytic flux and then promotes hepatic glucose metabolism and elevates the concentrations of malonyl coenzyme A, a substrate for *de novo* lipogenesis
APOC3	*APOC3* variants could increase the plasma concentrations of apolipoprotein C3. The apolipoprotein C3 could then inhibit the lipoprotein lipase reducing the clearance of triglycerides. Consequence of reduced clearance of triglycerides is the increase of chylomicron-remnant particles that confer a predisposition to both fasting and postprandial hypertriglyceridemia. Higher circulating levels of chylomicron-remnant particles are then especially cleared by the liver through a receptor-mediated process, resulting in NAFLD and hepatic insulin resistance
NCAN	*NCAN* encodes for a chondroitin sulfate proteoglycan thought to be involved in the modulation of cell adhesion and migration. *NCAN* is a risk factor for liver inflammation and fibrosis, suggesting that this locus is responsible for progression from steatosis to steatohepatitis
LYPLAL1	*LYPLAL1* encodes for a lysophospholipase and it is associated with increased hepatic steatosis probably preventing breakdown of triglycerides
PPP1R3B	This gene encodes the catalytic subunit of the serine/theonine phosphatase, protein phosphatase-1. The encoded protein is expressed in liver. It is associated with computer tomography–assessed liver attenuation but not histology-proven NAFLD
GC	*GC* gene is expressed predominately in the hepatocytes where it encodes for VDBP. VDBP is the main vitamin D carrier, which has been implicated in the development of obesity and diabetes. In fact, low vitamin D concentrations could increase adipocyte intracellular calcium, stimulating lipogenesis, whereas vitamin D supplementation improves insulin resistance and down-regulates inflammatory cytokines such as tumor necrosis factor-a and interleukin-6 in cell models. Vitamin D levels are influenced by GC genetic polymorphisms
LCP1	LCP-1 is an actin bundling protein expressed especially in hematopoietic cells and is involved in leukocyte activation and tumor cell proliferation. Its pathogenic role in NAFLD is unknown
SLC38A8 LPPR4	SLC38A8 protein product is a putative sodium-coupled neutral amino acid transporter whose expression is limited to the brain, whereas LPPR4 catalyzes the dephosphorylation of biologically active lipids and is expressed especially in the hypothalamus. While the functional significance of neuronally expressed genes such as SLC38A8 and LPPR4 with NAFLD is not apparent, there is convincing evidence that the nervous system and particularly the hypothalamus play an important role in lipid homeostasis in the liver
SAMM50	*SAMM50* gene encodes for Sam50 a protein that may be involved in mitochondrial dysfunction. The subsequent decreased removal of reactive oxygen species could lead to progression of NAFLD
PARVB	The *PARVB* gene encodes parvin-b, which forms integrin-linked kinase-pinch-parvin complex. Integrins are a large family of heterodimeric cell surface receptors that act as mechanoreceptors by relaying information between cells and from the ECM to the cell interior. Since integrin receptors directly bind to ECM components to control remodeling, they are thought to play a crucial role in the evolution and progression of liver fibrosis. Overexpression of parvin-b leads to a concomitant increase in lipogenic gene expression
FDFT1	The *FDFT1* gene, situated on chromosome 8, is an important modulator of cholesterol biosynthesis[67,68]. It codifies for the Squalene Synthase, an enzyme which converts two molecules of farnesyl pyrophosphate into squalene, a precursor to cholesterol. An hypothesis could be that this SNP is in linkage disequilibrium with a variant in the promoter of squalene synthase gene that through the enhancement of its expression, could lead to an increased activation of the enzyme and to the intra-hepatic accumulation of cholesterol

VDBP: Vitamin D binding protein; NAFLD: Non-alcoholic fatty liver disease; ECM: Extracellular matrix; LCP-1: Lymphocyte cytosolic protein-1; SLC38A8: Solute carrier family 38 member 8; LPPR4: Lipid phosphate phosphatase-related protein type 4 gene; GC: Group-specific component.

lipoprotein C3 could then inhibit the lipoprotein lipase reducing the clearance of triglycerides. Consequence of reduced clearance of triglycerides is the increase of chylomicron-remnant particles that confer a predisposition to both fasting and postprandial hypertriglyceridemia. Higher circulating levels of chylomicron-remnant particles are then especially cleared by the liver through a receptor-mediated process[61-63], resulting in NAFLD and hepatic insulin resistance. Other data available in literature on correlation between *APOC3* and NAFLD are contrasting[64,65]. The differences could be due to the anthropometric profile of subjects included in different researches. Petersen *et al*[60] studied a cohort without any risk factor of metabolic syndrome and with relatively lower body mass index (BMI) of 24.7 ± 3.6 and 24.1 ± 2.9 kg/m^2 in Indian and non-Indian groups, respectively; it is important to underline that a BMI of 24.7 may indicate overweight in Indians but normal body weight in European. In comparison, other works studied larger numbers of subjects with overweight/obesity, dyslipidemia and with the criteria of metabolic syndrome.

FDFT1 rs2645424

A SNP in the farnesyl-diphosphate farnesyltransferase 1 (*FDFT1*) gene has been associated with the degree of liver injury: the rs2645424. This variant, in fact, has been showed to be associated with NAFLD activity score by a GWAS study. This study examined 236 non-Hispanic white women with fatty liver[66]. The *FDFT1* gene, situated on chromosome 8, is an important modulator of cholesterol biosynthesis[67,68]. It codifies for the Squalene Synthase, an enzyme which converts two molecules of farnesyl pyrophosphate into squalene, a precursor to cholesterol. The rs2645424 is an intronic variant and therefore it is difficult to explain how it may affect the enzyme activity; an hypothesis could be that this SNP is in linkage disequilibrium with a variant in the promoter of squalene synthase gene that through

the enhancement of its expression, could lead to an increased activation of the enzyme and to the intra-hepatic accumulation of cholesterol. Evidence in animals has, in fact, shown that transient over-expression of the *FDFT1* gene in the liver of both wild-type and LDL Receptor knockout mice led to higher *de novo* cholesterol biosynthesis, over-secretion of cholesterol-rich LDL, increased cholesterol concentrations and a 37% enhancement in liver weight compared with controls related to hepatocyte proliferation[69]. This hypothesis would also be in agreement with recent researches demonstrating the role of intra-hepatic cholesterol accumulation in the pathogenesis of NASH[70].

NOVEL GENE VARIANTS ASSOCIATED WITH HEPATIC FAT CONTENT

More recently, more gene variants have been associated with fatty liver disease (Table 1), the mechanisms of action are summarized in Table 2.

Speliotes *et al*[71], in addition to *GCKR*, identified variants in novel loci *NCAN* and *LYPLAL1* associated with both increasing computer tomography (CT) hepatic steatosis and histological NAFLD and identified variants in another locus, *protein phosphatase 1 regulatory subunit 3B* (*PPP1R3B*), associated with CT steatosis but not histologic NAFLD[71]. Recently Kitamoto *et al*[72] found that *PNPLA3, sorting and assembly machinery component* (*SAMM50*), *parvin beta* (*PARVB*) genetic regions was significantly associated with NAFLD in the Japanese population. Adams *et al*[73] showed that SNPs in two genes expressed in liver were associated with NAFLD adolescents: *group-specific component (GC)* and *lymphocyte cytosolic protein-1 (LCP1)*. SNPs in two genes expressed in neurons were also associated with NAFLD: *lipid phosphate phosphatise-related protein type 4 (LPPR4)* and *solute carrier family 38 member 8 (SLC38A8)*[73].

FUTURE DIRECTIONS

Although the last few years have shed light in the pathogenesis of fatty liver, more researches are needed to be done and novel approaches to this problem are needed to be thought especially in the pediatric population. In fact, a limitation of this review is that several of the quoted studies have been performed in adults, but unfortunately accurate studies in pediatrics are quite limited also because the state of art techniques to assess hepatic fat content (MRI, MRS and liver biopsy) are difficult to perform in the pediatric population and very expensive.

One promising line of research that will allow to uncover novel mechanisms in the pathogenesis of fatty liver is the study of gut microbiome. Several lines of evidence, in fact, suggest a strong interaction between gut flora and liver. In fact, the liver receives 70% of its blood flow from the intestine through the portal vein and act as the first line of defence against gut-derived antigens, therefore it is one of the most exposed organs to gut-derived toxic factors[74]. The joint between gut microbiota and the development of fatty liver has been demonstrated both in murine model and in humans. In mice, Bäckhed *et al*[75] observed that the transplantation of normal caecal microbiota to germ-free mice induced, 15 d later, a 60% increase in body fat along with a more than two-fold increase in hepatic triglyceride content. For these reasons several researchers are now investigating the role of the gut flora in determining and influencing non-alcoholic fatty liver disease.

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TOPIC HIGHLIGHT

WJG 20th Anniversary Special Issues (12): Nonalcoholic fatty liver disease

Incretin based therapies: A novel treatment approach for non-alcoholic fatty liver disease

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Abstract

Non-alcoholic fatty liver disease is considered a hepatic manifestation of metabolic syndrome (MS). The current treatment of non-alcoholic fatty liver disease (NAFLD) principally includes amelioration of MS components by lifestyle modifications but the lack of success in their implementation and sustainment arises the need for effective pharmacological agent in fatty liver treatment. Incretins are gut derived hormones secreted into the circulation in response to nutrient ingestion that enhances glucose-stimulated insulin secretion. Glucagon-like peptide-1 (GLP-1) is the most important incretin. Its receptor agonist and inhibitors of dipeptidyl peptidase-4 (DPP-4) are used in treatment of type 2 diabetes mellitus. DPP-4 serum activity and hepatic expression are shown to be elevated in several hepatic diseases. There are several experimental and clinical trials exploring the efficacy of incretin based therapies in NAFLD treatment. They suggest that GLP-1 analogues might have beneficial effect on hepatic steatosis acting as insulin sensitizers and directly by stimulating GLP-1 receptors expressed on hepatocytes. The use of DPP-4 inhibitors also results in hepatic fat reduction but the mechanism of action remains unclear. There is growing evidence that incretin based therapies have beneficial effects on hepatocytes, however further study analysis are needed to assess the long term effect of incretin based therapies on NAFLD.

Key words: Non-alcoholic fatty liver disease; Insulin resistance; Glucagon-like peptide-1; Dipeptidyl peptidase-4; Metabolic syndrome

Core tip: Insulin resistance is considered a fundamental problem in the genesis of hepatic steatosis and the pathophysiology of its development. In this review we discussed the role of incretin based therapies, including glucagon like peptide-1 (GLP-1) analogues and dipeptidyl peptidase-4 (DPP-4) inhibitors as a potential novel agents in non-alcoholic fatty liver disease treatment comprising experimental and clinical data available so far which generally suggest that GLP-1 analogues as well as DPP-4 inhibitors might be involved in direct pathways of liver fat elimination. To the best of our knowledge, this is the first review comprising all the data about incretin based therapies in fatty liver treatment.

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INTRODUCTION

Non-alcoholic fatty liver disease (NAFLD) is one of the most common liver disease affecting up to 30% of the general population worldwide[1,2]. It is defined as a chronic liver condition characterized by hepatic fat accumulation in the absence of other identifiable cause such as alcohol abuse, viral or autoimmune hepatitis, alpha-1 antitrypsin deficiency, medications like corticosteroids and estrogens, and other conditions[3]. In a certain percentage of patients it might occur in a more serious form of the disease, non-alcoholic steatohepatitis (NASH) and 10%-15% of the patients with NASH develop cirrhosis or even hepatocellular carcinoma[4-6]. NAFLD, previously named also as a diabetic hepatitis, is highly associated with several components of metabolic syndrome (MS), particularly obesity, increased plasma lipid levels (primarily triglycerides), insulin resistance and concomitant glucose intolerance and type 2 diabetes mellitus (T2DM)[4,7,8]. The current treatment of NAFLD principally includes ameliorating MS components including weight loss and insulin sensitivity improvement by lifestyle modifications[9-11]. The use of insulin sensitising agents such as metformin and thiazolidinediones has been investigated in numerous clinical trials whose results suggest that this approach has modest efficacy for NAFLD treatment[12,13]. A recent meta-analysis by Musso *et al*[14] suggests that the use of TZDs might improve hepatic steatosis both in non-diabetic and diabetic patients but they also emphasise the possible limit of TZDs action in histological liver injuries more severe than NAFLD, such as NASH and also arise the question about importance of insulin sensitivity improvement and its histological benefits *per se*. Moreover, several studies indicate that the use of antioxidants such as vitamin E could improve histological features of NAFLD and NASH[15,16] which was recently confirmed by Sanyal *et al*[17]. Their study results suggest the vitamin E might have better effects on NASH than placebo and even when compared to TZDs but also that TZDs might be superior to vitamin E when NAFLD is concerned. According to all, the Association for the Study of Liver Diseases (AASLD) guideline for diagnosis and NAFLD treatment states that metformin has no significant effect on liver histology and it is not recommended in treatment in adults with NASH, while the use of pioglitazone (TZD) is recommended with a certain degree of precaution because of the unknown long-term outcomes[18]. Thus, the increasing prevalence of NAFLD and the lack of success in implementing and sustaining lifestyle modifications arises the need for effective pharmacological agent in fatty liver treatment and incretin based therapies might fulfil its role.

The incretines are intestinal mucosa-derived hormones which are secreted into the circulation in response to nutrient ingestion that enhance glucose-stimulated insulin secretion[19]. Glucose-dependent insulinotropic polypeptide (GIP) and glucagon-like peptide-1 (GLP-1) are two identified incretine hormones so far. They each contribute equally to incretine effect and fully account for such activity in humans[20]. The incretine effect comprises enhancement of insulin biosynthesis and secretion in glucose-dependent manner, pancreatic β cell mass increasment, appetite suppression and delay in gastric emptying[21]. However, as it was established that metabolic effects of GIP are blunted in T2DM and the GLP-1 effects are preserved, only GLP-1 remains in a great interest of T2DM and related disorders treatment[20]. As circulating GLP-1 has a half-life about 1-2 min due to rapid degradation by enzyme dipeptidyl peptidase-4 (DPP-4), GLP-1 receptor agonists with increased DPP-4 resistance and DPP-4 inhibitors have been developed[22]. Recent studies suggest that GLP-1 receptor agonists and DPP-4 inhibitors might be agents of great interest for hepatic fat reducement so in this review we present the pleiotropic effect of incretin based therapies for NAFLD.

GLP-1 RECEPTOR AGONISTS

GLP-1 receptor agonists represent a novel class of therapies for T2DM treatment that reflect glucoregulatory effects of endogenous incretin peptide GLP-1. This class of agents usually includes twice daily and once weekly formulation of exenatide and once daily liraglutide which are already implemented in routine clinical practice as well as several agents in earlier stages of clinical development such as lixisenatide, taspoglutide and albiglutide[23-26]. Exenatide was the first GLP-1 receptor agonist introduced in the clinical practice. It is originally derived from the salivary glands of the Gila monster and has 53% homology with human GLP-1. It is administered up to 60 min before morning and evening meals as indicated as an adjunct to diet and exercise or as an add-on ⩾ 1 oral hypoglycaemic agent (OHA) therapy[27]. Clinical trials with twice-daily exenatide demonstrated reductions of glycated haemoglobin A1c (HbA1c) averaging 1.1%[28-30]. Results from four separate open-label studies comparing exenatide therapy with starter insulin suggest that twice-daily exenatide at doses of 10 μg was similarly effective to either titrated basal or analog mix insulin[31-33]. Once weekly exenatide therapy results in a greater mean HbA1c reduction compared to twice-daily formulation as well as when compared to titrated insulin glargine[34].

The once-daily human GLP-1 receptor agonist liraglutide was approved by US Food and Drug Administration (FDA) in the 2010[35]. It has 97% homology with human GLP-1 with a single amino acid substitution extending its half-life up to 13 h[24]. Data from Liraglutide Effect and Action in Diabetes (LEAD) program have demonstrated that liraglutide, when used either as monotherapy or as an add-on in combination with ⩾1 OHA, lowers HbA1c by 0.84% to 1.48%[36,37]. Moreover, data from LEAD-6, the head-to-head trial versus exenatide, 1.8 mg liraglutide once daily resulted in a significant greater reduction in HbA1c than exenatide 10 μg bid (-1.1% *vs* -0.8%, $P < 0.0001$)[38]. So far only exenatide is FDA approved for the use in combination with basal

insulin. A case-controlled analysis of addition exenatide to insulin therapy resulted in a mean HbA1c decrease of 0.6% but also enabled the reduction in insulin dose which resulted in weight reduction[39]. Body weight reduction, beneficial itself, might also exert favorable effects on insulin action, cardiovascular disease risk factors such as blood pressure and plasma lipids and hepatic fat accumulation. A 52-wk study with once-weekly exenatide showed a 6-mm Hg systolic blood pressure reduction and improvements in the lipid profile in patients with T2DM[40]. Sathyanarayana *et al*[41] examined the effects of combined pioglitazone (peroxisome proliferator-activated receptor-γ (PPAR-γ) agonist) and exenatide therapy on hepatic fat content in patients with T2DM and suggested that combined pioglitazone and exenatide therapy was associated with a significantly greater decrease in hepatic fat (12.1% ± 1.7% to 4.7% ± 1.3%) and plasma triglyceride (38%) *vs* pioglitazone therapy despite the lack of a significant change in body weight (Δ = 0.2 kg). However, whether these favourable effects on MS components are associated with body weight reduction or are a result of treatment alone warrants further study investigation. Furthermore, although glucagon-like peptide-1-based therapy is now routinely used therapy for T2DM, there are concerns about risks for pancreatitis and pancreatic and thyroid cancer. Previous larges administrative database studies in the United States reported that treatment with the GLP-1-based therapies sitagliptin and exenatide was associated with increased odds of hospitalization for acute pancreatitis[42,43]. GLP-1 receptors are expressed in the exocrine pancreas, and GLP-1 based therapy has been shown to increase pancreatic ductal turnover and acinar to ductal metaplasia[44,45]. In addition, low-grade chronic pancreatitis, which increases risk of pancreatic cancer, was noted in rats treated with exenatide[46,47]. However, in a recent study GLP-1 analog liraglutide did not induce pancreatitis in mice, rats, or monkeys when dosed for up to 2 years and at exposure levels up to 60 times higher than in humans[48]. In addition, study including patients with adenocarcinoma and with and without diabetes found that insulin-stimulating medications such as incretin mimetics did not appear to accelerate pancreatic adenocarcinoma development[49]. There was also an increase in reported thyroid cancer as an adverse event related to exenatide or sitagliptin therapy compared to other oral therapies. In animal models, GLP-1 therapy has been shown to lead to C-cell hyperplasia, but it is unknown what effects GLP-1 therapy has on the human thyroid gland[43].

DPP-4 INHIBITORS

DPP-4 inhibitors are being used clinically in combination with most other oral antidiabetic agents (including sulfonylureas, thiazolidinediones, and metformin) in patients failing to achieve adequate glycaemic control, or who wish to limit weight gain. A number of inhibitors of the enzyme DPP-4, which regulates the bioactivity of native GLP-1, have been developed but only few of these agents (sitagliptin, vildagliptin, saxagliptin, linagliptin, alogliptin) are available for clinical use[50]. Either as monotherapy or an add-on to oral agents, DPP-4 inhibitors reduce mean HbA1c by approximately 0.5% to 0.8%[51-55], a clinical effect somewhat less than that reported with the GLP-1 receptor agonists. DPP-4 inhibitors lower blood glucose without a significant increase or reduction in body weight (0.2 to 0.8 kg)[54,55]. Data demonstrating extraglycemic effects of DPP-4 inhibitors such as benefits on lipids, blood pressure, or markers of inflammation are very limited. There is no evidence that DPP-4 inhibitor therapy results in significant body weight, appetite, or food intake reductions. Trials with vildagliptin have shown modest improvements in triglycerides and high-density lipoprotein cholesterol (4.8% and 10.6%, respectively, in combination with a thiazolidinedione), as well as reductions in systolic and diastolic blood pressure[55]. On the opposite to GLP-1 receptor agonists, DPP-4 inhibitor therapy is generally well tolerated, with no significant gastrointestinal or systemic side effects having been reported in clinical trials. A favorable tolerability profile means that DPP-4 inhibitor therapy can be safely administered to patients with a range of comorbidities. However, dosage adjustments of sitagliptin are recommended in patients with moderate- to end- stage renal disease[56], because it is cleared by the kidney while both sitagliptin and vildagliptin are contraindicated in patients with severe hepatic dysfunction but no sitagliptin dosage adjustment is needed in patients with mild-to-moderate hepatic insufficiency[57]. Postmarketing reports of sitagliptin-associated serious hypersensitivity reactions, including anaphylaxis, angioedema, Stevens-Johnson syndrome, and hepatic enzyme elevations have been noted[50,57]. On the other hand, the elimination of another oral DPP-4 inhibitor linagliptin, that was approved in the United States and Europe, is primarily non-renal and dose adjustment is not required even in patients with mild, moderate or severe hepatic impairment[50].

PATHOGENESIS AND METABOLIC CHANGES IN NAFLD

The pathogenesis of NAFLD comprises a spectrum of genetic factors in combination with obesity and consumption of products with high glycaemic index and rich in saturated fat which also represents a underlying mechanism in insulin resistance development[58-60]. Inability of insulin to suppress the lipolysis in white adipose tissue is defined as insulin resistance and is closely associated with hepatic fat accumulation[61,62]. The lack of lipolysis suppression leads to increased plasma concentration of free fatty acids (FFA) which then disrupts the hepatic balance in FFA influx and oxidation and becomes the main source of hepatic triglycerides in NAFLD[63]. Therefore, insulin resistance in white adi-

pose tissue might contribute to hepatic fat accumulation. Additionally, the use of insulin sensitizers thiazolidnediones, the peroxisome-proliferator-activated receptors γ (PPARγ) agonists has shown to decrease liver fat content by 40% despite the unaltered liver PPARγ in NAFLD[64]. Hepatocytes in human NAFLD contain high amount of saturated fatty acids and saturated fatty acid containing triglycerides which cannot all be contributable to FFA increased influx[65] but also to hepatic *de novo* lipogenesis which is significantly increased in NAFLD[63]. Higuchi *et al*[66] revealed that hepatic gene expression of sterol regulatory element-binding protein (SREBP) 1c, which is the key transcriptional activator of lipogenic genes as well as acetyl-CoA carboxylases (ACCs) and fatty acid synthetase (FAS), is increased in subjects with as compared to those without NAFLD while Kotronen *et al*[65] reported that the activity of lipogenic enzyme stearoyl-CoA desaturase 1 (SCD1) is increased which leads to decrease in long polyunsaturated fatty acid content.

In addition to white adipose tissue and skeletal muscle, the liver is the main site of insulin action[67]. Insulin resistance in liver usually accompanies insulin resistance in white adipose tissue and can be observed in the glucose and lipid metabolism. In the fasting state insulin restrains hepatic glucose production in order to maintain glucose concentration homeostasis. In insulin resistance state the ability of insulin to inhibit hepatic glucose production is impaired leading to higher fasting plasma glucose concentration and consequent hyperinsulinemia[67,68]. Another insulin action in liver is to restrain the production of very low density lipoprotein (VLDL)[69] so the insulin resistant liver overproduces triglyceride rich VLDL in the fasting state[61,70] which then leads to hypertriglyceridemia and low high density lipoprotein (HDL) concentration that can be found in subjects with MS and NAFLD[71,72]. In accordance with previous, insulin resistance is the central pathophysiological phenomenon of MS associated with the development of type 2 diabetes and NAFLD[73]. Moreover, although insulin resistance is not a underlying cause of some other disease like autoimmune type 1 diabetes, the presence of insulin resistance in those subjects are independently associated with markers of NAFLD[74] (Figure 1).

INCRETIN BASED THERAPIES-AN INDIRECT ACTION

GLP-1 receptor agonists (exenatide, liraglutide) are agents developed for T2DM treatment with a primarily role of maintenance of glucose homeostasis. However, their pleiotropic effect on appetite, weight, blood pressure, cardiovascular function and central nervous system have been reported[75]. The indirect action of GLP-1 receptor agonists on NAFLD pathophysiology might be observed from at least two aspects: the appetite suppression and weight reduction as well as direct and indirect improvement in insulin sensitivity since it has been proposed that weight reduction might improve insulin sensitivity[36,76-78]. GLP-1 receptor activation in hypothalamus reduces appetite and leads to weight loss[79]. When administered as mono-therapy, exenatide is associated with weight loss of approximately 3 kg over 24 wk of treatment in obese T2DM population[80] while 52-wk liraglutide monotherapy provided dose dependent reduction in mean body weight of 1.9 and 2.3 kg, respectively[36]. Similar effect was observed with non-diabetic healthy population treated with exenatide[81] and it was shown that even modest weight loss of 5%-10% of body weight decreases liver fat up to 40%-80% in non-diabetic subjects and T2DM patients[82-85]. On the opposite, DPP-4 inhibitors show no significant effect on weight loss[52,84] and the assumption that they cannot provide this indirect beneficial effect on hepatic fat reduction becomes obvious. As we already discussed, insulin resistance in white adipose tissue is one of the main steps in NAFLD development. Data from the study of Klonoff *et al*[85] as well as Buse *et al*[86] suggest that exenatide treatment in T2DM patients leads to significant alanine aminotransferase (ALT) and intrahepatic fat reduction in correlation to insulin resistance decreasment.

There are several studies suggesting that GLP-1 receptor analogues directly affect peripheral insulin sensitivity; Egan *et al*[77] used modified clamp and demonstrated that GLP-1 infusion increases glucose uptake without significant rise independent of changes in insulin secretion in ten healthy obese volunteers while the experimental results suggest the neutral action on lipolysis in white adipose tissue which then diminishes the possibility of their beneficial effect on NAFLD pathogenesis *per se*[87]. Treatment with DPP-4 inhibitors vildagliptin and sitagliptin have also demonstrated favourable effects on postprandial elevations of triglycerides, ApoB-48 and FFA levels[88,89]. The DPP-4 inhibition was also reported to enhance the antilipolytic effect of neuropeptide Y (NPY) in human abdominal adipocytes which could have indirect mechanism on hepatic fat reducing FFA influx in hepatocytes[90]. However, it should be kept in mind that NPY causes abdominal fat accumulation, one of the major steps in hepatic insulin resistance development[91]. It is possible that DPP-4 inhibitors might have indirect mechanism in inhibition of hepatic fat accumulation serve as local endogenous GLP-1 concentration enhancer because they have no influence on gastric emptying or weight loss (Figure 1).

INCRETIN BASED THERAPIES-A DIRECT ACTION

The implementation of GLP-1 agonists in clinical practice, primary in treatment of T2DM, revealed data suggesting their potency in intrahepatic liver fat and biomarker reduction independently of glycaemic or weight reduction[40,92,93]. However, it was unclear whether this effect could be contributable to GLP-1 agonists itself because there was no evidence of molecular mechanism that could explain this beneficial effect of GLP-1

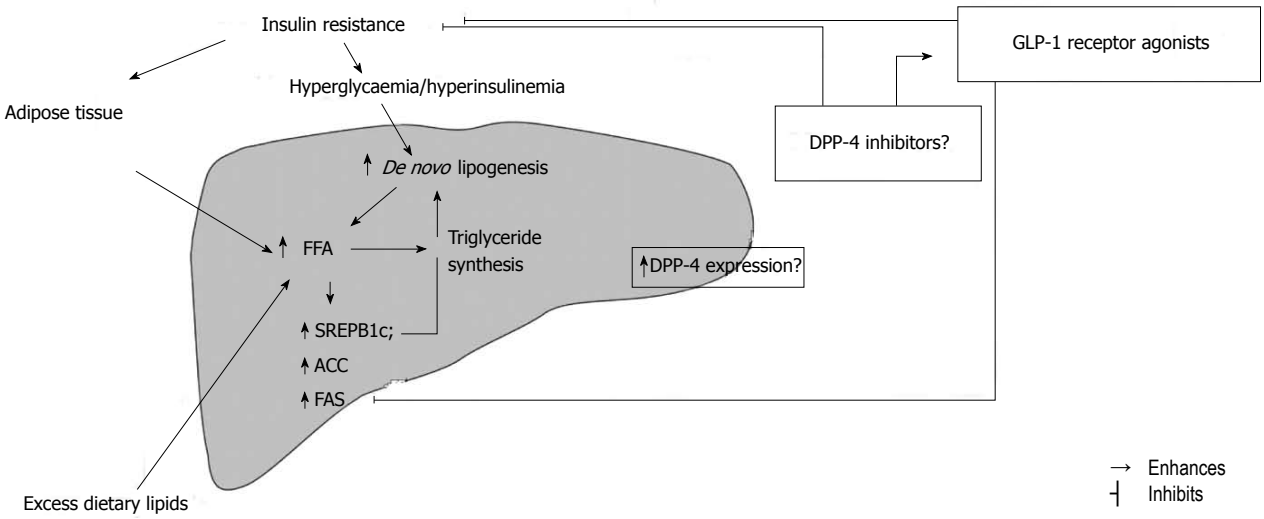

Figure 1 The pathogenesis of non-alcoholic fatty liver disease and the possible effect of incretin system components in treatment. Modified according to Dowman *et al*[63]. ACC: Acetyl-CoA carboxylase; DPP-4: Dipeptydil peptidase-4; GLP-1: Glucagon-like peptide-1; FFA: Free fatty acid; FAS: Fatty acid synthetase; SREPB1c: Sterol regulatory element-binding protein.

agonists. Several mouse models of NAFLD, based on genetic mutations or diet, were developed in order to serve as a preclinical platform to evaluate GLP-1 actions in liver[94]. Ding *et al*[95] suggested that GLP-1 analogue exendin-4 improves insulin resistance in *ob/ob* mice and significantly reduces hepatic lipid stores evaluated by histological improvement and improved of ALT values. They also revealed that exendin-4 has direct action on hepatocytes and subsequently results in a gene profile that is conducive in reduction in fatty acid synthesis and triglyceride storage in hepatocytes: increased mRNA for both PPARα along with decreased mRNA expression for SREBP-1c and ACC1. In support to those data Samson *et al*[96] demonstrated in diet induced obesity model mice that exendin-4 treatment reverses hepatic steatosis and decreases hepatic expression of genes involved in de novo fatty acid synthesis, including ACC1, fatty acid synthase (FAS) and SCD1. The question arises whether GLP-1 impairs hepatocyte *de novo* lipogenesis and/or enhances β-oxidation of fatty acids. Svegliati-Baroni *et al*[97] reported that exendin-4 treatment improves the expression of PPARα and its downstream target genes: acyl-Coenzyme A oxidase (ACOX) and carnitine palmitoyltransferase 1 A (CPT1A) in hepatocytes isolated from rats with non-alcoholic steatohepatitis. ACOX is the rate limiting enzyme in peroxisomal β oxidation while CPT1A is the key enzyme in initial transport of fatty acids into mitochondria for β oxidation. However, it was still unclear whether GLP-1 receptors were present in human liver and whether they were biologically active. Lee *et al*[98] reported that exendin-4 increases expression of GLP-1 receptor in a dose dependent manner in human hepatoma cell lines while Gupta *et al*[99] demonstrated that GLP-1 receptor is present in human hepatocytes and also provided molecular mechanism to explain the signal effectors of GLP-1 in its potential role in hepatocyte TG reduction by up regulating key elements of the insulin receptor substrate-2 (IRS-2) in hepatocytes. They suggest that GLP-1 based proteins should be analysed as insulin sensitizing agents in hepatocytes and that higher dose distribution of GLP-1 analogues should be considered in T2DM patients in order to reduce hepatic steatosis. There is also evidence that GLP-1 receptor agonists could improve hepatic steatosis by modulating fibroblast growth factor-21 (FGF-21) signalisation. In rodents, FGF21 is predominantly produced in the liver, where it enhances hepatic fat oxidation reduces triacylglycerol levels and hepatic steatosis but also increases adipocyte insulin sensitivity and regulates lipolysis in white adipose tissue, and improves glucose tolerance[100]. It has been suggested that these changes may occur through the effects of FGF21 on AMP-activated protein kinase (AMPK) activity. In the liver AMPK activates fatty acid oxidation via activation of PGC1α as well as its effects on other enzymes of lipogenesis described above (*e.g.*, SREBP and FAS)[101]. Dushay *et al*[102] have shown that liver FGF21 protein levels and RNA are increased in association with hepatic steatosis in obese humans with NAFLD. In mouse models of obesity, circulating FGF21 levels are elevated and FGF21 signalling in the liver and white adipose tissue is impaired[103]. Samson *et al*[104] suggested that exenatide treatment in T2DM patients and a diet induced obese mouse is associated with a decrease in FGF21 and hepatic fat, and an increase in hepatic AMPK and ACC phosphorylation explained as a possible sign of improved FGF21 resistance in the liver. Therapy with another GLP-1 agonist, liraglutide, in subjects with metabolic syndrome and T2DM is also associated with improvement of liver inflammation, alteration of liver fibrosis, and reduction of body weight[105,106]. Recent data indicate that endoplasmic stress is a major player in the progression of fatty liver to more aggressive lesions and treatment with liraglutide reduces steatosis and endoplasmic stress in high fat diet fed mice and

enhance lipoautophagy in liver[107].

Firneisz *et al*[108] reported a link between the serum DPP-4 activity and NAFLD in a small cross-sectional study whose results showed correlation of sDPP-4 activity with liver tests ALT, γGT, and less significantly ALP. Surprisingly, they reported that sDPP-4 activity was not increased in the T2D patients and there was no correlation between sDPP-4 and HbA1C values nor between sDPP-4 and fasting plasma glucose that were reported earlier in T2DM patients[109,110]. The positive correlation found among γGT, ALT and serum DPP-4 activities in NAFLD suggested that the excess DPP-4 found in the serum is of hepatic origin. Later on hepatic DPP-4 mRNA expression levels was analysed by real-time PCR using liver biopsy samples from 17 NAFLD patients and 10 healthy subjects by Miyazaki *et al*[111]. They showed that hepatic DPP-4 mRNA expression was significantly greater in NAFLD patients than in control subjects and that DPP-4 expression levels negatively correlated with homeostasis model assessment-estimated insulin resistance (HOMA-IR) index and positively correlated with serum cholesterol levels which clearly implicated hepatic DPP-4 in this disease. In addition, serum DPP-4 activity and hepatic DPP-4 expression are shown to be correlated with hepatic steatosis and NAFLD grading which indicates that it might be associated with hepatic lipogenesis and liver injury[112]. Iwasaki *et al*[113] evaluated the effect of sitagliptin administered in dose of 50 mg/body per day for 4 mo in 30 NAFLD patients with T2DM and reported a significant improvement of the serum levels of HbA1c and liver enzymes at 4, 8, 12 and 16 wk after the start of sitagliptin treatment and therefore concluded that sitagliptin might be considered as a novel therapeutic agent for NAFLD treatment. Moreover, it was recently reported that sitagliptin, DPP-4 inhibitor, ameliorated hepatic steatosis in 67-year-old Asian woman with refractory NAFLD after 3 mo treatment[114]. Considering some previously reported data about DPP-4 deficient rats showing lower levels of hepatic proinflammatory and profibrotic cytokines which often represents a "second hit" in NAFLD development[115], the role of DPP-4 inhibition in NAFLD treatment seams uncontested whether based on direct effect of DPP-4 inhibition or enhancement of endogenous GLP-1 action[116] (Figure 1).

CONCLUSION

In this review we described that insulin resistance was a fundamental problem in the genesis of hepatic steatosis and the pathophysiology of its development. We discussed the role of incretin based therapies, including GLP-1 analogues and DPP-4 inhibitors as a potential novel agents in NAFLD treatment comprising experimental and clinical data available so far. GLP-1 analogues are known to improve insulin resistance and are even more attractive because they have anorexigenic potential leading to weight loss, serum glucose and lipids improvement. Those, as well as DPP-4 inhibitors might also be involved in direct pathways of liver fat elimination. A growing body of literature suggests that GLP-1 and DPP-4 activity have numerous effects on the cells of various organs. The data regarding GLP-1 and DPP-4 inhibitors action on hepatocytes are convincing, but so far only T2DM patients with NAFLD/NASH have been studied with incretin analogues or DPP-4 inhibitors, therefore, the conclusions of this review only can be applied to T2DM patients suffering for NASH, and not for the nondiabetic-NAFLD patients. further study analysis are needed in order to aces the long term effect of incretin based therapies on NAFLD.

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TOPIC HIGHLIGHT

WJG 20th Anniversary Special Issues (12): Nonalcoholic fatty liver disease

Effects of resveratrol and other polyphenols in hepatic steatosis

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Abstract

Non-alcoholic fatty liver disease covers a wide spectrum of liver pathologies which range from simple steatosis to non-alcoholic steatohepatitis. Polyphenols are members of a very large family of plant-derived compounds that can have beneficial effects on human health, and thus their study has become an increasingly important area of human nutrition research. The aim of the present review is to compile published data concerning the effects of both isolated polyphenols as well as polyphenol extracts, on hepatocyte and liver fat accumulation under different steatosis-inducing conditions. The results reported clearly show that this group of biomolecules is able to reduce fat accumulation, but further studies are needed to establish the optimal dose and treatment period length. With regard to the potential mechanisms of action, there is a good consensus. The anti-lipidogenic effect of polyphenols is mainly due to reduced fatty acid and triacylglycerol synthesis, increased in fatty acid oxidation, and reduced of oxidative stress and inflammation. As a general conclusion, it can be stated that polyphenols are biomolecules which produce hepatoprotective effects. To date, these beneficial effects have been demonstrated in cultured cells and animal models. Thus, studies performed in humans are needed before these molecules can be considered as truly useful tools in the prevention of liver steatosis.

Key words: Polyphenols; Resveratrol; Quercetin liver; Steatosis; Non-alcoholic fatty liver disease

Core tip: Recently the beneficial effects of polyphenols in the prevention and treatment of liver steatosis have been reported. These biomolecules present hepatoprotective effects because they reduce liver fat accumulation, mainly by reducing lipogenesis and by increasing fatty acid oxidation, and decrease oxidative stress and inflammation, the main factors responsible for liver damage. To date, these beneficial effects have been demonstrated in cultured cells and animal models. Thus, studies performed in humans are needed before these molecules can be considered as truly useful tools in the prevention of liver steatosis.

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INTRODUCTION

Non-alcoholic fatty liver disease (NAFLD) encompasses a wide spectrum of liver pathologies which range from simple steatosis to non-alcoholic steatohepatitis (NASH). NASH is characterized by steatosis plus features of cellular injury, such as inflammation and hepatocyte ballooning. Some patients with NAFLD develop liver fibrosis, with a proportion progressing to cirrhosis and its complications of liver failure, portal hypertension, and hepatocellular carcinoma. Currently, cirrhotic stage NAFLD represents the third or fourth most common indication for liver transplantation in the United States, and the second most common indication for liver transplantation in large transplantation centers[1-3]. In addition, the prevalence of NAFLD-related cirrhosis has markedly increased in recent years as the underlying liver disease among patients transplanted for hepatocellular carcinoma in the United States. These data reflect the high prevalence of NAFLD in the general population, putting a substantial proportion of individuals at risk of NAFLD-associated morbidity and mortality[4-6].

Current prevalence estimates for NAFLD range from 20% to 75% depending on ethnicity, body mass index (BMI) and the presence of others diseases such as diabetes mellitus or dyslipemia[7,8]. Data suggest that 10% to 30% of NAFLD patients meet the criteria for NASH, with an overall prevalence ranging from 3% up to 25%, depending on the population studied[9].

The long-term prognosis for individuals with NAFLD is not the same across the spectrum of the disease. Steatosis when not associated with cellular injury or fibrosis, follows a relatively benign clinical course, with an overall mortality similar to the general population of the same age and sex. For instance, < 1% of patients with simple steatosis progressed to cirrhosis or died from liver-related complication after a mean follow-up of 15 years in a pooled analysis of several reported series[6]. However, patients with NASH, particularly those with increased fibrosis, have a worse prognosis as compared to an age-and sex-matched population. The prevalence of cirrhosis and death related to liver complications is about 11% and 7%, respectively, in patients with NASH during the first 15 years of follow-up[5]. The most frequent etiology of NAFLD is overweight and obesity. More than 90% of patients with NAFLD are overweight or obese[10].

Drug-induced liver disease (for example, amiodarone, tamoxifen, corticosteroids, methotrexate, *etc.*), autoimmune or viral hepatitis, and cholestatic or metabolic/genetic liver disease can also cause NAFLD.

PHYSIOPATHOLOGY OF LIVER STEATOSIS

The exact pathogenic mechanisms of liver steatosis are not yet fully known. Steatosis, liver inflammation and fibrosis has been associated with an excessive triglyceride accumulation in the liver, insulin resistance and increases in visceral adipose tissue, mediated by increased free radical formation and free oxygen radical species, and modulated by genetic susceptibility[11,12]. There is evidence supporting the theory that these genetic factors account for considerable variability in susceptibility to NAFLD. Since the introduction of genome-wide association studies (GWASs) to investigate genomic variations, there have been significant advances in our understanding of human genome and its clinical effects over a range of diseases. A large number of single nucleotide polymorphisms (SNPs) related to NAFLD has been documented by candidate gene studies. The SNPs may increase or decrease the function of the target genes and their encoding proteins. Genes such as patatin-like phospholipase domain-containing protein 3 (PNPLA3), neurocan core protein (NCAN), glucokinase regulatory protein (GCKR) and lysophospholipase-like protein 1 (LYPLAL1) have been implicated in an increased risk of NAFLD[13]. Lipid peroxidation and free oxygen radical species can deplete antioxidant enzymes (glutathione, vitamin E, beta-carotene and vitamin C) and activate proinflammatory citokines, inflammatory mediators and activation of natural killer cells among others. Others factors as iron, leptin, adiponectin and resistin may contribute to the NAFLD. In the last years intestinal microbes have been implicated as a potential source of hepatotoxic oxidative injury. Intestinal bacterial overgrowth and increased intestinal permeability were observed in patients with NAFLD[14]. Mechanisms by which intestinal bacteria may contribute to hepatocellular injury include endotoxin production, deconjugation of bile salts and inactivation of hepatic lipotropes, such as choline.

As explained above, the most frequent etiology of NAFLD is overweight and obesity. In the following lines an attempt will be made to explain the steps involved in the progression of NAFLD to NASH under this metabolic situation. Adipocyte insensitivity to insulin (insulin resistance), frequently observed in obese patients, overrides the brake to lipolysis in adipose tissue, thus leading to the release of a large amount of free fatty acids. The excessive supply of free fatty acids to the liver is the primary mechanism of NAFLD production. Furthermore, increased amounts of insulin usually found under insulin resistance conditions produce a decrease in hepatic synthesis of apolipoprotein B-100 and the increase in hepatic synthesis of fatty acids. Consequently, the amount of triacylglycerols produced and stored in the liver increases.

NAFLD patients show increased prevalence of TNF-α 238 polymorphism, which induces the over-expression of TNF-α in adipose tissue and, in turn, alterations in the insulin receptor, which causes greater resistance to insulin[15]. Furthermore, several pro-inflammatory adipokines produced by adipose tissue are elevated in obese patients, and can contribute to systemic inflammation and liver damage. By contrast, adiponectin an anti-inflammatory adipokine which antagonizes excess lipid storage in the liver and protects it from inflammation and fibrosis[16], is reduced in these patients and is even lower in patients with hepatic steatosis or NASH.

Free fatty acids increase the expression of cytochrome P-450 2E1 (CYP 2E1)[17], an enzyme involved in the beta-oxidation of long-chain and very long-chain fatty acids, which causes the formation of reactive oxygen metabolites in the liver[18]. On the other hand, several long chain free fatty acids are metabolized in the peroxisomal beta-oxidation. This oxidation produces hydrogen peroxide in the presence of iron and causes hydroxyl radicals, which are reactive oxygen metabolites. This excess of reactive oxygen metabolites consumes antioxidant molecules, such as glutathione and vitamin E, in the liver and thereby generates an oxidative stress, which leads to lipid peroxidation[19]. In turn, lipid peroxidation causes a lesion of the membranes and organelles of hepatocytes, resulting in the phenomena of hepatocellular degeneration and necrosis. The injury caused by lipid peroxidation in mitochondria modifies their morphology (megamitochondria), alters the electron transfer along the respiratory chain and produces more reactive oxygen metabolites, which close the circle, thus generating more oxidative stress[19,20].

The end products of lipid peroxidation, malondialdehyde and 4-hydroxynonenal, have chemoattractant properties, activate proinflammatory cytokines (TNF-α, TGF-β, IL-6, IL-8) and stellate cell stimulatory collagen-producer in the liver. This results in a mixed lesion with hepatocyte degeneration and necrosis, fibrosis and inflammatory infiltrates, in addition to steatosis (NASH)[21]. Also, malondialdehyde and 4-hydroxynonenal covalently bind proteins and produce aggregates of proteins that promote immune response. Secondly, antibodies which can cause an antibody-mediated hepatocellular injury (autoimmune hepatitis) are produced[22]. The perpetuation of oxidative stress and lipid peroxidation causes sustained collagen production which in turn leads to a progression of fibrosis and thus to hepatic cirrhosis. Cirrhosis is the basis of the development of complications such as liver failure and hepatocellular carcinoma[21,23].

EFFECTS OF POLYPHENOLS ON HEPATIC STEATOSIS

Polyphenols are members of a very large family of plant-derived compounds that show an extensive variety of chemical structures. They are classified as flavonoids and non-flavonoids. Among the flavonoids, various groups can be distinguished: flavonols, flavan-3-ols, flavones, isoflavones, flavanones, proanthocyanidins and anthocyanidins. Non-flavonoids included stilbenes and phenolic acids[24].

Although not essentials as vitamins or minerals, polyphenols can have beneficial effects on human health, and thus their study has become an increasingly important area of human nutrition research. A large number of epidemiological studies have shown that the consumption of diets rich in fruits and vegetables is associated with a reduction in the risk of suffering chronic diseases, such as cardiovascular diseases, specific cancers or neurodegenerative diseases[25]. Recently the beneficial effects of polyphenols in the prevention and treatment of liver steatosis have been reported.

EFFECTS OF RESVERATROL ON HEPATIC STEATOSIS

Resveratrol (*trans*-3,4',5-trihydroxystilbene) is a stilbene occurring naturally in several plants and provided in the diet by various food stuffs such as grapes, berries, red wine and nuts. It is well known for its health benefits, such as improvement of insulin sensitivity and glucose tolerance, reduction of plasma lipids, enhancement and suppression of inflammation and oxidative stress[26]. In recent years, it has proved able to modify lipid metabolism, and more specifically to induce a reduction in liver triacylglycerol content. In this context, both *in vitro* and *in vivo* studies have been carried out in order to show its effects on the prevention and the treatment of liver steatosis (Tables 1 and 2).

In vitro studies

Few studies address the potential hepatoprotective action of resveratrol under *in vitro* conditions. In these studies different cell models have been used. In hepatocytes isolated from rat liver and incubated with 25 μmol/L resveratrol for 30 min, Gnoni and Paglialonga[27] observed 40% reduction in triacylglycerols and decreased acetyl-CoA carboxylase (ACC) activity, without changes in fatty acid synthase (FAS) activity. Based on these results, the authors suggested that resveratrol reduced *de novo* lipogenesis, thus decreasing the availability of fatty acids, and consequently the synthesis of triacylglycerols.

Wang *et al*[28] used a cell model of steatosis induced by palmitate treatment. This treatment induces maximal fat over-accumulation with minimal cytotoxicity. The inclusion of 40 μmol/L of resveratrol for 24 h in the incubation medium reduced triacylglycerol accumulation induced by palmitate almost to basal values, due to a decrease in both the expression and the activity of sterol regulatory element binding protein 1c (SREBP-1c), which is the most important transcription factor for *de*

Table 1 ***In vitro*** **studies performed with polyphenols**

Ref.	Animal model	Polyphenol dose and treatment period length	Effects	Mechanisms
Gnoni *et al*[27], 2009	HepG2 cells	25 μmol/L resveratrol 30 min	↓ Triacylglycerols	↓ ACC activity = FAS activity
Zang *et al*[29], 2006	HepG2 cells	10 μmol/L resveratrol 24 h	Prevent lipid accumulation	↑ phosphorylation AMPK (activation) in liver ↑ phosphorylation ACC (inhibition) in liver
Shang *et al*[30], 2008	HepG2 cells	50 μmol/L resveratrol 24 h	↓ Triacylglycerols	↑ phosphorylation AMPK liver (activation) ↓ mRNA de SREBP1c and FAS liver
Wang *et al*[28], 2009	Human HepG2 cells	40 μmol/L resveratrol 24 h	↓ Triacylglycerols	↓ SREBP 1c ↑ SIRT 1
Vidyashankar *et al*[53], 2013	Hep G2 cells	10 μmol/L quercetin 24 h	↓ Triacylglycerols ↓ Insuline resistance ↓ Oxidative stress ↑ Superoxide dismutase, catalase and glutathione peroxidase activities	
Guo *et al*[60], 2011	Hep G2 cells	Anthocyanin Cy-3-g 1, 10, 100 μmol/L 24 h	↓ Triacylglycerols	Inhibit translocation of GPAT1 ↓ GPAT, mtGPAT1 activity
Baselga-Escudero *et al*[61], 2012	FAO cells	Proanthocyanidins 10, 25, 50, 100 mg/L 1 h	↓ MiR-122 at 25,50, 100 mg/L ↓ MiR.122, FAS with time (1, 3 h. 25 mg/L)	25 mg/L, ↓ FAS (5 h) protein expression
Pil Hwang *et al*[62], 2013	Hep G2 cells	3-caffeoyl, 4-dihydrocaffeoylquinic acid 1, 3, 10 μmol/L 1 h	↓ Fat accumulation in a dose-dependent manner	↓ SREBP1c, FAS mRNA and protein expression = LXRα mRNA expression ↑ Activating AMPK ↑ SIRT1
Liu *et al*[65], 2011	HepG2 cells	Blueberry extract 20, 40, 60, 80 and 100 μg/mL 24 h	↓ Triacylglycerols accumulation 80 μg/m L ↓ 60% of triacylglycerols accumulation	
Lee *et al*[67], 2012	BALB/c normal liver cells	Extract of *Hibicus sabdariffa L* 0.05, 0.1, 0.5, 1 mg/mL 48 h	↑ Cell viability	↓ p-JNK and AIF, tBid and Bax protein expression ↓ Lipid peroxidation ↑ Catalase and GSH
	Steatosis produced by acetaminophen			
Wang *et al*[66], 2012	HepG2	Extract of *Ginkgo biloba* 200 μg/mL *in vitro* 24 h	↓ Triacylglycerols	↑ CPT-1a, ACO mRNA expression ↓ FAS, Acac-β mRNA expression ↑ CPT-1a protein expression

↓: Decrease; ↑: Increase. RSV: Resveratrol; ACC: Acetyl CoA carboxylase; FAS: Fatty acid synthase; AMPK: AMP-activated kinase; SREBP1c: Sterol regulatory element binding protein 1c; SIRT1: Sirtuin 1; Q: Quercetin; GPAT1: Glycerol-sn-3-phosphate acyltransferase 1; mtGPAT1: Mitochondrial glycerol-sn-3-phosphate acyltransferase 1; LXRα: Liver X receptor α; pJNK: Activated c-Jun N-terminal kinase; AIF: Apoptosis inducing factor; GSH: Glutathione; CPT-1a: Carnitine palmitoyltransferase 1a; ACO: Acyl-coenzyme A oxidase 1; Acacβ: Acetyl-coenzyme A carboxylase β.

novo lipogenesis, *via* deacetylase sirtuin 1 (SIRT1).

Other studies have been performed using human cells. With similar results to those observed in rat hepatocytes, Zang *et al*[29] showed that incubation of cultured human HepG2 hepatocytes exposed to high concentrations of glucose, with 10 μmol/L of resveratrol, a concentration lower than those used in other studies conducted in rat hepatocytes, for 24 h prevented lipid accumulation. The authors found a decreased activity of the lipogenic enzyme ACC. Moreover, they observed an activation of AMP-activated protein kinase (AMPK), and concluded that this activation was required for the lipid lowering effect of resveratrol. These results are in good accordance with the study published by Shang *et al*[30] where HepG2 hepatocytes were exposed to high concentrations of glucose and insulin to get a cell steatosis model. A dose of 50 μmol/L of resveratrol reduced triacylglycerol accumulation. This polyphenol prevented the decline of phosphorylated AMPK induced by steatosis, followed by the down-regulation of SREBP-1c and FAS.

In summary, the studies performed in different models of rodent and human hepatocyte steatosis demonstrate that resveratrol show anti-lipidogenic effects at doses in the range of 10-50 μmol/L. There is a good consensus concerning the mechanism of action underlying this effect because all the above mentioned reports

Table 2 ***In vivo*** **studies carried out with resveratrol**

Ref.	Animal model	Resveratrol dose and treatment period length	Effects	Mechanisms
Baur *et al*[31], 2006	Male C57BL/6NIA mice fed a high-fat diet	0.04% in the diet (estimated 22.4 mg/kg *bw* per day) 6 wk	↓ Lipid droplets in liver	↓ Acetylation status of PGC-1α protein in liver
		186 mg kg *bw* per day 6 wk	↑ Mitochondrial number	
Kasdallah-Grissa *et al*[39], 2006	Male Wistar rats fed a standard diet 3 g ethanol/kg *bw* per day	0.04% in the diet (estimated 300-450 mg/kg *bw* per day) 6 wk	↓ Oxidative stress ↓ Lipid peroxidation	
Ahn *et al*[32], 2008	Male C57BL6/J mice fed an atherogenic diet	0.0125% in the diet (estimated 10 mg/kg *bw* per day) 8 wk	↓Total lipids and triacylglycerols in liver Ameliorated necroinflammation	↓ Expression lipogenic enzymes ↑ Expression enzymes involved in fatty acid oxidation ↑ SIRT1 mRNA expression
Ajmo *et al*[40], 2008	Male C57BL/6J mice fed a low-fat diet Ethanol added to account 29% of total calories	200 mg/kg *bw* per day 400 mg/kg *bw* per day 4 wk	↓ Liver weight ↓ Lipid droplets ↓ Triacylglycerols	↓ SREBP-1c mRNA and protein expressions ↓ FAS, SCD, ACC, ME mRNA ↑ PGC-1α mRNA ↑ ACO, CPT-1a mRNA ↑ Fatty acid oxidation Activation AMPK/SIRT1 axis
Bujanda *et al*[37], 2008	Male Wistar rats Steatosis induced by feeding rats ad libitum for four days per week and then fasting them the remaining three days	10 mg/kg *bw* per day 4 wk	↓ Liver fat infiltration ↓ Oxidative stress ↓ ALT	
Kim *et al*[33], 2008	C57BL/6J mice a high fat diet	0.4% in the diet (estimated 400 mg/kg *bw* per day) 10 wk	↓ Liver weight ↓ Triacylglycerols	
Shang *et al*[30], 2008	Male Wistar rats fed a high-fat, high-sucrose diet	100 mg/kg *bw* per day 10 wk	↓ Triacylglycerols ↓ Lipid droplets	↑ AMPK phosphorylation (activation) ↓ SREBP-1c and FAS mRNA
Rivera *et al*[42], 2009	Male *fa/fa* Zucker rats fed standard diet	10 mg/kg *bw* per day 8 wk	↓ Triacylglycerols	↓ ACC activity AMPK activation
Cho *et al*[34], 2012	Male C57BL/6J mice fed a high-fat diet	0.005% in the diet (0.5 mg/kg *bw* per day) 10 wk	0.5 mg/kg *bw* per day ↓ Triacylglycerols ↓ Number and size of liver fat droplets	↓ PAP = Enzymes involved in fatty acid oxidation ↓ FAS and ME activities
		0.02% in the diet (2 mg/kg *bw* per day) 10 wk	2 mg/kg *bw* per day ↓ Triacylglycerols ↓ Number and size of liver fat droplets	↓ PAP = Enzymes involved in fatty acid oxidation
Gómez-Zorita *et al*[43], 2012	Male *fa/fa* Zucker rats fed a standard diet	15 mg/kg *bw* per day 45 mg/kg *bw* per day 6 wk	15 and 45 mg/kg *bw* ↓ Liver weight ↓ Triacylglycerols ↓ Oxidative stress 15 mg/kg *bw* ↓ Transaminases	= Lipogenic enzyme activity ↑ Activity of enzymes involved in fatty acid oxidation
Poulsen *et al*[35], 2012	Male Wistar rats fed a high-fat diet	100 mg/kg *bw* per day 8 wk	Normalized triacylglycerols No hepatic inflammation	↑ UCP2 ↑ Mitochondria number
Alberdi *et al*[36], 2013	Male Sprague-Dawley rats fed a high-fat, high-sucrose diet	30 mg/kg of *bw* per day 6 wk	↓ Triacylglycerols	↑ Activity of enzymes involved in fatty acid oxidation = FAS, G6PDH, ME activities ↑ Phosphorylated ACC/total ACC (inhibition) ↑ Phosphorylated AMPK/total AMPK (activation) = PPAR-α, SREBP-1c, SIRT1, PGC-1α, TFAM, COX2, and HNF-4α, ↓ Acetylated PGC-1α /total PGC-1α (activation)

Franco *et al*[38], 2013	Lactating Wistar rats	30 mg/kg per day 4 wk	↓ Triacylglycerols ↓ Oxidative stress

↓: Decrease; ↑: Increase. BW: Body weight; PGC-1α: Peroxisome proliferator-activated receptor-c coactivator 1 α; SIRT1: Sirtuin 1; SREBP-1c: Sterol regulatory element binding protein 1c; FAS: Fatty acid synthase; SCD: Stearoylcoenzyme A desaturase; ACC: Acetyl CoA carboxylase; ME: Malic enzyme; ACO: Acyl-coenzyme A oxidase 1; CPT-1a: Carnitine palmitoyltransferase 1a; AMPK: AMP-activated kinase; ALT: Alanine aminotransferase; PPAR-γ: Peroxisome proliferator-activated receptor γ; C/EBP: CCAAT/enhancer binding protein; LXR: Liver X receptor; PAP: Phosphatidate phosphohydrolase; UCP2: Mitochondrial uncoupling protein 2; G6PDH: Glucose-6-phosphate dehydrogenase; PPAR-α: Peroxisome proliferator-activated receptor α; TFAM: Mitochondrial transcription factor A; COX2: Cyclooxygenase 2; HNF-4 α: Hepatocyte nuclear factor 4 α.

show decreased *de novo* lipogenesis.

In vivo studies

The *in vivo* studies reported have been carried out in rodents (mice and rats) by using different models of liver steatosis (diet-induced steatosis, ethanol-induced steatosis, genetic steatosis, *etc.*). These studies have revealed that this polyphenol reduces liver weight and triacylglycerol content. To explain this effect several mechanisms of action have been proposed.

One of the most frequently used models of liver steatosis is high-fat feeding. The first study conducted in this rodent model was reported by Baur *et al*[31]. C57BL/6NIA mice were treated with 22.4 mg resveratrol/kg body weight per day for 6 wk. Histological examination of liver showed that resveratrol administration reduced the accumulation of large lipid droplets. In another cohort of mice supplemented with 186 mg resveratrol/kg body weight per day for 6 wk the authors observed that treated animals showed increased number of mitochondria than the controls. This effect was mediated by deacetylation, and thus activation, of PGC-1α the co-activator of PPAR-α, the transcription factor which regulates fatty acid oxidation.

Ahn *et al*[32] described that the addition of 0.0125% of resveratrol to an atherogenic diet (a type of high-fat diet) led to a reduction in total lipids and triacylglycerols in liver from C57BL/6J mice after 8 wk of treatment. This amount of resveratrol in the diet corresponded to a dose of 10 mg/kg body weight per day in this study, lower than that used by Baur *et al*[31]. Histological analysis of liver sections confirmed that resveratrol significantly ameliorated both hepatic steatosis and necroinflammation. These changes were accompanied by a reduction in the expression of genes related to lipogenesis and an increase in the expression of genes related to fatty acid oxidation. Moreover, resveratrol increased hepatic expression of SIRT1. Therefore, the authors suggested that the beneficial effects of resveratrol on liver lipid metabolism can be exerted by SIRT1 activation.

Kim *et al*[33] also reported a reduction in liver weight and triacylglycerols in C57BL/6J fed a high-fat diet supplemented with 0.4% resveratrol for 10 wk. This amount of resveratrol represented 400 mg/kg body weight per day in this study, a very high dose. Potential mechanisms of action for resveratrol concerning this action were not proposed in this study.

Cho *et al*[34] compared the effects of two doses of resveratrol in C57BL/6J mice fed a high-fat diet. Animals received 0.5 mg/kg body weight per day or 2 mg/kg body weight per day for 10 wk. Resveratrol significantly reduced hepatic triacylglycerol content, although not in a dose-dependent manner. Consistent with these results haematoxylin and eosin staining of liver sections indicated that resveratrol caused a marked decrease in the number and size of liver fat droplets. In this case the lower dose appeared to be more effective than the higher dose. In order to examine the mechanism of action of resveratrol under these experimental conditions the activity of hepatic lipid-regulating enzymes was assessed. The high dose of resveratrol significantly decreased the hepatic activity of fatty acid synthase and glucose-6-phosphate dehydrogenase, two lipogenic enzymes and both doses decreased the activity of phosphatidate phosphohydrolase, an enzyme that catalyzes the synthesis of triacylglycerols. The enzymes involved in fatty acid oxidation remained unchanged.

By using a high-fat feeding model, but in this case working with rats instead of mice, Shang *et al*[30] observed that resveratrol, at a dose of 100 mg/kg body weight per day, orally administered, reduced hepatic triacylglycerol content after 10 wk of treatment. This effect was confirmed by histopathological analysis. Moreover, rats treated with the polyphenol showed increased AMPK phosphorylation and reduced SREBP-1c and FAS gene expressions. The authors concluded that resveratrol protected the liver from NAFLD and that the activation of AMPK was involved in the mechanism underlying the reduction in triacylglycerol accumulation.

By using the same dose of resveratrol (100 mg/kg body weight per day), Poulsen *et al*[35] reported that resveratrol prevented liver triacylglycerol accumulation induced by high-fat feeding in rats after 8 wk of treatment. The semi-quantitative microscopical steatosis grading revealed severe microvesicular steatosis in the high-fat fed group, but only slight changes in rats treated with resveratrol. These findings were consolidated by chemical extraction of hepatic lipid, as the triglyceride content was significantly lower in resveratrol-treated rats than in control animals. This effect was related to the increase in liver mitochondria number. The authors also observed an increase in uncoupling protein 2 (UCP_2), as results that seems to be related to reduced oxidative stress.

Another study conducted in rats fed a high-fat diet, but using clearly lower dose of resveratrol, was that reported by Alberdi *et al*[36]. Rats receiving this polyphenol in the diet in amounts that assured a dose of 30 mg/kg body weight per day for 6 wk, showed increased activi-

ties of palmitoyl transferase 1a (CPT-1a) and acyl-CoA oxydase (ACO), two enzymes involved in fatty acid oxidation, and decreased activity of the lipogenic enzyme ACC. The conclusion was that resveratrol partially prevented the increase in liver fat by increasing fatty acid oxidation and reducing lipogenesis. The potential involvement of the AMPK/SIRT1 axis was proposed.

High-fat feeding is not the only model of dietary-induced steatosis. Bujanda *et al*[37] analyzed the effects of resveratrol in a model obtained by feeding rats *ad libitum* for four days per week and then fasting them the remaining three days. This cycle was repeated four times. After 4 wk of treatment, resveratrol, administered by the oral route through an orogastric catheter, at a dose of 10 mg/kg body weight per day significantly reduced fat infiltration in liver. Moreover, oxidative stress, which is believed to play an important role in the pathogenesis of NAFLD, was significantly reduced in resveratrol-treated rats. Finally, serum concentrations of alanine aminotransferase (ALT), an indicator of hepatocyte damage, were significantly reduced in treated rats.

Franco *et al*[38], worked with another model of steatosis in rat pups. At birth, lactating rats were randomly assigned to each one of these groups: early weaning group (pups from dams which were wrapped with a bandage to interrupt lactation in the last 3 d of lactation) or control group (dams whose pups had free access to milk throughout lactation for 21 d). Pups from the early weaning group developed fatty livers among other metabolic alterations. The administration of resveratrol, by gavage, at a dose of 30 mg/kg body weight per day for one month significantly reduced liver triacylglycerols and oxidative stress.

Ethanol-induced steatosis is another commonly used model of fatty liver. Kasdallah-Grissa *et al*[39] induced steatosis in rats by administering intraperitoneally 3 g ethanol/kg body weight, a dose which shows moderate toxicity. Rats were fed a diet supplemented with 0.5% of resveratrol for 6 wk. Taking body weight and food intake of the animals into account the dose of resveratrol was approximately 300-450 mg/kg body weight per day. Resveratrol treatment attenuated lipid peroxidation induced by ethanol which indicates that resveratrol reduced oxidative stress and thus showed hepatoprotective properties.

Along the same lines, Ajmo *et al*[40] reported that supplementation with resveratrol at doses of 200 and 400 mg/kg body weight per day protected against ethanol-induced steatosis, reducing liver weight and hepatic triacylglycerols in male C57BL/6J mice. With regard to the potential mechanisms of action underlying these effects, the authors reported that resveratrol reduced gene and protein expression of SREBP-1c. This change was paralleled by a reduction in FAS, stearoyl-CoA desaturase (SCD), ACC and malic enzyme (ME). These results demonstrate that reduction in lipogenesis was involved in the anti-lipidogenic effect of resveratrol. Futhermore, resveratrol increased mRNA levels of peroxisome proliferator-activated receptor-c coactivator 1 α (PGC-1α), ACO and CPT-1a. When fatty acid oxidation was measured, mice treated with resveratrol showed increased values, as expected. Finally, adiponectin, an adipokine that promotes fatty acid oxidation, was also increased. These results show that increased fatty acid oxidation also participated in the effect of resveratrol on liver fat reduction. All these effects were observed with both doses with no dose-response pattern. The authors suggested that this protective action of resveratrol was, in whole or in part, mediated throughout the up-regulation of SIRT1-AMPK signaling system.

Another model of liver steatosis is the *fa/fa* Zucker rat, a genetically obese rat, which shows many human metabolic syndrome features such as insulin resistance, dyslipidemia, hyperinsulinemia and hypertension. Peripheral insulin resistance in obese Zucker rats enhances the mobilization of peripheral fat and the serum level of free fatty acids. However, liver oxidation or utilization of free fatty acids is inhibited. Thus, the liver in this rodent model synthesizes an excess of triacylglycerols and oxidizes a small amount of fatty acids, leading to strong fat infiltration of the hepatic parenchyma[41].

By using this animal model, Rivera *et al*[42] demonstrated that the administration of resveratrol at a dose of 10 mg/kg body weight per day for 8 wk induced a decrease in liver triacylglycerol accumulation. The authors suggested that this effect was related to the increase of phosphorylation of AMPK and ACC.

In previous studies from our group, also conducted in obese Zucker rats, assessed the effect of two doses of resveratrol (15 and 45 mg/kg body weight) on liver triacylglycerol content as well as on the activity of enzymes involved in two key metabolic pathways in the control of hepatic fat accumulation, lipogenesis and fatty acid oxidation[43]. After 6 wk of treatment, liver weight and triacylglycerols were decreased, oxidative enzyme activities were increased and lipogenic enzyme activities remained unchanged in both resveratrol-treated groups, with no differences between them. These results suggest that the decrease in liver steatosis was due to increased fatty acid oxidation. In this study, resveratrol significantly decreased hepatic thiobarbituric acid reactive substances (TBARS) formation, indicating an antioxidant effect and protection from the oxidative stress induced by obesity and steatosis in Zucker rats. Also, the high dose was able to diminish the amount of oxidized glutathione (GSSG) as well as to increase the GSH/GSSG ratio, a sensitive and reliable measure of the overall level of oxidative stress. These results suggest that the glutathione redox state became less pro-oxidizing due to supplementation with resveratrol. However, the reactive oxygen species (ROS) scavenging enzyme superoxide dismutase (SOD) did not seem to be involved in the resveratrol-induced reduction of oxidative stress.

Summary

There is a general consensus concerning the positive ef-

fects of resveratrol on liver steatosis in animal models. The range of doses used in experiments carried out on mice has been very huge (0.5-400 mg/kg per day). All the doses analyzed have been shown to be effective. In the experimental design of the studies described above, while the lowest doses (0.5 and 2 mg/kg body weight per day) were used in longer treatments (10 wk), the highest doses (200-400 mg/kg body weight per day) were used for shorter periods (4 wk). Thus, based on the reported data it is not possible to known whether low doses would be effective over shorter periods of treatment. As far as rats are concerned, the range of doses used has also been huge, although less than in mice (10-450 mg/kg body weight per day). The treatment periods were similar to those used in mice (4-10 wk). Due to the fact that very low doses have not been used in rats it is not possible to know whether this animal species in less responsive to resveratrol than the mouse. All these results suggest that more studies are needed to establish the best combination of dose-experimental period.

In the vast majority of the experiments described in the present review resveratrol was administered to animals at the same time as the factor which induced liver steatosis (high-fat feeding, ethanol intake, *etc.*). This means that resveratrol is able to prevent liver steatosis; this effect was observed independently of the etiology of this metabolic situation. Moreover, this polyphenol also ameliorated steatosis when this alteration was prior to the treatment, as observed in genetically obese rats. These rats showed steatosis before being treated with resveratrol and then showed reduced liver fat accumulation. These facts demonstrate that resveratrol is useful not only in the prevention of liver steatosis but also in its treatment.

Only two of all the studies reported used more than one dose in the same experiment. In the case of Ajmo *et al*[40] who used 200 and 400 mg/kg body weight per day, no dose-response effect was found. By contrast, in the case of Cho *et al*[34], the low dose (0.5 mg/kg body weight per day) appeared to be more effective than the higher dose (2 mg/kg body weight per day), most likely a phenomenon of hormesis.

Altogether data reported concerning the mechanisms of action of resveratrol underlying its liver anti-lipidogenic effect demonstrate that this polyphenol decreases *de novo* fatty acid synthesis, as well as triacylglycerol synthesis, and increases fatty acid oxidation. Moreover, the reduction of oxidative stress also contributes to this positive effect. The activation of AMPK and SIRT1 mediates these changes.

Human studies

With regard to human beings, there is only one published study so far. Healthy, obese, male volunteers without a history of diabetes or any other disorder received 150 mg resveratrol/d or placebo for 1 mo in a randomized double-blind crossover design. Plasma ALT concentration was significantly lower after resveratrol treatment compared to the placebo group. Intrahepatic lipid content was lower after 30 d of resveratrol supplementation in comparison to placebo. This was paralleled by lower plasma ALT value, both indicating improved liver function[44].

EFFECTS OF QUERCETIN ON STEATOSIS

Quercetin is a natural polyphenol belonging to a group with a variable structure, known as flavonoids. It is found in onions, broccoli, tomatoes, apples and berries[45].

It has been reported that quercetin exhibits a wide range of biological functions, including antioxidant, anticarcinogenic and anti-inflammatory activities[46-50]. More recently, beneficial effects on blood pressure and heart disease have been described[50-52]. Since 2006, several studies have shown the interesting properties of this flavonoid in the prevention of liver steatosis (Tables 1 and 3).

In vitro studies

Vidyashankar *et al*[53] carried out a study with HepG2 cells rendered steatosis by incubation with oleic acid-bovine serum albumin complex. These cells were then treated with a dose of 10 μmol/L of quercetin for 24 h. Decreased triacylglycerol accumulation, insulin resistance and inflammatory cytokine secretion, and increased cellular antioxidants were observed. The study suggested that quercetin was an effective molecule reversing the symptoms of NAFLD.

In vivo studies

As in the case of resveratrol, different animal models have been used in *in vivo* studies. Ying *et al*[54] used gerbils fed a high-fat diet as a model of steatohepatitis, and treated them with three doses of quercetin, 15, 30 and 60 mg/kg body weight per day for 2 wk. The lowest dose was ineffective. By contrast, both 30 and 60 mg/kg body weight per day reduced liver triacylglycerols, liver lipid droplet size, serum transaminases and proinflammatory mediators, such as TNF-α and IL-6 in a dose-response pattern. Only the highest dose reduced liver collagen.

The rest of the studies reported were conducted in mice. Kobori *et al*[55] conducted an experiment in BALB/c mice showing streptozotocin (STZ)-induced diabetes. When quercetin was added to the diet at 0.5% for 2 wk, animals showed a reduction in oxidative stress, as well as in liver injury produced by STZ.

The same research group carried out another study by means of a different experimental design[56]. C57BL/6J mice were a high-fat diet supplemented with 5 g quercetin/kg diet for 20 wk. Treated mice showed a reduction in liver triacylglycerol accumulation. Moreover, the increase induced by the diet in the expression of peroxisome proliferator activated receptor γ (PPAR-γ), cluster of differentiation 36 (CD36), SREBP-1c and FAS, genes which promote lipid accumulation, was normalized.

Table 3 *In vivo* studies carried out with quercetin

Ref.	Animal model	Quercetin dose and treatment period length	Effects	Mechanisms
Kobori *et al*[55], 2009	BALB/c mice with STZ-induced diabetes and steatosis fed a standard diet	0.5% in the diet 2 wk	↓ Oxidative stress ↓ Liver damage	
Kobori *et al*[56], 2011	C57BL/6 J mice fed a high-fat diet	0.5% in the diet 20 wk	↓ Triacylglycerols ↓ Oxidative stress	Normalized gene expression of PPAR-γ, SREBP-1c, FAS, CD36 ↑ PPAR-α mRNA expression
Marcolin *et al*[58,59], 2012, 2013	Male C57BL/6J mice fed a diet deficient in methionine and choline	0.005% in the diet 4 wk	↓ Macro/micro vesicular steatosis ↓ Balloning ↓ Serum transaminases ↓ Oxidative stress ↓ DNA damage	↓ Proinflammatory ond profibritic gene expression
Panchal *et al*[57], 2012	Male Wistar rats fed a high-fat diet	0.8% in the diet (50 mg/kg *bw* per day) 8 wk	Attenuated steatosis	↑ Nrf2 ↓ NF-kβ ↑ CPT-1a
Jung *et al*[50], 2013	C57B1/6 mice fed a high-fat diet	0.025% in the diet 8 wk	↓ Liver weight ↓ Triacylglycerols ↓ Lipid droplet size	↓ FAS, Acaca, Apoa4, Abcg5, Fdft1 and GPAM mRNA expressions
Ying *et al*[54], 2013	Gerbils fed a high-fat diet	15, 30, 60 mg/kg *bw* per day 2 wk	All doses ↓ Triacylglycerols ↓ Lipid droplets size ↓ Serum transaminases 60 mg/kg *bw* per day ↓ Liver collagen	

↓: Decrease; ↑: Increase. STZ: Streptozotocin; PPAR-γ: Peroxisome proliferator activated receptor γ; SREBP-1c: Sterol regulatory element binding protein 1c; FAS: Fatty acid synthase; CD36: Cluster of differentiation 36; PPAR-α: Peroxisome proliferator activated receptor α; BW: Body weight; CPT-1a: Carnitine palmitoyltransferase 1a; Acaca: Acetyl-coenzyme A carboxylase α; Apoa4: Apolipoprotein A-Ⅳ; Abcg: ATP-binding cassette, subfamily G, member 5; Fdft1: Farnesyl-diphosphate farnesyltransferase 1; Gpam: Glycerol-3-phosphate acyltransferase, mitochondrial.

Another interesting effect was the increase in the expression of peroxisome proliferator activated receptor α (PPAR-α), a transcription factor which control fatty acid oxidation, reduced by high-fat feeding. Finally, oxidative stress was reduced by quercetin.

Panchal *et al*[57], also working with a model of steatosis induced by high-fat feeding, observed attenuated steatosis in rats treated for 8 wk with quercetin at a dose of approximately 50 mg/kg body weight per day (0.08% quercetin in the diet). The authors suggested that this effect was related to the down-regulation of NF-κB, a transcriptional factor that stimulates inflammation, and the up-regulation of Nrf2, which prevents oxidation. Moreover, quercetin treatment resulted in increased expression of CPT-1a, a key enzyme of fatty acid oxidation.

Jung *et al*[50] reported a reduction in liver weight due to a decrease in the amount of triacylglycerols and lipid droplets in C57BL/6J mice fed a high-fat diet supplemented with 0.025% of quercetin for 8 wk. In this study the authors also analyzed the effects of quercetin on the expression of genes related to lipid metabolism in liver, such as FAS (involved in *de novo* lipogenesis), Acetyl-coenzyme A carboxylase α (Acaca), apolipoprotein A-Ⅳ (Apoa4), ATP-binding cassette, subfamily G, member 5 (Abcg5), Fdft1, farnesyl-diphosphate farnesyltransferase 1 (Fdft1) (involved in the synthesis of saturated fatty acids) and glycerol-3-phosphate acyltransferase mitochondrial (GPAM), (involved in triacylglycerol synthesis and related to SREBP-1c gen). All these genes were down-regulated in quercetin-treated mice.

Marcolin *et al*[58] analyzed the effect of quercetin (0.005% in the diet) in the protection against steatosis induced in C57BL/6J mice fed a diet deficient in methionine and choline. In this study, a lower degree of steatosis and a reduction in transaminases and oxidative stress were observed in mice treated with quercetin for 4 wk. Moreover, proinflammatory and profibrotic gene expression was reduced. Later on, in the same cohort of animals, they observed a reduction in macro/microvesicular steatosis, ballooning and DNA damage induced by quercetin treatment[59].

Due to fact that the authors of the studies described above did not provide data concerning food intake, it is not possible to know the dose (mg/kg body weight per day) provided to animals in order to be compared with other reports.

Summary

The number of studies performed with quercetin is lower than that of studies carried out with resveratrol. The most commonly used animal model in these studies was mice. It is important to point out that a low dose of this polyphenol (0.005%) is able to prevent liver steatosis in a quite short experimental period (4 wk). The only study which analyzed different doses of quercetin[54] showed a dose response pattern. As far as the potential mechanisms of this polyphenol are concerned, quercetin similarly to resveratrol decreases de novo fatty acid synthesis.

Table 4 ***In vivo*** **studies carried out with other polyphenols**

Ref.	Animal model	Polyphenols dose and treatment period length	Effects	Mechanisms
Guo *et al*[60], 2011	Male KKAy mice fed a standard diet	Anthocyanin Cy-3-g 0.01% in the diet 12 wk	↓ Triacylglycerols ↓ Lipid droplets	↓ GPAT1 activity
Baselga-Escudero *et al*[61], 2012	Male Wistar rats fed a standard diet and 2.5 mL of lard oil/kg BW	Proanthocyanidins 250 mg/kg BW 3 h	↓ Triacylglycerols	↓ FAS mRNA ↑ miR-122 mRNA
Luo *et al*[64], 2012	Male C57BL/6 mice fed a methionine and choline-deficient high-fat diet	Theaflavin 30 mg/kg BW by intraperitoneal injection 48, 24, and 2 h before induction of steatosis by ischemia-reperfusion	↓ Cell ballooning ↓ Microvesicular and macrovesicular steatosis ↓ ALT ↓ Oxidative stress ↓ Hepatocyte apoptosis ↓ F4/80-positive cells (inflammatory cells)	
Yoshimura *et al*[63], 2013	KKAy mice fed a high-fat diet	Ellagic acid 0.1% in the diet 68 d	↓ Serum ALT, AST, ↓ Macrovesicular steatosis ↓Triacylglycerols	↑ FAS mRNA expression = Acaca, SREBP-1c mRNA expression = ACO mRNA expression ↑ CTP-1a, PPAR-α mRNA expression

↓: Decrease; ↑: Increase. GPAT1: Glycerol-sn-3-phosphate acyltransferase 1; BW: Body weight; FAS: Fatty acid synthase; ALT: Alanine aminotransferase; AST: Aspartate aminotransferase; Acaca: Acetyl-CoA carboxylase α; SREBP-1c: Sterol regulatory element binding protein 1c; ACO: Acyl-coenzyme A oxidase; CPT-1a: Carnitine palmitoyltransferase 1a; PPAR-α: Peroxisome proliferator activated receptor α.

EFFECTS OF OTHER POLYPHENOLS IN HEPATIC STEATOSIS

In addition to resveratrol and quercetin, other polyphenols such as antocyanin Cy-3-g, proanthocyanidins, teaflavin (a flavan-3-ol) and ellagic acid (a tannin) have studied as potential agent for both prevention and treatment of hepatic steatosis (Tables 1 and 4).

In vitro studies

Guo *et al*[60] performed a study in HepG2 cells incubated with 3 doses of anthocyanin cy-3-g (1, 10, 100 μmol/L) for 24 h. A reduction in triacylglycerol content was observed due to an inhibition in glycerol-sn-3-phosphate acyl transferase 1 (GPAT1), a key enzyme in the synthesis of triacylglycerols.

In FAO cells, a rat hepatoma cell line, Baselga-Escudero *et al*[61] studied the effect of proanthocyanidins on hepatic lipid metabolism. 25 mg/L of these polyphenols reduced FAS and miR-122 expression. miR-122 is a novel class of non-coding RNA that regulates genes involved in fatty acid and triacylglycerol synthesis. Moreover, the protein expression of FAS was also decreased.

Pil Hwang *et al*[62] conducted a study where HepG2 cells were treated with 3 doses (1, 3 and 10 μmol/L) of 3-Caffeoyl, 4-dihydrocaffeoylquinic acid for 1 h. The study showed that this polyphenol inhibited fat accumulation in a dose dependent manner due to a decrease in *SREBP-1c* and *FAS* gene and protein expressions, through the activation of AMPK and SIRT1.

In vivo studies

After analyzing the *in vitro* effects of anthocyanin Cy-3g, Guo *et al*[60] conducted an *in vivo* study with KKAy mice fed a standard diet supplemented with 100 mg/kg of this anthocyanin or not, for 12 wk. The study showed a reduction in triacylglycerol content and lipid droplets due to an inhibition in translocation of GPAT1 and a reduction in the synthesis of triacylglycerols. These results are in good accordance with their own results obtained in cultured cells.

In the same animal model, but using a high-fat diet supplemented with 0.1% ellagic acid or not for 68 d, Yoshimura *et al*[63] observed a decreased in serum free fatty acids, triacylglycerol, ALT, aspartate transaminase (AST) and resisitin concentrations, without changes in leptin and adiponectin. Moreover, a decrease in macrovesicular steatosis and hepatic triacylglycerol was shown. CPT-1a and PPAR-α mRNA expression was increased but Acaca, SREBP-1c and ACO mRNA expression remained unchanged. Surprisingly, the mRNA expression of FAS was increased.

Luo *et al*[64] carried out a study with C57BL/6 mice fed a methionine and choline deficient high-fat diet and treated with 30 mg of theaflavin /kg body weight by intraperitoneal injections 2, 24 and 48 h before induction of steatosis by ischemia-reperfusion. They observed that theoflavin reduced cell ballooning, micro and macrovesicular steatosis, hepatocyte apoptosis, oxidative stress and inflammatory cells in liver, as well as serum transaminase concentrations.

Baselga-Escudero *et al*[61] conducted a study with Wistar rats fed *ad libitum* with a standard diet. The rats were orally gavaged with lard oil (2.5 mL/body weight) or 250 mg of proantocyanindins dissolved in the lard oil. After 3 h a reduction in serum and hepatic triacylglycerol content

was observed. Similar to what these authors observed in cultured cells, increased miR-122 mRNA levels accompanied by decreased FAS mRNA levels were found.

Summary

It is not possible to compare the above described studies because important differences in terms of type of polyphenol used, dose and animal model exist among them. Nevertheless, the reported results show that all the polyphenols tested, belonging to different families, show an anti-lipidogenic effect. As in the case of resveratrol, the potential mechanisms that justify this effect are decreased synthesis of fatty acids and triacylglycerols and increased fatty acid oxidation.

EFFECTS OF POLYPHENOL EXTRACTS IN HEPATIC STEATOSIS

In addition to studies conducted with individual polyphenols, several works have used polyphenol extracts with different origins and compositions. Although using polyphenol extracts makes it quite complicated to assign the beneficial effects observed to a specific molecule, they have two clear advantages. On the one hand, they better mimic the real situation in our dietary patterns. On the other hand, additive or synergic effects can be observed. In this context, sometimes combinations of molecules present beneficial effects that are not shown when they are administrated separately (Tables 1 and 5).

In vitro studies

There are few studies analyzing the effect of polyphenols extracts *in vitro*. Liu *et al*[65] studied the effect of blueberry phenolic compounds (anthocyanins, flavanols) at different doses (20-120 μmol/L) in HepG2 cells for 24 h. An inhibitory effect was observed in triacylglycerols accumulation in a dose-dependent manner. The maximum inhibitory value (approximately 60%) was reached at a concentration of 80 μmol/L.

Wang *et al*[66] analyzed the effect of 200 μmol/L of a *Ginkgo biloba* extract (quercetin, kaempfenol) for 24 h in HepG2 cells. This extract reduced triacylglycerols content due to the up-regulation of CPT-1a, ACO, FAS and Acetyl-CoA carboxylase β (Aca-β) gene expression and CPT-1a protein expression.

Lee *et al*[67] studied the effect of an extract of *Hibiscus sabdariffa L.* (including 8.83% protocatechuic acid, 9.97% catechin, 10.23% epigallocatechin, 20.20% epigallocatechin gallate, 18.10 caffeic acid) in BALB/c liver cells damaged with acetaminophen. Cells were treated with different doses of this polyphenol extract (0.05, 0.1, 0.5 or 1 mg/mL) for 48 h. The results showed that *Hibiscus sabdariffa L.* reduced triacylglycerol content. Moreover, it was able to eliminate the release of intermembrane proteins and to reduce cell death.

In vivo studies

Lee *et al*[67], in the study described in the previous section, also conducted an *in vivo* experiment in BABL/c mice showing liver damage produced by acetaminophen. Mice were treated with 3 doses of extract of *Hibicus sabdariffa L.* (0.01, 0.02 or 0.03% in the diet) for 2 wk. The study showed that this polyphenol extract reduced transaminases in a dose-dependent manner, and oxidative stress. Moreover, decreased liver damage and steatosis were shown by histopathological analysis.

Beltrán-Debón *et al*[68] administered an aqueous extract of *Aspalathus linearis L.* (rooibos; aspalathin, orientin, rutin) (10 g/L drinking water) for 14 wk to LDLr-/- mice, which is a model of metabolic alterations that resembles human metabolic syndrome in some aspects. This extract reduced serum triacylglycerols and free fatty acids. Moreover, histopathological analysis showed that steatosis degree was also lower in mice supplemented with rooibos extract.

Db/db mice fed a standard diet were used in the study reported by Tsuruta *et al*[69]. Animals were supplemented with an extract of *Nelumbo nucifera L.* (lotus root; proanthocyanidins, carechin, gallocatechin) at a dose of 0.5% in the diet for 3 wk. Lotus root extract reduced liver weight by 15% and triacylglycerol accumulation by 62%, but these changes did not reach statistical significance. Transaminases, which are hepatic injury markers, also tended to be lower (-24% ALT and -17% AST). In addition, the activity of lipogenic enzymes FAS and ME were decreased and CPT-1a, an enzyme related to fatty acid oxidation, remained unchanged.

In order to assess the effect of green tea, Axling *et al*[70] carried out a study with C57BL/6J mice fed a high-fat diet supplemented with 4% for 11 and 22 wk. Green tea (catechins) reduced serum triacylglycerols and ALT. In liver, this polyphenol extract reduced weight and the amount of triacylglycerols. All these results were observed during both treatment periods. In order to analyze the potential mechanisms of action, gene expression of SREBP-1c, PPAR-γ and ACC was assessed, but only in the group treated for 22 wk. All these genes showed down-regulation induced by green tea.

The effects of an extract of grape skin were tested by Park *et al*[71]. C57BL/6J mice were fed a high-fat diet supplemented with 0.15% of this extract for 10 wk. They showed decreased serum free fatty acids and leptin and increased adiponectin concentrations. With regard to liver, a reduction in triacylglycerols was observed. In order to determine the potential mechanisms of action, gene expression and activity of enzymes related to hepatic triacylglycerol metabolism were analyzed. Both the expression and the activity of enzymes involved in *de novo* lipogenesis, such as FAS, glucose-6-phosphate dehydrogenase (G6PDH) and ME and triacylglycerol synthesis, such as phosphatidate phosphohydrolase (PAP), were decreased. Accordingly, gene expression of PPAR-γ was also reduced. CPT-1a and PPAR-α mRNA levels, as well as β-oxidation were increased. Surprisingly, no changes were observed in CPT-1a activity.

Feillet-Coudray *et al*[72] carried out a study using Wistar rats fed a high-fat diet supplemented (0.2%) with

Table 5 ***In vivo*** **studies carried out with polyphenol extracts**

Ref.	Animal model	Polyphenols extract, dose and treatment period length	Effects	Mechanisms
Feillet-Coudray *et al*[72], 2009	Male Wistar rats fed a high-fat diet	Provinol®, a polyphenol extract obtained from red wine 0.2% in the diet 6 wk	↓ Macroesteatosis ↓ Lipid droplets ↓ Lipid peroxidation	
Aoun *et al*[73], 2010	Male Wistar rats fed a high fat diet	Provinol®, a polyphenol extract obtained from red wine 0.2% in the diet 6 wk	↓ Triacylglycerols ↓ Macroesteatosis = Fatty acid composition	↑ SIRT protein expression = SCD1, pAMPK, SREBP-1c, FAS, HNF-4 α, PGC1α and CPT-1a protein expression
Beltrán-Debón *et al*[68], 2011	LDLr-/- mice fed a standard diet	*Aspalathus linearis L.* (rooibos) 10 g/L drinking water 14 wk	Lower steatosis degree	↑ AMPK protein expression
Tsuruta *et al*[69], 2011	*db/db* mice fed a standard diet	*Nelumbo nucifera L.* (lotus root) 0.5% in the diet 3 wk	↓ 15% Liver weight (tendency) ↓ 62%Triacylglycerols (tendency) ↓ Transaminases (tendency)	= CPT-1a activity ↓ FAS, ME activity
Axling *et al*[70], 2012	C57BL/6 J mice fed a high fat diet	Green tea 4% in the diet 11 and 22 wk	↓ Liver weight ↓ Triacylglycerols ↓ Serum ALT	↓ SREBP-1c, PPAR-γ, ACC mRNA (22 wk)
Lee *et al*[67], 2012	BABL/c mice fed a standard diet Steatosis produced by acetaminophen	Extract of *Hibicus sabdariffa L* 0.01%, 0.02% or 0.03% in the diet 2 wk	↓ Liver damage ↓ Liver steatosis ↓ Serum ALT, AST (dose dependent) ↓ Oxidative stress	↓ p-JNK and AIF, tBid and Bax protein expression
Wang *et al*[66], 2012	Male Wistar rats fed a high-fat diet	Extract of *Ginkgo biloba* 0.25% in the diet 12 wk	↓ Triacylglycerols	↑ CPT-1a activity ↑ CPT-1a, Acaa1, Slc25a20, Hadh, ACO, PPAR-α, RXR-α mRNA expression, ↓ FAS mRNA expression
Park *et al*[71], 2013	C57BL/6 J mice fed a high-fat diet	Extract of grape skin 0.15% in the diet (160 mg/kg *bw* per day) 10 wk	↓ Triacylglycerols ↓ Serum leptin ↑ Serum adiponectin	↓ FAS, SCD1, PAP ↑CPT-1a, PPAR-α mRNA expressions and activities ↓ PPAR-γ, ↑ PPAR-α, CPT-1a mRNA expressions ↑ β oxidation, = CPT-1a activity
Yui *et al*[74], 2013	OLETF rats fed a standard diet	*Humulus lupulus L.* (hop pomace) 1% in the diet 70 d	↓Liver weight (tendency) ↓ Triacylglycerols (tendency)	↓ *de novo* lipogenesis = ACO, CPT-1a activity

↓: Decrease; ↑: Increase. SIRT: Sirtuin 1; SCD1: Stearoyl-CoA desaturase; AMPK: AMP-activated kinase; pAMPK: Phosphorylated AMP-activated kinase; SREBP-1c: Sterol regulatory element binding protein 1c; FAS: Fatty acid synthase; HNF-4α: Hepatocyte nuclear factor 4 α; PGC-1α: Peroxisome proliferator-activated receptor-c coactivator 1α; CPT-1a: Carnitine palmitoyltransferase 1a; ME: Malic enzyme; ALT: Alanine aminotransferase; PPAR-γ: Peroxisome proliferator activated receptor γ; ACC: Acetyl CoA carboxylase; pACC: Phosphorylated acetyl CoA carboxylase; AST: Aspartate aminotransferase; p-JNK: Activated c-Jun N-terminal kinase; AIF: Apoptosis inducing factor; Acaa1: acetyl-coenzyme A acyltransferase 1; Slc25a20: Solute carrier family 25, member 20; Hadh: Hydroxyacyl-coenzyme A dehydrogenase; ACO: Acyl-coenzyme A oxidase; PPAR-α: Peroxisome proliferator activated receptor α; RXR-α: Retinoid X receptor α; PAP: Phosphatidate phosphohydrolase.

of Provinol® (46.0% proanthocyanidols, 21.0% prodelphinidol, 6.1% anthocyanins, 3.8% cathechin, 3.0 % epicatechin gallate, 1.8% OH cinnamid acid, 1.4% quercetol; 0.15% resveratrol, 0.09 free anthocyanins) which is a polyphenol extract obtained from red wine for 6 wk. The histological analysis revealed a decrease in macroesteatosis and fat droplets in treated animals. A reduction in hepatic lipid peroxidation was also observed. The potential mechanisms of action underlying these effects were further reported by this research group[73]. Provinol® increased protein expression of SIRT1, without changing SCD1, SREBP-1c, FAS, hepatocyte nuclear factor 4 α (HNF-4α), PGC-1α, CPT-1a and phosphorylated AMPK. Moreover, a decrease in phosphorylated ACC was observed. Therefore, the authors suggested that the reduction in liver triacylglycerol accumulation was, at least in part, regulated by the inhibition of ACC, the limiting enzyme in *de novo* lipogenesis, probably through the activation of SIRT1 deacetylase.

Finally, Yui *et al*[74] carried out a study with OLEF rats fed a standard diet supplemented with 1% *Humulus lupulus L.* (hop pomace; flavonoids, procyanidins) for 70 d. Liver weight and hepatic triacylglycerol content showed a tendency towards reduced values. A reduction in *de novo* lipogenesis was observed, without changes in ACO and CPT-1a activity.

Summary

All the polyphenol extracts analyzed were able to reduce liver fat accumulation. As expected, the mechanisms underlying this effect were those reported in studies carried out with isolated polyphenols.

CONCLUSION

As a general conclusion, it can be stated that polyphenols are biomolecules which present hepatoprotective effects because they reduce liver fat accumulation and decrease oxidative stress and inflammation, the two main factors responsible for liver damage. To date, these beneficial effects have been demonstrated in cultured cells and animal models. Thus, studies performed in humans are needed before these molecules can be considered as truly useful tools in the prevention of liver steatosis.

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TOPIC HIGHLIGHT

WJG 20th Anniversary Special Issues (12): Nonalcoholic fatty liver disease

Role of gut microbiota and Toll-like receptors in nonalcoholic fatty liver disease

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Abstract

Emerging data have shown a close association between compositional changes in gut microbiota and the development of nonalcoholic fatty liver disease (NAFLD). The change in gut microbiota may alter nutritional absorption and storage. In addition, gut microbiota are a source of Toll-like receptor (TLR) ligands, and their compositional change can also increase the amount of TLR ligands delivered to the liver. TLR ligands can stimulate liver cells to produce proinflammatory cytokines. Therefore, the gut-liver axis has attracted much interest, particularly regarding the pathogenesis of NAFLD. The abundance of the major gut microbiota, including *Firmicutes* and *Bacteroidetes*, has been considered a potential underlying mechanism of obesity and NAFLD, but the role of these microbiota in NAFLD remains unknown. Several reports have demonstrated that certain gut microbiota are associated with the development of obesity and NAFLD. For instance, a decrease in Akkermansia muciniphila causes a thinner intestinal mucus layer and promotes gut permeability, which allows the leakage of bacterial components. Interventions to increase *Akkermansia muciniphila* improve the metabolic parameters in obesity and NAFLD. In children, the levels of Escherichia were significantly increased in nonalcoholic steatohepatitis (NASH) compared with those in obese control. Escherichia can produce ethanol, which promotes gut permeability. Thus, normalization of gut microbiota using probiotics or prebiotics is a promising treatment option for NAFLD. In addition, TLR signaling in the liver is activated, and its downstream molecules, such as proinflammatory cytokines, are increased in NAFLD. To data, TLR2, TLR4, TLR5, and TLR9 have been shown to be associated with the pathogenesis of NAFLD. Therefore, gut microbiota and TLRs are targets for NAFLD treatment.

Key words: Nonalcoholic fatty liver disease; Nonalcoholic steatohepatitis; Gut microbiota; Toll-like receptor; Probiotics; Prebiotics

Core tip: The gut-liver axis has attracted much interest particularly regarding the pathogenesis of nonalcoholic fatty liver disease (NAFLD) because gut microbiota contribute to nutritional absorption and storage. In addition, gut microbiota are a source of Toll-like receptor (TLR) ligands, which can stimulate liver cells to produce proinflammatory cytokines. To date, TLR2, TLR4, TLR5, and TLR9 have been shown to be associated with the pathogenesis of NAFLD. The present article reviewed the current understanding of gut microbiota and TLR signaling in NAFLD and potential treatment targeted at gut microbiota and TLRs.

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INTRODUCTION

The gut-liver axis has attracted much interest regarding the pathogenesis of nonalcoholic fatty liver disease (NAFLD), in which the balance between nutritional absorption and energy storage and expenditure is impaired. The gut is an organ that absorbs a variety of nutritional components from food; gut microbiota plays an important role in humans as well as rodents[1-3]. In addition, gut microbiota contribute to energy storage in the liver. Bäckhed *et al*[4] clearly showed that conventionally raised mice had a 42% higher body fat as well as hepatic triglyceride content than germ-free mice despite the fact that conventionally raised mice consuming fewer calories. Supporting the role of gut microbiota in nutritional absorption, germ-free mice in which gut microbiota from conventionally raised mice were transplanted produced a 57% increase in body fat within 2 wk. Certain gut bacteria are able to ferment complex carbohydrates, which are not digested by mammalian enzymes. Short-chain fatty acids (SCFAs), which are digested products of complex carbohydrates, account for 10% of dairy energy intake[5] and also stimulate *de novo* lipogenesis[6]. Thus, gut microbiota contribute to the development of NAFLD.

In addition to nutritional absorption and energy storage, the gut microbiota are a source of Toll-like receptor (TLR) ligands, which induce inflammation under certain conditions. Although bacterial components are potent TLR ligands, the liver has a high tolerance to TLR ligands because hepatic cells express minimal TLRs in normal liver. In contrast, TLR signaling is activated and downstream molecules are increased in NAFLD because the tolerance has been disrupted[7]. Altered gut microbiota and increased gut permeability are potential causes of the breakdown of tolerance. Indeed, circulating bacterial components and hepatic TLR expression are increased in human NAFLD patients as well as in animal models[8-11]. Thus, gut microbiota and TLRs are potential targets for NAFLD treatment.

The exact mechanisms by which gut microbiota contribute to NAFLD are poorly understood, although the role of gut microbiota in the development of NAFLD is well documented. Here, we first review the role of TLRs that are associated with NAFLD. Then we describe the function of gut microbiota observed in metabolic syndromes including NAFLD.

TLRS ARE ASSOCIATED WITH THE DEVELOPMENT OF NAFLD

TLRs are pattern recognition receptors that perceive bacterial and viral components[12,13]. TLR signaling is suppressed in healthy liver but is activated when pathogenic microorganisms and bacteria-derived molecules are delivered to the liver. This TLR signaling is the first line of defense against the invading pathogens through the production of anti-bacterial and anti-viral cytokines such as tumor necrosis factor (TNF) α, interleukin (IL)-1β, and interferons. However, sustained elevation of these cytokines injures the host; thus, continuous stimulation of TLR signaling does not always provide a benefit for the host. Recent data demonstrate that TLR signaling enhances hepatic injury in NASH, alcoholic liver disease, and chronic viral hepatitis[14-16]. Among the 13 TLRs identified in mammals, the pathogenesis of NASH is associated with TLRs including TLR2, TLR4, TLR5, and TLR9[14,17-20], which recognize lipopolysaccharide (LPS), peptidoglycan, flagellin, and bacterial DNA, respectively. Table 1 summarizes the results of TLR mutant mice fed a diet that induce NAFLD. Although other TLRs may contribute to the development of NAFLD, no solid data are available.

TLR4 is a receptor for LPS, which is a cell component of Gram-negative bacteria. Circulating LPS levels are elevated in rodent NAFLD induced by a high-fat (HF) diet, fructose-rich diet, methionine/choline-deficient (MCD) diet or choline-deficient amino acid-defined (CDAA) diet[9,11,19,21]. Although the mechanism by which these diets induce steatosis is different, these diets modify the gut microbiota and gut permeability[22,23]. Wild type (WT) mice on these diets show steatosis/steatohepatitis with increased expression of TLR4 and proinflammatory cytokines. LPS injections in NAFLD mice further increased proinflammatory cytokines and promoted liver injury[24,25]. Even in WT mice on standard laboratory chow, continuous infusion of low-dose LPS resulted in hepatic steatosis, hepatic insulin resistance, and hepatic weight gain[21]. Supporting the role of the LPS-TLR4 pathway in the development of NAFLD, TLR4 mutant mice are resistant to NAFLD[9,19,26], even though LPS levels are equivalent to those in WT mice. Consistent with histological findings in the liver, the expression of proinflammatory cytokines was suppressed in TLR4 mutant mice. Because 80% of intravenously injected LPS accumulates in the liver within 20-30 min[27,28], the liver is a target of LPS. In humans, plasma LPS levels are also elevated in metabolic syndromes including diabetes[29,30] and in NAFLD patients[31,32]. As in rodents, an HF diet elevates plasma endotoxin concentrations and endotoxin activity in humans[33,34]. Total parenteral nutrition and intestinal bypass surgery can increase plasma LPS levels. Under these conditions, hepatic steatosis occured without metabolic syndrome[35-37]. Antibiotics treatment to kill Gram-negative bacteria decreased plasma LPS levels and attenuated the steatosis in these patients[35-37]. Thus, LPS is closely associated with the development of NAFLD, and TLR4 signaling is a key pathway for the progression of NAFLD in humans as well as rodents.

TLR9 recognizes DNA containing an unmethylated-CpG motif, which is rich in bacterial DNA[12,13]. TLR9 expression in the liver is increased in several types of nonalcoholic steatohepatitis (NASH) models[14,38,39], and bacterial DNA is detected in blood and ascites samples from advanced cirrhosis patients[40,41]. We have demonstrated that bacterial DNA is detectable in the blood in CDAA-fed mice but not in control diet-fed mice[14]. To

Table 1 Toll-like receptor mutant mice and nonalcoholic fatty liver disease

Mice	Diet	Duration	Steatosis	Inflammation	Fibrosis	Ref.
TLR2 KO	MCD	5 wk	Identical	Worsen	N/A	[17]
TLR2 KO	MCD	8 wk	Worsen	Worsen	N/A	[18]
TLR2 KO	CDAA	22 wk	Identical	Improved	Improved	[48]
TLR2 KO	HF	20 wk	Improved	Improved	N/A	[46]
TLR2 KO	HF	5 wk	Improved	Improved	N/A	[47]
TLR4 mu	MCD	3 wk	Improved	Improved	N/A	[9]
TLR4 KO	MCD	8 wk	Improved	Improved	Improved	[19]
TLR4 mu	HF	22 wk	Improved	N/A	N/A	[26]
TLR4 mu	Fru	8 wk	Improved	Improved	N/A	[10]
TLR5 KO	ST		Worsen	Worsen	N/A	[20]
TLR9 KO	CDAA	22 wk	Improved	Improved	Improved	[14]

Assessment of toll-like receptor (TLR) mutant mice were compared with control (WT) mice. CDAA: Choline-deficient amino-acid defined; Fru: Fructose-rich; HF: High fat; MCD: Methionine and choline deficient.

investigate the role of TLR9, WT mice and TLR9 deficient mice were fed a CDAA diet to induce steatohepatitis. TLR9 deficient mice on the CDAA diet showed less steatosis, inflammation, and liver fibrosis compared with their WT counterparts. In addition, insulin resistance and weight gain induced by the CDAA diet were suppressed in TLR9 deficient mice[14]. A TLR9 ligand evokes inflammasome-associated liver injury[42,43], which is activated in human NASH compared with chronic hepatitis C[44]. Consistent with the *in vivo* experiments results, TLR9 signaling is associated with inflammasome expression in WT macrophages[14,45], resulting in the production of IL-1β. These data indicate that TLR9 signaling promotes the progression of NASH.

TLR2 perceives components of Gram-positive bacterial cell walls such as peptidoglycan and lipoteichoic acid[12,13]. The levels of Firmicutes, which are Gram-positive bacteria and a major component of the gut microbiota, are increased in mice on an HF diet, suggesting that TLR2 ligands are rich in gut microbiota in obese mice. Blockade of TLR2 signaling prevents insulin resistance induced by an HF diet in mice[46,47]. We have shown that TLR2 deficient mice are resistant to CDAA-induced steatohepatitis[48]. TLR2 deficient mice on a CDAA diet showed lower expression of pro-inflammatory cytokines such as TNFα and IL-1β. In *in vitro* experiments, TLR2 ligands induced proinflammatory cytokines in WT macrophages.

In contrast, TLR2-deficient mice on an MCD diet exhibit equivalent or more severe steatohepatitis as a result of hypersensitivity to LPS[17,18]. Although the MCD diet induces typical features of steatohepatitis, metabolic parameters are completely different; mice on MCD lose weight with increased insulin sensitivity, whereas mice on an HF or CDAA diet gain weight accompanied by insulin resistance. The difference in gut microbiota may account for the contrasting results in the role of TLR2 ligands.

TLR5 is a receptor for bacterial flagellin. Although the role of hepatic TLR5 expression remains unknown, its expression on intestinal mucosa plays critical roles in the development of metabolic syndrome. The first report on TLR5 showed that a lack of TLR5 in mice resulted in spontaneous colitis[49], indicating that TLR5 plays a protective role in the intestinal epithelium. A rederived line of TLR5 KO mice developed obesity and steatosis[20]. A striking finding in TLR5 KO mice is the alteration in gut microbiota at the species level. Transfer of TLR5 KO microbiota to WT germ-free mice reproduced the metabolic syndrome. On the other hand, TLR5 deficient mice from different animal colonies show no basal inflammation and metabolic syndrome under normal conditions[50]. These data suggest that the interplay between TLR5 and specific gut microbiota contributes to the development of metabolic syndrome.

PROINFLAMMATORY CYTOKINES IN NAFLD

Proinflammatory cytokines such as TNFα and IL-1β are downstream targets of TLRs and have been shown to promote the progression of NAFLD in animal models. For instance, TNFα signaling deficiency was resistant to NAFLD induced by an HF diet[51] or MCD diet[52]. Additionally, mice that were deficient in IL-1β signaling were protected from HF diet-induced fatty liver[53] or CDAA diet-induced NASH[14]. In addition, mice that were deficient in inflammasome components and caspase-1, which converts the pro-form of IL-1β to its active form, were also resistant to steatosis/steatohepatitis in NAFLD models[54,55]. These data indicate that TNFα and IL-1β are important mediators in the development of NAFLD. Because NAFLD patients show increased expressions of these cytokines as well as their receptors[56-58], these molecules are potential targets for NAFLD treatment.

TNFα regulates lipid metabolism and hepatocyte cell death. TNFα impairs insulin signaling by inhibiting insulin receptors and insulin receptor substrate-1[59], resulting in insulin resistance with elevated insulin levels. Insulin resistance increases fatty acid release from adipose tissue and inhibits free fatty acid (FFA) uptake in adipocytes. On the other hand, elevated insulin concen-

Table 2 Classification of gut microbiota based on Gram staining

Gram-positive bacteria	Gram-negative bacteria	Unclassified
Actinobacteria	*Bacteroidetes*	*Deferribacteres*
Firmicutes	*Cyanobacteria*	*Tenericutes*
TM7	*Verrucomicrobia*	

tration facilitates FFA flux into hepatocytes and hepatic lipogenesis[60]. Moreover, TNFα promotes cholesterol accumulation in hepatocytes by increasing cholesterol uptake through LDL receptors and by decreasing the efflux through lipid transporting genes such as ABCA1[61]. Lipid-accumulated hepatocytes are more sensitive to TNFα-mediated cell death[62,63]. Although TNFα does not induce apoptosis in normal hepatocytes by inducing nuclear factor κB (NF-κB)-related anti-apoptotic genes[64], excessive lipid levels in hepatocytes alter the cell survival signals. For instance, lipid-accumulated hepatocytes generate reactive oxygen species[62] and show increased gene expression of ASK-1 and c-Jun N-terminal kinase (JNK)[63], which drive cell death signaling.

IL-1β also mediates the features of NAFLD including steatosis[14,53] and hepatocyte death[14]. IL-1β regulates lipid metabolism by suppressing PPARα and its downstream molecules, resulting in hepatic accumulation of triglycerides[65]. On the other hand, IL-1β increases the expression of diacylglycerol acyltransferase 2, an enzyme that converts diglycerides to triglycerides[14]. Thus, IL-1β promotes triglycerides accumulation in hepatocytes. IL-1β contributes to hepatocyte death when hepatocytes are laden with lipids. Pro-apoptotic genes such as Bax are induced in lipid-accumulated hepatocytes upon IL-1β stimulation, whereas anti-apoptotic genes are increased in normal hepatocytes[14].

A major source of these proinflammatory cytokines is macrophages in the liver because macrophage depletion by liposomal clodronate causes low expression of TNFα and IL-1β[9,66]. In addition, mice deficient in TLR2, TLR4, and TLR9 exhibit low expression of proinflammatory cytokines even when these mice were fed a CDAA or MCD diet[9,14,48]. For a detailed analysis of hepatic macrophages, we generated chimeric mice in which WT mice and TLR2 deficient mice were reconstituted with TLR2 deficient macrophages and WT macrophages, respectively. Using a combination of macrophage depletion and bone marrow transplantation, more than 90% of the macrophages were successfully reconstituted by transplanted macrophages[11,15,67]. Chimeric mice reconstituted with TLR2 deficient macrophages reduced inflammation and liver fibrosis[48]. These data indicate that TLR2 on macrophages contribute not only to inflammation but also to liver fibrosis. Recent data show that TNFα and IL-1 produced by hepatic macrophages contribute to liver fibrosis by promoting the survival of activated hepatic stellate cells[68]. Indeed, IL-1β induces pro-fibrogenic genes in hepatic stellate cells[14,69,70]. These data indicate that hepatic macrophages contribute to the pathogenesis of NAFLD by TLR-mediated proinflammatory cytokine production.

COMPOSITIONAL CHANGE IN GUT MICROBIOTA IN OBESITY AND NAFLD

Because gut microbiota are a source of TLR ligands, their compositional change is a potential trigger in the activation of TLR signaling in the liver. Thus, there has been extensive research aimed at identifying the specific bacteria changes that lead to NAFLD. At least following nine microbacteria phyla reside in the gut: *Actinobacteria*, *Bacteroidetes*, *Cyanobacteria*, *Deferribacteres*, *Firmicutes*, *Proteobacteria*, *Tenericutes*, TM7, and *Verrucomicrobia*. Of them, Bacteroidetes and Firmicutes are major components of gut microbiota at the phylum level in rodents and humans[71]. Table 2 shows the classification based on the Gram staining. *Proteobacteria*, *Actinobacteria*, and *Verrucomicrobia* are minor phyla compared with *Bacteroidetes* and *Firmicutes*. Currently, there is insufficient information on TM7, *Deferribacteres*, *Cyanobacteria* and *Tenericutes* in metabolic syndrome.

Most studies have shown that the levels of Firmicutes are increased whereas those of *Bacteroidetes* are decreased in obesity and its related diseases[72-74] in humans as well as rodents; thus, an increased Firmicutes/Bacteroidetes ratio is a potential phenotype of obesity. In addition, the levels of *Bacteroidetes* were increased by interventions aimed at weight reduction, including prebiotics treatment[75] and Roux-en-Y gastric bypass (RYGB) surgery[76] in obese mice. These data suggest that Bacteroidetes are likely to have beneficial effects on obesity. On the other hand, transplantation of commensal *Bacteroides* thetaiotaomicron into germ-free mice induced a 23% increase in body fat[4]. It remains unclear whether the compositional change is a cause or result of obesity. To date, the role of Bacteroidetes in metabolic syndrome remains unknown. If a high *Firmicutes*/*Bacteroidetes* ratio is a feature of obesity, one may speculate that a larger amount of TLR2 ligands is delivered to the liver because *Firmicutes* are Gram-positive bacteria. Indeed, TLR2 deficient mice were resistant to NAFLD induced by an HF diet, which increases Firmicutes. On the other hand, mice on an MCD diet, a NASH model that exhibits weight loss, showed an increase in the levels of Gram-negative bacteria of the *Bacteroidetes fragilis* group[22], suggesting that TLR4 ligands are increased. As expected, TLR4 mutant mice were protected from NASH induced by an MCD diet[9,19]. Although the *Firmicutes*/*Bacteroidetes* ratio is likely to be correlated with the amount of TLR2 and TLR4 ligands, the association between gut microbiota and TLRs is not so simple. For instance, TLR4 deficient mice are also resistant to NAFLD induced by an HF diet, which increases the levels of Gram-positive bacteria. Detailed analysis showed that an HF diet increased the abundance of some minor Gram-negative bacteria such as *Desulfovibrionaceae* and *Enterobacteriaceae*[21,77]. Although both of these bacteria belong to a minor phylum, Proteobacteria, they are a potential

source of LPS[78,79]. In addition, Desulfovibrionaceae can disrupt the gut barrier[80], suggesting that these bacteria contribute to the pathogenesis of NAFLD, even at low levels. *In vitro* experiments indicate that LPS stimulates TLR4 at low concentrations compared with a TLR2 ligand. Thus, minor populations of gut microbiota may participate in the hepatic inflammation in the setting of an HF diet.

Proteobacteria, a phylum of Gram-negative bacteria, includes several pathogenic bacteria such as *Escherichia coli*, *Salmonella*, *Vibrio parahaemolyticus*, and *Helicobacter pylori*. In obese humans and mice, the levels of Proteobacteria are increased in their abundance. On the other hand, the *Proteobacteria* phylum was also increased after RYGB surgery[76]. Because the *Proteobacteria phylum* includes both non-harmful and pathogenic bacteria, further investigation is required to determine the role of *Proteobacteria* in the development of NAFLD.

The *Verrucomicrobia phylum* includes mucin-degrading bacteria *Akkermansia muciniphila* residing in the mucus layer of the intestine, which represents 3%-5% of the microbial community of healthy humans[81,82]. Recent studies showed that the proportion of Akkermansia muciniphila was decreased in the obese and was inversely correlated with body weight in rodents and humans[75,83-85]. Cani *et al*[75] intensively investigated the role of *Akkermansia muciniphila* in obese mice. Probiotic treatment significantly increased the abundance of *Akkermansia muciniphila* and improved metabolic parameters in obese mice models. In addition, *Akkermansia muciniphila* treatment reversed fat gain, serum LPS levels, gut barrier function, and insulin resistance by increasing endocannabinoids and gut peptides. Shin *et al*[86] reported that metformin, an anti-diabetic agent, increased the abundance of *Akkermansia muciniphila*, in which Treg cells improve insulin signaling. Furthermore, RYGB surgery increases the levels of *Akkermansia muciniphila*[76]. These data suggest that Akkermansia muciniphila has potential as a probiotics.

GUT MICROBIOTA IN OBESE CHILDREN

The incidence of NAFLD in children is also considerably increasing worldwide[87]; therefore, examination of gut microbiota has been extended to children. Mixed data were shown regarding *Firmicutes* and *Bacteroidetes* between normal and obese children: Xu *et al*[88] reported an increased levels of *Firmicutes* and an increased *Firmicutes*/*Bacteroidetes* ratio in obese individuals, whereas Zhu *et al*[89] showed increased levels of *Bacteroidetes* and an increased *Bacteroidetes*/*Firmicutes* ratio. These studies were conducted in different countries, *i.e.*, China and the United States. A report from Egypt further demonstrated different results[90], suggesting that the composition of gut microbiota may depend on the environment, particularly in children.

Zhu *et al*[89] further investigated the compositional changes in gut microbiota and focused on the function of the *Proteobacteria phylum*. Among the *Proteobacteria phylum*, the levels of *Escherichia* were significantly increased in NASH compared with those in obese children. They also found higher plasma ethanol levels in NASH children. They speculated that *Escherichia* produced ethanol in the gut because *in vitro* experiments showed that *Escherichia* could generate ethanol. However, it is unclear whether an increase in *Escherichia* is a common mechanism of adult NASH. RYBS surgery increased the abundance of *Escherichia* in the gut, although obesity and metabolic parameters were improved. Thus, the effect of *Escherichia* on the development of NASH may be different between children and adults. Similarly, the abundance of *Desulfovibrio*, a source of LPS, was decreased in obese children[84] whereas this species was increased by an HF diet[21,77].

PROBIOTICS AND NAFLD

Probiotics are live microorganisms that have beneficial effects on health. *Bifidobacterium* and *Lactobacillus* are widely used as probiotics because these bacteria can inhibit an expansion of Gram-negative pathogenic bacteria by producing lactic acid and other antimicrobial substances. Although these probiotic bacteria generally reside in the gut, the population of probiotic bacteria decreases in pathogenic conditions. Indeed, the levels of *Bifidobacterium*, a member of the *Actinobacteria phylum*, are decreased in rodent NAFLD models[21,22,77] as well as in humans[89]. Thus, probiotic supplementation is expected to reverse the phenotype of gut microbiota, leading to improved health. There are many reports on the beneficial effects of probiotics such as Bifidobacterium spp. in rodents. Administration of *Bifidobacterium* spp improves metabolic parameters including cholesterol levels, visceral fat weight, and insulin resistance[91,92]. The *Lactobacillus* casei strain Shirota, a member of Firmicutes, protects against NASH induced by an MCD diet in mice[22] and steatosis induced by a fructose-rich diet[93]. VSL#3 is a probiotic that consists eight strains of bacteria including *Lactobacillus* and *Bifidobacterium* species. VSL#3 administration ameliorates the grade of NAFLD in ApoE$^{-/-}$ mice or HFD-fed rats[94,95]. Probiotics suppress inflammatory indicators including serum LPS levels and hepatic TNFα expression in rodents[22,94,95]. In addition to compositional changes in gut microbiota, probiotics regulate gut permeability, which is enhanced in NAFLD. There are several junctions between intestinal epithelial cells to control barrier functions, including tight junctions, adherence junctions, gap junctions, and desmosomes. Of them, the tight junction is thought to play a central role in intestinal barrier function[5]. The expression of tight junction proteins such as ZO-1 and occludin decreased in murine models of NAFLD[96,97]. Several probiotic bacteria can strengthen barrier function by increasing the expression of tight junction proteins. For instance, the probiotics Bifidobacterium lactis 420, *Escherichia coli* Nissle 1917, and *Lactobacillus plantarum*

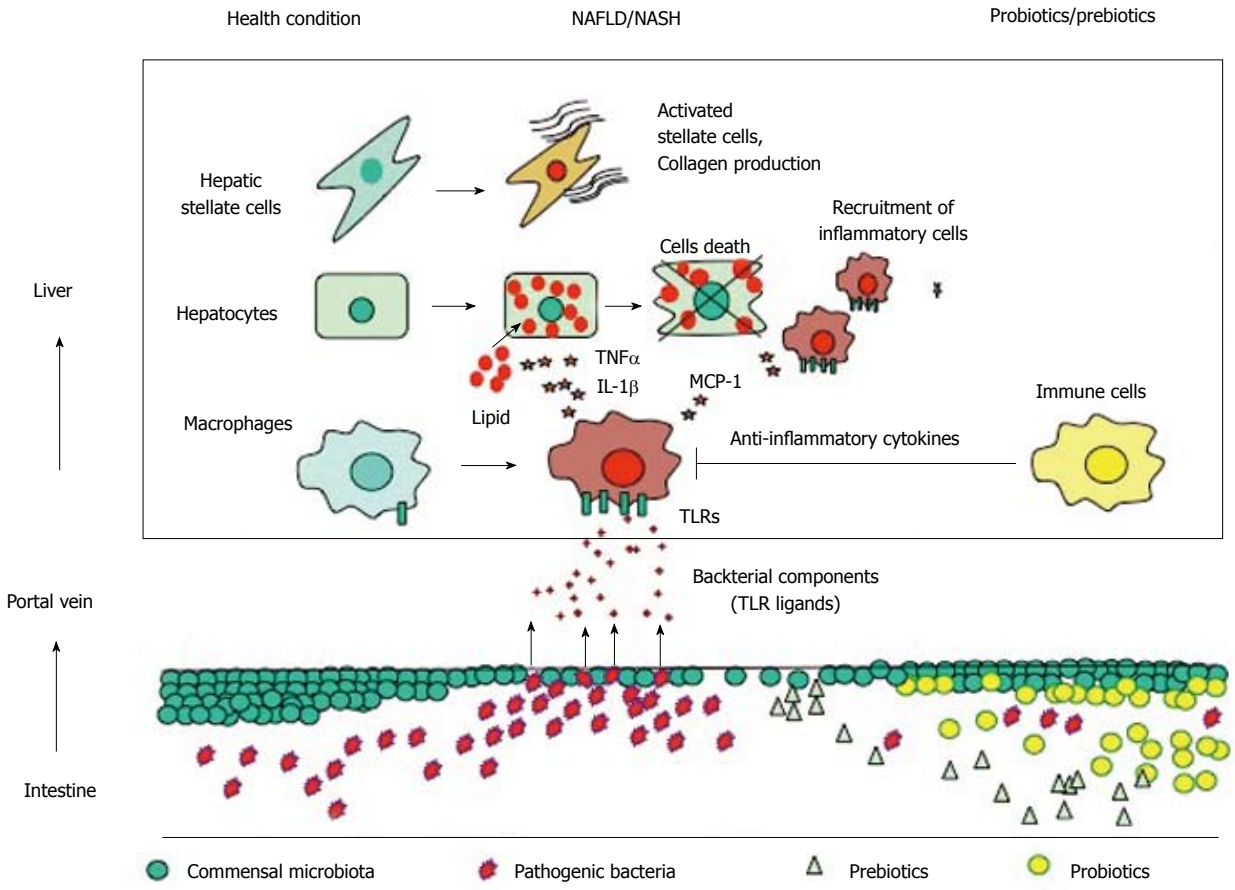

Figure 1 Gut-liver axis in the development of nonalcoholic fatty liver disease. Under healthy conditions, commensal microbiota inhibit the expansion of pathogenic bacteria and maintain the barrier function of the intestinal epithelium. In nonalcoholic fatty liver disease (NAFLD), the levels of pathogenic bacteria may increase, and the barrier function is disrupted by multiple mechanisms, leading to a translocation of bacteria components [toll-like receptor (TLR) ligands] into the portal vein. TLR ligands stimulate TLR expressing cells, such as macrophages, to produce proinflammatory cytokines including tumor necrosis factor α (TNFα) and interleukin-1b (IL-1b), which promote lipid accumulation as well as hepatocyte cell death. TLR ligands also stimulate macrophages to produce chemokines such as MCP-1, which recruits inflammatory macrophages. These proinflammatory cytokines and certain TLR ligands directly stimulate hepatic stellate cells to produce fibrogenic factors. In contrast, treatments with probiotics or prebiotics protects against the translocation of TLR ligands and the expansion of pathogenic bacteria. In addition, probiotics/prebiotics stimulate immune cells to produce anti-inflammatory cytokines.

increased tight junction proteins and preserved barrier function in DSS-induced colitis[98-100]. Probiotics also suppress the production of proinflammatory cytokines including TNFα, IL-1, and IFN-γ, which can disrupt tight junctions[101].

A question arises as to whether probiotic treatment may also supply TLR ligands including TLR2 and TLR9. *Lactobacillus* and *Bifidobacterium* are Gram-positive bacteria and contain TLR2 ligands such as peptidoglycan and lipoteichoic acid. Interestingly, probiotic treatment increased anti-inflammatory cytokines in a TLR2-dependent manner[102]. *Clostridium butyricum* induced IL-10 production from intestinal macrophages in acute experimental colitis through TLR2[103]. These data suggest that TLR2 has a dual function: TLR2 ligands from probiotic bacteria direct an anti-inflammatory state, whereas TLR2 ligands from obesity-related bacteria induce inflammation. Probiotic bacteria also contain an unmethylated-CpG motif, which is a TLR9 ligand. Indeed, the CpG-motif, which is abundant in *Bifidobacterium* species, can drive a murine macrophage cell line to produce TNFα and MCP-1[104], which are mediators that promote the progression of NASH[66]. On the other hand, most probiotic bacteria are not able to produce TLR9-mediated IFN-γ in myeloid dendritic cells except for limited strains[105], suggesting that the response to TLR9 ligands in immune cells may differ among bacteria. Collectively, the TLR ligands derived from probiotics may suppress inflammation partially through the production of anti-inflammatory cytokines.

PREBIOTICS AND NAFLD

Prebiotics are indigestible food ingredients including inulin and fructooligosaccharides, which have beneficial effects by altering the composition of gut microbiota, lipid metabolism, and gut barrier function. Although mammalian enzymes cannot digest complex carbohydrates, certain gut microbiota are able to ferment the carbohydrates to SCFAs such as acetate, propionate, and butyrate. These SCFAs are used as energy[106,107] as well as molecules to stimulate lipogenesis and gluconeogenesis. Interestingly,

SCFAs protect mice from obesity induced by diet or gene modification[108-110]. Acetate is a substrate for middle- to long- chain fatty acids[107] that stimulates hepatic lipogenesis, and the incorporation of acetate to these fatty acids did not occur under fasting conditions[111].

SCFAs can strengthen the barrier function of the intestine. For instance, butyrate restores the mucosal barrier in heat- or detergent-induced colonic injury[112]. In addition, treatment with MIYARI 588, a butyrate-producing probiotics, suppressed gut permeability by increasing the expression of tight junction proteins in mice fed a CDAA diet. As a result, elevation of LPS was inhibited, and steatohepatitis was ameliorated[23]. The probiotic *Lactobacillus plantarum* 299v showed beneficial effects by elevating butyrate concentrations in patients with recurrent *Clostridium difficile*-associated diarrhea. Although the levels of butyrate-producing bacteria in NASH remain unknown, the relative proportion of butyrate-producing bacteria is decreased in type 2 diabetes[113,114].

PERSPECTIVES

Accumulating evidence demonstrates that gut microbiota and TLR signaling are closely associated with the development of NAFLD. Figure 1 summarizes the association between gut microbiota and TLRs and potential effects of prebiotics and probiotics in NAFLD. To data, inconsistent data have been generated regarding the composition of gut microbiota at the phylum level in NAFLD patients because of environmental and interindividual diversity. In addition, studies that show beneficial effects of probiotics and prebiotics are based on small sample sizes. Because certain gut microbiota are likely to contribute to the development of NAFLD by regulating the intestinal barrier function, additional analyses should be performed to confirm their role in NAFLD. In the near future, further information will be provided by metagenomic analysis of gut microbiota in NAFLD. This information will inform NAFLD treatments through worldwide trials.

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TOPIC HIGHLIGHT

WJG 20th Anniversary Special Issues (12): Nonalcoholic fatty liver disease

Radiologic evaluation of nonalcoholic fatty liver disease

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Abstract

Nonalcoholic fatty liver disease (NAFLD) is a frequent cause of chronic liver diseases, ranging from simple steatosis to nonalcoholic steatohepatitis (NASH)-related liver cirrhosis. Although liver biopsy is still the gold standard for the diagnosis of NAFLD, especially for the diagnosis of NASH, imaging methods have been increasingly accepted as noninvasive alternatives to liver biopsy. Ultrasonography is a well-established and cost-effective imaging technique for the diagnosis of hepatic steatosis, especially for screening a large population at risk of NAFLD. Ultrasonography has a reasonable accuracy in detecting moderate-to-severe hepatic steatosis although it is less accurate for detecting mild hepatic steatosis, operator-dependent, and rather qualitative. Computed tomography is not appropriate for general population assessment of hepatic steatosis given its inaccuracy in detecting mild hepatic steatosis and potential radiation hazard. However, computed tomography may be effective in specific clinical situations, such as evaluation of donor candidates for hepatic transplantation. Magnetic resonance spectroscopy and magnetic resonance imaging are now regarded as the most accurate practical methods of measuring liver fat in clinical practice, especially for longitudinal follow-up of patients with NAFLD. Ultrasound elastography and magnetic resonance elastography are increasingly used to evaluate the degree of liver fibrosis in patients with NAFLD and to differentiate NASH from simple steatosis. This article will review current imaging methods used to evaluate hepatic steatosis, including the diagnostic accuracy, limitations, and practical applicability of each method. It will also briefly describe the potential role of elastography techniques in the evaluation of patients with NAFLD.

Key words: Nonalcoholic fatty liver disease; Nonalcoholic steatohepatitis; Liver steatosis; Magnetic resonance spectroscopy; Magnetic resonance imaging; Ultrasonography; Computed tomography; Elastography

Core tip: Ultrasonography is a cost-effective imaging technique for the diagnosis of hepatic steatosis in clinical practice. Magnetic resonance spectroscopy and magnetic resonance imaging are the most accurate and reliable methods of quantifying liver fat, especially for longitudinal follow-up of patients with nonalcoholic fatty liver disease. Ultrasound elastography and magnetic resonance elastography are promising imaging methods to evaluate the degree of liver fibrosis and to differentiate nonalcoholic steatohepatitis from simple hepatic steatosis.

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INTRODUCTION

Nonalcoholic fatty liver disease (NAFLD) is one of the most common causes of chronic liver diseases in Western countries, occurring in approximately 30% of the general population[1,2]. NAFLD consists of a spectrum of diseases, including simple steatosis, nonalcoholic steatohepatitis (NASH), liver fibrosis, and liver cirrhosis[3,4]. Although the exact risk or incidence of progression from simple hepatic steatosis to advanced stages of fatty liver disease has yet to be determined, the progression of simple hepatic steatosis to cirrhosis through the development of steatohepatitis (NASH) and fibrosis has been established[2,5-11]. NASH, characterized by hepatocyte injury, inflammation, and fibrosis, is a clear risk factor for progression to cirrhosis, and such progression has been reported in up to 25% of patients[6,7,9]. NASH is also associated with an increased risk of liver cancer and death from cardiovascular diseases or liver-related causes[2,6,9,10,12]. NAFLD is closely related to obesity, insulin resistance, hypertension, and dyslipidemia and is now regarded as a hepatic manifestation of the metabolic syndrome[4,13,14]. NAFLD also adversely affects disease progression and response to treatment in patients with viral hepatitis C[15] and has negative effects on the prognosis of hepatic transplant recipients[16].

Liver biopsy is regarded as the gold standard for the assessment of NAFLD and is the only reliable method for differentiating NASH from simple steatosis. This method, however, is invasive and is, therefore, unsuitable for screening large numbers of subjects at risk, or for follow-up of patients with NAFLD after therapeutic intervention. Furthermore, as liver biopsy samples are small in size, they are subject to sampling variability[17,18]. The clinical importance of NAFLD and the limitations of liver biopsy have increased the need for accurate and noninvasive imaging methods to evaluate NAFLD. To date, various imaging methods have been utilized to evaluate patients with NAFLD, including ultrasonography (US), computed tomography (CT), magnetic resonance imaging (MRI), and magnetic resonance spectroscopy (MRS), with these methods mostly used to quantify hepatic steatosis. Each imaging method has its own advantages and disadvantages which are summarized in Table 1. More recently, several imaging methods that measure liver stiffness have been investigated for their usefulness in assessing inflammation and fibrosis in patients with NAFLD. This article will review the imaging methods currently utilized for the evaluation of NAFLD and discuss their practical applicability.

US FOR EVALUATING HEPATIC STEATOSIS

Hepatic steatosis on US appears as a diffuse increase in hepatic echogenicity, or "bright liver", due to increased reflection of US from the liver parenchyma, which is caused by intracellular accumulation of fat vacuoles.

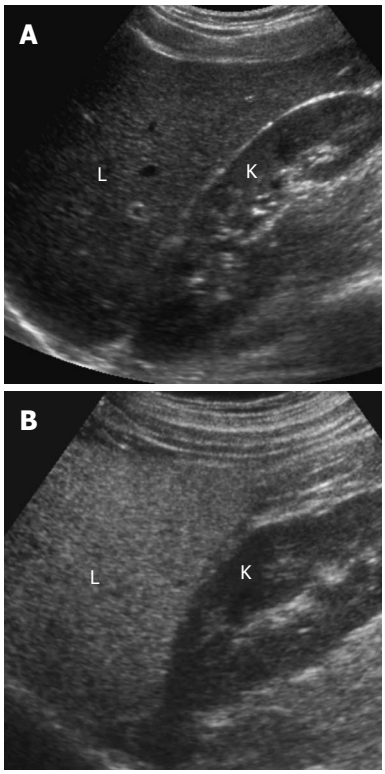

Figure 1 Ultrasonography evaluation of hepatic steatosis. A: Ultrasonography (US) image of a normal liver, showing that the echogenicity of liver parenchyma (L) and kidney cortex (K) is similar; B: US image of a steatotic liver, showing increased echogenicity of the liver parenchyma (L) which is clearly brighter than the kidney cortex (K).

US evaluation of hepatic steatosis typically consists of a qualitative visual assessment of hepatic echogenicity, measurements of the difference between the liver and kidneys in echo amplitude, evaluation of echo penetration into the deep portion of the liver, and determination of the clarity of blood vessel structures in the liver (Figure 1). Severity is usually graded clinically using a four-point scale, as follows: normal (grade 0), mild (grade 1), moderate (grade 2), and severe (grade 3)[19-21]. The diagnostic performance of US in detecting hepatic steatosis has been reported to vary, depending on the exact definition of steatosis and the presence of coexisting chronic liver disease. In patients without coexisting liver disease, US offers a fairly accurate diagnosis of moderate-to-severe hepatic steatosis (*i.e.*, defined as histologic degree $\geqslant$ 30% or 33%), with reported sensitivity ranging from 81.8% to 100.0% and specificity as high as 98%[19,20]. In contrast, US was not accurate in diagnosing hepatic steatosis when all degrees of steatosis were considered (*i.e.*, $\geqslant$ 3% or 5%), with a reported sensitivity ranging from 53.3% to 66.6% and specificity ranging from 77.0% to 93.1%[19,21-23]. As hepatic fibrosis may also increase hepatic echogenicity[24,25], the presence of underlying chronic liver disease may reduce the accuracy of US in the diagnosis of hepatic steatosis. For example, one study that included hepatitis C patients[25] found that US had a sensitivity of 60% and a specificity of 73% in detecting histologically proven moderate-to-severe he-

Table 1 Advantages and disadvantages of imaging techniques for evaluating hepatic steatosis

Techniques	Advantages	Disadvantages	Clinical applications
US	Widely available, easy to perform, less expensive	Operator dependency, limited accuracy in diagnosing mild hepatic steatosis, rather qualitative nature	Population screening, initial examination for subjects with suspected nonalcoholic fatty liver disease
CT	Widely available, easy to perform	Potential radiation hazard, limited accuracy in diagnosing mild hepatic steatosis	Detecting moderate-to-severe hepatic steatosis in donor candidates for liver transplantation
MRI	Highly accurate and reproducible for measuring hepatic fat	High cost, long examination time	Follow-up of response after therapy in practice or clinical trials
MRS	Highly accurate and reproducible for measuring hepatic fat	High cost, long examination time, evaluation of small portion of the liver, expertise required for data acquisition and analysis	Follow-up of response after therapy in practice or clinical trials

US: Ultrasonography; CT: Computed tomography; MRI: Magnetic resonance imaging; MRS: Magnetic resonance spectroscopy.

patic steatosis.

One major limitation of US is the substantial intra- and inter-observer variability. A retrospective study of 168 US examinations showed intra- and inter-observer agreements of 54.7%-67.9% and 47.0%-63.7%, respectively, when assessing the severity of hepatic steatosis using the traditional four-point visual grading system[26]. These findings are consistent with the results of a prospective study of 161 potential liver transplant donors[19], in that the results of 21.7% US examinations differed between two independent readers, with the two radiologists differing significantly in diagnosing hepatic steatosis by US[19]. These results indicate that US is highly dependent on the operator. Another limitation of US is the qualitative nature of the current four-point grading system. Although this grading system is the most widely used for US evaluation of hepatic steatosis in practice, it is too simplistic to account for small alterations in steatosis severity on follow-up. Thus, US may be inadequate for evaluating patients with NAFLD after therapeutic intervention. To overcome the limitations of US, computer-assisted quantitative US techniques were developed for the assessment of hepatic steatosis[27-29]. These techniques employ dedicated post-processing software programs to analyze US echo amplitude, attenuation, and/or texture-based information. The most robust parameter is the computerized hepatorenal index, defined as the ratio of the echo intensities of the liver and renal cortex. The results of two related studies were very promising, with this index demonstrating sensitivities of 92.7% and 100% and specificities of 91% and 92.5% in diagnosing hepatic steatosis $\geqslant$ 5%[28,29].

In summary, US is an established imaging technique for screening subjects at risk of NAFLD, with acceptable sensitivity and specificity in detecting moderate-to-severe hepatic steatosis. As US is easy to perform and less costly than other imaging methods, US is probably currently the most widely used imaging method for detecting hepatic steatosis in asymptomatic patients with elevated liver enzymes and suspected NAFLD[30]. However, because of its low accuracy in detecting mild steatosis, its operator dependency, and its qualitative nature in the absence of dedicated image post-processing, US may not be an adequate tool for monitoring NAFLD patients after therapeutic interventions. Computerized quantitative analysis methods for US may be able to overcome these limitations, but they require further clinical validation.

CT FOR EVALUATING HEPATIC STEATOSIS

CT evaluation of hepatic steatosis is based on the attenuation values of the liver parenchyma, evaluated as Hounsfield units (HUs), and dependent on tissue composition. As the attenuation value of fat (*i.e.*, approximately -100 HU) is much lower than that of soft tissue, hepatic steatosis lowers the attenuation of liver parenchyma. Although a few studies reported that contrast-enhanced venous phase CT and unenhanced CT scan had comparable diagnostic accuracy in the diagnosis of hepatic steatosis[31,32], unenhanced CT scans are usually preferred to avoid the potential errors in contrast-enhanced CT caused by variations in liver attenuation related to contrast injection methods and scan timing. Several quantitative CT indices have been used to assess hepatic steatosis, with the two most frequently used being the absolute liver attenuation value (*i.e.*, HU_{liver}) and the liver-to-spleen difference in attenuation (*i.e.*, CT_{L-S}) (Figure 2). Despite HU_{liver} showing a stronger correlation with histologic degree of hepatic steatosis than CT_{L-S}, HU_{liver} may be subject to errors resulting from variations in attenuation values across CT scanners from different vendors[33,34]. This error can be avoided, however, by using CT_{L-S}, which incorporates spleen attenuation as an internal control[33].

Although the accuracy of CT in diagnosing hepatic steatosis was found to vary, CT was quite accurate for the diagnosis of moderate-to-severe steatosis but was not as accurate for detecting mild steatosis. The threshold values of CT indices for the diagnosis of hepatic steatosis were also quite variable, depending on the methods and populations used[19,21,35]. In one study, which included 154 potential living liver donor candidates[35], a threshold CT_{L-S} value of -9 had a specificity of 100% and a sensitivity of 82% in detecting moderate-

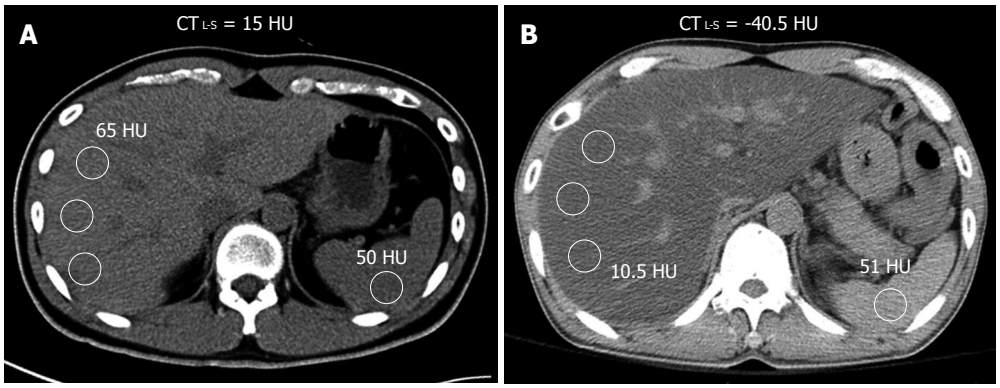

Figure 2 Computed tomography evaluation of hepatic steatosis using computed tomography$_{L-S}$ index. A: Computed tomography (CT) image of a normal liver, showing that its attenuation (65 HU) measured using regions-of-interest (white circles) was higher than that of the spleen (50 HU), and the CT_{L-S} value was 15 HU, which lies within the normal reference range; B: CT image of a steatotic liver, showing hepatic attenuation (10.5 HU) much lower than that of the spleen (51 HU), making the CT_{L-S} value -40.5 HU, far below the normal reference range and indicating moderate-to-severe hepatic steatosis.

to-severe hepatic steatosis. Another study reported that a threshold CT_{L-S} of 3.2 had a sensitivity of 72.7% and a specificity of 91.3%[19]. The variability in these reported threshold values limits the ability to generalize from the results of previous studies. To establish a more generalized threshold value of CT indices for the diagnosis of hepatic steatosis, a normal reference range for CT_{L-S} (1-18 HU) was established using histologically proven, nonsteatotic healthy livers[36]. An HU_{liver} of 48 and a CT_{L-S} of -2 were found to be threshold values for a 100% specific diagnosis of moderate-to-severe hepatic steatosis.

Several factors other than hepatic fat can influence liver attenuation on CT, including the presence of excess iron in the liver and the ingestion of certain drugs such as amiodarone[33,36,37]. Unlike conventional CT, dual-energy CT can differentiate among several chemical components in tissue, by using X rays at two different energy levels. This method has been applied to the evaluation of hepatic steatosis because it may more accurately evaluate hepatic steatosis in the absence of other factors affecting CT hepatic attenuation. To date, however, the theoretical advantage of dual-energy CT has not been established clinically. A recent study in animals using an up-to-date, dual-source, dual-energy CT scanner reported that the use of duel-energy CT did not improve the accuracy of conventional single-energy CT in assessing hepatic steatosis[38], reconfirming the results of a similar study in humans[39].

The low accuracy of CT in detecting a mild degree of hepatic steatosis suggests that this method may not be suitable for the evaluation of NAFLD because patients with NAFLD frequently have a mild degree of steatosis[9,40]. Moreover, the potential hazard of ionizing radiation makes CT unsuitable for use in children or for longitudinal monitoring of patients with NAFLD. CT for longitudinal follow-up of hepatic steatosis is also uncertain, due to a lack of knowledge about the reproducibility of serial CT measurements and the assay sensitivity of CT indices in detecting small changes in the severity of hepatic steatosis. Therefore, CT may not be appropriate for the evaluation of NAFLD, although it may be useful in evaluating hepatic steatosis in specific clinical scenarios. For example, CT can be used successfully to detect moderate-to-severe hepatic steatosis in donor candidates for liver transplantation[35,36,41], and CT measurement of fat in the liver may be useful for patients at risk of metabolic syndrome[42,43].

MAGNETIC RESONANCE METHODS FOR EVALUATING HEPATIC STEATOSIS

Unlike CT and US, which evaluate hepatic steatosis through proxy parameters (echogenicity and attenuation, respectively), MRI and MRS can more directly measure the quantity of hepatic fat. MRI and MRS both measure proton density fat fraction (PDFF), defined as the amount of protons bound to fat divided by the amount of all protons in the liver, including those bound to fat and water. The basic magnetic resonance (MR) physics used in both techniques to differentiate protons in fat from those in water is the chemical-shift phenomenon, *i.e.*, the difference in MR frequency between the protons in fat and water. The chemical-shift effect is directly visible on MRS spectra, displaying signals at their respective resonance frequencies. Moreover, the chemical-shift effect is used in a number of MRI techniques to generate MR images, with signal intensities reflecting the magnitude of protons bound to fat. Accurate quantitative measurement of hepatic steatosis using MRS and MRI premises that MR signal intensities from fat and water are entirely created by proton densities of fat and water without any influence from other factors. However, in reality, the differences in T1, T2, and T2* relaxation times between fat and water inevitably affect the signal intensities of fat and water on MRS and/or MRI. Therefore, various techniques have been developed to minimize the confounding effects. Several clinically feasible MRS and MRI techniques are introduced in the following sections.

MR spectroscopy: Technical aspects

MRS measures proton signals as a function of their res-

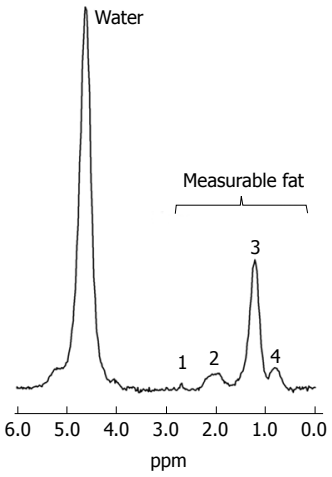

Figure 3 Magnetic resonance spectroscopy spectrum of hepatic fat. Water and fat peaks are displayed at different frequencies; water appears as a single peak at 4.7 ppm, whereas fat appears as four peaks, including the dominant methylene (CH_2) peak at 1.3 ppm (3), a methyl (CH_3) peat at 0.9 ppm (4), an α-olefinic and α-carboxyl peak at 2.1 ppm (2), and a diacyl peak at 2.75 ppm (1); the areas of these four fat peaks and the water peak can be measured by spectral tracing. Proton density fat fraction can be calculated as (sum of fat peaks) ÷ (sum of fat peaks + water peak)[45,82].

onant frequency and displays multiple peaks at different locations, according to the chemical structure of protons in these frequency domains. On MRS spectra of the liver, where fat and water are the most abundant proton-containing materials, most of the identifiable peaks are derived from water and fat, with water appearing as a single peak at 4.7 ppm and fat as multiple peaks due to the presence of various chemical bonds between the protons and adjacent atoms in fat, *e.g.*, a methylene (CH_2) peak at 1.3 ppm and other smaller peaks at various locations (Figure 3). The signal intensities of fat and water peaks can be directly quantified by the spectral tracing of each peak, and PDFF can be calculated as the ratio of the sum of the signal intensities of the fat-derived peaks divided by the sum of the signal intensities of all fat- and water-derived peaks.

For hepatic fat quantification, MRS data is usually collected from a single voxel (typically 2 cm × 2 cm × 2 cm to 3 cm × 3 cm × 3 cm in size), manually placed in the liver parenchyma using 3-plane localizing images. Shimming is necessary to achieve a homogeneous magnetic field across the voxel. Either a stimulated echo acquisition mode (STEAM) or a point-resolved spectroscopy (PRESS) sequence can be used to acquire MRS spectra, with PRESS sequences providing a higher signal-to-noise-ratio (SNR) than STEAM sequences. STEAM, however, is considered more appropriate for fat quantification, as this sequence is less susceptible to a J-coupling effect and results in more reliable PDFF quantification[44,45]. As water and fat peaks are acquired, water or fat suppression must not be used to quantify liver fat using MRS. Unlike brain MRS, which requires multiple acquisitions of data to achieve a sufficiently high SNR to detect minute metabolites, MRS of the liver can be performed successfully with a single acquisition[45,46]. Therefore, MRS of the liver with a single acquisition can be performed in a short time during a single breath-hold, effectively avoiding respiratory movement-related problems; this method is currently preferred[47-51].

For unbiased fat quantification, MRS sequences should be optimized to minimize relaxation effects. A long repetition time (TR), *i.e.*, typically longer than 3000 ms at 1.5T, can minimize T1-relaxation effects. T2-relaxation effects can be reduced by using the shortest possible echo times (TEs). However, multi-echo MRS, which corrects for T2-relaxation effects using multiple spectra acquired at different TEs, allows for a more complete T2 correction[49,52]. Multi-echo MRS techniques are typically performed within a single breath-hold, with five single averaged spectra acquired at five different TEs[47,48,50,52].

MR imaging: Technical aspects

Several different MRI methods have been introduced to quantify hepatic fat, including chemical-shift imaging (CSI), fat saturation, and fat-selective excitation approaches[45,53,54]. The CSI approach is most widely used because of its easy applicability and higher accuracy. Unlike MRS, which shows signals from fat and water at different locations on frequency domains, MRI displays the signal intensity of an image pixel as the vector sum of all signals from fat and water. CSI techniques separate MR signals into water and fat components based on the same MR physics as MRS (*i.e.*, the chemical shift between fat and water), but in a different way by using the chemical-shift-induced signal interference between the protons in fat and water.

The difference in resonance frequency between the dominant fat peak (*i.e.*, the methylene peak at 1.3 ppm) and the water peak (4.7 ppm) is 3.4 ppm, indicating that the water peak resonates 3.4 ppm faster than the methylene peak. Therefore, the protons in both methylene and water oscillate regularly and are positioned in opposed phase (OP) or in in-phase (IP) at certain TEs. The TEs corresponding to OP and IP depend on field strength: at 1.5T, the first OP and IP occurs at 2.3 ms and 4.6 ms, respectively, and OP and IP repeat at multiples of 4.6 ms after their first occurrence. At IP, the signals of methylene and water add constructively but, at OP, their signals cancel each other. Therefore, the difference in signal intensities between OP and IP images reflects the amount of fat (Figure 4).

Since their initial description[55], OP and IP image-based CSI techniques have improved. Dual-echo CSI utilizes a pair of OP and IP images for fat quantification. Although this technique is widely used for clinical MR imaging of the liver, fat quantification using dual-echo CSI is subject to bias from T1-and T2*-relaxation effects. In addition to the proton densities of fat and water, the difference in T1-relaxation times between fat and water affects the signal intensities on IP and OP images. Because of the difference in TEs between OP and IP, T2*-related signal decay during the interval from OP

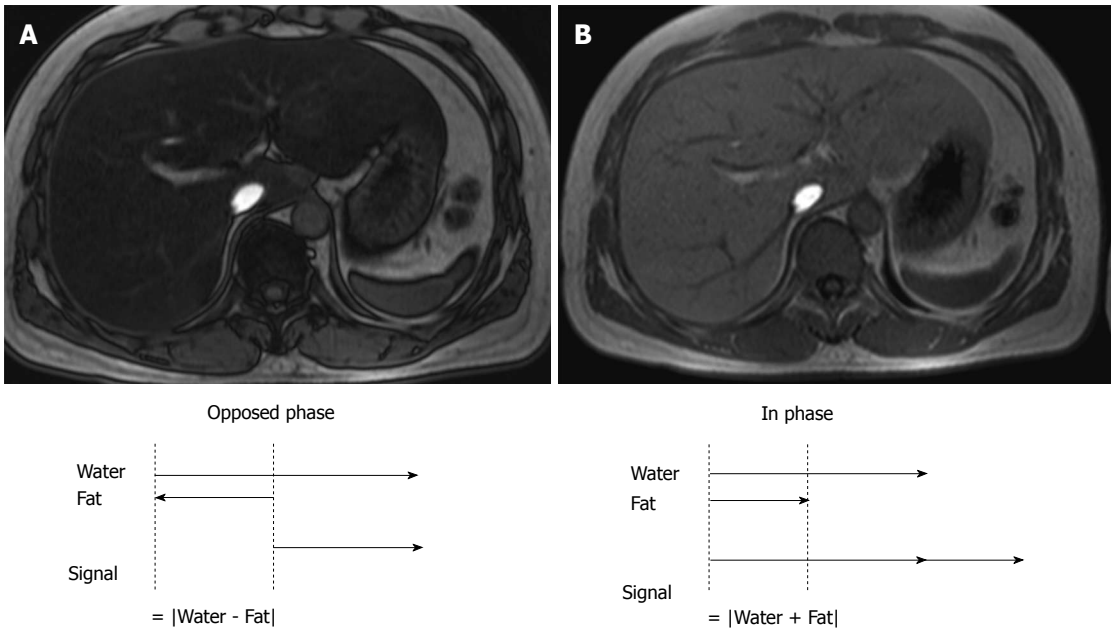

Figure 4 Dual-echo opposed-phase and in-phase chemical shift images of steatotic liver. A: At opposed-phase (OP) (echo time = 2.3 ms at 1.5T), the protons in water and those in methylene (the largest fat moiety) are placed in opposite directions, so that the signals of these two components cancel each other. Therefore, the liver appears dark (*i.e.*, decreased signal); B: At in-phase (IP), the protons in water and those in methylene are positioned in the same direction so that their signals are added. Liver fat fraction can be calculated based on signal intensities on OP and IP images as (signal at IP - signal at OP) ÷ 2 × signal on IP; the signal fat fraction calculated with dual-echo chemical shift images was not corrected for the T2* effect, and therefore may not accurately determine proton density fat fraction.

to IP also causes signal differences between OP and IP images. As these relaxation effects may lead to inaccurate quantification of fat[47,56,57], various techniques have been developed for correction. The T1 effect can be minimized with a low flip angle, whereas the T2*-effect can be corrected with triple or multiple echo acquisitions. Triple-echo CSI acquires a second IP echo in addition to the pair of first OP and IP echoes. The signal intensities of the first OP and IP echoes are corrected for the T2* effect using the T2* time estimated from the signal decay between the first and second IP echoes, followed by a calculation of the T2*-corrected PDFF. Multiple-echo CSI acquires three or more consecutive pairs of OP and IP echoes. Through signal modeling of multiple echoes, this technique allows for the estimation of the T2* time of the liver and T2*-corrected PDFF. These T1-independent, T2*-corrected CSI methods have shown higher accuracies in fat quantification than the classic dual-echo CSI method, resulting in unbiased fat quantification even in the presence of excess hepatic iron deposition[47,50-52,58,59]. Recently, an algorithm for accurate spectral modeling of fat was developed and implemented in the T1-independent T2*-corrected multi-echo CSI technique. This technique is based on fat having a complex chemical spectrum, consisting of multiple peaks with different resonance frequencies, and models the signal intensities on OP and IP images using the signal interferences among water and multiple fat peaks, not between water and a single methylene peak. Since all the aforementioned OP and IP image-based CSI methods use only the signal intensity information on images without phase information, they cannot determine whether fat or water is dominant in tissues. Thus, the signal intensities of OP and IP images are nearly the same for tissues containing 30% and 70% fat. Therefore, the dynamic range of PDFF is 0%-50% hepatic steatosis for these OP and IP image-based CSI methods.

The IDEAL (iterative decomposition of water and fat with echo asymmetry and least-squares estimation) method is a chemical-shift-based, water-fat separation method using both magnitude and phase information. To separate water and fat signals, this technique measures the local field map and demodulates it from the signal in the source images using three or more echoes at different TEs. Although technically complex, the use of phase information for the IDEAL method allows PDFF to be measured over a full dynamic range of 0%-100% hepatic steatosis. Following its initial development, the algorithms for reducing T1- and noise-related bias, for T2*-correction, and for spectral modeling of fat, were implemented with the IDEAL method, allowing for T1-independent, T2*-corrected estimation of PDFF[48,60-62].

CSI with MRI and MRS measures the same physical quantity (*i.e.*, PDFF) for the assessment of hepatic steatosis. Therefore, provided that CSI with MRI and MRS are correctly performed and interpreted, the PDFFs measured by the two techniques should be the same. As MRS estimates PDFF by directly measuring each water and fat peak, whereas CSI indirectly estimates PDFF using the signal interference between water and fat peaks, MRS has been considered more accurate than CSI in measuring PDFF. The feasibilities and accuracies of CSI methods were, therefore, initially validated by comparison with PDFF measured with MRS as the reference

standard. The results of these studies demonstrated that PDFF estimated using CSI techniques with T2*-correction and spectral fat modeling algorithms resulted in the most perfect agreement with MRS-derived PDFF, for both image-based and IDEAL-based approaches. Dual-echo CSI has been reported to generally underestimate PDFF, especially when excessive iron deposition is present[50-52,63]. These findings were recently reconfirmed by comparing PDFFs measured by CSI techniques and MRS with the histologic degree of hepatic steatosis[47,64]. This comparison found that multiple-echo CSI with T2*-correction and spectral fat modeling was as accurate as MRS in fat quantification, with no confounding effects of subjects' demographic factors and coexisting histologic abnormalities[47,64]. In contrast, dual-echo CSI was less accurate than MRS and multi-echo CSI in fat quantification and is confounded by the degree of hepatic iron deposition[47].

Clinical application of the MR techniques

Previous studies have compared the accuracies of MR techniques and other imaging modalities in the assessment of hepatic steatosis, with histologic grading as the reference standard[19-21]. These studies consistently demonstrated that MRS and MRI outperform CT and US in the diagnosis and grading of hepatic steatosis, even when MRS and MRI were performed without any of the sophisticated corrective methods described above (*i.e.*, correction of T2 or T2* effects)[19,21]. The MRI sensitivities and specificities in detecting histologic steatosis $\geqslant$ 5% were 76.7%-90.0% and 87.1%-91%, respectively, and the corresponding MRS performances were 80.0%-91.0% and 80.2%-87.0%, respectively[19,21]. MRS and MRI have several additional advantages over CT and US in the assessment of hepatic steatosis. MRS and MRI can evaluate hepatic steatosis in an objective manner using the quantitative index (*i.e.*, PDFF). PDFF measurements using MRS and MRI have been reported very reproducible[1,50,51,65]. In one study, the standard deviation of PDFF values over repeated measurement was less than 1% for both MRS and MRI[51]. Another study found that the reproducibility of PDFF measurements was high across scanners with different field strengths and from different vendors: the 95% Bland-Altman limits-of-agreement between MRI-determined PDFF on 1.5 and 3.0T scanners were approximately 2%-4%[65].

Although histologic degree of hepatic steatosis has been used as the "gold standard" for comparisons, recent studies suggest that MRS- and MRI-derived PDFF can actually serve as a better reference standard for the amount of fat in the liver than histological evaluation, due to the high accuracy and reproducibility of these MR techniques[66-68]. Studies assessing fat content in liver samples by computerized analysis of microscopic images or biochemical lipid assays found that the fat content in these liver samples was better correlated with MRI- or MRS-determined PDFF than with the pathologist's assessment of hepatic steatosis[66-68]. Histologic assessment of steatosis, including visual determination of percent hepatocytes containing fatty vacuoles or percent hepatic parenchymal area replaced by fat, is subject to large inter-observer variability[17] and may not accurately reflect the physical quantity of hepatic fat[66-68]. In addition, the traditional histological cutoffs categorizing the severity of steatosis (5%, approximately 30%, and approximately 60%) may be too blunt, especially in longitudinal follow-up. These findings and the inherent limitations of liver biopsy, including its invasiveness and ability to obtain very small samples, suggest that MRS and MRI may be the methods of choice, both as reference standards in research studies and in clinical practice, especially in the longitudinal follow-up of patients with hepatic steatosis after therapeutic intervention[69-74]. A recent study has validated the efficacy of MRI- or MRS-determined PDFF as an imaging biomarker to quantify changes in the amount of liver fat and to assess the effects of drug therapy in patients with NAFLD[71].

From a practical viewpoint, MRI appears to have several advantages over MRS. The acquisition and analysis of MRS data requires expertise and is time-consuming. Single-voxel MRS, the typical MRS method use to assess hepatic steatosis, collects data from a small portion of the liver (within a voxel $\leqslant$ 3 cm $\times$ 3 cm $\times$ 3 cm), which may be subject to sampling error, although it is much larger than a biopsy sample. By comparison, MRI is widely available, easily applicable, and can evaluate the entire liver within a short breath-hold. Since the scale of MRS- or MRI-determined PDFF (%) differs from the histologic degree (%) of hepatic steatosis (although both use percentages), clinical thresholds for MRS- or MRI-determined PDFF are needed. The largest MRS study to date, involving 2349 subjects in a general population, suggested that a PDFF value of 5.56% was the upper normal margin, as determined from the 95th percentile of PDFF in 345 subjects with no identifiable risk factors for hepatic steatosis[1].

IMAGING DIAGNOSIS OF NASH AND ELASTOGRAPHY

Hepatic steatosis can progress to fibrosis and cirrhosis through a development of steatohepatitis (NASH), which is a clear risk factor for liver cirrhosis and liver-related mortality[9,10,75]. Therefore, it is clinically important to diagnose the development of steatohepatitis in patients with NAFLD. In general, no imaging examinations have been found to accurately diagnose NASH, making liver biopsy the only reliable method of distinguishing NASH from simple steatosis. US elastography and MR elastography, however, are emerging as promising methods to diagnose NASH. US elastography and MR elastography evaluate liver stiffness by measuring the velocity of shear wave using US (US elastography) or MRI (MR elastography). Several US elastography techniques have been described, which differ in methods of shear wave generation and/or detection, including transient

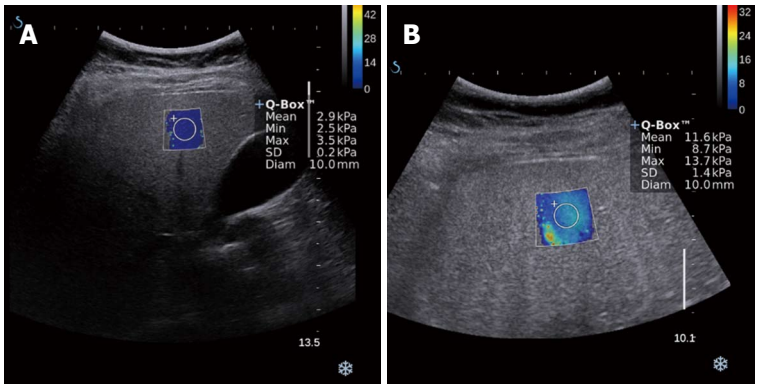

Figure 5 Supersonic shearwave elastography of simple steatosis *vs* nonalcoholic steatohepatitis. A: Supersonic shearwave elastography image of the liver with simple steatosis shows a mean liver stiffness value of 2.9 kPa, which lies within the normal reference range; B: Supersonic shearwave elastography image of the liver with nonalcoholic steatohepatitis shows an elevated mean liver stiffness value of 11.6 kPa.

elastography, acoustic radiation force impulse elastography, supersonic shearwave elastography (Figure 5), and real-time tissue elastography. These techniques were first applied to the evaluation of liver fibrosis in patients with chronic viral hepatitis, and their clinical application has recently been expanded to other liver diseases, including NAFLD. US elastography techniques have demonstrated very promising results for the diagnosis of liver fibrosis in NAFLD[76-80]. They have shown a stepwise increase in liver stiffness as the severity of histologic liver fibrosis increased, and have been highly accurate in differentiating advanced liver fibrosis from mild liver fibrosis, with sensitivities ranging from 88.9% to 100% and specificities ranging from 75.0% to 100%[76-80]. Liver stiffness value did not correlate with the degree of hepatic steatosis or with hepatic inflammation[76-80], indicating that US elastography can assess hepatic fibrosis associated with steatosis without confounding by steatosis but would not be able to assess hepatic inflammation[76-80]. A study of MR elastography in 58 patients with NAFLD showed that liver stiffness in patients with steatosis and lobular inflammation was significantly higher than in patients with steatosis only, and significantly lower than in patients with steatosis and fibrosis[81]. Taken together, these results indicate that US elastography or MR elastography may play a potential role in screening for NASH and/or advanced fibrosis in patients with NAFLD.

CONCLUSION

US is a well-established and cost-effective imaging technique for screening subjects at risk of NAFLD with a reasonable sensitivity and specificity in detecting moderate and severe hepatic steatosis, despite its limited accuracy for mild hepatic steatosis and operator dependency. CT is inaccurate in detecting mild hepatic steatosis and involves a potential radiation hazard, making it inappropriate for assessing hepatic steatosis, especially for longitudinal follow-up of patients with NAFLD. CT, however, may be effective in specific clinical situations, such as the evaluation of hepatic donor candidates for transplantation. MRS is currently the most accurate imaging method used to diagnose hepatic steatosis. MRI, if performed and analyzed correctly, has a comparable accuracy to MRS, is more practical, and can cover the entire liver. Technical optimization of MRS and MRI may result in accurate and unbiased hepatic fat quantification. Both MRS and MRI are very reproducible and accurate in quantifying hepatic fat and may replace liver biopsy as the reference standard for research studies. US elastography and MR elastography can diagnose liver fibrosis associated with NAFLD and may play a role in identifying NASH or NAFLD patients who are at greater risk of progressive liver disease.

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REVIEW

Mycobacterium avium subspecies *paratuberculosis* causes Crohn's disease in some inflammatory bowel disease patients

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Abstract

Crohn's disease (CD) is a chronic inflammatory condition that plagues millions all over the world. This debilitating bowel disease can start in early childhood and continue into late adulthood. Signs and symptoms are usually many and multiple tests are often required for the diagnosis and confirmation of this disease. However, little is still understood about the cause(s) of CD. As a result, several theories have been proposed over the years. One theory in particular is that *Mycobacterium avium* subspecies *paratuberculosis* (MAP) is intimately linked to the etiology of CD. This fastidious bacterium also known to cause Johne's disease in cattle has infected the intestines of animals for years. It is believed that due to the thick, waxy cell wall of MAP it is able to survive the process of pasteurization as well as chemical processes seen in irrigation purification systems. Subsequently meat, dairy products and water serve as key vehicles in the transmission of MAP infection to humans (from farm to fork) who have a genetic predisposition, thus leading to the development of CD. The challenges faced in culturing this bacterium from CD are many. Examples include its extreme slow growth, lack of cell wall, low abundance, and its mycobactin dependency. In this review article, data from 60 studies showing the detection and isolation of MAP by PCR and culture techniques have been reviewed. Although this review may not be 100% comprehensive of all studies, clearly the majority of the studies overwhelmingly and definitively support the role of MAP in at least 30%-50% of CD patients. It is very possible that lack of detection of MAP from some CD patients may be due to the absence of MAP role in these patients. The latter statement is conditional on utilization of methodology appropriate for detection of human MAP strains. Ultimately, stratification of CD and inflammatory bowel disease patients for the presence or absence of MAP is necessary for appropriate and effective treatment which may lead to a cure.

Key words: *Mycobacterium paratuberculosis*; Crohn's disease; Culture; PCR; Johne's disease; Inflammatory bowel disease

Core tip: The review manuscript describes the past, present and predicted future research accomplishments in the area of Crohn's disease and *Mycobacterium avium* subspecies paratuberculosis. This is a highly debated area and Dr. Naser's thoughts described in this review will fuel interest and discussions in inflammatory bowel disease research. The manuscript has been in preparation for a couple of years and it is of high quality.

Naser SA, Sagramsingh SR, Naser AS, Thanigachalam S. *Mycobacterium avium* subspecies *paratuberculosis* causes Crohn's disease in some inflammatory bowel disease patients. *World J Gastroenterol* 2014; 20(23): 7403-7415 Available from: URL: http://www.wjgnet.com/1007-9327/full/v20/i23/7403.htm DOI: http://dx.doi.org/10.3748/wjg.v20.i23.7403

INTRODUCTION

One of the earliest documented descriptions of Crohn's disease (CD) was described in 1769 by Giovanni Battista, an Italian physician. He described the results of an autopsy of a man who had suffered from chronic bowel movements throughout his life and subsequently died from diarrhea and fever[1]. This may have been the first account of granulomatous inflammatory bowel disease (IBD). Several years later in 1813, Combe and Saunders observed a patient who suffered from an abnormally narrow and thickened ileum[1] and Abercrombie had reported a case in 1828 whereby a patient suffered from an inflamed ileum (ileitis) as well as skip lesions affecting certain segments of the ascending colon and cecum[1]. There were several medical publications made in the 20th century which provided further insight into the characteristics and features of CD. For example, some such cases were reported by Braun (1901), Koch (1903), Lesniowski (1903), Wilmanns (1905), Moynihan (1907), and Proust (1907)[1]. In 1913 a surgeon by the name of Dalziel (1861-1924) had reported the symptoms of several of his patients that closely resembled clinical manifestations in cattle suffering from Johne's disease[2]. He is credited as being the first scientist to hypothesize that the causative agent of Johne's disease, *Mycobacterium avium* subsp. *paratuberculosis* (MAP), may in fact be responsible for chronic intestinal inflammation observed in the intestines of humans. In 1923 Moschcowitz and Wilensky[3] had reported four cases of young patients suffering from non-specific granulomata of the intestine. In these patients they observed what appeared to be tumor-like masses that were hard, thick, and associated with all four coats of the large intestine which caused stricture of the lumen. Based on these observations it was originally believed that these structural changes were confined to the colon. However, it was found that such lesions could also be located in the small intestine which was later seen in one patient[4]. In fact, it is interesting to note that all four of the patients had a history of appendicitis and appendectomy.

In 1932 acknowledgement of CD as an official medical entity was as a result of an article published by Drs. Burrill Crohn, Leon Ginzburg, and Gordon G. Oppenheimer, who all worked at Mount Sinai Hospital in New York at the time. Their article entitled "Regional ileitis: A Pathological and Clinical Entity" had appeared in the *Journal of the American Medical Association* in October 1932[5]. The title "Crohn's disease," has been coined after Dr. Burrill B. Crohn, a gastroenterologist who presented the above named paper at the annual American Medical Association in May 1932. The study described a disease that exclusively affected the terminal ileum of 14 patients from a pathological and clinical standpoint[5]. The patients were primarily young adults of ages ranging from 17 to 52 years, but only two of them were older than 40 years[5]. It was expressed that this was a moderately acute disease that was associated with inflammation characterized by rapid necrosis throughout the affected tissue, and by inflammation associated with scar tissue[5]. Furthermore, it was indicated that the disease is clinically represented by certain common symptoms similar to ulcerative colitis-fever, diarrhea, and even weight loss[5]. Dr. Crohn *et al*[5] also witnessed that the ulcers associated with the mucosa were accompanied by a non-uniform connective tissue reaction of the remaining walls of the involved intestine, a process which frequently leads to the narrowing of the lumen of the intestine, and this has been known to be associated with the formation of multiple fistulas. Other physicians reported of similar concurrent observations, but these reports cited the involvement of a number of different parts of the gastrointestinal (GI) tract. For instance, the first case reporting evidence of inflammation present in the colon and not just in the ileum was by Colp in 1934[6]. His report is considered as the first case detailing of ileocolitis which described that this inflammatory process could also extend to the cecum and the ascending colon[6]. In addition, several years later granulomatous lesions were also found in the skin[7]. As a result, it was becoming quickly apparent that CD is a chronic inflammatory disease that can affect any region of the GI tract ranging from the mouth to the anus, the ileum being the most commonly targeted site. In 1938, Penner and Crohn observed that 8 out of 50 analyzed patients suffering from regional ileitis displayed anal fistulae as a possible complication. They were initially unaware that anal and perianal fistulae could present such a complication of ileitis[8]. In 1952, Wells[9] introduced the term *segmental colitis* while delivering a lecture at the Royal College of Surgeons of England. According to Wells, this condition is associated with the formation of fibrous tissue on the bowel wall leading to its increased thickening as well as the presence of ulcers of the mucosa. These ulcers have a patchy pattern of spreading and are therefore known as "skip lesions"[9]. Most importantly this condition was observed in patients without lesions present in the ileum or jejunum. Wells presumed that segmental colitis is a form of colonic CD, but this was never acknowledged by Crohn himself[9].

At present, research on CD has partitioned it into three categories: inflammatory, obstructive, and fistulating[10]. The inflammatory and obstructive types tend to occur simultaneously and cause obstructions of the bowel due to thickening of the intestinal wall as a result of inflammation. The fistula types are commonly associated with erosion of the bowel walls including perianal fistulas and enteroenteric fistulas[10]. Depending on the locations of these erosions the disease is called Crohn's or granulomatous colitis if symptoms occur in the large intestine, Crohn's enteritis if symptoms occur in the small intestine, or Crohn's ileitis if symptoms occur in the ileum[11]. Furthermore, it has been documented that some patients suffer with inflammation of fat cells under the skin (erythema nodusum), or in large joints (peripheral arthritis)[10]. It is important to note that CD is quite similar to another IBD known as ulcerative colitis (UC). The latter affects only the colon whereas CD can affect any region of the GI tract[12].

WHO IS AFFECTED BY IBD?

Statistical evidence has indicated that the highest prevalence of CD and UC is in North America, northern Europe, and the United Kingdom. These diseases are beginning to rise in southern Europe, Asia, Africa, and Latin America. In fact, as much as 1.4 million persons in the United States and approximately 2.2 million individuals worldwide cope with IBD on a daily basis[13]. However, in one epidemiological study of CD based on ethnicity, it was revealed that CD is least prevalent in African Americans, Asians, and Hispanics. The rate of prevalence for African Americans was 29.8 per 100000, for Hispanics it was 4.1 per 100000, and for Asians it was 5.6 per 100000[14]. It has also been found that the occurrence of IBD is higher in industrialized countries such as North America and Europe *vs* under developed or developing countries. Therefore, this suggests that the pathogenesis of IBD may be caused by certain environmental factors[15]. This indicates that genetic susceptibility alone cannot account for the prevalence of CD. In addition, the incidence and prevalence of CD is essentially equal among men and women. Unfortunately, CD is a lifelong debilitating disease which can start in early childhood and continue into late adulthood. Most cases of CD are usually reported or initially diagnosed when the patient is in his or her late teens or early twenties. Recent studies have indicated that in the last few decades the number of CD patients diagnosed before the age of 40 years has increased to 80%[16]. This therefore emphasizes the young adult and adolescent age group as a primary target of this disease. Understanding the etiology of CD may facilitate the development of rapid and cost effective methods for disease diagnosis.

DIAGNOSIS OF CD

The most accurate and effective examination for the diagnosis of CD is a full colonoscopy along with intubation of the ileum[17]. This type of endoscopic examination allows the physician to clearly visualize the colon, ileum, and even certain parts of the lower regions of the small intestine. Physicians can also take multiple biopsies of all the segments of the colon as well as the terminal ileum[17]. Dye-based chromoendoscopy is an advanced imaging technique which allows for the visualization of subtle changes in the lining of the intestine. An alternative imaging method that can be utilized is capsule endoscopy, which is usually selected when there is no evidence of stricture or stenosis[18]. Other technology detects inflammation of the distal ileum such as enhanced gadolinium magnetic resonance imaging which has been proven to be very effective in distinguishing between inflammatory diseases of the GI tract, is non-invasive, and does not produce any radiation[19].

WHAT IS THE ETIOLOGY OF CD?

Unfortunately, the etiology or cause(s) of CD are still unknown. However, there have been several theories that have been proposed to explain this phenomenon. For example, the leading theories suggest that CD can be caused by certain environmental factors or by a dysregulated immune response in a genetically susceptible host. Many believe a milieu of environmental factors such as diet and certain infectious agents may trigger this disease. For example, it has been found that a diet of refined sugars, fatty acids, fast foods, and minimal consumption of fruits, vegetables, and fibers can contribute to triggering the disease[20]. Certain foods play a pivotal role in influencing the microbiome composition of the human gut. In fact, a "Westernized" diet is believed to change the microbiological environment such that there is an increased susceptibility for the development of intestinal bowel disease[20]. Some of the infectious causative agents studied in connection with CD include viruses, yeast, and bacteria including *Escherichia coli, Listeria monocytogenes, Chlamydia trachomatis, Pseudomonas maltophilia, Bacteroides fragilis, Mycobacterium kansasii, and* MAP. Fortunately, it is almost universally accepted that a host genetic predisposition is critical for development of CD[21].

In recent years, the amount of interest and research data in support of a possible infectious etiology for CD has been well noted. More specifically, the forerunner of the proposed infectious causative agents is MAP. However, there are several critics and skeptics who still discredit this theory. Therefore the goal of this review article is to shed light on this current predicament with the intention to further clarify our understanding of the pathogenesis of CD from the perspective of an infectious agent such as MAP.

MAP AND JOHNE'S DISEASE

It was in 1895 when Johne and Frothingham first identified MAP as the causative agent of chronic inflammation in the gut of a cow[22]. Johne's disease was later coined after Johne for his work in identifying this chronic inflammatory enteric disease in cattle, but this disease has also been observed over the years in several different animals such as sheep, goats, rabbits, monkeys, and even chimpanzees[22]. MAP belongs to the *Mycobacterium avium* complex (MAC) which consists of at least *M. avium* and *M. intracellulare*[23]. Through DNA sequence analysis it is possible to evaluate the similarities and differences among mycobacterial strains. It has been documented that MAP shares certain sequence similarities with other strains of MAC. For example there is a 16S-23S rDNA internal transcribed spacer (ITS) that is approximately a 280 base paired region located on the rRNA operon of mycobacteria[24]. It was found that ITS sequence analysis of MAP taken from 3 different mammalian species-bovine, primate, and human did not indicate much sequence variation between them and in 17 strains of MAC[24]. Thus, the connection between MAP and other mycobacterial strains is observed through this highly conserved sequence similarity. In addition, mycobacteria can be broadly classified as either an environmental or parasitic

species on the basis of their epidemiological and pathological nature[25]. Environmental mycobacteria such as the other strains of MAC can be considered as opportunistic bacteria that are found in a variety of habitats. Some of these environments include wet soil, rivers, agricultural slurry, the intestines of birds, ruminants, humans, and even within protists[26,27].

MAP is an obligate parasitic mycobacterium that causes chronic inflammation in the gut of several mammalian species and is considered to have three major genetic differences that serve to separate it from other non-pathogenic MAC. These differences are the presence of an insertion element designated as IS900, the presence of a genetic element known as "GS", and the presence of a unique MAP gene (*hsp*X) located in a specific genomic region. MAP contains a highly conserved insertion sequence (IS) or IS element referred to as IS900, which is repeatedly found in its genome approximately 15-20 times[28]. IS900 contains 1451 base pairs and harbors neither terminal inverted repeats nor flanking direct repeats normally found in other classical IS elements[28]. As a result, IS900 is grouped in a family of insertion elements that is specifically found in certain microorganisms. Some of these IS elements include IS901 and IS902 found in *Mycobacterium avium* subsp. *silvaticum*[29], IS116 present in *Streptomyces clavuligerus*[30], and IS1110 located in *M. avium*[31]. It has been documented that the pathogenic nature of several microorganisms has been linked to the presence of IS elements[32]. IS900 is able to take control of the translational machinery of MAP and thereby affects the expression of certain genes. It achieves this task by encoding for a putative transposase of 399 amino acids in size called p43 on one strand[33]. On the complementary strand IS900 encodes for a very unique gene called the *hed* (host-expression-dependent) gene[34]. This gene is quite unique in that upon entry into the MAP genome it requires a promoter, termination codon, and ribosome binding site (RBS). Previous studies have indicated that IS900 enters the genome of MAP at a specific consensus target sequence such that it is located between the RBS and start codon of the target gene in one specific direction[33]. As a result of this alignment, the *hed* ORF comes under the control of the mycobacterium host promoter thereby allowing for the translation of the Hed protein[33]. Thus, this is one probable explanation for how the insertion element IS900 may assist in the pathogenic phenotype of MAP compared with the other strains of MAC.

The second major genetic difference between MAP and other mycobacterial strains of MAC is that MAP contains a genetic element designated as "GS", which contains a low guanosine and cytosine (G + C) content[35]. GS is a 6496 bp element which possesses six genes-*gsa*, *gsb*A, *gsb*B, *gsc*, *gsd*, and *mpa*[36]. In addition, it has been found that the *mpa* gene of the GS element in MAP is a putative acetyltransferase, and has *mpa* homologues present in other microorganisms such as *oaf*A and *oac* of *Salmonella typhimurium* and *Shigella flexneri,* respectively[37-39]. Other virulence regions including "pathogenicity islands" or Pais have been reported on MAP chromosome[37] and have been found to be similar to a few protein-coding genes found in *Mycobacterium tuberculosis*. These protein-coding genes are *drr*A, *drr*B, and *drr*C, are located at Rv2936-Rv2938, and have been commonly associated with the pathogenic phenotype observed in *M. tuberculosis*[38].

TRANSMISSION OF MAP TO HUMANS THROUGH COW'S MILK

The real concern for the transmission of MAP from cattle to humans is that MAP-infected cows remain asymptomatic in a lengthy subclinical phase[39]. As a result of this, infected cows are not removed and may continue to be harvested for milk and meat, and the spread of MAP can go unnoticed through fecal matters to the rest of the herd[40]. There have been several cases reporting the culture and isolation of MAP from the milk of subclinical or asymptomatic cows[39].

There has been a plethora of documentation about the number of cases in several countries reporting outbreaks of human illness due to improper 'heat-treated' milk and dairy products. It has been observed that certain pathogens such as *Campylobacter* species, *Salmonella* species, *L. monocytogenes,* and even *Y. enterocolitica* have been found in pasteurized milk, powdered milk, and even cheese, thereby contaminating these products and causing human illness[41]. Thus, it is apparent that milk can serve as a means of transmission of these pathogens. Similarly because MAP is found to a large extent in dairy herds and domestic livestock, it can be inferred that it may be present in raw milk. It is assumed that the pasteurization process will destroy any viable pathogens including MAP. However, there have been numerous case studies indicating the thermal-resistant characteristics of MAP thereby enabling its survival after pasteurization. Chiodini and Hermon-Taylor simulated pasteurization methodologies under laboratory conditions as defined by the Public Health Service, US Food and Drug Administration[42]. They performed the high-temperature, short-time (HTST) method of pasteurization in which the milk samples were heated to 72 ℃ for 15 s in accordance with commercial pasteurization techniques[43]. The results indicated that approximately 3%-5% of strains of MAP survived this process. Also, the pasteurization of MAP obtained from human tissues suspended in milk showed to have a higher survival rate (38.7% and 26.2%) than the bovine samples (8.7% and 9.0%)[43]. Grant *et al*[44] reported that MAP was not completely destroyed after pasteurization if it was already present in the milk at a concentration greater than 10^4 cfu/mL. In other studies, Sung *et al*[45] were able to statistically determine the *D* values for various strains of MAP tested which estimated that MAP can survive HTST pasteurization methods when initially present at a concentration greater than 10^1 organisms/mL of milk. However, there have been some critics who have dismissed the validity of the previous studies because they claimed that the HTST pasteuriza-

tion method performed in the laboratory setting cannot simulate commercial pasteurization conditions such as the turbulent flow of milk[44]. For example, Stabel *et al*[46] challenged the validity of these previous studies and reported that there was no evidence indicating the presence of viable MAP after the performance of HTST pasteurization simulated with an Armfield HTST laboratory pasteurizer. However, Grant *et al*[44] defended the studies previously performed in the field and criticized the methodology selected by Stabel *et al*[46]. She indicated that Stabel *et al*[46]. had frozen and sonicated MAP prior to its addition to raw milk. Grant *et al*[44] also expressed that MAP will not under normal circumstances naturally undergo such treatments prior to contaminating milk samples. Furthermore, freezing and sonicating MAP will only make it more susceptible to heat shock As already indicated by Richards *et al*[47] in 1977, freezing (-70 ℃) of bovine fecal samples contaminated with MAP substantially reduced the viability of MAP. Also, it was Sung *et al*[45] who reported the decreased thermal resistance of declumped MAP cells compared to clumped MAP cells, thereby highlighting the changes caused by the sonication of MAP cells. It is without a doubt that milk can serve as a vehicle for the transmission of MAP from animals to humans through consumption of diary and meat products from infected animals.

PREVALENCE OF MAP IN THE ENVIRONMENT AND IN WATER

One of the major contributors to the spread of MAP in the environment is through the feces of infected cattle. Both subclinically and clinically infected cows excrete massive amounts of MAP through their feces on various pastures and farmlands[48,49]. This is a serious problem because it has already been documented that MAP can persist in the environment for long durations[50]. MAP is capable of surviving in fecal matter and in the soil for up to 12 wk[51]. Muskens *et al*[52] conducted a study investigating whether infected cattle could transmit MAP to other animals such as sheep grazing on the same pastures. They reported that 20% (10/50) of sheep showed evidence for the presence of MAP in their tissues. Subsequently, MAP can spread and infect other animals which come in contact with infected cattle. Furthermore, the prevalence of MAP in the environment is not only due to infected cattle, but can be due to other infected animals such as rabbits and deer which can also spread MAP abundantly through their feces[53]. Unfortunately, this is only part of the problem. In most cases the cow's fecal matter is used to make manure which is subsequently distributed across agricultural lands as fertilizer and thus contaminating ground water, rivers, and other surface bodies of water[36]. It will be just a matter of time before the accidental host (human population) is infected with MAP. MAP has been shown to resist chlorine disinfection treatment at concentrations similar to those used to disinfect public drinking water systems[54]. Clearly, it is apparent that water is a very potent vehicle for the transmission of MAP to humans.

MAP CHALLENGES IN THE LABORATORY

From the outset, MAP is an obligate intracellular bacterium which presents multiple challenges in the laboratory with respect to its cultivation from tissue samples from both CD patients and even Johne's disease in animals. Unfortunately this fastidious bacterium is very slow-growing and often requires the cultures to be incubated for an extended period as much as 16 wk at a time[55]. As a result it has become quite problematic over the years to isolate and culture it through conventional means. Furthermore, MAP has very specific growth requirements which must be met for its survival. For example, this intracellular bacillus is unable to synthesize iron-chelating compounds, and therefore its host must provide iron for MAP to survive. Furthermore, due to its high mycolic content mycobacteria can easily adapt to intracellular growth in macrophages and may even become drug resistant[56]. In addition, MAP in CD assumes a cell-wall deficient spheroplast-like form which complicate culture requirement and void the use of the golden standard Ziehl-Neelsen mycobacterial staining test. For this reason, MAP in its spheroplastic form cannot be identified by light microscopy which adds to the challenges of confirming its presence in a laboratory setting[57]. Due to these difficulties, MAP-specialized scientists looked towards better techniques for the detection and characterization of microorganisms. This led them to the utilization of IS900 polymerase chain reaction (PCR) for the detection of MAP and later on the development of appropriate culture media. Nevertheless, some challenges remain including standardization of the methodology, and most importantly spreading the awareness to clinicians and scientists that standard methodology is not appropriate for the detection of MAP in humans[58].

INVESTIGATING MAP ASSOCIATION WITH CD

It was in 1913, when Dalziel (1861-1924), a surgeon at Glasgow reportedly characterized 13 cases of chronic intestinal enteritis in humans[2]. Upon histological and clinical examination of nine patients, Dalziel specifically noticed that different parts of the gastrointestinal tract were affected: the jejunum, transverse and sigmoid colon, as well as the mid-ileum[2]. He reported that these symptoms closely paralleled clinical findings observed in cattle suffering from Johne's disease, a chronic inflammatory disease of the gut. As a result, Dalziel speculated that *paratuberculosis*, the then known causative agent of Johne's disease, could be a potential etiological agent responsible for the observed symptoms in his patients[2]. It was not until 1932 when Crohn's disease was first introduced as a clinical entity was it possible to connect the pathological and clinical findings described in CD to Dalziel's observations in 1913. However, much skepticism and uncertainty

Table 1 Studies supporting *mycobacterium avium* subspecies *paratuberculosis* association with Crohn's disease by Culture *n* (%)

Study	Crohn's disease	Control
Bull *et al*[63]	14 (33)	3 (33)
Chiodini *et al*[82]	16 (26)	13 (26)
Chiodini *et al*[59]	3 (100)	NP
Collins *et al*[64]	15 (19)	3 (6.3)
Gitnick *et al*[65]	4 (14.8)	1 (1.8)
Kirkwood *et al*[66]	4 (40)	0 (0)
Markesich *et al*[61]	12 (50)	1 (7.7)
Mendoza *et al*[67]	30 (100)	0 (0)
Moss *et al*[68]	6 (33.3)	1 (16.7)
Naser *et al*[69]	2 (100)	0 (0)
Naser *et al*[58]	14 (50)	0 (0)
Schwartz *et al*[62]	10 (37)	2 (5.6)
Sechi *et al*[57]	19 (63.3)	3 (10.3)
Singh *et al*[70]	4 (80)	6 (27.3)
Singh *et al*[71]	29 (50)	5 (12.5)
Wall *et al*[72]	6 (20)	0 (0)

NP: Not performed.

Table 2 Studies not supporting *mycobacterium avium* subspecies *paratuberculosis* association with Crohn's disease by Culture *n* (%)

Ref.	Crohn's disease	Control
Clarkston *et al*[83]	0/21 (0)	NP
Dumonceau *et al*[105]	0/31 (0)	0/22 (0)
Graham *et al*[84]	6/19 (31.5)	7/17 (41)
Kallinowski *et al*[75]	0/21 (0)	0/24 (0)
Kreuzpaintner *et al*[85]	0/23 (0)	0/23 (0)
Parrish *et al*[73]	0/130 (0)	0/130 (0)
Ricanek *et al*[74]	2/75 (2.7)	2/135 (1.5)

NP: Not performed.

persisted with respect to the etiology of Crohn's disease. Furthermore, confidence in this mycobacterial hypothesis over the years has suffered tremendously due to the substantial difficulty and failure in culturing mycobacteria from CD tissues and the reliance on methodology which were not appropriate for MAP from humans. MAP association with CD theory was revived when Chiodini *et al*[59] in 1984 reported the isolation of uncharacterized mycobacteria from tissues of three CD patients. They proposed that the bacterium existed in a cell-wall defective form which was later characterized as MAP[60]. Similar results were reported from studies out of David Graham and John Hermon-Taylor laboratories (discussed below). Advancements in cultural techniques and PCR assays unique to MAP by Naser's team (discussed below) fueled and renewed interest in investigating a possible etiological connection between MAP and CD.

CULTURE OF MAP FROM CD PATIENTS

In this review, data from a total of 23 peer review studies which investigated the presence of MAP in CD specimens using culture techniques were reviewed. As shown in Table 1, the results from 16 (70%) studies supported the association between MAP and CD. Only 7 (30%) studies did not support such association (Table 2). Much of the difficulty in culturing or isolating MAP stems from the fact that this fastidious organism has very specific nutritional requirements and is a very slow growing bacterium[59,61,62]. Culture of MAP in liquid or agar-based media requires weeks to months of laboratory incubation[22]. The presence of MAP in a cell wall-deficient spheroplastic form in humans adds additional challenges to growing it in the laboratory. Many investigators reported the recovery of MAP in a cell wall-deficient form from the tissues of CD patients at a higher occurrence than control groups consisting of non-IBD patients[59,61-72]. Certainly, the advent of PCR, RT-PCR and nested PCR had facilitated the detection of MAP IS900 in cultures from CD patients[53,57,58,64,66,68,69,72]. Table 1 lists a total of 16 studies which strongly support the association between MAP and CD. The development of mycobacterial growth indicator tube (MGIT) sparked a new wave of interest led by Saleh Naser team who supplemented MGIT media with additives essential for survival of cell wall-deficient *in vitro* and restoration of the cell wall. Consequently, Schwartz *et al*[62] reported a higher frequency of MAP in CD patients at 37% (10/27) *vs* healthy controls at 5.6% (2/36). What is truly insightful in this study is the fact that MAP was found at a higher percentage (86%) in surgically resected tissue samples than in tissue biopsies (20%) taken from CD patients[62]. These results alluded to the supposition that MAP may in fact be located below the mucosal layer instead of found on the apical surface area[62]. Naser *et al*[69] further employed the same culture condition to study whether or not MAP is present in human milk. They reported the presence of MAP in 100% (2/2) of breast milk samples taken from lactating CD mothers who had just given birth, compared to 0% (0/5) of healthy lactating controls. Thus, this study provides critical evidence to support the similarity between Johne's disease and MAP infection in CD. MAP was later on detected from breast milk from additional CD patients (data not shown). Most interestingly, Naser *et al*[58] were able to culture viable MAP from the buffy coat of blood sampled from CD patients at a significant percentage 50% (14/28). These intriguing results are further substantiated based on the fact that there was no evidence for the culture of MAP from the blood of the healthy control groups 0% (0/15). Other scientists reported the presence of MAP in 14/33 (42%) bowel-pinch biopsies of CD patients (14/33) compared to 3/33 (9%) non-IBD controls. It was Kirkwood *et al*[66] who sought to investigate if there was an association between MAP and CD in children who were symptomatic of this disease at an early stage. They revealed that 40% (4/10) of the cultured mucosal biopsies from the CD patients contained viable MAP, whereas 0% (0/4) of the healthy non-IBD controls showed no evidence for the presence of MAP. Consequently, these findings clearly indicate the possible association between MAP and CD, and according to Kirkwood *et al*[66] these results

Table 3 Studies supporting *mycobacterium avium* subspecies *paratuberculosis* association with Crohn's disease by polymerase chain reaction *n* (%)

Ref.	Crohn's disease	Control
Autschbach *et al*[76]	52 (100)	5 (100)
Bentley *et al*[86]	122 (33.8)	43 (21.5)
Bull *et al*[63]	34 (92)	9 (26)
	14 (42)	3 (9)
Collins *et al*[64]	15 (19)	3 (6.3)
Dell'Isola *et al*[87]	13 (72)	7 (29.2)
Erasmus *et al*[88]	10 (38)	4 (11)
Fidler *et al*[89]	4 (12.9)	0 (0)
Gan *et al*[90]	17 (47.2)	3 (15)
Ikonomopoulos *et al*[91]	7 (35)	NP
Kirkwood *et al*[66]	22 (39)	6 (15)
	8 (16)	0 (0)
Lisby *et al*[92]	11 (46)	3 (11)
Mendoza *et al*[67]	18 (60)	0 (0)
Mishina *et al*[78]	8 (100)	0 (0)
Moss *et al*[68]	6 (33.3)	1 (16.7)
Murray *et al*[93]	2 (22)	0 (0)
Naser *et al*[69]	2 (100)	0 (0)
Naser *et al*[58]	13 (46)	3 (20)
Romero *et al*[77]	10 (83)	1 (17)
Ryan *et al*[94]	6 (50)	0 (0)
Sanderson *et al*[95]	26 (65)	5 (12.5)
Scanu *et al*[96]	20 (87)	3 (15)
Sechi *et al*[57]	25 (83.3)	3 (10.3)
Singh *et al*[70]	4 (80)	5 (22.7)
Singh *et al*[71]	28 (96.6)	NP
Tiveljung *et al*[97]	3 (27)	0 (0)
Tuci *et al*[98]	21 (68)	11 (48)
Wall *et al*[72]	6 (20)	0 (0)

NP: Not performed.

imply that MAP maybe implicated with the early-onset of CD in children. Sechi *et al*[57] also reported a particularly strong association between MAP and CD based on their population study which involved the analysis of people in Sardinia diagnosed with and without CD. According to their results it was found that MAP DNA was detected in intestinal mucosal biopsies of approximately 63% (19/30) of CD patients compared to 10.3% (3/29) of control patients.

Contrary to the above data, there have been some studies providing evidence for the dismissal of MAP as a causative agent of CD (Table 2). For example, Parrish *et al*[73] conducted a study analyzing blood samples taken from 260 individuals who consisted of 130 CD patients and 130 healthy individuals. After culturing MAP, the results revealed that none of the CD patients 0% (0/130) as well as the healthy controls 0% (0/130) showed evidence for the presence of MAP[73]. Only one patient was reported having a positive result by PCR[73]. Due to the fact that MAP and MAP DNA are present in the food chain and the fact that MAP DNA has been detected in the blood of patients with CD and type I diabetes mellitus and in less frequency in the blood of healthy controls, most scientists in the field may question the protocol used in this study. In another study, Ricanek *et al*[74] collected bowel biopsies from 321 individuals, of which 75 of these biopsies were collected from CD patients and 135 were collected from non-IBD patients. After long-term culture of MAP it was reported that only 2.7% (2/75) of CD patients and 1.5% (2/135) of non-IBD patients showed the presence of MAP[74]. Similarly, Kallinowski *et al*[75] documented the inability to culture MAP from a variety of sources such as stool, sera, and even gut tissue samples. They reported that 0% (0/21) of CD patients and 0% (0/24) of healthy controls had MAP through culture[75]. The results from these studies should not be surprising since MAP is extremely fastidious and requires specialized culture media to grow which is contrary to culture media used in these studies. Other studies which failed to detect MAP in CD have depended on traditional standard methodology designed to culture and detect bacillary MAP from Johne's disease animals or other *Mycobacterium species*. It is important that investigators realize that *M. avium subspecies paratuberculosis* is not the same as *M. avium* or *M. tuberculosis*. Moreover, tissue and blood specimens collected from patients with active antibiotic treatment should be used for attempts to culture MAP in the laboratory. Rarely did the studies described in Table 2 allotted to whether the subjects used in their studies had antimicrobial agents prior to submission of the specimens.

DETECTION OF MAP DNA BY PCR

A total of 52 studies investigating MAP DNA in CD have been reviewed. Table 3 lists a total of 27 studies providing evidence in support of MAP association with CD by PCR. On the contrary. Table 4 lists 25 studies which present data in contradiction of MAP-CD association.

One of the studies showing a strong connection between MAP and CD has been performed by Autschbach *et al*[76]. They reported that a staggering 52% (52/100) of tissue from CD patients were found positive for the presence of MAP DNA compared to only 5% (5/100) of the non-IBD patients. Similarly, Romero *et al*[77] had examined several surgical tissue samples from 20 individuals by performing nested PCR specific for the IS900 sequence. The results from Naser's lab indicated that a substantially high percentage 83% (10/12) of CD patients were positive for the presence of MAP, while a much smaller percentage 17% (1/6) of non-IBD patients were positive for MAP[77]. In addition, there was another compelling study conducted by Bull *et al*[63] in 2003 in John Hermon-Taylor's laboratory, which presented data in support of MAP as a causative agent for CD. Fresh ileocolonic mucosal biopsies were collected and analyzed for the presence of MAP by the performance of PCR specific for IS900. The results revealed that 92% (34/37) of CD patients were positive for the presence of MAP DNA compared to a significantly diminished number of healthy controls 26% (9/34)[63]. In this same study Bull *et al*[63] had cultivated MAP using MGIT cultures described by Naser *et al*[58] and Schwartz *et al*[62]. After twelve weeks of incubation, PCR was performed with these cultures which again indicated

Table 4 Studies not supporting *mycobacterium avium* subspecies *paratuberculosis* association with Crohn's disease by polymerase chain reaction *n* (%)

Ref.	Crohn's disease	Control
Al-Shamali *et al*[99]	0 (0)	0 (0)
	0 (0)	0 (0)
Baksh *et al*[100]	0 (0)	NP
Bernstein *et al*[101]	0 (0)	6 (21.4)
Cellier *et al*[102]	2 (4)	2 (10)
	0 (0)	0 (0)
Chiba *et al*[103]	0 (0)	0 (0)
Clarkston *et al*[83]	1 (4.8)	0 (0)
Dumonceau *et al*[104]	17 (47)	13 (57)
	0 (0)	0 (0)
Domonceau *et al*[105]	0 (0)	0 (0)
Ellingson *et al*[106]	0 (0)	0 (0)
Frank and Cook[81]	0 (0)	0 (0)
Gibson *et al*[107]	0 (0)	0 (0)
Kallinowski *et al*[75]	0 (0)	0 (0)
Kanazawa *et al*[108]	0 (0)	0 (0)
Kreuzpaintner *et al*[85]	0 (0)	0 (0)
Lozano-Leon *et al*[109]	0 (0)	0 (0)
Parrish *et al*[73]	0 (0)	1 (0.77)
Ricanek *et al*[74]	0 (0)	1 (0.28)
Riggio *et al*[110]	0 (0)	0 (0)
Quirke[21]	0 (0)	0 (0)
Rowbotham *et al*[80]	0 (0)	1 (3.8)
Sasikala *et al*[79]	0 (0)	0 (0)
Suenaga *et al*[111]	10 (100)	14 (87.5)
	10 (100)	14 (87.5)
Toracchio *et al*[112]	1 (5)	NP
Tzen *et al*[113]	0 (0)	3 (27.3)
Wu *et al*[114]	0 (0)	NP

NP: Not performed.

a higher frequency of CD patients 42% (14/33) positive for MAP DNA *vs* only 9% (3/33) of healthy controls[63]. This data strengthens the support of MAP in connection with CD. Mishina *et al*[78] analyzed mucosal specimens using RT-PCR for the detection of MAP RNA where they found MAP in 100% (8/8) of CD patients and 0% (0/2) in non-IBD. This study is of particular importance because MAP RNA was amplified (without culture) adding more support to the presence of viable MAP in CD[78].

At the same time, many studies based on PCR techniques have failed to detect MAP DNA in CD and concluded the lack of association between MAP and CD (Table 4). For example, Sasikala *et al*[79] indicated that 0% (0/93) of CD patients showed the presence of MAP and 0% (0/97) of healthy controls were also negative for the presence of MAP. Similarly, Rowbotham *et al*[80] reported that none (0/68) of CD patients were positive for the presence of MAP and just 3.8% (1/26) of healthy controls had MAP. Lozano-Leon *et al*[109] indicated the absence of MAP in the blood of 73 CD patients and 73 healthy controls. Frank and Cook in 1996 also reported the absence of MAP in both CD and control subjects[81]. The investigators in these studies should be commended on their interest to question whether or not MAP is associated with CD, and for including impressive numbers of specimens in their studies. Due to the fact that MAP and MAP DNA are found in the food chain including dairy and meat products as well as in drinking water, it is difficult to accept that MAP or MAP DNA is not detected even accidently in some specimens. The methodology used in many of these studies must have lacked essential steps to recover the low abundance of MAP in CD specimens and must have not been able to reduce the laboratory loss of some MAP or MAP derivatives. Whether the loss of MAP occurred at the specimen collection level or during the analysis, it should be avoided. Tissue specimens must be collected appropriately and adequately from active ulcerated sites. Specimens should be transported promptly and appropriately by avoiding freezing and use of anti-microbial solutions. Blood should be withdrawn into tubes with anticoagulants, transported without freezing, and promptly, to avoid lysis of leukocytes and loss of MAP. DNA extraction conditions should be optimized to recover single MAP genome which is also free from PCR inhibitors such as hemoglobin. Earlier study in our laboratory suggested that MAP from CD patients contained limited IS900 copies compared to bovine MAP strains. Nested PCR consisting of two amplification rounds is necessary for sufficient detection of MAP DNA. For reasons mentioned above and other unknown factors, standard PCR based on a single amplification should not be used for detection of MAP in CD.

CONCLUSION

In this review, data has been presented in the form of tables providing evidence for and against an association between MAP and CD by PCR and culture. It was revealed that MAP can be detected and isolated from the tissues, blood, and milk of many CD patients. Based on this information, MAP is definitively involved in the pathogenesis of some CD cases even though other studies have not acknowledged this association as represented in Tables 2 and 4. It must be emphasized that much of the controversy concerning MAP and CD stems from the inconsistent methodologies that have been used in the detection and isolation of MAP, which have questioned the causal relationship between this bacterium and CD. These observed discrepancies result from the fact that the methods that were designed for the detection of MAP in animals with Johne's disease are inappropriate for the detection of MAP in humans. Consequently, the need for more sophisticated and optimized methodologies are required so that there can be accurate detection and isolation of MAP in CD patients. One such methodology has been developed in our laboratory, and success has been achieved based on key principles shown in Figure 1. Other factors that may also limit the detection of MAP in clinical samples from some CD patients include the stage of the disease, and prior treatment with antibiotics or drugs with antimicrobial activity. For example, negative detection of MAP in peripheral blood samples could be correlated with a localized intestinal CD com-

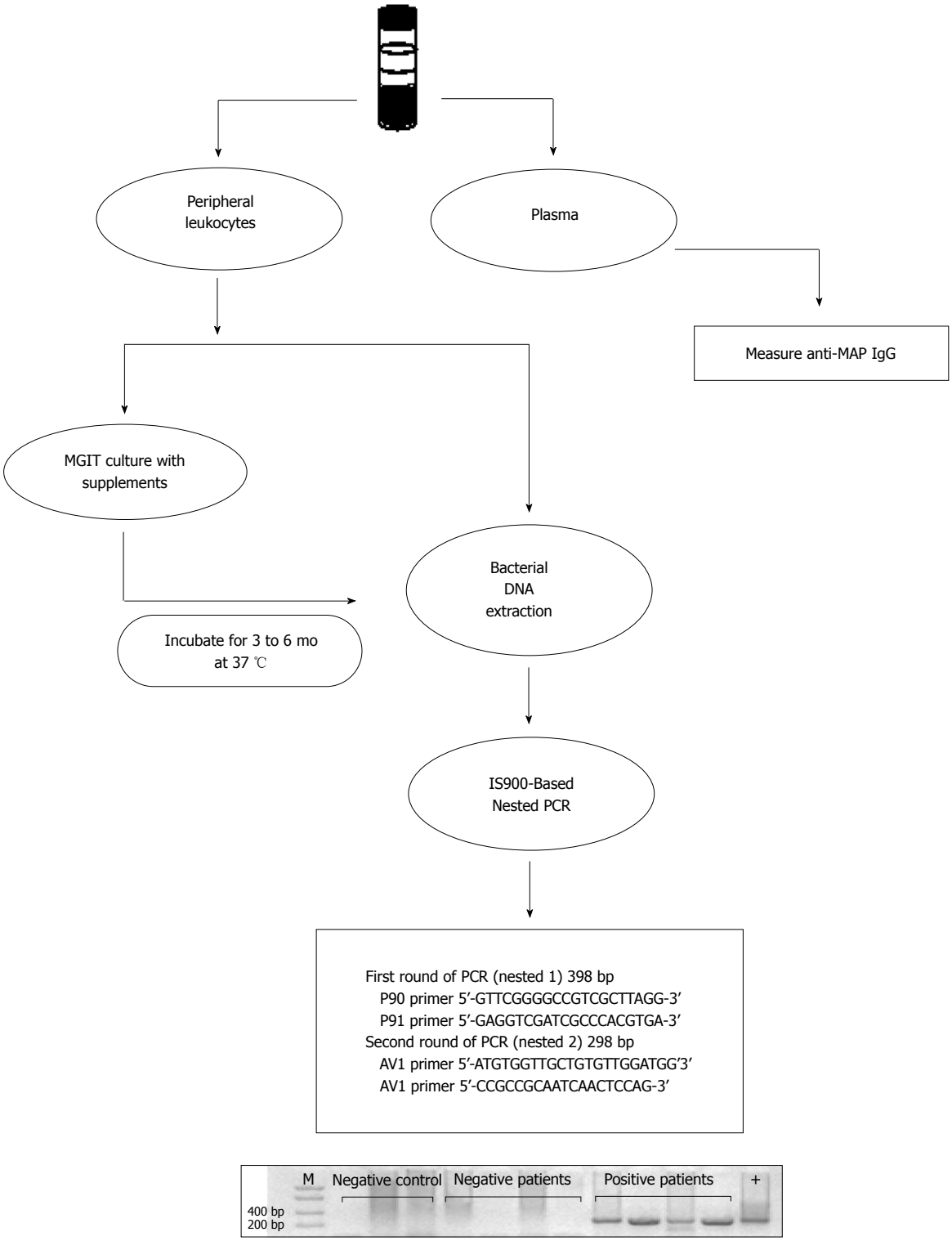

Figure 1 Schematic illustration of successful *Mycobacterium avium* subspecies *paratuberculosis* detection in clinical samples. Coded EDTA blood samples were collected from patients for investigating the presence of *Mycobacterium avium* subspecies *paratuberculosis* (MAP). Blood plasma was analyzed by measuring the concentration of anti-MAP IgG antibodies. Peripheral leukocytes were analyzed for the presence of MAP. In the first method, DNA was extracted followed by IS900-based nested polymerase chain reaction (PCR) using MAP-specific primers. In the second method a mycobacterium growth indicator tube (MGIT) liquid culture system with supplements was used to culture MAP lacking cell wall followed by 3 to 6 mo incubation and IS900-based nested PCR analysis.

pared to cases with advanced disease associated with systemic complications. The latter is most likely to lead to the presence of MAP in circulation.

Finally, it is also worth noting that it is a fact that CD is a syndrome with multifactorial etiology. It is very possible that lack of detection of MAP in clinical samples from some CD patients may be due to the absence of MAP role in these patients. The latter statement is con-

ditional on utilization of methodology appropriate for detection of human MAP strains. Stratification of CD and IBD patients for the presence or absence of MAP is necessary for appropriate and effective treatment which may lead to a cure.

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REVIEW

Recurrent *Clostridium difficile* infections: The importance of the intestinal microbiota

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Abstract

Clostridium difficile infections (CDI) are a leading cause of antibiotic-associated and nosocomial diarrhea. Despite effective antibiotic treatments, recurrent infections are common. With the recent emergence of hypervirulent isolates of *C. difficile*, CDI is a growing epidemic with higher rates of recurrence, increasing severity and mortality. Fecal microbiota transplantation (FMT) is an alternative treatment for recurrent CDI. A better understanding of intestinal microbiota and its role in CDI has opened the door to this promising therapeutic approach. FMT is thought to resolve dysbiosis by restoring gut microbiota diversity thereby breaking the cycle of recurrent CDI. Since the first reported use of FMT for recurrent CDI in 1958, systematic reviews of case series and case report have shown its effectiveness with high resolution rates compared to standard antibiotic treatment. This article focuses on current guidelines for CDI treatment, the role of intestinal microbiota in CDI recurrence and current evidence about FMT efficacy, adverse effects and acceptability.

Key words: *Clostridium difficile* infection; *Clostridium difficile* recurrence; Fecal microbiota transplantation; Stool transplantation; Microbiota

Core tip: Despite current antibiotic treatments, *Clostridium difficile* infection (CDI) is a growing epidemic with increasing rates of recurrence, severity and mortality. The treatment of recurrent CDI thus represents a real challenge. This article simultaneously focuses on current guidelines for CDI treatment, the role of gut microbiota in CDI recurrence and current evidence about fecal microbiota transplantation (FMT) efficacy, adverse effects and acceptability. According to studies published to date, FMT use for recurrent CDI is associated with high resolution rates compared with standard antibiotic treatment. Further studies are needed to confirm FMT effectiveness, and to determine the long-term consequences and good administration practices.

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INTRODUCTION

Clostridium difficile (*C. difficile*) infection (CDI) are the leading cause of nosocomial diarrhea, representing 20%-30% of diarrhea caused by antibiotics, and mortality is estimated at 2%[1,2]. Recent data from 28 community hospitals in the United States suggest that *C. difficile* has become the leading cause of healthcare-associated infection ahead of methicillin-resistant *Staphylococcus aureus*[3]. The increasing incidence of CDI among hospitalized and outpatients is

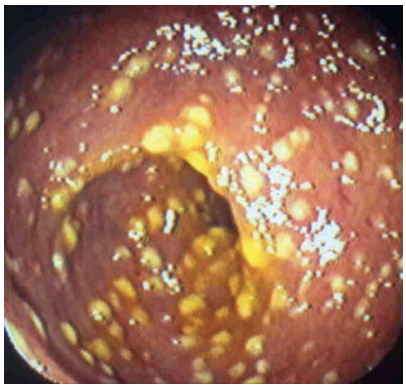

Figure 1 Colonoscopy showing typical yellow pseudomembranes that cover superficial mucosal ulcerations.

a real public health challenge with an increasing incidence from 30 per 100000 in 1996 to 84 per 100000 in 2005 in American acute care hospitals[4]. Indeed this is associated with an annual cost in the United States of an estimated 1 billion dollars[5]. Recent occurrence of severe *C. difficile* disease and higher mortality rates have been associated with the emergence of strains with increased virulence, the so-called "hypervirulent" isolates that belong to the BI/NAP1/027 category and which are fluoroquinolone-resistant[6].

The main *C. difficile* virulence factors are two exotoxins, the enterotoxin TcdA and the cytotoxin TcdB: their actions on the cytoskeleton and tight junctions result in decreased transepithelial resistance, fluid accumulation, and destruction of the intestinal epithelium. They also cause the release of inflammatory cytokines and promote neutrophil chemotaxis, thereby contributing to the mucosal injury[7].

Regardless of the treatment provided, and despite its effectiveness, more than 25% of patients will have a recurrence within 1 to 3 mo[8]. Taken together, CDI treatment today represents a therapeutic challenge because of the high prevalence of CDI, a significant rate of recurrence, and the recent emergence of the hypervirulent strain BI/NAP1/027. Considering the recent better understanding of gut microbiota and the importance of dysbiosis in the pathophysiology of recurrent CDI, there is growing interest in alternative therapeutic approaches, such as fecal microbiota transplantation (FMT) for patients in whom standard antibiotic therapy has failed. In this article we will review the current guidelines for CDI treatment, the importance of gut microbiota and its imbalance in CDI, and current evidence about FMT use in CDI treatment.

CLOSTRIDIUM DIFFICILE INFECTION

Risk factors

A retrospective study published in 2003 identified independent risk factors for CDI occurrence, as listed in Table 1[9]. Among them, the use of antibiotics was identified as the most important. Indeed, over 90% of patients with CDI received an antibiotic within 14 d prior to infection.

Table 1 Independent risk factors for *Clostridium difficile* infections (adapted from[9])

Antibiotic use (especially 3rd generation cephalosporins, fluoroquinolones)
Patients older than 60 yr
Admission in previous 60 d
Use of proton pump inhibitors and histamine-2 blockers
Use of anti-motility agent
Mechanical ventilation
Hypoalbuminemia

Table 2 Clinical presentation of *Clostridium difficile* infections (adapted from[44])

	Clinical manifestations	Laboratory and imaging studies
C. difficile diarrhea	Diarrhea Abdominal pain +/- fever	Colonoscopy: unremarkable
C. difficile colitis	Diarrhea Abdominal pain Fever	Leukocytosis Colonoscopy: patchy or diffuse erythematous colitis without pseudomembranes
Pseudomembranous colitis	Diarrhea Abdominal pain Fever	Leukocytosis Colonoscopy: pathognomonic pseudomembranes (yellow plaques 2-20 mm)
Fulminant colitis	Profuse diarrhea or ileus Abdominal pain Fever +/- signs of shock	Leukocytosis (sometimes white blood cell count > 4×10^9/L) Elevated serum lactate Sigmoidoscopy: pseudomembranes Abdominal computed tomography scanner: megacolon, +/- bowel perforation

C. difficile: *Clostridium difficile*.

Fluoroquinolones and beta-lactams are particularly associated with CDI, and the risk increases with antibiotic duration and dose[9].

Clinical presentation of CDI

The diagnosis of CDI is based on (1) clinical evidence (presence of moderate to severe diarrhea or ileus); and (2) microbiological detection of *C. difficile* in stool [*C. difficile* toxin detection by PCR (sensitivity 90%; specificity 96%) or stool culture] or compatible endoscopic appearance or histopathologic evidence[1] (Figure 1). The presence of diarrhea should raise suspicion of CDI and further investigations should only be undertaken in the case of strong clinical suspicion owing to the high prevalence of asymptomatic carriers among hospitalized patients (7%-20%)[2].

C. difficile infections include a broad spectrum of clinical presentations. Assessing the severity of an episode is of particular importance because it will determine the choice of treatment (Tables 2 and 3). There is no consensus about the definition of a severe episode, but American and European experts agree that a severe CDI is associated with one or more of the following features:

Table 3 Severity of *Clostridium difficile* (adapted from[1,10,11])

Severity criteria according to American experts	Non severe CDI	White blood cell count < 15 × 10^9/L and creatinine level < 1.5 × baseline
	Severe CDI	White blood cell count > 15 × 10^9/L or creatinine level > 1.5 × baseline
	Severe and complicated CDI	Hypotension or shock or ileus or megacolon
Severity criteria according to European experts	Severe CDI	Age > 65 yr or severe comorbidities or intensive care admission or immunodeficiency or Presence of ⩾ 1 of the following criteria: Fever ⩾ 38.5 ℃ Shivering Hemodynamic instability Signs of peritonitis Signs of ileus White blood cell count > 15 × 10^9/L Creatinine level > 1.5 × baseline Elevated serum lactate Pseudomembranous colitis (colonoscopy) Distension of large intestine (computed tomography, CT scan) Colonic wall thickening (CT scan) Pericolonic fat stranding (CT scan) Ascites not explained by other causes

CDI: *Clostridium difficile* infection; CT: Computed tomography.

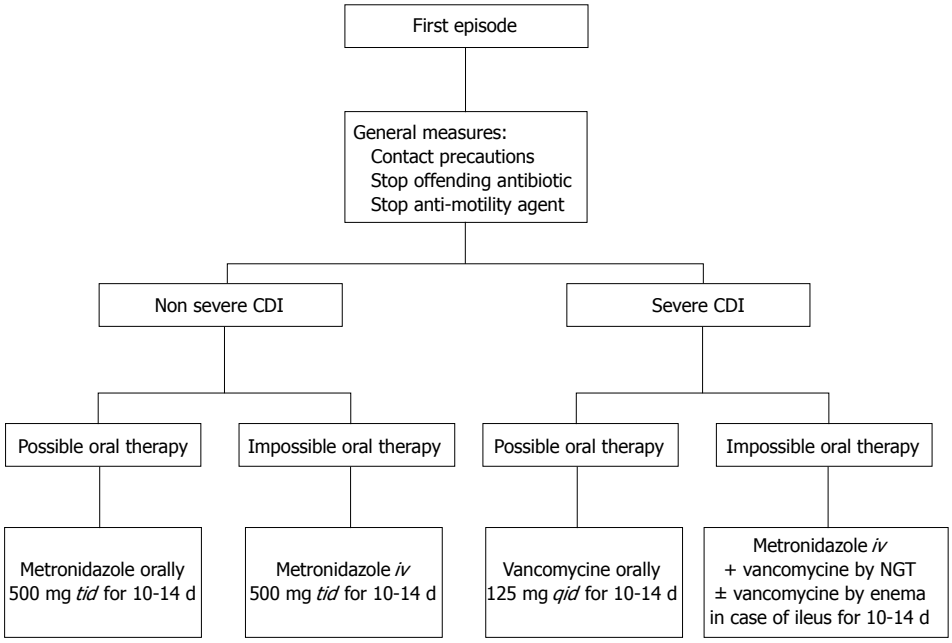

Figure 2 Algorithm for treatment of *Clostridium difficile* infections (adapted from[1,10,11]). *iv*: Intravenously; NGT: Nasogastric tube; CDI: *Clostridium difficile* infection.

clinical signs of severe colitis, an increase in serum creatinine of more than 50% of baseline, leukocytosis greater than 15 × 10^9/L, advanced age (⩾ 65 years old) and serious comorbidities[1,10].

Treatment response is present when either stool frequency decreases or stool consistency improves, parameters of disease severity improve and no new signs of severe disease develop; treatment response should be evaluated after at least three days[11]. After clinical response, it may take weeks for stool consistency and frequency to become entirely normal. After resolution of an episode, CDI recurs in about 25% of cases, regardless of the treatment provided (metronidazole or vancomycine) and its effectiveness[8]. Recurrence is defined as the return of symptoms within 8 wk after successful treatment[1]. Some factors are associated with a high risk of recurrence: patient's age (older than 65 years), further use of antibiotics, a low rate of anti-toxin A IgG, and a prior episode of CDI (the risk increases with the number of recurrences: 45% following the second episode and 65% after the third)[8].

Current treatment

The European Society of Clinical Microbiology and Infectious Diseases and the Infectious Disease Society of America have proposed recommendations for CDI treatment[1,10,11]. First, they offer some general measures such as stopping any offending antibiotic and anti-motility

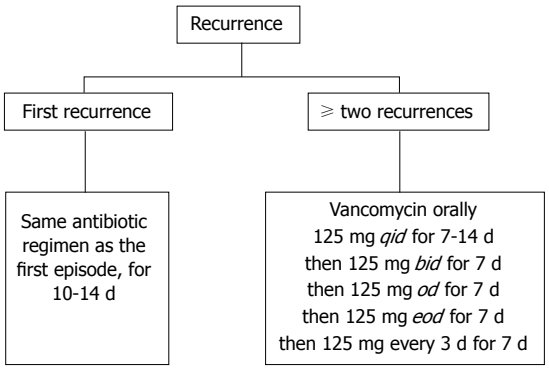

Figure 3 Algorithm for treatment of recurrent *Clostridium difficile* infections (adapted from[1,10,11]). eod: Every other day.

agents and establishing contact precautions[1,10]. For the treatment of a first non-severe episode, metronidazole is the first-line treatment. Indeed, randomized controlled trials have shown that metronidazole is as effective as vancomycin, and its use would prevent the appearance of vancomycin-resistant *Enterococcus*[12,13] (Figure 2). For the treatment of a first severe episode of CDI, vancomycin is used[14].

According to guidelines, the first recurrence should be treated with the same antibiotic as for the first episode[15] (Figure 3). For the second and any subsequent recurrences, metronidazole should not be used because of its potential neurotoxicity, so a taper regimen of vancomycin is employed for 35 to 42 d. This later recommendation is based primarily on the results of an observational study of 163 patients with recurrent CDI which compared metronidazole and different regimens of vancomycin. The highest success rate was achieved with a taper regimen of vancomycin (69% *vs* 30%-57% for other treatments)[16].

Recently, the FDA approved the use of fidaxomycin for the treatment of recurrent CDI. Fidaxomycin is a macrocyclic antibiotic characterized by little or no systemic absorption after oral administration and a narrow spectrum of activity against Gram-positive aerobic and anaerobic bacteria. This treatment is comparable to vancomycin in terms of resolution (88% *vs* 86%, respectively), but is associated with a lower risk of recurrence 4 wk after cessation of treatment (13%-15% *vs* 25%-27%)[17]. However, there is no prospective randomized controlled trial that investigated fidaxomicin's efficacy in patients with multiple recurrences of CDI; vancomycin is preferably administered using tapered regimen[11].

Concerning the use of probiotics, a meta-analysis concluded that probiotics composed of *Saccharomyces boulardii* or *Lactobacilli* could be used to prevent antibiotic-associated diarrhea[18]. A Cochrane systematic review concluded that even if the efficacy of using probiotics together with antibiotics seems to be superior for CDI treatment, there is not yet sufficient evidence to systematically recommend their use[19].

The importance of microbiota in CDI recurrence

The pathophysiologic features of recurrent CDI are not fully understood but likely involve two mechanisms: the resistance of *C. difficile* to metronidazole and vancomycin, and most importantly, the phenomenon of dysbiosis. The risk of recurrence is approximately 25% after a first CDI episode and dramatically increases with subsequent CDI recurrences[8]. Half of cases is considered as a relapse (*C. difficile* spores are not destroyed by antibiotics and can germinate to vegetative forms after antibiotic therapy), and the other half as a re-infection (infection by a new strain)[8].

Until recently, the lack of resistance of *C. difficile* to vancomycin and metronidazole seemed to be well demonstrated. However, recent studies have shown some resistance mechanisms of *C. difficile* thanks to new analytic methods able to stabilize and study *C. difficile* taken out of the gut[20].

Gut microbiota and its imbalance, called dysbiosis, has a crucial role in the pathophysiology of CDI recurrence. Over the last decade, an emerging consensus has formed about the importance of the intestinal microbiota, which has been considered similar to an organ. Gut microbiota is composed of more than 100 to 1000 microbial species that live in a host-microbe symbiotic relationship[21]. Among all gut bacterial phyla, *Bacteroides* and *Firmicutes* predominate[22]. The main functions of the microbiota can be classified as protective (commensal bacteria offer a resistance to colonization by enteric pathogens), metabolic (*e.g.*, some bacteria contribute to the catabolism of carbohydrates and to the synthesis of some vitamins), and immunologic (*e.g.*, some bacteria can activate regulatory T cells and induce a tolerance to innocuous antigens)[21,23,24].

Dysbiosis is associated with a number of diseases such as antibiotic-associated diarrhea, irritable bowel syndrome, inflammatory bowel diseases (IBDs)[25] and CDI recurrence.

Concerning CDI recurrence, antibiotics generate dysbiosis that is characterized by a reduced diversity of the microbiota, development of opportunistic species (*e.g.*, *Escherichia coli, Proteus mirabilis,* and *Enterococcus faecalis*), loss of resistance to colonization and increased synthesis of pro-inflammatory cytokines[26]. These disturbances promote colonization and infection with *C. difficile*, which further contributes to the dysbiosis (Figure 4)[27]. Standard antibiotic treatments and recurrent episodes contribute to the development of a vicious cycle.

Although there is an association, but no clear cause-effect relationship between dysbiosis and some gastrointestinal diseases, there is great interest in therapeutic approaches that could restore the equilibrium of the gut microbiota and improve these conditions. Probiotics are defined as "live micro-organisms which, when administered in adequate amounts as part of food, confer a health benefit on the host" (Joint FAO/WHO Expert consultation 2001)[28]. A meta-analysis concluded that the use of probiotics in combination with antibiotics in CDI treatment had no clear benefit in terms of recurrence risk compared to antibiotics alone[18]. However, according to preliminary results of an ongoing randomized placebo-

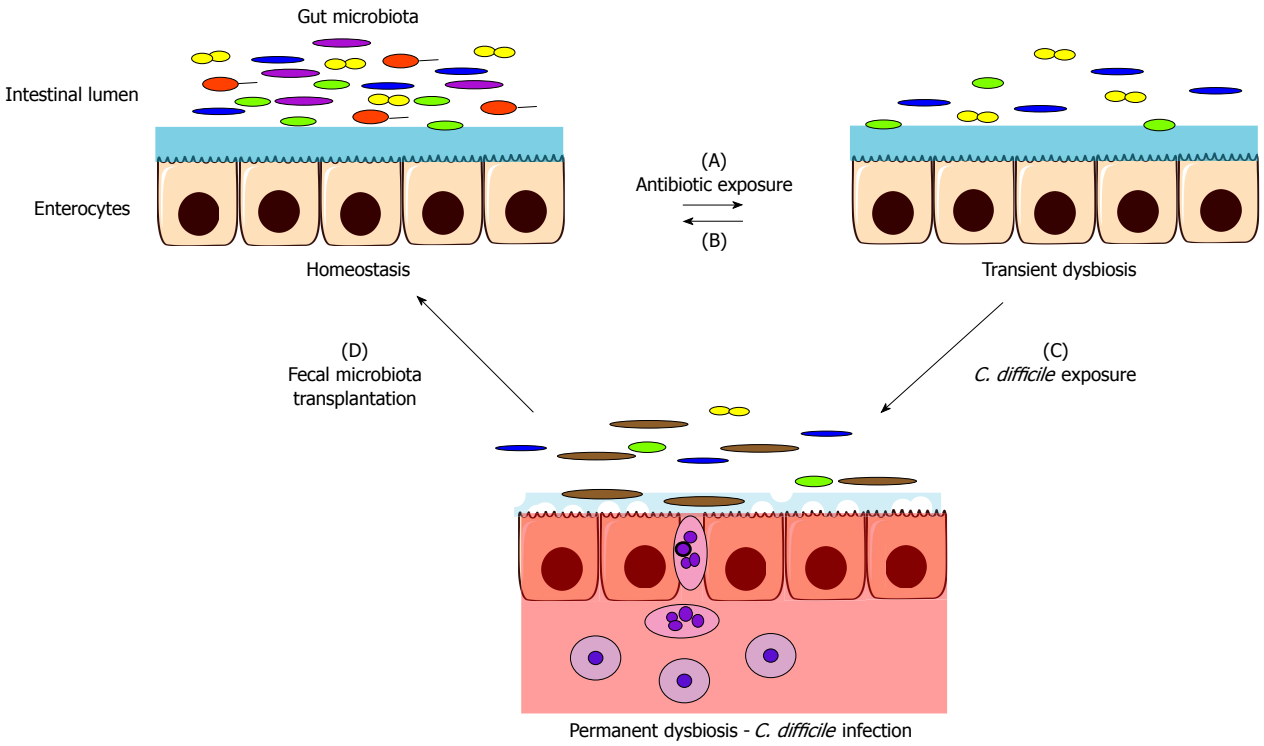

Figure 4 Perturbation of intestinal microbiota by antibiotics allowing *Clostridium difficile* infection and fecal microbiota transplantation effect (adapted from[27]). A: Antibiotic use destroys some sensitive bacteria and reduces the microbiota diversity and resistance to colonization by opportunistic pathogens; B: In the absence of opportunistic infection, microbiota usually recover its homeostasis; C: *Clostridium difficile* (*C. difficile*) infection can lead to persistent dysbiosis; D: Fecal microbiota transplantation restores microbiota diversity and colonization resistance and allows the elimination of *C. difficile*.

controlled trial, patients do appear to have less recurrent *C. difficile* diarrhea and early symptomatic improvement when using the probiotic *Lactobacillus* GG in combination with standard antiobiotics[29]. As for probiotics, the purpose of FMT is to resolve dysbiosis by restoring the phylogenetic diversity of intestinal flora and the resistance to colonization by *C. difficile*, thus allowing a return to normal colonic function (Figure 4). Unlike probiotics, which are only associated with a short-term change of the microbiota (10-14 d), FMT is able to significantly modify the recipient microbiota for at least 24 wk[30].

FECAL MICROBIOTA TRANSPLANTATION

Definition

FMT consists in the instillation of a suspension of stool from a healthy donor *via* the upper gastrointestinal route (usually nasoduodenal or nasojejunal tube) or lower gastrointestinal route (colonoscopy or retention enema).

Indications

Current indications of FMT for CDI treatment are[31]: (1) recurrent CDI: at least 3 episodes of mild to moderate CDI and failure of a 6- to 8-wk taper regimen of vancomycin, with or without an alternative antibiotic (*e.g.*, rifaximin, nitazoxanide); or at least 2 episodes of severe CDI resulting in hospitalization and significant morbidity; (2) moderate CDI not responding to standard therapy (vancomycin) for at least a week; and (3) severe (and perhaps even fulminant *C. difficile* colitis) with no response to standard therapy after 48 h.

Fecal microbiota transplantation procedure

To date, there is no standardized protocol for microbiota transplantation although the Fecal Microbiota Transplantation Workgroup published some recommendations in 2011[31].

Donors are screened for exclusion criteria such as antibiotic use during the last 3 mo, intestinal infection, inflammatory bowel disease, a history of neoplasia and presence of some infectious diseases (in particular, stool testing for *C. difficile*, *Salmonella* and *Shigella* and serologic testing for human immunodeficiency virus, hepatitis B virus, and hepatitis C virus)[31]. Donors are usually relatives or household members, as there is likely to be reduced risk of transmission of an infectious agent (since donors and recipients should share the same infectious risks). One systematic review showed that this strategy was associated with a higher resolution rate (93%) compared to the use of stools from an unrelated donor (84%)[32]. However, contrary to this report, a recent meta-analysis showed that there was no significant difference whether the donor was a relative or not[33].

Donor stools are collected within 6 h before transplantation; they are generally mixed with a saline solution and the supernatant is filtered. After having received a bowel lavage solution, the recipient receives *via* upper or lower gastrointestinal route 500 mL of the suspension (given in small amounts of 25-50 mL). Lower gastrointestinal delivery *via* colonoscopy or enema seems to be more ef-

Table 4 Characteristics of some recent studies concerning fecal microbiota transplantation in recurrent *Clostridium difficile* infection treatment

Ref.	Study type	Patients (*n*)	FMT delivery modality	Success rate	Follow-up
Garborg *et al*[35], 2010	Retrospective study	40	Gastroscope Colonoscope	73% after 1 instillation 83% after 2 instillations	10 wk
Burke *et al*[36], 2013	Review	115	Naso-enteric tube Gastroscope Colonoscope Retention enema	89.6%	2 mo to 5 yr
Gough *et al*[32], 2011	Review	317	Naso-enteric tube Gastroscope Colonoscope Retention enema	89% after 1 instillation 92% after ⩾ 2 instillations	3 d to 5 yr
Kassam *et al*[33], 2013	Meta-analysis	273	Naso-enteric tube Gastroscope Colonoscope Retention enema	89%	2 wk to 8 yr
Van Nood *et al*[38], 2013	Randomized controlled trial	43	Naso-duodenal tube	81.3% after 1 instillation 93.8% after 2 instillations	10 wk

FMT: Fecal microbiota transplantation.

fective[33]. The amount of stool has not been standardized. Transplantation of more than 50 g of stool seems to be associated with a higher resolution rate than transplantation of less than 50 g (86% and 82% respectively)[32]. Similarly, administration of more than 500 mL may also be associated with a higher resolution rate than administration of less than 200 mL (97% and 80% respectively)[32].

Current evidence about fecal microbiota transplantation

From 1958, when the use of FMT for treatment of pseudomembranous colitis was first described by Eiseman *et al*[34], until 2011, published studies on the effectiveness of FMT have largely consisted of case reports or reviews on case series[35]. These studies suggest that FMT is effective for treating relapsing CDI in adults and children[36,37].

A systematic review published in 2011 studied 317 patients treated with FMT for recurrent CDI between 1958 and 2011[32] (Table 4). This review showed that 85%-90% of patients treated with FMT did not develop recurrence during the follow-up period (which varied from 3 d to 5 years), again pointing to FMT as an effective treatment for recurrent CDI[32].

A meta-analysis published in 2013 confirmed the efficacy of FMT for recurrent CDI, showing resolution in 89% of cases, while a subgroup analysis showed a trend towards significant higher resolution rate when FMT was provided *via* lower gastrointestinal route[33] (Table 4). Another retrospective study confirmed a high resolution rate after a follow-up of 90 d[26].

In 2013, Van Nood *et al*[38] published the first multicentric, prospective, open-label, randomized controlled trial that included 43 patients with CDI recurrence (Table 4). The primary outcome was resolution without recurrence within 10 wk after treatment. FMT *via* naso-duodenal tube, in association with a shortened treatment of vancomycin (5 d), was significantly more effective than vancomycin alone for 14 d (resolution rate 81% *vs* 31%). Three patients experienced recurrence despite one infusion and a second transplantation allowed resolution (increasing resolution rate to 94%)[38]. The diversity of recipients' gut microbiota after FMT was significantly improved, with an increase of *Bacteroides* and some *Clostridium* species and a decrease of *Proteobacteria*[38]. Note that there is an association between the modification of gut microbiota composition, the resolution of dysbiosis and the resolution of recurrent CDI.

Adverse effects, safety issues

Concerning short term adverse effects, Van Nood *et al*[38] observed diarrhea (94% of patients), cramping (31% of patients) and belching (19% of patients) immediately after FMT. During the subsequent weeks of follow-up, 19% of patients reported constipation. A recent case report described a flare of ulcerative colitis after treatment of recurrent CDI with FMT[39].

There are still unanswered questions regarding the short and long term consequences of FMT. The few published studies describe microbiota modifications after a short follow-up period (10 wk in Van Nood *et al* study[38], 24 wk in Grehan *et al*[30]). So far, we still do not know whether FMT could pose a risk for the development of some diseases from the donor. Even if there is no clear cause-effect relationship but only associations between gut microbiota composition and some diseases (cardiovascular diseases, IBDs, diabetes, non alcoholic fatty liver disease, obesity for example), to date no study has assessed the risk of developing one of these conditions after FMT. While donors are primarily screened for infectious diseases or digestive neoplasia, we still do not know whether they should also be screened for other diseases (immunologic or cardiovascular diseases for example).

Despite encouraging results of FMT in recurrent CDI, further studies are needed to confirm its efficacy and also to define "good practices" for donor selection,

stool preparation, the method of administration, and the indications of this treatment. Thus, because FMT meets the legal definition of a drug and a biological product, the FDA is attempting to regulate the multiple steps of FMT[40].

Future challenge and future directions

Given its effectiveness, 97% of patients who received FMT would repeat the treatment[26]. A recent survey conducted among 192 healthy patients confirmed that in a hypothetical case of recurrent CDI, 81% would choose FMT over antibiotics alone when informed of the effectiveness of each treatment. This rate rose to almost 90% if the administration of feces was odorless or given as a pill[41].

Despite this high acceptance rate, the development of an optimal formulation and pharmaceutical form is a current challenge. There is growing interest in fecal extracts or multistrain preparations. Petrof *et al*[42] developed a stool substitute preparation, containing 33 bacterial isolates, made from purified intestinal bacterial culture from a healthy donor. With this preparation, they successfully treated recurrent CDI in 2 patients. A recent retrospective study showed that use of a multistrain mixture of probiotics in combination with antibiotics could allow complete resolution of CDI[43]. The best composition of stool substitutes or multistrain mixture of probiotics and their efficacy still needs to be confirmed.

CONCLUSION

The incidence of CDIs and their recurrences are increasing despite effective treatment. Recurrence risk is about 25% after the first CDI episode and more than 45% after the first relapse. Metronidazole and vancomycin are recommended for the treatment of a first episode, and their efficacy has been well demonstrated in non severe and severe cases respectively. The recommended treatment of the second and subsequent recurrences is a taper regimen of vancomycin. Considering the high recurrence rate of CDI and the associated morbidity and mortality, there is growing interest in developing new therapeutic approaches. The association between gut microbiota imbalance, dysbiosis, and CDI recurrence has motivated the use of FMT to restore the microbiota equilibrium and resolve recurrent CDI. According to studies published to date, resolution rates of recurrent CDI seem to be higher when using FMT associated with antibiotics than antibiotics alone. The effectiveness of this treatment is promising, but further studies are needed to confirm these results, to define "good practices" of FMT and to identify any long term effects.

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REVIEW

Capsule endoscopy in patients refusing conventional endoscopy

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Abstract

Capsule endoscopy is nowadays the diagnostic technique of choice in the study of small bowel pathologies, allowing the non-invasive study of the entire mucosa. This has led, together with new technical advances, to the creation of two new models (PillCam ESO and PillCam Colon) for the study of esophageal and colonic diseases. These two new capsules offer an interesting alternative to conventional endoscopy in the study of the upper and lower digestive tracts, because traditional endoscopy is often unpleasant and uncomfortable for the patient, can be painful, often requires moderate or deep sedation and is not without complications (hemorrhage, perforation, *etc*.). PillCam Colon is particularly important for its usefulness in the diagnosis of colonic polyps, and is a potentially useful tool in cases of incomplete colonoscopy or in colorectal cancer screening, even more when most patients are reluctant to undergo screening programs due to the said disadvantages of conventional colonoscopy. This article discusses the advantages of capsule endoscopy over conventional endoscopy, its current application possibilities and indications in routine clinical practice. In the various sections of the work, we assess the application of endoscopic capsule in different sections of the digestive tract (esophagus, stomach, and colon) and finally the potential role of panendoscopy with PillCam Colon.

Key words: Conventional endoscopy; Capsule endoscopy; Esophageal capsule endoscopy; Colon capsule endoscopy; Panendoscopy

Core tip: Upper gastrointestinal endoscopy and colonoscopy are the techniques of choice for the study of the pathologies of the upper and lower digestive tracts. Despite their many advantages, these techniques can be unpleasant and uncomfortable for the patient and may even require sedation, with the potential disadvantages that might imply. In this scenario, capsule endoscopy (PillCam ESO for the study of the esophagus and stomach and PillCam Colon mainly for the study of colonic diseases) is an alternative to conventional endoscopy, as it has demonstrated its usefulness, an adequate diagnostic yield and good tolerance by patients.

Romero-Vázquez J, Argüelles-Arias F, García-Montes JM, Caunedo-Álvarez Á, Pellicer-Bautista FJ, Herrerías-Gutiérrez JM. Capsule endoscopy in patients refusing conventional endoscopy. *World J Gastroenterol* 2014; 20(23): 7424-7433 Available from: URL: http://www.wjgnet.com/1007-9327/full/v20/i23/7424.htm DOI: http://dx.doi.org/10.3748/wjg.v20.i23.7424

INTRODUCTION

Upper gastrointestinal endoscopy (UGE) and colonoscopy represent the gold standard and the preferred endoscopic techniques for the study of diseases of the upper and lower digestive tracts. Their advantages and diagnostic and therapeutic yield have been clearly demon-

strated. However, although minimally invasive, they can be unpleasant/painful for the patient, require sedation and are not free of complications; these are why they are not indicated for some patients in many diagnostic procedures. This is particularly important in colorectal cancer screening, where the rate of adherence to such programs is low, given the reluctance of patients to have colonoscopy performed.

In 2000, capsule endoscopy (CE), a new non-invasive method that allowed the complete and direct study of the small bowel (SB), was born[1]. This technique has revolutionized the diagnosis and therapeutic algorithm of intestinal pathologies, so that, today, it is considered the technique of choice to study the diseases of the small bowel[2-8].

Its development contributed later to the birth of other devices. Since October 2004 there has been a new capsule available, the esophageal capsule endoscope (PillCam ESO), capable of studying esophageal diseases in detail.

Years later, as the study of colonic diseases through colonoscopy can be unpleasant/painful for the patient, incomplete in 5%-20% of cases and not free of potential complications in up to 2% of procedures (due to perforations, hemorrhage, infections, vasovagal responses, *etc*.), a new device, the colonic capsule, was created in order to allow the study of colonic diseases by capsule endoscopy.

The different models of capsule endoscopy mentioned above and currently available on the market (SB capsule, esophageal capsule and colon capsule) are unique in being able to explore different areas of the gastrointestinal tract; they are direct, noninvasive, painless for patients and without need for sedation; they offer also a high diagnostic yield and high reliability, which make them a diagnostic method chosen by many patients refusing conventional endoscopy.

In this article we discuss whether the different models of capsule endoscopy can be an alternative to conventional endoscopy in patients who refuse the latter.

ESOPHAGEAL CAPSULE ENDOSCOPY

Initial studies of the esophagus performed with the SB capsule had a low diagnostic yield[9], with the exception of the study carried out by Ramirez *et al*[10] with a string-capsule using the PillCam SB, that achieved a high diagnostic yield (close to 100%), although it was not validated by subsequent studies.

For this reason, a new capsule, esophageal capsule endoscope (ECE), was specifically created to study this section of the digestive tract; it was named PillCam ESO, by Given Imaging, a company based in Yokneam, Israel; the dimensions of the capsule are 26 mm × 11 mm, with two lenses and a higher capacity to capture images. Due to the usefulness of the capsule, it was soon recommended to study chronic gastroesophageal reflux disease (GERD) [mainly for diagnosis purposes and for the management of Barrett's esophagus (BE)] as well as for the screening of esophageal varices in portal hypertension (first using PillCam ESO1, that captured 2 images per second at each end, and later with the use of PillCam ESO2, capturing seven images per second).

Sanchez-Yague *et al*[11] showed that esophageal capsule could be useful in patients with suspected esophageal diseases who refused conventional endoscopy.

Most published series comparing PillCam ESO to UGE establish a high specificity and negative predictive value of the capsule for the screening of BE. However, the sensitivity was remarkably low, with high interobserver variability and low yield in short-segment BE, making it impossible to recommend this technique for these patients[12,13].

A subsequent meta-analysis of more than 600 patients with GERD concluded that ECE had a moderate sensitivity and specificity for the diagnosis of BE and that UGE should remain the gold standard technique in patients with BE[14].

Later, Chavalitdhamrong *et al*[15] showed, in a study including more than 500 patients, that esophageal capsule obtained high quality images from patients with symptoms of GERD in a non-invasive way, demonstrating that it could be an alternative screening test for the diagnosis of BE.

The main disadvantages of esophageal capsule in this subgroup of patients are the following: (1) difficulty to fully visualize the Z-line, partially improved with the patient in right lateral decubitus position; (2) inability to use local staining techniques, unlike those used with UGE; and (3) inability to take biopsies and therefore to know the degree of dysplasia associated with intestinal metaplasia.

In short, although patients mostly prefer the PillCam ESO to conventional endoscopy, larger studies are needed with larger number of patients (probably only possible through multicenter studies) to assess the actual role of ECE in patients with BE. Until then, UGE, preferably with magnification techniques and histological examination of the biopsies obtained, should be considered the gold standard technique for these patients[16].

With regard to the diagnostic yield for the screening of esophageal varices in patients with portal hypertension, the initial studies and meta-analysis of esophageal capsule proved its usefulness in the assessment of esophageal varices, in the detection of varices with significant size suggestive of primary prevention of variceal bleeding and in the diagnosis of portal hypertensive gastropathy[17-20].

Subsequently, Ishiguro *et al*[21] evaluated the role of esophageal capsule in the detection of varices, red spots and high risk varices in Japanese cirrhotic patients; the results showed that the capsule had a higher diagnostic yield than conventional endoscopy, indicating that the capsule is a useful technique for the screening and management of this population.

Table 1 shows the main studies published on the role of PillCam ESO in the screening/management of esophageal varices in cirrhotic patients, its sensitivity, specificity

Table 1 Main studies published on PillCam Colon in esophageal varices

Ref.	*n*	S	E	Accuracy of treatment[1]
Eisen *et al*[17], 2006	32	100%	89%	NA
de Franchis *et al*[18], 2008	288	88%	84%	91%
Lapalus *et al*[19], 2009	120	77%	86%	92%
Ishiguro *et al*[21], 2012	29	95%	84%	85%

[1]Accuracy for indicated prophylactic treatment.

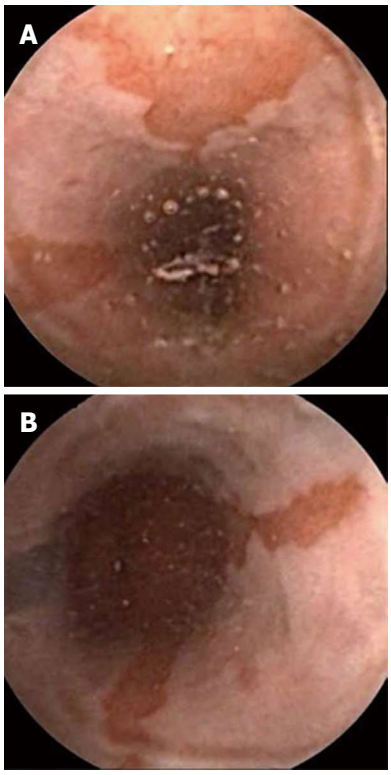

Figure 1 Suspected Barrett's esophagus seen with PillCam ESO. A: Suspected long Barrett's esophagus; B: Ectopic tissue mucosa ascending from Z line.

and safety in predicting patients requiring prophylactic treatment. Some representative images of our experience with PillCam ESO are shown in Figures 1 and 2.

As a result of the publishing of the previously mentioned studies, nowadays, the only clearly accepted indication for esophageal capsule (until the appearance of new technological advances) is the screening and management of esophageal varices, given its role in patients with GERD being more controversial.

GASTRIC AND ESOPHAGEAL CAPSULE ENDOSCOPY

Regarding the gastric cavity, nowadays, there is no capsule specifically designed to study gastric diseases. While using a capsule at this level could help to obtain many images and to diagnose existing pathologies, the morphology of the gastric cavity as well as its high volume makes it impossible to be sure that all areas of the stomach would be visualized, especially the gastric fundus, and we could

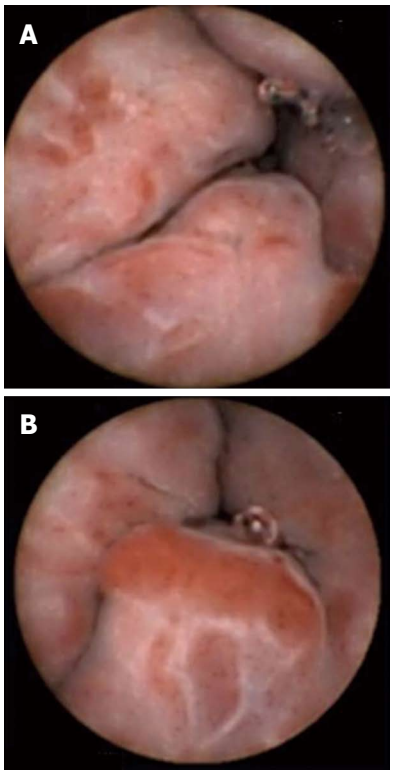

Figure 2 Esophageal varices seen with PillCam ESO. A: Large esophageal varices with red spots; B: Large esophageal varices in distal esophagus.

be leaving relevant pathologies undiagnosed. Thus, the exploration of the entire stomach using an EC is impossible with the currently available models.

However, if we were able to remotely control the endoscopic capsules available, we would be able to visualize the entire gastric mucosa. This control would allow us to take more pictures, from more angles and at different distances, improving the diagnostic yield of the EC in the stomach[22].

Initial experimental studies performed in animals have shown that the remote control of EC is a possibility that can be developed first in healthy volunteers and then in patients[23,24].

Thus, Swain *et al*[25] evaluated the effectiveness of remotely controlling a PillCam Colon modified with the inclusion of magnetic materials, in the esophagus and stomach of a healthy volunteer, by means of an external magnetic field repeatedly moved and rotated in different directions, observing that a larger number of images could be obtained. The authors demonstrated that an EC could be externally controlled with a magnetic field in a simple, safe and effective way, controlling its movements by viewing through a flexible gastroscope. There were no complications and the procedure was not painful or unpleasant for the patient.

Later, Rey *et al*[26] evaluated the diagnostic accuracy of a new magnetically driven EC (Olympus-Siemens) in healthy volunteers and patients with epigastric pain and/or symptoms of reflux. Low level magnetic fields were used to control a dual sensor capsule in the stomach of the patients included in the study with an air-water

interface provided by the ingestion of 1300 cc of water one hour before the test time. In analyzing the results, they found that the gastric mucosa could be technically visualized in all cases except one (98%). In the remaining 52 patients, the antrum, body, fundus and cardia could be completely visualized in 98%, 96%, 73% and 75% of cases, respectively. There were no complications.

Similarly, Keller *et al*[27] studied the gastric mucosa by means of a PillCam Colon modified by the inclusion of magnetic material to allow for remote control in 10 healthy volunteers. The entire gastric mucosa could be observed in 75% of cases. As limitations to this experimental technique, it could be noticed that small amounts of fluid limited the visibility of small areas in the most apical parts of the fundus and gastric distension produced was not enough to evaluate all folds. Therefore, the visualization of the gastric mucosa in the patients included in the said study was good, although not complete in all of them.

Undoubtedly, these studies open the door to new technological developments that will likely succeed in the future to explore the whole of the gastric mucosa in an easy, safe and effective way[28].

Thus, new especially designed capsules could be created to study the esophagus, stomach and the first portions of the duodenum, having in this case a "new PillCam ESO" modified for remote operation.

Recently, two studies have proposed the use of the esophageal capsule for two pathologies previously considered to be "alien" in publications related with the PillCam ESO. Chandran *et al*[29] assessed its role in stratifying the risk of upper gastrointestinal bleeding, noticing that its major limitation was the low rate of duodenal visualization and the discord between the capsule and conventional endoscopy. However, when the PillCam ESO made proper assessments of the duodenum, the concordance between both tests was excellent. Moreover, Shah *et al*[30] concluded after studying a small group of patients who were to undergo bariatric surgery that there were no significant differences between the findings made by PillCam ESO and those observed by conventional endoscopy.

COLON CAPSULE ENDOSCOPY

PillCam COLON capsule endoscopy (CCE) (Given Imaging Ltd, Yoqneam, Israel) is a new capsule that has been designed to explore the colon. Two models have been developed: the first generation of CCE (CC1) is similar to the conventional capsule but has two cameras which are able to record video images from both ends. The device measures 31 by 11 mm and acquires images at a rate of 4 frames per second. The pre-programmed "sleep" mode allows recording of images from the esophagus and the stomach for 3 min and after the capsule switches to sleep mode for 1 h 45 min, so that it saves battery. During this period, the capsule is likely to transit most of the small bowel and reaches approximately the level of terminal ileum. Recording and downloading of data are similar to those for small-bowel capsule endoscopy[31].

Recently, a second-generation colon capsule (CC2) has been developed to improve the sensitivity for detection of colonic changes. The new PCC-2 is bigger (11.6 mm × 31.5 mm) and two new characteristics have been introduced: (1) the view angle from both the imagers has been widened to 172 degrees; and (2) in order to further enhance the colon coverage, the capsule is equipped with an adaptable image acquisition rate depending on the speed of progression of the capsule along the colon; CC-2 captures 35 frames per second while it is moving and 4 frames per second when it is stationary. Also, there is a new data recorder that guides the medical staff and the patient through the procedure. In fact, it buzzes and vibrates and displays instructions to alert the patient to continue the preparation according to the protocol previously explained to the patient. The new RAPID software develops a flexible spectral imaging color enhancement (FICE) technology to allow a more detailed analysis of the mucosal surface and also has a polyp size estimation tool.

Colorectal cancer (CRC) is the second most frequent cause of cancer-related death in Western countries. Nevertheless, no more than 25% of compliance has been achieved in screening programs, because of different problems but, without any doubt, because of people´s resistance to conventional colonoscopy[32]. The high-priority objective and indications for CCE are the "screening" of colorectal cancer in the risk population. In addition, it appears to be a promising new modality for colonic evaluation, not only adenomas, and it could be a good alternative in patients refusing conventional colonoscopy, to complete colon examination in patients with no conclusive incomplete colonoscopy, or when it is contraindicated[33].

In order to assess the sensitivity and specificity of CCE1 compared to colonoscopy in screening colorectal cancer, some studies were performed[34-36]. The most important study published about CCE is a prospective, multicenter study comparing capsule endoscopy with colonoscopy in the detection of colorectal polyps or cancer in a group of patients with known or suspected colonic diseases[34]. A total of 328 patients were included in the study. Sensitivity and specificity of CCE to detect polyps of 6 mm in size or larger were 64% and 84%, respectively. It is important to comment that of 19 cancers detected by colonoscopy, 14 were detected by capsule endoscopy. These results are similar to those of the two European studies published[35,36]. In these studies, the sensitivity was 69% and 76%, specificity was 81% and 64%, the positive predictive value (PPV) was 74% and 83% and the negative predictive value (NPV) was 78% and 54% for polyp detection. We recently published our results that are similar to the above mentioned, although specificity is lower[37]. Compared to colonoscopy, the rate of agreement was 75.6%, the sensitivity was 84%, the specificity was 62.5%, PPV was 77.7% and NPV was 71.4%. Two meta-

Table 2 Results of main colon trials using PillCam Colon 1

PillCam Colon C1	Year	*n*	S	E	PPV	NPV
Results for polyps (any size)						
Eliakim *et al*[35]	2006	91	69%	81%	74%	78%
Schoofs *et al*[36]	2006	41	76%	64%	83%	54%
Van Gossum *et al*[34]	2009	328	64%	84%	60%	86%
Results for significant polyps (> 6 mm or > 3 polyps > 3 mm)						
Eliakim *et al*[35]	2006	91	63%	94%	67%	91%
Schoofs *et al*[36]	2006	41	60%	73%	46%	83%
Van Gossum *et al*[34]	2009	328	64	84		

PPV: Positive predictive value; NPV: Negative predictive value.

Table 3 Results of main colon trials using PillCam Colon 2

PillCam Colon C2	Year	*n*	S	E
Results for significant polyps (> 6 mm or > 3 polyps > 3 mm)				
Eliakim *et al*[40]	2009	98	89%	84%
Spada *et al*[41]	2011	109	76%	64%
Results for significant polyps (> 10 mm)				
Eliakim *et al*[40]	2009	98	88%	89%
Spada *et al*[41]	2011	109	88%	95%

analyses have been recently published and confirm that CCE is a reasonable method for screening asymptomatic individuals for colorectal polyps. It may be particularly useful for patients with "incomplete" colonoscopy, those with contraindications for conventional colonoscopy, and those unwilling to undergo colonoscopy because of its perceived inconvenience and discomfort[38,39].

To date, two studies have evaluated CCE-2. An Israeli multicenter trial was the first one[40]. In this study, CCE-2 was prospectively compared with conventional colonoscopy as the gold standard. Colonoscopy was independently performed after capsule ingestion. A total of 98 patients were enrolled. Patients were considered to have a significant finding when polyps at least 6 mm in size or masses were detected. Sensitivity for polyps at least 6 mm in size was 89%, and at least 10 mm in size was 88%, with specificities of 76% and 89%, respectively. Recently a European, prospective, multicenter trial including eight European sites was published by Spada *et al*[41]. A total of 109 patients were enrolled. Sensitivity for polyps at least 6 mm in size was 84%, and at least 10 mm in size was 88%, with specificities of 64% and 95%, respectively. CCE-2 correctly classified 35 and 28 of these patients, corresponding to a detection rate of 90% for neoplasia at least 6 mm in size, and 93% for adenomas at least 10 mm in size. Similarly to the Eliakim study, in this study the low specificity for polyps at least 6 mm in size was explained by a substantial rate of false-positive polyps because of size mismatch. It must be considered important that the CCE-2 is able to detect more small lesions than colonoscopy.

Tables 2 and 3 summarize the main studies on PillCam Colon 1 and PillCam Colon 2 in the detection of colonic polyps, respectively.

Although the main objective of CCE must be colon cancer screening, CE should be considered a new technique able to detect colonic lesions in patients with special indications for colorectal cancer screening such as ulcerative colitis (UC) or patients who refuse conventional colonoscopy or with incomplete colonoscopy. CCE after incomplete colonoscopy appears to yield significant findings, guide further workup, and have high patient acceptance[42].

UC is a chronic inflammatory condition that causes continuous mucosal inflammation of the colon. Visualization of the mucosa affected in UC is essential for many aspects of disease management (including drug dosing and duration and the decision to deliver intravenous medication or undergo surgery) and can predict recurrence rates and complications related to UC according to the degree of mucosal healing[43]. Also, patients with UC have an elevated risk of developing colon cancer, which can be attributed to the length of time since disease onset and the extent of colon affected by UC[44]. As such, it is recommended in patients who have had UC for 8-10 years (or 15 years of disease in patients with left-sided colitis) that annual or biannual surveillance colonoscopy should be conducted[45,46]. Consequently, CCE could play a role in patients with UC. To date, a few preliminary studies have demonstrated the feasibility of CCE for the management of patients with known UC, suggesting that CCE may be useful to monitor inflammation and to screen for colorectal cancer in patients with UC[47,48]. Nevertheless, Meister *et al*[49] showed a significantly better assessment of disease activity by standard colonoscopy than by CCE. Furthermore, compared with colonoscopy, the extent of UC was underestimated when evaluated by CCE. In contract, Ye *et al*[48] reported a good correlation in the severity (k = 0.751) between CCE and colonoscopy but a moderate correlation in the extent of the inflammation, perhaps because it is not easy to determine the precise location of disease by CCE and the experience of the physician with CCE evaluation in this study was limited. We performed a pilot study using CCE-2 and observed that there was a good correlation between CCE and colonoscopy in assessing the disease severity and extent of inflammation[50].

Some representative images of our experience with PillCam colon are shown in Figures 3, 4, 5 and 6.

On the other hand, CCE preparation must be exhaustive because all the faecal remains cannot be removed by CCE opposite to colonoscopy. Even small amounts of residual stool may prevent an accurate visualization of the colonic mucosa by CCE. The colon preparation for CCE has two aims: to provide a clean colon and clear images and to promote capsule propulsion first through the entire small bowel and then through the colon to the rectum. Preparation is really important because as Spada *et al*[51] have demonstrated when an adequate preparation is obtained, accuracy of CCE tends to be higher and comparable with that of colonoscopy.

To address the above issue, a specific preparation for CCE was designed using polyethylene glycol (PEG) and

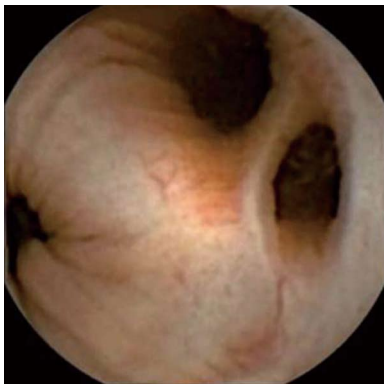

Figure 3 ***Diverticulosis coli.***

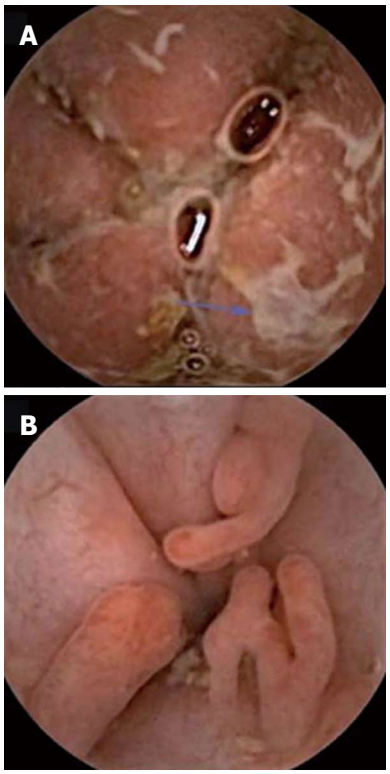

Figure 4 Severe (A) and pseudopolyps in inactive (B) ulcerative colitis.

two boosters of sodium phosphate (NaP). The main role of the NaP booster is to accelerate CCE transit through the small and the large bowel so that the colonic mucosa could be seen before the end of the battery. This conventional preparation was first evaluated in two initial pilot studies. In a study by Eliakim *et al*[35], the overall cleanliness of the colon was rated as excellent or good in 84.4% of the cases. In the second pilot study the results are better; an excellent or good preparation was achieved in 90% of the cases[36]. In a recent study published by our group with the same preparation the grade of cleanliness was good or excellent in 65.6%[37].

In recent times, for CCE-2, some changes of the regimen of CCE have been proposed. Low doses of NaP are now included in the regimen of preparation for CCE-2 to reduce the risk of adverse events (one booster of 30 mL

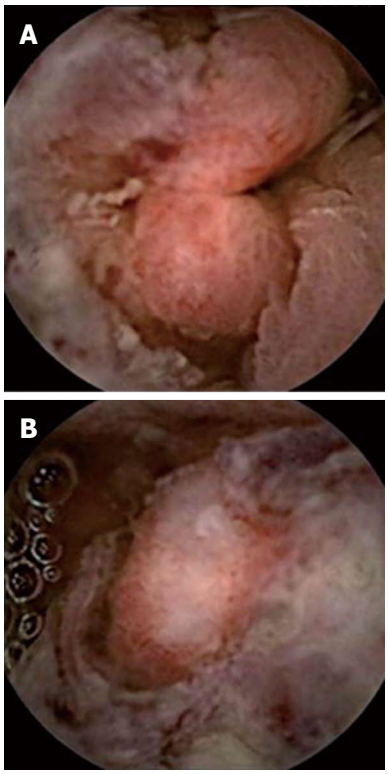

Figure 5 Colorectal cancer. A: Ulcerated sigmoid neoformation; B: Partially stenosing sigmoid neoformation.

NaP with 1 liter of water when the capsule has entered the small bowel, and a second booster of 15-25 mL NaP with 0.5 liter of water 3 h later if the capsule has not been excreted) or instead of this booster PEG booster has been proposed. Also the volume of PEG has been reduced[52].

PEG solutions are safe and effective, but require consumption of large volumes of fluid, generally 4 liters. The 2 L PEG solution plus ascorbic acid (PEG + Asc) is also effective and safe, and the volume is reduced. Some studies have studied these points. In a study by Ell *et al*[53], it is concluded that the PEG + Asc bowel preparation reduces the volume patients have to drink, so it was more acceptable to patients, and should, therefore, improve effectiveness in routine practice. In another study PEG + Asc provided effective bowel cleansing, which was equivalent to that of sodium picosulphate + magnesium citrate in terms of grading cleansing as overall success or failure[54]. Nevertheless, it is important to consider the split dose. In this sense the cleansing results are worse if patients receive the full dose PEG + Asc the evening before the procedure compared to the split dose[55]. A colon cleansing procedure using PEG + ascorbic acid for CCE yielded an adequate cleansing level in > 80% of patients, and good accuracy for detecting polyps[56]. This procedure may be considered as an alternative, particularly for patients in whom sodium phosphate-based preparations are contraindicated. Based on these data, we have performed a study that demonstrated the efficacy of 2 L PEG[57]. The main aim was to compare the level of cleansing with two different regimens. It was a prospective and blinded

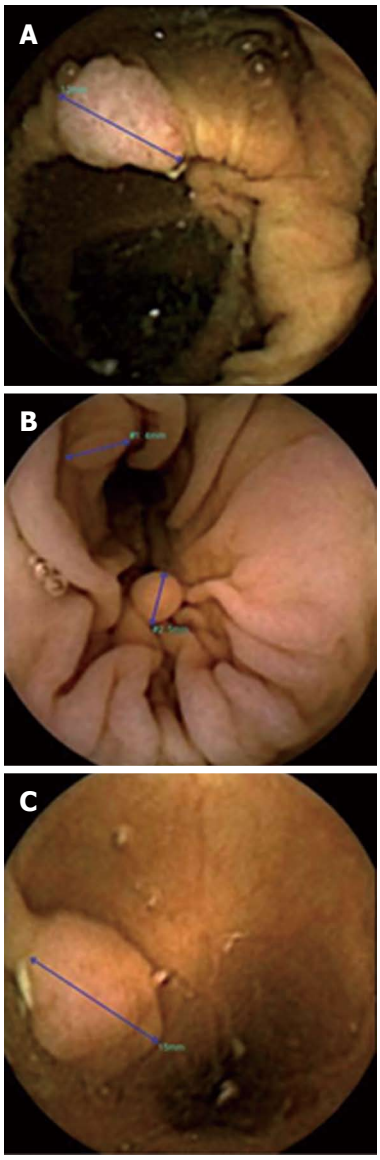

Figure 6 Polyps. PillCam Colon-2 using polyp size estimation tool. A: Senile ascending colon polyp; B: Millimetric descending colon polyps; C: Semi-pediculated polyp greater than 1cm in the sigmoid.

study. In the first group (A) patients were prepared with 2 L of PEG plus ascorbic acid and in the second group (B) 4 L of PEG was used. In group A, "excellent and good" preparation was more frequent than in group B, and also in the cecum, right colon and transverse colon, although there was no significant difference. We can conclude with these preliminary results that PEG 2 L could be better than PEG 4 L in the colonic preparation for patients that will undergo capsule colonoscopy, although more studies must be conducted with more patients.

Table 4 shows the main bowel preparation regimens used nowadays.

In conclusion, although many studies must be done to develop and improve the sensitivity and specificity of CCE, it is a new endoscopic tool that can be used for screening colon cancer in patients who refuse conventional colonoscopy, or in cases where it is contraindicated or it has been incomplete.

Table 4 Bowel preparation schedule

Schedule		Intake
Day-2		Sennosides 80-160 mg
Day-1	All day	Clear liquid diet
	Evening	2 L PEG or 1 L PEG + ascorbic acid
Exam Day	Morning	2 L PEG or 1 L PEG + ascorbic acid
	Approximately 10 am	Capsule Ingestion
	1st Boost Small bowel detection	30 mL NaP and 1 L water or 0.5 L PEG
	2nd boost 3 h after 1st boost	15 mL NaP and 0.5 L water or 0.5 L PEG
	Suppository 2 h after 2nd boost	10 mg bisacodyl

PANENDOSCOPY

As shown above, various studies published on PillCam Colon have demonstrated its usefulness in the detection of colonic polyps, with better sensitivity and specificity levels, as well as better positive and negative predictive values directly related to the increase in the learning curve and with the latest colonic capsule prototype, the PillCam Colon 2.

Moreover, by analyzing the different colonic studies we have been able to realize that thanks to the PillCam Colon ability to produce images of other extracolonic locations, it was able to identify lesions and pathologies at these locations.

By panendoscopy we mean the ability to obtain images of the digestive tract from the esophagus to the hemorrhoidal plexus without interruptions and, therefore, to explore the entire gastrointestinal tract wirelessly and noninvasively.

Our group has conducted a preliminary descriptive study (not yet published) in which, in view of the results obtained and acting cautiously given the absence of previously published data, PillCam Colon allows recording the entire digestive tract in most patients, making it possible to find relevant pathologies in other sections of the digestive tract, especially in the small bowel, although technical and procedural improvements are necessary to achieve the correct visualization of the stomach and esophagus (Figure 7).

Thus, in patients with indications for colonoscopy for suspected colonic disease who refuse to undergo this technique, the PillCam Colon helps explore not only this intestinal segment but also the whole digestive tract through panendoscopy.

CONCLUSION

In patients refusing conventional endoscopy, capsule endoscopy allows the direct and non-invasive study of the upper and lower digestive tracts. Unlike colonic segments,

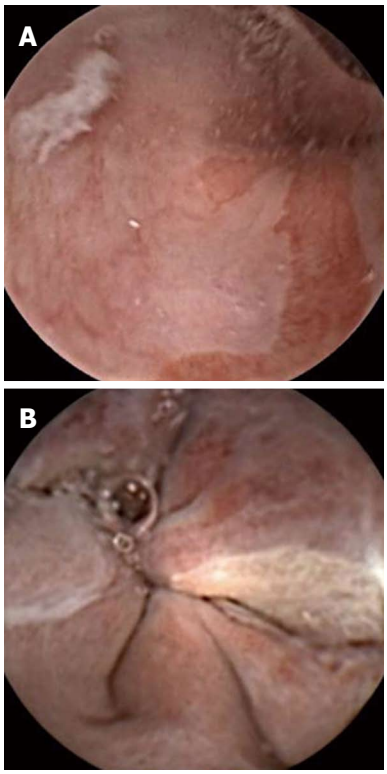

Figure 7 Suspected Barrett's esophagus (A) and esophageal varices seen (B) with PillCam Colon.

where PillCam Colon allows visualizing the mucosa with a high diagnostic yield, in esophageal and gastric sections (with the exception of the screening of esophageal varices) a larger number of studies and improvements in the procedures with capsule endoscopy are needed in order to achieve comparable efficacy to conventional UGE.

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REVIEW

Hepatitis B transmission by cell and tissue allografts: How safe is safe enough?

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Abstract

More than 2 million human tissue transplants (bone, tendon, cartilage, skin, cornea, amniotic membrane, stem cells, heart valve, blood vessel, *etc.*), are performed worldwide every year. Cells and tissues are shared between countries which have different regulations and laboratory equipment and represent a risk of hepatitis B virus (HBV) transmission that has become a global safety concern. While the risk of transfusion-transmitted HBV infection from blood donations has been estimated, the rate of HBV transmission from donors to recipients of allografts is unknown and varies between different tissues. There are various important ways of reducing the transmission risk, but donor screening and donor testing are still the main factors for preventing HBV transmission. HBV detection is included in the routine screening tests for cell and tissue donors. The standard test for preventing transplant-transmitted hepatitis B is the hepatitis B surface antigen. The implementation of methods involving nucleic acid amplification and the new generation of reactives to detect viral antibodies or antigens with an immunoassay, has increased the sensitivity and the specificity of the screening tests. The objective of our research was to review the literature and critically analyse the different steps for avoiding HBV transmission in cell and tissue donors, focusing on the screening tests performed.

Key words: Hepatitis B virus; Tissue bank; Tissue transplantation

Core tip: Human tissue transplantation is a current practice that still represents a risk for hepatitis B virus transmission (HBV). HBV detection is included in the routine screening tests for cell and tissue donors. The implementation of methods involving nucleic acid amplification has increased the sensitivity and specificity of the screening tests. The aim of this review is to update the knowledge of the risk of hepatitis transmission through tissue transplantation and critically analyze current screening tests.

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INTRODUCTION

More than 2 million human tissue transplants (bone, tendon, cartilage, skin, cornea, amniotic membrane, stem cells, heart valve, blood vessel, *etc.*), are performed worldwide every year. Cells and tissues are shared between countries with different regulations and laboratory equipment, and represent a risk for hepatitis B virus (HBV) transmission that has become a global safety concern. While the risk of transfusion-transmitted HBV infection per blood donations has been estimated[1], the rate of HBV transmission from donors to recipients of allografts is unknown and varies among different tissues.

Infectious disease transmission occurs in less than 1% of solid organ recipients and is believed to be at a lower rate for tissue and cell recipients[2,3]. In fact, the level of safety in tissue transplantation has been significantly increased and disease transmission can be considered a rare event when comparing reports of infection and the number of allografts transplanted per year. This outcome has been achieved as a result of the experience gained over the last 50 years in this field.

The presence of hepatitis B surface antigen (HBsAg) and HBV DNA has been described in corneas from HBsAg-positive donors. Nevertheless, controversial results have been reported on their potential for disease transmission[4-7]. Morris *et al*[8] showed in 1990 their experience using aortic valve allografts from HBsAg-positive donors. The reason for accepting these donors was the scarcity of heart valve donors and the relatively high prevalence of HBV infection in their own country. In the case of HBsAg-positive and anti-HBe-negative donors, they used prophylactic administration of hepatitis B immunoglobulin and/or hepatitis B vaccine. In their series, only 1 of 9 recipients seroconverted to HBV (positive for anti-HBc, anti-HBs and anti-HBe, but negative for HBsAg).

In a recent paper, Hinsekamp *et al*[9] have reviewed the adverse reactions and events related to musculoskeletal allografts which is the most demanded tissue. They analyzed medical literature, reports from professional organizations and tissue banks. Wang *et al*[10] used FDA's MedWatch reporting system to review reports on adverse events attributed to allografts of several kinds of tissue, during 2001-2004. No cases of hepatitis B transmission were reported. The review of the literature by Pruss *et al*[11] considered this risk as very rare. Nevertheless, Mallick *et al*[12], reported 9 cases of hepatitis B transmission from tissue allografts. This last article showed the importance of the implementation of Current Good Tissue Practice rules in the task of reporting infections.

In the last 20 years, stocks of human tissues in tissue banks have been significantly increased and refined strategies for the assessment of HBV transmission risk have been developed. A balance between safety and availability must be achieved but tissue availability must never jeopardizes biosafety. The adoption of algorithms for decision making must be based on medical evidence, avoiding the unjustified loss of products in tissue banks[13,14].

CRITICAL POINTS TO REDUCE HBV TRANSMISSION RISK BY CELL AND TISSUE TRANSPLANTATION

What are the most important areas in which particular attention has been given in order to reduce transmission risk? Figure 1 shows several potential sources of transmission: donor, tissue allograft, surgical team (during collection or transplant), processing team and other tissues (during processing or storage). However, donor screening and donor testing remain as the main issues for preventing HBV transmission.

Donor screening

This is the first factor for preventing hepatitis B transmission. It includes different sources of information to assess donor suitability: medical history, physical examination, social behaviour, and other available medical records considered as relevant risk factors[15]. HBV infectious risk is inferred from the donor's medical and social history and based on guidelines for preventing HIV transmission through tissue and organ transplantation[16].

Generally, for living donors when the femoral head (as a source of cancellous bone for use in impaction grafting procedures) is obtained from elderly patients undergoing hip surgery, the donor selection is assumed by health personnel in the orthopedic surgery department (nurses or surgeons who obtain data directly from the donor themselves), who are sometimes untrained in this task or with limited time available.

For deceased donors, this function is performed by the transplant procurement manager, who is a professional trained in the field of donor selection and responsible for the coordination of donation-transplantation activities, which implies health care professionals and services from a variety of specialties. Collecting data on medical and behavioural history is an essential step in analyzing risk factors, but, sometimes, in asymptomatic patients, hepatitis is difficult to detect.

Donor testing: General considerations

HBV transmission is due to the collection of cell and tissues from window period donors. However, in recent years, the HBV transmission risk has been significantly reduced with a greater accuracy in the performance of serological assays for infectious disease testing and the inclusion of DNA-based techniques. Human error has, therefore, become a major cause in cases of disease transmission and efforts addressed to avoid it must be increased.

The final objective of donor testing is the detection of potentially transmissible disease. This task will become ineffective if other relatively simple issues are not considered. Some additional important considerations related to the characteristics of the donor's blood sample include: (1) Quantity: insufficient sample volume to complete testing; and (2) Quality: hemolysed or samples that are too diluted, which may influence the reliability of the results, especially in phases when the viral load is low (*e.g.*, due to the infusion of blood, colloid and crystalloid solutions in patients with massive blood loss who finally become cadaveric tissue donors) and before the antibodies are detectable (window period)[11,14,17,18]. In the case of deceased donors, if the pre-mortem specimens are not available and the only alternative is to use post-mortem blood, an increase in false serological results can be expected (especially for false positive cases)[19]. Not all available systems for serological testing are validated for use with samples

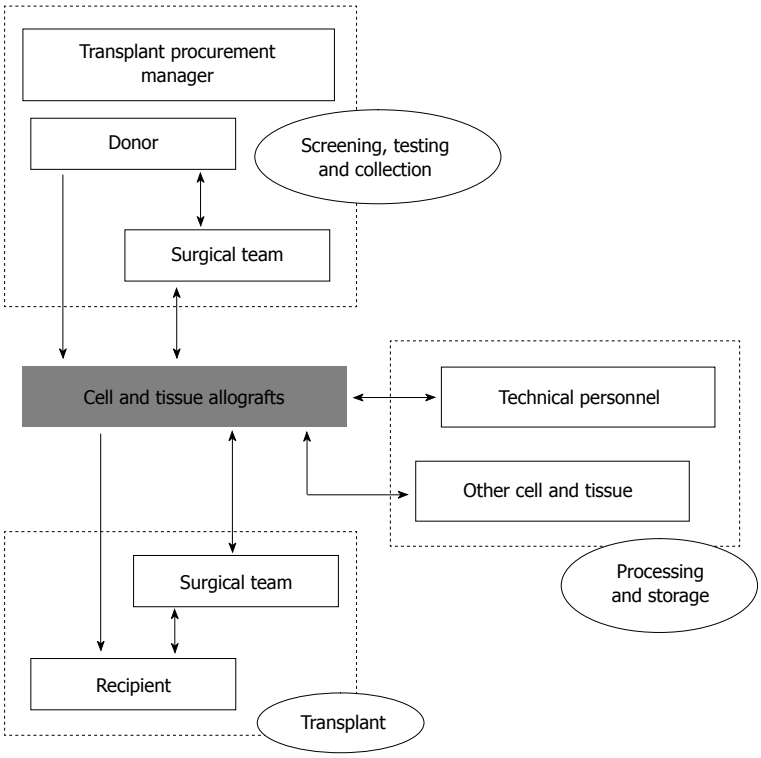

Figure 1 Activities and professionals to take into account when assessing risk factors for disease transmission associated to tissue transplantation. Arrow heads show the interactions among elements.

from post-mortem blood samples. Baleriola *et al*[20] compared the efficacy of viral marker detection in paired pre- and post-mortem samples and did not find a reduction of sensitivity between them. Traceability: unlabelled or not clearly identified samples cannot be associated with a donation.

In cases of deceased donors, an important number of tissues may be obtained from each one. The implementation of strategies to minimize the incidence of unsuitable samples will then improve the activity of the tissue bank. Kitchen *et al*[14] have proposed the use of a sequential serology screening algorithm in order to reduce the number of tissues which are rejected due to non-specific screen reactivity. Using this strategy, they have improved the specificity of serology screening, significantly decreasing screening losses. For these authors, when comparing blood donors with deceased tissue donors, in terms of infection risk, the latter should be considered as first time blood donors. Zou *et al*[21] reported a higher estimated probability of undetected viremia among tissue donors than among first-time blood donors but lower than those attributed to the general population. They point out that the donor selection procedure for tissue donors (medical history, physical examination, and interviews with the next of kin) is not as effective as the face-to-face interview that is carried out with blood donors.

Donor testing: Specific HBV tests

HBV detection tests are included in the routine screening tests for cell and tissue donors. The standard test for preventing transplant-transmitted hepatitis B is hepatitis B surface antigen (HBsAg)[11]. Since this immunoassay test was introduced in 1971 its analytical sensitivity has now improved dramatically, and the current tests can sense HBsAg at concentrations lower than 0.1 ng/mL and 0.62 ng/mL[22].

The probability of finding a tissue donor with undetectable viremia by immunoassay testing has been calculated from human immunodeficiency virus (HIV), hepatitis B virus (HBV) and hepatitis C virus (HCV)[23] using the incidence-window period model[24]. The risk of detecting a negative serology test in an infected tissue donor has been shown to be higher for these two viruses with lower incidence rates and longer window periods (HBV and HCV), than for the HIV virus (higher incidence rate and shorter window period (HIV)[21].

The implementation of methods involving nucleic acid amplification (NAT) and the new generation of reactives to detect viral antibodies or antigens with immunoassay, has increased the sensitivity and the specificity of the screening tests. The window period length for HBsAg test has fallen from 59 d[23] using enzymoimmunoassay (EIA) to 36-38 d[24] using the new EIAs and chemolumynoimmunoassay (ChLIA) tests. Anti-hepatitis B core antigen (anti-HBc) arises between one and two weeks later that HBsAg[25] while HBV DNA, measured by nucleic acid testing in individual donation (ID-NAT), arises around 21 d after the donor becomes infectious and 15 d before the serologic testing detects the HBV infection[26].

Despite the continuous progress in screening tests, three causes of transfusion and transplant-transmitted hepatitis B remain: window period donation, donor carrying an occult hepatitis B infection (OBI) and virus with mutations that are undetectable by the available screening tests[27]. NAT, especially ID-NAT, provides the shorter window period and defines the OBI[28] (HBV DNA presence, without HBsAg and with or without anti-HBc

and/or anti-hepatitis B surface antigen, anti-HBs). There are two types of window period (early infection and what occurs after acute infection, with undetectable HBsAg), three OBI types (only with DNA, with DNA and anti-HBc, with DNA and anti-HBs) and two chronic infection types (NAT^+ and NAT). Every variety of HBV related conditions, except chronic infection NAT, is detectable by NAT, while the HBsAg test does not detect five of the conditions, while the anti-HBc test does not detect three of them[27].

Once the advisability of HBV NAT testing for screening of the cell and tissue donations is well established, it is mandatory to decide whether it should be in ID-NAT or in minipool (MP-NAT). Since HBV NAT is available, we are aware that the repeat testing results of a sample with low DNA concentration are often discordant, even in the same watch[29]. Recently, a donation with west nile virus (WNV) and low concentration RNA included in a minipool with a positive result, was negative in individual retesting, and its transplantation caused WNV transmission with a fatal outcome[30]. It is well known during the last few years that the length of the window period with ID-NAT is shorter than MP-NAT[31]. Since the prudent attitude seems to be to wast every donations included in a MP-NAT that is initially reactive, NAT screening as ID-NAT must be performed.

Once established that HBV ID-NAT is mandatory, the need to maintain serologic tests is an interesting question. In the case of an affirmative answer, which serologic tests should be performed? In order to resolve this question, it has to be realized that the analytical sensitivity of VHB ID-NAT is 10 IU/mL (50 copies/mL)[32] and a concentration as low as 1 copy/20 mL can transmit HBV infection[26].

HBsAg becomes positive about two weeks later than ID-NAT tests and, by definition, is negative in OBI. So, one could envisage the scandalous possibility of removing HBsAg as a mandatory test in HBV screening if HBV ID-NAT is performed[33]. Both anti-HBc and ID-NAT tests cover serologic window periods and the vast majority of NAT negative VHB chronic infections. In countries with a prevalent high HBV infection it is feasible to minimize the tissue loss by adding the anti-HBs determination, so that donations ID-NAT negative, anti-HBc positive with anti-HBs > 10 IU/L can be delivered[34]. If ID-NAT is not performed, the anti-HBs concentration to allow tissue delivery must be > 100 IU/L[35].

In summary, the optimum screening HBV tests should be adapted to current knowledge. Until the HBV NAT with an analytical sensitivity around 1 copy/20 mL becomes available, anti-HBc must be performed, allowing for the delivery of NAT negative, anti-HBc negative and the NAT negative, and anti-HBc positive with anti-HBs > 10 IU/L donations. On the contrary, NAT positive and NAT negative with anti-HBc positive and anti-HBs < 10 IU/L donations must be discarded. The HBsAg determination is not useful if ID-NAT is routinely performed. In fact, several studies have concluded that NAT should be a routine test for donations and confirmed the value of maintaining anti-HBc for the detection of low-level HBV DNA-positive donors and observed that HBsAg screening showed no blood safety value[11,21,36,37].

In the case of living donors (*i.e.*, femoral head), if NAT testing is not used, tissue can be quarantined for at least 180 d[38], until the donor is retested for serological markers. Sometimes this retesting is not possible (it entails additional discomfort for elderly patients) and the tissue must be rejected. Westby *et al*[39] calculated the cost comparison between 180 d retesting and NAT testing, concluding that NAT implementation was much more cost effective.

Surgical teams

Tissue collection and tissue transplant are highly dependent on human intervention. The professionals participating in these activities can be the source for hepatitis B transmission. Some cases of hepatitis B transmission from a surgeon have been reported[40,41].

Processing

One of the most frequently reported acquired infections by laboratory staff is the HBV infection as it is several times greater in laboratory staff than the general population and is one of the most frequently reported laboratory acquired infections[42].

In the mid 50's, when the first tissue banks begun operating, the effect of freezing on viruses was not clear. It was suggested that after long-term storage (> 5 years) viruses could become inactive[43]. This last reference is considered as the first report of the transmission of hepatitis by frozen bone. Since then, it has been really difficult to find more cases of this disease due to tissue transplants. As an alternative to freezing, some tissues (such as cancellous bone) can be dehydrated, using freeze-drying or chemical agents. These dehydrated tissues have the advantage of being stored at room temperature. The dehydration process and the importance of water in the maintaining of a viral envelope could explain the reduction of viral infectivity observed in some cases, hindering the fusion with the cell membrane[44]. Several authors have reported cases of disease transmission with organ and fresh-frozen tissue transplantation but not with processed tissues from the same donor[45]. In addition, dehydrated/lyophillized tissues undergo secondary sterilization (*e.g.*, gamma irradiation). However, for some tissues, the level of radiation must be limited to avoid undesiderable effects on their biological and/or biomechanical properties[46-48].

Nevertheless, the low temperature and the addition of cryoprotective agents which maintain the stability of lipidic membranes (*e.g.*, albumin and sucrose), can enlarge the infective period (even for lyophillized tissues)[49,50]. DNA is more stable than RNA and, hence, more resistant to the effects of environmental conditions. Baleriola *et al*[51] have observed a significant loss of HBV load in samples stored at -70 ℃ for 9 years. However, the viral titter after storage was adequate for the detection of HBV nucleic acid with NAT. Bond *et al*[52] have deter-

mined that HBV can survive and remain infectious on environmental surfaces for up to 7 d.

Tissues are processed in tissue banks inside flow cabinets placed in clean rooms, with stringent environmental monitoring. During this phase, measures to avoid cross-contamination among tissues from different donations must be implemented.

Storage

Nitrogen is commonly used in cell and tissue banks in order to achieve ultralow temperatures to ensure long term storage, avoiding significant changes in the stored products. However, the liquid phase of nitrogen (-196 ℃) has been reported as a vehicle for hepatitis B transmission thus the vapour phase has been recommended to prevent it[53-55]. In addition, the use of double-bagging protects stored material against cross-contamination risk in case of leakage.

Information and quality system

Sometimes, the lack of a system to share data among professionals implied within the different activities performed from donation to transplant, is the cause of delivery of non-conforming tissues. The importance of rapid communication was showed in the case described by Tugwell *et al*[56] in which tissues from a hepatitis C positive donor were transplanted in spite of some tissues from this donor, who a year before had been shown as the origin of disease transmission (but these infections were not reported).

Considering the significant improvement achieved in the detection of viral markers, the incidence of human errors has emerged as the main risk for hepatitis B transmission. Taking advantage, therefore, of the broad experience accumulated in the field of allograft transplantation, the implementation of a quality system especially in tissue banks and surgical units, as well as a risk management system based on preventive action (proactive criteria), have contributed to minimizing this risk. Failure mode and effect analysis (FMEA, FMECA) are two widely used approaches to identify and eliminate events which can lead to adverse events, before they occur. Both strategies are based on the quantification of the ability to note the cause of the failure (detectability) and the probability of taking place (occurrence). The main difference between them is that FMECA incorporates a classification for the severity of the consequences. This system yields a hierarchization which enables decision making regarding the priorities to adopt. The EUSTITE (European Union Standards and Training for the Inspection of Tissue Establishments) Project has provided useful grading for the parameters to be considered in risk assessment.

FOLLOW-UP OF CELL AND TISSUE RECIPIENTS

In general, tissue banks have shown an inability to track the outcomes of the patients receiving their tissues, which is an important issue in order to detect adverse events from their use. Sometimes, the reason is the difficulty in confirming the association between an infectious disease with the transplanted tissue. The development of measures to solve the problem of this lack of communication requires efficiency on the part of the various participants: organ and tissue procurement organizations, tissue banks, transplant centers, professional organizations, public health agencies. Examples of this collaboration are the Transplantation Sentinel Network (TSN) in United States, and in Europe, the SOHOVS (Vigilance and Surveillance of Substances of Human Origin) Project[9,12,56-59].

As previously mentioned, the knowledge of adverse reactions and events associated with tissue allografts has led to risk management analysis and the implementation of corrective actions, which have significantly improved the safety of tissue transplantation. The systems for surveillance and the creation of communication networks for reporting incidences related to disease transmission have been the best source for acquiring the experience necessary in risk management. In 2010, as a consequence of the Sixty-third World Health Assembly, the World Health Organization launched Project Notify. The aim of this project was to provide a global interface for the vigilance and surveillance of substances of human origin[59].

Dhakal *et al*[60] have proposed an interest in using Medicare data to facilitate the access to information related to tissue allograft outcomes.

In this scenario, tissue banks appear as the link for providing the information generated from donation to transplantation, ensuring traceability in both trace-back and trace-forward analysis. Indeed, tissue processors are the main source of reports.

Authorization/inspection/audit

Public health care authorities have the competence to develop regulations regarding activities performed from donation to transplantation, with special attention given to cell and tissue banking. In addition, control measures must be implemented in order to ensure compliance with these regulations.

This task should be supported with the incorporation of ethical and technical standards developed from scientific associations involved in the different activities (donor selection, donor testing, tissue procurement, tissue processing, tissue storage, tissue transplant, quality control, data management, communication system, risk management).

As an example, the EQSTB (European Quality System for Tissue Banking) project has been developed with the aim of analysing the factors that may influence the quality and safety of cells and tissues for transplantation[61]. A useful guide for auditing tissue establishments was developed as a result of this project. In the United States, the FDA has edited guides for good tissue practices with recommendations.

CONCLUSION

HBV transmission risk remains as a problem in tissue and cell transplantation, whose incidence is unknown. Although recipient HBV infection can occur by different means, HBV transmission is mainly due to the collection of cell and tissues from window period donors. Donor screening protocols should be designed in order to minimize the infectious risk. The optimum screening HBV tests should be those adapted to the current knowledge.

In summary, we suggest the following strategies be considered in order to minimize the risk of hepatitis B transmission: Centralization of expert knowledge pertaining to the donor selection process in transplant procurement management and the use of stringent donor selection procedures. Centralizing donor testing, especially in cases of deceased donors, in an expert laboratory. Double bagging of products during storage. Avoiding pooling and cross-contamination during processing. Ensuring the use of assays suitable for samples from deceased donors. Implementing algorithms to distinguish true infections (confirmed positive results) of non-specific reactivity. Establishing a quarantine for at least two weeks for lyophillized tissues. Depending on epidemiology, to include anti-HBc, anti-HBs and HBV nucleic acid tests in donor testing. Establishing efficient communication systems to report adverse events. Developing of surveillance measures for data management: identification of donor, tissue and associated samples, and transcription of results. Developing programs for operator training. Establishing multidisciplinary work teams for risk assessment.

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ORIGINAL ARTICLE

Protective effect of bone marrow mesenchymal stem cells in intestinal barrier permeability after heterotopic intestinal transplantation

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Abstract

AIM: To explore the protective effect of bone marrow mesenchymal stem cells (BM MSCs) in the small intestinal mucosal barrier following heterotopic intestinal transplantation (HIT) in a rat model.

METHODS: BM MSCs were isolated from male Lewis rats by density gradient centrifugation, cultured, and analyzed by flow cytometry. The HIT models were divided into a non-rejection group, saline-treated rejection group (*via* penile vein), and BM MSC–treated group (*via* penile vein). Intestinal mucosal barrier injury was estimated by diamine oxidase (DAO) and *D*-lactic acid (*D*-LA) expression levels. Tumor necrosis factor-α (TNF-α), interferon-γ (INF-γ), interleukin-10 (IL-10), and transforming growth factor-β (TGF-β) were detected by enzyme-linked immunosorbent assay. Ultrastructural change of tight junctions (TJs) was observed under transmission electron microscope. Expression levels of the TJ proteins occludin and zona occludens (ZO)-1, affected by the inflammatory factors, were measured using real-time polymerase chain reaction and Western blotting.

RESULTS: The pathological score at each time point after surgery indicated significantly less serious injury in the BM MSCs-treated group than in the rejection group ($P < 0.05$). In the former, graft levels of DAO and *D*-LA were reduced, and TNF-α and INF-γ production was inhibited (at day 7: 10.6473 ± 0.0710 *vs* 17.2128 ± 0.4991, $P < 0.05$; 545.1506 ± 31.9416 *vs* 810.2637 ± 25.1175, $P < 0.05$). IL-10 and TGF-β production was increased greatly (at day 7: 125.7773 ± 4.7719 *vs* 80.3756 ± 2.5866, $P < 0.05$; 234.5273 ± 9.3980 *vs* 545.1506 ± 31.9416, $P < 0.05$). There was increased expression of occludin and ZO-1 protein (at day 7: 0.2674 ± 0.0128 *vs* 0.1352 ± 0.0142, $P < 0.05$; at day 5: 0.7189 ± 0.0289 *vs* 0.4556 ± 0.0242, $P < 0.05$) and mRNA (at day 7: 0.3860 ± 0.0254 *vs* 0.1673 ± 0.0369, $P < 0.05$; at day 5: 0.5727 ± 0.0419 *vs* 0.3598 ± 0.0242, $P < 0.05$).

CONCLUSION: BM MSCs can improve intestinal barrier permeability, repair TJs, and increase occludin and ZO-1 protein expression. With altered cytokine levels, they can protect the intestinal mucosa after transplantation.

Key words: Bone marrow mesenchymal stem cells; Small intestinal transplantation; Intestinal mucosal bar-

rier; Occludin; Zona occludens-1

Core tip: Rejection and sepsis after small intestinal transplantation (SITx) is a serious and common complication. The small intestinal mucosal barrier plays an important role in the progression of postoperative complications. This study demonstrated that in rats, implantation of recipient-derived bone marrow mesenchymal stem cells decreased intestinal permeability and preserved intestinal mucosal barrier function after SITx *via* a mechanism linked to the balance between graft inflammatory cytokine levels and increased expression of the intestinal tight junction proteins occludin and zona occludens-1.

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INTRODUCTION

Small intestinal transplantation (SITx) has become the definitive treatment for patients with end-stage intestinal failure who cannot tolerate parenteral nutrition[1]. However, SITx is difficult due to the strong expression of histocompatibility antigens, large numbers of resident leukocytes, and micro-organism colonization. Rejection and sepsis following SITx is a serious and common complication that affects both patient and graft survival[2].

Bone marrow mesenchymal stem cells (BM MSCs) are pluripotent adult stem cells. BM MSCs give rise to mesoderm cells[3,4] and differentiate into osteoblasts, chondrocytes, adipocytes, myocytes, and liver and neural cells[5-7], which have potential for use in treating various diseases. Allogeneic MSCs that were transplanted into primates *via* an intravenous route and distributed to the gastrointestinal tract proliferated[8]. Due to the secretion of several growth factors, BM MSCs also exhibit immunomodulatory capabilities[9-13]. BM MSCs reduce intestinal ischemia-reperfusion (I/R) injury in rats[14,15] and contribute to significant prolongation of composite tissue allotransplant survival[16] and promotion of graft revascularization[17].

The intestinal mucosa is the physical, chemical, immunological, and biological barrier against toxins and pathogens in the gut lumen. The intestinal mucosal barrier is composed of mucosal fluid, microvilli, epithelial mucosal cell tight junctions (TJs), and other special structures. TJs are the most important structures in the intestinal mucosal barrier. They are composed of multiple proteins, including transmembrane proteins such as occludin, tricellulin, claudins, and junctional adhesion molecule (JAM). The intracellular portions of these transmembrane proteins interact with cytoplasmic peripheral membrane proteins, including zona occludens (ZO)-1, -2, and -3[18-20], and two distinct transmembrane proteins: occludin and claudin[21,22], which are linked to the actin-based cytoskeleton[23]. TJs function as occlusion barriers by maintaining cellular polarity and homeostasis and by regulating paracellular space permeability in the epithelium[24]. Occludin and ZO-1 proteins play crucial roles in TJ assembly and maintenance[25-27]. In this study, we established a heterotopic intestinal transplantation (HIT) model in rats to explore the protective effect of BM MSC transplantation in the small intestinal mucosal barrier and the possible mechanisms thereof.

MATERIALS AND METHODS

Animals and rat HIT models

Seventy-six inbred specific pathogen-free Brown Norway (BN) and 30 Lewis (LEW) male rats weighing 200-220 g were used as donors in the homologous and isologous experimental groups, respectively. One hundred and six LEW male rats weighing approximately 220-250 g were used as recipients. Additionally, 25 male LEW rats weighing 100-120 g were used for BM MSC extraction. All animals were purchased from the Chinese Academy of Military Medical Sciences (Beijing, China). The use of animals and animal experimental procedures in this study was approved by the Ethics Committee of the Chinese Academy of Military Medical Sciences.

All rats were fasted for 12 h with free access to water before surgery and randomly assigned to a non-rejection group (A, LEW-LEW, n = 30); saline-treated rejection group (B, BN-LEW, *via* penile vein, n = 43); and BM MSC-treated group (C, BN-LEW, *via* penile vein, n = 33). The operations were performed using standard sterile technique under general anesthesia with 5% chloral hydrate (50 mL/kg).

Donor operation

The small intestine was visualized *via* a pubis-xiphoid midline incision. The small intestine was retracted to the left and packed in saline-moistened gauze. All tributary branches of the mesenteric artery and vein were visualized, ligated with 5-0 silk, and divided. The small intestine itself was cut just distal to the duodenum and proximal from the cecum. The mesenteric artery was freed from the surrounding tissue by dissecting the colon. The tissue connecting the small intestine to the colon was dissected. Lactated Ringer's solution (5 mL) containing 125 U heparin was then administered systemically to the donor animal before the graft was removed from the donor and placed in cold lactated Ringer's solution (0-4 ℃) after being flushed *via* the mesenteric artery with the same solution. Grafts were perfused with cold saline at low pressure.

Recipient operation

The abdomen was opened, and the small intestine of

the recipient packed in saline-moistened gauze inside the abdominal cavity, and retracted to the left. The aorta was freed distal to the left renal artery. An oval aperture was cut in the occluded part of the aorta, and an end-to-side anastomosis with the graft aorta attached to the mesenteric artery was created. In the same fashion, an anastomosis was created between the recipient portal vein and the donor postcaval vein. For both anastomoses, a continuous 10-0 silk suture was done under × 20 magnification. The proximal end of the donor small bowel was anastomosed to the stoma on the abdominal wall. Saline (1 mL, 0.9%) or culture media (1 mL) containing 5×10^6 BM MSCs was injected *via* the penile vein. The abdomen was closed and the animals were allowed to recover with free access to tap water and standard pellet rat chow. Only for surviving animals, HIT was included in the analysis. After transplantation, all animals were euthanized at 1, 3, 5, 7 and 10 d. The graft was removed from each animal and stored at -80 ℃ until analysis. Intestine samples were fixed for histopathological analysis and transmission electron microscopy.

Isolation and characterization of BM MSCs

BM MSCs were isolated from the femur and tibia of male LEW rats (100-120 g). Red blood cells were lysed using 0.1 mol/L NH_4Cl; the remaining cells were washed, resuspended, and cultured for 4 wk in DMEM/F12 (Gibco, Carlsbad, CA, United States) containing 100 U/mL penicillin, 100 mg/mL streptomycin, and 15% fetal bovine serum. BM MSCs were cultured in an incubator at 37 ℃ in 5% CO_2 with saturated humidity. The medium was changed every 72 h. When the third-passage cells reached 80% confluence, cells were trypsinized, washed, centrifuged, and resuspended at 1×10^7/mL in phosphate-buffered saline.

BM MSCs were stained using antibodies against CD29, CD90, RT1A, CD45, RT1B (BioLegend, San Diego, CA, United States), and CD34 (Santa Cruz Biotechnology, Santa Cruz, CA, United States), and analyzed by flow cytometry (FACSCalibur; BD Biosciences, Alaska, MN, United States). The proportion of $CD29^-$, $CD90^-$, and RT1A-positive cells and $CD34^-$, $CD45^-$, and $RT1B^-$ negative cells was > 98%. BM MSCs were also confirmed as spindle-shaped, plastic-adherent cells under standard culture conditions by microscopy. The purity of BM MSCs was > 95% (Figure 1).

Diagnosis and evaluation of rejection

A pathologist blinded to the source analyzed the slides. The degree of histopathological changes was graded semiquantitatively using the histological injury scale described by Chiu *et al*[28]: 0: normal mucosal villi; 1: development of a subepithelial space, usually at the villi apex, with capillary congestion; 2: extension of the subepithelial space with moderate epithelial lifting from the lamina propria; 3: massive epithelial lifting down the sides of the villi and ulceration at the villous tips; 4: denuded villi with dilated capillaries and increased cellularity of the lamina propria; and 5: degradation and disintegration of the lamina propria, hemorrhage, and ulceration. The summation of six randomly chosen fields from each rat was evaluated and averaged to determine the degree of mucosal injury.

Enzyme-linked immunosorbent assay

The graft levels of diamine oxidase (DAO), *D*-lactic acid (*D*-LA), tumor necrosis factor-α (TNF-α), interferon-γ (INF-γ), interleukin-10 (IL-10), and transforming growth factor-β (TGF-β) were determined using the kits from R&D Systems, Minneapolis, MN, United States, according to the manufacturer's protocol.

Detection and observation of intestinal mucosal ultrastructure

Ultrathin (70-nm) sections were prepared using standard techniques and examined under a transmission electron microscope (Hitachi H-600, Tokyo, Japan).

Western blotting of tissue occludin and ZO-1

Intestinal tissue samples were homogenized and lysed in buffer [50 mmol/L Tris-HCl (pH 8.0), 150 mmol/L NaCl, 100 μg/mL phenylmethylsulfonyl fluoride, 1% Triton X-100] for 30 min on ice. Then, 50 μg protein samples were boiled for 5 min in sample buffer, separated by 10% and 8% sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and transferred onto nitrocellulose membranes. Nonspecific reactivity was blocked using 5% non-fat dry milk in TBST [10 mmol/L Tris-HCl (pH 7.5), 150 mmol/L NaCl, 0.05% Tween-20] for 1 h at room temperature. The membrane was then incubated with a rabbit anti-rat polyclonal occludin and ZO-1 antibody (1:500; Santa Cruz Biotechnology) at 4 ℃ overnight. After three washes in TBST, the membranes were incubated with horseradish peroxidase-conjugated goat anti-rabbit IgG (1:2000; Santa Cruz Biotechnology) for 2 h at room temperature. Reactive protein was detected using an ECL chemiluminescence system (Boster, Wuhan, China).

Real-time PCR detection of occludin and ZO-1 mRNA

RNA was isolated with TRIzol (TakaRa, Japan). Reverse transcription (RT) to complementary DNA was performed with 12 μg RNA by Moloney murine leukemia virus-RT at a final volume of 10 μL according to the manufacturer's instructions. The reaction volume was increased to 20 μL and the dilutions used in the reactions were always performed in accordance with the β-Actin content. Product specificity was determined using 2% agarose gel electrophoresis. The sequences of the primers used are listed in Table 1.

Statistical analysis

SPSS version 13.0 (SPSS, Chicago, IL, United States) was used for statistical analysis. Values reported are the mean ± SD. Different groups of data were compared by analysis of variance. $P < 0.05$ was considered statistically

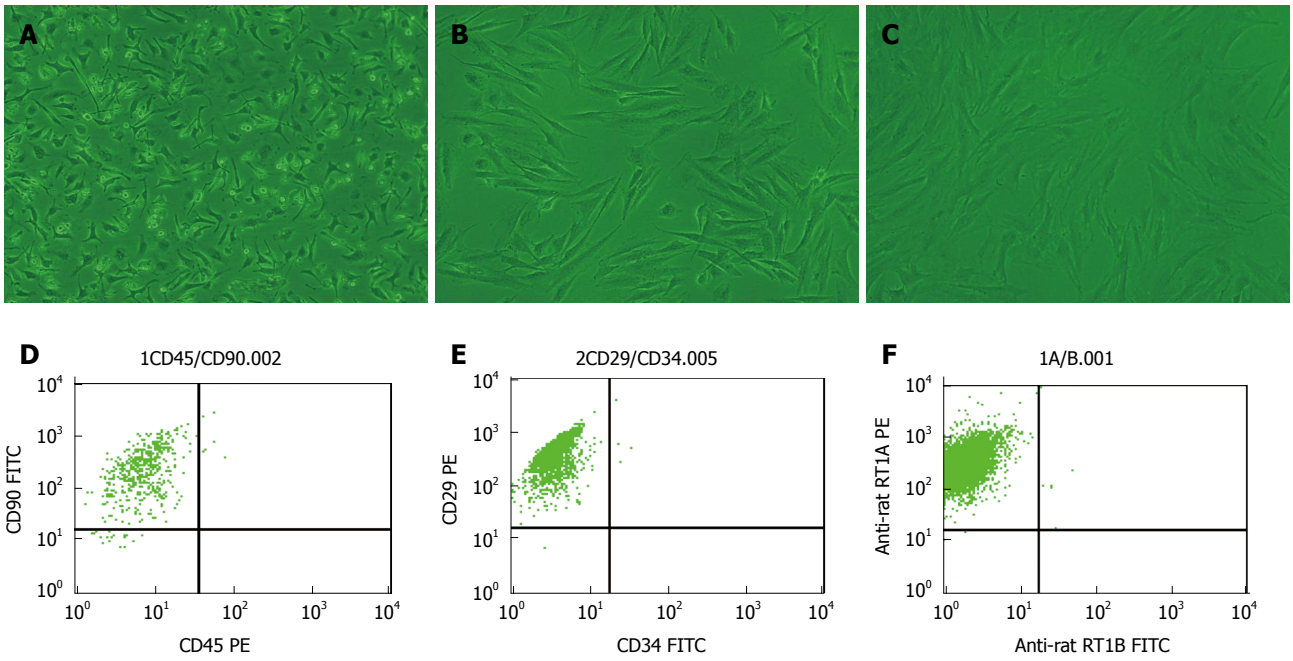

Figure 1 Morphology and flow cytometry results of Lewis-derived bone marrow mesenchymal stem cells. A: First-passage bone marrow mesenchymal stem cells (BM MSCs); B: Second-passage BM MSCs; C: Third-passage BM MSCs (× 200 magnification); D: The proportion of $CD90^+$ and $CD450^-$ cells was approximately 95.48%; E: The proportion of $CD29^+$ and $CD34^-$ cells was approximately 99.82%; F: The proportion of $RT1A^+$ and $RT1B^-$ cells was approximately 99.87%.

Table 1 Sequences of primers for occludin, zona occludens-1, and β-actin

Tested gene product		Primer sequences	Fragment
Occludin	Upstream	5′-CCTGTTTAGTTAGGTGAAG-3′	156 bp
	Downstream	5′-TTCCTGAGAAGGGTTATG-3′	
ZO-1	Upstream	5′-GGGGGATTTATAACTTGGG-3′	321 bp
	Downstream	5′-CTGGTTGGATGTCTGTGG-3′	
β-Actin	Upstream	5′-GCGTGACATTAAAGAGAAGCTG-3′	500 bp
	Downstream	5′-AGAAGCATTTGCGGTGCAC-3′	

ZO-1: Zona occludens-1.

significant.

RESULTS

BM MSC extraction

Cells were confirmed as BM MSCs based on their spindle-shaped morphology, adherence to plastic, and flow cytometry results (Figure 1). Most of the third-passage adherent cells were $CD90^-$, $CD29^-$, and RT1A-positive and negative for the MSC markers CD45, CD34, and RT1B. Furthermore, the percentage of $CD90^+$ and $CD45^-$ cells rapidly increased from 80% to > 98% over the first three passages.

General condition of the rats, graft histopathology, and grade of intestinal mucosal injury after HIT

Following small intestinal transplantation, all rats in group A survived for 10 d; in group B, two rats died by day 5, four rats died by day 7 and six rats died by day 10; and in group C, one rat died by day 7 and two rats died by day 10. The 10-d death rate was 0 (0/30), 30.23% (13/43), 9.09% (3/33) in groups A, B, and C, respectively. We observed no significant abnormal activities; the rats were only slightly sluggish 1 d after surgery. Subsequently, they became frequently irritable, and there was an abnormal increase in secretions from the nose and mouth, reddened ears, and liquid stools at 3 d after surgery. On day 5, there were obvious pathological symptoms of severe rejection, relative sluggishness, weight loss, liquid stools, and abdominal mass; physical examination revealed a macerated peristomal site with surrounding erythema, induration, and serosanguineous drainage. The condition of the rats worsened over time (Figure 2). The general condition improved at each time point in group C, and the pathological scores were mitigated (Table 2).

Small intestinal mucosal barrier function changes following transplantation

At each time point, the graft DAO levels in group B increased more than twofold compared to that in group A ($P < 0.05$). However, the graft DAO levels in group C were significantly lower than that in group B at each time point ($P < 0.05$) (Table 3).

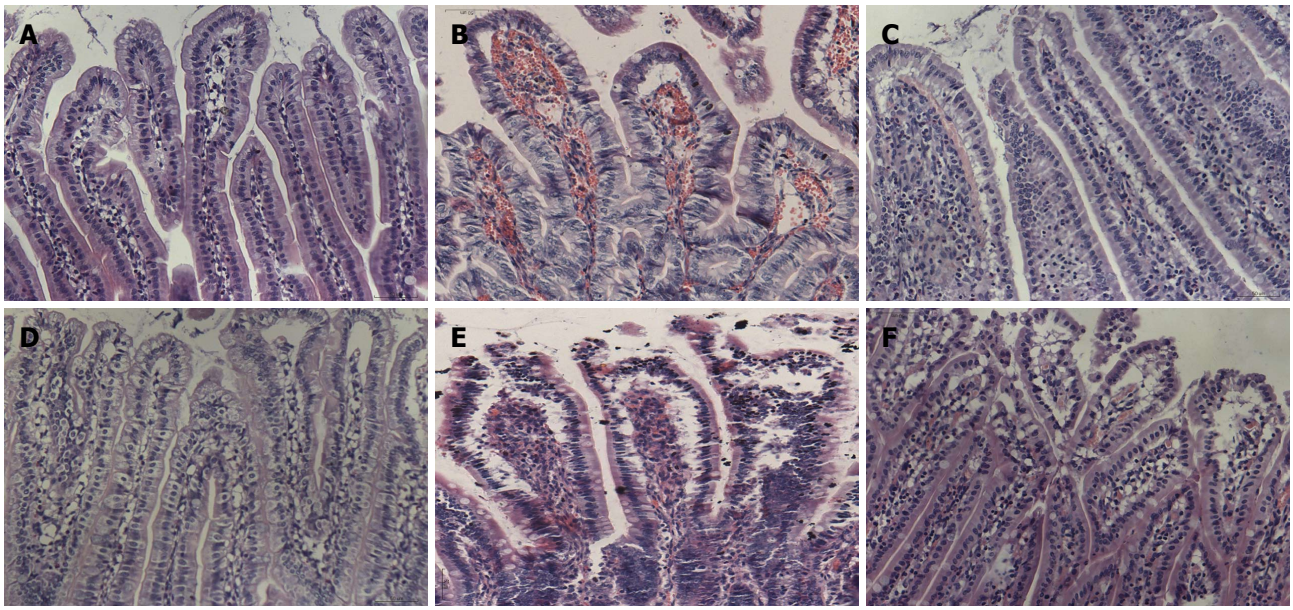

Figure 2 Graft histopathology at different time points after heterotopic intestinal transplantation (HE staining, × 200). A: Normal intestine with normal villous architecture and glands in group A; B: Intestinal mucosa degradation and hemorrhage of the lamina propria, ulceration, decreased ratio of villus height and crypt height, aggravated lymphocyte infiltration, partial gland epithelial necrosis in group B at day 5 after operation; D: There were decreased intestinal mucosal villi with mild deformity and interstitial infiltration of inflammatory cells; E: The condition was aggravated 7 d after heterotopic intestinal transplantation, and there was epithelial degeneration, intestinal wall thinning and necrosis, and interstitial inflammatory cell infiltration in great quantities; C, F: Recovery of the damaged mucosa in group A at (C) day 5 and (F) day 7.

Table 2 Grade of intestinal mucosal injury following heterotopic intestinal transplantation

Group	D 1	D 3	D 5	D 7	D 10
A	8.26 ± 0.65	9.37 ± 0.92	10.36 ± 0.78	12.65 ± 0.65	13.14 ± 0.86
B	17.4 ± 1.76[a]	30.64 ± 3.62[a]	41.6 ± 2.27[a]	67.2 ± 2.57[a]	81.74 ± 3.52[a]
C	12.17 ± 1.17[c]	24.00 ± 1.54[c]	30.17 ± 0.41[c]	41.83 ± 2.93[c]	52.33 ± 1.03[c]

All values are mean ± SD (n = 6; the summation of six randomly chosen fields from each rat were evaluated and averaged to determine the degree of mucosal injury). A: Non-rejection group; B: Rejection group; C: Bone marrow mesenchymal stem cells therapy group. [a]$P < 0.05$ *vs* group A; [c]$P < 0.05$ *vs* group B.

Ultrastructural characteristics of the intestinal mucosa and TJs

In group A, the epithelial cells and TJs remained intact. By contrast, there was TJ and villi disruption, loose microvilli, and swollen organelles with decreased electron density in group B at 5 d after surgery. At the same time point, the TJs and endothelial cell mitochondria and microvilli in group C remained undisrupted (Figure 3).

Expression of occludin and ZO-1 protein following HIT

Occludin and ZO-1 expression decreased more significantly in group B than in group A; however, occludin and ZO-1 expression was significantly higher in group C than in group B (Figure 4).

Expression of occludin and ZO-1 mRNA following HIT

The expression of occludin and ZO-1 mRNA decreased more significantly in group B than in group A. Occludin and ZO-1 mRNA expression was significantly higher in group C than in group B (Figure 5).

Graft TNF-α, IFN-γ, IL-10, and TGF-β levels

Compared to group B, graft TNF-α, IFN-γ, IL-10, and TGF-β levels were decreased significantly at day 7 in group A ($P < 0.05$); graft TNF-α and IFN-γ levels were decreased significantly and graft IL-10 and TGF-β levels were increased significantly at day 7 in group C ($P < 0.05$) (Figure 6).

DISCUSSION

Although the HIT model does not represent the physiological state of small intestinal function, it is used for investigating immunological reactions such as rejection. Additionally, the survival rate of the HIT model is higher and it involves a simple technique[29]. In this study, we explored the mechanism of intestinal mucosal barrier protection following HIT in rats through BM MSC implantation. In injured tissues, BM MSC transmigration across the endothelium is a useful tool for cellular therapy. MSCs develop tight cell-cell contacts and integrate into the endothelial wall of the capillary vessel[30]. Based on its simplicity and safety, saline or BM MSCs were injected *via* the penile vein postoperatively.

Postoperatively, the general condition of the rats worsened over time. At the same time, the grade of

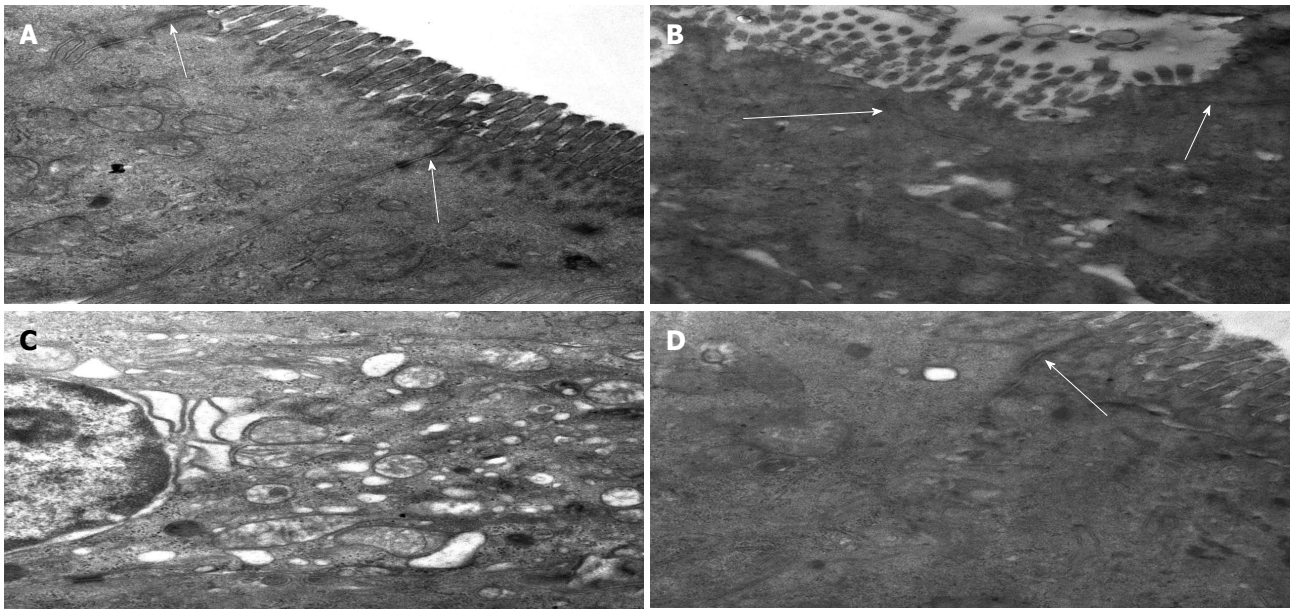

Figure 3 Ultrastructural characteristics of intestinal mucosa and tight junctions following heterotopic intestinal transplantation (magnification × 30000). A: Epithelial cells and tight junctions (TJs) (arrows) were intact in group A; B: Intestinal microvilli and TJs (arrows) were disrupted, and some microvilli were loose; C: Organelles were swollen with reduced electron density at day 5 after transplantation in group B; D: Microvilli and mitochondria of the endothelial cells were almost normal and TJs (arrow) were not disrupted at day 5 after transplantation in group C.

Table 3 Levels of graft diamine oxidase and *D*-lactic acid following heterotopic intestinal transplantation

	Group	D 1	D 3	D 5	D 7	D 10
DAO (U/mL)	A	10.15 ± 1.10	12.86 ± 1.35	15.76 ± 1.33	18.55 ± 1.77	21.83 ± 1.21
	B	22.36 ± 2.82[a]	34.74 ± 5.59[a]	44.54 ± 2.77[a]	51.61 ± 1.70[a]	68.02 ± 2.46[a]
	C	18.69 ± 2.17[c]	29.79 ± 2.49[c]	36.15 ± 3.98[c]	40.13 ± 1.21[c]	59.87 ± 4.34[c]
D-LA (g/L)	A	4.12 ± 0.53	6.13 ± 0.57	8.62 ± 1.67	10.78 ± 0.72	11.67 ± 1.56
	B	6.32 ± 0.46[a]	8.65 ± 0.62[a]	10.46 ± 0.98[a]	12.74 ± 0.68[a]	17.74 ± 1.75[a]
	C	3.08 ± 0.32[c]	4.08 ± 0.30[c]	5.031 ±0.18[c]	7.25 ± 0.51[c]	12.81 ± 0.47[c]

All values are mean ± SD (n = 6). A: Non-rejection group; B: Rejection group; C: Bone marrow mesenchymal stem cells therapy group. [a]P < 0.05 *vs* group A; [c]P < 0.05 *vs* group B. DAO: Diamine oxidase; D-LA: *D*-lactic acid.

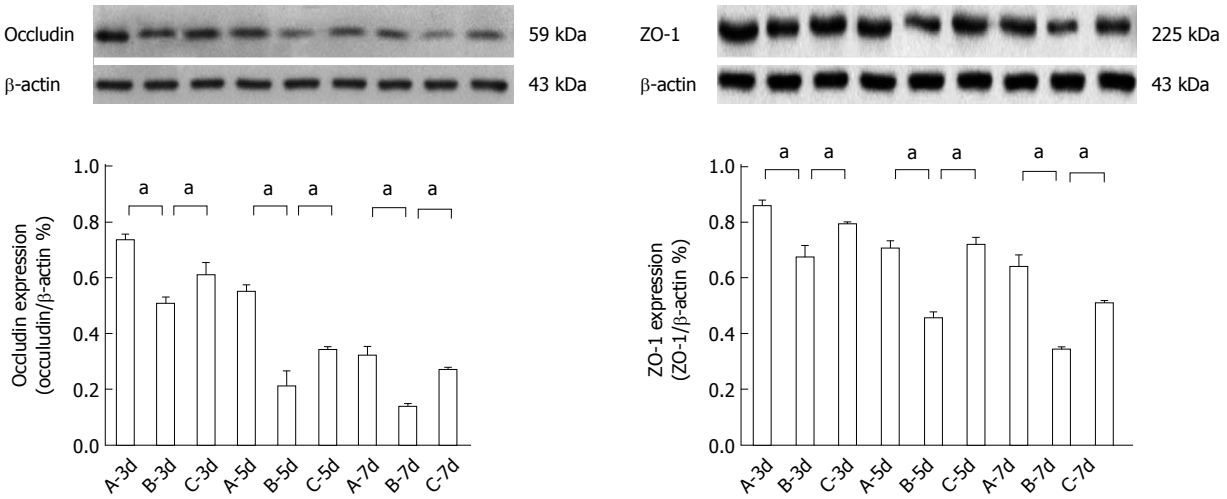

Figure 4 Occludin and zona occludens-1 protein expression after heterotopic intestinal transplantation. Occludin and zona occludens (ZO)-1 protein expression was significantly lower in group B than in group A ([a]P < 0.05 *vs* group B at day 5: 0.2082 ± 0.0582 *vs* 0.5477 ± 0.0284; [a]P < 0.05 *vs* group B at day 7: 0.3415 ± 0.0128 *vs* 0.6387 ± 0.046). Occludin and ZO-1 protein expression was significantly higher in group C than in group B ([a]P < 0.05 *vs* group B at day 7: 0.2674 ± 0.0128 *vs* 0.1352 ± 0.0142; [a]P < 0.05 *vs* group B at day 5: 0.7189 ± 0.0289 *vs* 0.4556 ± 0.0242). A: Group A (non-rejection); B: Group B (rejection); C: Group C (bone marrow mesenchymal stem cells therapy). β-actin was used as the loading control. Values shown are the mean ± SD.

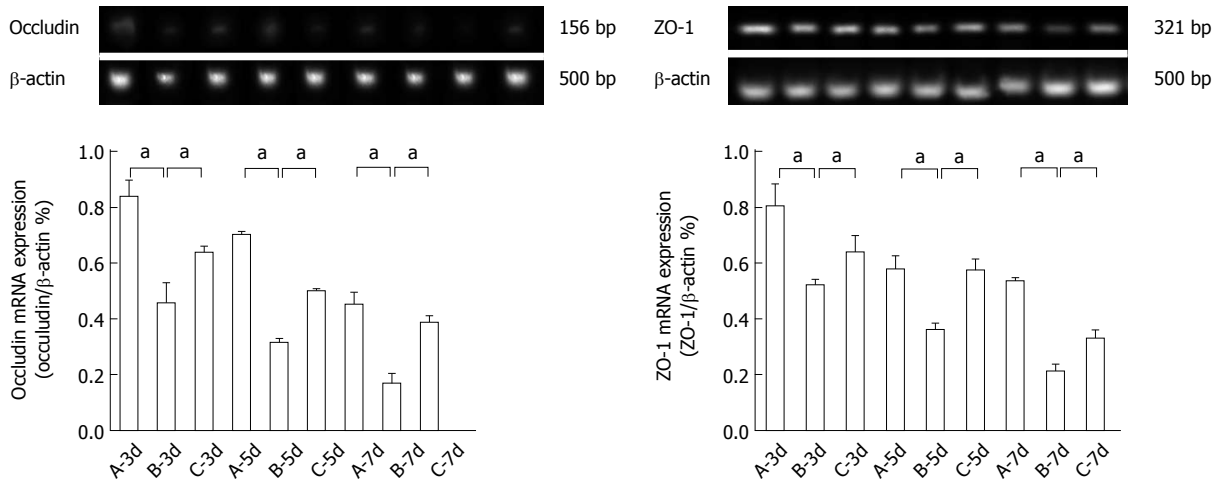

Figure 5 Occludin and zona occludens-1 mRNA expression after heterotopic intestinal transplantation. Occludin and zona occludens (ZO)-1 mRNA expression was significantly lower in group B than in group A ($^aP < 0.05$ *vs* group B at day 5: 0.3135 ± 0.0168 *vs* 0.7011 ± 0.0128; $^aP < 0.05$ *vs* group B at day 7: 0.2101 ± 0.0279 *vs* 0.5345 ± 0.0136). Occludin and ZO-1 mRNA expression was significantly higher in group C than in group B at day 5 ($^aP < 0.05$ *vs* group B at day 7: 0.3860 ± 0.0254 *vs* 0.1673 ± 0.0369; $^aP < 0.05$ *vs* group B at day 5: 0.5727 ± 0.0419 *vs* 0.3598 ± 0.0242). A: Group A (non-rejection); B: Group B (rejection); C: Group C (bone marrow mesenchymal stem cells therapy). β-actin was used as the loading control. Values shown are the mean ± SD.

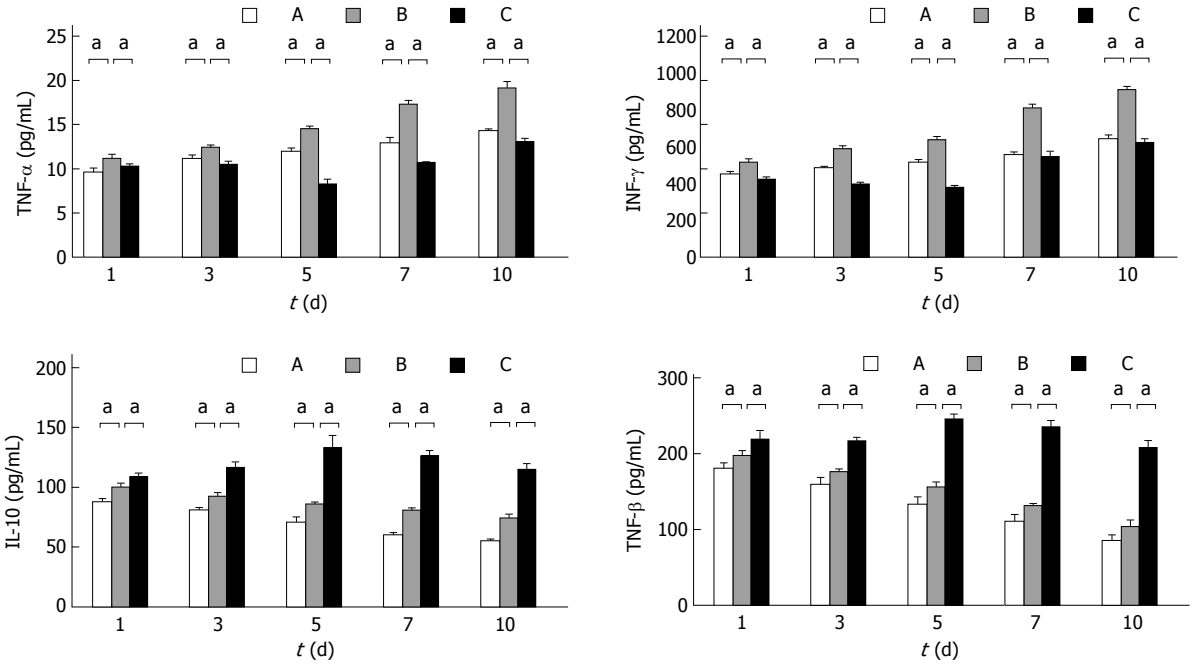

Figure 6 Graft tumor necrosis factor-α, interferon-γ, interleukin-10, and transforming growth factor-β levels. Graft tumor necrosis factor-α (TNF-α), interferon-γ (INF-γ), interleukin-10 (IL-10), and transforming growth factor-β (TGF-β) levels in group B were significantly decreased compared to that in group A at day 7 (17.2128 ± 0.4991 *vs* 12.8364 ± 0.7131, $P < 0.05$; 810.2637 ± 25.1175 *vs* 555.3763 ± 17.9702, $P < 0.05$; 80.3756 ± 2.5866 *vs* 59.9878 ± 2.1521, $P < 0.05$; 130.8756 ± 3.3428 *vs* 109.9878 ± 9.7968, $P < 0.05$). Graft TNF-α and IFN-γ levels in group C were significantly decreased compared to that in group B at day 7 (10.6473 ± 0.0710 *vs* 17.2128 ± 0.4991, $P < 0.05$; 545.1506 ± 31.9416 *vs* 810.2637 ± 25.1175, $P < 0.05$); graft IL-10 and TGF-β levels in group C were significantly increased compared to that in group B at day 7 (125.7773 ± 4.7719 *vs* 80.3756 ± 2.5866, $P < 0.05$; 234.5273 ± 9.3980 *vs* 545.1506 ± 31.9416, $P < 0.05$). A: Group A (non-rejection); B: Group B (rejection); C: Group C (BM MSC therapy). All values are mean ± SD ($n = 6$). $^aP < 0.05$ *vs* group B.

histopathological changes increased. The graft levels of DAO and *D*-LA increased with the rejection reaction, indicating impaired intestinal barrier function. DAO and *D*-LA are sensitive markers of intestinal permeability[31]. Furthermore, we observed disrupted intestinal microvilli and TJs, some loose microvilli, and swollen organelles with reduced electron density (Figure 3). However, the clinical symptoms improved after the BM MSC injection. The DAO and *D*-LA levels in group C also decreased more significantly than that in group B.

TJs are multi-protein complexes composed of transmembrane proteins, peripheral membrane (scaffolding)

proteins, and regulatory molecules that include kinases. The most important transmembrane protein is occludin, which defines several aspects of TJ permeability. Peripheral membrane proteins such as ZO-1 and ZO-2 are crucial to TJ assembly and maintenance, partly because these proteins contain multiple domains for interaction with other proteins such as claudins, occludin, and actin[32]. Occludin and ZO-1 expression decreased more significantly in group B than in group A, particularly at 5 and 7 d; however, occludin and ZO-1 protein and mRNA expression was significantly higher in group C than in group B, particularly at days 7 and 5 (Figures 4 and 5), and was consistent with the ultrastructural changes. Based on the above results, we concluded that BM MSCs can protect the intestinal mucosal barrier, improve TJ permeability, repair TJ ultrastructure, and promote occludin and ZO-1 protein and mRNA expression.

BM MSCs do not express costimulatory molecules and are potent inhibitors of T cell proliferation in mixed lymphocyte cultures, prolonging allograft survival in rodent models[33-37]. BM MSC treatment favors the reestablishment of cellular homeostasis by both increasing endogenous proliferation processes and inhibiting the apoptosis of small intestinal epithelial cells. The effects of BM MSCs stem from their ability to improve the renewal capability of the small intestinal epithelium[38]. BM MSCs produce growth factors that play a critical role in healing damaged tissues. Growth factor production by BM MSCs in response to the wound microenvironment suggests that they might augment wound healing through the responsive secretion of growth factors that enhance angiogenesis and promote wound repair[39]. TNF-α can increase intestinal epithelial cell paracellular permeability[40], reduce expression of the ZO-1 gene promoter[41] and ZO-1 protein[42], and can cause abnormal distribution of ZO-1 protein. TNF-α can cause TJ relaxation and depolymerization, and exhibits a synergistic effect with IFN-γ[43]. TGF-β can both stimulate and inhibit cell proliferation by a two-way regulatory function[44]. After BM MSC injection, graft TNF-α and IFN-γ levels decreased significantly, and that of graft TGF-β and IL-10 increased significantly. Therefore, we inferred that BM MSCs protect and repair damaged intestinal barrier function following HIT by activating paracrine IL-10 and TGF-β, and that IL-10 and TGF-β exhibit sufficient immunomodulatory capabilities and protect intestinal epithelial cells and TJs, suppressing paracrine TNF-α and INF-γ.

In conclusion, BM MSCs can protect and repair damaged intestinal mucosal barrier function following HIT, and cytokines are involved in this effect. The mechanism of BM MSCs is complex and requires further study.

COMMENTS

Background

Small intestinal transplantation (SITx) has become the definitive treatment for patients with end-stage intestinal failure who cannot tolerate parenteral nutrition. Even after years of development, however, the rate of postoperative infection remains high. According to the Intestinal Transplant Registry, the cause of death after SITx is 51% within one year and remains 41.7% after one year. Under physiological conditions, the small intestinal mucosa is a barrier against toxins and pathogens in the gut lumen. Ischemia-reperfusion (I/R) and rejection damage of the intestinal mucosa after heterotopic intestinal transplantation (HIT) easily lead to bacterial translocation and endotoxemia. Bone marrow mesenchymal stem cells (BM MSCs) reduce intestinal I/R injury in rats and contribute to significant prolongation of graft survival. However, the mechanism is unclear. Although previous studies have provided insight into the molecular structure of tight junctions (TJs), considerably less is known about their functionality under physiological or pathophysiological conditions. Few studies have described the intestinal mucosa ultrastructure or TJ changes after HIT. In this study, the authors used a rat model of HIT to investigate the effect of BM MSCs on intestinal mucosa ultrastructure, with emphasis on the mechanisms of intestinal barrier dysfunction.

Research frontiers

In this study, the authors demonstrated that BM MSC implantation decreased intestinal permeability and preserved intestinal mucosal barrier function after HIT in rats. The mechanism was linked to reduced tumor necrosis factor-α and interferon-γ levels, increased interleukin-10 and transforming growth factor-β levels, and increased protein and mRNA expression of the intestinal TJ proteins occludin and zona occludens (ZO)-1.

Innovations and breakthroughs

This is believed to be the first study to report that BM MSCs reduce rejection after HIT, occludin and ZO-1 downregulation, and TJ disruption *via* a cytokine-regulated mechanism.

Applications

By understanding how BM MSCs protect the intestinal mucosal barrier, this study may represent a future strategy for therapy or prevention of rejection and sepsis after SITx, which is a serious and common complication.

Terminology

TJs are the most important structures in the mucosal barrier. They are composed of multiple proteins, including transmembrane proteins such as occludin, tricellulin, claudins, and junctional adhesion molecule. The intracellular portions of these transmembrane proteins interact with cytoplasmic peripheral membrane proteins, including ZO-1, -2, -3, and two distinct transmembrane proteins: occludin and claudin, which are linked to the actin-based cytoskeleton. TJs function as occlusion barriers by maintaining cellular polarity and homeostasis and by regulating paracellular space permeability in the epithelium.

Peer review

This paper demonstrates the impact of BM MSCs on rat small intestine after HIT. This study will be of interest and the paper is clearly written.

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Inhibitory effects of rapamycin on the different stages of hepatic fibrosis

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Abstract

AIM: To investigate and compare the inhibitory effects of rapamycin in the different stages of liver fibrosis.

METHODS: We performed bile duct ligation (BDL) in male Wistar rats (n = 24). The experimental rats were classified into four groups: the BDL^{+}/Rapa^{-} group (un-treated control, n = 4), the BDL^{+}/Rapa^{+} group (treated 14 d after BDL, n = 8), the BDL^{+}/Rapa^{++} group (treated on the day after BDL, n = 8), and the BDL^{-}/Rapa^{-} group (un-treated, sham -operated control, n = 4). The BDL^{+}/Rapa^{+} and BDL^{+}/Rapa^{++} groups were administered rapamycin (2 mg/kg) for 28 d. The liver tissues were tested by immunohistochemical staining for α-smooth muscle actin (α-SMA) and cytokeratin.

RESULTS: The liver mRNA levels of transforming growth factor (TGF)-β1 and platelet-derived growth factor (PDGF) were measured using the polymerase chain reaction. The protein levels of liver p70s6K and p-p70s6k were determined using Western blotting. α-SMA expression was lowest in the BDL^{+}/Rapa^{++}group. TGF-β1 and PDGF expression levels in the rapamycin-treated group were lower than those in the un-treated group and higher than those in the control groups (TGF-β1: 0.23 ± 0.00 *vs* 0.34 ± 0.01, 0.23 ± 0.0 *vs* 0.09 ± 0.00, P < 0.0001; PDGF: 0.21 ± 0.00 *vs* 0.34 ± 0.01, 0.21 ± 0.0 *vs* 0.09 ± 0.00, P < 0.0001). The p70s6k and p-p70s6k levels decreased in the treated groups and were lowest in the BDL^{+}/Rapa^{++}group (p70s6k: 1.05 ± 0.17 *vs* 1.30 ± 0.56, 0.40 ± 0.01 *vs* 1.30 ± 0.56, P < 0.0001; p-p70s6k: 1.40 ± 0.5 *vs* 1.67 ± 0.12, 0.70 ± 0.01 *vs* 1.67 ± 0.12, P < 0.0001).

CONCLUSION: The results of our study indicate that rapamycin has inhibitory effects on liver fibrosis, and the treatment is most effective in the early stages of fibrosis.

Key words: Liver cirrhosis; Sirolimus; Transforming growth factor beta; Platelet-derived growth factor; Ribosomal protein S6 kinases

Core tip: Liver cirrhosis is a serious disease causing significant mortality, but a curative treatment has not yet been developed. Therefore, there is great interest within the field of drug development in developing agents capable of inhibiting the progression of hepatic fibrosis. Rapamycin is an immunosuppressive agent that is also expected to attenuate the progression of liver fibrosis. We therefore aimed to investigate the inhibitory effects of rapamycin in the early and late stages of fibrosis, with the goal of contributing to the development of novel fibrosis treatments.

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INTRODUCTION

Liver cirrhosis, which is the end result of the fibrosis that accompanies several chronic liver diseases, can yield deadly complications in affected patients.is accompanied by complications that are often the cause of death of cirrhosis patients. Bile duct ligation (BDL) has been widely used as a model of liver fibrosis in animal models[1-4].

Transforming growth factor beta 1 (TGF-β1) and platelet-derived growth factor (PDGF) are key cytokines involved in the healing process that occurs following acute or chronic liver damage[5]. TGF-β1, which is a 25-kDa dimeric protein that is secreted in its latent form, is converted into its active form after liver injury. This molecule is a pro-fibrogenic cytokine, the serum and tissue levels of which increase in models of chronic liver disease. In various tissues, such as the liver, kidney, lung, bone marrow, and skin, TGF-β1 is involved in the recovery of damaged tissues, playing an important role in fibrotic processes[6-8].

PDGF is a potent proliferative cytokine that is involved in the promotion of cell division and angiogenesis. It is associated with fibrosis, atherosclerosis, and malignant disease[9-12].

The 70-kDa ribosomal S6 kinase (p70s6k), which is activated by growth factors and hormones through the phosphatidylinositol 3-kinase (PI3K)-dependent signaling pathway, is a downstream molecule of the mammalian target of rapamycin (mTOR) and is involved in protein synthesis and cell cycle control. Phospho-p706k (p-p70s6k) is the active form of p70s6k and plays an important role in the G1/S cell cycle transition. The role of p70s6k in cell cycle control has been assessed in previous studies in the presence of rapamycin, also known as sirolimus[13].

Rapamycin is a bacterial macrolide antibiotics that blocks cell proliferation by inhibiting the G1/S transition in several cell types. In particular, several studies have shown that rapamycin delays the G1/S transition of fibroblasts, making it of potential use in the treatment of fibrotic diseases. Rapamycin is used clinically as an important immunosuppressive drugs to prevent rejection after organ transplantation. It also inhibits the growth of mammalian cells, such as B and T lymphocytes, by suppressing the activation of p70s6k and inhibiting mTOR[14-18].

The levels of alpha smooth muscle actin (α-SMA), a marker of activated hepatic stellate cells (HSCs) and myofibroblasts, increase after chronic liver damage. Therefore, α-SMA can be used to identify and quantify activated HSCs in liver fibrosis[19-21].

Cytokeratin 19 (CK 19), is involved in cytoskeleton formation, is expressed in bile duct cells and their related carcinomas. Although this protein shows almost no expression in hepatocytes, it has emerged as an important marker of liver stem cells[22].

The aim of our study was to investigate the inhibitory effects of rapamycin in BDL-induced liver fibrosis in rats. We classified the treatment animals into 2 groups, the BDL and control groups, and administered rapamycin to both groups to determine the efficacy of its inhibitory effects according to the degree of liver fibrosis.

MATERIALS AND METHODS

Animals and ethics

Normal male Wistar rats (approximately 100 g body weight) were kept in 12-h light/dark cycles with free access to food and water. All the animal experiments were approved by a state-appointed animal ethics board. All institutional and national guidelines for the care and use of laboratory animals were followed.

Induction of fibrosis and sham surgery

The BDL procedure was performed on 20 rats. Before surgery, the rats were anesthetized using 0.2 mL of a 1:4 mixture of ketamine (Huons Co., Korea) and Rompun (Byer Co., Germany). In the BDL group, the middle part of the bile duct was cut and the 2 ends were tied. The control group ($BDL^-/Rapa^-$) rats (n = 4) underwent sham laparotomies.

Rapamycin administration in the treatment groups

Four of the 20 rats that underwent BDL were allocated to the $BDL^+/Rapa^-$ group(non-treatment group), and the remaining 16 rats were given rapamycin. A sonde was used for the daily oral administration of the drug at a certain time, and the administration occurred at different time points. Eight rats were given with 2 mg/kg Rapamune (Wyeth Co., Puerto Rico, United States) 14 d post-BDL ($BDL^+/Rapa^+$, 2 wk post-BDL), and the other 8 rats given the drug immediately after BDL ($BDL^+/Rapa^{++}$, immediately after BDL). The drug administration period was 28 d for all subjects.

Hepatic tissue and blood sample collection

The survival times of the 4 groups were 42 d and all rats were sacrificed after the treatment of the $BDL^+/Rapa^+$ group was completed. The hepatic tissue was stored at -70 °C for the polymerase chain reaction (PCR) and Western blot analyses. Blood samples were collected by puncturing the heart under anesthesia and were centrifuged (3000 rpm, 10 min). Aspartate transaminase (AST), alanine transaminase, total protein (TP), albumin (Alb), and total bilirubin (TB) levels were measured using a measuring device (FUJI FILM, DRI-CHEM 4000I, Japan).

Haematoxylin and eosin staining and masson trichrome staining

For the hematoxylin and eosin (HE) staining, the tissues were fixed in formalin, stained with Harris's hematoxylin for 5 min, washed with tap water for 5 min, treated with 1% acid alcohol for 30 s, and then washed under running water for 1 min. This procedure was followed by staining with eosin for 2 min, dehydrating with alcohol, and sealing with xylene.

For the Masson Trichrome (MT) staining, the tissue that was fixed in formalin was incubated for 30 min with Bouin's fixatives at 56 ℃, stained with Weigert's hematoxyline for 10 min for nuclear staining, and washed with running water for 10 min. Biebrich scarlet-acid fuchsin was used to initiate the reaction or 10 min, after which time each sample was incubated in phosphomolybdic-phosphotungstic acid for 10 min for mordant treatment and discoloration. The tissue was incubated with aniline blue for 10 min and, then treated with acetic acid for 3 min to allow time for the discoloration of the uncombined aniline blue. Finally, the tissue was washed in running water for 2 min.

Immunohistochemistry

α-SMA was used for the staining of the monoclonal mouse anti-human smooth muscle actin (DAKO Co., Denmark). First, paraffin-removed tissue sections were treated with a 0.1 mol/L citric acid solution (pH 6.0) for antigen retrieval. The antibody was treated with dilutions in multiples of 1:40-1:80.

CK 19 also involved the use of paraffin-removed tissue sections for antigen retrieval with a PT link (DAKO Co., Denmark). This antibody (DAKO Co.) was diluted in multiples of 1:250 and then stained using the DAKO stain.

RNA extraction, cDNA generation and PCR

To extract the total RNA from the control and BDL rat livers, 1 mL of Trizol (Invitrogen, CA, United States) was added to the tissues, and the resulting samples were homogenized. The homogenates were mixed with 200 μL of chloroform. After incubation for 5 min at room temperature, the homogenates were centrifuged at room temperature for 10 min at 13200 *g*. The supernatants were transferred to clean tubes containing 1000 μL of isopropyl alcohol (Sigma-Aldrich, St. Louis, MO, United States) followed by centrifugation at 13200 rpm for 30 min. The resulting supernatant was mixed with 500 μL of DEPC-treated water and centrifuged at room temperature for 10 min at 13200 *g*. The resulting supernatant was discarded, and the pellet was dried at room temperature, dissolved in DEPC-treated water (Sigma-Aldrich, St. Louis, MO, United States), and stored at -75 ℃. The quality and integrity of the RNA were confirmed by agarose gel electrophoresis. A total of 1 μg of the RNA was used to prepare the cDNA by random priming using a First-Strand cDNA Synthesis Kit (Enzynomics, Daejeon, Korea). The PCR conditions were as follows: initial denaturation at 95 ℃ for 5 min followed by 35 cycles at 95 ℃ for 25 s, 54.5 ℃ for 25 s, and 72 ℃ for 25 s, followed by a final extension step of 72 ℃ for 5 min. The PCR products were analyzed by electrophoresis on 1.5% agarose gels. GAPDH was used as a housekeeping gene control. The primer sequences used are listed in Table 1.

Table 1 Primer sequences for the polymerase chain reaction

Gene		Primer sequence	Product size (bp)
TGF-β1	Forward	5'-TACAGGGCTTTCGCTTCAGT-3'	394
	Reverse	5'-TGGTTGTAGAGGGCAAGGAC-3'	
PDGF	Forward	5'-GTCGAGTCGGAAAGCTCATC-3'	416
	Reverse	5'-GTCACCCGAGTTTGAGGTGT-3'	

TGF-β1: Transforming growth factor-β1; PDGF: Platelet derived growth factor.

Western blotting

The frozen tissue was pulverized with a precooled mortar and pestle and then homogenized at 4 ℃ in 1 × radioimmunoprecipitation assay buffer (Sigma-Aldrich). Following centrifugation of the homogenates at 13000 *g* for 20 min at 4 ℃, the pellets were discarded and the supernatants were either used immediately or stored at -70 ℃. The TP was measured using a Bradford dye-binding protein assay kit (Thermo Scientific, MA, United States). An aliquot of the supernatant was kept for protein determination and Laemmli sample buffer (Bio-Rad, CA, United States) containing β-mercaptoethanol was added to the remainder of the supernatant. The samples were boiled for 5 min, and 50 μg of the TP samples were loaded onto a 10% polyacrylamide gel after cooling. The samples were electrophoresed in a Mini-Protean Tetra-Cell electrophoresis assembly (Bio-Rad) under constant voltage. The proteins were then transferred to polyvinylidene fluoride membranes using a Mini-Protean trans-blot semidry transfer cell for 2 h at 4 ℃. Non-specific binding sites were blocked by incubation in 1 × phosphate buffered saline (PBS) containing 5% skim milk and 0.1% Tween 20 (PBSTM) for 1 h. The membranes were then incubated overnight at 4 ℃ with an antibody targeting p70s6k (1:250) or, p-p70s6k (1:250). After washing with 1 × PBSTM, the membrane was incubated for 1 h at room temperature with goat anti-rabbit IgG-horseradish peroxidase (1:2000) and then washed for 5 min in 1 × PBSTM a total of four times. The membranes were also probed with an anti-actin monoclonal antibody (1:1000) as an internal control. Immunoreactive proteins were detected and visualized with a chemiluminescence reagent (Daeillab service, Seoul, Korea) and the scanned films were quantified using a gel documentation system (Dongjinsa, Seoul, Korea). All antibodies were purchased from Santa Cruz Biotechnology (CA, United States) or Cell Signaling Technology (MA, United States).

Statistical analysis

After measuring the total weight and liver weights, calcu-

Table 2 Basal characteristics and results of blood chemistry

Treatment group	$BDL^+/Rapa^-$ (n = 4)	$BDL^+/Rapa^+$ (n = 7)	$BDL^+/Rapa^{++}$ (n = 7)	$BDL^-/Rapa^-$ (n = 4)	P value[1]
TBWt (g)	323.25 ± 85.84	351.28 ± 55.42	338.57 ± 21.16	276.25 ± 15.9	0.136
Liver Wt (g)	20.49 ± 2.68[a]	23.55 ± 4.90[a]	21.53 ± 3.10[a]	10.85 ± 0.38	< 0.0001
Liver Wt/TBWt	0.065 ± 0.011[a,c]	0.069 ± 0.017[a]	0.064 ± 0.010[a,c]	0.040 ± 0.003[c]	0.010
AST (U/L)	254.50 ± 143.79	244.43 ± 97.56	293.57 ± 89.67	86.75 ± 10.69	0.023
ALT (U/L)	47.00 ± 17.87	50.86 ± 21.97	52.00 ± 17.58	34.25 ± 2.63	0.429
TB (mg/dL)	9.15 ± 2.16[a]	8.74 ± 0.92[a]	11.17 ± 2.11[a]	0.50 ± 0.14	< 0.0001
TP (g/dL)	5.48 ± 1.28	5.37 ± 0.33	5.74 ± 0.61	5.48 ± 0.38	0.773
Alb (g/dL)	2.65 ± 0.85[a]	2.81 ± 0.41[a]	3.43 ± 0.43[a]	3.83 ± 0.33	0.008

[1]Statistical significances were tested by one-way analysis of variances among groups and statistically significant results are indicated by P values. [a]$P < 0.05$ *vs* $BDL^-/Rapa^-$; [c]$P < 0.05$ *vs* $BDL^+/Rapa^+$ based on Scheffe multiple comparison test. Basal characteristics and results of blood chemistry in bile duct ligation ($BDL^+/Rapa^-$), bile duct ligation with rapamycin ($BDL^+/Rapa^+$, $BDL^+/Rapa^{++}$) and sham-operated ($BDL^-/Rapa^-$) groups. Each values indicate mean ± SD. TBWt: Total body weight; Wt: Weight; TP: Total protein; Alb: Abumin; AST: Aspartate transaminase; ALT: Alanine transaminase; TB: Total bilirubin; Rapa: Rapamycin; $Rapa^+$: Rat administered rapamycin for 28 d from the 14th day post-BDL; $Rapa^{++}$: Rat administered rapamycin for 28 d beginning the first day post-BDL.

lating the ratio of the total weights to the liver weights, and averaging the blood test results, a one-way analysis of variance (ANOVA) was performed to examine the between-group significance. PCR and Western blot bands were examined using the IMT *i*-solution program to calculate the multiples of the densities and areas. In addition, we measured the ratio of GAPDH (control protein) to actin twice. The results are reported as the mean ± SE. SPSS 20.0 was used for the one-way ANOVA. We compared the *P*-values to assess the statistical significance. A P value of less than 0.05 was considered significant.

RESULTS

The baseline characteristics and blood chemistry of the experimental groups

One rat in each of the rapamycin-treated groups (the $BDL^+/Rapa^+$ and $BDL^+/Rapa^{++}$) died on the second and third days after the 28 d of drug administration. Thus, 4 rats in $BDL^+/Rapa^-$, 7 rats in the $BDL^+/Rapa^+$, 7 rats in the $BDL^+/Rapa^{++}$, and 4 rats in the $BDL^-/Rapa^-$ group completed the experiment. A total of 22 rats underwent anesthesia for blood collection, and their total weights and liver weights were measured before performing the autopsies. The experimental animals were killed 6 wk after BDL, and the liver tissues were collected. According to the one-way ANOVA, there was no difference in the total weights between groups (P = 0.136), but a significant difference was found in the ratio of the liver weights to the total weights (liver wt/TB wt) between the groups (P = 0.010). Similarly, the AST levels were significantly different between the groups (P = 0.023), as were the TB and Alb levels ($P < 0.0001$ and, P = 0.008, respectively) (Table 2).

HE and MT staining

HE staining demonstrated that the portal areas of the $BDL^+/Rapa^-$ rats were markedly expanded with evidence of ductular proliferation and a severe inflammatory reaction (moderate to severe periportal activity). The $BDL^+/Rapa^+$ rats, which received drug treatment 14 d after BDL, exhibited a moderate expansion in their portal area, with marked ductular proliferation and a mild to moderate inflammatory reaction (mild to moderate periportal activity). The $BDL^+/Rapa^{++}$ rats, which received drug treatment immediately after BDL, exhibited a mild in their portal areas, with mild ductular proliferation and a mild inflammatory reaction (mild periportal activity) (Figure 1A). The MT staining results revealed showed marked portal-portal fibrotic septa formation and collagen deposition (blue) in the $BDL^+/Rapa^-$ group. Reduced fibrotic septa formation was observed in the $BDL^+/Rapa^+$ and $BDL^+/Rapa^{++}$ groups, and the $BDL^+/Rapa^{++}$ group exhibited the lowest levels of fibrotic septa formation and collagen deposition (Figure 1B).

Immunohistochemical staining of α-SMA and CK 19

The results of the α-SMA staining in the $BDL^+/Rapa^-$ rat livers revealed strong staining (dark brown) in the portal-portal area compared with the $BDL^-/Rapa^-$ rat livers. The treated $BDL^+/Rapa^+$ and $BDL^+/Rapa^{++}$ livers showed relatively decreased expression, especially in the $BDL^+/Rapa^{++}$ group, in which the rapamycin was administered immediately following BDL (Figure 1C). CK 19 protein staining confirmed the presence of greater bile duct proliferation in the $BDL^+/Rapa^-$ group (dark brown) compared with the $BDL^-/Rapa^-$ group, as well as a decreased ductular reaction in the $BDL^+/Rapa^+$ and $BDL^+/Rapa^{++}$ groups (Figure 1D).

TGFβ1 mRNA expression

The inhibitory effect of rapamycin on fibrosis was confirmed by the expression levels of TGFβ1 mRNA, which increased with fibrosis. According to the results of the one-way ANOVA, the TGFβ1/GAPDH ratios exhibited significant differences between the groups, as shown in the figures and by the densitometry results ($P < 0.0001$). According to Scheffe's multiple comparison test, the $BDL^+/Rapa^-$ rats exhibited significantly

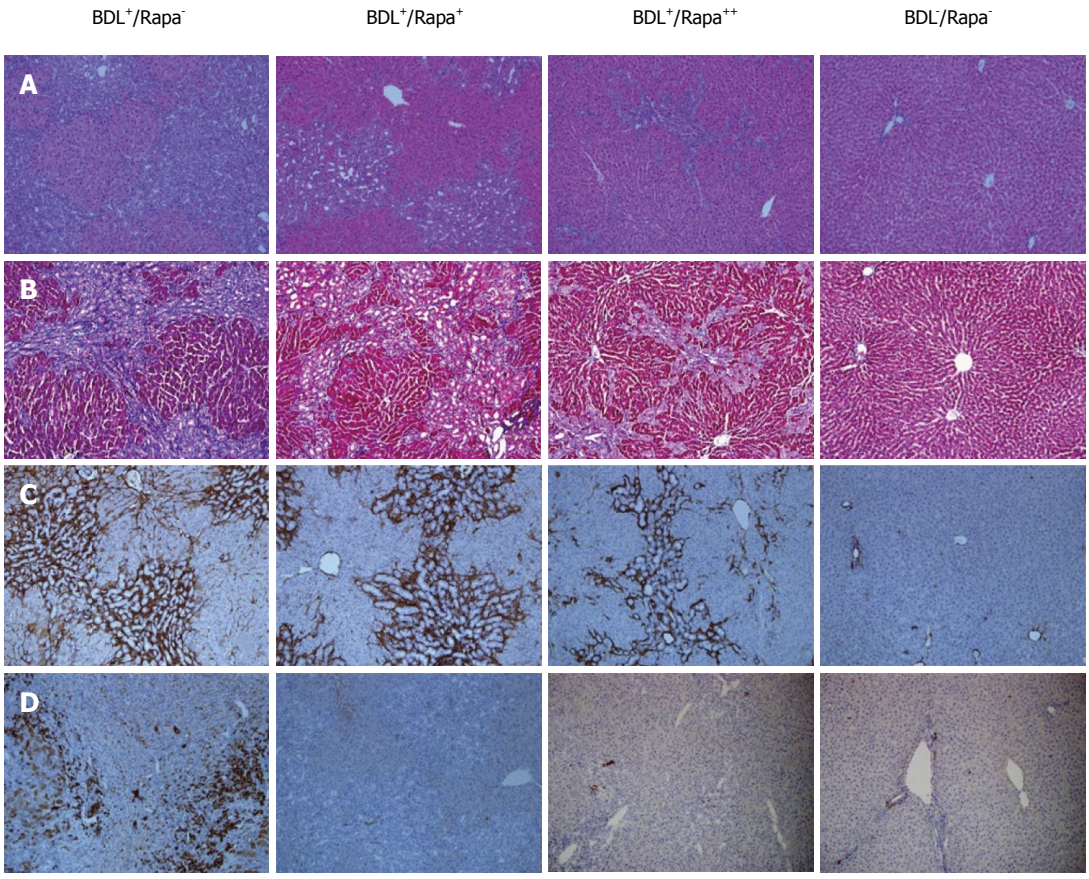

Figure 1 Comparison of histological findings between the groups that underwent bile duct ligation (BDL) with/without rapamycin treatment and the control. A: Hematoxylin and eosin (HE) staining. The bile duct ligation (BDL)$^{+}$/Rapa^{++} group showed the smallest expansion of portal areas, with the least amount of ductular proliferation and the most mild inflammatory reaction among the BDL groups; B: Masson-Trichrome staining. The BDL^{+}/Rapa^{++} group showed the lowest degree of fibrotic septa formation and deposition of collagen among BDL groups (blue); C: α-smooth muscle actin (α-SMA) staining (dark brown). The BDL^{+}/Rapa^{++} group showed the lowest amount of staining among BDL groups; D: Cytokeratin 19 (CK 19) protein expression (dark brown). The BDL^{+}/Rapa^{++} group showed the lowest amount of staining among BDL groups. Rapa: Rapamycin; Rapa^{+}: Rat administered rapamycin for 28 d from the 14th day post-BDL; Rapa^{++}: Rat administered rapamycin for 28 d beginning the first day post-BDL. Original magnification: × 100(^{a}-d).

increased expression compared with the controls ($P <$ 0.0001), and the difference in expression between the BDL^{+}/Rapa^{+} and BDL^{+}/Rapa^{++} rats was also significant ($P < 0.001$ and $P < 0.0001$). Decreased expression was found in the BDL^{+}/Rapa^{+} and BDL^{+}/Rapa^{++} groups compared with the BDL^{+}/Rapa^{-} group, further, suggesting that rapamycin has an inhibitory effects on fibrosis (Figure 2A).

PDGF mRNA expression

In the PDGF band and density one-way ANOVA, the PDGF/GAPDH values showed statistically significance differences between the groups ($P < 0.0001$). The BDL^{+}/Rapa^{+} and BDL^{+}/Rapa^{++} group showed significantly decreased level of PDGF expression compared with the BDL^{+}/Rapa^{-} group ($P = 0.001$, $P = 0.001$) (Figure 2B).

mTOR down-stream molecules p70s6k and p-p70s6k

The protein p-p70s6k is the active form of p70s6k and is involved in the mTOR signaling pathway, the action site of rapamycin, cell cycle control, and HSCs proliferation. Its expression was therefore compared with that of a control protein (actin).

The BDL^{+}/Rapa^{+} and BDL^{+}/Rapa^{++} groups showed a significant decrease in the expression of p70s6k compared with the BDL^{+}/Rapa^{-} group ($P < 0.013$, $P <$ 0.0001). The BDL^{+}/Rapa^{++} group was found to have a significantly decreased p70s6k expression level compared with the BDL^{+}/Rapa^{-} group (with no drug administration) and the BDL^{+}/Rapa^{+} group (with drug administration at a different time) ($P < 0.0001$, $P < 0.0001$).

Based on the analyses of p-p70s6k protein expression, the treated BDL^{+}/Rapa^{++} group showed a significantly decreased expressions compared with the BDL^{+}/Rapa^{+} ($P = 0.005$) and BDL^{+}/Rapa^{-} ($P = 0.001$) groups. These results demonstrate that p-p70s6k has different expression levels according to the time of drug administration, making the effects of drug administration at each time predictable (Figure 3).

DISCUSSION

In our study, we aimed to determine whether rapamycin

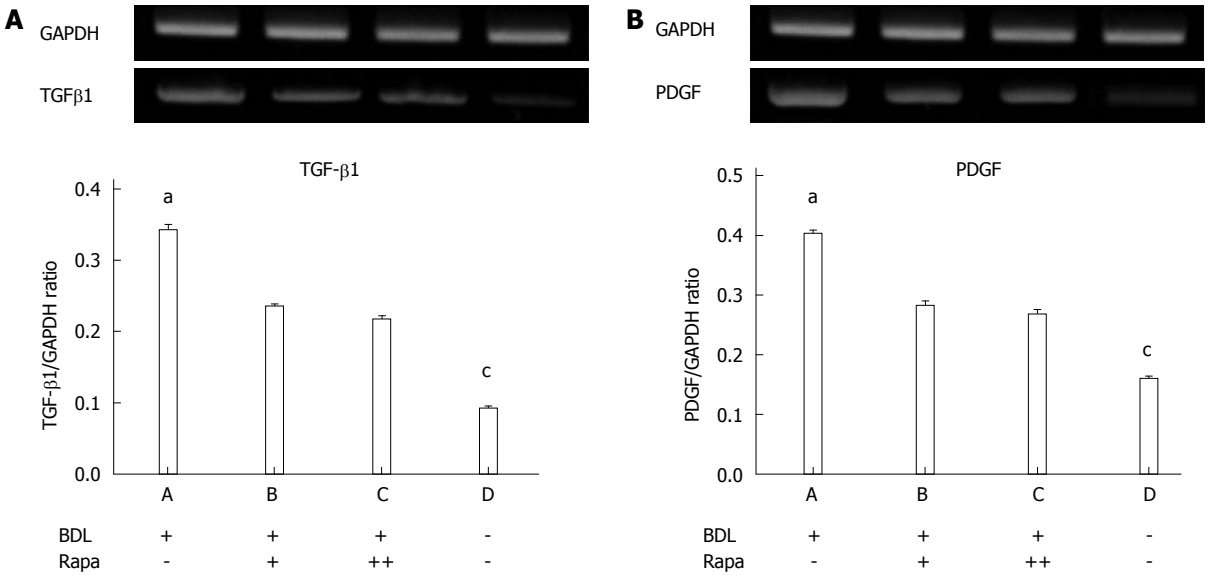

Figure 2 Transforming growth factor β1 and platelet-derived growth factor mRNA expression was assessed by polymerase chain reaction analysis. A: Transforming growth factor β1 (TGFβ1); B: Platelet-derived growth factor (PDGF). BDL: Bile duct ligation; Rapa: Rapamycin; Rapa^{+}: Rat administered rapamycin for 28 d starting the 14th day after BDL; Rapa^{++}: Rat administered rapamycin treatment for 28 d starting the 1st day after BDL; GAPDH: Glyceraldehyde 3-phosphate dehydrogenase. $^{a}P < 0.05$ *vs* other group; $^{c}P < 0.05$ *vs* BDL^{+}/Rapa^{++} group.

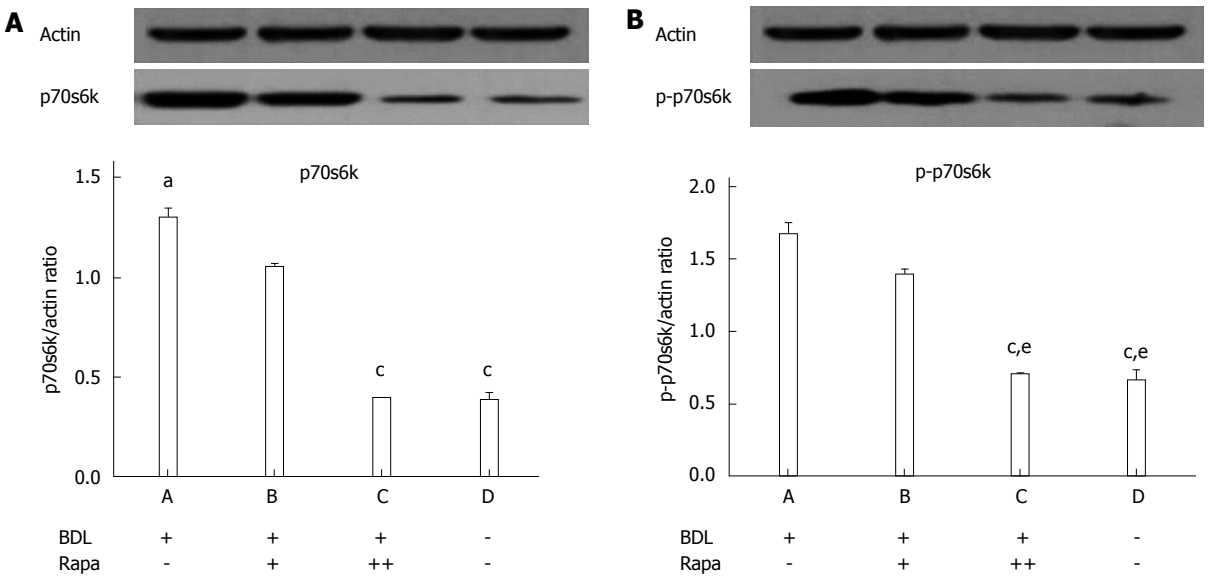

Figure 3 Western blot analyses showed the p70s6k and p-p70s6k expression levels in the advanced liver fibrosis samples. A: 70-kDa ribosomal S6 kinase (p70s6k); B: p-p70s6k. BDL: Bile duct ligation; Rapa: Rapamycin; Rapa^{+}: Rat administered rapamycin for 28 d beginning the 14th day post-BDL; Rapa^{++}: Rat administered rapamycin for 28 d beginning the first day post-BDL. $^{a}P < 0.05$ *vs* other group; $^{c}P < 0.05$ *vs* BDL^{+}/Rapa^{+} group; $^{e}P < 0.05$ *vs* BDL^{+}/Rapa^{-} group.

has an inhibitory effects on liver fibrosis. We examined changes in relevant cytokines when this drug was administered to early and more progressed cases of liver fibrosis in animal models of liver fibrosis that was induced by BDL. We administered rapamycin for 28 d during the different stages of fibrosis.

Liver fibrosis develops from the excessive deposition of extracellular matrix as proliferation-promoting cytokines and growth factors lose their regulatory control. This occurs after chronic liver damage as in the cases of viral hepatitis, alcoholic fatty liver, and non-alcoholic steatohepatitis[23,24]. Liver cirrhosis is the most advanced stage of liver fibrosis and can yield complications such as esophageal varices, hepatic encephalopathy, and peritonitis. Cirrhosis patients eventually die because of these complications. Rapamycin is mainly used to prevent rejection after transplantation; it inhibits cell proliferation and is used in coronary artery stenting to treat cardiovascular diseases[25,26]. Following additional studies on the inhibitory effects of rapamycin on the liver, the lung,

and renal fibrosis, it is expected that rapamycin will be deemed suitable as next anti-fibrosis treatment[27]. Several human studies have evaluated the antifibrotic effects of rapamycin on fibrosis and cirrhosis in humans. McKenna *et al*[28] described the impact of sirolimus in reducing the extent and progression of fibrosis in liver transplant recipients with recurrent HCV[29]. Kelly *et al*[30] suggested that sirolimus-based immunosuppression is associated with a lower risk of significant graft fibrosis following liver transplantation in HCV-infected recipients.

In this study, we confirmed the effects of rapamycin on the inhibition of fibrosis in a BDL rat model. In the previous studies of rapamycin, the drug was administered immediately after BDL through either intraperitoneal or subcutaneous injections. Non-oral administration has different absorption routes compared with the current route for rapamycin in humans. Our study used the same drug delivery mechanism as that used in humans to compensate for this limitations. The rapamycin used in this study was ground into a powder, mixed with water, and provided through a small-diameter tube (sonde) at the same time every day directly into the stomach of the rats. Drug administration was initiated at a different time points to enable partial progression of fibrosis.

Liver tissues stained with HE and MT were used for the analysis, and depositions of the extracellular matrix in the treated $BDL^+/Rapa^+$ and $BDL^+/Rapa^{++}$ groups were found to be decreased compared with those in the $BDL^+/Rapa^-$ group. In particular, the $BDL^+/Rapa^{++}$ group exhibited less collagen deposition than the $BDL^+/Rapa^+$ group. This result demonstrates the potential of rapamycin to inhibit liver fibrosis. In addition, the effects were greater in the animals in earlier stages of fibrosis.

HSCs, portal fibroblasts, and myofibroblasts are involved in liver fibrosis. α-SMA is a marker of HSCs and myofibroblasts, which were compared in this study to confirm the difference in their expression levels in the portal veins and interface zone. The $BDL^+/Rapa^-$ group was found to exhibit strong staining, and the treated $BDL^+/Rapa^+$ and $BDL^+/Rapa^{++}$ groups showed significantly decreased staining compared with the $BDL^+/Rapa^-$ group (Figure 1C). This finding is interpreted as a demonstration of the anti-fibrosis capacity of rapamycin.

CK 19 is expressed in normal epithelial cells, and its expression is increased during ductular reactions in biliary sclerosis[31]. Decreased CK 19 staining was found in the $BDL^+/Rapa^+$ and $BDL^+/Rapa^{++}$ groups compared with the $BDL^+/Rapa^-$ groups (Figure 1D).

TGF-β1 is a marker of the active form of HSCs and functions as a key cytokine in the progression liver fibrosis. Once tissue healing after damage is complete, its production is terminated for an unknown reason; however, in the case of chronic and repeated damage, this self regulation is lost[32]. The results of this study also showed increased expression in the $BDL^+/Rapa^-$ group compared with the control. However, the $BDL^+/Rapa^+$ and $BDL^+/Rapa^{++}$ groups were found to have decreased expression compared with the $BDL^+/Rapa^-$ group, thus confirming the inhibitory effects of rapamycin (Figure 2A). The *P* values of the $BDL^+/Rapa^+$ and $BDL^+/Rapa^{++}$ groups compared to the $BDL^+/Rapa^-$ group were statistically significant, at 0.001 and 0.0001, respectively. These results correspond to those of Bierker *et al*[18], suggesting that rapamycin has inhibitory effects on cell proliferation following liver damage.

PDGF-β is the strongest known mitogen and is auto-secreted in HSCs. In our comparison of the density of PCR bands between the experimental groups, the $BDL^+/Rapa^-$ group showed the greatest increase in PDGF mRNA expression ($P < 0.0001$), and the treated groups ($BDL^+/Rapa^+$ and $BDL^+/Rapa^{++}$) showed a statistically significant decrease in the expression of PDGF mRNA compared to the $BDL^+/Rapa^-$ group ($P = 0.001$). These results demonstrate that rapamycin stops the production of cytokines and decreases their expression in chronic liver damage.

p70s6k is a downstream protein of the PI3K-Akt signaling system, and its secretion is stimulated by various hormones and growth factors. p70s6k controls protein synthesis, proliferation, and the cell cycle. Activation of p70s6k occurs when serine or threonine is phosphorylated, and this active form is inhibited by rapamycin[33-35]. In our experiment, p70s6k/Actin showed significantly decreased expression levels in the $BDL^+/Rapa^+$ and $BDL^+/Rapa^{++}$ groups compared with the $BDL^+/Rapa^-$ group ($P = 0.013$, $P < 0.0001$). The $BDL^+/Rapa^{++}$ group exhibited significantly decreased expression compared with the $BDL^+/Rapa^+$ group ($P < 0.0001$). With regard to the p-p70s6k expression, the $BDL^+/Rapa^+$ group showed slightly decreased expression levels compared with the $BDL^+/Rapa^-$ group, although this finding was not statistically significant ($P = 0.117$). However, compared with the $BDL^+/Rapa^{++}$ group, there was a statistically significant decrease in the expression ($P = 0.005$). The $BDL^+/Rapa^{++}$ group was observed to have decreased expression level compared with the $BDL^+/Rapa^-$ group ($P = 0.001$) the $BDL^+/Rapa^+$ group ($P = 0.005$), (Figure 3).

The observed differences in p70s6k and p-p70s6k protein expression are consistent with the results of previous studies investigating the effects of rapamycin inhibition on p70s6k phosphorylation. However, the differences in p70s6k expression between the $BDL^+/Rapa^+$ and $BDL^+/Rapa^{++}$ groups are not consistent with the results of Biecker *et al*[18] and Zhu *et al*[36]. These authors predicted that different mechanism was involved in the mTOR pathway. The inhibition of these two proteins is believed to inhibit cell proliferation. The $BDL^+/Rapa^{++}$ and $BDL^+/Rapa^+$ groups showed significant differences in their expression levels of p70s6k and p-p70s6k, suggesting that the administration of rapamycin is more effective in the early stages of fibrosis.

We confirmed the inhibitory effects of rapamycin on fibrosis, including partially progressed fibrosis, and determined that these effects were greater during the

early stages of fibrosis. Therefore, the results of this study should be useful in developing drugs to inhibit or reduce fibrosis in patients with chronic liver diseases. One limitation of this study is the fact that rapamycin is insoluble in water. We therefore ground it into a powder mixed with water to enable administation directly into the stomach of the rats through small-diameter tubes. Given this experimental setup, it is possible that residual drug remained inside the tube, potentially decreasing the dosage to less than 2 mg/kg per day. However, this possible error was minimized by injecting air into the tube after drug administration.

In conclusion, this study confirmed that rapamycin, which is an immunosuppressant used in the treatment of transplant patients, has inhibitory effects on liver fibrosis. Importantly, these effects are more pronounced when the drug is administered immediately after the start of liver fibrosis or before progression of fibrosis. Our results showed that better treatment effects can be expected when rapamycin is administered during the early stages of fibrosis. However, administration of this drug after the progression of fibrosis is also effective. No severe side effects or adverse events due to the administration of rapamycin were noted in this study.

Therefore, the treatment of chronic liver disease patients with rapamycin is likely to inhibit the progression of liver cirrhosis while improving fibrosis is more progressed cases.

ACKNOWLEDGMENTS

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COMMENTS

Background

Liver cirrhosis is the endpoint of the fibrogenic process that accompanies chronic liver disease. Complications arising from cirrhosis frequently result in death. Thus, the inhibition of fibrosis progression is associated with improvements in the survival rate. Rapamycin is used as an immunosuppressant agent but is also believed to be an anti-fibrotic drug. Indeed, several studies have reported that rapamycin inhibits fibrosis.

Research frontiers

Rapamycin is a bacterial macrolide with immunosuppressive properties. Many studies have reported that rapamycin had an inhibitory effect on fibrogenesis in the lung, skin, and liver. In the field of of liver disease, one research area of interest in the development of a drug that can ameliorate or prevent fibrosis.

Innovations and breakthroughs

In previous studies of the ability of rapamycin to inhibit liver fibrosis, rapamycin was administered by peritoneal injection or by mixing it with drinking water in animal models. However, these are not the modes of delivery in humans. Authors therefore designed our study to be more consistent with actual treatment, such that the rapamycin was ground into a powder and mixed with water for direct injection into the rat stomach through small-diameter tubes. They also divided their treatment groups into a group treated 14 d after bile duct ligation (BDL) and a group treated 1 d after BDL. They offer a comparison of the inhibitory effects of rapamycin in the different stages of liver fibrosis.

Applications

The results suggest that rapamycin is a potential therapeutic drug that could be used to inhibit liver fibrosis.

Terminology

Fibrosis represents is the endpoint of a fibrogenic process that accompanies chronic liver injury in cases of viral hepatitis and other diseases. The complications of fibrosis are the cause of death in many cirrhotic patients. Rapamycin is a bacterial macrolide antibiotics that blocks cell proliferation. It is a clinically important immunosuppressive drugs used to prevent rejection after organ transplantation.

Peer review

The authors examined the immunohistochemical staining of α-smooth muscle actin in liver tissues, transforming growth factor and platelet-derived growth factor mRNA expression levels, and p70s6k and p-p70s6k protein levels in the experimental groups. The treated groups ($BDL^+/Rapa^{++}$ and $BDL^+/Rapa^+$) showed decreased staining and expression levels compared with the un-treated BDL group ($BDL^+/Rapa^-$). These results suggested that rapamycin has an inhibitory effect on liver fibrosis and is therefore a potential treatment drug.

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CASE CONTROL STUDY

Clinical significance of expression of tissue factor and tissue factor pathway inhibitor in ulcerative colitis

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Abstract

AIM: To investigate the clinical significance of expression of tissue factor (TF) and tissue factor pathway inhibitor (TFPI) in ulcerative colitis (UC).

METHODS: Thirty UC specimens taken by colonoscopy from patients with active UC treated at the Department of Pathology, Central Hospital Affiliated to Shenyang Medical College from February 2010 to January 2012 were included in an experimental group, and 30 normal colon tissue samples taken by colonoscopy from non-UC patients were included in a control group. Expression of TF and TFPI in UC and normal colon tissue samples was detected by immunohistochemistry.

RESULTS: The positive rate of TF in UC was significantly higher than that in normal colon tissue (63% *vs* 33%, $\chi^2 = 5.41$, $P < 0.05$). The positive rate of TFPI in UC was also significantly higher than that in normal colon tissue (43% *vs* 17%, $\chi^2 = 5.08$, $P < 0.05$).

CONCLUSION: Positive rates of TF and TFPI expression in UC are significantly higher than those in normal colon tissue. TF and TFPI may play an important role in the pathogenesis of UC.

Key words: Ulcerative colitis; Tissue factor; Tissue factor pathway inhibitor

Core tip: Recent research showed that tissue factor (TF) and tissue factor pathway inhibitor (TFPI) are associated with inflammatory reactions, and they are expressed in many kinds of tissue and cells. There are few reports about the relationship between TF and TFPI and ulcerative colitis (UC). In this study, we performed immunohistochemical staining for TF and TFPI expression in tissue of UC and normal colon, to determine their association with UC and their significances in pathogenesis. The study results suggest that TF and TFPI may play an important role in the pathogenesis of UC.

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INTRODUCTION

Ulcerative colitis (UC) is a chronic, nonspecific intestinal inflammatory disease of unknown causes, with the main clinical symptoms being abdominal pain, diarrhea, and blood and mucus in the stool. The etiology and pathogenesis of UC is very complex and not yet completely understood. A better understanding of the pathogenesis of UC will bring new clues to UC treatment. In recent years, many studies have shown that there is a correlation between UC and hypercoagulable states. Microthrombus formation may be one of the important mechanisms

responsible for the pathogenesis of UC, and ongoing hypercoagulable states may promote the development and progression of inflammation and be related to UC progression[1]. Studies have found that the pathological process and clinical complications of diseases with thrombosis are closely associated with tissue factor (TF) and tissue factor pathway inhibitor (TFPI)[2,3]. Several studies have demonstrated the presence of TF and TFPI in many human diseases with thrombosis[4,5]. Recent studies revealed a correlation between TF and TFPI and inflammation[6]. TF and TFPI are expressed in a variety of tissues and cells[5]. Currently, there have been few reports on the correlation between TF and TFPI and UC. In the present study, immunohistochemistry was used to detect the expression of TF and TFPI in UC to reveal their relationship with UC and to elucidate their role in the pathogenesis of UC.

MATERIALS AND METHODS

Subjects

Thirty UC specimens taken by colonoscopy from patients with active UC treated at Department of Pathology, Central Hospital Affiliated to Shenyang Medical College from February 2010 to January 2012 were included in an experimental group, and 30 normal colon tissue samples taken by colonoscopy from non-UC patients were included in a control group. The pathological diagnosis of UC was made by a pathologist. The experimental group contained 12 males and 18 females, and the control group contained 16 males and 14 females. The subjects ranged in age from 35 to 52 years, with a mean age of 49 years. In the experimental group, there were 3 cases of entire colon lesions, 8 cases of left-sided colon lesions, 15 cases of sigmoid lesions, and 4 cases of rectal lesions. UC was diagnosed based on the diagnostic criteria in "Analysis on Chinese Consensus on Standard Diagnosis and Treatment of Inflammatory Bowel Disease" developed by the Inflammatory Bowel Disease Collaborative Group of the Chinese Society of Gastroenterology[7]. The study protocol was approved by the Ethics Committee of Central Hospital Affiliated to Shenyang Medical College, and all subjects signed informed consent.

Main reagents and equipment

Rabbit anti-human TF monoclonal antibody and mouse anti-human TFPI monoclonal antibody were purchased from Boermei (Shenyang, China). SP kit and AEC kit were purchased from Zhongshan Golden Bridge Technology (Beijing, China). An optical microscope (Olympus BX40) and microtome (Shandon AS325) were also used.

Intestinal mucosal specimen collection

Intestinal mucosal tissues were obtained during colonoscopy from the lesion having the most severe inflammation in patients with active UC. For patients in the control group, tissue samples were taken from a site showing no abnormalities (25 cm from the anus) in the sigmoid colon. Four tissue samples were taken from each patient and stored in a refrigerator.

Immunohistochemistry

After dewaxing and hydration, paraffin sections were soaked in phosphate-buffered saline for 5 min. Antigen retrieval was then achieved using the microwave method, and endogenous peroxidase activity was inhibited by incubation with an endogenous peroxidase blocking agent (reagent A in the SP Kit) at room temperature for 10 min. After washing with PBS 3 times for 3 min each time, nonspecific binding was blocked by incubation with normal goat serum (reagent B in the SP Kit) at room temperature for 10 min. Sections were then incubated with primary antibody TF or TFPI overnight at 4 ℃. After washing, sections were incubated with a biotinylated secondary antibody (reagent C in the Universal SP Kit) at room temperature for 10 min, followed by incubation with horseradish peroxidase-labeled streptavidin (reagent D in the Universal SP Kit) at room temperature for 10 min. After washing again, the signal was developed by incubation with AEC reagent for 5-10 min. Nuclei were counterstained with hematoxylin, and mounted on slides.

Immunostaining evaluation

(1) evaluation criteria for positive TF expression: TF was localized in the membrane and cytoplasm of cells in the mucosal tissue around the ulcer, and positive immunohistochemical signals were brown. Ten typical high power fields (HPF × 400) were selected from each slide for evaluation. The extent of the immunohistochemical signal was divided into 4 levels: 0%-5% (-), 5%-10% (+), 10%-40% (++), > 40% (+++); (2) evaluation criteria for positive TFPI expression: TFPI was localized in the cytoplasm of cells in the mucosal tissue around the ulcer, and positive immunohistochemical signals were yellowish brown. Ten typical HPF (× 400) were selected from each slice for evaluation. The extent of the immunohistochemical signal was divided into 4 levels as above; and (3) positive rate: Ten HPF were observed for each case. The percentage of positive cells were divided into 4 levels as above.

Statistical analysis

Statistical analyses were performed using SPSS 11.0 for Windows software (SPSS Inc., Chicago, IL, United States). Percentages were compared using the χ^2 test. P values < 0.05 were considered statistically significant.

RESULTS

Expression of TF in UC and normal colon tissues

The positive rate of TF expression in UC was 63% (19/30), significantly higher than that in normal colon tissues (33%, 10/30) ($P < 0.05$, Table 1, Figure 1A and B).

Expression of TFPI in UC and normal colon tissues

The positive rate of TFPI expression in UC was 43% (13/30), significantly higher than that in normal colon tissues (17%, 5/30) ($P < 0.05$, Table 2, Figure 2A and B).

Table 1 Expression of tissue factor in ulcerative colitis and normal colon tissues

Group	Number of total cases	Number of positive cases	Number of negative cases	Positive rate
Experimental	30	19	11	63%
Control	30	10	20	33%

The data indicated that the positive rate of tissue factor expression in ulcerative colitis was higher than that in normal colon tissues. $\chi^2 = 5.41$, $P < 0.05$ *vs* control.

Table 2 Expression of tissue factor pathway inhibitor in ulcerative colitis and normal colon tissues

Group	Number of total cases	Number of positive cases	Number of negative cases	Positive rate
Experimental	30	13	17	43%
Control	30	5	25	17%

The data indicated that the positive rate of tissue factor pathway inhibitor expression in ulcerative colitis was higher than that in normal colon tissues. $\chi^2 = 5.41$, $P < 0.05$ *vs* control.

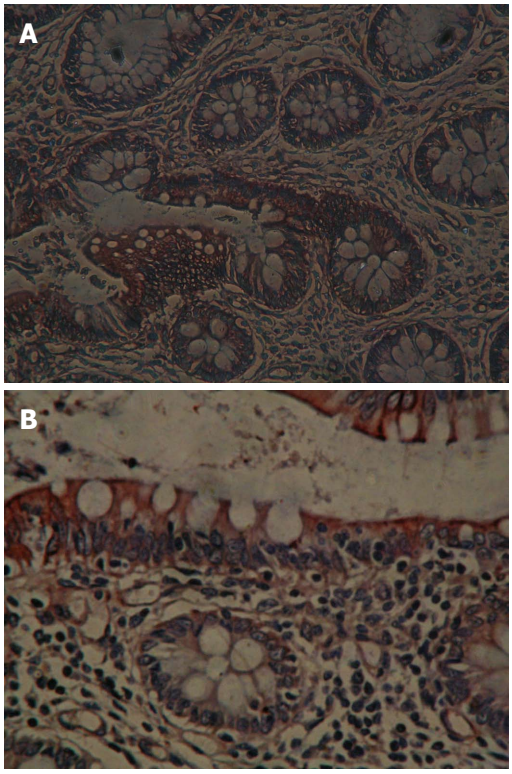

Figure 1 Positive expression of tissue factor in ulcerative colitis (A) and normal colon tissue (B). Magnification, × 400.

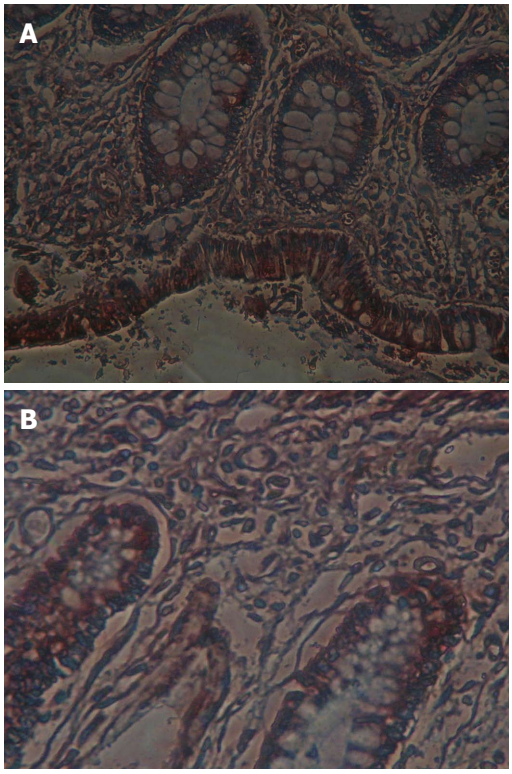

Figure 2 Positive expression of tissue factor pathway inhibitor in ulcerative colitis (A) and normal colon tissue (B). Magnification, × 400.

DISCUSSION

The etiology and pathogenesis of UC are very complex and are still not very clear. It is currently believed that inflammatory processes elicited by mucosal immune system abnormalities caused by multiple factors, including environmental, immunological and genetic factors, play an important role in the pathogenesis of UC[8,9]. In recent years, the incidence of UC has increased, and there are more reported cases of critically ill patients. Clinical studies indicate that there exist hypercoagulable states and a potential risk of thrombosis in UC patients. UC complicated with thromboembolic diseases are not uncommon, and autopsies revealed that up to 39% of UC patients have thromboembolic diseases. Thromboembolic diseases have become the third leading cause of death in patients with UC[10,11]. Intestinal multifocal infarction caused by microvascular inflammation has become an important mechanism in the pathogenesis of UC. Persistent hypercoagulable states may be related to the clinical progression of UC[1], and have a role in promoting the development and progression of inflammation. In this study, immunohistochemistry was used to detect the expression of molecular biomarkers TF and TFPI in UC tissues to provide further information in the elucidation of the pathogenesis of UC.

Studies have found that the pathological process and clinical complications of diseases with thrombosis are closed associated with TF and TFPI. Studies have demonstrated the presence of TF and TFPI in many human diseases with thrombosis. Recent studies have also found that there exists an association between TF and TFPI and inflammation.

TF is a protein with 236 amino acids, and contains an extracellular domain, a transmembrane domain and a cytoplasmic domain. It is now clear that the extracellular

domain is critical for the binding to FVII and the initiation of the extrinsic coagulation pathway, and has procoagulant activity and proteolytic functions[12]. The TF-initiated coagulation process was formerly known as the extrinsic coagulation pathway. Research advances over the past decade have shown that this is a start-up phase of coagulation, and the intrinsic coagulation pathway belongs to the maintenance phase of coagulation. TF has important significance in physiological hemostasis and pathological thrombosis and participates in the inflammatory response. The transmembrane domain may also play a role in signal transduction, but the function of the intracellular domain has been a hotspot of TF research in recent years. The phosphorylation of 3 serine residues in the terminus of the cytoplasmic domain can mediate intracellular signal transduction and promote the transcription and synthesis of vascular endothelial growth factor (VEGF)[13,14]. Ollivier *et al*[15] found that, in human pulmonary fibroblasts, TF-dependent VEGF expression requires binding between FVII and TF, and binding of FVII with inactivated active sites and TF inhibits VEGF production. Bulut *et al*[16] suggested that VEGF is involved in the pathogenesis of UC and is related to disease severity. TF binding to its receptor FVIIa triggers intracellular signal transduction mechanisms and induces the upregulation of matrix metalloproteinases (MMPs), which are involved in tissue repair and have very important significance in inflammatory diseases. A large number of studies have confirmed that MMPs are involved in basal inflammatory tissue repair, angiogenesis and leukocyte chemotaxis in UC patients, and MMPs are highly expressed in UC tissues and are enhanced with the aggravation of inflammation[17,18]. The results of the present study showed that the positive rate of TF expression in UC tissue (63%) was significantly higher than that in normal colon tissue (33%) ($P < 0.05$), indicating that the expression level of TF differs between UC tissue and normal colon tissue. This finding suggests that TF may be associated with the occurrence of UC, and hypercoagulable states and intestinal microthrombosis may mediate the role of TF in UC. In addition, TF, as an indirect inflammatory mediator to stimulate the release of other inflammatory mediators and induce inflammatory responses, may be involved in this process.

As the only physiological inhibitor of TF-FVIIa complex, TFPI inhibits TF-induced thrombus formation. It depends on the feedback inhibition of FXa to regulate the extrinsic coagulation pathway, preventing the unlimited expansion of clotting[19]. TFPI plays an important role not only in the extrinsic coagulation pathway[20,21], but also in inflammatory responses. In the process of inflammatory responses, when endothelial cells contact with tumor necrosis factor (TNF), endotoxin and thrombin, TFPI output on the cell surface significantly increases, and TF activity is increased[22]. Animal experiments and clinical studies showed that TFPI has anti-inflammatory effects, and it can inhibit leukocyte activation as well as the synthesis and expression of inflammatory mediators including TNF-α, interleukin (IL)-6 and IL-1. These inflammatory mediators have a clear association with the occurrence of UC and are important in the pathogenesis of UC[23,24]. TFPI not only has inhibitory effects on the TF-FVIIa complex, FXIa and cathepsin G[25], as well as serine proteases, plasmin, trypsin, chymotrypsin, plasma kallikrein and FXa[26], but also is an indirect inhibitor of MMPs in the extracellular matrix[27]. Studies have demonstrated that the expression of MMP-3 and MMP-9 is significantly increased in the mucosal inflammatory area in UC patients, and is enhanced with aggravation of inflammation. Clinical trials showed that blood levels of TFPI were significantly higher in UC patients than in normal controls, and in patients with active UC than in those with the disease in the remission period. The results of the present study showed that the positive rate of TFPI in UC tissue (43%) was significantly higher than that in normal colon tissue (17%) ($P < 0.05$), indicating that the expression level of TFPI differs between UC tissue and normal colon tissue. This finding suggests that TFPI may have a role in the occurrence of UC, possibly by inhibition of TF-induced thrombosis and suppression of inflammatory mediator release to inhibit inflammation. Therefore, the application of recombinant TFPI to inhibit the activation of inflammatory cells and the release of inflammatory mediators can be considered a strategy to treat UC[28], and this may provide a new avenue for the treatment of UC.

In the present study, immunohistochemistry was used to investigate the expression of TF and TFPI in UC tissue, and the results may help understand the molecular mechanisms of pathogenesis of UC. Clinical detection of TF and TFPI may be helpful in the diagnosis of UC. The present study explored the mechanisms of pathogenesis of UC from another perspective, and the results obtained are expected to be able to guide the selection of reasonable treatment regimens and provide new ideas for the clinical treatment of UC. However, due to the limitations of experimental conditions and funding, the present study did not conduct a deeper exploration of UC pathogenesis. Future studies are required to further explore the pathogenesis, diagnosis, treatment and prognosis of UC.

COMMENTS

Background

Ulcerative colitis (UC) is associated with a blood hypercoagulable state and microthrombus formation may be one aspect of the pathogenesis of UC. Thrombus formation is closely associated with tissue factor (TF) and tissue factor pathway inhibitor (TFPI).

Research frontiers

A persistently hypercoagulable state may be related to the clinical progression of UC, which can promote inflammation take place and progress. Recent research showed that TF and TFPI expression was associated with thrombus formation and also inflammatory reactions, with expression in many kinds of tissue and cells.

Innovations and breakthroughs

There are few reports about the relations between TF and TFPI and UC. In this study, authors performed immunohistochemical staining for TF and TFPI expression in tissue of UC and normal colon, and found significantly higher expression in UC tissue.

Applications

The study results suggest that TF and TFPI may play an important role in the pathogenesis of UC. TF and TFPI expression in tissue can help diagnosis of UC TFPI and TF research may provide new ideas for clinical treatment.

Peer review

This is a good study in which authors analyze the expression of TF and TFPI in tissue of UC. The results are interesting and suggest that TF and TFPI may play an important role in the pathogenesis of UC.

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CASE CONTROL STUDY

Association of *MYO9B* gene polymorphisms with inflammatory bowel disease in Chinese Han population

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Abstract

AIM: To explore the association of *MYO9B* gene polymorphisms with clinical phenotypes and intestinal permeability of individuals with inflammatory bowel disease (IBD) in China.

METHODS: A total of 442 IBD patients and 402 healthy volunteers were genotyped for two single nucleotides (rs962917 and rs1545620) using the ligase detection reaction and polymerase chain reaction. Allelic and genotype frequency analyses were performed for the two groups. Intestinal permeability was evaluated using lactulose (L) and mannitol (M) excretion. The association of *MYO9B* gene polymorphisms with intestinal permeability between the normal and high intestinal permeability groups was analyzed.

RESULTS: Overall, there was no significant difference in the genotypic and allelic frequencies of *MYO9B* between IBD patients and controls. Although no association was found with ulcerative colitis in the comparison between the subgroups, the frequencies of rs962917 and rs1545620 were different in the Crohn's disease (CD) subgroup with ileocolitis (CC *vs* CT and TT, P = 0.014; and AA *vs* AC and CC, P = 0.022, respectively). rs1545620 variants appear to be the genetic susceptibility factor for perianal disease in CD patients (AA *vs* AC CC, P = 0.029). In addition, the L/M ratio was significantly higher in IBD patients than in controls (0.065 ± 0.013 *vs* 0.020 ± 0.002, P = 0.02), but no association was found between the *MYO9B* gene and the L/M ratio in IBD patients.

CONCLUSION: *MYO9B* gene polymorphisms may influence the sub-phenotypic expression of CD in China. No association between these *MYO9B* polymorphisms and intestinal permeability in IBD patients was found.

Key words: Inflammatory bowel disease; Crohn's disease; Ulcerative colitis; MYO9B; Genetic susceptibility; Intestinal permeability

Core tip: An association between *MYO9B* gene polymorphisms and inflammatory bowel disease (IBD) in the Chinese Han population has not yet been confirmed. The authors aimed to explore the association of *MYO9B* gene polymorphisms with the clinical phenotypes and intestinal permeability of IBD in China. The results suggested that *MYO9B* gene polymorphisms may influence the sub-phenotypic expression of Crohn's disease but failed to confirm an association between the *MYO9B* polymorphisms and intestinal permeability in Chinese Han IBD. These findings indicate that the *MYO9B* gene may differ among IBD patients of various races from various regions.

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INTRODUCTION

Inflammatory bowel disease (IBD), which includes Crohn's disease (CD) and ulcerative colitis (UC), is a chronic recurrent inflammation of the gastrointestinal tract of unknown origin[1]. Environmental influences, immunological factors, and genetic background may play important roles in its etiopathogenesis[2-4]. Since the initial identification of *CARD15* as a CD susceptibility gene in 2001, genetic studies have shown that there are numerous genetic susceptibility factors for IBD[5-10]. The genetic background of IBD may be different in Asian individuals compared with Western populations. For example, variations in *OCTN* or *CARD15* are generally accepted to be associated with susceptibility to CD in Western populations[11,12]. However, these associations have not been confirmed in Chinese individuals[13].

Studies have provided evidence that IBD may result from a genetic predisposition that leads to defects in mucosal immune regulatory cells, barrier leakage, and susceptibility to environmental triggers, including luminal bacteria and specific antigens[14,15]. The complex interaction of genetic, microbial, and environmental factors may result in continuous activation of the mucosal immune system, leading to IBD[15]. *MYO9B* variants have been reported to potentially be involved in IBD pathogenesis[16]. The *MYO9B* gene, encoding myosin IXB, was first identified as a susceptibility gene for celiac disease in a Dutch cohort study[17]. This gene is a single motor protein with a Rho GTPase activating domain, and is involved in epithelial cell tight junction assembly and cytoskeletal remodeling[18,19]. Cooney *et al*[16] recently genotyped 8 *MYO9B* single nucleotide polymorphisms (SNPs) in 652 CD patients, 650 UC patients, and 1190 controls and reported a significant association between genetic variants in *MYO9B* and IBD, which indicated that *MYO9B* variants may be involved in IBD pathogenesis. This involvement may be due to defects in MYO9B-dependent intestinal epithelial cells because IBD is often characterized by increased permeability of the intestinal epithelium[20-22].

These findings have not been confirmed in China. Therefore, it is necessary to explore the association of *MYO9B* gene polymorphisms with IBD in the Chinese Han population and to assess the impact of *MYO9B* genetic variations on intestinal permeability in IBD. Most studies on the *MYO9B* gene polymorphisms associated with intestinal permeability mainly investigated rs962917 and rs1545620[23,24]. Therefore, our study genotyped these two *MYO9B* SNPs to investigate the association of *MYO9B* gene polymorphisms with IBD clinical features and with the permeability of the intestinal mucosa in the Chinese Han population.

Table 1 Demographic and clinical characteristics of inflammatory bowel disease patients and controls, *n* (%)

	CD	UC	Controls
Total (*n*)	207	235	402
Sex (F/M)	91/116	106/129	196/206
Age at diagnosis			Mean age 40.21 ± 5.37
A1 (< 16 yr)	10 (4.8)	4 (1.7)	
A2 (17-40 yr)	133 (64.3)	127 (54.0)	
A3 (> 40 yr)	64 (30.9)	104 (44.3)	
Disease location, CD			
L1 (terminal ileum)	75 (36.2)	Proctitis 71 (30.2)	
L2 (colonic location)	42 (20.3)	Left-sided 103 (43.8)	
L3 (ileocolitis)	90 (43.5)	Extensive 61 (26.0)	
L4[1] (upper gastrointestinal tract)	18 (8.7)		
Disease behavior, CD			
B1 (inflammatory disease)	63 (30.4)		
B2 (structuring disease)	85 (41.1)		
B3 (penetrating disease)	59 (28.5)		
P[2] (perianal disease)	47 (22.7)		

[1]L4 is a modifier that can be added to L1-L3 when concomitant upper gastrointestinal disease is present; [2]Perianal disease was categorized as *P*, that could be added to B1-B3 when concomitant perianal disease is present. CD: Crohn's disease; UC: Ulcerative colitis.

MATERIALS AND METHODS

Patients and controls

IBD patients were consecutively recruited from the Department of Gastroenterology, the First Affiliated Hospital of Anhui Medical University between February 2006 and May 2012. Diagnosis of IBD was based on established clinical, endoscopic, radiological, and histological criteria[1]. The study cohort consisted of 235 UC patients (129 men; mean age: 42.14 ± 10.69 years) and 207 CD patients (116 men; mean age: 37.15 ± 9.25 years). The phenotype of these patients was classified based on age at diagnosis, location, and behavior of disease according to the Montreal classification of IBD[25]. Demographic and clinical characteristic data are presented in Table 1. The control group (206 men; mean age: 40.21 ± 5.37 years) was recruited from healthy individuals from the medical examination center. There were no significant differences between the case and control groups with respect to age or sex. The Han ethnic group, with a population of 1225932641 (according to the 6th Population Survey of China in 2010), lived in most provinces of China. In this study, both patients and controls were of Han ancestry and were unrelated inhabitants in Anhui province. Approval of the protocol was obtained from the Ethics Committee of the First Affiliated Hospital of Anhui Medical University.

Measurements gene determination method

Venous blood (5 mL) was collected from each patient and control. DNA was extracted in accordance with the kit's instructions (Axygen Corp., CA, United States) and

preserved at -20 ℃. Two mononucleotide polymorphic sites of the *MYO9B* gene, rs962917 and rs1545620, were detected by polymerase chain reaction (PCR)/ligase detection reaction (LDR). Primers and probes were synthesized by Shanghai Biotech Corp. (Shanghai, China). For rs962917, the sequence of the forward primer was 5'-CCTCCTGCCTCATACCGTAA-3', and the sequence of the reverse primer was 5'-AATCCACGTCACGAGACGAC-3'; the LDR probe set included a fluorescent probe (P-CGTCACCTGTTTATTGCTGCTTTTTTTTT TTTTTTTTT-FAM) and a pair of detection probes (5'-TTTTTTTTTTTTTTT TTGCAGGGCTCAGCGACTCCCTCCG-3' and 5'-TTTTTTTTTTTTTTTTTTTG CAGGGCTCAGCGACTCCCTCCA-3'). For rs1545620, the sequence of the forward primer was 5'-GCGGATGATGCTCTGTTTCT-3', and the sequence of the reverse primer was 5'-AAGTAGACGTCCTTCACGG-3'; the LDR probe set included a fluorescence probe (P-CGTCACCTGTTTATTGCTGCTTTTTT TTTTTTTTTTTT-FAM) and a pair of detection probes (5'-TTTTTTTTTTTTTT TTTTTGGCTGCCGTGTACCTCCAGGCCT-3' and 5'-TTTTTTTTTTTTTTTTTT TTTGGCTGCCGTGTACCTCCAGGCCG-3'). Then, 20 μL of the multiple PCR mixture was prepared for multiple PCR amplification, which included 2 μL of 1× buffer solution, 3.0 mmol/L $MgCl_2$, 2 mmol/L dNTPs, 0.4 μL each of the positive and negative primers, 0.4 μL of Tag polymerase 1U (Qiagen Corp., Hilden, Germany), 4 μL of 1× Q-solution, and 50 ng of genomic DNA. Double distilled water was added to the final volume. Then we performed initial denaturation at 95 ℃ for 2 min, 35 cycles of 94 ℃ for 30 s, 62 ℃ for 90 s, and 72 ℃ for 60 s, and final extension at 72 ℃ for 10 min. 3% agarose gel electrophoresis was used to detect the PCR products.

In addition, 10 μL of multiple LDR mixture was prepared for the multiple LDR, which included 1 μL of 1× buffer solution, 1 μL of the probe mix (0.05 pmol/L/each), 0.05 μL of Taq DNA ligase (NEB Corp., Beijing, China), and 2 μL of the PCR products (50 ng/μL). Double distilled water was added to the final volume. After sufficient mixing, the solution was centrifuged for the LDR, which consisted of the following steps: initial denaturation at 95 ℃ for 2 min, followed by 30 cycles of denaturation at 94 ℃ for 15 s and annealing at 50 ℃ for 25 s. The LDR product was sequenced with a 377 DNA Sequencer (ABI Corp., United States).

Evaluation of intestinal permeability

Intestinal permeability was evaluated using the lactulose (L) and mannitol (M) excretion test in all patients and healthy controls. After an overnight fast, the subjects drank 100 mL of the test solution containing 10 g of L and 5 g of M. No food or drink other than water was allowed until completion of the test. Urine samples over the following 6 h were collected in a plastic tube containing 2% thimerosal as a preservative. The total volume was recorded, and 20 mL of each sample was stored at -20 ℃ until analysis. The urinary concentrations of L and M were measured by high-pressure liquid chromatography with pulsed electrochemical detection (HPLC-PED)[26]. Intestinal permeability was evaluated based on the ratio of the concentrations of L and M (L/M) in the urine[27]. As previously described, intestinal permeability was considered normal when the L/M ratio was less than 0.03[23].

Statistical analysis

SPSS 13.0 (SPSS Inc., Chicago, IL, United States) statistical software was used for data analysis. Qualitative variables are expressed as percentages, and quantitative variables are calculated as the means. To compare groups, we used χ^2 tests. Haploview Software ver. 3.2 (http://www.broad.mit.edu/mpg/haploview) was used for testing the Hardy-Weinberg equilibrium, linkage disequilibrium, and transmission disequilibrium. Logistic regression was applied to model the association of SNPs with the sub-phenotypes. Odds ratios (OR) with 95%CIs were determined. For all allelic and genotype analyses, Bonferroni's correction for the number of SNPs tested was used to correct for multiple testing and a P value < 0.05 indicated statistical significance.

RESULTS

Demographics and clinical features

A total of 442 patients (245 male, 197 female) with IBD and 402 healthy controls, all of Han ancestry from China and none with a positive family history of IBD, were enrolled. There were no significant differences in age and sex between the two groups (Table 1). The age at diagnosis, disease location, and the behavior of IBD disease are shown in Table 1. According to the Montreal classification[25], the most frequent disease location in our UC patients was the left side of the colon (43.8%), followed by the rectum (30.2%) and extensive colon (26.0%). In the CD patients, 43.5% exhibited ileocolitis, 36.2% exhibited pure ileitis, 20.3% had disease in the colon region, and only 8.7% had upper intestinal tract involvement. In addition, 41.1% of the CD patients had stricturing disease, 30.4% had nonstricturing nonpenetrating disease, and 28.5% had a penetrating phenotype, with concomitant perianal disease in 22.7% patients.

Association of MYO9B variants with IBD

A total of 442 IBD patients and 402 controls were genotyped for two *MYO9B* gene polymorphisms, rs962917 and rs1545620 (Tables 2 and 3). Both allelic and genotypic frequencies in the IBD and control groups were evaluated for Hardy-Weinberg equilibrium ($P > 0.05$). In the IBD patients, the rs962917 genotype frequencies of CC, CT, and TT were 8.6%, 36.9%, and 54.5%, respectively. For rs1545620, the genotype frequencies of AA, AC, and CC were 14.3%, 28.9%, and 56.8%, respectively. No significant differences in the allelic and genotypic frequencies were observed between the controls and IBD patients for either SNP. When the CD and UC patients were analyzed separately, no association was found

Table 2 Allele and genotype frequencies of the MYO9B SNPs rs962917 in inflammatory bowel disease patients *vs* controls, *n* (%)

	Control (*n* = 402)	IBD (*n* = 442)			CD (*n* = 207)			UC (*n* = 235)		
			OR (95%CI)	P_{corr}		OR (95%CI)	P_{corr}		OR (95%CI)	P_{corr}
GF										
CC	27 (6.6)	38 (8.6)	1.23 (0.79-1.65)	0.64	21 (10.1)	1.52 (0.63-2.17)	0.09	17 (7.2)	1.09 (0.82-1.40)	0.75
CT	173 (42.5)	163 (36.9)	0.87 (0.52-1.24)	0.26	79 (38.2)	0.81 (0.47-1.33)	0.24	84 (35.8)	0.83 (0.69-1.14)	0.31
TT	207 (50.9)	241 (54.5)	1.07 (0.82-1.41)	0.97	107 (51.7)	1.02 (0.85-1.24)	0.83	134 (57.0)	1.12 (0.67-1.46)	0.48
AF										
C	227 (27.9)	239 (27.0)	0.92 (0.66-1.71)	0.52	121 (29.2)	1.06 (0.75-1.41)	0.66	118 (25.1)	0.86 (0.67-1.35)	0.38
T	587 (72.1)	645 (73.0)	1.02 (0.81-1.30)	0.77	293 (70.8)	0.98 (0.61-1.59)	0.49	352 (74.9)	1.14 (0.82-1.33)	0.29

IBD: Inflammatory bowel disease; GF: Genotype frequencies; AF: Allele frequencies; OR: Odds ratio; CI: Confidence interval; SNP: Single nucleotide polymorphism; CD: Crohn's disease. Genotype and allele frequencies in patients and controls were compared using the χ^2 test; P_{corr}: Corrected *P* value.

Table 3 Allele and genotype frequencies of the MYO9B SNPs rs1545620 in IBD patients *vs* controls, *n* (%)

	Controls (*n* = 402)	IBD (*n* = 442)			CD (*n* = 207)			UC (*n* = 235)		
			OR (95%CI)	P_{corr}		OR (95%CI)	P_{corr}		OR (95%CI)	P_{corr}
GF										
AA	42 (10.4)	63 (14.3)	1.47 (0.56-3.85)	0.43	26 (12.6)	2.01 (0.71-5.66)	0.21	37 (15.7)	1.26 (0.31-3.25)	0.61
AC	135 (33.6)	128 (28.9)	0.86 (0.62-1.94)	0.29	63 (30.4)	0.91 (0.27-2.49)	0.07	65 (27.7)	0.82 (0.57-2.61)	0.37
CC	225 (56.0)	251 (56.8)	1.03 (0.37-2.19)	0.71	118 (57.0)	1.35 (0.33-2.19)	0.57	133 (56.6)	1.14 (0.61-3.77)	0.84
AF										
A	219 (27.2)	254 (25.9)	0.96 (0.44-2.91)	0.61	115 (27.8)	1.09 (0.39-2.46)	0.37	139 (29.6)	1.16 (0.57-3.14)	0.57
C	585 (72.8)	727 (74.1)	1.07 (0.56-2.28)	0.77	299 (72.2)	0.92 (0.49-3.17)	0.42	331 (70.4)	0.92 (0.77-2.51)	0.39

IBD: Inflammatory bowel disease; GF: Genotype frequencies; AF: Allele frequencies; CD: Crohn's disease. Genotype and allele frequencies in patients and controls were compared using the χ^2 test; P_{corr}: Corrected *P* value.

Table 4 Genotype frequency of rs962917 and rs1545620 SNPs in Crohn's disease phenotypes, *n* (%)

CD (*n* = 207)	rs962917			rs1545620		
	CC (21)	CT (79)	TT (107)	AA (26)	AC (63)	CC (118)
Age, CD						
A1 (< 16 yr)	1 (4.7)	4 (5.1)	5 (4.7)	1 (3.9)	3 (4.7)	6 (5.1)
A2 (17-40 yr)	14 (66.7)	51 (64.6)	68 (63.6)	16 (61.5)	41 (65.1)	76 (64.4)
A3 (> 40 yr)	6 (28.6)	24 (30.3)	34 (31.7)	9 (34.6)	19 (30.2)	36 (30.5)
Disease location, CD						
L1	9 (42.8)	28 (35.4)	38 (35.5)	11 (42.3)	22 (34.9)	42 (35.6)
L2	6 (28.6)	15 (19.0)	21 (19.6)	7 (26.9)	12 (19.0)	24 (20.3)
L3	6 (28.6)[1]	36 (45.6)	48 (44.9)	8 (30.8)[2]	29 (46.0)	52 (44.1)
L4	2 (9.5)	7 (8.9)	9 (8.4)	2 (7.7)	6 (9.5)	10 (8.5)
Disease behavior, CD						
B1	6 (28.6)	24 (30.4)	33 (30.8)	8 (30.8)	19 (30.2)	36 (30.5)
B2	9 (42.8)	32 (40.5)	44 (41.1)	11 (42.3)	26 (41.2)	48 (40.7)
B3	6 (28.6)	23 (29.1)	30 (28.1)	7 (26.9)	18 (28.6)	34 (28.8)
P	5 (23.8)	18 (22.9)	24 (22.4)	3 (11.5)[3]	16 (25.3)	28 (23.7)

Genotype frequencies in patients and controls were compared using the χ^2 test; [1]*P* = 0.014 CC *vs* CT TT; [2]*P* = 0.022 AA *vs* AC CC; [3]*P* = 0.029 AA *vs* AC CC. CD: Crohn's disease.

for the two IBD phenotypes.

Association of MYO9B variants with disease phenotype

The contribution of the different genotypes of the two SNPs in IBD patients was investigated to verify whether the *MYO9B* variants affected the clinical features. In the CD patients, the genotype frequencies of rs962917 and rs1545620 appeared to be differently distributed in ileocolitis (CC *vs* CT and TT, *P* = 0.014; and AA *vs* AC and CC, *P* = 0.022, respectively). Of note, an association was identified between the rs1545620 genotypes and the subgroup of perianal disease (AA *vs* AC and CC, *P* = 0.029) in CD (Table 4). However, in the UC patients, no significant association with any specific sub-phenotype was observed.

Association of MYO9B variants with intestinal permeability in IBD

The L/M ratio was significantly higher in IBD patients than in controls (0.065 ± 0.013 *vs* 0.020 ± 0.002, *P* = 0.006). The IBD patients were divided into two groups according to intestinal permeability (L/M ⩾ 0.03 or

Table 5 Genotype frequencies of rs962917 and rs1545620 SNPs in ulcerative colitis phenotypes, *n* (%)

UC (*n* = 235)	rs962917			rs1545620		
	CC (17)	CT (84)	TT (134)	AA (37)	AC (65)	CC (133)
Age, UC						
A1 (< 16 yr)	0 (0)	1 (1.1)	3 (2.2)	1 (2.7)	1 (1.5)	2 (1.5)
A2 (17-40 yr)	9 (52.9)	49 (58.4)	69 (51.5)	17 (45.9)	37 (56.8)	73 (54.9)
A3 (> 40 yr)	8 (47.1)	34 (40.5)	62 (46.3)	19 (51.4)	27 (41.5)	58 (43.6)
Disease location, UC						
Proctitis	4 (23.5)	23 (27.4)	44 (32.8)	11 (29.7)	20 (30.8)	40 (30.1)
Left-sided	7 (41.2)	39 (46.4)	57 (42.6)	16 (43.2)	28 (43.1)	59 (44.4)
Extensive	6 (35.3)	22 (26.2)	33 (24.6)	10 (27.1)	17 (26.1)	34 (25.5)

χ^2 test was applied to compare the number of genotypes between patients and controls; All *P* values were > 0.05.

Table 6 Association of MYO9B variants with intestinal permeability in IBD, *n* (%)

IBD		rs962917			rs1545620		
		CC (17)	CT (84)	TT (134)	AA (37)	AC (65)	CC (133)
UC (*n* = 235)	L/M ≥ 0.03	10 (58.8)	38 (45.2)	71 (53.0)	16 (43.2)	30 (46.2)	73 (54.9)
	L/M < 0.03	7 (41.2)	46 (54.8)	63 (47.0)	21 (56.8)	35 (53.8)	60 (45.1)
CD (*n* = 207)		CC (21)	CT (79)	TT (107)	AA (26)	AC (63)	CC (118)
	L/M ≥ 0.03	9 (42.9)	42 (53.2)	59 (55.1)	12 (46.2)	33 (52.4)	65 (55.1)
	L/M<0.03	12 (57.1)	37 (46.8)	48 (44.9)	14 (53.8)	30 (47.6)	53 (44.9)

χ^2 test was applied to compare the number of genotypes between patients and controls; All *P* values were > 0.05. IBD: Inflammatory bowel disease; CD: Crohn's disease.

L/M < 0.03), as previously described[23]. The analysis showed no significant differences in the genotype frequency between the normal and high intestinal permeability groups.

DISCUSSION

Genetic susceptibility is a key factor in the pathogenesis of IBD[28]. A focus on the genetic background of IBD in different geographic areas or races may provide insight into possible etiologic factors. Genetic variation in the *MYO9B* gene might produce an effect on epithelial cell tight junction assembly and cytoskeletal remodeling[29,30]. Because IBD is often characterized by increased permeability of the intestinal epithelium[21,22], *MYO9B* gene polymorphisms are a very valuable target for the study of IBD. Our research suggested that *MYO9B* gene polymorphisms influence the sub-phenotypic expression of CD. The frequencies of rs962917 and rs1545620 were significantly different in the CD subgroups according to disease location. In particular, when patients with CD were divided into perianal or non-perianal lesion groups, an association was identified between rs1545620 genotypes and the subgroup of perianal disease (*P* = 0.029) (Table 5). Although the L/M ratio was significantly higher in the IBD patients than in the controls, there was no association between the *MYO9B* gene and the L/M ratio in the IBD patients (Table 6).

The pathologic process of IBD involves many factors, including immune dysfunction of the intestinal mucosa, infection, heredity, and the environment[2-4]. Intestinal mucosal barrier dysfunction is an essential component of IBD pathogenesis[15]. The increase in intestinal mucosal permeability may lead to the displacement of intestinal antigenic substances, induce and aggravate intestinal inflammation and immunoreactivity, further damage the intestinal mucosal barrier, and increase intestinal mucosal permeability[31]. Büning *et al*[32] found that the permeability of the small intestine significantly increased in 89 UC cases in the remission stage. We used L and M to evaluate intestinal mucosal permeability and found that patients with CD or UC had markedly higher L/M ratios than controls, which indicates that intestinal permeability in IBD patients is increased.

The changes in intestinal permeability are associated with closely linked intestinal epithelial cells and myosin contraction[33]. *MYO9B*, the gene encoding myosin, has been shown to be associated with diseases in the digestive system[17]. van Bodegraven *et al*[34] studied eight SNPs in the *MYO9B* gene, including six loci associated with digestive diseases, and found that the *MYO9B* gene is closely related to IBD and that the rs1545620 locus has a marked association with UC. Nunez *et al*[35] also found that *MYO9B* gene polymorphisms were correlated with UC, but not with CD, in a study in a Spanish population. In a study in an Italian population, the *MYO9B* gene and IBD were closely associated, indicating that the rs1545620 and rs962917 genotypes may increase the susceptibility to IBD[23]. Few studies of *MYO9B* gene polymorphisms in IBD patients are available in China. Shi *et al*[36] studied candidate genes for UC in a Chinese Han population and selected two polymorphic loci of the *MYO9B* gene. Their study observed an association of the TT genotype of rs1545620 in *MYO9B* with UC (*P* = 0.0169, OR = 0.29, 95%CI: 0.11-0.78). However, our study failed to find a significant difference in the

genotype frequency and allele frequency distributions of rs962917 and rs1545620 between IBD patients and normal controls. *rs962917* and *rs1545620* gene polymorphisms were not distributed differently in the UC clinical subgroup in our study. Furthermore, the study by Amundsen *et al*[37] failed to support the notion that *MYO9B* is a susceptibility gene in UC.

An association study of the *MYO9B* gene in Italian patients with IBD reported that the allele frequencies of *MYO9B* SNPs were different in CD subgroups according to disease location, with a trend towards an increased frequency of upper gastrointestinal involvement ($P = 0.057$) and perianal disease ($P = 0.042$)[23]. Our research also found that the frequencies of rs962917 and rs1545620 were significantly different in the CD subgroups according to disease location.

Whether *MYO9B* gene polymorphisms affect intestinal permeability remains unknown. Latiano *et al*[23] reported that *MYO9B* gene polymorphisms were not significantly related to intestinal mucosal permeability. We used L and M to evaluate intestinal mucosal permeability. These sugars are not involved in metabolism and are urinated in prototype. Their excretion rate in the urine can reflect changes in intestinal mucosal permeability. HPLC-PED, which was adopted to detect the concentrations of L and M, is highly efficient and sensitive. This study confirmed that patients with IBD have markedly higher L/M ratios than controls, indicating increased intestinal permeability in IBD patients. However, we failed to find a correlation of *MYO9B* genotypes with intestinal permeability.

In summary, a significant association between genetic variants in *MYO9B* and IBD has been reported, which indicates that *MYO9B* variants may be involved in IBD pathogenesis in Western populations. However, our study suggested that *MYO9B* gene polymorphisms may influence the sub-phenotypic expression of CD but did not find an association between these *MYO9B* polymorphisms and intestinal permeability in IBD patients from a Han population in China. These findings indicate that IBD patients from different races and regions may express distinct clinical IBD characteristics and that the influence of *MYO9B* gene polymorphisms differs.

COMMENTS

Background

Genetic variation in the *MYO9B* gene might predispose individuals to inflammatory bowel disease (IBD) according to studies performed in Western populations. Furthermore, IBD is often characterized by increased permeability of the intestinal epithelium.

Research frontiers

The association of *MYO9B* gene polymorphisms with IBD has been studied in Western countries, but the conclusions from these studies have not been confirmed in China.

Innovations and breakthroughs

This study explored the association of *MYO9B* gene polymorphisms with the clinical phenotypes and intestinal permeability of IBD in China. This results suggested that *MYO9B* gene polymorphisms may influence the sub-phenotypic expression of Crohn's disease but did not find an association between *MYO9B* polymorphisms and intestinal permeability in IBD patients from a Han population in China. These findings indicate that *MYO9B* gene polymorphisms may play a small role in changing the intestinal mucosal permeability in Chinese Han IBD patients.

Applications

The conclusions of this study involving *MYO9B* gene polymorphisms in IBD patients from a Han population in China were different from those of studies in Western populations. Studies of the genetic background of IBD in different geographic areas or races may provide insights into possible etiologic factors.

Peer review

This is a study from China aiming to explore the association of *MYO9B* gene polymorphisms with the clinical phenotypes and intestinal permeability of IBD. The study, which possesses a logical presentation of facts with regard to the description of the patient cohort, the performance of experiments, and the analysis of data, will provide more information about *MYO9B* gene polymorphisms in IBD to other authors.

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RETROSPECTIVE STUDY

Clinical characteristics and corticosteroid therapy in patients with autoimmune-hepatitis-induced liver failure

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Abstract

AIM: To investigate the clinical features, response to corticosteroids, and prognosis of autoimmune hepatitis (AIH)-induced liver failure in China.

METHODS: A total of 22 patients (19 female and 3 male; average age 51 ± 15 years) with AIH-induced liver failure treated in our hospital from 2004 to 2012 were retrospectively analyzed. Clinical, biochemical and pathological characteristics of the 22 patients and responses to corticosteroid treatment in seven patients were examined retrospectively. The patients were divided into survivor and non-survivor groups, and the clinical characteristics and prognosis were compared between the two groups. The *t* test was used for data analysis of all categorical variables, and overall survival was calculated by the Kaplan-Meier method.

RESULTS: At the time of diagnosis, mean IgG was 2473 ± 983 mg/dL, with three (18.8%) patients showing normal levels. All of the patients had elevated serum levels of antinuclear antibody (⩾ 1:640). Liver histology from one patient showed diagnostic pathological changes, including massive necrosis and plasma cell infiltration. Four patients survived (18.2%) and 18 died (81.8%) without liver transplantation. The results showed that patients with low admission Model for End-Stage Liver Disease (MELD) scores (21.50 ± 2.08 *vs* 30.61 ± 6.70, $P < 0.05$) and corticosteroid therapy (100% *vs* 16.7%, $P < 0.05$) had better prognosis. A total of seven patients received corticosteroid therapy, of whom, four responded and survived, and the other three died. Survivors showed young age, shorter duration from diagnosis to corticosteroid therapy, low MELD score, and absence of hepatic encephalopathy at the time of corticosteroid administration. Six patients who were administered corticosteroids acquired fungal infections but recovered after antifungal therapy.

CONCLUSION: Early diagnosis and corticosteroid therapy are essential for improving the prognosis of patients with AIH-induced liver failure without liver transplantation.

Key words: Autoimmune hepatitis; Liver failure; Autoantibody; Prognosis; Corticosteroid therapy

Core tip: We describe the clinical characteristics and prognosis of autoimmune-hepatitis-induced liver failure in China. Early diagnosis and initiating corticosteroid therapy at an early stage may be essential for improving the prognosis of patients with AIH-induced liver failure without liver transplantation.

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with autoimmune-hepatitis-induced liver failure. *World J Gastroenterol* 2014; 20(23): 7473-7479 Available from: URL: http://www.wjgnet.com/1007-9327/full/v20/i23/7473.htm DOI: http://dx.doi.org/10.3748/wjg.v20.i23.7473

INTRODUCTION

Autoimmune hepatitis (AIH) is a liver disease of unknown etiology that is characterized by chronic hepatic inflammation, presence of autoantibodies, and hypergammaglobulinemia[1]. AIH has protean manifestations that often affect young women, with the majority of patients presenting with subclinical or chronic disease. In many cases, cirrhosis is already established when diagnosis is made at the first presentation of symptoms. The disease may also occur acutely with jaundice in some patients; a subset of whom may develop acute and subacute liver failure[2,3].

Although there are a large number of AIH patients, only a few cases develop liver failure. In a survey in the United States carried out between 1998 and 2008, the major etiology of fulminant hepatic failure (FHF) in 1147 patients was acetaminophen overdose (46%), with only 5% of cases being induced by AIH[4]. Similar data were reported from Europe where 2%-5% of cases of FHF were induced by AIH[5,6] There were 19 (0.5%) patients with liver failure induced by AIH in our survey from 2002-2011, due to a high incidence of chronic hepatitis B virus (HBV) infection[7]. Although the incidence of AIH-induced liver failure is low, the prognosis of these patients remains poor, with a reported survival rate of only about 20% in those who do not undergo liver transplantation[7-9]. However, there are insufficient data for the clinical features and prognosis of AIH-induced liver failure in China.

Preferred treatment for patients with AIH is immunosuppression, including corticosteroids and azathioprine, and up to 70% of patients can achieve remission. Long-term treatment with azathioprine, with or without prednisolone, can prevent relapse[10,11]. However, the usefulness of immunosuppressive therapy in AIH-induced liver failure has not been well demonstrated. The management of corticosteroid therapy is controversial in these patients. Earlier studies have established the beneficial effects of corticosteroids in patients with acute severe (fulminant) AIH[12-14]. Responders to corticosteroids have been shown to have improvement or stabilization of bilirubin and International Normalized Ratio. In contrast, a survey from Ichai *et al*[15] reported that the initiation of corticosteroid therapy did not prevent liver transplantation in most patients, and empirical therapy in acute liver failure may delay or complicate liver transplantation. Moreover, the decision to initiate immunosuppressant drugs must be counterbalanced by the risk of septic complications[16]. Unfortunately, data on the use of corticosteroid therapy for patients with AIH-induced liver failure remain poorly described in China.

In this regard, the clinical features and prognosis of patients with AIH-induced liver failure were retrospectively analyzed in our hospital from 2004 to 2012. The management of corticosteroid therapy was also examined.

MATERIALS AND METHODS

Patient population

The retrospective study was carried out at the Liver Failure Treatment and Research Center of 302nd Military Hospital, Beijing, China between January 2004 and December 2012. A diagnosis of AIH was made based on the presence of anti-nuclear antibody (ANA) and/or anti-smooth muscle antibody (ASMA), and on the criteria defined by the International Autoimmune Hepatitis Group (IAIHG)[17,18]. A definite diagnosis required a pretreatment score > 15, while a probable diagnosis required a score between 10 and 15. The criteria for liver failure were used according to the Diagnostic and Treatment Guidelines of Liver Failure suggested by the Liver Failure and Artificial Liver Group, Chinese Society of Infectious Diseases of Chinese Medical Association[19]. Acute liver failure (ALF) was defined as the presence of coagulopathy [prothrombin activity (PTA) < 40%] and hepatic encephalopathy (grade 2) within 2 wk of the first symptoms without previous underlying liver disease. The patients with subacute liver failure had similar syndromes as acute liver failure (ALF) patients, but the duration of disease was 2-26 wk. Acute on chronic liver failure (ACLF) was defined as acute liver decompensation on the basis of chronic liver disease with mandatory jaundice [total bilirubin (TBil) > 171.0 μmol/L or a rapid rise > 17.1 μmol/L per day], coagulopathy (PTA < 40%), and recent development of complications.

For all patients, there was no evidence of concurrent HBV, hepatitis C virus, hepatitis D virus, hepatitis E virus, or human immunodeficiency virus (HIV) infection, and no evidence of drug-induced, alcoholic liver disease or Wilson's disease. Other conditions that can lead to liver failure were excluded in this study. All of the patients were followed up until 24 wk or death. A total of 22 patients (19 female and 3 male; average age 51.1 ± 14.5 years) were enrolled in this study.

The protocol was approved by the Ethical Committee of 302 Military Hospital. All procedures were performed in accordance with the ethical standards of the responsible committee on human experimentation (institutional and national) and with the Helsinki Declaration of 1975, as revised in 2008 Written informed consent was obtained from each patient before entering the study protocol.

Before corticosteroid therapy was initiated, an absence of sepsis was confirmed by negative cultures of blood samples, ascites fluids, and urine specimens, and by chest X-ray.

Clinical parameters evaluation

History of alcohol consumption, drug intake, blood transfusion, and medication in all patients was carefully

Table 1 Clinical features of patients at the time of diagnosis

Case	Age/sex (yr)	TBil (mg/dL)	PTA	ALT (IU/L)	ALP (IU/L)	Creatinine (mg/dL)	MELD	Liver cirrhosis	HE	SIRS	Liver failure type
1	36/F	13.9	28%	300	185	0.77	22	Yes	No	No	ACLF
2	50/F	10.1	27%	326	107	0.63	29	Yes	No	No	ACLF
3	66/F	21.3	12%	452	275	0.77	30	Yes	No	Yes	ACLF
4	48/F	26.6	38%	374	158	1.37	27	Yes	No	No	ACLF
5	44/F	21.7	32%	280	134	0.83	32	No	Yes	No	Subacute
6	42/M	32.8	39%	791	144	1.32	31	No	Yes	Yes	Subacute
7	33/F	29.0	30%	139	148	0.93	24	No	No	No	Subacute
8	19/F	15.6	33%	234	439	2.87	36	Yes	No	Yes	ACLF
9	67/M	25.5	19%	145	54	0.87	29	Yes	Yes	No	ACLF
10	58/F	27.9	36%	274	212	1.23	30	No	Yes	No	Subacute
11	59/F	24.0	20%	138	150	1.50	41	Yes	No	No	ACLF
12	75/F	19.6	30%	725	173	0.86	23	Yes	No	No	ACLF
13	56/F	29.8	31%	268	420	0.80	31	No	Yes	No	Subacute
14	41/F	32.5	5%	99	81	1.87	44	Yes	Yes	No	ACLF
15	72/M	27.4	19%	400	195	1.54	38	No	Yes	No	Acute
16	45/F	11.1	39%	512	162	0.58	22	Yes	No	No	ACLF
17	45/F	19.0	38%	557	290	0.87	22	No	No	No	Subacute
18	39/F	23.0	26%	147	35	0.75	21	Yes	Yes	No	ACLF
19	72/F	23.3	23%	337	123	1.41	36	Yes	Yes	No	ACLF
20	52/F	17.1	38%	197	168	0.76	19	No	No	No	Subacute
21	65/F	17.7	35%	134	32	0.66	19	No	No	Yes	Subacute
22	40/F	27.1	31%	76	92	1.67	31	Yes	No	No	ACLF

HE: Hepatic encephalopathy; SIRS: Systemic inflammatory response syndrome; TBil: Total bilirubin; PTA: Prothrombin activity; ALT: Alanine aminotransferase; ALP: Alkaline phosphatase; MELD: Model for end-stage liver disease; ACLF: Acute on chronic liver failure.

reviewed. Serum autoantibodies, including ANA, ASMA, liver kidney microsomal antibody (LKM)-1 and antimitochondrial antibody (AMA), were tested using indirect immunofluorescence with the standard methods (EUROIMMUN, Germany), with a dilution of ≥ 1:100 considered as positive. Immunoglobulin assay was performed with the method of immunological turbidimetry (Roche Diagnostics GmbH, Germany). The standard biochemical tests for the assessment of liver diseases, including alanine transaminase (ALT), alkaline phosphatase (ALP), serum creatinine, plasma PTA, and TBil were routinely performed in the Central Clinical Laboratory of the 302nd Military Hospital. Liver biopsy was performed in one case for definite diagnosis, and the biopsy specimen was examined in the Pathology Department of the 302nd Military Hospital.

Statistical analysis

The results were expressed as mean ± SD. Continuous variables were compared using Student's *t* test. Categorical data were compared using Fisher's exact test. $P < 0.05$ was considered statistically significant. Data processing was carried out with SPSS for Windows (SPSS Inc., Chicago, IL, United States).

RESULTS

Clinical features of enrolled patients

The clinical features of the 22 patients at the time of diagnosis are shown in Table 1. Thirteen (59.1%) of the patients had ACLF, one (4.5%) had ALF, and eight (36.4%) had subacute liver failure. All of the patients with ACLF had liver cirrhosis. At admission, nine (41%) patients suffered from hepatic encephalopathy. Four patients had systemic inflammatory response syndrome (SIRS). Laboratory data at admission reflected severe hepatic dysfunction, with mean TBil of 22.5 ± 6.5 mg/dL, ALT of 317 ± 236 IU/L, and PTA of 29% ± 8%. The average serum creatinine level was 1.13 ± 0.54 mg/dL.

All of the patients underwent ultrasound (US) examination, and 16 had computed tomography (CT). Both hepatic necrosis and liver regeneration were present in those patients who showed hypoattenuation and hyperattenuation areas on US and/or CT scans. Thirteen patients showed characteristics of liver cirrhosis, including echo coarseness, liver surface nodularity, and splenomegaly.

Immunoserological features and AIH scoring

The immunoserological features of patients are shown in Table 2. All of the patients had positive ANA (≥ 1:100): > 1:1000 in 16 (72.7%), 1:640 in six (27.3%). AMSA was positive (≥ 1:100) in six (27.3%) patients. Two patients were positive for LKM-1. A total of 16 patients underwent immunoglobulin G (IgG) assay. The average serum level of IgG was 2473 ± 983 mg/dL, which was about 1.5-fold higher than normal (1660 mg/dL). The IgG level was normal in three (18.8%) patients. Percentage of γ-globulins was available in cases 3, 5, 8, 9 and 17 enrolled before 2008.

The AIH scoring system proposed by the International Autoimmune Hepatitis Group[17] was used to score all patients. The AIH score ranged from 11 to 19 (14.5 ± 1.9) before treatment. Four (18.2%) patients were diagnosed with definite AIH and 18 (81.8%) with probable AIH. Seven patients (31.8%) were administered corticosteroid therapy.

Table 2 Immunoserological features and outcomes of enrolled patients

Case	ANA (fold)	ASMA (fold)	LKM-1 (fold)	AMA	IgG (mg/dL)	AIH score	Simplified AIH score	Corticosteroid therapy	Outcome	Weeks from onset to death
1	> 1000	-	-	-	2807	17	6	Yes	Survival	/
2	640	-	-	-	5140	16	6	No	Death	11
3	> 1000	-	UD	-	36%	15	6	No	Death	10
4	> 1000	-	UD	-	1140	13	4	No	Death	11
5	640	-	-	-	20%	13	4	No	Death	3
6	640	80	320	-	1300	11	6	No	Death	6
7	640	-	-	-	2118	15	6	Yes	Survival	/
8	> 1000	160	-	-	54%	15	6	No	Death	5
9	> 1000	-	-	-	30%	12	6	No	Death	5
10	> 1000	1000	UD	-	2026	14	6	No	Death	8
11	> 1000	-	-	-	2358	14	6	No	Death	3
12	> 1000	-	UD	-	1983	14	6	Yes	Death	22
13	> 1000	-	-	-	3014	15	6	No	Death	2
14	> 1000	320	-	-	2167	14	6	No	Death	3
15	> 1000	-	UD	-	UD	12	4	No	Death	2
16	> 1000	160	UD	-	2823	15	4	Yes	Death	19
17	> 1000	-	-	-	23%	13	5	No	Death	6
18	> 1000	-	-	-	3553	18	6	Yes	Survival	/
19	> 1000	1000	-	-	3474	19	8	Yes	Death	11
20	640	-	160	-	1456	14	6	Yes	Survival	/
21	640	-	-	-	1768	14	6	No	Death	12
22	1000	-	-	-	2776	15	6	No	Death	8

Cases 3, 5, 8, 9 and 17 presented percentage of γ-globulins. UD: Undetermined. ANA: Anti-nuclear antibody; ASMA: Anti-smooth muscle antibody; LKM: Liver kidney microsomal antibody; AMA: Antimitochondrial antibody; AIH: Autoimmune hepatitis.

Outcomes and corticosteroid therapy in patients with AIH-induced liver failure

The outcomes of patients were evaluated at 24 wk. Four (18.2%) patients survived, and 18 (81.8%) died without liver transplantation. The average time from onset to death was 8.2 ± 5.6 wk (Table 2). Possible factors associated with survivors and non-survivors are shown in Table 3. The survivors were younger than non-survivors (40.0 ± 8.37 years *vs* 53.56 ± 14.58 years, $P = 0.041$). The Model for End-Stage Liver Disease (MELD) score was 21.50 ± 2.08 and 30.61 ± 6.70 in survivors and non-survivors, respectively ($P = 0.015$), suggesting that non-survivors had a higher disease severity at the time of diagnosis. Comparison between survivors and non-survivors showed that corticosteroid treatment significantly improved survival rate ($P = 0.002$). However, there were no significant differences between survivors and non-survivors in biochemical parameters, ANA titer, IgG level, plasma exchange, and complications at the time of diagnosis. It should be mentioned that in non-survivors, eight patients had hepatic encephalopathy at the time of diagnosis and 10 had hepatic encephalopathy during the 24-wk follow-up. In survivors, only one patient had hepatic encephalopathy at the time of diagnosis of liver failure, with no increase during follow-up.

Seven patients were administered an initial dose of 20-50 mg/d prednisolone or methylprednisolone (Table 4). In order to improve treatment efficiency, azathioprine was added in cases 1, 12 and 16. Other patients were not treated due to hemocytopenia. The survival curve of the relationship with corticosteroid therapy is shown in Figure 1. The median survival time was significantly increased in patients with corticosteroid therapy compared with those who were not treated ($P = 0.007$). The survival rates were 57.1% and 0% in patients with or without corticosteroid therapy, respectively ($P = 0.0218$), suggesting that patients with corticosteroid therapy had better prognosis.

Clinical characteristics of patients receiving corticosteroid therapy

The clinical characteristics and outcomes of the seven patients who received corticosteroid therapy are summarized in Table 4. All of the patients were female. Four (57.1%) patients survived and three (42.9%) died without liver transplantation. The mean age was 41 years (range, 33-52 years) and 64 years (range, 45-72 years) in the survivors and non-survivors, respectively. The median period from diagnosis to corticosteroid application was 26 d (range, 16-35 d) and 91 d (range, 67-105 d) in survivors and non-survivors, respectively. The average MELD score before corticosteroid treatment was 21 and 29 in survivors and non-survivors, respectively. Infection occurred in six patients during corticosteroid therapy, including oral and pulmonary fungal infection. The patients recovered after newly developed antifungal therapy (caspofungin and voriconazole).

Only one patient underwent liver biopsy. Diagnostic pathological changes, including interface hepatitis, massive necrosis, and plasma cell infiltration were observed in that patient.

DISCUSSION

There is a paucity of published data on patients with AIH-induced liver failure; consisting mostly of small case

Table 3 Possible factors associated with survivors and non-survivors

Variables	Survivor (n = 4)	Non-survivor (n = 18)	P value
Age (yr)	40.00 ± 8.37	53.56 ± 14.58	0.041[1]
SIRS (yes), n (%)	0 (0)	4 (22.2)	-
Cirrhosis (yes), n (%)	2 (50)	11 (61)	0.685[2]
Acute/subacute/ACLF	0/2/2	1/6/11	-
TBil (mg/dL)	20.75 ± 6.67	22.94 ± 6.58	0.496[1]
ALT (IU/L)	195.75 ± 74.09	340.11 ± 205.69	0.268[1]
PTA (%)	30 .50 ± 5.26	28.17 ± 9.87	0.996[1]
MELD score	21.50 ± 2.08	30.61 ± 6.70	0.015[1]
Hepatic encephalopathy (yes), n (%)	1 (25)	8 (44)	0.463[2]
Ascites (yes), n (%)	2 (50)	14 (78)	0.280[2]
ANA (> 1:100)	4 (100)	18 (100)	-
IgG (mg/dL)	2483.50 ± 901.45	2344.88 ± 1119.46	0.591[1]
Corticosteroid treatment	4 (100)	3 (16.7)	0.001[2]
Plasma exchange, n (%)	1 (25)	3 (16.7)	1.000[2]

[1]Mann-Whitney test; [2]χ^2 test. ANA: Anti-nuclear antibody.

Table 4 Clinical characteristics of patients receiving corticosteroid treatment

Case	Treatment	Loading dose of corticosteroid (mg/d)	Duration (d)[1]	MELD[2]	Hepatic encephalopathy[2]	Creatinine (mg/dL)[2]	Adverse events	Outcome
1	mPSL	30	16	23	None	0.77	None	Survival
7	PSL	50	26	24	None	0.93	Oral fungal infection	Survival
18	mPSL	32	35	19	None	0.75	Oral fungal infection	Survival
20	mPSL	32	21	20	None	0.76	Oral fungal infection, pulmonary fungal infection	Survival
12	mPSL	24	105	25	None	0.86	Oral fungal infection, pulmonary fungal infection	Death
16	mPSL	24	101	26	None	0.58	Oral fungal infection	Death
19	PSL	30	67	36	Yes	1.41	Oral fungal infection	Death

[1]Days from diagnosis to corticosteroid therapy; [2]At corticosteroid application. PSL: Prednisolone; mPSL: Methylprednisolone.

series[13,15,20]. Thus, the clinical characteristics, response to immunosuppressants, and outcomes without liver transplantation of this cohort remain poorly described. Unfortunately, the previous criteria[17,18] were designed to differentiate AIH from other causes of chronic liver disease, rather than to address diagnostic considerations of ALF. Recently, clinical and histological criteria for autoimmune ALF have been suggested by groups in the US and Japan[21,22]. In our study, the patients who met the IAIHG criteria for AIH and the criteria for liver failure were diagnosed with AIH-induced liver failure.

The prevalence of AIH-induced liver failure differs according to geographical location. From 2004 to 2012, 22 patients were diagnosed with AIH-induced liver failure in our hospital. The 22 patients only accounted for 0.6% of liver failure in our survey. The incidence was significantly lower than that reported before[6,23], due to a high incidence of chronic HBV infection and cryptogenic liver failure[7]. Our previous study showed that AIH comprised 5.3% of patients with cryptogenic liver diseases[24]. The American Association for the Study of Liver Disease (AASLD) suggests performing liver biopsy when AIH is suspected as the cause of ALF and autoantibodies are negative[25]. However, liver biopsy may be difficult or impossible for patients with liver failure because of the severity of the hepatic insult. Thus, it is likely that the cases of AIH-induced liver failure were confused with those of unknown etiology.

Our study showed that, similar to other studies[9,14], AIH-induced liver failure was more common in female patients, with a female:male ratio of roughly 6: 1. Serum autoantibodies have been established as biomarkers for the diagnosis of AIH. In our study, all patients had a high titer of serum ANA. IgG was the predominant immunoglobulin that was elevated in the serum of AIH patients. Thirteen of 16 patients had elevated levels of IgG, with an average of 2473 ± 983 mg/dL, which was about 1.5-fold higher than the normal serum level. A previous study has reported that immunoparesis is commonly seen in patients with liver failure in whom both autoantibodies and/or elevated IgG concentrations may be absent[26]. However, elevated serum levels of ANA and/or IgG were still critical markers for diagnosis of AIH-induced liver failure when liver biopsy was not performed in our study.

The outcome of liver failure induced by AIH is poor. In a recent series of 12 patients with fulminant forms of AIH reported by Fujiwara *et al*[9], two (17%) survived without liver transplantation, one (8%) survived with liver transplantation, and eight (67%) died without liver transplantation. In a Japanese nationwide survey between 1998 and 2003, the survival rate of patients with fulminant hepatitis and late-onset hepatic failure without liver

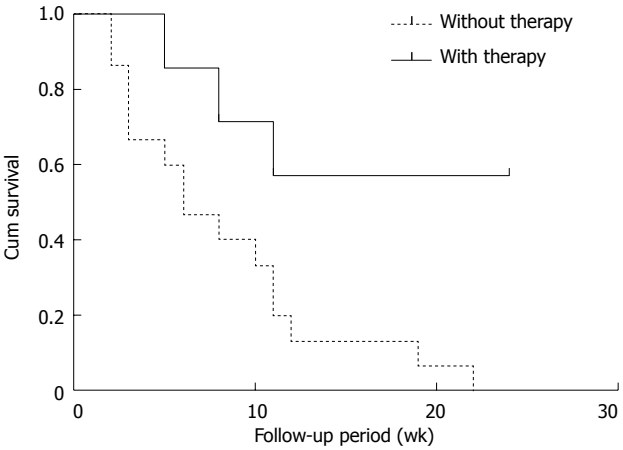

Figure 1 Survival curves for patients receiving or not receiving corticosteroid therapy by Kaplan-Meier method. Total, *n* = 22; with therapy, *n* = 7; without therapy, *n* = 15.

transplantation was 17.1% in AIH[27]. In accordance with previous studies, the survival rate was 18.2% (4/22) in patients without liver transplantation in our study. The results also showed that patients with low admission MELD scores and corticosteroid therapy had better prognosis.

Whether corticosteroids increase the risk of septic complications in patients with severe liver disease is subject to an ongoing debate because liver failure itself is associated with an increased risk of bacterial and fungal infections[28]. In the study of Ichai *et al*[15], 42.3% of patients developed a septic event. In our study, among seven patients who received corticosteroid therapy, six developed fungal infections: of which, there was oral fungal infection in six and pulmonary fungal infection in two patients. These patients with fungal infections recovered because of early diagnosis using serum (1, 3)-β-*D*-glucan determination and use of newly developed antifungal agents, such as caspofungin and voriconazole[29,30]. Fungal infections seemed not to be related to prognosis in our study. Therefore, it is necessary to determine potential infections in the context of corticosteroid therapy and initiation of effective antifungal therapy as soon as possible.

In conclusion, patients with AIH-induced liver failure comprise a small proportion of those with liver failure in China. We should be aware of the possibility that cryptogenic liver failure is induced by AIH. The outcomes of patients with AIH-induced liver failure without liver transplantation are poor. Their prognosis might be improved by the introduction of sufficient immunosuppressive therapy either at an early stage or in patients with less disease severity. Multicenter studies with a large number of patients are also needed to clarify the clinical features of AIH-induced liver failure and define the treatment strategies.

COMMENTS

Background

Autoimmune hepatitis (AIH) is a liver disease of unknown etiology that often affects young women. The majority of patients present with subclinical or chronic disease. However, a subset of patients may develop acute and subacute liver failure. Although the incidence of AIH-induced liver failure is low, the prognosis of these patients remains poor in the absence of liver transplantation.

Research frontiers

The use of corticosteroid therapy is controversial. To date, there are insufficient data regarding the clinical features, response to corticosteroids, and prognosis of AIH-induced liver failure in China.

Innovations and breakthroughs

The authors described the clinical characteristics and prognosis of AIH-induced liver failure in China. Twenty-two patients with AIH-induced liver failure from 2004 to 2012 were enrolled. The results showed that AIH-induced liver failure is a life-threatening liver disease with a survival rate of 18% without liver transplantation. Seven patients received corticosteroid therapy, of whom, four responded and survived and three died. Survivors showed young age, shorter duration from diagnosis to corticosteroid therapy, low Model for End-Stage Liver Disease score, and absence of hepatic encephalopathy at the time of corticosteroid administration.

Applications

The results suggest that early diagnosis and initiation of corticosteroid therapy at an early stage may be essential for improving the prognosis of patients with AIH-induced liver failure without liver transplantation.

Terminology

A diagnosis of AIH-induced liver failure was based on the presence of antinuclear antibody and/or anti-smooth muscle antibody, and the criteria defined by the International Autoimmune Hepatitis Group. The criteria for liver failure were those of the Diagnostic and Treatment Guidelines of Liver Failure suggested by the Liver Failure and Artificial Liver Group, Chinese Society of Infectious Diseases of Chinese Medical Association.

Peer review

This was a good descriptive study with the strengths of a large sample assessed over a long period of time. It is the first study of the field done in China. The conclusions drawn from the analysis are reasonable and well considered.

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PROSPECTIVE STUDY

Synchronous and metachronous neoplasms in gastric cancer patients: A 23-year study

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Abstract

AIM: To determine the prevalence and characteristics of additional primary malignancies in gastric cancer (GC) patients.

METHODS: GC patients (862 total; 570 men, 292 women; mean age 59.8 ± 12.8 years) diagnosed at the Department of Gastroenterology at Pomeranian Medical University over a period of 23 years were included in this retrospective analysis of a prospectively maintained database. Mean follow-up time was 31.3 ± 38.6 mo (range 1-241 mo). The following clinicopathological features of patients with synchronous tumors were compared to those with metachronous tumors: age, sex, symptom duration, family history of cancer, tumor site, stage (early *vs* advanced), histology, and blood group. GC patients with and without a second tumor were compared in terms of the same clinicopathological features.

RESULTS: Of 862 GC patients, 58 (6.7%) developed a total of 62 multiple primary tumors, of which 39 (63%) were metachronous and 23 (37%) synchronous. Four (6.9%) of the 58 multiple GC patients developed two or more neoplasms. The predominant tumor type of the secondary neoplasms was colorectal (n = 17), followed by lung (n = 9), breast (n = 8), and prostate (n = 7). Age was the only clinicopathological feature that differed between GC patients with synchronous *vs* metachronous malignancies; GC patients with synchronous neoplasms were older than those with metachronous neoplasms (68.0 ± 10.3 years *vs* 59.9 ± 11.1 years, respectively, P = 0.008). Comparisons between patients with and without a second primary cancer revealed that the only statistically significant differences were in age and blood group. The mean age of the patients with multiple GC was higher than that of those without a second primary tumor (63.4 ± 11.4 years *vs* 59.5 ± 13.0 years, respectively, P = 0.026). GC patients with a second primary tumor were more commonly blood group O than those without (56.2% *vs* 31.6%, respectively, P = 0.002).

CONCLUSION: GC patients may develop other primary cancers; appropriate preoperative and postoperative diagnostic modalities are thus required, particularly if patients are older and blood group O.

Key words: Gastric cancer; Multiple primary cancers; Synchronous; Metachronous; Blood groups

Core tip: In our study, the incidence of second primary

malignancies in gastric cancer (GC) patients was 6.7%. The predominant tumor type of the secondary neoplasms was colorectal cancer, followed by lung, breast, and prostate. GC patients with synchronous neoplasms were older than those with metachronous neoplasms. GC patients with second primary tumors were significantly more likely to be blood group O and older than those without. This suggests a need for additional procedures, such as colonoscopy, chest X-ray, mammography and computed tomography, particularly for those who are older and blood group O.

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INTRODUCTION

The first systematic study of this of multiple malignancies phenomenon was published in the 1930s by Warren and Gates[1]. These authors proposed the first working definition of multiple primary neoplasms: (1) both tumors should be confirmed histologically as malignant; (2) each cancer must be anatomically separate and distinct; and (3) the second tumor must not be a recurrence or metastasis of the first cancer. Multiple tumors may develop synchronously or metachronously. The phenomenon of multiple primary neoplasms is increasingly being discussed in the literature due to the increased survival time of cancer patients after treatment and because of advances in diagnostic methods. It is estimated that multiple neoplasms affect about 10% of all cancer patients[2-4]. Estimates of the incidence of multiple primary neoplasms in patients with gastric cancer (GC) range from 1.7% to 8.0%[5-12]. A few reports suggest that the development of multiple primary neoplasms in patients with GC is even more frequent. Green *et al*[13] reported that this phenomenon affects approximately 8% of advanced GC patients and 32% of early GC patients. Studies in Japan and Italy estimated that 9%-11% of early GC patients develop other malignancies[14,15]. Kim *et al*[16] described 113 patients with multiple primary cancers at three or more sites; 41 (36.3%) of these patients had GC.

Most studies of multiple malignancies have found colorectal cancer as the second tumor in GC patients[6-8,10,16,17]. The other sites of second tumors include breast, lung, prostate, uterus, small intestine, liver, esophagus, and kidney[6,8-11,16,17]. Many studies of multiple malignancies have been conducted in Asia; only a few have been conducted in Europe. To the best of our knowledge, none have been conducted in Poland, where GC is diagnosed in > 5000 people every year. The purpose of the present study was to determine the prevalence and characteristics of additional primary malignancies in GC patients in a Polish population and to compare GC patients with and without second primary tumors.

MATERIALS AND METHODS

Subjects

Between January 1988 and December 2011, data were collected from 862 patients with histopathologically confirmed GC who were diagnosed at the Department of Gastroenterology at the Pomeranian Medical University in Szczecin, Poland. The following clinical and pathological data were collected: patient sex, age, family history of cancer, duration of symptoms, tumor site, stage (early *vs* advanced), histology, blood group, and previously or subsequently histologically verified second primary malignancy other than GC. In the current study, we retrospectively analyzed a prospective maintained database.

Tumor classification and follow-up

The stage and histological type of GC were assessed by routine histopathological examination. Histological types were classified according to the Lauren classification[18]. Early GC was defined as invasive cancer that invades no more deeply than the submucosa, irrespective of lymph node metastasis. In patients who did not undergo surgery and distal metastases or tumor infiltration were confirmed by diagnostic procedures (*e.g.*, computed tomography, ultrasonography, biopsy) the stage was classified as advanced. The criteria of Warren *et al*[1] were used to classify synchronous and metachronous tumors. If the time interval between the appearance of the two neoplasms did not exceed 6 mo, they were defined as synchronous, and if the interval time was longer than 6 mo, they were classified as metachronous. Tumor location was classified as proximal (cardiac region) or other (truncus, antrum, entire stomach, or anastomosis).

The mean follow-up time for the GC patients was 31.3 ± 38.6 mo (range 1-241 mo). The group of metachronous GC patients (n = 34) was compared to the synchronous GC patient group (n = 22). Two patients were excluded from this comparison because they developed both metachronous and synchronous cancers. The group with multiple GC (n = 58) was compared to the group without a second cancer (n = 804).

Statistical analysis

For statistical analysis, we used χ^2 or Fisher's exact tests for categorical variables, and Student's t-test and Mann-Whitney U-test for continuous variables. $P < 0.05$ was considered to be statistically significant. All statistical analyses were performed with the statistical software STATISTICA 10.

RESULTS

Characteristics of the gastric cancer patients

The baseline characteristics of the GC patients included

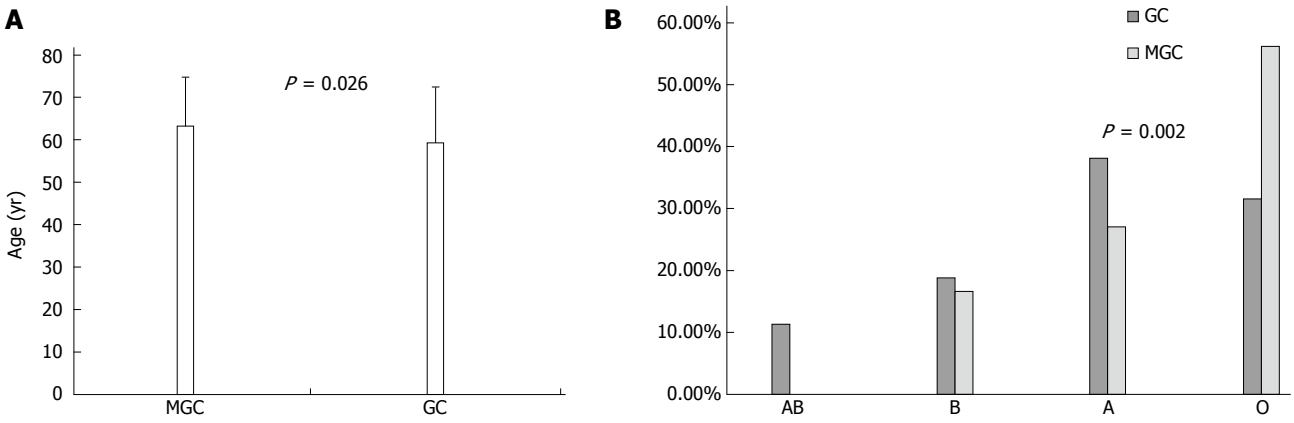

Figure 1 Mean ages (A) and blood groups (B) of the gastric cancer patients with (multiple gastric cancer) and without (gastric cancer) multiple tumors. GC: Gastric cancer; MGC: Multiple gastric cancer.

Table 1 Comparison of clinicopathological features of gastric cancer patients with and without a second primary cancer *n* (%)

Characteristic	Total	Gastric cancer	Multiple gastric cancer	*P* value
Gender				0.499
Male	570 (66.1)	534 (66.4)	36 (62.1)	
Female	292 (33.9)	270 (33.6)	22 (37.9)	
Total	862	804	58	
Age[1] (yr)	59.8 ± 12.8	59.5 ± 13.0	63.4 ± 11.4	0.026
DS (mo)	25.1 ± 46.4	25.1 ± 46.5	26.3 ± 46.3	0.433
FHC				0.806
No	369 (52.6)	342 (52.7)	27 (50.9)	
Yes	333 (47.4)	307 (47.3)	26 (49.1)	
Total	702			
Location[2]				0.467
Proximal	181 (21)	171 (21.3)	10 (17.2)	
Other location	681 (79)	633 (78.7)	48 (82.8)	
Total	862			
Histology				0.116
Intestinal	260 (43.4)	234 (42.2)	26 (57.8)	
Diffuse	289 (48.3)	272 (49.1)	17 (37.8)	
Mixed	50 (8.3)	48 (8.7)	2 (4.4)	
Total	599			
Stage				0.100
Early	119 (16.4)	106 (15.7)	13 (24.5)	
Advanced	608 (83.6)	568 (84.3)	40 (75.5)	
Total	727			
Blood group				0.002
A	211 (37.2)	198 (38.1)	13 (27.1)	
B	106 (18.7)	98 (18.9)	8 (16.7)	
O	191 (33.7)	164 (31.6)	27 (56.2)	
AB	59 (10.4)	59 (11.4)	None	
Total	567			

[1]Data are expressed as mean ± SD; [2]Tumor location. DS: Duration of symptoms; FHC: Family history of cancer.

in the study are shown in Table 1. The median age of all 862 patients (570 men and 292 women) was 59.8 ± 12.8 years (range 15-89 years). The mean duration of symptoms from the first alarming symptoms was 25.1 mo (range 0-480 mo). In 21 cases, there were no symptoms and gastric cancer was diagnosed during emergency endoscopy due to gastrointestinal bleeding. Among GC patients with a family history positive for cancer, 40.2% reported gastric cancer in first- or second-degree relatives, of whom 35.8% also had neoplasms other than gastric cancer. In 18% of patients with a family history positive for cancer, cancers of the gastrointestinal tract excluding the stomach (*e.g.*, colon, pancreas, and esophagus) were noted. In addition, 41.8% of patients reported other cancers (*e.g.*, leukemia, uterus, skin breast, lung, and larynx) in close family members. Twenty six (49%) of 53 multiple GC patients reported cancer in their first- or second-degree relatives; almost half (46.2%) of these relatives had GC.

GC surgery was performed on 598 patients; one underwent mucosectomy and the rest underwent exploratory surgery or were treated nonsurgically because of advanced GC or general contraindications. Of the total patients with GC and a known stage of disease (n = 727), 119 (16.4%) had early GC. Of the 119 patients with early GC, 13 (10.9%) had multiple GC tumors.

In 181 (21%) of the total 862 cases, the tumor site was the cardia and fundus or the cardia and the upper part of the truncus (classified as the proximal site). In the remaining patients, the tumor site was classified as other localization, as follows: 376 (43.6%) truncus, 253 (29.4%) antrum, 39 (4.5%) entire stomach. In 13 (1.5%) cases, the tumor was located in the anastomosis after a previous operation to treat ulcers.

The main histological type of gastric cancer was diffuse (48.3%), followed by intestinal (43.4%) and mixed (8.3%). The most common blood group in all GC cases was group A (37.2%), followed by group O (33.7%), group B (18.7%), and group AB (10.4%).

A comparison between the groups of patients with or without a second primary tumor indicated that the only significant differences occurred in age and blood group. The mean age of multiple GC patients was higher than that of those without a second primary tumor (63.4 ± 11.4 years *vs* 59.5 ± 13.0 years, respectively, P = 0.026) (Figure 1A).

GC patients with a second primary tumor were more commonly blood group O than those without a second primary tumor (56.2% *vs* 31.6%, respectively, P = 0.002) (Figure 1B). The following characteristics were not sig-

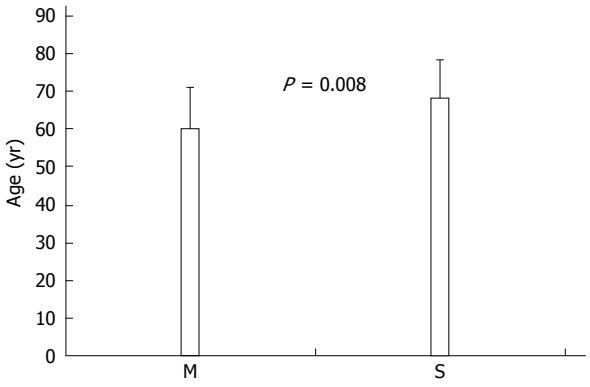

Figure 2 Mean ages of the gastric cancer patients with metachronous and synchronous neoplasms. M: Metachronous; S: Synchronous.

nificantly different between the two groups: sex, duration of symptoms, family history of cancer (negative history *vs* positive history), disease stage (early *vs* advanced), site of GC (proximal *vs* other localization), and histology (Table 1).

Characteristics of multiple gastric cancer patients

The baseline characteristics of the patients with multiple GC who participated in the study are shown in Table 1. Fifty-eight (6.7%) of the 862 GC patients developed another primary malignancy. Of the 58 multiple GC patients, 36 (62.1%) were men and 22 (37.9%) were women. The median age at diagnosis for these GC patients was 63.4 years ± 11.4 years (range 33-89 years). Four (6.9%) of the 58 patients developed two or more additional neoplasms, yielding a total of 62 multiple primary tumors other than GC, including 39 (63%) metachronous and 23 (37%) synchronous neoplasms. Of the 58 cases, 22 (38%) had synchronous and 34 (58.6%) had metachronous malignancies, while two patients developed two metachronous neoplasms. Two patients (3.4%) were diagnosed with both metachronous and synchronous cancers. One was diagnosed with three tumors (synchronous skin cancer and metachronous colon cancer at two different sites), while the second patient developed synchronous lung cancer and metachronous prostate cancer.

Of the 39 diagnosed metachronous neoplasms, 26 (66.7%) malignances were diagnosed before GC and 13 (33.3%) were diagnosed after GC. Of the patients with metachronous tumors, 25% developed a stomach malignancy after receiving chemotherapy or radiotherapy to treat the first primary neoplasm. Seven patients (19%) developed metachronous neoplasms after chemotherapy to treat GC, and one patient developed metachronous neoplasms after radiotherapy alone.

A comparison of the clinicopathological features of GC patients with synchronous and metachronous neoplasms yielded a statistically significant difference for patient age. GC patients with synchronous neoplasms were older than those with metachronous neoplasms (68.0 ± 10.3 years *vs* 59.9 ± 11.1 years, respectively, $P = 0.008$) (Figure 2). The following characteristics were not significantly different between the synchronous and metachronous neoplasm groups: sex, duration of symptoms, family history of cancer (negative history *vs* positive history), disease stage (early *vs* advanced), site of GC (proximal *vs* other localization), histology, and blood group (Table 2).

Table 2 Comparision of the clinicopathological features of gastric cancer patients with metachronous and synchronous tumors *n* (%)

Characteristic	Total	Metachronous	Synchronous	*P* value
Gender				0.139
Male	34	18 (52.9)	16 (72.7)	
Female	22	16 (47.1)	6 (27.3)	
Total[1]	56	34	22	
Age[2] (yr)	63.1 ± 11.3	59.9 ± 11.1	68.0 ± 10.3	0.008
DS (mo)	27.3 ± 46.9	31.7 ± 44.0	22.1 ± 54.0	0.217
FHC				0.249
No	26	14 (53.8)	12 (46.2)	
Yes	25	18 (72.0)	7 (28.0)	
Total	51			
Location[3]				0.724
Proximal	10	7 (70.0)	3 (30.0)	
Other location	46	27 (58.7)	19 (41.3)	
Total	56			
Histology				0.835
Intestinal	24	16 (66.7)	8 (33.3)	
Diffuse	17	12 (70.6)	5 (29.4)	
Mixed	2	1 (50.0)	1 (50.0)	
Total	43			
Stage				0.553
Early	13	7 (53.8)	6 (46.2)	
Advanced	38	24 (63.2)	14 (36.8)	
Total	51			
Blood group				0.675
A	13	9 (69.2)	4 (30.8)	
B	8	4 (50.0)	4 (50.0)	
O	25	15 (60.0)	10 (40.0)	
AB	None	None	None	
Total	46			

[1]Two patients were excluded from this comparison because they developed both metachronous and synchronous cancers; [2]Data are expressed as mean ± SD; [3]Tumor location. DS: Duration of symptoms; FHC: Family history of cancer.

Site distribution of second cancer

An analysis of the site distribution in the 58 GC patients with multiple cancers ($n = 62$) showed that the most common site was colorectal ($n = 17$, 27.4%), followed by lung ($n = 9$, 14.5%), breast ($n = 8$, 12.9%), and prostate ($n = 7$, 11.3%) (Figure 3). In men, the most common site was colorectal ($n = 10$), lung ($n = 8$), and prostate ($n = 7$). In women, the most common site was breast ($n = 8$) and colorectal ($n = 7$). Among the 23 synchronous tumors, the most common primary sites were colorectal ($n = 10$, 43.5%), lung ($n = 4$, 17.4%), and prostate ($n = 3$, 13.1%). For the 39 metachronous cancers, the dominant types were colorectal cancer ($n = 7$, 17.9%) and breast cancer ($n = 7$, 17.9%), followed by lung cancer ($n = 5$, 12.8%) (Table 3).

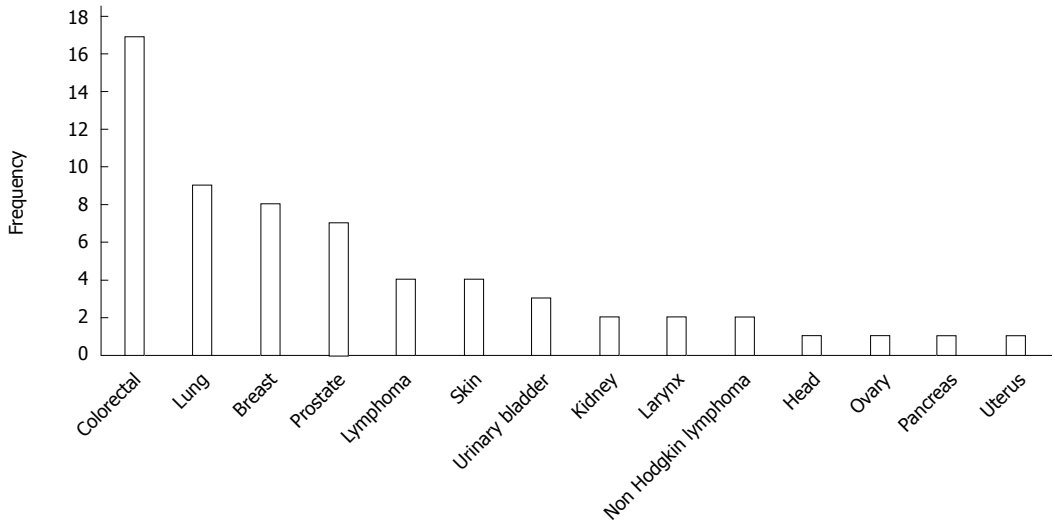

Figure 3 Site distribution of additional primary cancers in 58 gastric cancer patients.

Table 3 Sites of additional synchronous and metachronous cancers in gastric cancer patients *n* (%)

Site	No. tumors *n* = 62	Metachronous *n* = 39	Synchronous *n* = 23
Colorectal	17 (27.4)	7 (17.9)	10 (43.5)
Lung	9 (14.5)	5 (12.8)	4 (17.4)
Breast	8 (12.9)	7 (17.9)	1 (4.3)
Prostate	7 (11.3)	4 (10.3)	3 (13.1)
Lymphoma	4 (6.5)	2 (5.1)	2 (8.7)
Skin	4 (6.5)	2 (5.1)	2 (8.7)
Urinary bladder	3 (4.9)	3 (7.7)	-
Kidney	2 (3.2)	1 (2.6)	1 (4.3)
Larynx	2 (3.2)	2 (5.1)	-
Non Hodgkin Lymphoma	2 (3.2)	2 (5.1)	-
Head	1 (1.6)	1 (2.6)	-
Ovary	1 (1.6)	1 (2.6)	-
Pancreas	1 (1.6)	1 (2.6)	-
Uterus	1 (1.6)	1 (2.6)	-

DISCUSSION

The main finding of the current study was a rate of 6.7% for the occurrence of multiple cancers in a group of GC patients in Poland. This rate is slightly higher than those reported by studies conducted in Italy, Portugal, and Sweden, in which the estimated rates were 1.9%-3.4%[9,10,19] in analyses of groups that included more than 34000 patients. In a study of more than 4500 patients in Korea who underwent surgery for GC, Eom *et al*[8] reported an incidence of multiple cancers of approximately 3.4%. Luciani *et al*[3] looked at 1503 consecutive GC patients and estimated that the overall prevalence of multiple malignances was 10% and that it was higher in patients ⩾ 70 years old compared to younger patients (15% *vs* 6%, respectively). Our findings are similar to those of Kim *et al*[20], who analyzed 5778 patients with GC in Korea and found that 423 (7.3%) had been diagnosed with synchronous and metachronous double primary cancers.

A group of Japanese researchers[21] reported that about 5% of 1070 early GC patients developed multiple malignancies after surgical treatment; however, the authors described only metachronous tumors in their prospective study. Similarly, other Japanese researchers[22] reported ten (9.1%) patients who died due to primary cancers other than the original GC in a group of 109 early GC cases. We found that the incidence of multiple tumors in early GC cases was 10.9%, which remains consistent with data from Asia. Because early GC patients have a longer survival and thus are more likely to develop other neoplasms, it seems that multiple tumors would be expected to occur more frequently in early GC patients. The data from Green *et al*[13] confirms this. The authors estimate that this phenomenon may affect up to 32% of early GC patients, which seems to be a very high percentage considering that the authors analyzed only 28 patients.

Our data show that GC patients with synchronous neoplasms were older than those with metachronous neoplasms (68.0 ± 10.3 years *vs* 59.9 ± 11.1 years, respectively, $P = 0.008$). However, there were no statistically significant differences between these two groups in terms of sex, family history of cancer, duration of symptoms, tumor site in the stomach, histology, disease stage (early *vs* advanced), or blood group. This finding is consistent with the findings of Dinis-Ribeiro *et al*[10], who analyzed a group of 2668 GC patients and found 78 (3.4%) cases with primary tumors other than GC. They also found that patients with synchronous neoplasms were older than those with metachronous neoplasms. They, too, found no statistically significant differences with regard to sex, GC location, and TNM staging. Taken together, these results indicate that after a diagnosis of GC, older patients in particular should be investigated for second malignancies.

In our series, the most common types of synchronous and metachronous neoplasms were colorectal cancer, followed by cancers of the lung, breast, and prostate. Our data seems to be similar to the findings of

others, who also reported that colorectal cancer is the most frequent neoplasm in GC patients with multiple malignancies[6,8,10,17,20,23], followed by lung, uterus, breast, and prostate cancers[6,8,10,11,17,24].

Our observations are somewhat surprising when one considers that in the Polish population, the most common type of cancer is lung cancer, while colon and rectal cancer are the fourth and seventh most common types (according to registration of new cases of cancer sites in men). However, in women, lung cancer is the second most common cancer after breast cancer, while colon cancer is the fifth and rectal is the eighth most common cancer (according to registration of new cases)[25]. The high incidence of colorectal cancer in GC patients may be due to the same environmental factors that affect the gastrointestinal tract; strikingly, however, in our study group, none of the GC patients had esophageal carcinoma. In contrast, other researchers have found that the esophagus is one of the more common sites for second primary tumors in GC patients[6,21,23]. It is difficult to interpret this difference in findings among studies, because carcinogenesis is such a complex and poorly understood process. Previous anticancer therapy is considered to predispose patients to developing additional malignancies. In the current study, 27.6% of the GC patients with multiple metachronous primary cancers had been treated with cytotoxic agents or radiation therapy for a first cancer regardless of whether GC was the first or second tumor.

A comparison between the groups of patients with or without a second primary tumor indicated that the only significant differences were in blood group and age. GC patients with second primary tumors were more commonly group O than those without (56.2% *vs* 31.6%, respectively, $P = 0.002$). However, out of our entire group of patients with GC, only 4.8% had both blood group O and multiple tumors. In GC patients with multiple malignancies, more than half were blood group O, 27% were blood group A, and almost 17% were blood group B. None of the patients were AB, although 40% of the Polish population are blood group A, 32% are O, 19% are B, and 9% are AB. In a previous study, we found that of 195 GC patients, 45% were blood group A, 27% were blood group O, 18% were blood group B, and 10% were blood group AB[26]. Previous studies reported that blood group A is associated with GC[27-29]. Furthermore, Wang *et al*[30] indicated a significantly higher risk of GC in individuals with blood group A; moreover, these patients were more likely to be infected with *Helicobacter pylori* than individuals with other ABO blood types. Other studies have found associations between pancreatic cancer and breast cancer and blood groups[31,32]; thus, this may not be a random association, and it may merit further investigation. To the best of our knowledge, our results represent the first investigation of these associations, and they indicate that patients with GC who have blood group O should be controlled for the development of second primary tumors.

The mean age of patients with multiple tumors was higher than that for patients without a second primary tumor (63.4 ± 11.4 years *vs* 59.5 ± 13.0 years, respectively, $P = 0.026$).

Ikeda *et al*[17] found that patients with a second tumor tended more frequently to be males and elderly than those without a second tumor. Eom *et al*[8] indicated that the mean age of patients and the proportion who had early GC were both higher in patients with a second cancer than in those without. Both studies were conducted in Asia and were carried out in larger groups.

Comparisons of clinicopathological features other than age and blood groups indicated that GC patients with and without a second cancer did not differ in terms of sex, tumor site, family history of cancer, stage, and histology. Despite the lack of statistically significant differences between GC patients with and without a second tumor in terms of family history of cancer, one observation is noteworthy: Almost half (49%) of the GC patients with multiple malignancies reported cancer in their first- or second-degree relatives; almost half (46.2%) of these relatives had GC. The only previous report of a similar finding came from Muela Molinero *et al*[19], who observed that 56% of GC patients diagnosed with multiple malignant primary neoplasms had a history of cancer in first-degree relatives. In our previous study of a group of 218 GC patients, a positive family history of cancer was noted in 36% of cases; of these cases, 46% had GC[26].

In conclusion, we confirmed the phenomenon of multiple malignancies in a group of GC patients in Poland. Specifically, we found that almost 7% of GC patients in our population had additional neoplasms. The most common additional malignancies were colorectal, breast, lung, and prostate cancers. Based on our findings, we believe that after a diagnosis of GC, the attending physician should be vigilant and perform additional procedures as appropriate, such as colonoscopy, mammography, chest X-ray, and abdominopelvic computed tomography. This may be particularly relevant for GC patients who are blood group O and are older.

COMMENTS

Background

The phenomenon of multiple primary neoplasms is increasingly being discussed due to the increased survival time of cancer patients after treatment and because of advances in diagnostic methods. It is estimated that multiple neoplasms affect about 10% of all cancer patients. In the study, 6.7% developed a total of 62 multiple primary tumors. The predominant tumor type of the secondary neoplasms was colorectal, followed by lung, breast, and prostate. The present paper is one of only a few European reports, it is the only study from Poland to explore this subject.

Research frontiers

Gastric cancer patients may develop other primary cancers; appropriate preoperative and postoperative diagnostic modalities, such as colonoscopy, mammography, chest X-ray, and abdominopelvic computed tomography are thus required, particularly if patients are older and blood group O. It seems that future investigation will decide on their clinical suitability.

Innovations and breakthroughs

The current study shows not only the occurrence of multiple tumors in patients with gastric cancer, as has been reported by others, but also investigates as-

sociations between this phenomenon and the following clinical features: blood groups, duration of symptoms, and family history of cancer. The results represent the first investigation of these associations, and they indicate that patients with gastric cancer who have blood group O should be controlled for the development of second primary tumors.

Applications

Patients with gastric cancer patients may develop other primary tumors, particularly those who are blood group O and of advanced age. Appropriate additional preoperative and postoperative diagnostic modalities may be needed for patients with gastic cancer who have a higher likelihood of developing synchronous and metachronous tumors.

Peer review

The authors presented the data of prevalence and clinical characteristics of gastric cancer patients with additional primary malignancies and found that patients with older age and blood O were more likely to develop other primary cancers beyond gastric cancers. Those findings were not reported in other similar articles. This is an interesting observations and study has been performed nicely. However, it would be more useful for clinical practice to find more clinical parameters valuable in identifying gastric cancer patients with multiple cancers.

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PROSPECTIVE STUDY

Complications after ileal pouch-anal anastomosis in Korean patients with ulcerative colitis

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Abstract

AIM: To investigate the outcomes of treatments for complications after ileal pouch-anal anastomosis (IPAA) in Korean patients with ulcerative colitis.

METHODS: Between March 1998 and February 2013, 72 patients (28 male and 44 female, median age 43.0 years ± 14.0 years) underwent total proctocolectomy with IPAA. The study cohort was registered prospectively and analyzed retrospectively. Patient characteristics, medical management histories, operative findings, pathology reports and postoperative clinical courses, including early postoperative and late complications and their treatments, were reviewed from a medical record system. All of the ileal pouches were J-pouch and were performed with either the double-stapling technique (n = 69) or a hand-sewn (n = 3) technique.

RESULTS: Thirty-one (43.1%) patients had early complications, with 12 (16.7%) patients with complications related to the pouch. Pouch bleeding, pelvic abscesses and anastomosis ruptures were managed conservatively. Patients with pelvic abscesses were treated with surgical drainage. Twenty-seven (38.0%) patients had late complications during the follow-up period (82.5 ± 50.8 mo), with 21 (29.6%) patients with complications related to the pouch. Treatment for pouchitis included antibiotics or anti-inflammatory drugs. Pouch-vaginal fistulas, perianal abscesses or fistulas and anastomosis strictures were treated surgically. Pouch failure developed in two patients (2.8%). Analyses showed that an emergency operation was a significant risk factor for early pouch-related complications compared to elective procedures (55.6% *vs* 11.1%, $P < 0.05$). Pouchitis was related to early (35.3%) and the other late pouch-related complications (41.2%) ($P < 0.05$). The complications did not have an effect on pouch failure nor pouch function.

CONCLUSION: The complications following IPAA can be treated successfully. Favorable long-term outcomes were achieved with a lower pouch failure rate than reported in Western patients.

Key words: Ulcerative colitis; Ileal pouch-anal anastomosis; Complications; Pouch failure; Pouch function

Core tip: There has been a recent increase in the number of ileal pouch-anal anastomosis (IPAA) procedures conducted to treat ulcerative colitis in Asian countries, including Korea. However, the reports on the outcomes of IPAA have been lacking. This study investigated the treatment outcomes for complications associated with

ileal pouch formation, including pouch failure. The early postoperative complications included pouch bleeding, pelvic abscess, and anastomosis rupture. The late complications included pouchitis, pouch-vaginal fistula, perianal abscess or fistula, and anastomosis stricture. The post IPAA complications were treatable either medically or surgically with a lower pouch failure rate than previously reported from Western countries.

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INTRODUCTION

Ulcerative colitis (UC) is characterized by a chronic course of recurrent relapse and remission and the need for long-term medical management. However, 20%-30% of patients undergo surgical treatment and are successfully treated by total colectomy[1]. Since the first introduction of ileal pouch-anal anastomosis (IPAA) in 1978, the procedure has become a standard surgical method for treatment of UC while avoiding a permanent ileostomy[2]. The J-pouch is a popular method, and many surgeons have accepted IPAA as a safe procedure with favorable functional results and improvements in quality of life[3-6].

Many complications may be encountered after IPAA and several reports presented significant morbidity and mortality rates[7,8]. Early postoperative complications, such as small bowel obstruction, pouch bleeding, leakage and pelvic sepsis, may be detrimental to the patient's health. Late complications can often cause troublesome results, reducing the satisfaction of surgical treatments. Pouchitis is one of the late complications and readministration of anti-inflammatory treatment is required. Pouch stricture or fistulas can develop, resulting in functional problems such as frequency, incontinence or sexual dysfunction, leading to further distress. These complications are difficult to manage and require complex treatments including various medications and surgical procedures. In 5%-10% of the patients, complications cannot be resolved resulting in a pouch failure[9,10].

In Asian countries, including Korea, there has been an increase in the number of UC cases, consequently increasing the number of surgical treatments[11]. However, there has been a lack of reports regarding the long-term treatment outcomes for complications of IPAA. This study describes the early and late complications associated with IPAA for UC, as well as the outcomes of the corresponding treatments, including the rate of pouch failure.

MATERIALS AND METHODS

Patient population and surgical procedure

Between March 1998 and February 2013, 72 consecutive patients underwent IPAA for UC by a single surgeon (Park KJ) at our institution with extensive experience in the colorectal division. The prospective registered patient cohort was analyzed retrospectively. This study was approved by our Institutional Review Board (IRB approval number: H-1107-044-368). Patient characteristics, medical management histories, operative findings, pathology reports, postoperative clinical courses, including complications and their treatments, and stool frequency presented as bowel movements/d were reviewed from an electronic medical record system. The criteria for elective operations were medical intractability, dysplasia or malignancy. The criteria for emergency operations were massive bleeding, fulminant colitis or toxic megacolon. Medical intractability was defined as an inability to remain in remission despite long periods of medical anti-inflammatory treatment. Dysplasia or malignancy was confirmed by routine colonoscopy. Massive bleeding was defined by a continuous necessity for more than four units of packed red blood cells in 24 h or bleeding that put the patient at risk of developing shock. Fulminant colitis was suspected when sepsis resulted from an aggravation of the colitis and peritonitis despite the initial attempts at medical management. Toxic megacolon was diagnosed by radiologic examinations. The surgical procedure was composed of a proctocolectomy and IPAA with or without a loop ileostomy. All of the ileal pouches were generated by the J-pouch procedure, and most of the anastomoses were performed by the double-stapling technique. A few patients required hand-sewn anastomoses, and all of these procedures were performed from the anal side. Only after confirming the security of IPAA, through radiological imaging of the loopogram using a water-soluble dye, was the loop ileostomy taken down.

Classification of complications

Postoperative complications were classified into three categories based upon the post-surgery period in which they occurred. The first category included early complications, which occurred during the postoperative recovery period after IPAA, usually within 30 d of surgery. The second category included complications related to the ileostomy takedown, and the third category included late complications that occurred during the follow-up period. Pouch failure was defined as excision of a total pouch or a nonfunctioning pouch requiring permanent diversion with ileostomy as a result of failure to manage the pouch-related complications.

Statistical analysis

The treatments and outcomes of the postoperative complications were investigated, and the risk factors for complications were analyzed using SPSS for Windows,

Table 1 Clinical characteristics of the patients and operations *n* (%)

Characterisitics	
Sex	
Male	28 (38.9)
Female	44 (61.1)
Age	
⩾ 40	47 (65.3)
< 40	25 (34.7)
BMI	
⩾ 25	10 (13.9)
< 25	62 (86.1)
Co-morbidity	21 (29.2)
Hypertension	12 (16.7)
Diabetes	5 (6.9)
Liver disease	4 (5.6)
Cerebrovascular disease	2 (2.8)
Idiopathic thrombocytopenic purpura	1 (1.4)
Disease extent	
Left colon	18 (25.0)
Transverse colon	27 (37.5)
Whole colon	27 (37.5)
Operation	
Elective	63 (95.8)
Emergent	9 (12.5)
Indications	
Medical intractability	56 (77.8)
Dysplasia or malignancy	7 (9.7)
Fulminant colitis	5 (6.9)
Toxic megacolon	2 (2.8)
Massive bleeding	2 (2.8)
Anastomosis	
Double stapling	69 (95.8)
Hand-sewing	3 (4.2)

BMI: Body mass index.

version 18.0 (SPSS Inc., Chicago, IL, United States). Univariate analysis was performed using Pearson's χ^2 test and Fisher's exact test. Multivariate analysis was performed using logistic regression for the factors with *P* values of < 0.25 by univariate analysis. A Mann-Whitney test was performed for comparison of the values of stool frequency. Data are presented as median ± the standard deviation. Statistical significance was accepted for *P* values of < 0.05.

RESULTS

Clinical characteristics

The median age at surgery was 43.0 ± 14.0 years [range: 11-67 years; interquartile range (IQR): 33.5-55.5]. The median age at diagnosis of UC was 39.0 ± 14.0 years (range: 5-67 years; IQR: 26.0-49.0), and the median duration from the diagnosis to surgery was 52.0 ± 65.0 mo (range, 1.0-260.0 mo; IQR: 23.3-118.8). All of the patients had taken at least one anti-inflammatory drug, such as 5-aminosalicylic acid, azathioprine or steroids. The median body mass index (BMI) was 21.7 ± 3.6 (range: 12.9-31.1; IQR: 19.4-24.2). The clinical characteristics of the patients are described in Table 1.

Table 2 Early complications after ileal-pouch anal anastomosis *n* (%)

Complications	
Early complications	31 (43.1)
Pouch related	12 (16.7)
Pouch bleeding	6 (8.3)
Pelvic abscess	4 (5.6)
Anastomosis rupture	3 (4.2)
Pouch unrelated	26 (36.1)
Wound infection	13 (18.1)
Ileus	6 (8.3)
Intra-abdominal bleeding	6 (8.3)
Pneumonia	2 (2.8)
Deep vein thrombosis	2 (2.8)

Operations

Total proctocolectomy was performed on 71 (98.6%) patients. Remnant proctectomy was performed on one (1.4%) patient who underwent total colectomy and ileorectal anastomosis due to the uncertainty in a preoperative diagnosis of colitis. This patient was eventually diagnosed with UC and underwent remnant proctectomy and IPAA. Most of the patients underwent IPAA using a double stapling technique, but hand-sewn anastomosis was also performed in three (4.2%) patients. Of these three, two patients had serious tearing on the distal stump subsequent to stapling, and the other patient had a negative margin of low rectal cancer. Diverting loop ileostomy was performed in 71 (98.6%) patients. One patient underwent surgery for dysplasia and inflammation of the colon followed by a loop ileostomy a month later due to the development of a pouch-vaginal fistula. The characteristics of operations are described in Table 1.

Treatments for early postoperative complications after IPAA

Thirty-one (43.1%) patients experienced early postoperative complications (Table 2). One patient (1.4%), who underwent emergency surgery, died due to pneumonia. Pouch-related complications occurred in 12 (16.7%) patients. Most of the early complications were treated conservatively, for example with transfusion for pouch bleeding, antibiotics or percutaneous drainage for pelvic abscess or anastomosis rupture. One patient was treated surgically with irrigation and drainage through the anal side to treat a pelvic abscess and an anastomosis rupture.

Treatments for complications related to ileostomy takedown

Diverting loop ileostomies were closed in 71 (98.6%) patients after a median period of 4.4 ± 3.4 mo (range: 2.0-23.0 mo; IQR: 3.6-6.7). Only one patient died due to an early postoperative complication after IPAA. The 12 (16.9%) patients with pouch-related early complications underwent an ileostomy takedown after 7.1 ± 5.4 mo (range: 4.0-23.0 mo; IQR: 4.8-7.7), whereas the remaining patients underwent takedown after 4.3 ± 2.5 mo (range:

Table 3 Complications related to ileostomy take-down *n* (%)

Complications	
Complications at ileostomy take-down	17 (23.6)
Wound infection	9 (12.5)
Ileus	8 (11.1)
Enterocutaneous fistula	2 (2.8)
Intra-abdominal abscess	1 (1.4)
Cardiac problems	1 (1.4)

2.0-13.0 mo; IQR: 3.4-6.0) ($P < 0.05$). Seventeen (23.9%) patients had complications related to ileostomy takedown (Table 3) that were managed conservatively. No patients were lost following the ileostomy takedown.

Treatment for late complications during the follow-up period

Seventy-one patients were followed-up and the median follow-up period was 82.5 ± 50.8 mo (range: 2.0-179.0 mo; IQR: 35.5-136). Twenty-seven (38.0%) patients had late complications, and complications related to IPAA were found in 21 (29.6%) patients (Table 4). Pouchitis was the most common late complication, and occurred at a median of 15.0 ± 29.4 mo (range: 2.0-90.0 mo; IQR: 5.9-46.8) after IPAA. Pouchitis was managed by antibiotics or anti-inflammatory drugs. Pouch-vaginal fistulas developed at 17.0 ± 22.7 mo (range: 1.0-58.0 mo; IQR: 3.0-42.0), and were treated by a transanal advancement flap in three patients or diverting loop ileostomy in two patients. One patient, who had undergone a transanal advancement flap experienced recurrent fistulas, and an ileostomy was consequently performed. However, a fistula recurred again after the ileostomy was taken down, and a transanal rectal advancement flap was repeated. The patient eventually underwent pouch removal and reformation of the IPAA, but suffered from a recurrent pouch-vaginal fistula and severe incontinence. As a result, a pouch excision with permanent end ileostomy was performed. One of the patients, who had a rectovaginal fistula treated with an ileostomy, underwent an ileostomy takedown after closure of the fistula, while another patient requested a permanent ileostomy. Perianal abscesses or fistulas were treated by conventional procedures of incision and drainage, fistulotomy or seton placement. Anastomosis stricture was managed by anal strictureplasty or manual dilatation. As a result, pouch failure developed in two (2.8%) patients due to pouch excision following a re-pouch formation for recurrent pouch-vaginal fistula and a refusal of ileostomy takedown, respectively. The outcomes of treatments for the pouch-related complications are described in Figure 1.

Risk factors for pouch-related complications and pouch failure

In a univariate analysis, emergency operation was a statistically significant risk factor for pouch-related early complications ($P < 0.05$), which were related to the development of pouchitis ($P < 0.05$). In a multivariate analysis, emergency operation and pouchitis were significantly related to pouch-related early complications (HR = 19.1, 95%CI: 3.1-119.7, $P < 0.05$, and HR = 6.4, 95%CI: 1.4-30.5, $P < 0.05$, respectively) (Table 5). Pouchitis was a significant risk factor for other late pouch-related complications in univariate ($P < 0.05$) and multivariate analyses (HR = 10.0, 95%CI: 2.1-47.0, $P < 0.05$). There was no significant risk factor for pouch failure.

Table 4 Late complications after ileal-pouch anal anastomosis *n* (%)

Complications		Development (mo, median)
Late complications	27 (37.5)	
Pouch related	21 (29.2)	
Pouchitis	17 (23.6)	15
Pouch vaginal fistula	5 (6.9)	17
Perianal abscess or fistula	4 (5.6)	11
Anastomosis stricture	2 (2.8)	21.5
Pouch unrelated	12 (16.7)	
Ileus	10 (13.9)	17
Incisional hernia	2 (2.8)	18.5

Pouch function at the last follow-up

The median stool frequency, an indication of pouch function, was 6.0 ± 2.2 bowel movements/d (range: 3-10 movements/d; IQR: 5.0-8.0) at the last follow-up visit. Patients with early pouch-related complications ($n = 12$) had 7.0 ± 2.3 bowel movements/d (range: 3-10 times/d; IQR: 4.3-8.5), and early pouch-related complication-free patients ($n = 59$) had 5.0 ± 2.2 bowel movements/d (range: 3-10 movements/d; IQR: 5.0-8.0) (Figure 2A). Patients with late pouch-related complications ($n = 21$) had 7.0 ± 2.4 bowel movements/d (range: 3-10 movements/d; IQR: 5.0-10.0), whereas late pouch-related complication-free patients ($n = 50$) had 5.0 ± 2.1 bowel movements/d (range: 3-10 movements/d; IQR: 4.8-8.0) (Figure 2B).

DISCUSSION

IPAA is considered a safe procedure due to low mortality (< 1.0%) with most patients having a long-term satisfactory functional pouch and a pouch failure rate of 5%-10%[12]. However, the morbidity rate varies greatly (30%-60%) with pouch-related complications distressing both the patient and surgeon. In some cases, a permanent ileostomy is needed for severe refractory pouchitis, fistula, incontinence or stenosis[13]. Recently, laparoscopy has been reported as a safe procedure with a significantly lower rate of early complications. However, laparoscopy is a challenging procedure, and long-term results should be further evaluated[14].

In this study, early postoperative complications occurred in 43.1% of patients, and late complications occurred in 38.0% of patients. Pouch failure developed in 2.8% of patients, with complete pouch failure developing in one patient who developed recurrent pouch-vaginal fistulas. The pouch failure rate was relatively lower than

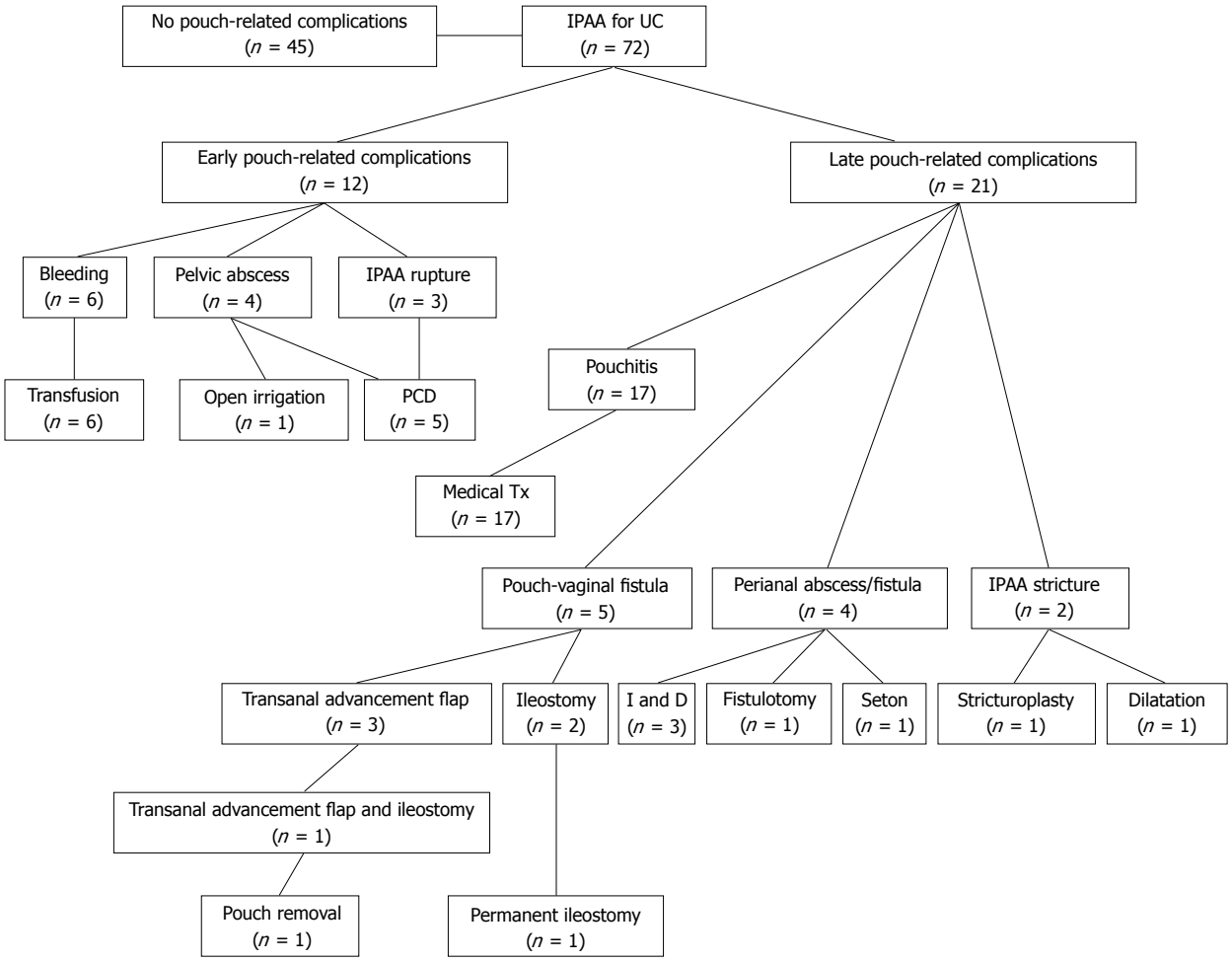

Figure 1 Treatment outcomes for pouch-related complications following ileal pouch-anal anastomosis in Korean patients with ulcerative colitis. Most of the pouch-related complications were treated successfully and pouch failure developed in two patients. IPAA: Ileal pouch-anal anastomosis; UC: Ulcerative colitis; PCD: Per-cutaneous drainage; Tx: Treatment; I and D: Incision and drainage.

in some Western reports despite a similar incidence of complications. Although the pouch failure rate can increase during the duration of the follow-up period, our long-term results were still comparable to those of Western counterparts. A meta-analysis study published by a Dutch team, which pooled data from more than 40 Western studies, reported a pouch failure rate of 6.8% in a 36.7-mo follow-up period. The pouch failure rate increased to 8.5% in a follow-up period of > 5 years[8]. The differences in pouch failure rates between the present and Western studies may be due to ethnic and dietary differences between Asian and Western patients. Earlier Japanese studies also reported a lower rate of pouch failure, suggesting that Asian countries might achieve better outcomes with IPAA treatment of UC. However, further studies are necessary to confirm this statement. Another explanation for the lower pouch failure rates in this study is a lack of patients with indeterminate colitis. The IPAA treatment outcomes for indeterminate colitis or Crohn's disease are poorer than for preoperatively confirmed UC[15]. The rate of success in this study may be due to the aggressive and methodical treatments for the complications. Most of the complications were treated successfully, though some of the patients with pouch failure still had unresolved late pouch-related complications. Thus, prompt and targeted treatment for pouch-related complications is important to prevent future pouch failure.

Early pouch-related postoperative complications were significantly related to pouchitis, which was also a significant risk factor for the other late pouch-related complications in this study. One explanation is that extensive delays of diverting loop ileostomy takedown for treatment of pouch-related complications could lead to inflammation of the mucosa. The inflammation may be a result of changes in pH, bacterial flora or oxygen content, and consequently increase the rate of chronic pouchitis[16]. Patients with early pouch-related complications delayed ileostomy longer than others in this study. Pouch bleeding may also be an early sign of acute pouchitis[17,18], but in this study, the acute pouch bleedings during the immediate postoperative periods most likely originated from the ileal mucosa of the anastomosed staple lines. Based on the correlation with subsequent pouch-related complications, previous reports have recommended early and long-standing maintenance and treatment for pouchitis[19]. Although both antibiotics and topical steroid en-

Table 5 Analysis of risk factors for pouch related complications and pouch failure

Variables	Early complication	Late complication	Pouch failure	*P* value (univariate) (early/late/failure)	*P* value (multivariate) (early/late)
Sex				0.755/0.249/0.518	/0.998
Male	4 (14.3)	6 (21.4)	0 (0.0)		
Female	8 (18.2)	15 (34.1)	2 (4.5)		
Age				0.524/0.140/1.000	/0.534
⩾ 40	9 (19.1)	11 (23.4)	1 (2.1)		
< 40	3 (12.0)	10 (40.0)	1 (4.0)		
Overweight				1.000/0.713/1.000	
BMI ⩾ 25	1 (10.0)	2 (20.0)	0 (0.0)		
BMI < 25	10 (17.7)	19 (30.6)	2 (3.2)		
Co-morbidities				0.737/0.618/0.501	
Yes	4 (19.0)	7 (33.3)	1 (4.8)		
No	8 (15.7)	14 (27.5)	1 (2.0)		
Disease extents				1.000/0.653/1.000	
Lt colon	3 (16.7)	6 (33.3)	0 (0.0)		
Beyond lt colon	9 (16.7)	15 (27.8)	2 (3.7)		
Operation				0.005/1.000/1.000	0.002/
Emergent	5 (55.6)	2 (22.2)	0 (0.0)		
Elective	7 (11.1)	19 (30.2)	2 (3.2)		
Anastomosis				0.426/1.000/1.000	
Double stapling	11 (15.9)	20 (29.0)	2 (2.9)		
Hand sawing	1 (33.3)	1 (33.3)	0 (0.0)		
Pouchitis				0.028/0.002[1]/0.419	0.016/0.002[1]
Yes	6 (35.3)	7 (41.2)[1]	1 (5.9)		
No	6 (10.9)	4 (7.3)	1 (1.8)		
Early complications				/0.095/1.000	/0.998
Yes		6 (50.0)	0 (0.0)		
No		15 (25.0)	2 (3.3)		
Late complications					
Yes			2 (9.5)	/0.082	
No			0 (0.0)		

[1]Analysis with late complications except for the pouchitis. BMI: Body mass index.

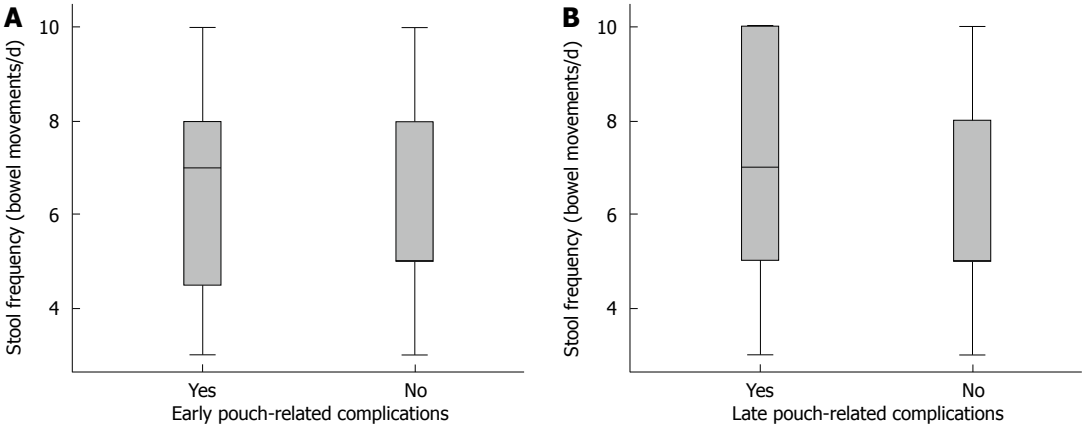

Figure 2 Stool frequencies after ileal pouch-anal anastomosis. A: Bowel movements/day was compared between patients with early pouch-related complications (n = 12) and early pouch-related complication-free patients (n = 59) (7.0 ± 2.3 movements/d *vs* 5.0 ± 2.2 movements/d); B: Bowel movements/day was compared between patients with late pouch-related complications (n = 21) and late pouch-related complication-free patients (n = 50) (7.0 ± 2.4 movements/d *vs* 5.0 ± 2.1 movements/d).

emas have been shown to effectively treat pouchitis, anti-inflammatory or immunosuppressive drugs might still be required in severe cases. Recently, infliximab was shown to be effective in some patients[20], and probiotics may also be beneficial in acquiring a prophylactic effect[21].

Cuffitis is a complication comparable to pouchitis that can arise from the remnant columnar cuff 2-3 cm above the anal margin after double-stapling IPAA[22]. Cuffitis, which can cause pouch-related symptoms, can be treated with topical anti-inflammatory suppositories[23]. We performed most of the IPAAs using the double-stapling technique because of its simplicity and feasibility compared to hand-sewn anastomosis. Furthermore, the double-stapling technique is known to have lower post-operative complications with better functional outcomes against anal incontinence[24]. As minimal cuff length is

the critical factor for the prevention of cuffitis and development of dysplasia or cancer, a surgeon should make every effort to avoid insufficient resection of the distal rectum.

Pouch-vaginal fistula is one of the most devastating complications and has a high recurrence rate following local repair[25]. In certain cases, either a long-standing diverting ileostomy might be necessary, or more a complex procedure, such as a gracilis muscle flap, should be performed[26]. Pouch-vaginal fistulas are more frequent and more complex in Crohn's colitis than in UC, resulting in a 48% IPAA failure rate[27-29]. However, there are reports of pouch-vaginal fistulas developing in UC patients with pelvic sepsis or when a surgeon encountered technical problems[4,30]. Thorough histologic reviews, performed on the five patients who had pouch-vaginal fistulas, confirmed UC. The conclusion was based on the presence of diffuse chronic inflammation without ileal inflammation and diffuse crypt changes with atrophy, distortion or villiform surface, all favoring a diagnosis of UC over Crohn's disease[31]. Most of our patients presented with relatively simple fistulas and were treated with either a transanal advancement flap or ileostomy. Only one patient had a pouch failure from recurrent fistulas despite reformation of the IPAA resulting in a complete pouch excision. We hypothesize that the fistula may have been due to severe pouchitis. Although favorable results have been reported in some cases, a re-pouching procedure may be difficult due to intra-abdominal adhesions from previous laparotomies, severe inflammation around the pouch and difficulty in finding the deep lower fistula opening[32,33].

The performance of an emergency operation was found to be a risk factor for early pouch-related complications, and was conducted in patients with a more severe status. Massive bleeding, fulminant colitis or toxic megacolon could deteriorate the general physical conditions of the patients, and many reports have asserted that pouch surgery should be avoided under these circumstances[34]. However, we performed pouch procedures in emergency surgeries with low mortality despite the significant morbidity of early pouch-related complications. Emergency operations were not related to late pouch-related complications, and there were no pouch failures in these patients. Although there are few reports stating that IPAA is a safe procedure in severe emergency cases[35,36], we considered IPAA to be a reasonable procedure if performed by an experienced colorectal surgeon. Nonetheless, further studies are necessary to verify its safety.

Long-term pouch function and quality of life have been reported to be satisfactory in many patients, and we previously reported good functional outcomes of IPAA[37,38]. In this study, stool frequency in the patients with early and late pouch-related complications was slightly higher, but there was no significant difference when compared to pouch-related complication-free patients. The conclusion is that successfully treated complications can lead to good pouch function and, therefore, better quality of life. The two major limitations of this study are retrospective data analysis and a small sample pool. Nevertheless, due to an increase in number of UC cases in Korea, it is important to investigate the outcomes of IPAA and identify treatments for long-term follow-up periods.

In conclusion, conducting IPAA for UC is a safe and successful procedure despite the potential for complications. Early detection and aggressive medical and surgical treatment for pouch-related complications are critical in achieving lower rate of pouch failure and better functional outcomes. Furthermore, meticulous effort by the surgeon is required to prevent recurrent complications, especially during technically demanding procedures.

COMMENTS

Background

Ileal pouch-anal anastomosis (IPAA) is a safe procedure for treatment of ulcerative colitis (UC). However, the reported rate of complications from this procedure is 30%-60%, with pouch failure developing at a rate of 5%-10% in Western patients.

Research frontiers

There has been a recent increase in the number of UC cases diagnosed in Asian countries. However, studies on the long-term outcomes of IPPA have been lagging. In this study, the authors evaluated the outcomes of treatments for complications after ileal-pouch formation in Korean UC patients, including pouch failure, and compared the findings to previous Western studies.

Innovations and breakthroughs

Although similar incidences of complications were reported, the rate of pouch failure during the long-term follow-up periods was relatively lower in the present study as compared to Western reports. Both aggressive surgical and medical treatment of the complications are necessary to lower the rate of pouch failure and obtain better functional outcomes.

Applications

Differences between the Korean and Western patient outcomes may be due to differences in ethnic or dietary factors. Furthermore, Japanese studies also had lower incidences of pouch failure suggesting that if ethnic and dietary factors are the key players, similar outcomes may be achieved in other Asian countries. Nevertheless, further research is necessary to elucidate the role of these factors in pouch failure.

Terminology

Early complications occur during the IPAA postoperative recovery periods, usually within 30 d after surgery. Late complications occur during the follow-up periods. Pouch failure was defined as an excision of the total pouch or a non-functioning pouch, which required permanent diversion with ileostomy.

Peer review

This is an intriguing study as the authors were able to achieve a very low rate of pouch failure with aggressive treatment for the complications following IPAA of UC. The results are interesting and encouraging in that patients undergoing IPAA can expect good results with surgeons being able to successfully treat complications, which may arise following IPAA of UC.

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SYSTEMATIC REVIEWS

Association between orofacial granulomatosis and Crohn's disease in children: Systematic review

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Abstract

AIM: To review pediatric cases of orofacial granulomatosis (OFG), report disease characteristics, and explore the association between OFG and Crohn's disease.

METHODS: We conducted a systematic review according to the PRISMA guidelines. We searched Medline, LILACS, Virtual Health Library, and Web of Knowledge in September 2013 for cases of OFG in the pediatric age range (< 18 years), with no language limitations. All relevant articles were accessed in full text. The manual search included references of retrieved articles. We extracted data on patients' characteristics, disease characteristics, association with other diseases, and treatment. We analyzed the data and reported the results in tables and text.

RESULTS: We retrieved 173 reports of OFG in children. Mean age at onset was 11.1 ± 3.8 years (range: 2.0-18 years). Prevalence in males was significant higher than in females (P < 0.001), with a male:female ratio of 2:1. Gastrointestinal signs or symptoms were present in 26.0% of children at the time of OFG diagnosis. Overall, 70/173 (40.4%) children received a concomitant diagnosis of Crohn's disease. In about half (51.4%) of the cases the onset of OFG anticipated the diagnosis of Crohn's disease, with a mean time between the two diagnoses of 13.1 ± 11.6 mo (range: 3-36 mo). Overall, 21/173 (12.1%) of the children with OFG had perianal disease, while 11/173 (6.4%) had a family history of Crohn's disease. Both perianal disease and a family history of Crohn's disease were significantly associated with a higher risk of Crohn's disease diagnosis in children with OFG [relative risk (RR) = 3.10, 95% confidence interval (CI): 2.46-3.90; RR = 2.74, 95%CI: 2.24-3.36, P < 0.0001 for both). Treatment of OFG included steroids (70.8% of children) and other immunosuppressive drugs (42.7%), such as azathioprine, thalidomide and infliximab.

CONCLUSION: High prevalence of Crohn's disease in children with OFG suggests that OFG may be a subtype of Crohn's disease.

Key words: Orofacial granulomatosis; Melkersson-Rosenthal syndrome; Cheilitis granulomatosa; Crohn's disease; Children; Systematic review

Core tip: This systematic review of children with orofacial granulomatosis (OFG) resulted in the following main findings: (1) 40.4% of children with OFG were affected by Crohn's disease during their life; (2) 12.1% of children with OFG had perianal disease; (3) 6.4% had a positive family history for Crohn's disease; (4) both OFG and Crohn's disease were more prevalent in boys; and (5) both diseases had a long-term course, and treatment resembled the treatment used for Crohn's disease. Taken together, these findings suggest that OFG may be a subtype of Crohn's disease.

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INTRODUCTION

The term orofacial granulomatosis (OFG) is conventionally used to describe patients with granulomatous lesions affecting the orofacial tissues[1,2]. The disease is uncommon but is increasingly being recognized. Lip swelling and facial swelling are the most common clinical signs of OFG, often presenting with a spectrum of other features (Figure 1)[3,4]. Over time the majority of patients tend to develop additional lesions, and the lip or facial swelling can become indurated, permanent, and significantly debilitating[1-4].

The pathogenesis of OFG is still uncertain. Different theories suggested a possible role for allergy, infections, and genetic predisposition[1,2].

More recently, it has been hypothesized that OFG may be a subtype of Crohn's disease. This hypothesis is based on the following data: (1) histologically OFG is characterized by noncaseating epithelioid cell granulomas that are indistinguishable from the features of Crohn's disease[1,2]; (2) concurrent intestinal Crohn's disease has been described in 20-50% of adult patients with OFG[5,6]; and (3) both OFG and Crohn's disease have a similar clinical course, that is, long-term with a series of recurrent attacks[1-4].

To the best of our knowledge, no systematic review of pediatric cases of OFG has previously been published. The objective of this work was to systematically review pediatric cases of OFG, and evaluate the association between OFG and Crohn's disease.

MATERIALS AND METHODS

This systematic review was conducted according to the PRISMA guidelines[6]. We searched Medline, LILACS, Virtual Health Library, and Web of Knowledge in September 2013 for cases of OFG in the pediatric age range (< 18 years), with no language limitations. The search strategy is reported in Table 1. All relevant articles were accessed in full text. The manual search included references of retrieved articles. We extracted data on patients' characteristics, disease characteristics, association with other diseases, and treatment. We analyzed the data and reported the results in the tables and text. Cases of granulomatous perioral dermatitis, Melkersson-Rosenthal syndrome (*i.e.*, cases characterized by facial nerve palsy), and OFG-like lesions after organ transplant were excluded from this review.

RESULTS

The process of study selection is reported in Figure 2. We retrieved 18 case series[7-23] and 35 case reports[24-56], for a total of 173 children with OFG. One article could not be found in full text[57].

The mean age at OFG onset was 11.1 ± 3.8 years (range: 2.0-18 years). Although not all reports detailed the sex of the patients, OFG appeared to be significantly more prevalent in boys than in girls ($P < 0.001$, Table 1), with a male to female ratio of 2:1. The disease was reported more commonly in children of Caucasian origin.

The primary clinical feature of children with OFG was lip swelling (93.3%), with involvement of either one or both lips (Table 2). About half of the children presented some intraoral manifestations, such as ulcers, gingival hyperemia or hypertrophy, oral cobble-stoning lesions, or tongue abnormalities. Both angular cheilitis and perioral swelling were each present in about 20% of children. Gastrointestinal signs or symptoms (perianal disease, and/or abdominal pain, diarrhea, or intestinal bleeding) were present in 26.0% of the cases at time of OFG diagnosis.

The diagnosis of OFG was made more often by a team comprising different specialists (65.3%), rather than by one single type of physician (24.7%, $P < 0.001$, Table 3). Stomatologists and dentists were the specialists who were more frequently involved in the diagnosis of OFG (64.7%), followed by gastroenterologists (49.7%), while dermatologists and pediatricians were involved in the diagnosis in only 22.0% and 18.5% of cases, respectively. A consistent delay (months to years) in reaching the final diagnosis of OFG was frequently reported, with a single patient reporting up to 5 years delay[18,42,52].

Differential diagnosis of Crohn's disease was considered in 79.2% of children, while sarcoidosis and tuberculosis were investigated in 20.8% and 14.4% of cases, respectively. Crohn's disease was diagnosed in 70/173 (40.4%) children with OFG, either at time of presentation of OFG, or during the following months or years (mean time: 13.1 ± 11.6 mo, range: 3-36 mo). In contrast, only three children (1.7%) were diagnosed with tuberculosis[33-35], two (1.1%) with sarcoidosis[16], and 19 (10.9%) with allergy/atopy.

Overall, 21/173 (12.1%) of the children with OFG had perianal disease, while 11/173(6.4%) had a family history of Crohn's disease. Both perianal disease and a family history of inflammatory bowel disease were significantly associated with an increased risk of Crohn's disease in children with OFG [relative risk (RR) = 3.10, 95% confidence interval (CI): 2.46-3.90; RR = 2.74, 95%CI: 2.24-3.36, $P < 0.0001$ for both, Table 4].

There was heterogeneity in the reported incidence of Crohn's disease and of perianal disease among different case series, with those reported by gastroenterologists[7,11], describing a significantly higher incidence of Crohn's disease than other series. In Campbell *et al*[7], the largest OFG case series available including both adults and children (207 patients, of which 22% were children), the incidence of Crohn's disease was similar in children and adults with OFG, but in children the occurrence of OFG anticipated the onset of Crohn's disease significantly

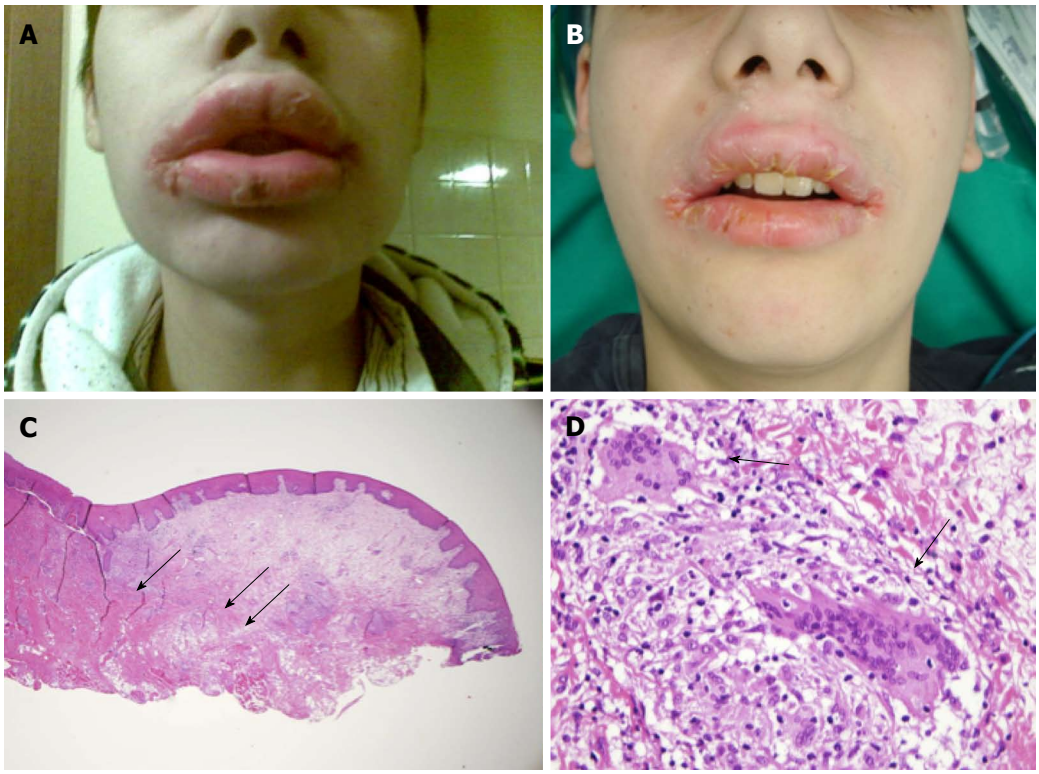

Figure 1 Case 1, swelling of both lips and gingival lesions (A and B); Histological examination showing typical noncaseating granulomas (arrows) and inflammatory infiltrate (C and D).

Table 1 Demographic characteristic of children with orofacial granulomatosis *n* (%)

Patient characteristics	Value (*n* = 173)	*P* value
Age, mean ± SD (range, yr)	11.1 ± 3.8 (2-18)	
Sex		
Male	69 (39.9)	< 0.001[1]
Female	34 (19.6)	
Unspecified	70 (40.5)	
Ethnic group		
Caucasian	19 (11.0)	< 0.001[1]
African	3 (1.7)	
Indian	2 (1.2)	
Unspecified	149 (86.1)	

[1]Indicate similarities with Crohn's disease.

more often than in adults (84% *vs* 27%, $P = 0.0003$)[7]. Ulcers and raised C-reactive protein were more frequent in patients with OFG and Crohn's disease than in those with OFG alone (respectively 49% *vs* 15%, $P = 0.001$ and 73% *vs* 49%, $P = 0.01$)[7]. Cases with intraoral involvement, perianal disease and intestinal Crohn's disease were more likely to occur in those with childhood onset of OFG compared to those with adult onset (44% *vs* 24%, $P = 0.09$)[7].

When Crohn's disease was diagnosed in children with OFG, oral and perioral lesions were not considered a metastatic manifestation of Crohn's disease, but an expression of the disease in the digestive tract.

Although the duration of follow-up was short in most reports, overall, 26.4% and 17.6% of children received two and three or more treatment attempts, respectively (Table 5). A combination of two or more drugs in the same treatment attempt was reported in 53.7% of children. Steroids were prescribed in 70.8% of children. Other immunosuppressive or immunomodulatory drugs were administered to 42.7% of children, and among these, azathioprine/6-mercaptopurine (6MP; 12.3%), thalidomide (10.1%) and infliximab (9.1%) were the most frequently prescribed. Eight (9.1%) children underwent surgery.

DISCUSSION

This review of the pediatric literature highlights the fact that OFG is a disease that can occur during childhood and adolescence. The exact prevalence of the disease in children is unknown, and cannot be derived from this review. Reports from specialized centers suggest that the real prevalence of the disease may be higher than what has been reported so far in the literature[7]. Results from the present review also suggest that OFG in children is still not a well-known disease among physicians, and that there is uncertainty in respects to its diagnosis, with possible diagnostic delay.

This review highlights some important findings regarding Crohn's disease and OFG. First, Crohn's disease was diagnosed in 40.2% of children with OFG. This rate could be underestimated due to several factors: (1) Crohn's disease was not systematically assessed in all children with OFG; (2) on average, children were followed

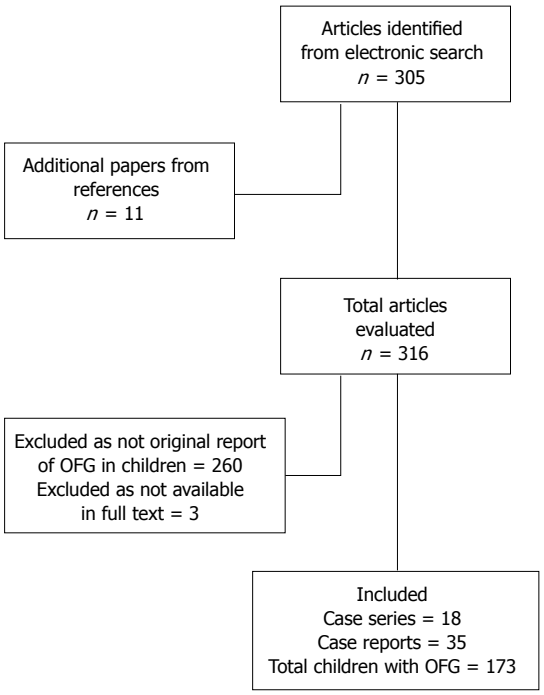

Figure 2 Flow diagram of studies selection. OFG: Orofacial granulomatosis.

up for a short time, while this review highlights that gastrointestinal signs and symptoms may appear at a later stage (on average, 13 mo later); (3) Crohn's disease may be subclinical or silent, as observed in reports in adults, showing that a considerable percentage of patients with OFG without intestinal symptoms were diagnosed with Crohn's disease after ileoscopy or radiolabeled white cell screening[58]; (4) patients with OFG were treated with steroids or other immunosuppressive drugs that could have silenced the intestinal symptoms of Crohn's disease; (5) publication bias may have affected the characteristics of reported cases; and (6) there is some confusion between the diagnosis of OFG and oral Crohn's disease[59], and this may have affected the number of cases reported as OFG. Although some of the above-mentioned factors may have affected the prevalence of Crohn's disease in OFG by overestimating it, most factors may have led to an underestimation of the actual prevalence of Crohn's disease in OFG.

Second, 6.4% of children with OGF also presented a family history of inflammatory bowel disease. Such a percentage is much closer to what is reported in the population with Crohn's disease, than in those not affected by inflammatory bowel disease. Perianal disease, which is already a recognized subset of Crohn's disease, was detected in 12.1% of children with OFG overall. Intra-oral lesions and perianal disease often occur together in Crohn's disease[60], and cohort studies have showed that both manifestations are important predictors of Crohn's disease severity[59,60].

Third, this review highlights other similarities between Crohn's disease and OFG: both OFG and Crohn's disease[58-60] are more prevalent in boys; both diseases have a

Table 2 Clinical manifestations in children diagnosed with orofacial granulomatosis *n* (%)

Clinical features	Value (*n* = 104)[2]
Lips	
Present	104 (100.0)
Lip swelling	97 (93.3)
Only upper lip	27 (26.0)
Only lower lip	15 (14.4)
Both lips	26 (25.0)
Preset but unspecified	29 (27.9)
Angular cheilitis	22 (21.1)
Intra-oral manifestation[1]	
Present	50 (48.1)
Oral ulcerations	24 (23.1)
Gingival hyperemia/hypertrophy	22 (21.1)
Tongue abnormalities	8 (7.7)
Oral cobble-stoning lesions	8 (7.7)
Facies	
Perioral/cheek swelling	19 (18.3)
Neck	
Cervical lymphadenopathy[1]	6 (5.8)
Gastrointestinal	
Present	27 (26.0)
Perianal disease[1]	21 (20.2)
Abdominal pain[1]	7 (6.7)
Diarrhea[1]	7 (6.7)
Intestinal bleeding[1]	6 (5.8)
Nutritional status	
Impaired growth[1]	5 (4.8)
Obesity	1 (1.0)
Ocular	
Conjunctivitis[1]	1 (1.0)
Genitalia	
Vulvar oedema[1]	2 (2.0)
Scrotal swelling[1]	1 (1.0)

[1]The asterisks indicate similarities with Crohn's disease; [2]All cases with a detailed description of clinical signs and symptoms are reported in the table.

long-term course; and treatments used for OFG resemble those used for Crohn's disease. Even the list of other diseases diagnosed in association with OFG (*i.e.*, erythema nodosum, and alopecia), although the number of cases was limited, resembles the list of immunological diseases usually associated with Crohn's disease.

All the above findings suggest that OFG and Crohn's disease may be two variants, if not just two different localizations, of the same chronic inflammatory disease. Other authors have proposed this hypothesis, based on the high (20%-50%) reported prevalence of Crohn's disease in adults with OFG[5,6].

To the best of our knowledge no systematic review of adult cases of OFG has been carried out so far, and cases reported in adults suffer from the same risk of bias as cases reported in children (*e.g.*, short follow-up time). More studies in an adequate sample of patients with OFG (both children and adults) with a systematic evaluation for Crohn's disease and long follow-up time are needed to explore further the hypothesis that OFG is a subtype of Crohn's disease or even one of its manifestations.

So far, based on existing literature, Crohn's disease

Table 3 Diagnostic process in children reported with orofacial granulomatosis *n* (%)

Characteristics	Value (*n* = 173)
Physicians involved in the diagnosis	
Stomatologist/dentist/maxillo surgeon	112 (64.7)
Gastroenterologist	86 (49.7)
Dermatologist	38 (22.0)
Pediatrician	32 (18.5)
Otorhinolaryngologist	13 (7.5)
Allergologist	4 (2.3)
Plastic surgeon	3 (1.7)
Internal Medicine	1 (0.6)
Composition of the team	
One single specialty	60 (34.7)
More than one specialty	113 (65.3)
Differential diagnosis considered at time of OFG presentation	
Crohn's disease	137 (79.2)
Sarcoidosis	36 (20.8)
Tuberculosis	25 (14.4)
Allergy	19 (11.0)
Infection	17 (9.8)
C1q esterase deficiency	10 (5.8)
Melkersson–Rosenthal syndrome	4 (2.3)
Autoimmunity	3 (1.7)
Vasculitis	1 (0.6)
Foreign body	1 (0.6)
Enteropathic acrodermatitis	1 (0.6)

OFG: Orofacial granulomatosis.

Table 4 Concomitant diseases diagnosed in children with orofacial granulomatosis and associated characteristics *n* (%)

Disease	Value (*n* = 173)	RR (95%CI), *P*
Crohn's disease		
Total children	70 (40.4)	
At presentation	34 (19.6)	
During follow-up	36 (20.8)	
Time from OFG diagnosis to Crohn's diagnosis (mean ± SD, range)	13.1 ± 11.6 (3-36)	
Presence of perianal disease		
Total	21/173 (12.1)	3.10
In children with Crohn's	21/70 (30.0)	(2.46-3.90),
In children without Crohn's	0/103 (0)	0.0001
Familiarity for inflammatory bowel diseases		
Total	11/173 (6.4)	2.74
In children with Crohn's	11/70 (15.7)	(2.24-3.36),
In children without Crohn's	0/103 (0)	0.0001
Allergy/atopy		
Any allergy	19 (10.9)	
Asthma	7 (4.0)	
Atopy	6 (3.5)	
Rhinitis/rhinoconjunctivitis	6 (3.5)	
Eczema	6 (3.5)	
Hives	1 (0.6)	
Other diagnosis		
Tuberculosis	3 (1.7)	
Sarcoidosis	2 (1.1)	
Other diseases	10 (5.7)	
Erythema nodosum	3 (1.7)	
Insulin dependent diabetes	1 (0.6)	
Celiac disease	1 (0.6)	
Alopecia	1 (0.6)	
Low CD4/CD8 ratio	1 (0.6)	
Epilepsy	1 (0.6)	

Table 5 Treatments of children with orofacial granulomatosis *n* (%)

Treatment characteristics	Value (*n* = 104)[1]
A treatment was prescribed[2]	
Yes	89 (96.7)
No	3 (3.2)
Unspecified	12 (11.5)
Number of treatment attempts reported[2]	
One	38 (55.8)
Two	18 (26.4)
Three or more	12 (17.6)
Unspecified	21 (23.5)
More than one drug in the same treatment attempt[2]	
Yes	36 (53.7)
No	31 (46.2)
Unspecified	22 (24.7)
Type of treatments prescribed[2]	
Antibiotics	26 (29.2)
Anti-histaminic	10 (11.2)
Steroids-total	63 (70.8)
Topical	27 (30.3)
Intralesional	24 (27.0)
Oral	39 (43.8)
Other immunosuppressive-total	38 (42.7)
Azathioprine/6MP	11 (12.3)
Thalidomide	9 (10.1)
Infliximab	8 (9.0)
Dapsone	4 (4.5)
Tacrolimus (topic)	4 (4.5)
Methotrexate	3 (3.4)
Tacrolimus (systemic)	3 (3.4)
Hydroxychloroquine/chloroquine	2 (2.2)
Colchicine	2 (2.2)
Other treatments	
5ASA	13 (14.6)
Chlorhexidine (topic)	4 (4.5)
Enteral nutrition	2 (2.2)
Fumaric acid esterase	1 (1.1)
Surgery	8 (9.0)

[1]All cases that provided a detail description of clinical signs are reported in the table; [2]Percentages are calculated on the number of children for which the treatment was specified. 5-AZA: 5-aminosalicylic acid.

should be considered in the differential diagnosis of every child with signs of OFG; in particular, if other signs of systemic, gastrointestinal or perianal involvement are present. If Crohn's disease is not diagnosed at the time of OFG presentation, patients with OFG should be closely followed up for any sign of intestinal Crohn's disease, including perianal disease.

If OFG and Crohn's disease are two different clinical entities, more precise diagnostic criteria should be developed to differentiate between the two diseases.

In conclusion, OFG has been considered a different entity from Crohn's disease due to its presentation (lip and buccal involvement) and to the absence of systemic signs and symptoms[1-5]. The findings of our systematic review on children with OFG suggest that OFG may be a subtype of Crohn's disease. Mores studies with a systematic evaluation of patients and with adequate follow-up are needed to confirm this hypothesis. Based on the existing evidence, Crohn's disease should be considered

in the differential diagnosis of children with unexplained OGF. These children should be also followed up in the long term, because intestinal Crohn's disease may develop after several years.

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COMMENTS

Background

The term orofacial granulomatosis (OFG) is conventionally used to describe patients with granulomatous lesions affecting the orofacial tissues. So far, OFG had been considered a different entity from Crohn's disease, although the hypothesis that OFG may be a subtype of Crohn's disease has been proposed.

Research frontiers

To the best of our knowledge, no systematic review of pediatric cases of OFG has previously been published. The objective of this study was to review systematically all pediatric cases of OFG, report on the disease characteristics, and evaluate the association between OFG and Crohn's disease.

Innovations and breakthroughs

Crohn's disease was diagnosed in approximately 40% of children with OFG, either at time of presentation of OFG, or during the following months or year (mean time: 13.1 ± 11.6 mo, range: 3-36 mo). Such a high prevalence of Crohn's disease in children with OFG, and other common features between the two diseases, suggest that OFG may be considered a subtype of Crohn's disease.

Applications

Crohn's disease should be considered in the differential diagnosis of children with OFG. Children with OFG should be also followed up in the long term, because intestinal Crohn's disease may develop after several years.

Terminology

OFG is a rare granulomatous disease that affects the orofacial tissues. Lip and facial swelling are the most common clinical signs, often presenting with a spectrum of other features. Over time the majority of patients tend to develop additional lesions, and the lip or facial swelling can become indurated, permanent, and significantly debilitating.

Peer review

This is a well-documented review article regarding the association between OFG and Crohn's disease in children.

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META-ANALYSIS

Interferon-associated retinopathy risk in patients with diabetes and hypertensive hepatitis C

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Abstract

AIM: To investigate the association of hypertension and diabetes mellitus (DM) with interferon-associated retinopathy (IAR) risk in chronic hepatitis C (CHC).

METHODS: Two investigators independently searched PubMed and Embase for eligible articles published prior to December 2013; additional studies were identified by reviewing the bibliographies. Only case-control or cohort studies that evaluated the association between hypertension and/or DM and IAR incidence in CHC patients were included. IAR was characterized by the presence of cotton-wool spots and/or retinal hemorrhage, and was defined as the primary efficacy measure. Pooled relative risks (RRs) with 95% confidence intervals (CIs) were estimated using data extracted from papers based on random-effects models.

RESULTS: Eight eligible studies were included in the present meta-analysis. The outcomes showed that patients with CHC and hypertension were at higher risk of IAR (48/189 *vs* 96/455, RR = 1.90; 95%CI: 1.15-3.15, $P < 0.05$). Patients with DM receiving interferon (IFN)-based therapy for CHC infection may be at higher risk for IAR (18/72 *vs* 60/256, RR = 1.56, 95%CI: 1.11-2.20, $P < 0.05$); however, the outcome was not stable. There was no significant difference in IAR risk between genotype-1-infected patients and non-genotype-1-infected patients (RR = 1.09, 95%CI: 0.64-1.87, $P > 0.05$). Comparable incidences of IAR were also found between patients treated with pegylated interferon (PIFN) α-2a and those treated with PIFN α-2b (RR = 0.84, 95%CI: 0.56-1.24, $P > 0.05$) and between patients treated with IFN α and those treated with PIFN α (RR = 1.04, 95%CI: 0.72-1.50, $P > 0.05$).

CONCLUSION: Patients with hypertension have a higher risk of retinopathy when receiving IFN-based therapy for CHC.

Key words: Hepatitis C infection; Interferon-associated retinopathy; Hypertension; Diabetes mellitus; Interferon

Core tip: This meta-analysis demonstrated that patients with hypertension were at higher risk for developing retinopathy when receiving interferon-based therapy for chronic hepatitis C infection. Further studies are needed to clarify the association between diabetes mellitus and interferon-associated retinopathy.

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INTRODUCTION

Chronic hepatitis C (CHC) infection, which affects > 170 million people worldwide[1], may eventually lead to cirrhosis and/or hepatocellular carcinoma (HCC)[2,3]. According to recent guidelines, the combination of pegylated interferon (PIFN) α and ribavirin is still regarded as the standard chemotherapy for CHC[4]. Two new direct-acting antiviral agents (DAAs), telaprevir and boceprevir, specifically inhibit the activity of the hepatitis C virus (HCV) NS3/4A protease and have been recently approved for HCV genotype 1 infection[5,6]. This new treatment regimen clearly led to a 20%-30% increase in the sustained viral response (SVR) rate of genotype-1-infected patients[7,8]. However, triple therapy that includes interferon (IFN) α and the two DAAs has some limitations. First, triple therapy is only used in genotype-1-infected patients. Second, the cost of treatment is increased, and 40% of the treated patients show additional side effects, such as cutaneous rash and anemia[7,8]. Moreover, triple therapy is associated with the rapid onset of drug resistance[9,10]. Although many other direct antiviral therapies and IFN-free regimens are in development, these therapies are unlikely to reach clinical application in the next few years. For the reasons given above, we may safely conclude that IFN α plus ribavirin will remain the central therapeutic option for several years[5,6]. Thus, the clinicians must continue to manage side effects related to treatment with IFN α.

Various adverse effects, including ophthalmological side effects[11-17] have been reported with the use of IFN. The most commonly documented ocular complication is retinopathy which is characterized by cotton-wool spots and/or retinal hemorrhage[11,13,16]. Several studies have investigated the possible risk factors for retinopathy in patients with CHC during antiviral therapy using IFN α and/or PIFN α. However, the results have been controversial. Some studies have suggested that diabetes mellitus (DM) and hypertension are possible risk factors for interferon-associated retinopathy (IAR)[11,18,19], and others have not identified these risk factors[20-22]. Therefore, the question of whether ophthalmologic screening should be recommended for CHC patients with hypertension or DM before, during, and after treatment is controversial. To address this issue, we performed a meta-analysis of studies that assessed the association of hypertension and/or DM and IAR risk among CHC patients.

MATERIALS AND METHODS

Search strategy

Two investigators independently searched PubMed and Embase (up to December 31, 2013) to collect all eligible papers. The search strategies for PubMed and Embase were as follows: ("retinal diseases" [MeSH Terms] OR "retinal diseases" [All Fields] OR "retinopathy" [All Fields]) AND ("interferons" [MeSH Terms] OR "interferons" [All Fields] OR "interferon" [All Fields]) AND ("hcv" [All Fields] OR "hepatitis c" [MeSH Terms] OR "hepatitis C" [All Fields]), and ("retinal diseases"/exp OR "retinal diseases" OR "retinopathy"/exp OR "retinopathy") AND ("interferons"/exp OR "interferons" OR "interferon"/exp OR "interferon") AND ("hepatitis C"/exp OR "hepatitis C"), respectively. In addition, we also reviewed the bibliographies of relevant articles that were not found by database searches. Disagreements were resolved by discussion and consensus between the two reviewers.

Inclusion and exclusion criteria

The following inclusion criteria were used when collecting published studies: (1) evaluation of the association between IAR incidence and hypertension and/or DM; (2) a case-control or cohort study; (3) sufficient information for estimating the relative risks (RRs) and their 95% confidence intervals (CIs); and (4) English or Chinese publications. The exclusion criteria were as follows: (1) a case report, review, conference abstract, comment or editorial letters; (2) a lack of control groups; (3) overlapping articles or articles with duplicate data; and (4) an inability to obtain the necessary data.

Data extraction and definition of end-points

Two investigators independently extracted the following information from each study: name of first author, year of publication, ethnicity, type of IFN, numbers of cases and controls, length of follow-up, and end-points and risk estimates (or the relevant data needed to calculate them). We calculated the duration of follow-up from the start of IFN therapy and discarded pre-existing retinopathy at baseline. Whenever possible, we contacted the authors to inquire about insufficient data. Any disagreement was resolved by consensus between the reviewers.

Retinopathy was used as the only end-point for this analysis, which was defined as the presence of any of the following lesions: cotton-wool spots, retinal hemorrhage, or microaneurysms.

Quality assessment

For observational cohort studies, the methodological quality was assessed independently by two authors using the Newcastle-Ottawa Scale (NOS)[23] based on the following criteria: (1) selection of cases and controls (or cohort); (2) comparability of cases and controls (or cohort); and (3) ascertainment of exposure/outcome. Studies with an overall score ≥ 6 were classified as high quality.

Statistical analysis

The RRs and corresponding 95%CIs were used as the effect measurements. All unadjusted RRs were calculated using available data. To combine crude risk estimates, a quantitative meta-analysis was performed using STATA version 12.0 (STATA Corporation, College Station, TX, United States). Both Cochran's Q test and I^2 measure-

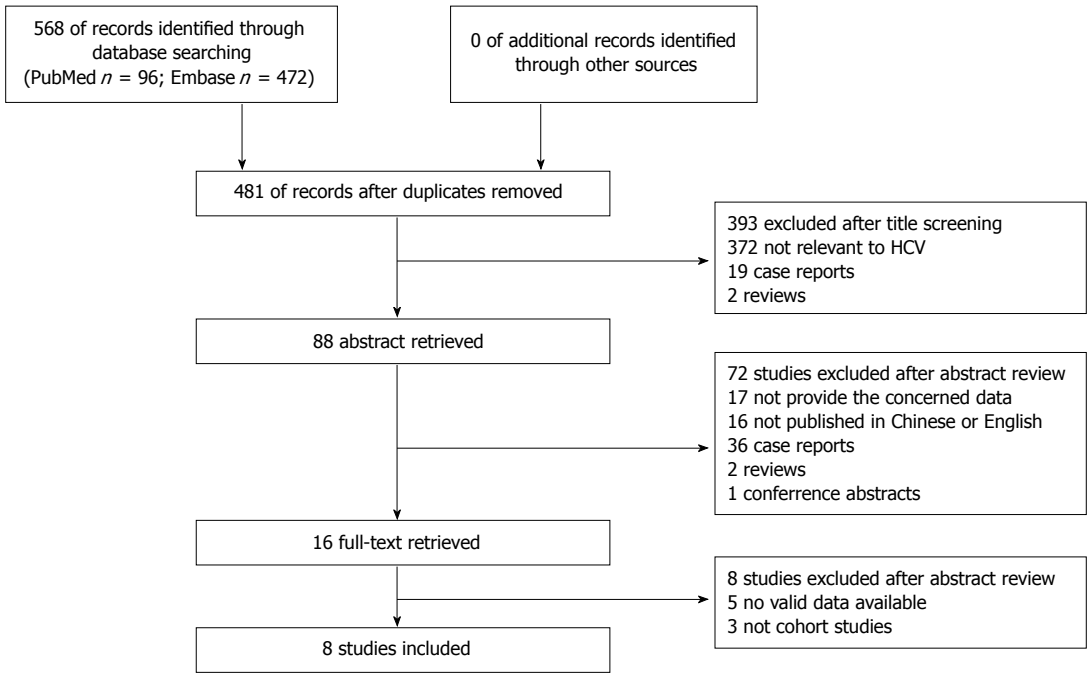

Figure 1 Flow chart illustrating the process of article selection.

ments were performed to evaluate intra-study heterogeneity. Substantial heterogeneity was indicated if the *P* value was $\leqslant 0.10$ or I^2 was $\geqslant 50\%$. However, irrespective of the presence of significant heterogeneity, a random-effect model was utilized to allow comparisons among different pooled risk estimates. Publication bias was evaluated by Egger's test[24]. $P \leqslant 0.10$ indicated the presence of publication bias. Sensitivity analysis was performed to evaluate the validity and reliability of the primary meta-analysis. Subgroup analysis was also conducted to evaluate the effects of study design, ethnicity, and treatment duration on the incidence of IAR among CHC patients with or without hypertension or DM. We also pooled the unadjusted risk estimates of IAR for age, type of IFN, and HCV genotypes to evaluate whether old age, PIFN treatment, or genotype 1 infection portends an increased risk of IAR.

RESULTS

Search results and characteristics of included studies

Of the 568 references identified, 87 duplicates were deleted. Screening of the title, abstract, and full text yielded eight studies involving 606 patients (Figure 1)[11,18-22,25,26]. Among these studies, two[20,21] were retrospective cohort studies, and the remainder[11,18,19,22,25,26] were prospective cohort studies. Two studies each were conducted in Japan[11,19] and the United States[20,21], and one report each was published in Egypt[22], Canada[26], France[25] and South Korea[18]. Conventional IFN, PIFN, and a combination of IFN and PIFN were used in two[11,19], three[20,22,26], and three[18,21,25] studies, respectively. The patients in one study[19] were followed for 24 wk, while the patients in the other studies[11,18,20-22,25,26] had a longer follow-up. The mean follow-up time was 46.50 ± 13.51 wk. Ophthalmic examinations were performed by ophthalmologists in all the studies except one[19], which did not provide the information on who performed the examination. Baseline ophthalmic examination in one[20] study was performed within 12 wk of initiating therapy, while in the remaining studies it was performed before the start of therapy[11,18,19,21,22,25,26]. Follow-up ophthalmic examinations varied among the studies. The two retrospective studies[20,21] might have underestimated the incidence of retinopathy, because only patients with reported symptoms underwent a follow-up eye examination. According to the scoring system, five studies[19,20,22,25,26] were of high methodological quality and three[11,18,21] were not. All the articles were published in English as full-text articles (Table 1).

Hypertension and IAR

The incidence of IAR was compared between CHC patients with and without hypertension in all studies. Although four studies[11,20-22] did not demonstrate a significantly increased risk of IAR in patients with CHC and concomitant hypertension, the remaining four[18,19,25,26] reported a significantly increased risk of IAR in patients with CHC and concomitant hypertension. The meta-analysis showed that the incidence of IAR was significantly higher in CHC patients with than in those without hypertension (48/189 *vs* 96/455, RR = 1.90, 95%CI: 1.15-3.15, $P = 0.013$) (Figure 2A). Significant intra-study heterogeneity was observed among the included studies ($I^2 = 70.6\%$, $P = 0.001$) (Figure 2A). No evidence of publication bias was found by Egger's test ($P = 0.28$).

Significant intra-study heterogeneity was observed among the included studies, therefore, the data were subgrouped with the aim of removing the heterogeneity. In the subgroup analysis of the association between hypertension and IAR in CHC patients by study design,

Table 1 Main characteristics of the studies included in the meta-analysis

Ref.	Year	Country	Study design	Type of IFN	No. of HCV (+) IAR (+) cases/HCV (+) IAR (-) controls	Reported end-points	Follow-up	Study quality
Kawano *et al*[11]	1996	Japan	Prospective cohort	Natural IFN α, recombinant IFN α-2a/2b	17/49	Retinopathy	48 wk	5
Okuse *et al*[19]	2006	Japan	Prospective cohort	Recombinant IFN α-2b	18/56	Retinopathy	24 wk	6
d'Alteroche *et al*[25]	2006	France	Prospective cohort	IFN α, PIFN α	11/133	Retinopathy	72 wk	6
Panetta *et al*[21]	2009	United States	Retrospective cohort	Consensus IFN, PIFN α-2a/2b	114/48	Retinopathy	48 wk	5
Mehta *et al*[20]	2010	United States	Retrospective cohort	PIFN	26/39	Retinopathy	48 wk	4
Kim *et al*[18]	2010	South Korea	Prospective cohort	PIFN, conventional IFN	13/22	Retinopathy	36 wk	8
Vujosevic *et al*[26]	2012	Canada	Prospective cohort	PIFNα-2a/2b	22/66	Retinopathy	36/60 wk	8
Mousa *et al*[22]	2013	Egypt	Prospective cohort	PIFN α-2a/2b	40/42	Retinopathy	48 wk	6

IFN: Interferon; PIFN: Pegylated interferon.

the pooled RR was only significant in prospective studies (RR = 2.38, 95%CI: 1.46-3.87, P = 0.000) and not in retrospective studies (RR = 0.74, 95%CI: 0.16-3.33, P = 0.690) (Table 2). The subgroup analysis by ethnicity showed that the pooled RR was significant only in Asians (RR = 1.56, 95%CI: 1.07-2.27, P = 0.021) and not in Caucasians and Africans (for Caucasians: RR = 1.96, 95%CI: 0.78-4.92, P = 0.154; for Africans: RR = 2.21, 95%CI: 0.15-33.50, P = 0.567) (Table 2). Subgroup analysis by ethnicity was not reliable for Africans due to the fact that only one study was performed. In addition, the subgroup analysis by ethnicity indicated that studies conducted on Caucasians were the main source of heterogeneity (I^2=84%, P = 0.000) (Table 2). Finally, we stratified the studies by treatment duration and found that the pooled RR was significant only in patients treated for 24 wk (RR = 1.56, 95%CI: 1.07-2.27, P = 0.021) and not in patients treated for 48 wk (RR = 2.00, 95%CI: 0.85-4.68, P = 0.112) (Table 2).

DM and IAR

The incidence of IAR was compared between patients with CHC with and without DM in six studies[11,18-22]. The meta-analysis showed that there was an increased risk of IAR among CHC patients with DM (18/72 *vs* 60/256, RR = 1.78, 95%CI: 1.33-2.38, P = 0.000) (Figure 2B). This association remained significant after the removal of three small studies (RR = 1.55, 95%CI: 1.04-2.32, P = 0.033). No significant heterogeneity was observed among the included studies (I^2 = 0.0%, P = 0.518). The sensitivity analysis showed that the result changed significantly (RR = 1.50, 95%CI: 0.82-2.76, P = 0.192) after the study of Kawano *et al*[11], which clearly carried the most weight, was omitted from the analysis, suggesting that the outcome was not stable.

HCV genotypes and IAR

Three studies[18,19,25] provided sufficient data for evaluating the effect of HCV genotypes on the development of IAR. We found comparable incidences of IAR between genotype-1-infected and non-genotype-1-infected patients (RR = 1.09, 95%CI: 0.64-1.87, P = 0.746) (Figure 2C). Significant heterogeneity was found (I^2 = 84.7%, P = 0.001) in these studies. Egger's test did not detect the presence of publication bias (P = 0.335).

Type of IFN and IAR

The incidence of IAR was compared between the PIFN α-2a and PIFN α-2b treatment groups in three studies[11,20,21], and four studies[11,18,21,25] had results on the incidence of IAR in patients who received IFN α and PIFN α therapies. Comparable incidences of IAR were observed between patients treated with PIFN α-2a and those treated with PIFN α-2b (RR = 0.84, 95%CI: 0.56-1.24, P = 0.374) and between patients treated with IFN α and those treated with PIFN α (RR = 1.04, 95%CI: 0.72-1.50, P = 0.845) (Figure 2D). No substantial heterogeneity was found (PIFN α-2a *vs* PIFN α-2b: I^2 = 8.6%, P = 0.335; IFN α *vs* PIFN α: I^2 = 0%, P = 0.792) in these groups. No evidence of publication bias was found by Egger's test (PIFN α-2a *vs* PIFN α-2b: P = 0.694; IFN α *vs* PIFN α: P = 0.458).

Age and IAR

One study[27] identified age as an independent predictor of IAR by multiple logistic regression analysis. However, only three[18,19,26] of the included studies reported that the adjusted odds ratios (ORs) or hazard risks (HRs), which could not be combined in our meta-analysis because there were only two ORs (one study did not provide the 95%CI) and one HR. Although the three studies reported a positive association between hypertension and IAR risk, no association was found between DM or age and IAR risk when adjusted by factors such as sex, levels of viremia, levels of alanine aminotransferase (ALT), and response to therapy. Moreover, none of the studies included in this meta-analysis were age-matched studies. Therefore, we could not evaluate the association of age with IAR in CHC patients with hypertension or DM.

DISCUSSION

Ocular side effects are well-recognized complications of the current standard chemotherapy for hepatitis C; the most common of which is ischemic retinopathy. Most patients developed retinopathy within 2 mo after IFN

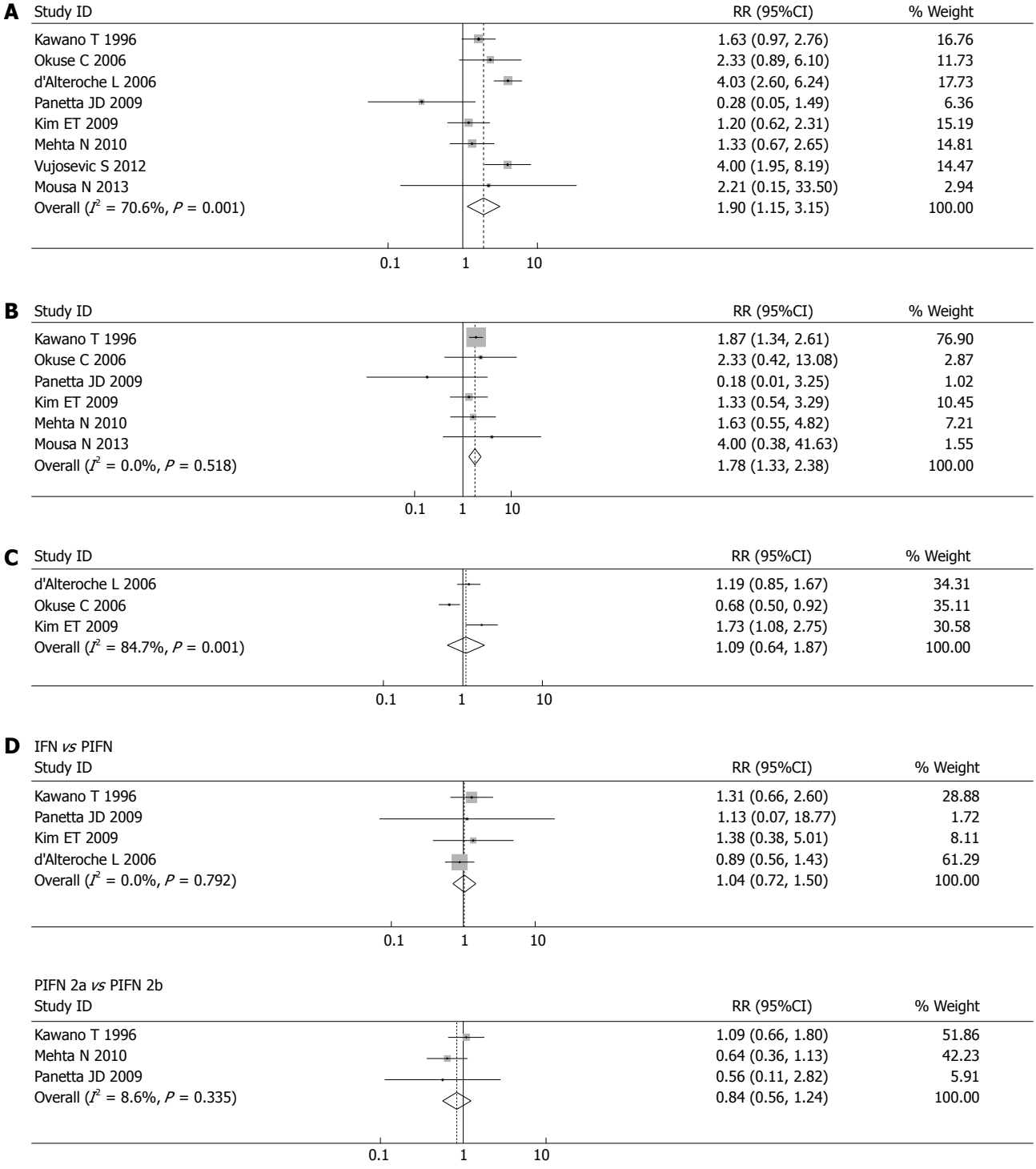

Figure 2 Forest plot. A: RRs for the association of IAR and hypertension. Squares represent the study-specific RR. Diamonds represent the summary relative risks (SRRs). Horizontal lines represent 95%CIs. A random-effect model was used. An overall tendency toward the right side of the reference line (RR = 1) indicated that hypertension could increase the incidence of IAR; B: RRs for the association of IAR and DM. Squares represent the study-specific RR. Diamonds represent the SRRs. Horizontal lines represent 95%CIs. A random-effect model was used. An overall tendency towards the right side of the reference line (RR = 1) indicated that DM could increase the incidence of IAR; C: RRs for the association of IAR and HCV genotypes. Squares represent the study-specific RR. Diamonds represent the SRRs. Horizontal lines represent 95%CIs. A random-effect model was used. The contact of overall diamond with the reference line (RR = 1) indicated that there was no difference in IAR incidence between genotype-1-infected and genotype-2-infected patients; D: Effect of different types of IFN on IAR risk. Squares represent the study-specific RR. Diamonds represent the SRRs. Horizontal lines represent 95%CIs. A random-effect model was used. The contact of overall diamond with the reference line (RR = 1) indicated that there was no difference in IAR incidence between the PIFN α-2a group and the PIFN α-2b group or between the IFN α group and the PIFN α group.

treatment[11-14,28,29]. Several studies[13,16,30] showed that no retinal lesions were detected in any sample from patients with CHC who were not undergoing IFN therapy. These results further support the hypothesis that IFN treatment induces retinopathy in patients with CHC. Cotton-wool spots and retinal hemorrhage were the most common

Table 2 Summary relative risks for the association of hypertension and interferon-associated retinopathy risk by study design, ethnicity

	Subgroup	Ref.	RR (95%CI)	Z (*P* value)	Heterogeneity of study design		
					χ^2	Df (*P* value)	I^2
HTN	Study design						
	Prospective	[11,18-20,25,26]	2.38 (1.46-3.87)	3.49 (0.000)	13.62	5 (0.018)	63.30%
	Retrospective	[20,21]	0.74 (0.16-3.33)	0.40 (0.690)	2.99	1 (0.084)	66.50%
	Ethnicity						
	Asian	[11,18,19]	1.56 (1.07-2.27)	2.31 (0.021)	1.33	2 (0.514)	0.00%
	Caucasian	[20,21,25,26]	1.96 (0.78-4.92)	1.43 (0.154)	18.69	3 (0.000)	84.00%
	African	[22]	2.21 (0.15-33.50)	0.57 (0.567)	-	-	-
	Treatment duration						
	24 wk	[11,18,19]	1.56 (1.07-2.27)	2.31 (0.021)	1.33	2 (0.514)	0.00%
	48 wk	[20-22,25,26]	2.00 (0.85-4.68)	1.59 (0.112)	18.67	4 (0.001)	78.60%
	Overall	[11,18-22,25,26]	1.90 (1.15-3.15)	2.50 (0.013)	23.82	7 (0.001)	70.60%

HTN: Hypertension; -: Could not be calculated.

manifestations of IAR[11,18-22,25,26], whereas decreased visual acuity and subjective symptoms were rare. In most patients who have retinopathy, the treatment can safely be continued in close collaboration with an ophthalmologist. However, serious complications, such as a severe decrease in visual acuity due to retinal vein occlusion and vitreous hemorrhage, have been reported in some cases[31-33], especially in individuals with risk factors for retinopathy. Patients with retinopathy were reported to drop out of IFN treatment in all the included studies. The present study examined the association between hypertension, DM, HCV genotype, type of IFN, and risk of IAR in CHC patients receiving IFN therapy.

Our meta-analysis revealed that the risk of IAR in CHC patients with hypertension was elevated 1.90-fold, as compared with that in CHC patients without hypertension, even when any individual study was removed. However, significant heterogeneity was found among the included studies. The subgroup analysis by study design suggested that the two retrospective studies[20,21] underestimated the incidence of IAR, because these studies did not include appropriate eye examinations. One of the two studies[20] described the performance of the ophthalmic examinations within 12 and 24 wk of initiation of therapy. Another limitation of the study was that patients who did not undergo follow-up evaluations after their eye examinations were not actively pursued. The other study[21] conducted ophthalmic examinations only when the patients complained of symptoms. However, retinopathy occurred in most patients, which was often asymptomatic.

As stated previously, the prevalence of chronic hepatitis is higher in Asia, including South Korea and Japan, than in Europe or the United States[18]. Additionally, it appears that there may be geographic differences in the incidence of IAR[34]. Therefore, we stratified the included studies by ethnicity to evaluate the effect of ethnicity on the incidence of IAR among CHC patients with or without hypertension. The results showed that the association between hypertension and IAR was significant in Asians but not in Caucasians or Africans. This result may be attributed to the limited number of studies. Only a single study was performed on Africans, thus, the subgroup analysis on ethnicity might not have been reliable for the African population. Additional studies based on African patients should therefore be performed to re-evaluate the association between hypertension and risk of IAR in this population. According to the results of the subgroup analysis by treatment duration, the association between hypertension and IAR was significant in patients who were treated for 24 wk, but not in those treated for 48 wk. However, the only studies including patients who were treated for 24 wk were conducted on Asian patients. Therefore, the effect of treatment duration on IAR incidence could not be determined. The subgroup analyses revealed that the substantial heterogeneity might be due to the studies performed in non-Asian populations. Taken together, our results suggest that hypertension was associated with a significantly increased risk of IAR in CHC patients.

Six studies compared the incidence of IAR among CHC patients with or without DM. However, the number of patients with DM in three[18-20] of the six studies was too small. The pooled results revealed that CHC patients with DM have a significantly high risk of IAR. When several small studies were removed, the high risk of IAR in CHC patients with DM remained very significant. However, the overall trend was altered when the study of Kawano *et al*[11] was excluded. These results suggest that the outcome is not credible, therefore, the analyses should be reinvestigated in the future.

Additionally, our meta-analysis revealed that PIFN did not increase the incidence of IAR compared to IFN, which was different from the results of d'Alteroche *et al*[25]. Further studies are needed to identify the underlying cause of this inconsistency. This result may help us to exclude the influence of the different types of IFN used in each study on the incidence of IAR in patients with hypertension or DM. We also found that HCV genotype had no effect on the development of IAR. However, we cannot exclude the effect of patient age on IAR incidence among patients with hypertension or DM, due to a lack

of data.

IAR likely occurs due to disturbances in retinal microcirculation[35]. Guyer *et al*[36] speculated that IFN therapy may cause deposition of immune complexes in the retinal vasculature; this leads to leukocyte infiltration with subsequent retinal ischemia, which then leads to capillary non-perfusion, retinal hemorrhage, and cotton-wool spot formation. An ischemic insult, similar to that observed in patients with hypertension and diabetes, could be regarded as a potential mechanism. Therefore, previous retinal arteriole and venule anomalies may constitute susceptibility to retinopathy. Endothelial cells are known to play an important role in microcirculation[11]. There is evidence that IFN inhibits the proliferation and migration of vascular endothelial cells *in vitro* and inhibits experimental intraocular neovascularization[37,38]. Additionally, studies[30,39] have demonstrated high circulating levels of plasma-activated complement component 5 (C5a), which is a potent intravascular aggregator of granulocytes in patients receiving IFN therapy for hepatitis C. Complement activity associated with high C5a levels may cause retinal capillary infarction, manifesting as capillary non-perfusion, cotton-wool spots, and retinal hemorrhage. Compared with each individual factor, a combination of these factors may lead to greater effects during IFN α therapy.

Our study showed that patients with hypertension were at particular risk for developing retinopathy during IFN therapy, and this is most likely related to the pre-existing disturbances in their retinal microcirculation. Chronic hypertension is associated with thickening of the arterial and small arteriolar walls[40]. Therefore, systemic hypertension predisposes patients to IAR. The fact that hypertensive retinopathy induces the formation of flame-shaped hemorrhage and white cotton-wool spots, which are also observed in IAR, indicates that systemic hypertension and IAR may be related to each other. Consequently, in these high-risk patients, severe retinal damage carries a risk of visual loss; thus, ophthalmic evaluations should be recommended prior to and during IFN therapy.

There were several limitations to this study. First, heterogeneity and confounding factors, such as patient age and response to chemotherapy, might have affected our meta-analysis. However, we are unable to account for these differences because of a lack of data. Second, we primarily studied the risk of IAR in CHC patients with DM in this meta-analysis. However, the association between DM and IAR in CHC patients remains unclear, owing to the limited data available in published studies. Therefore, further studies should be conducted to assess the association between DM and IAR in CHC patients. Finally, the effect of different antihypertensive drugs and the compliance of patients on IAR could not be evaluated in the current study due to the lack of data. In our meta-analysis, although some of the included studies indicated that hypertension and DM were well controlled, others did not provide the related information.

In conclusion, our meta-analysis suggests that patients with hypertension are more susceptible to the development of retinopathy. The influence of ethnicity and treatment duration on the incidence of IAR among CHC patients with or without hypertension should be re-evaluated. Moreover, further studies are needed to clarify the association between DM and IAR in patients with CHC.

COMMENTS

Background

Retinopathy is an adverse effect of interferon (IFN)-based therapy for chronic hepatitis C (CHC) infection; cotton wool spots and retinal hemorrhage are the most common manifestations of interferon-associated retinopathy (IAR). Some studies have suggested that hypertension and diabetes mellitus (DM) are possible risk factors for IAR, and others have not identified these risk factors. Therefore, the question of whether ophthalmic screening should be recommended for CHC patients with hypertension or DM before, during, and after treatment is controversial.

Research frontiers

Over the past two decades, many studies have been performed to identify the possible risk factors for retinopathy in patients with CHC undergoing antiviral therapy with IFN α and/or pegylated IFN (PIFN) α. These studies aimed to determine whether ophthalmic screening should be recommended for CHC patients with hypertension or DM before, during, and after treatment.

Innovations and breakthroughs

Discordant results have been reported regarding the influence of hypertension and DM on the development of IAR. To address this issue, authors performed a meta-analysis of studies that assessed the association of hypertension and/or DM with IAR risk among CHC patients. Authors showed that patients with hypertension are at higher risk for developing retinopathy when receiving IFN-based therapy for CHC infection. However, further studies are needed to clarify the association between DM and IAR.

Applications

This study provides a theoretical basis for determining whether ophthalmic screening should be recommended for CHC patients with hypertension or DM before, during, and after treatment.

Terminology

IAR is an adverse effect caused by the use of IFN, which manifests as cotton-wool spots and petechiae of the retina, perfusion abnormalities in the capillary system, microaneurysms, and retinal vein occlusion. Clinically, decreased visual acuity and subjective symptoms are rare. By contrast, in some case reports, cotton-wool spots (indicating a precapillary arteriolar occlusion) were symptomatic or were associated with other symptomatic ischemic signs of retinopathy, such as papilledema, retinal artery occlusion, and retinal vein thrombosis, and were sometimes responsible for a definitive decrease in visual acuity.

Peer review

This meta-analysis aimed to assess the association of hypertension and/or DM with IAR risk among CHC patients. The results are interesting and the issue is important. The authors found that patients with hypertension, but not those with DM, are at higher risk for developing retinopathy when receiving IFN-based therapy for CHC infection. This study was the first meta-analysis of relevant studies to examine retinopathy in patients with hypertension, and the results will be helpful for determining whether an ophthalmic screening should be recommended for CHC patients with hypertension or DM before, during, and after treatment.

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CASE REPORT

Case of acute pancreatitis associated with *Campylobacter* enteritis

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Abstract

A 25-year-old man was admitted with the chief complaints of right flank pain, watery diarrhea, and fever. Blood tests revealed high levels of inflammatory markers, and infectious enteritis was diagnosed. A stool culture obtained on admission revealed no growth of any significant pathogens. Conservative therapy was undertaken with fasting and fluid replacement. On day 2 of admission, the fever resolved, the frequency of defecation reduced, the right flank pain began to subside, and the white blood cell count started to decrease. On hospital day 4, the frequency of diarrhea decreased to approximately 5 times per day, and the right flank pain resolved. However, the patient developed epigastric pain and increased blood levels of the pancreatic enzymes. Abdominal computed tomography revealed mild pancreatic enlargement. Acute pancreatitis was diagnosed, and conservative therapy with fasting and fluid replacement was continued. A day later, the blood levels of the pancreatic enzymes peaked out. On hospital day 7, the patient passed stools with fresh blood, and *Campylobacter jejuni/coli* was detected by culture. Lower gastrointestinal endoscopy performed on hospital day 8 revealed diffuse aphthae extending from the terminal ileum to the entire colon. Based on the findings, pancreatitis associated with *Campylobacter* enteritis was diagnosed. In the present case, a possible mechanism of onset of pancreatitis was invasion of the pancreatic duct by *Campylobacter* and the host immune responses to *Campylobacter*.

Key words: Acute pancreatitis; *Campylobacter*; Enteritis; Bacteria; Infectious colitis

Core tip: A 25-year-old man was admitted with infectious enteritis. During the hospital stay, although the symptom of enteritis was improved, the patient developed epigastric pain and increased blood levels of the pancreatic enzymes, and was diagnosed acute pancreatitis. The patient passed fresh blood stools, and *Campylobacter jejuni/coli* was detected by culture. Based on the findings, pancreatitis associated with *Campylobacter* enteritis was diagnosed. Bacteria that cause gastroenteritis can also be causative agents for pancreatitis. When upper abdominal pain or increased levels of pancreatic enzymes not consistent with the course of gastroenteritis are observed, we need to consider concomitant pancreatitis.

Kobayashi R, Matsumoto S, Yoshida Y. Case of acute pancreatitis associated with *Campylobacter* enteritis. *World J Gastroenterol* 2014; 20(23): 7514-7517 Available from: URL: http://www.wjgnet.com/1007-9327/full/v20/i23/7514.htm DOI: http://dx.doi.org/10.3748/wjg.v20.i23.7514

INTRODUCTION

Infection is one of the diverse causes of pancreatitis.

Bacteria that cause gastroenteritis can also be causative agents of pancreatitis. We encountered a rare case in which acute pancreatitis occurred during the course of Campylobacter enteritis.

CASE REPORT

A 25-year-old man was admitted with a 3-d history of right flank pain and watery diarrhea. He had eaten chicken liver and steamed chicken at a restaurant 4 d earlier. On the day prior to admission, he had developed a fever of approximately 38 degrees C, and Clostridium butyricum preparations and acetaminophen had been prescribed at a neighborhood clinic. However, because of the persistence of diarrhea at a stool frequency of more than 20 times per day and fever, the patient was referred and admitted to our hospital for detailed examination. None of his friends who had eaten the same meal with him had similar symptoms.

On admission, a fever of 39.1 degrees C and tenderness of the right flank were noted; however, there was no rebound tenderness or muscle guarding. The white blood cell count was elevated to 13120/μL, and the serum C-reactive protein level was increased to 11.58 (Table 1). A stool culture obtained on admission revealed no growth of any significant pathogens. As abdominal ultrasonography (US) revealed thickening of the wall of the ascending colon, infectious enteritis was diagnosed, and conservative therapy with fasting and fluid replacement was undertaken. Although no antimicrobial agent was administered, the fever resolved by day 2 of admission. The frequency of defecation and the white blood cell count began to decrease, and the right flank pain began to subside. On hospital day 4, the stool frequency decreased to approximately 5 times per day, and the right flank pain resolved completely. However, the patient developed epigastric pain. The plasma levels of pancreatic amylase and lipase were elevated to 341 IU/L and 660 IU/L, respectively (Table 2). Computed tomography (CT) revealed mild pancreatic enlargement (Figure 1). Acute pancreatitis was diagnosed, and conservative therapy with fasting and fluid replacement was continued. On the following day, the pancreatic enzyme levels peaked. On hospital day 6, oral intake was resumed. On hospital day 7, the patient passed stools containing fresh blood, and a stool culture yielded growth of *Campylobacter jejuni/coli*. Lower gastrointestinal endoscopy performed on hospital day 8 revealed diffuse aphthae extending from the terminal ileum to the entire colon (Figure 2). Subsequently, the bloody stools resolved, and the clinical course was favorable. The patient was discharged on hospital day 12.

Table 1 Hematological examination on admission

WBC	13120/μL	γ-GTP	25 U/L
Neut	94.0%	CK	132 U/L
Lymp	1.0%	P-AMY	24 U/L
Hb	15.8 g/dL	Lipase	660 U/L
Plt	22.2 × 10^4/μL	CRP	11.58 mg/dL
TP	7.4 g/dL	BUN	10 mg/dL
Alb	4.5 g/dL	Cr	1.1 mg/dL
T-Bil	0.66 mg/dL	Na	133 mmol/L
D-Bil	0.21 mg/dL	K	3.6 mmol/L
AST	22 U/L	Cl	97 mmol/L
ALT	13 U/L	Tcho	89 mg/dL
LD	229 U/L	TG	106 mg/dL
ALP	222 U/L	BS	118 mg/dL

WBC: White blood cell; Neut: Neutrophil; Lymp: Lymphocyte; Hb: Hemoglobin; Plt: Platelet; TP: Total protein; Alb: Albumin; T-Bil: Total bilirubin; D-Bil: Direct bilirubin; AST: Aspartate transaminase; ALT: Alanine aminotransferase; LD: Lactate dehydrogenase; ALP: Alkaline phosphatase; γ-GTP: Gamma-glutamyl transpeptidase; CK: Creatinine phosphokinase; P-AMY: Pancreatic amylase; CRP: C-reactive protein; BUN: Blood urea nitrogen; Cr: Creatinine; Na: Sodium; K: Potassium; Cl: Chloride; Tcho: Total cholesterol; TG: Triglyceride; BS: Blood sugar.

Table 2 Hematological examination on hospital day 4

WBC	5420/μL	γ-GTP	28 U/L
Neut	76.0%	CK	83 U/L
Lymp	14.0%	P-AMY	341 U/L
Hb	14.8 g/dL	Lipase	438 U/L
Plt	27.9 × 10^4/μL	CRP	4.07 mg/dL
TP	7.0 g/dL	BUN	7 mg/dL
Alb	4.1 g/dL	Cr	0.94 mg/dL
T-Bil	0.52 mg/dL	Na	134 mmol/L
D-Bil	0.19 mg/dL	K	4.0 mmol/L
AST	24 U/L	Cl	96 mmol/L
ALT	16 U/L		
LD	231 U/L		
ALP	186 U/L		

WBC: White blood cell; Neut: Neutrophil; Lymp: Lymphocyte; Hb: Hemoglobin; Plt: Platelet; TP: Total protein; Alb: Albumin; T-Bil: Total bilirubin; D-Bil: Direct bilirubin; AST: Aspartate transaminase; ALT: Alanine aminotransferase; LD: Lactate dehydrogenase; ALP: Alkaline phosphatase; γ-GTP: Gamma-glutamyl transpeptidase; CK: Creatinine phosphokinase; P-AMY: Pancreatic amylase; CRP: C-reactive protein; BUN: Blood urea nitrogen; Cr: Creatinine; Na: Sodium; K: Potassium; Cl: Chloride.

DISCUSSION

Infection is one of the diverse causes of pancreatitis. Viruses that are known to cause pancreatitis include mumps virus, coxsackievirus, hepatitis B virus, cytomegalovirus, varicella-zoster virus, herpes simplex virus, and human immunodeficiency virus. The bacteria reported to cause pancreatitis include Mycoplasma, Legionella, and Leptospira species, as well as those causing gastroenteritis, such as *Salmonella typhi*, *Campylobacter jejuni*, Yersinia enterocolitica, and Yersinia pseudotuberculosis[1]. Although the mechanisms by which bacteria cause pancreatitis remain unknown, possible mechanisms include the direct spread of inflammation from adjacent organs, such as the small intestine, to the pancreas[2]; invasion of the bile duct[1] and pancreatic duct[3] by bacteria; dissemination through the blood and lymphatic vessels[1]; and immune responses of the host to bacterial invasion of the pancreas[2].

In regard to pancreatitis caused by Campylobacter enteritis, the reported age at onset ranges widely, from 9 to

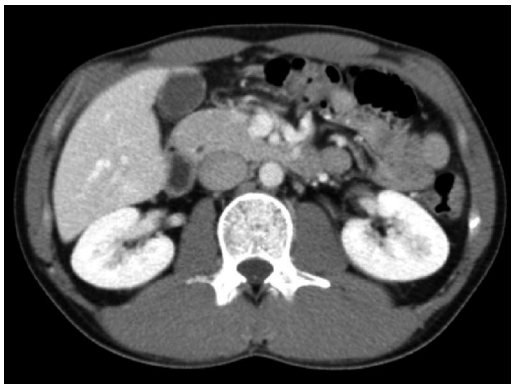

Figure 1 Abdominal computed tomography on hospital day 4 revealed mild pancreatic enlargement.

88 years, and there is no gender difference. The interval from the onset of enteritis to the occurrence of pancreatitis ranges from 3 to 7 d. In terms of the severity, there are many cases of mild pancreatitis. *Campylobacter* enteritis has been reported to be both treated and not treated with antimicrobial agents[2-8]. However, it remains unclear whether antimicrobial agents are effective for the treatment of pancreatitis associated with Campylobacter enteritis.

In the present case, there was no apparent cause of acute pancreatitis, except for *Campylobacter* enteritis. The patient drank socially and was not on any regular medication. No gallstones were detected by US or CT. Additionally, there was no evidence of hypertriglyceridemia. Thin-slice CT revealed no apparent abnormalities of the pancreatobiliary junction. Even a single administration of acetaminophen can cause pancreatitis, but according to previous case reports, pancreatitis occurs within 24 h of taking the drug[9]. In our case, because pancreatitis occurred 5 d after the patient had taken acetaminophen, acetaminophen was unlikely to have been the cause of the pancreatitis. Moreover, because the levels of antinuclear antibodies and immunoglobulin G4 were within the normal range, autoimmune pancreatitis was unlikely. Thus, our patient was diagnosed as having pancreatitis associated with *Campylobacter* enteritis. As for the mechanism of the onset of pancreatitis in this patient, invasion of the bile duct by *Campylobacter* was unlikely because the blood levels of biliary enzymes were normal. CT revealed no increase in the adipose tissue around the pancreas, and the inflammatory findings around the intestine were also mild. These findings made the direct spread of the inflammation from the intestine to the pancreas also unlikely. Thus, it is assumed that invasion of the pancreatic duct by *Campylobacter* or an immune response of the host to invasion of the pancreas by *Campylobacter* was the mechanism of the onset of pancreatitis in our patient.

We encountered a rare case in which acute pancreatitis occurred during the course of *Campylobacter* enteritis. Bacteria that cause gastroenteritis can also be causative agents of pancreatitis. When upper abdominal pain or increased levels of pancreatic enzymes that are not consistent with the course of gastroenteritis are observed, concomitant pancreatitis needs to be kept in mind.

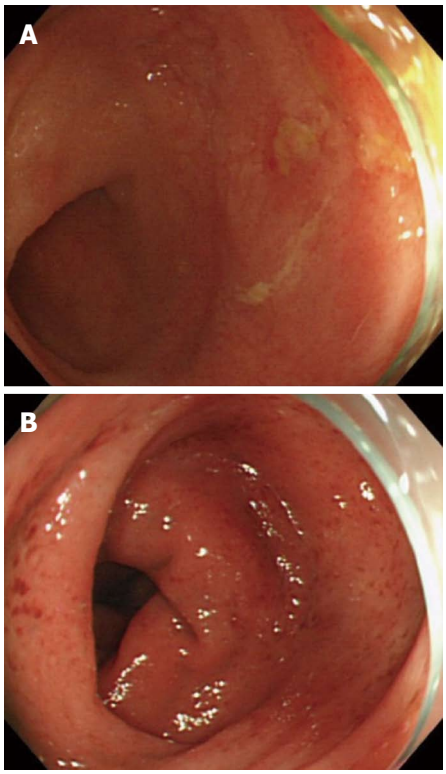

Figure 2 Lower gastrointestinal endoscopy performed on hospital day 8 revealed aphthae in the terminal ileum (A) and descending colon (B).

COMMENTS

Case characteristics

The patient was admitted with a 3-d history of right flank pain and watery diarrhea, and on hospital day 4, the symptom of enteritis was improved, but the patient developed epigastric pain.

Clinical diagnosis

Pancreatitis associated with *Campylobacter* enteritis was diagnosed.

Differential diagnosis

There was no apparent cause of acute pancreatitis, except for *Campylobacter* enteritis, because the patient did not have a history of alcohol abuse or regular medication, additionally, gallstones, abnormalities of the pancreatobiliary junction, hypertriglyceridemia, and high levels of antinuclear antibodies and immunoglobulin G4 were not detected.

Laboratory diagnosis

On admission, the plasma level of pancreatic amylase was not elevated; however, on hospital day 4, when the patient developed epigastric pain, the plasma levels of pancreatic amylase and lipase were elevated.

Imaging diagnosis

Lower gastrointestinal endoscopy performed on hospital day 8, which revealed diffuse aphthae extending from the terminal ileum to the entire colon.

Treatment

We performed conservative therapy with fasting and fluid replacement, no antimicrobial agent was administered for either *Campylobacter* enteritis or acute pancreatitis.

Experiences and lessons

When upper abdominal pain or increased levels of pancreatic enzymes that are not consistent with the course of gastroenteritis are observed, concomitant pancreatitis needs to be kept in mind.

Peer review

This was a rare case in which acute pancreatitis occurred during the course of *Campylobacter* enteritis. Infection is one of the diverse causes of pancreatitis. Many viruses and bacteria can cause acute pancreatitis. But pancreatitis caused by *Campylobacter* enteritis was rare. So in clinical work, when upper abdominal pain or increased levels of pancreatic enzymes not consistent with the course of gastroenteritis are observed, concomitant pancreatitis needs to be considered in mind.

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CASE REPORT

Cronkhite-Canada syndrome: Report of six cases and review of literature

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Abstract

Cronkhite-Canada syndrome (CCS) is a rare nonfamilial polyposis syndrome characterized by epithelial disturbances in the gastrointestinal tract and skin. The aim of this study was to investigate the clinical features and potential therapies for CCS. Six patients with CCS admitted from December 1992 to July 2008 to Peking Union Medical College Hospital were evaluated. All patients had clinical manifestation of nonhereditary gastrointestinal polyposis with diarrhea, skin hyperpigmentation, alopecia, and nail dystrophy. Fecal occult blood was positive in all six cases. Serum hemoglobin, potassium, calcium and protein were below the normal range in two cases. Anti-*Saccharomyces cerevisiae* and antinuclear antibodies were present in three cases. Multiple polyps were found in all patients by gastroscopy and colonoscopy, with only one in the esophagus. Histologically, there were hyperplastic polyps in five cases, tubular adenoma in three, and juvenile polyp in one with chronic inflammation and mucosal edema. Comprehensive treatment led by corticosteroids can result in partial remission of clinical symptoms, and long-term follow-up is necessary.

Key words: Cronkhite-Canada syndrome; Clinical feature; Etiology; Therapeutics

Core tip: Cronkhite-Canada syndrome (CCS) is a rare noncongenital gastrointestinal polyposis syndrome, characterized by skin hyperpigmentation, hair loss and nail atrophy, associated with high morbidity. This case report summarizes the characteristics of six CCS patients, and reviews the literature. Comprehensive treatment led by corticosteroids can improve prognosis, and long-term follow-up is necessary.

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INTRODUCTION

Cronkhite-Canada syndrome (CCS) is a rare noncongenital gastrointestinal polyposis syndrome, characterized by ectodermal dysplasia, skin hyperpigmentation, hair loss and nail atrophy. Since CCS was first reported in 1955[1], more than 400 cases have been reported worldwide. CCS has high morbidity but its etiology is still unknown and there is no consistently recommended treatment. In order to gain a detailed understanding of this rare disease, we reviewed the literature and reported on six CCS patients.

CASE REPORT

Data collection

We collected data from the patients with CCS admitted to Peking Union Medical College Hospital from December

1992 to July 2008, who were diagnosed by history, physical examination, endoscopic findings of gastrointestinal polyposis, and histology, including diffuse polyposis of the gastrointestinal tract; ectodermal dysplasia, cutaneous hyperpigmentation, dystrophic changes of fingernails, and alopecia; diarrhea, weight loss, and abdominal pain. All six patients met the diagnostic criteria, with adult onset and no family history of polyposis.

Patients with Peutz-Jeghers syndrome, juvenile polyps, familial polyposis of the colon, Gardner syndrome, Turcot syndrome; and Menetrier's disease were excluded.

General information

The six cases comprised three male and three female patients aged 24-69 years (mean: 54.67 years).

Clinical manifestations and physical examination

Three patients had used hair dye or topical pharmaceuticals, and two had intestinal ascariasis. The time between onset and diagnosis was 2-7 mo (mean: 4 mo). The most common symptoms were diarrhea (n = 5) and anorexia (n = 6, including 1 each with hypogeusia or dysgeusia) with different degrees of weight loss. Diarrhea often occurred several times per day, sometimes > 10 times. Patients also suffered hair loss (n = 6), nail changes (n = 6), and skin hyperpigmentation (n = 4) (Figure 1). Hair loss occurred on the scalp, eyebrows, eyelashes, axilla, pubic areas, and limbs. Scalp hair was the most predominant type of hair loss reported. Nail changes were described as thinning, splitting, and onycholysis. Skin hyperpigmentation was mainly manifest as brownish patches with a clear boundary with colored spots occurring on the limbs, face, body, palms, and soles of the feet. Two patients had lower limb edema.

Laboratory examination

Fecal occult blood was positive in all six cases; maw worm eggs were positive in the feces in two cases; and indistinguishable bacilli were cultivated in the feces from one case. Laboratory data indicated that all kidney and liver functions were normal. Serum hemoglobin, potassium, calcium and protein were below the normal range in two cases, and eosinophilic cells and IgE were elevated in one case. Circulatory anti-*Saccharomyces cerevisiae* antibody (ASCA) was positive in two cases, antinuclear antibody (ANA) was present in one case, and thyroid function decreased in one case.

Imaging and pathology

Multiple polyps were found in all patients by gastroscopy and colonoscopy, with only one in the esophagus, varying in size from 2 to 40 mm (Figures 2 and 3). Endoscopy results revealed diffuse sessile or pedunculated polyps with either a smooth or rough surface. Radiology of the gastrointestinal tract showed polyposis in the small bowel in two cases. Histology of these polyps showed hyperplastic polyps in five cases, tubular adenoma in three, and juvenile polyp in one with chronic inflammation and mucosal edema.

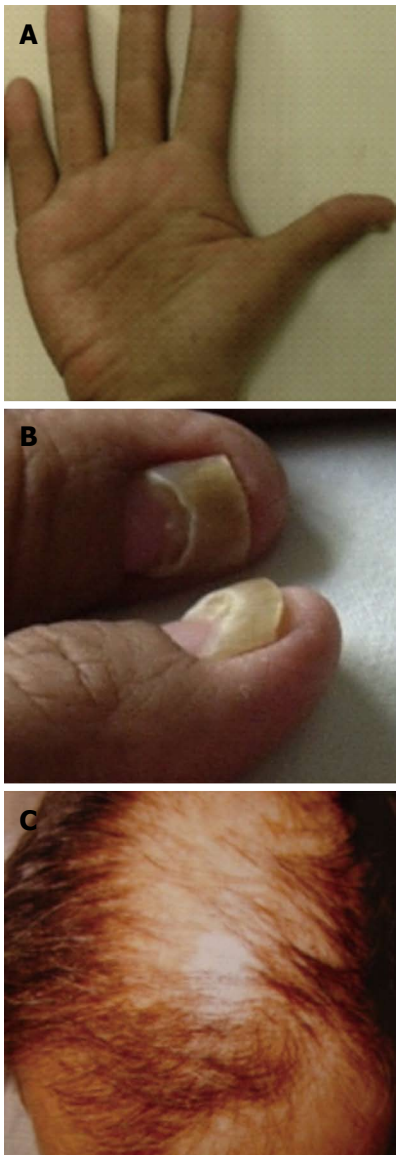

Figure 1 Clinical presence, cutaneous symptoms. A: Hyperpigmentation of palm; B: Onychodystrophy; C: Alopecia.

Treatment

All patients underwent comprehensive therapy, including glucocorticoids, nutritional support, antibiotics, and polypectomy. Five patients underwent a 2-wk to 1-mo course of prednisone at a dose of 1 mg/kg per day before reducing the dose if necessary. Within 2-8 wk after initiation of treatment, symptoms including nail atrophy, anorexia, diarrhea, and skin pigmentation gradually resolved. When prednisone was discontinued or decreased in dose, diarrhea relapsed in two patients but retreatment resulted in remission. In the course of therapy, endoscopy showed that polyps were unchanged in two cases, aggravated in two, and relapsed after remission in two. Polyps of 5-40 mm were resected by endoscopic polypectomy.

Four patients were treated with parenteral nutrition, such as replacement and supplement therapy with blood, albumin, vitamins, amino acids, and lipids with clear improvements. Three patients were treated with antibiotics

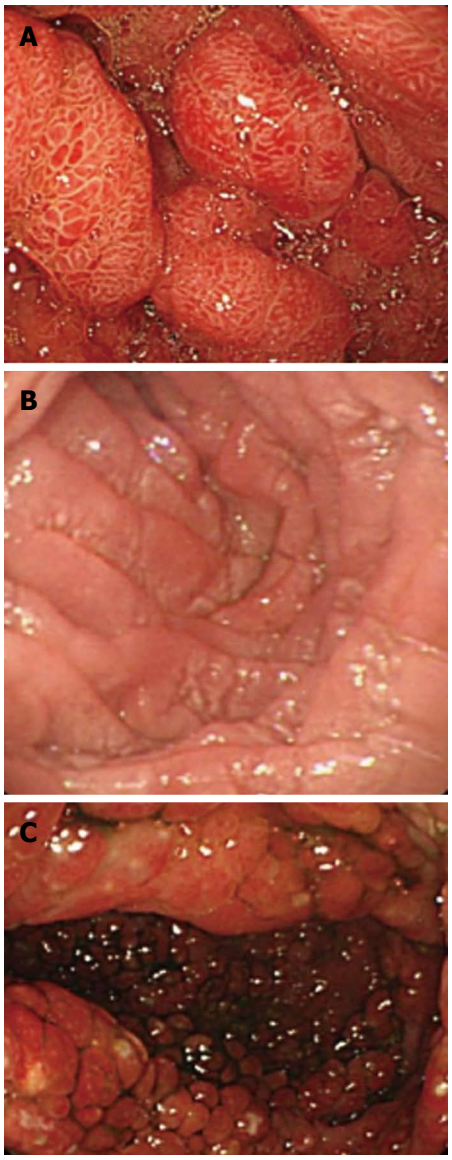

Figure 2 Endoscopy results. A: Numerous polyps in the stomach; B: Mucosal edema in the descendant duodenum; C: Multiple polyps in the colon.

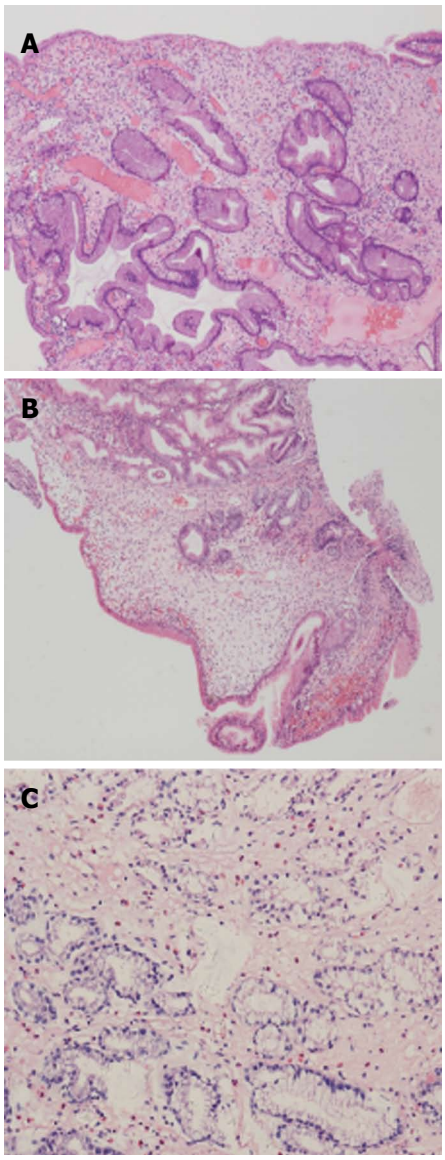

Figure 3 Histology of the biopsy (A), hyperplastic polyps (B) and juvenile polyps (C). The mucosa showed cystically dilated glands in the edematous stroma with eosinophilia and lymphocyte infiltrated.

(albendazole and sulfasalazine). No patient underwent surgery (Table 1).

DISCUSSION

CCS is a rare nonfamilial polyposis syndrome characterized by epithelial disturbances both in the gastrointestinal tract and epidermis. At present, the pathogenesis of CCS is unknown, but the following factors may be relevant. The first is immune abnormalities: many studies have shown that patients with CCS are positive for ANA[2]. All six cases in the present study had positive immune parameters; two were positive for ASCA and one was positive for ANA. Hormonal therapy was effective for all three patients, especially in a significantly shorter time for the ANA positive patient. For patients with CCS, their skin and nail changes were similar to the appearance of the ectoderm in autoimmune polyendocrinopathy-candidiasis-ectodermal dystrophy (APECED) syndrome. The efficacy of corticosteroids provides the strongest evidence of an inflammatory cause of CCS[3,4]. All the above signs indicated that autoimmunity might play an important role in CCS. The second factor is infection. Two of the present cases had combined infection with two or more pathogens, but the relationship of these pathogens with diarrhea and CCS was difficult to determine. It was suggested the Hp might be a causative factor[5]. The third factor is allergies; after stopping the use of inducers (hair dye and topical medications), IgE and eosinophils were decreased and symptoms improved.

CCS is reported worldwide, with 75% of cases in Japan. The average age of onset is 60 years and the male to female ratio is 3:2. With a relatively acute onset, it typically takes 3 mo to 1 year from onset to diagnosis[6]. There are no clear diagnostic tools for CCS. Diagnosis is based on history, physical examination, endoscopic findings of gastrointestinal polyposis, and histology. The most com-

Table 1 Therapy and prognosis for all six patients

Patient	Therapy	Prognosis
M/65	Prednisone, PPI, antibiotics, 5-ASA, *Lactobacillus*, and nutrition nutritional therapy	Diarrhea, skin hyperpigmentation, atrophic nail remission
F/54	Prednisone, nutritional therapy, and antacid drugs	Diarrhea, appetite, atrophic nail remission Gastrointestinal polyps unchanged 1 mo later
M/54	Prednisone, antibiotics, nutritional therapy, antacid drugs, and polypectomy	Diarrhea, abdominal pain remission Gastrointestinal polyps unchanged 1 mo later
F/69	Prednisone, nutritional therapy, and antacid drugs	Diarrhea, skin hyperpigmentation, atrophic nail remission, gastrointestinal polyps reduced 1 yr later
F/24	Albendazole , antibiotics and sulfasalazine	Diarrhea, appetite remission, atrophic nail, and alopecia aggravated
M/62	Prednisone, albendazole, antibiotics, sulfasalazine and polypectomy	Diarrhea, atrophic nail and polyps resolved 8 wk later Prednisone was discontinued after 3 yr, and all symptoms relapsed

5-ASA: 5-aminosalicylic acid.

mon clinical manifestations are diarrhea, gustatory loss, weight loss, hair loss, nail atrophy, skin hyperpigmentation, edema, anemia, and glossitis[7]. The condition of the present group of patients was generally consistent with that reported previously. Goto[6] has divided this disease into five types according to its onset. Type Ⅰ: diarrhea as initial symptom (35.4%); Type Ⅱ: abnormal gustatory sense as initial symptom (40.9%); Type Ⅲ: dominated by dry mouth (6.4%); Type Ⅳ: initial symptoms include hair loss and nail atrophy (9.1%); and Type Ⅴ: initial loss of appetite and general malaise, followed by nail atrophy, hair loss and abnormal gustatory sense but no diarrhea (8.2%). In our group of patients, Type Ⅰ dominated, and other types were relatively rare. One case was Type Ⅱ, which was mainly characterized by abnormal salt and sweet taste, accompanied by tongue atrophy.

CCS polyps are distributed through the whole digestive tract, being common in the stomach and colon, less common in the small intestine and rectum, and uncommon in the esophagus[3]. In most circumstances, the polyps are found simultaneously in the stomach and intestines. Polyps usually are nodular or irregular in shape, differently sized, and diffusely distributed. The pathology of CCS is not specific. There are four histological types: hyperplastic polyps, tubular adenomas, juvenile polyps, and inflammatory. The present group of patients met the characteristics above. Cancer arising from polyps is an important cause of death, and 12.5% of patients with polyps develop cancer[8], thus close monitoring or removal of polyps is important.

There were some limitations to our study. It was a retrospective study. CCS is a rare disease, so there are no prospective randomized controlled studies. From our experience and review of the literature, comprehensive treatment, mainly with glucocorticoids, is the most effective option at present. Currently, comprehensive treatment includes corticosteroids, polyp electrocision, and enteral/parenteral nutrition, and the recent literature suggests that it could improve prognosis and quality of life[4,9-11]. The currently recommended high-dose hormonal therapy (prednisone ⩾ 40 mg/d) was initiated[4], with a treatment course of 6-12 mo, with the longest maintenance period of up to 4 years. In most circumstances, a slow reduction of dosage is suggested. In 26 Chinese cases of CCS, 8 were treated with hormonal and nutritional therapy, seven of which showed varying degrees of improvement[9]. In our six patients, four achieved general remission after hormonal and nutritional therapy. However, the symptoms of CCS have different hormonal responses. In our group of patients, diarrhea and abdominal pain were more easily corrected in five cases, while the improvement of ectodermal symptoms was relatively slow. Hormones played a limited role in gastrointestinal tract polyps. Two cases achieved no improvement in polyps after treatment, one became worse, and one had recurrence after 3 years of full remission. Fossati *et al*[12] have reported that after 4 years of hormonal maintenance therapy, one patient had improved clinical symptoms, including ectodermal changes, but endoscopic review did not show improvement in polyps. At present, polyps are mainly subject to endoscopic resection to prevent cancerous development. Treatment is still challenged by repeated occurrence of CSS. Two of our six patients experienced recurrence at one-year follow-up. It was reported that immunosuppressants are useful in maintaining remission[3]. However, further clinical observation is needed.

Salazosulfapyridine and 5-aminosalicylic acid are used in patients with gastrointestinal symptoms. Nutritional support, antibiotics, histamine receptor antagonists, cromolyn sodium and surgery can be used to improve symptoms such as diarrhea, weight loss, and ectodermal symptoms[5]. Successful treatment by immunosuppressants for steroid-resistant CCS has also been recently reported[13].

In conclusion, CCS is a rare disease with major symptoms of gastrointestinal polyps, diarrhea, skin hyperpigmentation, and hair/finger (toe) nail atrophy. Comprehensive treatment led by hormonal therapy can lead to partial or full remission of clinical symptoms. Long-term follow-up is necessary for further results.

COMMENTS

Case characteristics

Cronkhite-Canada syndrome (CCS) is a rare nonfamilial polyposis syndrome characterized by epithelial disturbances in the gastrointestinal tract and skin.

Clinical diagnosis

CCS is diagnosed by nonfamilial adenomatous polyposis (diffuse polyposis of

the gastrointestinal tract); ectodermal dysplasia (cutaneous hyperpigmentation, dystrophic changes of fingernails, and alopecia); diarrhea, weight loss, and abdominal pain.

Differential diagnosis

Peutz-Jeghers syndrome, juvenile polyps, familial polyposis of the colon, Gardner syndrome, Turcot syndrome, and Menetrier's disease.

Laboratory diagnosis

Fecal occult blood was positive in all six cases. Serum hemoglobin, potassium, calcium and protein were below the normal range in two cases. Circulatory anti-*Saccharomyces cerevisiae* antibody and antinuclear antibody were present in three cases.

Imaging diagnosis

Multiple polyps were found in all patients by gastroscopy and colonoscopy, with only one in the esophagus.

Pathological diagnosis

Histological assessment showed hyperplastic polyps in five cases, tubular adenoma in three, and juvenile polyp in one with chronic inflammation and mucosal edema.

Treatment

All patients underwent comprehensive therapy, including glucocorticoids, nutritional support, antibiotics, and polypectomy.

Related reports

There is no prospective randomized controlled study. From their experience and review of the literature, comprehensive treatment mainly with glucocorticoids is the most effective treatment to date.

Term explanation

Comprehensive treatment includes corticosteroids, polyp electrocision, and enteral/parenteral nutrition *etc*.

Experiences and lessons

CCS has high morbidity, but detailed understanding and comprehensive treatment could improve prognosis and quality of life.

Peer review

This article describes the clinical features, pathophysiology and potential therapy of CCS.

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Hepatic venous pressure gradient measurement before TIPS for acute variceal bleeding

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Abstract

Hepatic venous pressure gradient (HVPG) is an independent predictor of variceal rebleeding in patients with cirrhosis. After pharmacological and/or endoscopic therapy, the use of a transjugular intrahepatic portosystemic shunt (TIPS) may be necessary in HVPG non-responders, but not in responders. Thus, HVPG measurement may be incorporated into the treatment algorithm for acute variceal bleeding, which further identifies the candidates that should undergo early insertion of TIPS or maintain the traditional pharmacological and/or endoscopic therapy. The potential benefits are to reduce the cost and prevent TIPS-related complications.

Key words: Acute variceal bleeding; Transjugular intrahepatic portosystemic shunt; Hepatic venous pressure gradient; Liver cirrhosis

Core tip: If hepatic venous pressure gradient could be measured before a transjugular intrahepatic portosystemic shunt for the treatment of acute variceal bleeding, the invasiveness of treatment strategy would be further decreased.

Qi XS, Fan DM. Hepatic venous pressure gradient measurement before TIPS for acute variceal bleeding. *World J Gastroenterol* 2014; 20(23): 7523-7524 Available from: URL: http://www.wjgnet.com/1007-9327/full/v20/i23/7523.htm DOI: http://dx.doi.org/10.3748/wjg.v20.i23.7523

TO THE EDITOR

In spite of increasing advances in the management of acute variceal bleeding in patients with cirrhosis, it often remains fatal with a 6-wk and 1-year mortality rate of approximately 20% and 60%, respectively[1]. In a comprehensive review recently published in the *World Journal of Gastroenterology*, Loffroy *et al*[2] summarized the key role of transjugular intrahepatic portosystemic shunt (TIPS) for the treatment of acute variceal bleeding. Notably, a randomized controlled trial indicated that an early decision for TIPS with polytetrafluoroethylene-covered stents could significantly reduce the incidence of variceal rebleeding and improve survival in cirrhosis patients with a Child-Pugh score of 7-13 (*i.e.*, high-risk patients) and acute variceal bleeding[3]. The survival benefit of the early use of TIPS was mainly because no fatal episode of early rebleeding occurred in the TIPS group, as compared with the pharmacotherapy-endoscopy group in which five patients died of recurrent variceal bleeding[3]. This remarkable finding has potentially challenged the therapeutic strategy recommended by the current practice guidelines that a combination of pharmacological and endoscopic therapy is the most rational approach in the treatment of acute variceal hemorrhage[4]. However, as described by Loffroy *et al*[2], TIPS is still not considered as a primary treatment option due to the limited evidence, but as a rescue treatment for bleeding esophageal varices that have failed pharmacological and endoscopic treatments. In line with their comments, the application of early TIPS in all

patients with acute variceal bleeding may be excessive, and combined drug and endoscopic treatment may be effective in a proportion of high-risk patients with acute variceal bleeding. Furthermore, hepatic venous pressure gradient (HVPG) measurement can be incorporated into the algorithm for identifying the candidates for early TIPS in the treatment of acute variceal bleeding.

HVPG measurement within 24 h after admission has been recommended as the best predictor of a poor outcome in patients with cirrhosis with variceal bleeding[1]. Patients with acute variceal bleeding are treated with pharmacological and endoscopic therapy, therefore, the risk of variceal rebleeding with the first 5 d of admission is four times greater in patients with HVPG $\geqslant$ 20 mmHg than in those with HVPG < 20 mmHg[5]. Based on these findings, the patients who experience rebleeding after combined drug and endoscopic therapy are considered HVPG non-responders and may be appropriate for early use of TIPS. By contrast, patients without rebleeding may be HVPG responders and inappropriate for early TIPS insertion. Accordingly, to achieve the goal of a minimally invasive treatment strategy, HVPG should be measured before TIPS placement is considered for the treatment of acute variceal bleeding. In this setting, the candidates for the early use of TIPS may be further identified, thereby decreasing the cost and avoiding the long-term complications of portosystemic shunting.

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3 **Tian D**, Araki H, Stahl E, Bergelson J, Kreitman M. Signature of balancing selection in Arabidopsis. *Proc Natl Acad Sci USA* 2006; In press

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6 21st century heart solution may have a sting in the tail. *BMJ* 2002; **325**: 184 [PMID: 12142303 DOI:10.1136/bmj.325.7357.184]

Volume with supplement

7 **Geraud G**, Spierings EL, Keywood C. Tolerability and safety of frovatriptan with short- and long-term use for treatment of migraine and in comparison with sumatriptan. *Headache* 2002; **42** Suppl 2: S93-99 [PMID: 12028325 DOI:10.1046/j.1526-4610.42.s2.7.x]

Issue with no volume

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9 Outreach: Bringing HIV-positive individuals into care. *HRSA Careaction* 2002; 1-6 [PMID: 12154804]

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10 **Sherlock S**, Dooley J. Diseases of the liver and billiary system. 9th ed. Oxford: Blackwell Sci Pub, 1993: 258-296

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Electronic journal (list all authors)

15 Morse SS. Factors in the emergence of infectious diseases. Emerg Infect Dis serial online, 1995-01-03, cited 1996-06-05; 1(1): 24 screens. Available from: URL: http://www.cdc.gov/ncidod/eid/index.htm

Patent (list all authors)

16 **Pagedas AC**, inventor; Ancel Surgical R&D Inc., assignee. Flexible endoscopic grasping and cutting device and positioning tool assembly. United States patent US 20020103498. 2002 Aug 1

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