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EDITORIAL

Contrast-enhanced ultrasound in differentiating malignant from benign portal vein thrombosis in hepatocellular carcinoma

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Abstract

Portal vein thrombosis (PVT) may occur in liver cirrhosis patients. Malignant PVT is a common complication in cirrhotic patients with concomitant hepatocellular carcinoma (HCC) and, in some cases, it may be even the initial sign of an undetected HCC. Detection of malignant PVT in a patient with liver cirrhosis heavily affects the therapeutic strategy. Gray-scale ultrasound (US) is widely unreliable for differentiating benign and malignant thrombi. Although effective for this differential diagnosis, fine-needle biopsy remains an invasive technique. Sensitivity of color-doppler US in detection of malignant thrombi is highly dependent on the size of the thrombus. Contrast-enhanced computed tomography (CT) and contrast-enhanced magnetic resonance (MRI) can be useful to assess the nature of portal thrombus, while limited data are currently available about the role of positron emission tomography (PET) and PET-CT. In contrast with CT, MRI, PET, and PET-CT, contrast-enhanced ultrasound (CEUS) is a fast, effective, well tolerated and cheap technique, that can be performed even in the same session in which the thrombus has been detected. CEUS can be performed bedside and can be available also in transplanted patients. Moreover, CT and MRI only yield a snapshot analysis during contrast diffusion, while CEUS allows for a continuous real-time imaging of the microcirculation that lasts several minutes, so that the whole arterial phase and the late parenchymal phase of the contrast diffusion can be analyzed continuously by real-time US scanning. Continuous real-time monitoring of contrast diffusion entails an easy detection of thrombus maximum enhancement. Moreover, continuous quantitative analyses of enhancement (wash in - wash out studies) by CEUS during contrast diffusion is nowadays available in most CEUS machines, thus giving a more sophisticated and accurate evaluation of the contrast distribution and an increased confidence in diagnosis in difficult cases. In conclusion, CEUS is a

very reliable technique with a high intrinsic sensitivity for portal vein patency assessment. More expensive and sophisticated techniques (*i.e.*, CT, MRI, PET, and PET-CT) should only be indicated in undetermined cases at CEUS.

Key words: Contrast-enhanced ultrasound; Hepatocellular carcinoma; Portal vein thrombosis; Benign thrombosis; Malignant thrombosis

Core tip: Portal vein thrombosis (PVT) may occur in liver cirrhosis patients. Malignant PVT is a common complication in cirrhotic patients with concomitant hepatocellular carcinoma (HCC) and, in some cases, it may be even the initial sign of an undetected HCC. Due to its high performance in characterization of PVT in cirrhotic patients, contrast-enhanced ultrasound should be considered as the gold standard method and, often, the only diagnostic tool in cirrhotic patients for differential diagnosis between malignant and benign PVT.

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Portal vein thrombosis (PVT) may occur in liver cirrhosis patients[1-6] with a prevalence ranging from 0.6% to 11%[2,6]. In addition, PVT is even more frequent in cirrhotic patients with concomitant hepatocellular carcinoma (HCC)[2]. However, PVT in liver cirrhosis may be also associated with inflammatory and infectious diseases (liver, bowel, pancreas), hypercoagulable states, endoscopic sclerotherapy of esophageal varices, and percutaneous ablation therapies[3-5].

Malignant PVT, so named for its neoplastic origin, is a common complication of HCC[1,2,4,5], and, in some cases, it may be even the initial sign of an undetected HCC[7,8].

Detection of malignant PVT in a patient with liver cirrhosis heavily affects the therapeutic strategy. Indeed, some Authors believe that HCC infiltration of the portal vein represents an exclusion criteria for liver transplantation, surgical resection, chemoembolization, and imaging-guided ablation, even in the presence of an uninodular lesion with a diameter lower than 5 cm[9,10].

Although conventional gray-scale ultrasound (US) is a highly sensitive technique for detection of PVT, it remains widely unreliable for differentiating benign and malignant thrombi[11]. Furthermore, although fine-needle biopsy (FNB) under US guidance proved to be effective for this differential diagnosis[7,8], it remains an invasive technique, relatively unsafe in cirrhotic patients, in which an impaired haemostatic balance is often reported.

HCC is a hypervascular malignancy with arterial intralesional flow. The latter is expression of tumoral neoangiogenesis and represents the cornerstone for the diagnostic approach[12]. Indeed, the demonstration of the neovascularization of the portal thrombus allows for a highly specific and non-invasive diagnosis of the malignant nature of PVT[13].

In keeping with this, detection of pulsatile arterial signals at color-doppler US (CDUS) inside the portal thrombi may be a fast and specific technique for assessment of malignant PVT[14,15]. These previous reports also suggested high sensitivity of CDUS for this purpose. However, these results have been challenged by other recent studies[13,16], showing a sensitivity lower than 20%. In reality, sensitivity of CDUS in detection of malignant thrombi is highly dependent on the size of the thrombus and the previous reports do not specify the size of the portal vein thrombi in their series.

The injection of contrast material in a peripheral vein allows for the detection of tissues microcirculation by most imaging techniques.

In 2006, we reported the first work focused on the evaluation of contrast-enhanced ultrasound (CEUS) as a tool for differential diagnosis between malignant and benign PVT. In a series of cirrhotic patients with PVT, we performed a comparative study between FNB of the thrombus, CDUS and CEUS for the differential diagnosis of benign and malignant PVT in cirrhotic patients[13]. In this study, CEUS showed the best performance with high sensitivity (88%) and specificity (100%).

These results were confirmed and extended in a subsequent study on a very large series of patients with hepatic cirrhosis in which we documented that CEUS showed a high sensitivity (94%) and specificity (96%) in differentiating malignant *vs* non-malignant PVT[17]. In the same year, Rossi *et al*[18] confirmed the high sensitivity and specificity of CEUS for that indication and, based on all these data, the European Federation of Societies for Ultrasound in Medicine and Biology (EFSUMB) included the "differential diagnosis between malignant and benign portal vein thrombosis" among indications for CEUS in the updated "EFSUMB Guidelines"[19].

In the last five years, several series of cirrhotic patients with PVT evaluated with CEUS were reported, substantially confirming the previous data[20].

Detection of thrombus enhancement by contrast-enhanced computed tomography (CT) and contrast-enhanced magnetic resonance (MRI) can assess the nature of portal thrombus[21,22]. In a series of 58 cirrhotic patients with PVT, Tublin *et al*[23] reported 100% specificity of multidetector CT (MDCT) in the

diagnosis of malignant thrombosis. However, this study showed a rather low overall sensitivity (43%) of MDCT in detecting thrombus neovascularity. In addition, Rossi *et al*[18] compared CEUS and MDCT as techniques for differential diagnosis of PVT and CEUS proved to be far superior to MDCT and showed a very high sensitivity for detection (100%) and characterization (98%) of PVT, while MDCT showed a rather lower sensitivity both for detection (68%) and characterization (67%).

In 2012, Qian *et al*[24] compared indexes obtained by correlation between thrombus and aorta or thrombus and patent portal vein in portal phase, using dual-energy spectral CT for characterization of benign and malignant PVT. Interestingly, they reported a very high sensitivity (100%) and specificity (91.7%) of CT.

However, there are several disadvantages of CT, which include higher costs than CEUS, radiation exposure and the use of contrast materials, with important risks of anaphylaxis and nephropathy.

Gadolinium-enhanced MRI angiography is a useful technique for detection and characterization of PVT[22]. However, in our best knowledge, there are no published data on its sensitivity and specificity, and, also for this technique, there are several disadvantages that are mainly high cost of the procedure, limited number of available equipments, and possible severe nephrogenic systemic fibrosis caused by gadolinium.

A very interesting report by Catalano *et al*[25] described a sophisticated technique using unenhanced diffusion-weighted (DW) MRI imaging in distinguishing bland thrombus from neoplastic thrombus in PVT. In a short series of selected patients with known PVT, using an appropriate cut-off, malignant PVT could be assessed with 100% specificity. However, apart from the costs and scarce availability of the equipment, also in this case there are several drawbacks. DW MRI is an indigenous procedure with relatively low resolution of T2*WI protocol that often misses detection of thrombus in small portal venous branches and needs long times of breath-hold acquisitions, sometimes not feasible in cirrhotic patients.

Although limited data are currently available[26,27] we have also to consider the emerging role of positron emission tomography (PET) and PET-CT in differentiating malignant from benign PVT.

In contrast with CT, MRI, PET, and PET-CT, CEUS is a fast, effective, well tolerated and cheap technique, that can be performed even in the same session in which the thrombus has been detected[28,29]. CEUS can be performed bedside and can be available also in transplanted patients. Moreover, CT and MRI only yield a snapshot analysis during contrast diffusion, while CEUS allows for a continuous real-time imaging of the microcirculation that lasts several minutes, so that the whole arterial phase and the late parenchymal phase of the contrast diffusion can be analyzed continuously by real-time US scanning. Continuous real-time monitoring of contrast diffusion entails an easy detection of thrombus maximum enhancement. In fact, some patients show only a transient and very early enhancement inside the malignant thrombi after injection of the contrast[30]. CT and MRI could miss thrombus neovascularity in these kind of patients if the arterial phase scans are not taken at the time of maximum enhancement. Moreover, continuous quantitative analyses of enhancement (wash in - wash out studies) by CEUS during contrast diffusion is nowadays available in most CEUS machines, thus giving a more sophisticated and accurate evaluation of the contrast distribution and an increased confidence in diagnosis in difficult cases.

In conclusion, CEUS is a very reliable technique with a high intrinsic sensitivity for portal vein patency assessment. CEUS shows significantly higher sensitivity than CT in both detection and characterization of PVT. Due to its high performance in characterization of PVT in cirrhotic patients, we think that CEUS should be considered as the gold standard method and, often, the only diagnostic tool in cirrhotic patients for differential diagnosis between malignant and benign PVT. In this clinical setting, CEUS can be considered the best method for assessing eligibility of cirrhotic patients with HCC and PVT to liver transplantation, surgical resection or percutaneous treatments, without resorting to invasive methods such as FNB. More expensive and sophisticated techniques (*i.e.*, CT, MRI, PET, and PET-CT) should only be indicated in undetermined cases at CEUS.

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Management of hepatitis delta: Need for novel therapeutic options

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Abstract

Hepatitis D virus (HDV) is the smallest single stranded RNA virus infecting humans. The hepatitis B surface antigen envelope protein protects the HDV nucleocapsid antigen and provides a means for the virus to enter and exit the hepatocyte. Hepatitis B and D viruses exploit the human sodium taurocholate co-transporting polypeptide (NTCP), a receptor, for their entry into hepatocytes. Prenylation of the large delta antigen is a critical determinant of HDV particle assembly. Treatment with pegylated interferon results in sustained virological response six months post-treatment in one fourth of the patients. Nucleos(t)ide analogs (NAs) have been widely tested in hepatitis delta, but they appear to be ineffective. Combination treatment of NAs with interferon also proved to be disappointing so there is a need for novel therapeutic options. The receptor function of NTCP is blocked by Myrcludex B, a synthetic N-acylated preS1 lipopeptide that competes with infectious virions for receptor binding. There are already some approved drugs available, including irbesartan, ezetimibe, and ritonavir and cyclosporin A, with documented inhibitory effects on NTCP's metabolic function. These drugs may have a role in HDV treatment. Interference with host-mediated post-translational changes of proteins that are crucial to the HDV life cycle, such as prenylation may become an important tool to control HDV infection and prevent replication. Lonafarnib, a prenylation inhibitor significantly reduces virus levels in hepatitis delta patients. Antisense oligodeoxynucleotides which are complementary to genomic HDV ribozyme self-cleavage site and stem I regions can inhibit genomic HDV ribozyme activity.

Key words: Hepatitis D virus; Hepatitis delta; Interferon; Lonafarnib; Prenylation inhibitors; Myrcludex

Core tip: The human sodium taurocholate cotransporting polypeptide (NTCP) has been identified as a functional, preS1-specific receptor for hepatitis B and D virus entry. New antiviral drugs will target this receptor to control hepatitis delta infection. There are

already a few approved drugs available in the market with inhibitory effects on NTCP. Interference with host-mediated post-translational changes of proteins that are crucial to the Hepatitis D virus (HDV) life cycle, such as prenylation may become an important tool to prevent HDV replication. These developments are important since pegylated interferon maintains a sustained virological response in just a quarter of all patients.

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INTRODUCTION

Hepatitis D virus (HDV) is the smallest single stranded RNA virus infecting humans. It is a defective virus that requires hepatitis B surface antigen (HBsAg) for transmission. The virus particle has a circular genomic RNA, encoding small and large delta antigens (S-HDAg and L-HDAg) and a surrounding lipid envelope embedded with HBsAg. S-HDAg is required for the initiation of viral genome replication, whereas L-HDAg serves as a principal inhibitor of replication and is essential for the assembly of new virion particles[1,2]. The HBsAg envelope protein protects the HDV nucleocapsid antigen and provides a means for the virus to enter and exit the hepatocyte. Hepatitis B and D viruses exploit human sodium taurocholate co-transporting polypeptide (NTCP) for species-specific entry into hepatocytes[3]. HBsAg is not necessary for the replication of HDV once it has entered the cell.

Worldwide, there are approximately 240 million individuals chronically infected with the hepatitis B virus (HBV), including 15-20 million coinfected with the hepatitis D virus[4]. The disease continues to be a major medical scourge in the Asia Pacific region, specially Pakistan, Mongolia and Eastern Europe[5]. Hepatitis delta (D) occurs either as coinfection with acute hepatitis B or as a superinfection in patients with pre-existing chronic HBV infection. Chronic hepatitis D is a serious form of chronic liver disease with an accelerated course leading to early cirrhosis, decompensation, and hepatocellular carcinoma[6].

INTERFERON BASED THERAPIES

No specific therapy exists for hepatitis D. Interferon (IFN) alpha, standard or pegylated, is the only approved option available so far. The rate of response is proportional to the dose of IFN, with 9 million units (MU) three times a week being more effective than 3 MU thrice weekly[7]. Conventional interferon given for one year results in end-of-treatment virological and biochemical response in one third of the patients. However, sustained virological response (SVR) at 6 mo post treatment is only seen in 17% of patients[8]. Treatment with pegylated interferon (PEG-IFN) results in SVR six months post-treatment in one fourth of the patients[6,9]. Weekly injection of PEG-IFN is currently used for 12 to 18 mo. SVR at six months correlates with absence of advanced fibrosis in most cases[10]. Late HDV RNA relapses may occur after PEG-IFN therapy of hepatitis delta and thus the term "sustained virological response" should be used with caution in HDV infection[11].

After initiation of therapy with PEG-IFN, a median delay of 9 d (interquartile range 5-15) was observed with no significant changes in HDV level. Thereafter, HDV declined in a biphasic manner, where a rapid first phase lasting for 25 d (inter quartile range: 23-58) was followed by a slower or a plateau second phase. None of the patients with flat second phase in HDV achieved complete virological clearance (CVR). The observation that a flat second phase in HDV and HBsAg kinetics was associated with failure to achieve CVR provides the basis to develop early stopping rules during PEG-IFN treatment in HDV-infected patients[12]. There is no impact of interferon lambda3 rs12979860 single nucleotide polymorphism on the long term drug induced hepatitis D clearance[13]. Response to the treatment can be predicted by the HDV RNA assessment at six months, which may give clues as to whether to stop treatment. Patients with negative HDV RNA at six months are more likely to have SVR[9] while non-responders could be identified by a less than 3 log decrease of HDV RNA at 6 mo of treatment[14]. Although no head to head comparison trials have been carried out using pegylated interferon alpha 2a or 2b, both therapeutic regimens appear to be equally effective.

Nucleos(t)ide analogs (NAs) have been widely tested in CDH, but they appear to be ineffective. Only one study has shown that Long-term exposure to tenofovir significantly reduced serum HDV-RNA apart from completely suppressing HBV-DNA in HIV-infected patients with hepatitis delta. This virological benefit was accompanied by significant improvements in liver fibrosis[15]. Combination treatment of NAs with IFNs does not provide any edge over PEG-IFN monotherapy in terms of suppressing HDV infection[16]. In the Hep-Net/International Delta Hepatitis Intervention Trial (HIDIT 1) PEG-IFN with Adefovir arm given for 48 wk showed significant decline in HBsAg titers compared to PEG-IFN with placebo[17] but HIDIT 2 trial using the more powerful nucleotide analogue, tenofovir, in combination with pegylated interferon with increased treatment duration of up to 96 wk, failed to reproduce the earlier results of HBsAg decline[18].

VIRUS ENTRY INHIBITORS

Inhibition of virus entry has become a major concept

in the development of new antiviral drugs. Hepatitis B immunoglobulins have long been used to neutralize infection by binding to the S-domain of HBV surface proteins and preventing reinfection of the graft after liver transplantation. Recently, the human sodium taurocholate cotransporting polypeptide (NTCP) has been identified as a functional, preS1-specific receptor for HBV and HDV entry into hepatocytes[19]. It is therefore important to study the therapeutic potential of virus entry inhibitors, especially when combined with strategies to induce immune-mediated killing of infected hepatocytes. The large envelope (L) protein on the surface of HBV and HDV particles has many different functions and is required for virus entry. The HBV L protein mediates attachment of virions to heparan sulfate proteoglycans on the surface of hepatocytes. The myristoylated N-terminal preS1 domain of the L protein subsequently binds to the NTCP. The receptor functions of NTCP and virus entry are blocked by Myrcludex B, a synthetic N-acylated preS1 lipopeptide that competes with infectious virions for receptor binding[20]. Ni *et al*[3] identified 2 short-sequence motifs in human NTCP that were required for species-specific binding and HBV and HDV entry *via* NTCP.

A recent ongoing study has recruited 24 patients with hepatitis delta (compensated liver disease; 12.5% cirrhosis) scheduled for 48 wk of PEG-IFN therapy. Eight hepatitis delta patients are receiving pre-treatment with 2 mg Myrcludex B alone for 24 wk (B1); Myrcludex B was added to PEG-IFNa for the first 24 wk to another 8 patients (B2) while 8 patients are getting treatment with PEG-IFNa alone (B3). 6/7 and 7/7 of patients, whom data is available, experienced greater than one log reduction in HDV RNA levels at week 24 during Myrcludex B monotherapy or combination therapy while this response was observed in 7/7 of patients with PEG-IFNa monotherapy group at week 12. However, at week 24, HDV RNA became negative in two patients who received Myrcludex B alone and five patients who received its combination with PEG-IFNa. ALT values declined at week 24 in 6/7 (B1), 4/7 (B2) and 3/7 (B3, week 12) patients. One patient in B1 and one in B2 had both negative HDV RNA and normal ALT at week 24[21]. This is a small study, with only interim results available. However, it is apparent that at least end of treatment virological response will be better in the combination arm.

There are already some approved drugs available with documented inhibitory effect on NTCP metabolic function[22]. These FDA approved molecules include irbesartan, ezetimibe, and ritonavir. Irbesartan is an angiotensin Ⅱ receptor antagonist used primarily for the treatment of hypertension. Ezetimibe lowers plasma cholesterol levels, while ritonavir is an antiretroviral drug from the protease inhibitor class used to treat HIV infection. Blanchet *et al*[23] investigated the ability of these drugs to impair viral entry using a HDV *in vitro* infection model based on a NTCP-expressing Huh7 cell line. They demonstrated the potential of three FDA approved molecules to alter HDV infection *in vitro*. Another drug, cyclosporin A inhibits the binding of HBV preS to NTCP[24]. Nevertheless, its effect on HDV transmission needs further study. Administration of an entry inhibitor, possibly used in combination with current HBV drugs, may improve patients' treatment outcome.

PRENYLATION INHIBITORS

Interference with host-mediated post-translational changes of proteins that are crucial to the HDV life cycle, such as prenylation may become an important tool to control HDV infection and prevent replication. Molecular genetic studies have implicated the host-mediated post-translational changes of proteins such as prenylation of large delta antigen (LHDAg) as a critical determinant of HDV particle assembly. Moreover, it has been shown that the isoprenylation of L-HDAg induces liver fibrosis through the modulation of TGF-beta-induced signal transduction pathway[25]. Prenylation is the addition of hydrophobic molecules to a protein or chemical compound. Protein prenylation involves the transfer of either a farnesyl or a geranyl-geranyl moiety to C-terminal cysteine(s) of the target protein[26]. The enzymes that carry out prenylation in the cell are farnesyl transferase and geranylgeranyl transferase. Prenylation plays an important role in mediating protein-protein interactions and protein-membrane interactions.

Delta antigen prenylation can be pharmacologically inhibited by the prenylation inhibitor BZA-5B. BZA-5B is an inhibitor of farnesyltransferase activity. Furthermore, BZA-5B specifically abolishes particle production in a dose-dependent manner[27]. FTI-277, another farnesyltransferase inhibitor, prevented the production of complete infectious HDV versions of two different genotypes[28]. Farnesyltransferase inhibitors thus represent an attractive potential class of novel antiviral agents for use against HDV. In a recent phase 2a study in which 12 patients were treated with 100 mg twice daily (n = 6; termed group 1) or 200 mg twice daily for 28 d of lonafarnib, a farnesyltransferase inhibitor (termed group 2). After a delay of approximately 1 d in which HDV remained at baseline levels, a biphasic viral decline was observed. The 1st phase lasted for 7 to 21 d with greater (P = 0.04) viral decline from baseline in group 2 (median 0.95; inter quartile range: 0.69; log IU/mL) compared to group 1. So a dose dependent effect of lonafarnib in blocking HDV release was observed with efficacies of 67% and 87% in the 100 mg and 200 mg twice daily lonafarnib dosing groups, respectively[29,30]. Thus, the treatment of chronic HDV infection with the prenylation inhibitor lonafarnib significantly reduced the virus levels in these patients. This reduction in viral load in a short period of three weeks is interesting and merits further studies with longer duration of therapy. There is an unmet need to explore the efficacy and safety of combining a

viral entry inhibitor with the prenylation inhibitor in the next phase of the studies.

ANTISENSE OLIGONUCLEOTIDES

An antisense oligonucleotide is a short strand of deoxyribonucleotide analogue that hybridizes with the complementary mRNA in a sequence-specific manner *via* Watson-Crick base pairing[31]. Antisense therapy is a form of treatment for genetic disorders or infections. It is possible to synthesize a strand of nucleic acid that will bind to the messenger RNA (mRNA) produced by that gene and inactivate it, effectively turning that gene "off". Alternatively, the strand might be targeted to bind a splicing site on pre-mRNA and modify the exon content of an mRNA. It has been shown that antisense oligodeoxynucleotides which are complementary to genomic HDV ribozyme self-cleavage site and stem I regions can inhibit genomic HDV ribozyme activity[32].

In conclusion, patients with hepatitis D should be treated with PEG-IFN for 12-18 mo. Long term monitoring is needed after the successful treatment to detect late relapses. Identification of NTCP as a functional, preS1-specific receptor for HBV and HDV is a great breakthrough and opens a new era of virus entry inhibitors. Delta antigen prenylation inhibitors are another group of drugs with potential to halt the spread of infection. It is therefore important to study the therapeutic potentials of these drugs, and evolve combined strategies to prevent viral entry, assembly, and transmission as well as induce immune-mediated killing of infected hepatocytes.

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TOPIC HIGHLIGHT

2015 Advances in Cirrhosis

Asymmetric dimethylarginine as a mediator of vascular dysfunction in cirrhosis

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Abstract

Cirrhosis is associated with marked abnormalities in the circulatory function that involve a reduction in systemic vascular resistance. An important cause of this vasodilatation is the increased production or activity of nitric oxide (NO) in the splanchnic circulation. During portal hypertension and cirrhosis an increased endothelial NO synthase (eNOS) activity is demonstrated in splanchnic vessels. In contrast, the activity of eNOS in the cirrhotic liver is decreased, which suggests a different regulation of eNOS in the liver and in the splanchnic vessels. Asymmetric dimethylarginine (ADMA) is an endogenous NO inhibitor and higher plasma levels of ADMA are related to increased cardiovascular risk in both the general population and among patients with cirrhosis. It has been demonstrated that the liver is a key player in the metabolism of ADMA. This observation was further supported by investigations in human patients, showing a close correlation between ADMA plasma levels and the degree of hepatic dysfunction. ADMA is degraded to citrulline and dimethylamine by dimethylarginine dimethylaminohydrolases (DDAHs). DDAHs are expressed as type 1 and 2 isoforms and are widely distributed in various organs and tissues, including the liver. In this review, we discuss experimental and clinical data that document the effects of dimethylarginines on vascular function in cirrhosis. Our increasing understanding of the routes of synthesis and metabolism of methylarginines is beginning to provide insights into novel mechanisms of liver disease and allowing us to identify potential therapeutic opportunities.

Key words: Dimethylarginine dimethylaminohydrolase; Nitric oxide; Liver diseases; Dimethylarginines; Endothelial function

Core tip: Several lines of evidence point out that the liver is an important organ clearing asymmetric dimethylarginine (ADMA), an endogenous inhibitor of nitric oxide synthase and a mediator of elevated intrahepatic vascular tone in cirrhosis. ADMA is degraded by dimethylarginine dimethylaminohydrolases that are expressed widely in the liver. Therefore, liver dysfunction could lead to alterations in the levels of ADMA and modifies nitric oxide bioavailability. Our increasing understanding of the routes of synthesis and metabolism of methylarginines is beginning to provide insights into novel mechanisms of liver disease and allowing us to identify potential therapeutic opportunities.

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SYNTHESIS OF NITRIC OXIDE

Nitric oxide (NO) is generated from the metabolism of L-arginine by three isoforms of NO synthase (NOS), namely endothelial NOS (eNOS), inducible NOS (iNOS) and neuronal NOS (nNOS)[1]. Endothelial NO accounts for the powerful vasodilator effects of endothelium-derived vasodilator factor[2-4] and consequently plays a decisive role in determining vasomotor tone. Upon its generation in endothelial cells, NO acts in part by stimulating soluble guanylate cyclase to produce second-messenger cyclic guanosine monophosphate (cGMP), which in turn mediates vasodilatation[5].

SYNTHESIS OF METHYLARGININES

Free guanidino-methylated (N^G) arginine residues occur endogenously as a result of proteolysis of methylated proteins[6,7]. The L-arginine analogues include N^G-methyl-L-arginine (L-NMMA), N^G, N^G-dimethyl-L-arginine (asymmetric dimethylarginine; ADMA) and N^G, N'^G-dimethyl-L-arginine (symmetric dimethylarginine; SDMA). ADMA and L-NMMA are competitive inhibitors of the NOS enzymes[8-10]. SDMA, a stereoisomer of ADMA, has no inhibitory effect on NOS, but may interfere with NO synthesis by competing with L-arginine for transport across cell membranes[11,12]. More recent data demonstrate that SDMA, but not ADMA, stimulates reactive oxygen species production of monocytes by acting on Ca^{2+} entry *via* store-operated calcium channels[13]. Since plasma concentrations of ADMA are approximately ten times higher than that of L-NMMA, it was postulated that ADMA is the main endogenous regulator of the L-arginine/NO pathway[11].

Protein methylation is a post-translational modification of intracellular proteins involving the addition of a methyl group, provided by S-adenosyl methionine, to arginine residues in the polypeptide chain by the action of protein arginine methyltransferases (PRMTs)[14]. PRMTs catalyze monomethylation, which initially leads to the synthesis of L-NMMA[14] or transfer two methyl groups in either a symmetric (leading to SDMA) or an asymmetric configuration (leading to ADMA)[15,16]. PRMTs are divided in two groups: types Ⅰ and Ⅱ. Type Ⅰ PRMTs produce ADMA and type Ⅱ produce SDMA. Both types are responsible for the production of L-NMMA[15,17].

Alterations in expression of PRMTs are associated with corresponding changes in ADMA release, thus suggesting that rates of ADMA formation in the vessel wall may be regulated in part through alteration in PRMTs expression[18]. The gene expression of PRMTs is increased in cultured endothelial cells after administration of both native low-density lipoprotein and oxidized low-density lipoprotein[18]. These compounds, similarly to glucose and homocysteine, are also responsible for a higher activity of PRMTs, which results in increased ADMA concentration[16,18-20].

Plasma levels of ADMA in healthy subjects are in the range of 0.3-0.5 μmol/L[11], but in some pathological states increase two- or even ten-fold, contributing to inhibit NO synthesis[21]. It is noteworthy that the concentration of ADMA inside cells is higher than outside. The intracellular levels of ADMA in endothelial cells obtained from rabbit carotid artery is ten times higher than in plasma[22].

Elimination of methylarginines

ADMA is excreted in part by the kidneys, but the main elimination pathway is throught the enzyme dimethylarginine dimethylaminohydrolase (DDAH Ⅰ and Ⅱ) to citrulline and dimethylamine[11,23]. DDAH is widely distributed in tissues, including the liver[24-26]. In contrast to ADMA, SDMA is eliminated by renal clearance and cannot be degraded by DDAH[23,27-29]. There has been increasing interest in the studies concerning plasma levels of NO, ADMA and SDMA in patients with liver dysfunction[26,30-34]. In experiments in Wistar rats, Nijveldt *et al*[26] demonstrated the potential role of the liver in the metabolism of ADMA as evidenced by a high net uptake and a considerable fractional extraction rate. In contrast to ADMA, SDMA was hardly affected by the liver[26]. Further studies showed markedly increased plasma levels of ADMA in multiple organ failure patients[31] and in patients developing hepatic failure after major hepatectomy[35]. Interestingly, Siroen and co-workers have shown that the human liver not only takes up ADMA, but also SDMA from the portal and systemic circulation, and suggested that high plasma levels of SDMA may have hemodynamic consequences similar to those reported by ADMA[36]. This finding is in contrast with the results in the rat in which SDMA was not affected by the liver[26]. This discrepancy between human and rat liver

has been ascribed to the presence of other, relatively unknown alternative metabolic pathways for both dimethylarginines[27], which may be more significant in the human liver in comparison with the rat liver[36].

DDAH Ⅰ appears to be the major isoform responsible for ADMA degradation[37], being the major isoform expressed in the hepatocytes[38]. Loss of DDAH Ⅰ activity, using specific inhibitors, leads to accumulation of ADMA. Both plasma and tissue levels of ADMA increase in *Ddah+/-* mice with deletion of the *Ddah1* gene. In *Ddah+/-* mice, some symptoms of endothelial dysfunction, including increased contraction in response to phenylephrine, reduced relaxation in response to acetylcholine or calcium ionophore A23187, and increased relaxation in response to sodium nitroprusside, are observed[39]. Hemodynamic effects such as increased mean arterial blood pressure, decreased cardiac output and heart rate, and elevated right ventricular pressure are also revealed. No increase in the expression of DDAH Ⅱ in these mice is noted[39].

ROLE OF ADMA IN SOME PATHOPHYSIOLOGICAL STATES

Elevated ADMA plasma levels have been found to be associated with impaired endothelium-dependent vasodilatation[40]. Elevated plasma levels of ADMA were detected in patients with cardiovascular diseases[41-44]. Oxidative stress is also responsible for increased synthesis and/or inhibition of catabolism of ADMA[45] that are observed in patients with hypercholesterolemia, hyperglycemia, hyperhomocysteinemia, diabetes, hypertension, and heart failure[18,43,46,47]. A high level of ADMA independently predicts future cardiovascular risk in patients with coronary artery disease[48] and adverse cardiovascular events in patients undergoing percutaneous coronary intervention[49]. The level of this compound also increases with aging[40]. In critically ill patients with clinical evidence of more than two organ failures, ADMA is a strong and independent risk factor of mortality[31]. Renal failure[11] and liver cirrhosis[30] are further examples of disorders with increased levels of ADMA as both organs are responsible for the elimination of ADMA. Significant correlation was observed between the concentration of ADMA in graft and liver function after transplantation[50].

Several lines of evidence indicate that elevated inflammatory biomarkers are closely associated with endothelial dysfunction and NO synthesis inhibition[51,52]. Systemic inflammation is linked to enhanced ADMA plasma levels and endothelial dysfunction, both in low-grade inflammation, such as atherosclerosis[53,54] and in chronic inflammatory diseases, such as rheumatoid arthritis[55], inflammatory bowel disease[56], and asthma[57].

ALCOHOLIC CIRRHOSIS

Excessive NO production[58] and cGMP plasma levels[59] seem to play a key role in determining the decrease in splanchnic vascular resistance observed in decompensated cirrhosis[58]. Conversely, the levels of NO in hepatic tissue are decreased, and this is probably due to reduced hepatic eNOS activity shown in experimental animals[60,61] and humans[62]. A decrease in NO production by sinusoidal endothelial cells in the cirrhotic liver is an important factor in the development and maintenance of portal hypertension[52,61-64].

Patients with decompensated alcoholic cirrhosis (class B and C according to Pugh classification)[65] exhibit greater plasma levels of ADMA and NO when compared with both patients with compensated alcoholic cirrhosis (class A of Pugh score) and healthy subjects, but SDMA and L-arginine levels are not different between groups[30]. There is a positive correlation between the clinical score of the patients and concentrations of ADMA and NO and a negative correlation between plasma ADMA and NO concentrations in the control group and compensated patients[30]. However, no correlation is observed between ADMA and NO in decompensated cirrhotic patients[30]. Therefore, the increase in ADMA and NO concentrations in decompensated patients may reflect a response to hepatocellular damage[30]. Since SDMA concentrations are not significantly different between groups, a high ratio ADMA/SDMA is observed in the decompensated group of patients, thus suggesting a decreased activity in DDAH[66,67]. It is conceivable that a rise in ADMA in the liver would decrease local production of NO and intrahepatic vascular resistance would increase. Elevated ADMA, attributable to reduced renal excretion, is unlikely because creatinine plasma levels in the three groups of patients were within normal values[30].

The increase in plasma levels of ADMA observed in alcoholic cirrhosis[30] and in acute decompensation of alcoholic liver disease[68] is moderate (twofold elevation in ADMA). The increase in plasma levels of ADMA in individuals with vascular risk factors[69] and in patients with coronary artery disease[70] is also small. Therefore it has been proposed that small changes in the plasma levels of ADMA may be sufficient to alter significantly NO production by endothelial cells[71]. Cellular studies demonstrate that ADMA accumulates inside endothelial cells reaching values 5-10 times higher than outside the cells[22]. Therefore, small changes in plasma levels of ADMA would be expected to have a large effect on the intracellular levels of this NOS inhibitor and cause inhibition of NO formation[22]. However, the intracellular levels of ADMA in endothelial cells under cirrhosis are not known.

In a rat carotid model of balloon injury it was observed that 28 d post injury there was an impairment of endothelium-dependent vascular relaxation that was

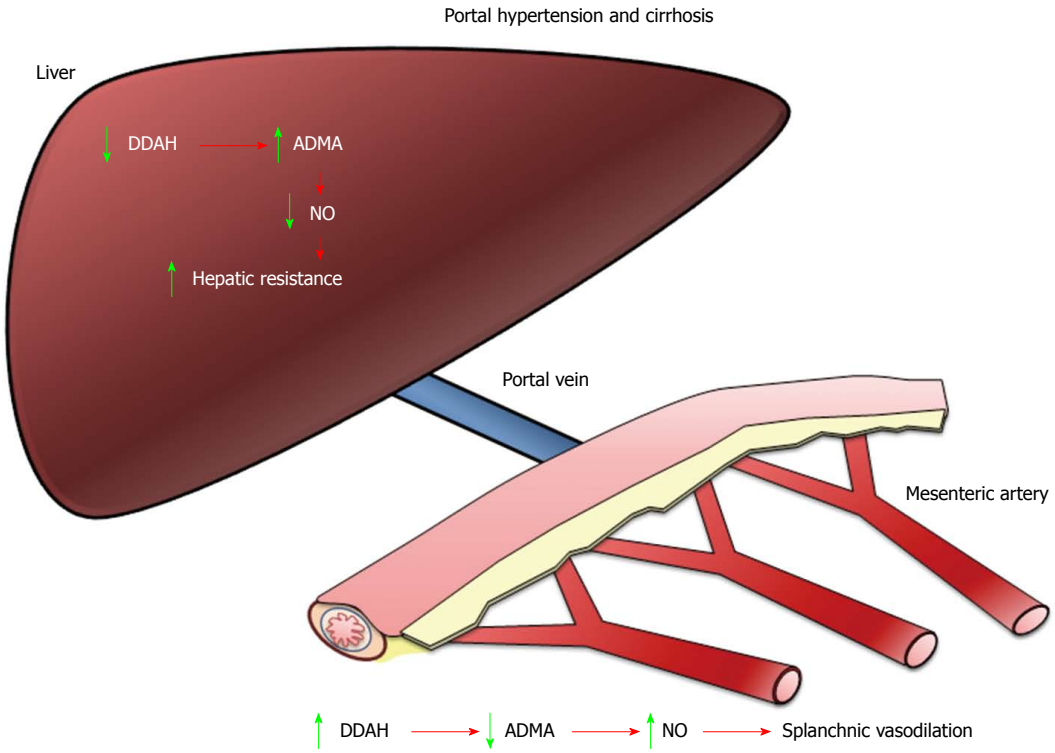

Figure 1 Different asymmetric dimethylarginine metabolism in liver and splanchnic circulation in portal hypertension and cirrhosis. A simplified scheme of different asymmetric dimethylarginine (ADMA) metabolism showing that dimethylarginine dimethylamohydrolase (DDAH) activity is reduced in the cirrhotic liver leading to accumulation of ADMA and decreased nitric oxide (NO) synthesis producing and increase of hepatic vascular tone. On the other hand, in splanchnic circulation portal hypertension and cirrhosis induce and increased expression and function of DDAHs, reducing ADMA levels and increasing NO bioavailability that could cause splanchnic vasodilation.

associated with a significant increase in intracellular levels of methylarginines (ADMA and L-NMMA)[22]. These results demonstrated for the first time that under these pathological conditions intracellular methylarginines reach concentrations sufficient to inhibit NO production and endothelium-dependent relaxation. Whether these observations can be applied to other pathological conditions is still unknown. The course of ADMA concentrations has been investigated in patients undergoing liver transplantation[32]. The results showed that preoperative plasma levels were significantly elevated, and ADMA plasma concentrations decreased on the day after transplantation, thus indicating that the liver graft is quickly capable of clearing ADMA[32]. Furthermore, in patients who experienced acute rejection, ADMA concentrations were higher compared with nonrejectors, indicating a reduced activity of the degrading enzyme DDAH in the liver[32]. A recent report has shown a significant reduction in hepatic DDAH-1 expression in cirrhotic livers that is associated with increased hepatic ADMA, reduced hepatic eNOS activity and elevated portal hypertension[38] (Figure 1).

A paradoxical situation has been described in the setting of alcoholic cirrhosis where an increased plasma concentration of ADMA, endogenous inhibitor of NOS, is linked to an increase of NO. Interpretation of these results is difficult but some possible explanations have been proposed. It has been demonstrated that DDAH activity is inhibited by high concentrations of NO[72]. This NO-induced DDAH inhibition would lead to accumulation of ADMA[72]. If this DDAH inhibition also occurs in cirrhosis, it may clarify why the enhanced synthesis of NO in cirrhosis can be associated with an inhibition of the DDAH activity and an increase in ADMA levels.

It has been shown that ADMA inhibits the basal release of NO from the endothelium and increases the tone of peripheral vessels[73]. This inhibitory effect on NO synthesis corrects the hyporeactivity to vasoconstrictors demonstrated in experimental models of portal hypertension[74]. Therefore, it is possible that a rise in ADMA plasma levels in cirrhosis might represent a compensatory mechanism to decrease NO overproduction and, accordingly, to counterbalance excessive peripheral vasodilatation[30].

The paradox is extended to the study of hyperdynamic circulation in cirrhosis where the mesenteric vasodilation established during cirrhosis is associated with an increase in systemic ADMA levels[30,68,75,76]. The increase in such levels does not inhibit NOS nor the excess NO in the splanchnic endothelial cells in contrast with the decreased generation of NO in the liver[77]. Most studies have paid attention to NOS activity and NO overproduction in splanchnic vessels disregarding the role of DDAHs in these vessels. In small mesenteric arteries from portal hypertensive and cirrhotic rats the low ability of ADMA to inhibit NOS is related to a higher expression of DDAHs and a larger ADMA degradation[78]. It has been

proposed that this greater DDAH expression could be a new mechanism involved in the increased basal release of NO and enhanced mesenteric vasodilation observed in the hyperdynamic circulation[78] (Figure 1).

In patients with acute alcoholic hepatitis ADMA, SDMA and their combined sum, which has been termed dimethylarginine score (DAS = ADMA + SDMA), are increased[68]. The calculated DAS value is significantly higher in nonsurviving patients with acute hepatic decompensation compared to survivors. Therefore the DAS value has been proposed as a clinically relevant predictive indicator when evaluating survival in acute hepatic decompensation[68]. DDAH protein expression is reduced and PRMT-1 increased in alcoholic hepatitis livers, thus indicating that the increase in ADMA may result from both decreased breakdown (decreased hepatic DDAH) and/or increased production (increased PRMT). The increase in SDMA is probably secondary to impairment of the renal circulatory bed. Increased ADMA has been associated with a decrease in renal plasma flow and increased renovascular resistance[79] which would lead to increased renal retention of SDMA.

CHRONIC HEPATITIS C

The role of NO in the pathophysiology of HCV infection still remains controversial. Clinical studies have failed to provide association between the NO plasma levels and chronic hepatitis C[80-82]. Considering data from the literature, patients with chronic hepatitis C have shown NO plasma levels decreased[81] or enhanced[82]. Furthermore, no significant differences were observed between NO, ADMA, and SDMA plasma levels in patients with chronic hepatitis C and in patients with sustained viral response after treatment with interferon (IFN) plus ribavirin[83]. One explanation for the discrepancy may be the grade of hepatocellular damage. Certainly, a previous study performed in our laboratory has demonstrated that in patients with compensated alcoholic cirrhosis (*i.e.,* Child-Pugh score below 7), no significant differences in ADMA, SDMA and NO plasma concentrations were observed as compared to healthy subjects[30]. However, in patients with decompensated alcoholic cirrhosis (*i.e.,* Child-Pugh score equal or above 7) or hepatorenal syndrome, ADMA and NO concentrations were significantly higher when compared to healthy subjects[30,84].

It remains unclear the mechanisms by which IFN mediates its anti-HCV effect *in vivo*. Mononuclear cells obtained from patients with hepatitis C treated with IFN-α show increased iNOS activity and NO synthesis, pointing out that induced NO may be associated to the antiviral actions of IFN in hepatitis C[85]. Although it has been demonstrated a widespread expression of iNOS in hepatocyte from patients with chronic hepatitis C, this increased expression of the enzyme is not accompanied by increased NO serum concentration[86]. Furthermore, in patients with hepatitis C after treatment with IFN for two weeks the NO serum levels were increased if HCV-RNA was eradicated, but if HCV-RNA was present the NO levels were not different from those in healthy subjects[82]. In another study, NO plasma levels were decreased in four patients treated with pegylated IFN-α-2b or IFN-α-2a plus ribavirin for 48 wk[87]. Consistent with these results, a previous study demonstrated that NO plasma levels in patients with chronic hepatitis C treated with INF-α for 18 mo did not differ from controls[88]. It remains to be clarified whether NO and ADMA levels change throughout the period of treatment of the HCV infection with IFN or another therapy for hepatitis C.

HEPATORENAL SYNDROME

Hepatorenal syndrome (HRS), a major complication of end-stage cirrhosis, is characterized by functional renal failure and severe alterations in the systemic circulation (for review see[89]). Impairment of kidney function is a consequence of marked reduction of renal blood flow probably caused by activation of specific vasoconstrictor systems including sympathetic nerves, renin-angiotensin, and arginine-vasopressin to counteract the vasodilatation of splanchnic circulation[89]. Nijveldt *et al*[90] hypothesized a causal role for ADMA in the progression of renal failure in advanced cirrhosis. They proposed that accumulation of ADMA, probably caused by impaired hepatic removal, may have detrimental effects on renal function by inhibiting NO synthesis, thereby interfering with renal blood flow and glomerular filtration[90]. ADMA elicits contractile effects on human renal arteries[91] and increased plasma levels of ADMA has been associated with a decrease in renal plasma flow and increase in renovascular resistance in humans[79]. Therefore, it has been proposed that increased ADMA might be involved in the decrease of renal perfusion and the initiation of functional renal failure in advanced cirrhosis[84].

In a previous study we investigated whether plasma levels of SDMA, ADMA and NO are elevated in patients with hepatorenal syndrome, compared with patients with cirrhosis without renal failure[84]. In the group of patients with hepatorenal syndrome, SDMA concentration was about fourfold higher compared to patients with cirrhosis with normal kidney function or healthy subjects. ADMA and NO concentrations were significantly higher in both groups of patients when compared to controls. In patients with hepatorenal syndrome, a significant positive correlation was observed between SDMA and serum creatinine but not between ADMA and creatinine.

SDMA plasma levels remain within normal values in alcoholic cirrhosis with normal renal function[30] and in patients with end-stage liver failure prior to liver transplantation[33]. This strongly suggests that the high levels of SDMA observed in patients with HRS are caused by impairment of renal function.

As a consequence, SDMA is not correlated with NO but it is significantly correlated with serum creatinine. A positive correlation between plasma SDMA concentrations and serum creatinine has been previously demonstrated in patients with chronic renal failure due to primary renal disease in the absence of liver dysfunction[11,66,92]. After kidney transplantation, the concentrations of SDMA returned to baseline values[92]. Kielstein *et al*[93] collected and analyzed data from 18 studies involving 2136 patients; their results showed that SDMA concentration correlated highly with inulin clearance as well as with serum creatinine. These results confirmed previous studies in humans[94-96] and experimental animals[97,98] showing a close relationship between SDMA and renal function. On the other hand, in our study we did not find correlation between plasma levels of ADMA and serum creatinine which indicates that the increase in plasma ADMA in patients with HRS was not due to a decrease in renal clearance of ADMA. In contrast to ADMA, SDMA is eliminated by renal clearance and cannot be degraded by DDAH[23]. The significance of high plasma levels of SDMA in HRS is uncertain because there is no evidence that it may inhibit NOS[11]. However, SDMA at high concentrations may interfere with NO production by blocking cellular L-arginine uptake[11,12,99]. These studies suggest that SDMA could have an inhibitory effect of NO synthesis by limiting arginine availability to NOS[12]. Indeed, in primary chronic renal failure, Fleck and coworkers pointed out the potential importance of SDMA and concluded that not only ADMA but also SDMA levels are likely responsible for hypertension in these patients, possibly by competition for reabsorption between SDMA and arginine in the kidney[92]. Therefore, SDMA is likely to be another factor responsible for the increased intrarenal vascular resistance observed in HRS.

The pathogenesis of renal vasoconstriction in cirrhosis is multifactorial[89]. Several lines of evidence suggest that splanchnic arterial vasodilatation due to an increased synthesis of NO plays a main role in the initiation of reduced renal perfusion. Intravenous administration of NOS inhibitors to cirrhotic patients increases renal blood flow and glomerular filtration rate, probably due to the increase in renal perfusion caused by an increment in systemic arterial pressure in these patients[100]. These findings led to the suggestion that NOS inhibitors might be useful in the treatment of ascites in cirrhosis[101]. However, experiments in cirrhotic rats raise the possibility that NO blockade may have deleterious effects by increasing intrahepatic vascular resistance[60]. Moreover, it is possible that the plasma concentrations of ADMA achieved in cirrhosis could be biologically effective in renal vessels. With regard to this, it has been proposed that increased ADMA in hepatic dysfunction plays an important role in the development of renal failure in patients with cirrhosis[90]. Indeed, ADMA increases renovascular resistance in humans[79] and elicits contractile effects on human renal[91] and cerebral[102] arteries. In addition, NOS inhibitors enhance vascular contractile responses to adrenergic agonists and sympathetic stimulation of human arteries[103-106]. Thus, elevated ADMA levels could promote renal vasoconstriction by blocking NO synthesis in the endothelium of renal vessels as well as potentiating the effects of perivascular sympathetic nerves. This would lead to impairment of renal function and SDMA retention.

CONCLUSION

Several basic and clinical studies have established that the liver is an important organ in the metabolism of ADMA. As ADMA is an endogenous inhibitor of NOS, changes in the liver function could affect ADMA levels and interfere with the NO synthesis. Despite this, there is still a concerning gap in the knowledge, understanding, and general awareness of mechanisms involved in the vascular dysfunction associated to cirrhosis. The regulation of methylarginine metabolism by modulating cellular PRMT or DDAH activity will therefore likely present a potential therapeutic option for the treatment of vascular dysfunction associated to liver diseases.

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TOPIC HIGHLIGHT

2015 Advances in Hepatitis C virus

Epidemiology of hepatitis C in Croatia in the European context

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Abstract

We analyzed prevalence, risk factors and hepatitis C virus (HCV) genotype distribution in different population groups in Croatia in the context of HCV epidemiology in Europe, with the aim to gather all existing information on HCV infection in Croatia which will be used to advise upon preventive measures. It is estimated that 35000-45000 of the Croatian population is chronically infected with HCV. Like in other European countries, there have been changes in the HCV epidemiology in Croatia over the past few decades. In some risk groups (polytransfused and hemodialysis patients), a significant decrease in the HCV prevalence was observed after the introduction of routine HCV screening of blood/blood products in 1992. Injecting drug users (IDUs) still represent a group with the highest risk for HCV infection with prevalence ranging from 29% to 65%. Compared to the prevalence in the

Croatian general population (0.9%), higher prevalence rates were found in prison populations (8.3%-44%), human immunodeficiency virus-infected patients (15%), persons with high-risk sexual behavior (4.6%) and alcohol abusers (2.4%). Low/very low prevalence was reported in children and adolescents (0.3%) as well as in blood donors (0%-0.009%). In addition, distribution of HCV genotypes has changed due to different routes of transmission. In the general population, genotypes 1 and 3 are most widely distributed (60.4%-79.8% and 12.9%-47.9%, respectively). The similar genotype distribution is found in groups with high-risk sexual behavior. Genotype 3 is predominant in Croatian IDUs (60.5%-83.9%) while in the prison population genotypes 3 and 1 are equally distributed (52.4% and 47.6%). Data on HCV prevalence and risk factors for transmission are useful for implementation of preventive measures and HCV screening.

Key words: Hepatitis C; Seroprevalence; Genotypes; Croatia; Europe

Core tip: As in other European countries, epidemiology of hepatitis C has changed in Croatia in last few decades. In addition, changes in the hepatitis C virus (HCV) genotype distribution were observed due to changes in prevailing routes of transmission. Although a decline in HCV prevalence was observed in some risk groups (polytransfused and hemodialysis patients), HCV prevalence is still high in injecting drug users (IDUs) (29%-65%), reaching 100% in older injectors and those reporting sharing injection equipment. In addition, a high HCV prevalence (8.3%-44%) was found in Croatian prisoners reflecting high proportion of IDUs within this population group. Since IDUs represent a group with the highest risk for HCV, strategies to reduce risk among IDUs should be considered.

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INTRODUCTION

Hepatitis C virus (HCV) represents a major public health problem. The World Health Organization (WHO) estimates that about 2.8% or 170 million of world's population has been infected with HCV, of whom 15 million people live in the WHO European region. In addition, 86000 hepatitis C-related deaths are reported per year in Europe[1]. The HCV prevalence varies markedly in different regions and populations. Injecting drug users (IDUs) and recipients of blood transfusions prior to 1992 are traditionally identified risk groups for HCV infection[2-4]. Variable HCV prevalence is reported in hemodialysis patients and prison populations[5-11]. Transmission of HCV also occurs through occupational, perinatal and sexual exposures[12-14]. However, the association between HCV transmission and high-risk sexual behavior is still controversial[15-17]. Tattooing has emerged in recent years as an additional route of HCV transmission[18,19]. In addition, some other unconventional risk factors for HCV transmission such as digestive endoscopy, abortions, acupuncture, beauty treatments, practice of contact sports and professional pedicure/manicure have been identified among HCV-seropositive persons[20].

In this review, the prevalence, risk factors and HCV genotype distribution in different population groups in Croatia were analyzed in the context of HCV epidemiology in Europe (Table 1).

HCV PREVALENCE AND TRANSMISSION RISK FACTORS IN CROATIA

It is estimated that between 35000 and 45000 of the Croatian population is chronically infected with HCV[21,22]. Prevalence of HCV infection in different population groups is presented in Figure 1.

Polytransfused patients and plasma product recipients

Before the introduction of routine HCV screening of blood/blood products in 1992, transfusion-associated HCV infections were common in Croatia. A study on 359 hemophilia patients in the period from 1993 to 1999 showed that 75.9% tested positive to anti-HCV antibodies, of whom all were infected through coagulation factor concentrates before 1990[23]. In a pilot study conducted in 1992, serologic evidence of HCV infection was found in 24.1% polytransfused patients[24]. With the current blood transfusion safety and the availability of recombinant clotting factors, these patients are no longer at risk for HCV infection[25].

Injecting drug users

IDUs represent the most common risk group for HCV infection in Croatia. There are several studies estimating prevalence of HCV infection among IDUs which showed positivity rate from 29% to 65%, according to geographical region[26-30]. Seroprevalence rates among IDUs in therapeutic communities were significantly higher compared to outpatients (60.66% *vs* 41.86%)[28]. Factors associated with an increased risk of HCV infection included age, duration of IDU and sharing injection equipment. A very high prevalence of 100% was observed among older injectors (40-49 years) compared to 46.5% in younger injectors (20-29 years)[29]. HCV-positive IDUs started using heroin at a significantly younger age than HCV-negative IDUs

Table 1 Prevalence of anti-hepatitis C virus and hepatitis C virus-RNA in different population groups in Europe (2004-2014)

Study population/country	Study area	Sample size	Anti-HCV	HCV RNA	Reference
Intravenous drug users					
Belgium	Limburg, Antwerp	310	66.2%-84.4%	-	Mathei *et al*[64], 2005
Bulgaria	Sofia	773	73.90%	-	Vassilev *et al*[65], 2006
Croatia	Brod-Posavina County	208	44.60%	-	Kolovrat *et al*[28], 2010
	Zadar County	327	59%	-	Medić *et al*[27], 2008
	Multicenter	76	51.30%	-	Vilibic-Cavlek *et al*[29], 2011
	Zagreb, Rijeka, Split	401	29%-65%	-	Kolarić *et al*[26], 2010
Greece	Multicenter	-	43.3%-61.3%	-	Raptopoulou *et al*[60], 2011
Hungary	Budapest	215	15%	-	Gyarmathy *et al*[66], 2011
Italy	Multicenter	1085	83.20%	-	Camoni *et al*[62], 2010
	-	1320	48.10%	-	Curcio *et al*[61], 2011
	Multicenter	543	63.90%	68.30%	Stroffolini *et al*[63], 2012
Lithuania	Vilnius	300	80%	-	Gyarmathi *et al*[66], 2011
Luxembourg	Nationwide	268	81.30%	-	Removille *et al*[67], 2011
Romania	Bucharest	45	88.90%	57.80%	Sultana *et al*[68], 2011
Russia	St. Petersburg	387	94.60%	-	Paintsil *et al*[3], 2009
Spain	Barcelona	1132	88%	-	Muga *et al*[2], 2006
Sweden	Stockholm	310	86.50%	-	Norden *et al*[58], 2013
The Netherlands	Amsterdam	497	60%	69%	Lindenburg *et al*[59], 2011
	Rotterdam	452	38.80%	-	Norden *et al*[58], 2013
United Kingdom	Multicenter	1058	27%-74%	-	Hickman *et al*[69], 2007
Hemodialysis patients					
Albania	Tirana	50	16.70%	56%	Vila Brunilda *et al*[79], 2014
Croatia	Zagreb	128	2.30%	-	Crnjaković-Palmović *et al*[34], 2005
	Istria County	157	3.40%	-	ICIPH[35], 2014
France	Multicenter	4718	7.70%	-	Saune *et al*[81], 2011
Germany	Multicenter	1633	5.80%	-	Kliem *et al*[83], 2008
Italy	Sicily	320	6.25%	-	Li Cavoli *et al*[82], 2011
Romania	Multicenter	174	39.26%	-	Voiculescu *et al*[78], 2010
Serbia	Nationwide	5208	12.70%	-	Djukanovic *et al*[80], 2012
[1]Persons with high-risk sexual behavior					
Croatia (MSM, CSW, STD)	Multicenter	821	4.60%	73.10%	Vilibic-Cavlek *et al*[17], 2009
(MSM)	Zagreb	360	3.00%	-	Bozicevic *et al*[92], 2009
Estonia (CSW)	Tallin	227	7.90%	-	Uuskula *et al*[99], 2008
Italy (CSW)	Verona	345	0.90%	-	Zermiani *et al*[100], 2012
Moldova (MSM)	Balti, Chisinau	397	1.2%-3.7%	-	Zohrabyan *et al*[95], 2013
Sweden (MSM)	Stockholm	1008	0.50%	-	Blaxhult *et al*[93], 2013
United Kingdom (MSM)	London	2309	0.65%	-	Scott *et al*[94], 2010
(MSM)	London	1121	1.20%	-	Price *et al*[91], 2013
Prisoners					
Croatia	Multicenter	3348	8.30%	-	Burek *et al*[37], 2010
	Multicenter	190	11%	-	Vilibic-Cavlek *et al*[18], 2011
France	Caen	597	4.90%	-	Verneuil *et al*[103], 2009
	Nationwide	60975	4.80%	79%	Semaille *et al*[102], 2013
	Multicenter	5957	5.20%	-	Roux *et al*[104], 2014
Hungary	Multicenter	4894	4.90%	-	Treso *et al*[9], 2012
Ireland	Regional (Northern)	1185	1.10%	-	Danis *et al*[113], 2007
Italy	Multicenter	973	38%	-	Babudieri *et al*[10], 2005
Macedonia	Prilep, Bitola	200	20%	-	Jovanovska *et al*[109], 2014
Portugal	-	445	11%	-	Barros *et al*[107], 2008
Spain	Multicenter	370	22.70%	-	Saiz de la Hoya *et al*[110], 2011
United Kingdom	Nationwide	10723	24.20%	-	Kirwan *et al*[106], 2011
	Scotland	5187	19%	-	Taylor *et al*[108], 2013
HIV-infected patients					
Croatia	Zagreb	120	15%	72.20%	Seme *et al*[38], 2007
Italy	Ancona	440	32.90%	-	Orsetti *et al*[118], 2013
Slovenia	Nationwide	356	10.70%	68.40%	Seme *et al*[114], 2009
	Nationwide	579	7.60%	75%	Škamperle *et al*[117], 2014
Spain	Regional (Southern)	520	69%	71%	Cifuentes *et al*[119], 2012
United Kingdom	London	1017	8.90%	-	Mohsen *et al*[115], 2005
Alcohol abusers					
Croatia	Istria County	167	2.40%	-	[2]ICIPH[35], 2014
Germany	Hamburg	463	5.20%	-	Schmidt *et al*[123], 2013
Spain	Salamanca	396	3.53%	-	Novo-Veleiro *et al*[126], 2013
Adult general population					
Bulgaria	Plovdiv	2211	1.08%	-	Atanasova *et al*[136], 2014

Croatia	Primorje-Gorski Kotar County	785	3.70%	-	Tićac *et al*[39], 2010
	Zagreb	451	2.20%	-	Serdar *et al*[40], 2013
	Multicenter	1950	0.90%	-	Vilibic-Cavlek *et al*[36], 2014
France	Paris	14416	0.84%	-	Meffre *et al*[133], 2010
Germany	Berlin, Frankfurt	28809	2.4%-3.5%	68%	Vermehren *et al*[144], 2012
Greece	Crete	876	2.20%	-	Drositis *et al*[145], 2013
Italy	Regional (Southern)	2195	2.60%	-	Cozzolongo *et al*[150], 2009
Kosovo	-	1287	0.50%	-	Quaglio *et al*[142], 2008
Latvia	Multicenter	1459	2.40%	71.40%	Tolmane *et al*[146], 2011
Macedonia	Skopje	4000	0.40%	-	Kiprijanovska *et al*[140], 2013
The Netherlands	Amsterdam	1364	0.60%	-	Baaten *et al*[138], 2007
	Arnhem, Nijmegen	2200	0.20%	-	Slavenburg *et al*[137], 2008
Norway	Oslo	-	0.55%	-	Vik *et al*[143], 2008
Poland	Multicenter	1652	0.90%	-	Ganczak *et al*[135], 2009
Romania	Nationwide	13460	3.23%	91%	Gheorghe *et al*[147], 2010
Spain	Multicenter	-	0.6%-1.6%	-	Munoz-Gamez *et al*[132], 2013
Pregnant women					
Croatia	Zagreb County	200	0.50%	-	Vilibic-Cavlek *et al*[17], 2009
	Istria County	930	1.30%	-	Kucinar *et al*[41], 2014
Greece	Piraeus	5497	0.80%	-	Panagopoulos *et al*[159], 2004
Poland	Warszaw	544	2.02%	-	Aniszewska *et al*[161], 2009
Russia	Cheboksary	150	3%	-	Asratian *et al*[163], 2009
Spain	Madrid	157	1%	-	Santiago *et al*[154], 2012
Switzerland	Multicenter	9057	0.71%	-	Prasad *et al*[153], 2007
Healthcare workers					
Bosnia and Herzegovina	Tuzla	1699	0.40%	73.40%	Ahmetagić *et al*[50], 2006
Italy	Regional (Central)	733	1.8%-4.7%	-	Catalani *et al*[176], 2004
Poland	Warsaw	961	1.70%	19%	Slusarczyk *et al*[175], 2012
	Multicenter	414	1.40%	-	Ganczak *et al*[135], 2012
	Lodz	520	0.80%	-	Rybacki *et al*[173], 2013
Blood donors					
Albania	Nationwide	52727	0.60%	-	Durro *et al*[182], 2010
Bosnia and Herzegovina	Tuzla	16082	0.08%	91.10%	Ahmetagić *et al*[177], 2009
Croatia	Multicenter	155634	0.04%	-	Grgičević *et al*[23], 2006
	Multicenter	-	0%-0.009%	-	Transfusion medicine newsletter[44]
Italy	Regional (South)	17912	0.50%		Sommese *et al*[181], 2014

[1]Persons with high-risx sexual behavior: Men who have sex with men-MSM, commercial sex workers-CSW, persons with sexually transmitted diseases-STD; [2]Istria County Institute of Public Health, Pula, Croatia. ICIPH: Istria County Institute of Public Health; HCV: Hepatitis C virus; HIV: Human immunodeficiency virus; IDU: Injecting drug users; STDs: Sexually transmitted diseases; CSW: Commercial sex workers; MSM: Men who have sex with men.

and reported a longer history of IDU. Young IDUs were found to be at higher risk for HCV infection because of their high-risk behavior patterns. They are usually less critical toward drugs, less cautious, and more easily influenced by others[27]. The frequency of sharing injection equipment was the most important risk factor for HCV transmission in this risk group[27-29]. The HCV seroprevalence rates ranged from 27.3% in IDUs who reported sharing needles/syringes occasionally to 100% in those who always shared injection equipment[29].

Hemodialysis patients

Hemodialysis patients also represent a risk group for HCV infection. In a pilot study conducted in 1992, 44% of hemodialysis patients showed anti-HCV antibodies[24]. A similar seropositivity rate (38%) was noted in 1994[31]. Two regional surveys from north-west Croatia (1997) and north Adriatic Coast (2003) reported prevalence rates of 26.1% and 23%, respectively[32,33]. A low prevalence (2.3%) was noted in 2005 in a Dialysis Center at one Zagreb hospital[34]. More recent data from Istria County (2007-2013) showed a similar prevalence of 3.2%[35].

Persons with high-risk sexual behavior

Persons with high-risk sexual behavior (persons with multiple sexual partners, men who have sex with men-MSM, commercial sex workers-CSW, persons with a history of sexually transmitted diseases-STDs) show a higher HCV prevalence (4.6%)[17] compared to the Croatian general population (0.9%)[36]. In a multicenter study from 7 cities (Zagreb, Split, Rijeka, Zadar, Osijek, Slavonski Brod and Dubrovnik) conducted during 2003-2006, the highest seroprevalence rate (8.5%) was found in patients with a history of STD compared to 6.5% in persons with multiple sex partners, 4.0% in CSW/clients of CSW and 2.9% in MSM. Among STD markers, a prior HBV infection and gonorrhea were shown to be risk factors associated with higher HCV prevalence. No other factors reflecting risky sexual behavior such as sexual orientation and number of sexual partners as well as number of risk behaviors correlated with HCV seropositivity. HCV-RNA

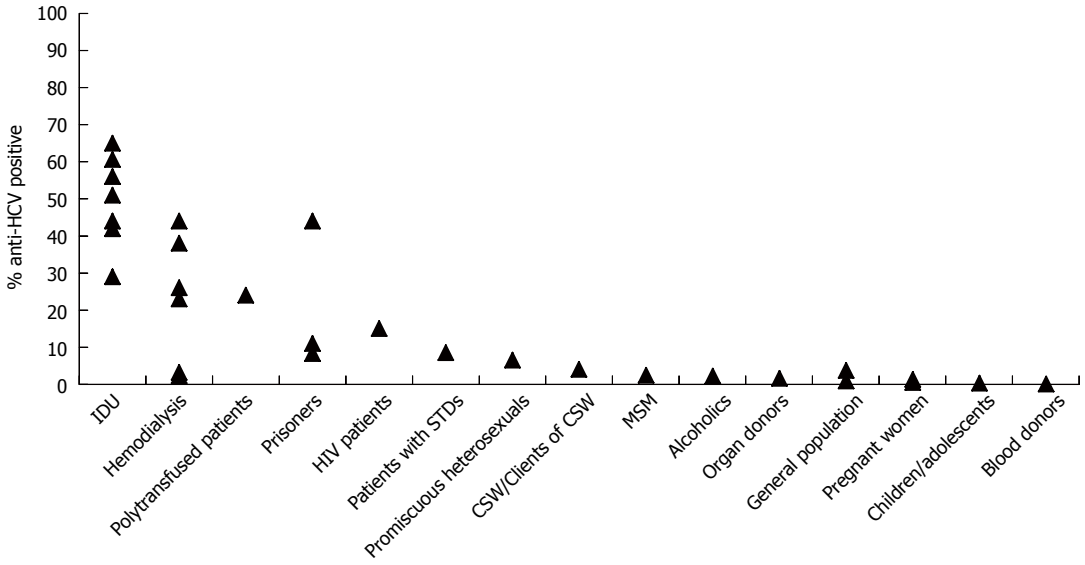

Figure 1 Hepatitis C virus seroprevalence among different population groups in Croatia. IDU: Injecting drug users; STDs: Sexually transmitted diseases; CSW: Commercial sex workers; MSM: Men who have sex with men.

was detected in 73.1% anti-HCV positive persons, but was also found in three seronegative cases ("window period")[17].

Prison population

Incarcerated persons accounted for 0.4% of a total Croatian population, among which IDUs comprise about 25%-30%. In a prison population, the overall HCV prevalence is reported to be 8.3%-44%[18,26,37]. HCV seropositivity in prisons also correlates significantly to IDU. HCV infection is most prevalent among IDUs (42%-52%) and relatively high among highly promiscuous persons (4.9%)[18,37]. Significant differences in seropositivity were found in prisoners who reported unprotected sexual activity compared to prisoners who used condoms (22% *vs* 4%). A history of tattoos was another risk factor associated with higher anti-HCV positivity in this population group. HCV-infected prisoners were significantly more likely to have a history of a tattoo exposure (27%) than HCV-uninfected prisoners (8%). However, it is not clear whether tattooing is a real risk factor for HCV transmission since many of anti-HCV positive prisoners reported other potential exposure to HCV (sharing injection equipment or risk sexual behavior). In addition, higher seroprevalence rates were found in prisoners who were unemployed and in those who resided in urban areas[18].

Human immunodeficiency virus-infected patients

One study addressed HCV prevalence in human immunodeficiency virus (HIV)-infected Croatian patients. Among 120 patients tested from 1985 to 2002, 15% were found to be anti-HCV positive and 72.2% of them were found to be viremic. A significant difference in the HCV prevalence was detected among blood and sexual exposure risk groups (66.7% *vs* 6.6%) with the highest prevalence reported in hemophiliacs (100%) and IDUs (58.3%)[38].

Alcohol abusers

Prevalence of HCV in alcohol abusers in Croatia is largely unknown. Regional data showed that HCV prevalence in alcoholic patients is higher compared to the Croatian general population. Among 167 consecutive samples from alcoholic patients tested at the Istria County Institute of Public Health between 2007 and 2013, four were confirmed positive for HCV antibodies (2.4%)[35].

General population

Two studies on the HCV prevalence were conducted in the Croatian general population. The 2011-2012 study included adult population undergoing routine preoperative check-up from 4/21 counties located in the Croatian mainland. Results of the study suggest that HCV is uncommon in both urban and rural general population. Eighteen of 1930 (0.9%) tested participants were found positive to HCV. No difference in seropositivity was found between genders and age groups[36]. An earlier study (2006-2007) from a Primorje-Gorski Kotar County, located on the north Adriatic coast, reported anti-HCV prevalence of 3.7% using enzyme-linked immunosorbent assay (ELISA). Age distribution of HCV-positive cases showed that majority of patients belong to the 21-30 age group (44%) and 31-40 age group (19%)[39]. Differences in the seroprevalence rates among the Croatian general population most probably reflect the methodological differences (line immunoassay-LIA and ELISA, selection of study participants) in these surveys.

In the period from 2002-2011, occupational exposures were monitored in one Zagreb hospital. Of 451 source patients, 2.2% were infected with

HCV. Majority of accidents were reported at surgical departments (63%), followed by departments of internal medicine (22.6%), and other departments such as dialysis, different laboratories, neurology, psychiatry and radiology[40].

Pregnant women

A few Croatian studies addressed HCV prevalence in pregnant women. Two regional studies (Zagreb County, 2003-2006 and Istria County, 2011-2012) showed HCV prevalence rates of 0.5% and 1.3%, respectively[17,41]. The Istrian study analyzed risk factors and revealed that 83.3% of seropositive women reported a history of IDU and 8.3% reported a former relationship with an IDU. HCV seropositivity increased with age from 0.3% to 3.1%, starting with 26-30 age group[41]. A prevalence of 49% was found in pregnant IDUs from Split region[42].

Children and adolescents

There is no published HCV prevalence research involving children in Croatia. In a group of 297 children and adolescents (up to 18 years) from Istria County tested in the period from 2007 to 2014, only one (0.3%) showed anti-HCV antibodies on two repeated testing. In one 6-mo-old child, anti-HCV antibodies were detected at initial testing while at age of 18 mo the result was negative (data from the Microbiology Service, Istria County Institute of Public Health). Mother's history of IDU was reported in both cases.

Organ/blood donors

Since 2006, the Croatian Institute of Transfusion Medicine has been providing mandatory testing of organ donors for bloodborne pathogens. Among 642 organ donor plasma samples tested between 2006 and 2012, 1.6% were found to be anti-HCV positive[43]. Blood donors represent a group with the lowest seroprevalence of HCV infection in Croatia. The frequency of confirmed positive donors continuously declined from 1.38% in 1992 to 0.035% in 1999[23,24], and thereafter remained stable. In the last decade (2004-2013), the anti-HCV seropositivity is reported to be 0%-0.009%)[44].

EPIDEMIOLOGY OF HCV INFECTION IN CROATIA IN THE CONTEXT OF HCV EPIDEMIOLOGY IN EUROPE

Polytransfused patients and plasma product recipients

Hemophilia patients who received clotting factor concentrates and recipients of blood transfusion before 1990 both represent high-risk groups for HCV infection. As expected, patients requiring multiple transfusions have a high prevalence of HCV infection. The prevalence of HCV among hemophiliacs correlates with the amount and type of product transfused. Nearly all hemophiliacs exposed to untreated commercial clotting factor concentrates before anti-HCV screening are HCV positive, while among those treated with cryoprecipitates, the anti-HCV positivity was about 66%[4]. Similar to seroprevalence in other European studies (59%-97%)[45-49], a Croatian study from 1990s showed a high HCV seropositivity in hemophilia patients (75.9%)[23]. Seroprevalence rate of 24.1% in Croatian polytransfused patients is within the range of the majority other studies (3%-21%)[48-52]. With the implementation of mandatory anti-HCV and HCV RNA screening of blood/blood donations, the risk of transfusion-associated hepatitis C has virtually been eliminated[53]. Rare cases of HCV transmission were reported from recently infected donors with serum HCV RNA level below the detection limit[54,55]. However, many European countries are facing the consequences of the past epidemic of transfusion-associated hepatitis C. In several European studies, patients with transfusion-associated HCV infection account for 20%-30% of patients older than 50 years with advanced chronic hepatitis, cirrhosis and hepatocellular carcinoma[25,56,57].

Injecting drug users

IDU is one of the most efficient routes for HCV transmission[4]. The prevalence of HCV infection among IDUs varies, although rates are continuously very high in most European countries. Recent studies have demonstrated HCV seroprevalence of 38.8%-60% in the Netherlands[58,59], 43.3%-61.7% in Greece[60], 48.1%-83.2% in Italy[61-63], 62.6% in Belgium[64], 73% in Bulgaria[65], 80% in Lithuania[66], 81.3% in Luxembourg[67], 86.5% in Sweden[58], 88% in Spain[2], 88.9% in Romania[68] and 94.6%-96% in Russia[3,65]. In Croatia, there are considerable geographical variations in HCV prevalence among IDUs (29%-65%)[26-30] similar to those observed in the United Kingdom (24%-74%)[69]. The efficiency of IDU in HCV transmission might be due to prolonged virus survival in contaminated syringes. A study from Doerrbecker *et al*[70] addressed HCV inactivation and stability profiles on inanimate surfaces to mimic viral cross-transmissions among IDUs. Viral infectivity on inanimate surfaces was detectable in the presence of serum for up to five days. Paintsil *et al*[71] analyzed the survival of HCV in syringes and the duration of potential infectiousness. The results of their study showed that HCV survived for up to 63 d in high void volume syringes. Besides syringes, sharing of drug injection paraphernalia such as drug preparation containers, cotton filters and rinse water poses a risk of transmitting the HCV[72]. HCV on a spoon as cooker can survive temperatures up to 65 ℃, confirming that virus survival on cookers could also be a potential source of HCV aside from syringes[70]. Other notable risk factors associated with increased risk of being HCV-infected in IDUs population include older age, unemployment, longer history of IDU and higher

number of rehabilitation treatment episodes[14,61].

Hemodialysis patients

The prevalence of HCV among hemodialysis patients varies widely between geographic areas as well as between centers within the same country. In the 1990s, high prevalence rates (20%-50%) in most of European dialysis population were attributed to frequent blood transfusions[5-7,73,74]. The introduction of sensitive ELISA tests for screening of blood and organ donations, use of erythropoietin in treatment of anemia and improvement in infection control practices have greatly decreased HCV infection among haemodialysis patients[25,75]. A European multicenter study suggested a decline in HCV seroprevalence among hemodialysis patients in majority, but not in all European countries. From 1991 to 2000, anti-HCV prevalence decreased in France (42% to 30%), Sweden (16% to 9%), Italy (28% to 16%), Hungary (26% to 15%) and Belgium (13.5% to 6.8%) and tended to decrease in the United Kingdom (7% to 3%)[76]. A similar trend was observed among hemodialysis patients in Croatia. HCV seroprevalence declined from 44% in 1992[24] to 23% in 2003[33]. More recent regional data showed low and stable seroprevalence rates in Croatian hemodialysis patients (2.3%-3.2%)[34,35]. There was no significant change in Germany (7%-6%) and Spain (5%-12%) by 2000[76]. However, another Spanish study from Cordoba showed a decrease in the HCV prevalence from 24% in 1992 to 9.2% by the end of 2002[77]. In contrast, Poland showed not only stable, but also very high HCV prevalence (42%-44%)[76]. In addition, a high prevalence rate was found in Romania (39.26%)[78]. Some more recently published studies showed prevalences of 16.7% in Albania[79], 12.7% in Serbia[80], 7.7% in France[81], 6.25% in Italy[82] and 5.8% in Germany[83]. The number of blood transfusions and the length of time on dialysis are the most important risk factors for HCV acquisition in hemodialysis patients[83,84]. Additional risks factors include IDU and a history of kidney transplantation[84].

Persons with high-risk sexual behavior

The role of sexual transmission in epidemiology of HCV infection is still controversial. In the past, sexual transmission has been considered a relatively inefficient route for HCV transmission. A risk of HCV transmission is extremely low among stable monogamous heterosexual partners[85,86]. However, the risk for sexual partners is significantly higher when the risk factor for the index case is IDU[87-89]. In the last decade HCV infection has emerged as a STD in some European countries, expecially among HIV-positive MSM. A recently published Dutch study showed an increase in HCV seroprevalence in HIV-positive MSM from 5.6% in 1995 to 20.8% in 2008. *Chlamydia trachomatis* infection, IDU, unprotected anal intercourse and older age were variables independently associated with HCV infection[90]. Another study conducted among British MSM showed an overal seroprevalence of 2.1%. The prevalence in HIV-negative MSM (1.2%) was higher, but not significantly higher, than that in the general population (0.67%). However, the prevalence was significantly higher in HIV-positive MSM (7.7%). Moreover, HCV infection was more common in MSM with a history of syphilis than in those without such history (12.2% *vs* 1.7%) and those who reported casual unprotected anal intercourse in the previous year than in those who did not report such intercourse (4.1% *vs* 1.2%)[91]. Two Croatian studies found a higher prevalence in HCV seropositivity in HIV-negative MSM (2.9% and 3%)[17,92] compared to the general population (0.9%)[36] but these diferences did not reach statistical significance. Similar findings were reported from other European studies among MSM that have controlled for IDU (Sweden, the Netherlands, United Kingdom, Moldova)[90,91,93-95]. Among Croatian persons with high-risk sexual behavior, the highest HCV seropositivity rates were detected in patients with a history of STD (8.5%) and persons with multiple sex partners (6.5%)[17]. Association between HCV seroprevalence and multiple sex partners was observed in several studies. However, the number of partners associated with infection risk varied among studies, ranging from one partner in the previous month to more than 50 partners in the previous year or lifetime[96,97]. In persons with multiple sex partners, there is an increased probability of having sex with an infectious partner[98]. In Croatian CSW and their clients, a prevalence of 4.0% was found. A higher prevalence of 7.9% was reported in Estonian CSW[99]. In contrast, prevalence of HCV in Italian CSW was as low as 0.9%, lower than in the general Italian population. The low HCV prevalence reflects the low prevalence of IDU in the analyzed cohort[100].

Prison population

Since IDUs constitute a substantial proportion of prison population in many European countries, HCV prevalence rates among prisoners are higher than in the general population[101]. The HCV seropositivity is reported to be 4.9% in Hungary[9], 4.8%-5.2% in France[102-104], 7%-24.2% in England and Wales[105,106], 11% in Portugal[107], 19% in Scotland[108], 20% in Macedonia[109], 22.7% in Spain[110] and 38% in Italy[10]. Different studies showed association between the HCV seroprevalence and history of IDU. Among prisoners who reported IDU, rates vary from 60.2% in Ukrainian[8], 69% in Portugese[107], 74.7% in Italian[10] to a high 87% among Danish prisoners[11]. In three Croatian studies conducted among prison population the seroprevalence ranged from 4.9% ih highly promiscuous persons to 52% in IDUs[18,26,37]. Some studies suggested that tattooing and piercing are risk factors HCV infections, especially those done

in nonprofessional settings[19,111]. In contrast, a Dutch study showed no evidence for an increased HCV seroprevalence among persons with multiple tattoos and/or piercings. The authors suggested that this might be due to the introduction of hygiene guidelines for tattoo and piercing shops in combination with the low observed prevalence HCV in the general population[112]. Compared to similar studies, the prevalence of HCV among prisoners in Northern Ireland is lower (1.1%) than in other European countries (only 11% of Irish prisoners reported ever injected drugs)[113].

HIV-infected patients

With the increased life expectancy of HIV-infected patients due to highly active antiretroviral therapy, HCV has recently emerged as an important pathogen in these patients[114]. Prevalence of HIV/HCV coinfection varies substantially according to route of transmission. About 50%-90% of HIV positive IDUs are co-infected with HCV[15,115,116], whereas the co-infection rate in HIV positive MSM is 3.5%-7%[89,115]. In Europe, prevalence of HIV/HCV coinfection is reported to be 7.6%-10.7% in Slovenia[38,117], 8.9% in the United Kingdom[115], 32.2% in Italy[118] and 58%-69% in Spain[119]. The reported prevalence in Croatian HIV-infected patients (15%)[38] is within the European range. HIV infection appears to adversely affect the outcome of hepatitis C, leading to increased viral persistence, higher levels of viremia, and accelerated progression of HCV-related liver disease[120,121].

Alcohol abusers

It is traditionally assumed that the prevalence of HCV infection in alcohol-dependent individuals is higher than in the general population, but the modes of transmission are not clearly understood[122-124]. Higher risk for trauma and accidents requiring blood transfusion could be a potential reason for a higher HCV prevalence in alcoholics[125]. Additionally, risky sexual behavior and IDU could be confounding factors for HCV seropositivity in this population[126]. A wide range of prevalence has been reported which could be related to a different distribution of risk factors among studies. Several earlier European studies showed prevalence rates of 14% in Sweden[127,128], 24.3% in Spain[129] and 31.7% in Italy[130]. History of IDU was reported by 58%-88.7% Swedish HCV-positive alcoholic patients. The prevalence of blood transfusions, number of hospital admissions, duration of alcohol dependence or presence of tattooing were not shown to be factors of importance for the HCV transmission[127,128].

Two recently published studies showed lower prevalence rates. A Spanish study analyzed a total of 396 patients with diagnosis of alcohol abuse/alcohol dependence consecutively attended at the alcoholism unit and found 3.53% to have chronic HCV infection. Variables independently associated with HCV infection were female gender, current or past IDU and the presence of alcoholic liver disease[126]. In a German study, anti-HCV antibodies were found in 5.3% alcohol-dependent patients. A history of IDU or nonprofessional tattooing emerged as potential risk factors[123]. Data from Norway (Oslo County) showed a prevalence of 4.4% in alcoholics[131]. HCV prevalence in Croatia was reported to be lower (2.4%)[35] compared to European data. However, these data are limited to a small number of tested subjects and probably do not reflect the prevalence of all alcoholic population.

General population

Data from the European countries indicate significant variations in HCV seroprevalence, even within the same country. It seems that HCV seroprevalence in the Croatian adult general population (0.9%)[36] echoes the prevalence rates of many European countries (Spain 0.6%-1.6%[132], France 0.84%[133], Belgium 0.87%[134], Poland 0.9%[135] and Bulgaria 1.08%[136]. Lower prevalence rates were reported in the Netherlands (0.2%-0.6%)[137,138], Sweden (0.37%)[139], Macedonia (0.4%)[140], Greece (0.5%)[141], Kosovo (0.5%)[142] and Norway (0.55%-0.7%)[131,143]. A German study conducted among adult population in two metropolitan emergency departments (Berlin, Frankfurt) during 2008-2010 found higher prevalence rates (2.4% and 3.5%, respectively). Authors suggested that a high HCV prevalence may be partly explained by the urban study setting as well as the fact that high-risk populations such as IDU and homeless people were not excluded from the study. Additionally, some other risk groups (*e.g.*, patients with coagulation disorders or liver transplant candidates) may even have been overrepresented which may have accounted for selection bias[144]. Similar HCV prevalence rates was found in the Cretan (2.2%)[145] and the Latvian general population (2.4%)[146]. A high overall seroprevalence rate (3.23%) was reported in a Romanian nationwide study (2006-2008), with significant differences between the main geographical regions (2.63%-4.25%) as well as between counties (0.56%-7.19%)[147]. Italy has a particular south-to-north prevalence gradient, with very high prevalence in south and central Italy (7.3% and 6.1%) and lower in the north (2.6%)[4,148,149].

The majority of European studies showed no difference in HCV seropositivity between genders[149,150] or a higher prevalence in males[77,146,151]. In contrast, a Romanian study has found higher HCV prevalence among females (3.51%) compared to males (2.85%)[147]. There was no significant difference in the HCV seropositivity between males (1.2%) and females (0.7%) in the Croatian population[36].

Although in some European regions age-specific seroprevalence generally increases with age[76,150,152], no difference in HCV prevalence was found among

different age groups in Croatia (0.7%-1.7%)[36]. Italian authors reported a bimodal distribution of HCV with the highest prevalence in subjects over 75 years of age[149]. Seroprevalence of anti-HCV could be considered bimodal in Croatian patients as well, with the highest prevalence in the 30-39 age group (1.7%)[36].

Pregnant women

Prevalence of HCV in pregnant women is similar to that in the general age-matched population. HCV seroprevalence in the Croatian pregnant women (0.5%-1.3%) is comparable to that reported in Switzerland (0.71%)[153] and Spain (1%-1.44%)[154,155]. Lower prevalence rates were reported in northern Europe (United Kingdom; 0.19%-0.22%[156], Scotland: 0.3%-0.4%[157]), while Italy, Greece, Poland, Slovakia and Russia reported higher HCV seropositivity (1.9%, 0.8-1.95%, 2.02%, 2.2% and 3%, respectively[158-163]. In a Polish study, the most commonly identified risk factors were history of blood products transfusion before 1992 (24%), hospitalization with surgical procedures (23%) and IDU (15%)[161]. In a Croatian study, all but one HCV seropositive pregnant women reported current or past IDU or a former relationship with an IDU (83.3% and 8.3%, respectively)[41]. HCV prevalence in Croatian pregnant IDUs (49%)[42] is similar to the overall prevalence among IDUs (51%)[27,29].

Children and adolescents

Before 1992, the mode of HCV acquisition in children was blood transfusion. Higher prevalence rates of 10%-20% have been reported in children with other potential exposures such as hemodialysis, malignancy and surgery for congenital heart disease[164-166]. The prevalence reported in Croatian children and adolescents (0.3%) is within the European range (0.05%-0.4%)[167,168]. Vertical (mother-to-child) transmission and adolescent high-risk behaviors (IDU) are now the major routes of HCV transmission in developed countries[169]. The average risk for vertical transmission is about 4% per birth[4,14]. Perinatal transmission is confined almost always to women with detectable HCV RNA[167]. Factors predisposing to HCV transmission are higher maternal viral load at the time of delivery, maternal history of IDU and untreated HIV infection[14]. Breastfeeding carries no further risk of HCV transmission[4,170].

Occupationally exposed groups

Occupational HCV transmission has been reported among healthcare workers (HCWs) who have sustained contaminated needle stick injuries[4]. Prevalence studies among HCWs indicate the low risk for HCV infection associated with occupational exposures. The HCV prevalence among HCWs was not found to significantly differ from that of the general population[171-173]. However, some differences in the prevalence among regions are observed. Very low overall HCV prevalences were reported in Bosnian and Herzegovinian and Belgian HCWs (0.4% and 0.41%, respectively)[50,174]. However, a Belgian study showed higher rates in three larger metropolitan hospitals (1.3%-2.3%)[174]. Three studies conducted in Poland showed prevalence rates 0.8%-1.7%[135,173,175]. Higher HCV prevalence was found in Italy. A study conducted in Pistoia (central Italy) analyzed samples from 511 HCWs engaged in direct clinical task and 222 clerical/nurse school attendees, of whom 3.8% and 1.8% were seropositive to HCV[176]. There are no published data on the HCV prevalence in the Croatian HCWs.

Blood donors

Blood donors' studies showed a decreasing trend in HCV prevalence across time. Data from European countries showed prevalence of 0.13% in Norway[143], 0.08%-0.26% in Bosnia and Herzegovina[177,178], 0.16%-0.32% in Germany[179], 0.4% in Hungary[180], 0.5% in Italy[181], 0.6% in Albania[182] and 0.3%-1.5% in Romania[183]. After 2000, HCV seroprevalence in Croatian blood donors was continuously very low (0.009%-0.03%)[44]. Since blood donors represent a strictly controlled group, it is expected that the HCV prevalence is lower than in the general population.

HCV GENOTYPES DISTRIBUTION IN CROATIA

HCV RNA was detected in 72.2%-82.7% Croatian HCV infected patients[17,32,38]. Prevalence of HCV genotypes varies by different population groups (Figure 2) as well as by regions. In the general population, genotype 1 is the most widely distributed (60.4%-79.8%), followed by genotype 3 (12.9%-47.9%)[184-186]. The most commonly detected subtype is 1b (37.4%)[184]. In a 10-year study (1995-2006) conducted in four geographical regions (two regions in Croatian mainland and two regions located on the Adriatic Coast), genotype 1 was predominant in three regions (north-west/north-east continental and north coastal area) with prevalence rates 60.4%-76.1% while in a south coastal area, the prevalence of both genotype 1 and genotype 3 was similar (46.9% and 47.9%, respectively). In other regions, genotype 3 was found in 18.3%-32.4% patients[184]. Another study conducted in north-east Croatia (2009-2011) detected genotype 1 in 79.8% and genotype 3 in 12.9% patients[187]. The difference in genotype 3 prevalence between regions could be attributed to different populations. The first study included residents of Split, second largest Croatian city with a large number of IDUs in whom genotype 3 is the most prevalent. Percentage of genotypes 2 and 4 was very low in both studies (0.8%-2.2% and 3.4%-6.5%, respectively), while genotypes 5 and 6 were not detected[184,187].

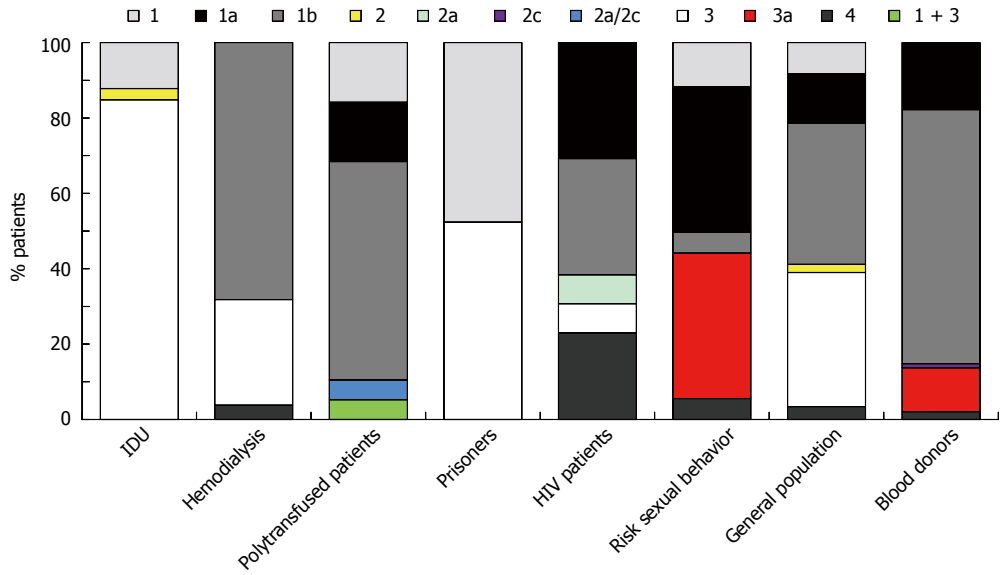

Figure 2 Hepatitis C virus genotypes distribution in Croatia. IDU: Injecting drug users; HIV: Human immunodeficiency virus.

A history of blood transfusion before 1992 was an independent predictor of HCV infection caused by genotype 1[187]. Genotype 1, subtypes 1a and 1b were detected in majority of Croatian polytransfused patients with HCV infection (12%, 12% and 44%, respectively) (data from the Department of Infectious Diseases, General Hospital "Dr Josip Bencevic", Slavonski Brod). In hemodialysis patients, subtype 1b was detected in 75% patients (33.3% received more than two blood transfusions) and type 3 in 20.8% patients[32]. Genotype 3 is predominant in Croatian IDUs (60.5%-83.9%)[184,185]. The most prevalent subtypes in this population group are 3a (60.5%) and 1a (23.7%)[184]. A study among Croatian male prisoners showed an equal distribution of genotype 3 (52.4%) and genotype 1 (47.6%)[185]. In persons with high-risk sexual behavior, genotype 1 is the most commonly detected (55.6%) followed by genotype 3 (38.9%). The HCV subtypes distribution is the following: 1a (38.9%), 3a (38.9%) and 1b (5.6%)[17].

HCV GENOTYPES DISTRIBUTION IN EUROPE

Understanding the HCV genotypes distribution is important as a part of a molecular clue for the spread of HCV. It is well-documented that genotype distribution is associated with the mode of transmission[4]. Available data indicate that genotypes 1 and 3 account for the majority of HCV infections in Europe. The most frequent subtype is 1b, detected in many countries in Central (Albania, Bosnia and Herzegovina, the Czech Republic, Hungary, Montenegro, Romania), Western (Austria, France, Greece, Italy, Portugal, Spain) and Eastern Europe (Belarus, Estonia, Lithuania, Latvia and Russia) with a wide range of prevalences (27.2%-92.6%, 29.7%-57.5% and 58.8%-87.7%, respectively). In Finland, Luxembourg, Norway and Switzerland, both subtypes 1b and 1a were equally prevalent, while in Denmark, Sweden and United Kingdom, subtype 1a is more commonly reported. The prevalence rates of genotype 3 varied from 6.6%-44.6% in Central, 3.6%-46.0% in Western and 9.2%-38.5% in Eastern Europe[188]. In southern Italy, genotype 2c is commonly found[189,190]. Genotype 4 prevalence is rising in Europe (detected in significant proportions in France, Germany, Greece, Italy, Poland, Portugal, Spain, Sweden and Switzerland) reflecting immigration patterns in these areas[4]. Other HCV genotypes such as genotype 5 and 6 are more geographically restricted. Genotype 5 was found in restricted areas of Belgium, Spain, France and Greece and is mainly transmitted by blood transfusion[191]. Genotypes/subtypes 1a and 3/3a are the most commonly identified in IDUs in Europe[9,138,192-196]. Genotypes 1b and 2 are linked to blood transfusion and unsafe medical procedures[197]. There are some regional differences in HCV genotypes among hemodialysis patients. Subtype 1b seems to be most frequent in the Netherlands and France while in Italian hemodialysis patients subtypes 2a and 3a predominated[198]. In the general population, genotypes 1 and 3 are the most commonly detected in majority of European countries with the prevalences reported to be 45.1%-79.3% and 19.7%-35.1%, respectively[199,200]. HCV genotype 1 is even more prevalent in Hungarian (85.5%) and almost exclusively present in Romanian (93.4%-99.1%) patients with chronic HCV infection[200,201]. In Italy, genotype 1b appears to be the most frequent (30.7%-60%), with genotype 2 following (21.3%-34.8%)[200].

FUTURE CHALLENGES

Over the past few decades, there have been remarkable

changes in hepatitis C epidemiology. The prevalence of genotypes has evolved with time due to changes in the predominant route of transmission[4]. However, challenges in HCV prevention remain. Since IDUs still represent a group with the highest risk of HCV transmission, strategies to reduce risk among IDUs should be considered.

From an epidemiological point of view, one of the main challenges regarding HCV infection is to identify infected individuals in order to offer timely treatment. In the last five years, an average of 200 newly discovered HCV infected persons per year are reported to the Reference Centre for Epidemiology, Croatian National Institute of Public Health. Based on a seroprevalence rate of 0.9% in the general population, we must assume that only a small part of the estimated 40000 Croatian HCV-positive citizens are aware of their HCV infection. This discrepancy emphasizes the need to provide testing for HCV infection to a larger proportion of the population.

Another challenge is to identify routes of transmission in individual cases of HCV infection. In routine reports on surveillance of communicable diseases, the country is expected to report the most probable route of infection to WHO and to the European Centre for Disease Prevention and Control (ECDC). In order to meet these requirements, HCV infection as a reportable disease under enhanced surveillance, which anticipates collecting additional information for each case of HCV infection using a standardized questionnaire, in this case, information on the most probable route of infection. A large quantity of information exists on patients with HCV, including clinical, epidemiological, behavioral information, laboratory parameters, but is scattered among different sections of the health system and should be collected at one place and linked to an individual patient. Ideally, a registry of HCV infected persons should be set up, which would not only allow to collect and record all the relevant information on each individual, but would also allow monitoring progression of infection as well as treatment outcomes of patients under treatment.

The origin of HCV is a challenge which has been target of virologists, epidemiologists and geneticists for years but has remained obscure. The majority of recent emerging infections in human populations represent zoonoses transmitted from wild animals and possibility of HCV cross-species transmission from animal species must be taken into consideration[202,203]. Although higher primates are susceptible to experimental infection, HCV naturally infects only humans[203]. Recently, a novel hepacivirus infecting a wild non-human primate, the black-and-white colobus (*Colobus guereza*), an Old world monkey from Uganda was discovered[204]. Animal origin of HCV is additionally supported by recent studies that have described related hepaci and pegiviruses in diverse animal species. In contrast to ongoing focus on primate for HCV origins, a virus related to HCV was described in domestic dogs in 2011[205]. In an effort to further investigate the host range of canine hepacivirus, serology-based approach was utilized to screen for the presence of the virus in mammalian species[206]. Serological evidence of hepacivirus infection was detected in horses with high prevalence while viral RNA was found in 7.8% seropositive horses. Equine hepacivirus (EHcV) is the most closely related animal hepacivirus to HCV described to date. Different studies confirmed EHcV infection in horses[207-209] and repeated sampling of viremic horses demonstrated viral persistence over at least 6-mo period and viral loads comparable to those observed in HCV infection[206]. Similar to HCV infections in humans, acute and chronic stages of EHcV infection in horses with viral RNA detectable predominantly within the liver was confirmed[210]. Several recently published studies demonstrated hepaciviruses and pegiviruses in rodents and bats[211-213].

Detection of multiple novel hepaciviruses in diverse mammalian species has highlighted the importance of further research to define distribution of hepaciviruses and their host range. Discovery of zoonotic source for the HCV would be an important step in understanding host relationship and adaptation and enhance the ability to study pathogenesis and immune response using susceptible animal models.

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TOPIC HIGHLIGHT

2015 Advances in Gastrointestinal Endoscopy

Recent advances in endoscopic ultrasonography-guided biliary interventions

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Abstract

Interventional endoscopic ultrasonography (EUS) based on EUS-guided fine-needle aspiration has rapidly spread as a minimally invasive procedure. Especially in patients with failed endoscopic retrograde cholangiopancreatography, EUS-guided biliary intervention is reported to be useful as salvage therapy. EUS-guided biliary interventions are carried out using three techniques: EUS-guided bilioenteric anastomosis, EUS-guided rendezvous procedure, and EUS-guided antegrade treatment. Although interventional EUS is not yet a standardized procedure, there have been recent advances in this field that address various biliary diseases. Here, we summarize the indications, techniques, clinical results of previous studies, and future perspectives.

Key words: Endosonography-guided biliary intervention; Choledochoduodenostomy; Hepaticogastrostomy; Rendezvous procedure; Antegrade treatment

Core tip: Endoscopic-ultrasonography-guided biliary drainage (EUS-BD) is widely accepted as salvage therapy for transpapillary treatment. However, there are few data comparing the clinical efficacy of EUS-BD and endoscopic retrograde cholangiopancreatography (ERCP) with regard to which is the treatment of choice. As EUS-BD is performed under direct visualization, it has the potential to replace ERCP. However, a prospective randomized study is necessary to confirm it.

Kawakubo K, Kawakami H, Kuwatani M, Haba S, Kawahata S, Abe Y, Kubota Y, Kubo K, Isayama H, Sakamoto N. Recent advances in endoscopic ultrasonography-guided biliary

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INTRODUCTION

The endoscopic transpapillary approach based on the endoscopic retrograde cholangiopancreatography (ERCP) technique has been a standard treatment for various biliary diseases, such as choledocholithiasis and biliary obstruction[1,2]. ERCP sometimes fails, however, for various reasons[3]. Recently, interventional endoscopic ultrasonography (EUS) based on EUS-guided fine-needle aspiration (EUS-FNA) has been developed as a salvage therapy for transpapillary treatment. In patients with failed ERCP, percutaneous or surgical interventions are mandatory but are associated with considerable morbidity and mortality[4-6]. Interventional EUS could manage these patients with minimal invasion. Although interventional EUS is not yet a standardized procedure, there have been recent advances in this field that address various biliary diseases.

EUS-GUIDED BILIARY INTERVENTION TECHNIQUES

EUS-guided biliary interventions are carried out using three techniques: EUS-guided bilioenteric anastomosis (EUS-BEA), EUS-guided rendezvous procedure (EUS-RV), and EUS-guided antegrade treatment (EUS-AT). EUS-guided biliary drainage is performed with the armamentarium available for EUS-FNA and ERCP-related procedures such as an EUS-FNA needle, a guidewire, and a biliary stent.

EUS-BEA

EUS-BEA is performed to create an artificial fistula between the intestine and the bile duct by placing a stent. One indication for this procedure is the palliation of obstructive jaundice in patients with malignant diseases who have failed biliary drainage by ERCP. The procedure is mainly divided into three methods according to the anatomical site of the fistula: EUS-guided choledochoduodenostomy (EUS-CDS), EUS-guided hepaticogastrostomy (EUS-HGS), and EUS-guided gallbladder drainage (EUS-GBD). In these cases, the echoendoscope is advanced into the upper gastrointestinal tract (stomach, duodenum and jejunum), after which, the biliary system (bile duct or gallbladder) is punctured by a needle. The guidewire is then advanced into the biliary system followed by dilation of the fistula. Finally, the stent is deployed between the biliary system and the gastrointestinal tract.

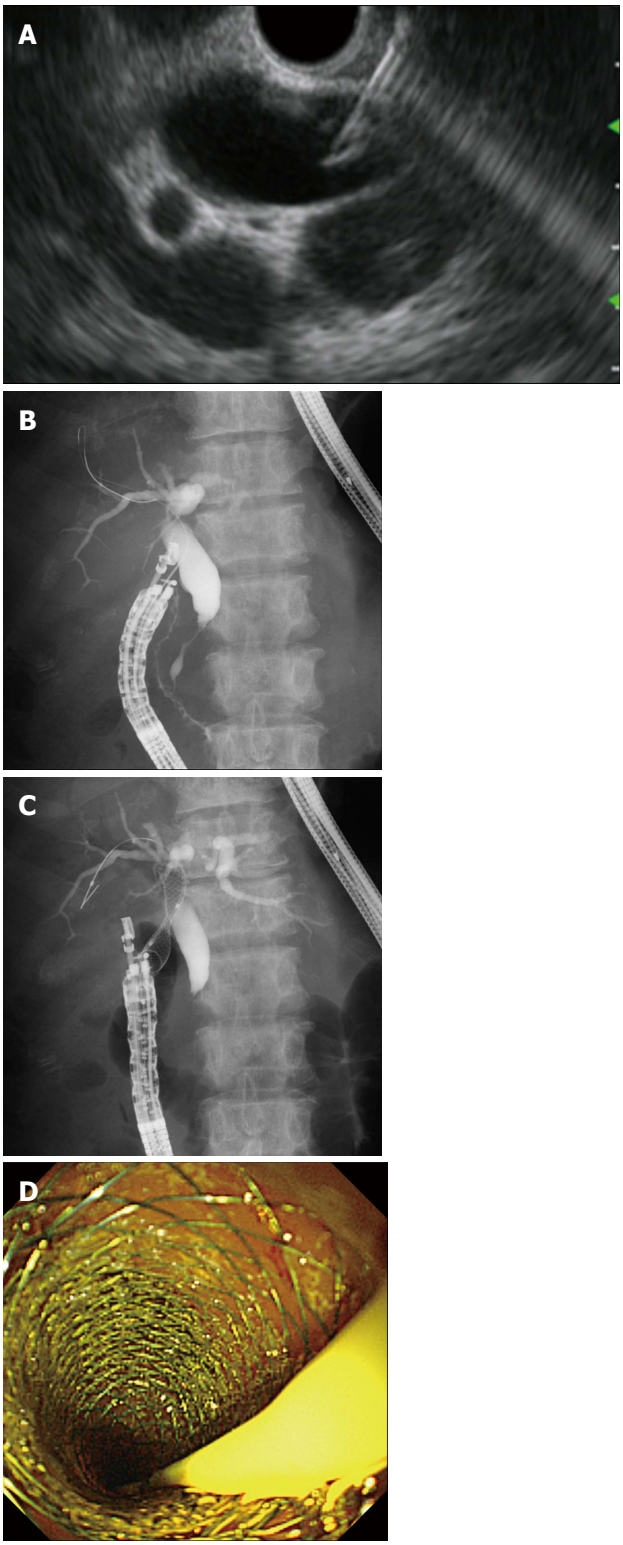

Figure 1 Endoscopic ultrasonography-guided choledochoduodenostomy. A: Endoscopic ultrasonography showing that extrahepatic bile duct was punctured by the needle; B: Guidewire was advanced through the needle into the right liver lobe; C: A self-expandable metallic stent (SEMS) was placed between the bile duct and the duodenum; D: Endoscopic view showing that the distal end of a SEMS was located at the duodenum.

EUS-CDS is undertaken to create a fistula between the extrahepatic bile duct and the duodenal bulb (Figure 1). Since the first report in 2001 by Giovannini

Table 1 Published data of endoscopic ultrasonography-guided choledochoduodenostomy (EUS-CDS or HGS) (n > 20)

Ref.	No. of patients	Study design	CDS or HGS	Stent	Technical success	Complication rate	Patency	Complication
Park *et al*[9]	55	P	CDS, HGS	PS, MS	92, 100	21, 19	152, 132	Peritonitis, pneumoperitoneum, Bleeding
Hara *et al*[11]	18	P	CDS	PS	94	17	272	Peritonitis, hemobilia
Khashab *et al*[25]	20	R	CDS, HGS	PS, MS	100	10	-	NA
Kawakubo *et al*[8]	64	R	CDS, HGS	PS, MS	95, 95	14, 30	-	Bile leak, stent misplacement, bleeding
Hara *et al*[12]	18	P	CDS	MS	94	11	NR	peritonitis
Vila *et al*[10]	65	R	CDS, HGS	NA	86, 65	15, 29	-	Biloma, bleeding, perforation, pancreatitis, cholangitis, hematoma, abscess, pseudocyst
Dhir *et al*[13]	30	R	CDS, HGS	MS	92.3	20	-	Cholangitis, perforation, bile leak, pneumoperitoneum, death

P: Prospective; R: Retrospective; CDS: Choledochoduodenostomy; HGS: Hepaticogastrostomy; PS: Plastic stent; MS: Metallic stent; NA: Not available; NR: Not reached.

et al[7], EUS-CDS has been widely accepted as an effective alternative therapy for palliation of distal malignant biliary obstruction after failed ERCP (Table 1)[8-13], although it is impossible to perform EUS-CDS in patients with altered upper gastrointestinal tract anatomy. Because of the anatomical proximity of the viscera, EUS-CDS is not technically challenging. The technical success rate is reported to be 86%-100%. Artifon *et al*[14] reported that the clinical success and complication rates were similar for EUS-CDS and percutaneous transhepatic biliary drainage (PTBD) for patients in whom ERCP failed. Plastic stents were mainly placed in the early cases of EUS-CDS, whereas more recently self-expandable metallic stents (SEMSs) have played a major role. EUS-CDS is mainly indicated for patients with distal malignant biliary obstruction in whom ERCP has failed. However, the feasibility of EUS-CDS as the first-line treatment for distal malignant biliary obstruction was recently reported[12]. The complication rate was reported at 9%-23%. Biliary peritonitis and pneumoperitoneum were the most frequent complications. Others were stent migration, bleeding, and cholangitis. There are no standardized techniques for managing these complications, so it is important to be ready to handle any situation[15-19]. Recently, the feasibility of performing EUS-CDS with specialized stents has been reported[20-22]. Widespread use of sophisticated SEMSs for EUS-CDS could reduce the incidence of complications.

EUS-HGS is performed to create a fistula between the left intrahepatic bile duct and the stomach (Figure 2). The indication for EUS-HGS is similar to that for EUS-CDS, that is, palliation of distal malignant biliary obstruction. However, EUS-HGS could be also performed in patients with altered upper gastrointestinal tract anatomy or gastric outlet obstruction. In addition, palliation of a left intrahepatic bile duct with a malignant hilar biliary obstruction is possible with EUS-HGS[23,24]. The technical success rate with EUS-HGS is 81%-100% (Table 1)[8,9,13,25]. EUS-HGS itself has serious complications, however, because the fistula traverses the peritoneum[26]. The complication rate ranges from 19% to 30%. Common complications are pneumoperitoneum, bleeding, bile leak, cholangitis, and stent migration. In particular, inward stent migration can be fatal. It is important to manage these complications[27,28]. To prevent inward stent migration, a longer stent might be better and it should be deployed not under fluoroscopic guidance but rather direct endoscopic visualization. Although some specialized SEMSs for EUS-HGS have been developed[22], highly dedicated SEMSs for EUS-HGS are necessary to reduce the complication rate. Recently, the feasibility of EUS-HGS for treating isolated right intrahepatic bile duct was reported[29,30]. Also, bilateral EUS-HGS for palliation of hilar malignant biliary obstruction could be feasible in the near future.

EUS-GBD is performed to create a fistula between the gallbladder and gastrointestinal tract (stomach or duodenum) (Figure 3). This procedure is indicated for drainage in patients with acute cholecystitis as an alternative to percutaneous transhepatic gallbladder drainage (PTGBD), and who are not suitable candidates for emergency cholecystectomy. The technical success rate is reported to be around 100% (Table 2)[31-33]. A plastic stent or nasobiliary tube is used in patients with temporary stent placement, whereas SEMSs are used for patients who are not suitable for future cholecystectomy. Jang *et al*[33] reported that the technical and clinical success rates for EUS-GBD were similar to those for PTGBD. Also, the pain scores were lower following EUS-GBD than after PTGBD in patients prior to cholecystectomy. A recent report indicated that, if a specialized SEMS is used, EUS-GBD is feasible for patients who are not suitable candidates for elective cholecystectomy[34]. In the future, EUS-GBD might be the treatment of choice for acute cholecystitis in high-risk patients.

EUS-RV

An indication for the EUS-RV is biliary cannulation in patients who have failed biliary cannulation in conventional ERCP. For EUS-RV, the bile duct is punctured and a guidewire is advanced through the

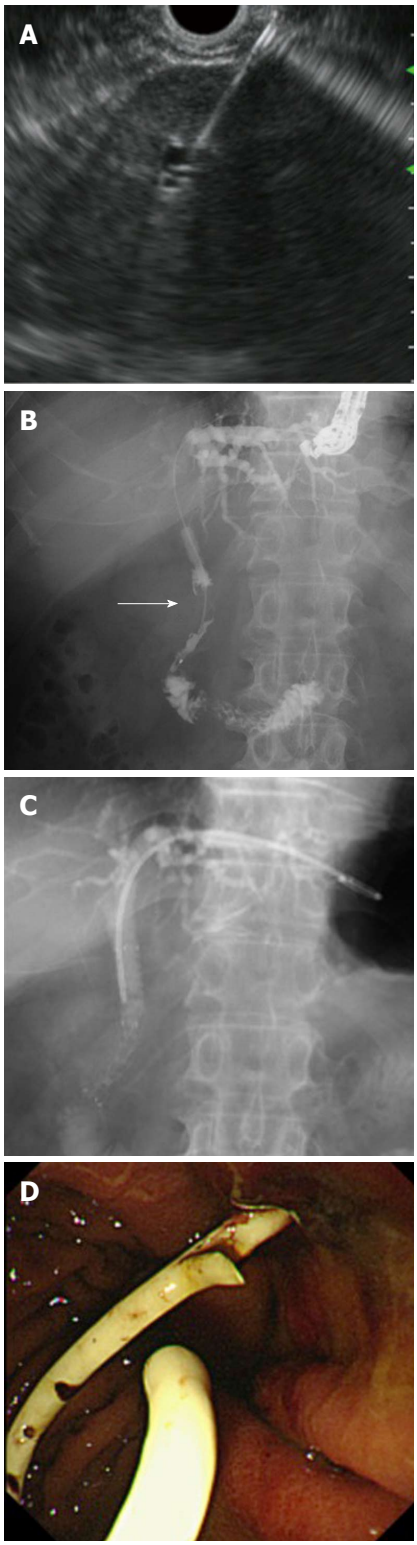

Figure 2 Endoscopic ultrasonography-guided hepaticogastrostomy. A: Left intrahepatic bile duct was punctured; B: Cholangiography showing distal malignant stricture (arrow); C: Following antegrade self-expandable metallic stent placement, a plastic stent was deployed between the intrahepatic bile duct and stomach; D: Endoscopic view showing that the distal end of the plastic stent was located at the stomach.

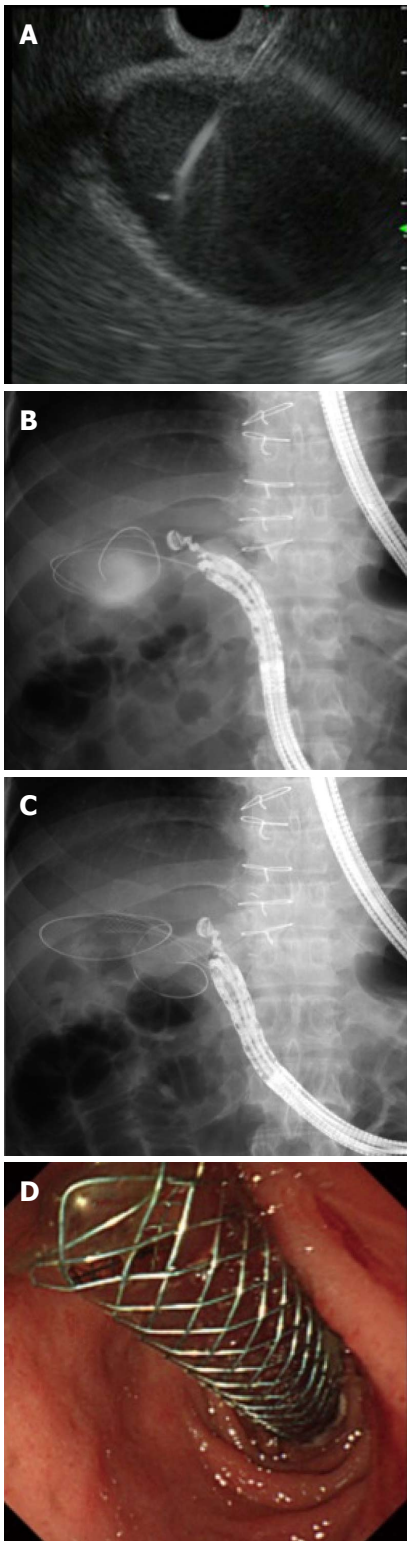

Figure 3 Endoscopic ultrasonography-guided gallbladder drainage. A: Endoscopic ultrasonography showing that the gallbladder was punctured by the needle; B: Guidewire was advanced through the needle into the gallbladder; C: A self-expandable metallic stent (SEMS) was placed between the gallbladder and the duodenum; D: Endoscopic view showing that distal end of a SEMS was located at the stomach.

Table 2 Published data of endoscopic ultrasonography-guided gallbladder drainage (n > 10)

Ref.	No. of patients	Study design	Stent	Technical success	Complication rate	Complication
Jang *et al*[33]	30	P	ENBD	97%	7%	Pneumoperitoneum
Choi *et al*[32]	63	R	MS	98%	5%	Perforation, pneumoperitoneum

P: Prospective; R: Retrospective; ENBD: Endoscopic nasobiliary drainage; MS: Metallic stent.

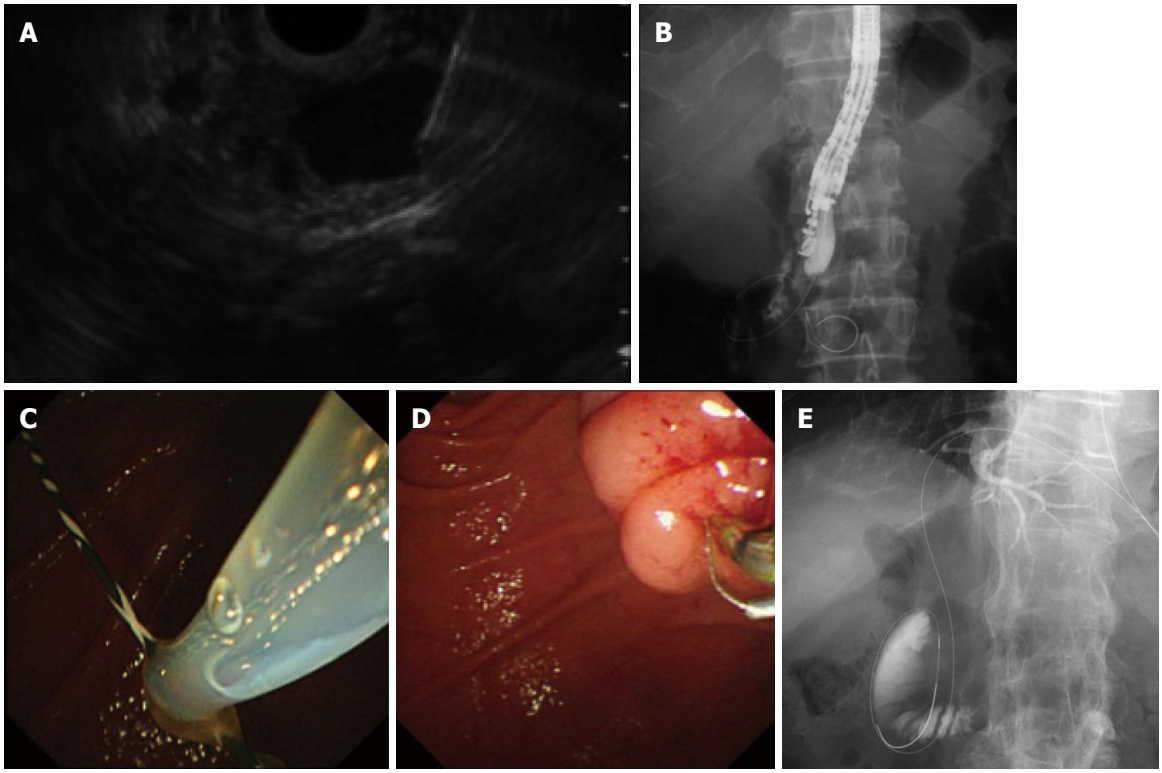

Figure 4 Endoscopic ultrasonography-guided rendezvous procedure. A: Endoscopic ultrasonography showing that extrahepatic bile duct was punctured by the needle; B: Radiography showing the guidewire was passed through the needle into the duodenum across the papilla antegradely. The endoscope was in the short position; C: Following echoendoscope withdrawal, the duodenoscopic view showed that a guidewire passing through the papilla was grasped by the snare; D: After the guidewire was pulled out through the accessary channel of the duodenoscope, a catheter was inserted into the bile duct over the existing guidewire; E: Transhepatic approach. Abdominal radiography shows that a guidewire was placed through the papilla into the duodenum.

needle to the duodenum. An ERCP-related procedure is then performed using the guidewire. EUS-RV is indicated when biliary access fails during ERCP. There are two biliary access routes depending on the site to be addressed: transhepatic and transduodenal (Figure 4). The transduodenal route is further divided according to the endoscope position: long (push) and short (pull)[35]. The short endoscopic position is referred to as the tip of the endoscope oriented in the caudal direction, while the endoscope is directed cranially in the long endoscopic position. The echoendoscope is advanced into the upper gastrointestinal tract (esophagus, stomach, duodenum and jejunum), and the bile duct is punctured by an FNA biopsy needle. The guidewire is then advanced through the needle antegradely across the papilla to the duodenum. In patients undergoing choledochojejunostomy, the guidewire is passed through the anastomosis to the jejunum. The echoendoscope is then pulled out, keeping the guidewire in place. The ERCP endoscope (duodenoscope or enteroscope) is then advanced to the papilla or the anastomosis where the guidewire has been placed. Biliary access is then achieved alongside or over the guidewire after grasping and withdrawing the instrument through the accessory channel of the endoscope. The biliary intervention is then begun. EUS-RV is indicated when biliary cannulation fails or a biliary stricture cannot be passed. The technical success rate varies from 63% to 98% (Table 3)[10,36-39]. The technical success rate for the transduodenal route is reported to be higher than that for the transhepatic route. Dhir *et al*[39] noted that the biliary cannulation success rate was higher with EUS-RV than that for the precut technique in patients with difficult ERCP cannulation. The complication rate was around 10%. The most common complications were biliary peritonitis and pancreatitis. Shah *et al*[38] reported that the transhepatic route could have a tamponade effect

Table 3 Published data of endoscopic ultrasonography-guided rendezvous procedure (n > 40)

Ref.	No. of patients	Access route	Technical success	Complication rate	Complication
Maranki *et al*[36]	49	TD, TH	63%	16%	Pneumoperitoneum, bleeding, peritonitis, aspiration pneumonia
Iwashita *et al*[37]	40	TD, TH	73%	13%	Pancreatitis, pneumoperitoneum, sepsis
Shah *et al*[38]	74	NA	74%	8%	Pancreatitis, bile leak, perforation
Dhir *et al*[39]	58	TD	98%	3%	Bile leak
Vila *et al*[10]	60	NA	68%	22%	Biloma, bleeding, Perforation, pancreatitis, cholangitis, hematoma, abscess, pseudocyst

NA: Not available; TD: Transduodenal route; TH: Transhepatic route.

Table 4 Published data of endoscopic ultrasonography-guided antegrade treatment (n > 10)

Ref.	No. of patients	Study design	Stent	Technical success rate	Complication rate	Complication
Shah *et al*[38]	16	R	MS	81%	6%	Hematoma
Park *et al*[41]	14	P	MS	57%	0%	-
Dhir *et al*[42]	35	R	MS	97%	23%	Bleeding, cholangitis, bile leak, pneumoperitoneum
Ogura *et al*[43]	12	R	MS	100%	8%	Pancreatitis

MS: Metallic stent; P: Prospective; R: Retrospective.

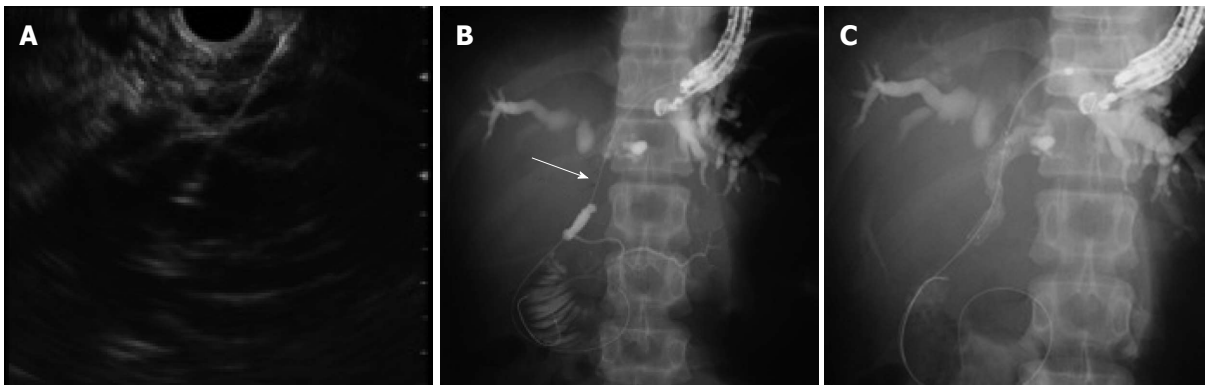

Figure 5 Endoscopic ultrasonography-guided antegrade treatment. A: Left intrahepatic bile duct was punctured; B: Guidewire was advanced through the needle across the stricture (arrow); C: A self-expandable metallic stent was placed antegradely across the stricture.

and protect against bile leakage, but a prospective study is needed to confirm their results.

EUS-AT

An indication for EUS-AT is biliary intervention in patients in whom conventional ERCP is impossible. EUS-AT is performed when it is necessary to approach the biliary intervention antegradely through a bilioenteric fistula. Antegrade is the opposite of retrograde in ERCP and is used to maintain bile flow. EUS-AT is indicated, for example, when biliary drainage is needed in patients whose upper gastrointestinal anatomy is altered, or who have malignant gastric outlet obstruction because it is impossible to perform conventional ERCP-related procedures in those patients. EUS-AT is usually performed *via* the transhepatic route (Figure 5). The echoendoscope is advanced into the upper gastrointestinal tract (esophagus, stomach and jejunum), and the left intrahepatic bile duct is punctured using an FNA biopsy needle. A cholangiogram is then obtained followed by antegrade guidewire insertion through the needle to the duodenum or jejunum across the papilla or the anastomosis. Finally, the biliary intervention is performed antegradely. It is thus possible to perform EUS-AT in patients with altered upper gastrointestinal anatomy[40]. There have been some small case series regarding the feasibility of EUS-AT to treat bile duct stones, malignant biliary obstruction, and anastomotic stricture. The technical success and complication rates for EUS-AT are 57%-100% and 0%-23%, respectively (Table 4)[38,41-43]. Although there are no large case series, the reported complications have been identified as bile leakage, pneumoperitoneum, and bleeding.

Table 5 Important features of each procedure

	EUS-BEA	EUS-RV	EUS-AT
Indication	Patients with malignant biliary obstruction after failed ERCP	Patients with failed biliary cannulation in ERCP	Patients with malignant biliary obstruction after failed ERCP
Advantage	Not traversing the malignant stricture	Leading to ERCP related procedure	Possible in patients with altered upper GI anatomy
Weak point	Necessity of fistula dilation Lack of dedicated stent	Difficult guidewire manipulation Difficulty in patient who was not accessible to the papilla	Difficult guidewire manipulation

ERCP: Endoscopic retrograde cholangiopancreatography; EUS-RV: Endoscopic ultrasonography-guided rendezvous procedure; EUS-BEA: Endoscopic ultrasonography-guided bilioenteric anastomosis; GI: Gastrointestinal; US-AT: Endoscopic ultrasonography-guided antegrade treatment.

WHICH TECHNIQUE SHOULD WE SELECT?

In patients with an accessible papilla, any EUS-BD procedure could be possible. Artifon *et al*[14] reported that the technical and clinical success rates for EUS-CDS and EUS-HGS for treating distal malignant obstruction were similar. Also, Khashab *et al*[25] reported that the clinical efficacy and safety were similar for EUS-CDS and EUS-RV. Although the complication rate for EUS-RV was relatively lower than that for EUS-BEA, more prospective studies are necessary. For patients with an inaccessible papilla, EUS-HGS and EUS-AT are possible.

There are no data concerning which procedure should be chosen. Hence, it is important to understand the characteristics of each procedure (Table 5).

FUTURE PERSPECTIVES

There are few data available that compare the clinical efficacy of EUS-BD and ERCP with regard to which is the treatment of choice. Dhir *et al*[42] reported that the short-term outcomes of EUS-BD (EUS-CDS and EUS-AT) were comparable with those achieved with ERCP. As EUS-BD is performed under direct visualization, it has the potential to substitute for ERCP. A prospective, randomized study is necessary, however, to confirm it. The future development of highly dedicated devices is mandatory.

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TOPIC HIGHLIGHT

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Endoscopic treatments for small gastric subepithelial tumors originating from muscularis propria layer

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Abstract

Minimally invasive endoscopic resection has become an increasingly popular method for patients with small (less than 3.5 cm in diameter) gastric subepithelial tumors (SETs) originating from the muscularis propria (MP) layer. Currently, the main endoscopic therapies for patients with such tumors are endoscopic muscularis excavation, endoscopic full-thickness resection, and submucosal tunneling endoscopic resection. Although these endoscopic techniques can be used for complete resection of the tumor and provide an accurate pathological diagnosis, these techniques have been associated with several negative events, such as incomplete resection, perforation, and bleeding. This review provides detailed information on the technical details, likely treatment outcomes, and complications associated with each endoscopic method for treating/removing small gastric SETs that originate from the MP layer.

Key words: Gastric subepithelial tumors; Endoscopic treatment; Submucosal tunneling endoscopic resection; Endoscopic muscularis excavation; Endoscopic full-thickness resection

Core tip: Minimally invasive endoscopic resection has become an increasingly popular method for small gastric subepithelial tumors (SETs) that originate from the muscularis propria (MP) layer. Currently, the main endoscopic therapies for patients with such tumors are endoscopic muscularis excavation, endoscopic full-thickness resection, and submucosal tunneling endoscopic resection. This review provides detailed information on the technical details, likely treatment

outcomes, and complications associated with each endoscopic method for treating small gastric SETs that originate from the MP layer.

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INTRODUCTION

Gastric subepithelial tumors (SETs) that originate from the muscularis propria (MP) layer are often asymptomatic and as a result are most frequently found incidentally during endoscopic procedures. A proportion of such gastric SETs are diagnosed as gastrointestinal stromal tumors (GISTs), which have the potential for malignancy[1]. Resection of gastric SETs can both aid in diagnosis and may at the same time be curative if the entire lesion is removed. Previously, surgical resection was the principal therapeutic option for removing small gastric SETs that originate from the MP layer[1-4]. However, surgical procedures are invasive and associated with certain complications, such as postoperative hemorrhage, gastroesophageal reflux, or late anastomotic stenosis[3], especially in asymptomatic patients with tumors less than 2.0 cm in diameter. According to the National Comprehensive Cancer Network guideline, endoscopic surveillance is another approach for GIST < 2 cm without high-risk EUS features[5]. However, repeated endoscopic examinations involve known issues associated with cost-effectiveness, patient compliance and risk related to delayed diagnosis of a malignancy.

Previously, endoscopic methods, such as snare polypectomy, band ligation, and endoscopic mucosal resection (EMR), were used to remove gastrointestinal SETs, but their use has generally been restricted to tumors located in the muscularis mucosae or submucosal layer[6-8]. Recently, with improvements in endoscopic submucosal dissection (ESD) technology, ESD is not only used to successfully treat superficial gastrointestinal (GI) lesions but also GI SETs that originate from the MP laye[9-12]. Currently, several studies have described various modalities of endoscopic resection that have been used to successfully treat gastric SETs that originate from the MP layer. Thus, this article is to review the current status of minimally invasive endoscopic treatments for such gastric SETs.

ENDOSCOPIC MUSCULARIS EXCAVATION

ESD is an effective endoscopic technique, which has made it possible to perform *en bloc* resections of superficial gastric cancers. With the development of ESD techniques and the application of new endoscopic accessories, several studies have recently evaluated the safety and effectiveness of ESD techniques in the treatment of small gastric SETs that originate from the MP layer. Lee *et al*[9] reported that ESD was successfully performed in 11 patients with a total of 12 gastric SETs that originate from the MP layer, with a complete resection rate of 75%, and without massive bleeding, perforation, or any other severe complications during hospitalization. In another study of 18 gastric SETs, Zhang *et al*[12] reported that the complete resection rate was 94.4%. During the same procedure, 2 patients developed perforations, which were successfully treated by endoscopic methods. These results demonstrate that ESD can be used for successful removal of small gastric SETs that originate from the MP layer, which may ultimately replace treatment by surgical resection (at least in some cases).

Although standard ESD procedures can be used safely for the resection of gastric SETs while providing an accurate pathologic diagnosis, this procedure is associated with some complications, including perforation, bleeding, and abdominal infection[10,11,13]. Among them, perforation is the main complication of such ESD procedures. Previous studies using ESD to treat gastric SETs have reported an incidence of perforation ranging from 0%-20%[10,11,14-16]. In addition, if tumors presenting with a tight connection to the underlying MP have extended over a large area, complete resection by ESD often fails[10,16]. In a recent study focusing on the use of ESD to treatment gastric SETs, Białek *et al*[10] reported that the complete resection rate was 100% when tumors had no connection to the underlying MP; yet when tumors presented with a narrow connection to the underlying MP, the complete resection rate was only 68.2%. Therefore, ESD has some limitations for the treatment of gastric SETs with a tight connection to the underlying MP tightly.

Recently, with improved ESD technology, there have been an increasing number of reports on the use of endoscopic resection methods for gastric SETs that originate from the MP layer. During this procedure, a circumferential muscularis excavation is usually made that is as deep as the MP layer around the tumor, which is used to peel the tumor from the MP layer. Due to this critical difference in procedure, this technology has been named endoscopic muscularis excavation (EME)[17]. Note that EME is similar to the technique of standard ESD, with the only difference in the depth of excavation.

In our center, EME is performed as follows (see Figure 1)[17]: After marking the lesion margins with a needle-knife, several milliliters of submucosal injection solution is injected into the submucosa around the lesion. Subsequently, a cross-incision is made inside the marker dots using the electric knife, and several

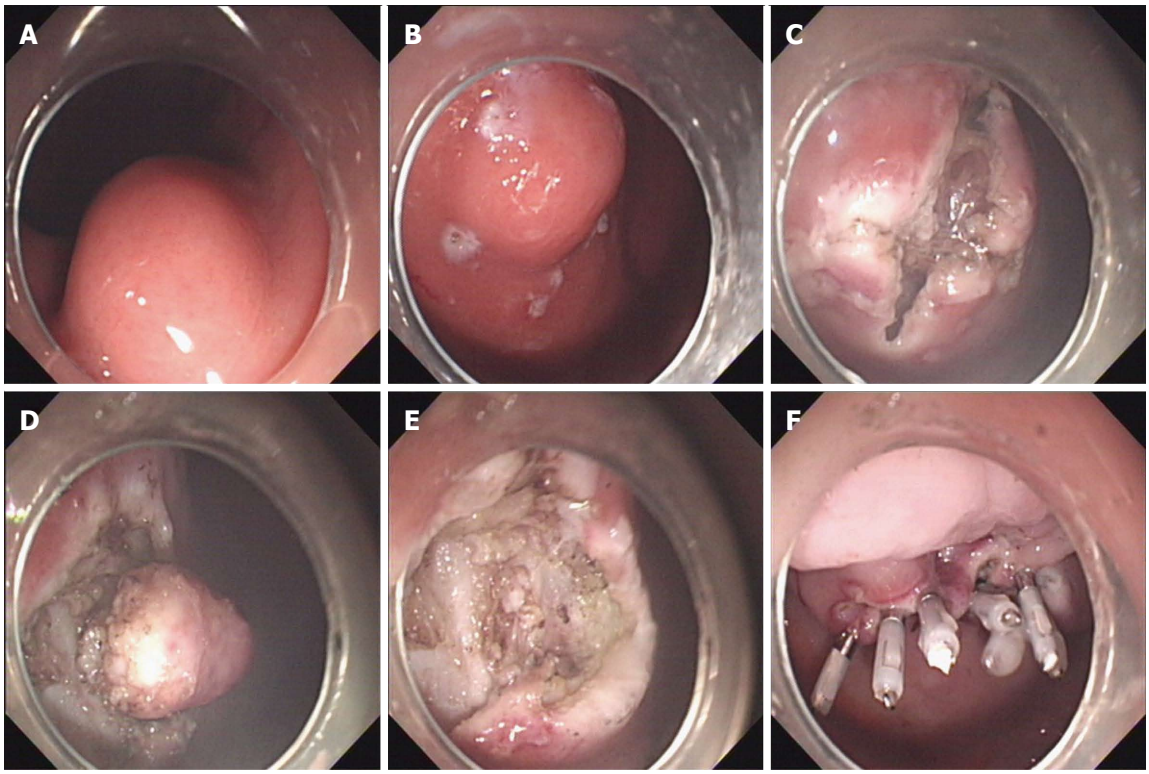

Figure 1 Endoscopic muscularis excavation. A: A subepithelial tumor was found at the posterior wall of the gastric body; B: Making several dots around the tumor; C: A cross-incision was made at the overlying mucosa of the tumor; D: Excavating the tumor from the muscularis propria layer; E: An artificial ulcer was observed after excavation; F: The artificial ulcer was closed with several clips.

milliliters of submucosal injection solution (above mentioned) is injected around the lesion to help distinguish the tumor mass from the gastric muscular layers and avoid tumor rupture during the excavation. A circumferential excavation is made along the edge of the tumor until the tumor is completely separated from the MP layer using the electric knife. After tumor removal and adequate hemostasis, the wound is closed with metallic clips.

Compared with standard ESD procedure, the primary advantage of EME is the improved complete resection rate. In our previous study using EME to remove gastric SETs that originate from the MP layer, the complete resection rate was 96.2% (204/212)[18]. In almost all similar studies reported to date, the complete resection rate has been higher than 90%, which reaches an acceptable level[19-21] (Table 1). In contrast, standard ESD for SETs originating from the MP layer has classically yielded a lower *en bloc* resection rate of 64%-75%[22], suggesting that EME may be a better technique for the resection of SETs that infiltrate the MP layer.

However, as a result of deeper dissection (as compared to ESD), the risk of complications associated with EME is significantly increased[22]. Massive bleeding is often caused by the accidental injury of the arteries that feed the tumor, which is one of the key factors that can affect procedure success or failure. Once massive bleeding is encountered, the endoscopic view may be affected, and the EME procedure might need to be discontinued[16]. Endoscopic hemoclips could be one effective method to control rapid bleeding. However, their use may possibly hamper subsequent endoscopic operations. It should be noted that although massive bleeding rarely happens, EME procedures should be halted and changed to open surgery or laparoscopic surgery when it cannot be managed using endoscopic methods. However, minor bleeding is not uncommon and can be treated successfully with argon plasma coagulation or coagulation forceps during the procedure.

Perforation is the considered major complication of EME treatment of gastric SETs originating from the MP layer. Some perforations can be avoided by repeated doses of submucosal injection solution and meticulous excavation. However, when the tumor is tightly attached to the MP layer or serosa, perforation is often inevitable. Several studies have reported that perforation may be associated with several factors, including histologic diagnosis, location, and origin of the tumor[18,19]. Our recent study demonstrated that perforation was more likely to occur with tumors located in the fundus compared to any other part of the stomach, which is also common for tumors originating in the deeper MP layers as opposed to those in the superficial MP layer. In addition, the rate of perforation was significantly higher for GISTs than for leiomyomas. Therefore, an EUS examination is necessary to evaluate the tumor features and the origin of the tumor to predict the risk of perforation

Table 1 Clinical outcomes of endoscopic muscularis excavation for gastric subepithelial tumors originating from the muscularis propria layer

Ref.	No. cases (tumors)	Location (details)	Mean tumor size (mm)	Pathology	Complete resection rate, *n* (%)	Mean operating time (min) and range(min)	Complications (details)	Mean follow-up time (mo) and recurrence
Jeong *et al*[19]	64 (65)	23 cardia 8 fundus 30 body 4 antrum	13.8	26 GISTs 32 leiomyomas 2 schwannomas 3 others	60 (92.3)	34.7	8 perforations	10.0 No recurrence
Chu *et al*[21]	16 (16)	1 cardia 3 fundus 9 body 3 antrum	26.1	14 GIST 2 leiomyoma	15 (93.8)	52.0	0	14.8 No recurrence
Liu *et al*[20]	31 (31)	14 esophagus 7 cardia 5 fundus 5 body	22.1	16 GISTs 15 leiomyomas	30 (96.8)	76.8	4 perforations	17.7 No recurrence
Zhang *et al*[18]	212 (212)	93 fundus 104 body 15 antrum	16.5	97 GISTs 115 leiomyomas	204 (96.2)	46.1	32 perforations 9 massive bleeding	26.0 No recurrence

GISTs: Gastrointestinal stromal tumors.

before the procedure[18]. Usually, perforations were relatively small and could not be directly visualized during the procedure. Therefore, the occurrence of subcutaneous emphysema during the procedure should be monitored as it may be used to denote such perforations. Fortunately, patients with perforations usually can be successfully managed by endoscopic methods and conservative treatment; few require surgical intervention[17-21].

ENDOSCOPIC FULL-THICKNESS RESECTION

During the application of EME for gastric SETs originating from the MP layer, our center found that when the tumor had extraluminal growth or was tightly connected to the underlying MP or serosa, it was necessary to resect the underlying MP or serosa that adhered to the tumor. This technique of creating an iatrogenic perforation of the gastric wall for the subsequent removal of the tumor was named endoscopic full-thickness resection (EFTR)[23-26] (Table 2). EFTR is performed as shown in Figure 2. Several milliliters of mixture solution are injected into the submucosa after dots are marked around the tumor with a needle-knife. Then, mucosal incision is made in the overlying mucosa to reveal the tumor. A circumferential excavation is then made as deep as the MP around the tumor with the electric knife. After the intraluminal side of the tumor is fully revealed, a small puncture is first made in the proximal seromuscular layer of the tumor with the electric knife, and then the resection is made along the puncture. Subsequently, snare resection is performed to completely remove the lesion after the electric knife resects three-quarters of the circumference of the tumor. Before performing snare resection, a dual-channel endoscope is used while grasping the tumor in the gastric cavity to avoid it falling into the peritoneum. Clips or clips combined with an endoloop are used for closure of the gastric wall defect.

With the development of EFTR, the indications of endoscopic resection may be further expanded. However, several problems need to be noted. One key problem with the EFTR procedure involves issues with completely closing the gastric wall defect after full-thickness resection to avoid secondary surgical intervention. Incomplete closure of the gastric wall defect is a dangerous adverse event, and may lead to serious morbidity. This is a probable major safety consideration for the clinical application of EFTR. A wide range of methods and devices for closure of gastric wall defects have been studied, but most techniques require complex or specialized equipment, which represents a significant technical challenge[25-27]. How to easily and safely close the defect is a problem that is worth further exploration. Previously, metal clips were widely used for closure of the iatrogenic perforation during the endoscopic procedure. Based on these experiences, some endoscopists elected to apply these clips as a closure technique for gastric wall defects after EFTR. In a recent study of 26 patients treated by EFTR, Zhou *et al*[23] reported that clips are an effective and safe method for closure of the gastric wall defect. However, some endoscopists contend that the edge of the gastric wall defect might exhibit edema for a long time after the procedure, and the clip can therefore only close the gastric mucosa. Thus, there is a risk of gastric leakage when only using clip closure after EFTR, especially for some large defects ($\geqslant$ 3 cm)[28]. In a recent study, our center reported an easier to operate "clips plus endoloop" method in which the defect was closed with clips in a "side-to-center" manner using an endoloop to trap and

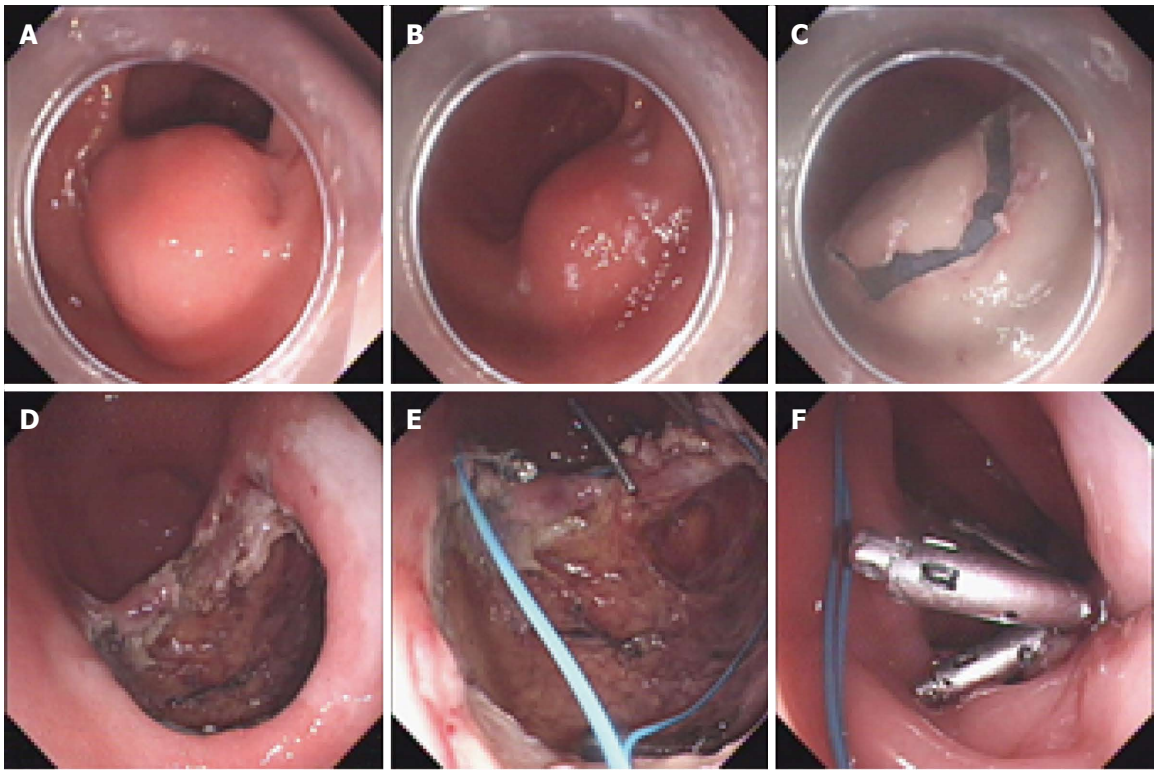

Figure 2 Endoscopic full-thickness resection. A: A subepithelial tumor was found at the greater curvature of the gastric antrum; B: Making several dots around the tumor; C: The mucosa incision was incised outside the marker dots; D: The omentum could be seen through the gastric wall defect after endoscopic full-thickness resection; E, F: Closure of the gastric wall defect using clips and an endoloop.

Table 2 Clinical outcomes of endoscopic full-thickness resection for gastric subepithelial tumors originating from the muscularis propria layer

Ref.	No. cases	Location (details)	Mean tumor size (mm)	Pathology	Complete resection rate, *n* (%)	Mean operating time (min)	Complications (details)	Mean follow-up time (mo) and recurrence
Guo *et al*[26]	23	11 fundus 9 body 3 antrum	12.1	19 GISTs 4 leiomyomas	23 (100)	40.5	2 localized peritonitis	3 No recurrence
Zhou *et al*[23]	26	12 fundus 14 body	28	16 GISTs 6 leiomyomas 3 glomus tumors 1 schwannoma	28 (100)	105	0	8 No recurrence
Schmidt *et al*[25]	31	3 cardia 4 fundus 13 body 11 antrum	20.5	18 GIST 2 leiomyomas 2 adenomyomas 3 ectopic pancreas 1 lipoma 1 schwannoma 4 others	28 (90.3)	60	12 bleeding	7 No recurrence
Ye *et al*[24]	51	22 fundus 28 body 1 antrum	24	30 GIST 21 leiomyoma	50 (98.0)	52	0	22.4 No recurrence

GISTs: Gastrointestinal stromal tumors.

tighten all clips. Compared with single clip closures, this closure method would reinforce the closure of the gastric defect and prevent postoperative gastric leaks and peritonitis[24]. In addition, it has the advantage of being a simple manipulation that does not require complex or specialized equipment. One thing to note is that closure by clips or clips plus endoloop only approximates the mucosal and submucosal layers, although this method appears to be safe and effective based on the present literature. However, these methods contradict accepted surgical safety principles, such that additional and more comprehensive randomized, controlled, and multicenter studies are required to confirm its safety and reliability.

Table 3 Clinical outcomes of submucosal tunneling endoscopic resection for gastric subepithelial tumors originating from the muscularis propria layer

Ref.	No. cases (tumors)	Location (details)	Mean tumor size (mm)	Pathology	Complete resection rate, *n* (%)	Mean operating time (min)	Complications (details)	Mean follow-up time (mo) and recurrence
Xu *et al*[30]	15 (15)	9 esophagus 3 cardia 2 body 1 antrum	19	5 GISTs 9 leiomyomas 1 glomus tumor	15 (100)	78.7	1 pneumoperitoneum 1 pneumothorax 1 SE	3.9 No recurrence
Liu *et al*[34]	12 (12)	7 esophagus 5 cardia	18.5	2 GISTs 9 leiomyomas 1 schwannoma	12 (100[1])	78.3	2 pleural effusion 4 pneumothorax 8 SE	7.1 No recurrence
Wang *et al*[36]	57 (57)	57 esophago-gastric junction	21.5	7 GISTs 46 leiomyomas 1 intramuscular lipoma 1 granular cell tumor 2 schwannomas	57 (100)	47.0	8 pneumothorax 3 pneumoperitoneum 12 pneumothorax and SE	12 No recurrence
Ye *et al*[32]	85 (85)	60 esophagus 16 cardia 9 stomach	19.2	19 GISTs 65 leiomyomas 1 calcifying fibrous tumor	85 (100)	57.2	2 pleural effusion 6 pneumothorax 4 pneumothorax 8 SE	8 No recurrence

[1]The 100% is *en-bloc* resection rates, no information on complete resection rate was given in this study. GIST: Gastrointestinal stromal tumors; SE: Subcutaneous emphysem.

Another key problem with the EFTR procedure is the potential risk of abdominal cavity infection, although the current literature reports no significant instances of peritonitis and abdominal cavity infection with EFTR. During the EFTR procedure, the abdominal cavity will be at risk of contamination by the gastric fluid, nonsterile endoscopes, and endoscopic accessories. Similarly, abdominal cavity infection is a serious problem, which is currently the subject of heated arguments in the field of natural orifice transluminal endoscopic surgery (NOTES). However, compared with NOTES, EFTR offers a shorter operation time and a lesser risk of contact with the adjacent tissue/organs. Thus, sterile endoscope/accessories and antiseptic gastric lavage are not likely required[29]. However, optimized bowel preparation, intravenous infusion of antibiotics, and gastrointestinal decompression are warranted. In addition, it is also very important to prevent the gastric juice from flowing into the abdominal cavity to minimize the risk of contamination. Thus, before gastric wall puncture, the operative field should be separated from the gastric fluid by changing the position to obtain a satisfactory view of the tumor and then suctioning the gastric fluid completely[23].

SUBMUCOSAL TUNNELING ENDOSCOPIC RESECTION

With the introduction of EME and EFTR, endoscopic resection techniques have evolved continuously. Recently, the emergence of NOTES marked the rise of a new branch of therapeutic endoscopy. Inspired by NOTES and peroral endoscopic myotomy, in 2012, Xu *et al*[30] reported a new technique, named submucosal tunneling endoscopic resection (STER), which can be implemented for upper gastrointestinal SETs originating from the MP layer. Since its introduction, there have been an increasing number of reports on the use of STER for upper gastrointestinal SETs originating from the MP layer[31-36] (Table 3).

The STER procedure is performed as follows (Figure 3)[31]: (1) to avoid losing the target while creating a submucosal tunnel, the space between the lesion and the mucosal incision is also located by methylene blue or indigo carmine injection, which provides guidance in creating a submucosal tunnel; (2) a 2 cm longitudinal mucosal incision is made 5 cm proximal to the tumor as the entry point with a needle-knife. Then, a submucosal tunnel to the SET is established using an electric knife between the submucosal and muscular layers. The tunnel ends 1-2 cm distal to the tumor to ensure enough working space for tumor resection. Subsequently, endoscopic resection of the tumor is then performed by ESD using the electric knife. During the tumor resection, repeated injections of saline solution help to differentiate the MP layer from the tumor mass and to avoid tumor capsule rupture during the excavation of the tumor from the MP layer; and (3) the mucosal incision site is then closed with several clips after tumor removal.

In contrast with EME or EFTR, STER has advantages in terms of preserving the integrity of the digestive tract mucosa and submucosa, while also promoting early wound healing. Moreover, a 5-cm-long submucosal tunnel has a good "leak-proofing" effect. If digestive tract leakage occurs during the procedure,

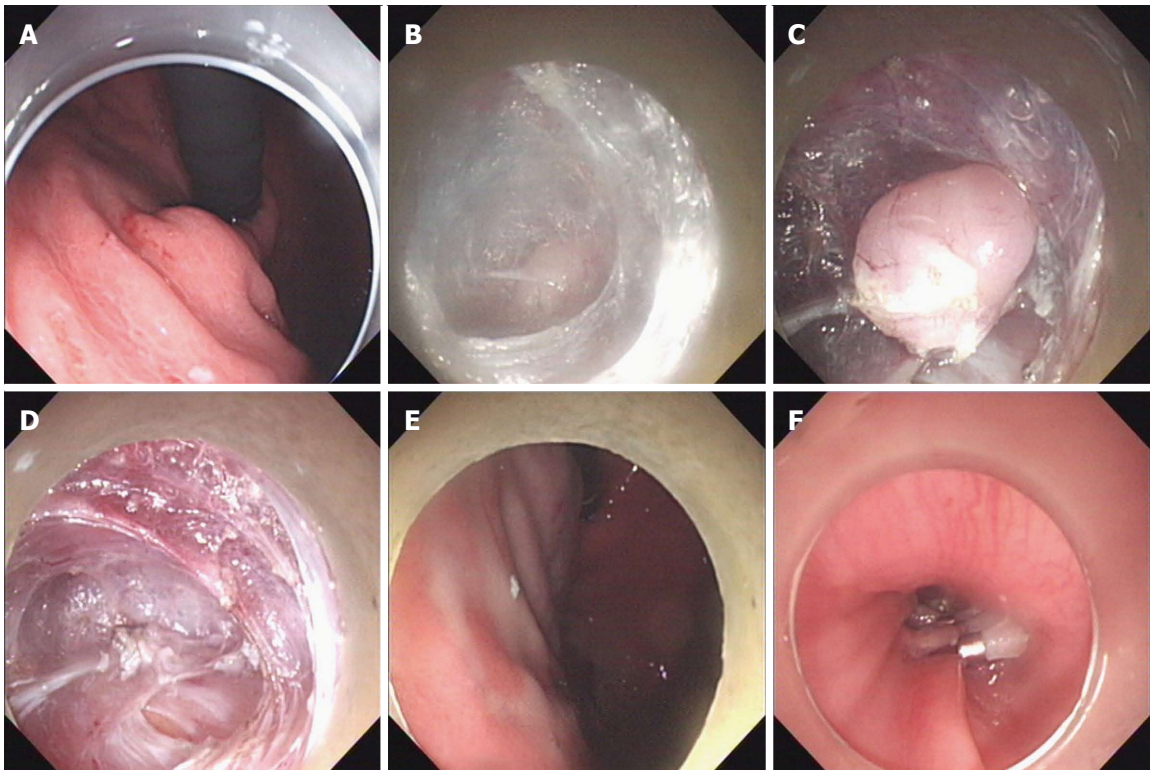

Figure 3 Submucosal tunneling endoscopic resection. A: A subepithelial tumor was found in the gastric fundus; B, C: Creating a submucosal tunnel to the tumor and then excavating the tumor from the muscularis propria layer *via* the submucosal tunnel; D: The omentum could be seen through a small gastric wall after tumor removal; E: The gastric mucosa was intact; F: The entrance of the submucosal tunnel was closed with several clips after tumor removal.

it reduces the risk of postoperative digestive tract fistula and pleural/abdominal infection[34]. Compared with EME and EFTR, STER seems to be a safer and more effective treatment for gastric SETs originating from the MP layer. However, some complications are frequently observed. The main complications of STER are subcutaneous emphysema, pneumoperitoneum pneumothorax, and pleural effusion[30-36]. Previous studies using STER have reported an incidence of complications ranging from 0%-20%[30-36]. However, these patients usually recover without any need for further endoscopic or surgical intervention. In addition, insufflation with CO_2 can significantly reduce the occurrence of post-procedural mediastinal emphysema[36].

There are still several technical challenges with the use of STER to treat gastric SETs originating from the MP layer[32]. First, one important challenge of this technique is the establishment of a submucosal tunnel to the lesion. General stomach features, such as a great space, extensibility, and mucosa hypertrophy, increase the difficulty in establishing a submucosal tunnel (as compared to the esophagus). Moreover, specific locations within the stomach present with varying levels of difficulty for establishing a submucosal tunnel. Compared with other parts of the stomach, in our experience, it is relatively simple to establish a submucosal tunnel in the cardia adjacent to the gastric fundus, the greater curvature of gastric antrum, and the lesser curvature of gastric body. Second, the tunnel directions can be more difficult to identify in the stomach, which increases the difficulty of establishing a submucosal tunnel to the tumor. The route between the tumor and the mucosal incision is first oriented by injection of indigo carmine or methylene blue before establishing a submucosal tunnel, which plays a guidance role for the operator. Third, keeping the tunnel mucosa intact is more difficult in the stomach than in the esophagus. "Leak-proofing" can only work effectively when the tunnel mucosa maintains its integrity. Forth, in some cases the lesion is more tightly connected to the underlying MP or serosal layer. It is then necessary to resect the lesion including the underlying muscularis propria and serosa. In this situation, care needs to be taken to avoid injury to the adjacent organs. However, STER is still a generally safe and effective technique for SETs that originate from the MP layer, so long as strict criteria for case selection and admittance of surgical operations are adopted.

INDICATIONS OF ENDOSCOPIC PROCEDURE

The optimal indication for endoscopic resection in gastric SETs should be less than 3.5 cm in diameter of tumor size. The reasons for this indication are as follows. First, when a tumor is > 3.5 cm in diameter, it is difficult to remove *via* an endoscopic approach

after *en bloc* resection because of the limitations of the cardia and esophageal cavity space. Second, for the STER procedure, it is also difficult to excavate a large tumor during the narrow submucosal tunnel, which is often associated with an obscured endoscopic view and a high risk of tunnel mucosa perforation. Third, for the EFTR procedure, resecting a large tumor will create a large gastric wall defect, which is extremely difficult to close by clips and associated with a potential risk of postoperative gastric leaks. Thus, at present, very few cases of SETs > 3.5 cm were reported in published literature and some of them resulted in partial or piecemeal resection[10,20]. However, partial or piecemeal resections, not an *en-bloc* resection, make the histologic evaluation very difficult. In addition, tumor capsule rupture might cause tumor recurrence or metastasis. Therefore, only tumors less than 3.5 cm in diameter should be removed *via* endoscopic procedures. This is a relatively strict rule for endoscopic management. Note that tumors with high-risk EUS features, such as irregular borders, cystic spaces, ulcerations, echogenic foci, or heterogeneity, are not suitable for those endoscopic treatments.

There is no standard for the selection of endoscopic operation methods for small gastric SETs that originate from the MP layer. Endoscopist experience and tumor characteristics, such as the size, depth, location, and extraluminal or endoluminal growth of the tumor, are the main factors in deciding which surgical method to employ. Generally, for gastric SETs with endoluminal growth, ESE is a favorable choice, whereas for extraluminal growth, EFTR is another favorable choice. In areas suitable for establishing a submucosal tunnel, such as in the greater curvature of the gastric antrum, the lesser curvature of gastric body, or the cardia adjoining to the gastric fundus, STER is also a favorable choice, but should be performed only by an experienced endoscopist.

CONCLUSION

Although there are some complications or adverse events associated with endoscopic operation, such as perforation, massive bleeding, and subcutaneous emphysema, endoscopic operation provides a new option for the management of gastric SETs that originate from the MP layer, which is superior to surgical operation in terms of keeping the normal structure and function of the stomach and improving the long-term quality of life. However, to successfully achieve complete resection and reduce the potential risk of complications, endoscopists need to skillfully master technical details of every endoscopic procedure and choose wisely according to the tumor characteristics, such as the size, depth, location, and extraluminal or endoluminal growth of the tumor. In addition, careful postoperative management and close follow-up, especially for some patients with complications or adverse events, are also vital to optimize treatment outcomes.

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REVIEW

Non-functional neuroendocrine tumors of the pancreas: Advances in diagnosis and management

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Abstract

Nonfunctional neuroendocrine tumors of the pancreas (NF-PNETs) are a heterogeneous group of neoplasms. Although rare, the incidence of NF-PNETs is increasing significantly. The classification of PNETs has evolved over the past decades and is now based on a proliferation grading system. While most NF-PNETs are slow growing, tumors with more aggressive biology may become incurable once they progress to unresectable metastatic disease. Tumors of higher grade can be suspected preoperatively based on the presence of calcifications, hypoenhancement on arterial phase computed tomography, positron emission technology avidity and lack of octreotide scan uptake. Surgery is the only curative treatment and is recommended for most patients for whom complete resection is possible. Liver-directed therapies (thermal ablation, transarterial embolization) can be useful in controlling unresectable hepatic metastatic disease. In the presence of unresectable progressive disease, somatostatin analogues, everolimus and sunitinib can prolong progression-free survival. This article provides a comprehensive review of NF-PNETs with special emphasis on recent advances in diagnosis and management.

Key words: Pancreas; Neuroendocrine tumor; Neuroendocrine carcinoma; Islet cell; Octreotide

Core tip: Pancreatic neuroendocrine tumors (PNETs) are a fascinating and diverse group of neoplasms. While the clinical features of functional PNETs are frequently discussed, the majority of PNETs are actually non-functional. Although typically slow growing, tumors with more aggressive biology may progress to unresectable metastatic disease. Surgery should be considered for all patients for whom complete resection is possible, while liver directed therapies are useful for managing hepatic metastases. For patients with progressive metastatic disease, strong evidence supports the use of somatostatin analogues, everolimus and sunitinib in prolonging survival. The purpose of this article is to provide a comprehensive review of NF-PNETs.

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INTRODUCTION

Pancreatic neuroendocrine tumors (PNET) are a rare heterogeneous group of neoplasms that arise from progenitor islet cells. PNETs may be classified as either functional (F-PNET) or non-functional (NF-PNET), depending on their ability to secrete biologically active hormones and elicit characteristic symptomatology. NF-PNETs exhibit a wide range of malignant potential, ranging from slow-growing and non-infiltrative tumors to locally invasive and rapidly metastasizing ones, thereby making standardization of the diagnosis, surgical and medical management, follow-up surveillance and prognosis challenging. Fortunately, significant advances in diagnostic modalities, tumor localization and therapeutic options have been made over the past decade. This article provides a comprehensive review of NF-PNETs and an update on advances in their diagnosis and management.

EPIDEMIOLOGY

Although neuroendocrine neoplasms can occur nearly anywhere in the body, gastroenteropancreatic neuroendocrine tumors (GEP-NET) and pulmonary neuroendocrine tumors comprise the majority. PNETs comprise approximately 7% of all NETs[1]. However, compared to other pancreatic pathology, PNETs are relatively rare, comprising only 1%-2% of all pancreatic neoplasms. The incidence of PNETs increases significantly after the age of 40 with a peak incidence around age 65[1]. There is only a slight male predominance[2]. Between 60%-90% of all PNETs are non-functional and given their frequently asymptomatic nature the majority of patients present with distant metastasis[2,3].

By all measures, the incidence of PNETs is increasing. The Surveillance, Epidemiology and End Results (SEER) program has shown that the incidence has increased from 0.17 per 100000 people in 1973 to 0.47 per 100000 people in 2007[1]. Likewise, a six-fold increase in the incidence was found in Ontario, Canada between 1994 and 2009 (from 0.1 to 0.6 per 100000 persons)[4]. Autopsy studies would also suggest that the prevalence of PNETs is higher than previously suspected[5]. Interestingly, this trend of increasing incidence of PNETs seems to be true of all neoplasms of neuroendocrine origin[4,6] and may be partly related to increased incidental discovery due to more frequent use and improving sensitivity of cross-sectional imaging.

STAGING AND PROGNOSIS

In 2000, the World Health Organization (WHO) first established guidelines that distinguished between well-differentiated tumors with benign behavior (localized to pancreas, size < 2 cm, low mitotic rate and Ki-67, no angioinvasion or perineural invasion), tumors with uncertain behavior (limited to the pancreas, angioinvasion or perineural invasion, size ⩾ 2 cm) and tumors with clearly malignant behavior (gross local invasion or distant metastases)[7]. In 2010, the WHO revised their previous grading system to a proliferation based grading system (Table 1). Based on mitotic counts and Ki-67 indices, well-differentiated tumors included those of low and intermediate grade while poorly differentiated tumors included high grade tumors. It was concluded that mitotic count and Ki-67 should be performed on all specimens and that the grade would reflect the higher value when discordant[8]. In fact, Ki-67 and differentiation has been found to be some of the most important factors in determining prognosis[9].

Based on results of a consensus conference in 2005, the European Neuroendocrine Tumour Society (ENETS) proposed a classification scheme for all foregut NETs that combined a TNM staging system with a histologic grading system[10]. The most commonly used staging system, however, is from the 7th edition American Joint Committee on Cancer (AJCC)[11]. Revised in 2010, this system applies to all neoplasms of the pancreas, both endocrine and exocrine, and is based on TNM staging (Table 1). Importantly, the AJCC system does not incorporate histological grading criteria.

Both the ENETS and the AJCC system have been validated and provide important prognostic information for patients with PNETs[12]. However, some have called into question whether the AJCC system provides adequate discriminatory value. Specifically, validation studies by Strosberg *et al*[13,14] showed no survival difference between stages Ⅰ and Ⅱ as well as stages Ⅲ and Ⅳ. In addition, Rindi *et al*[15] studied a large international cohort of resected PNETs and found no significant differences in survival between stage Ⅱ and Ⅲ. A large range of outcomes was seen in patients of all stages, suggesting poor discriminatory ability, and they concluded that the ENETS staging system was superior[15]. Qadan *et al*[16] utilized SEER to demonstrate that no significant survival differences could be replicated between stages Ⅱ and Ⅲ or Ⅲ and Ⅳ, and suggested a revised TNM staging system with potentially improved prognostic capabilities.

CLINICAL PRESENTATION

Unlike other solid tumors (including F-PNETs), NF-PNETs can remain asymptomatic before they reach

Table 1 Current pancreatic neuroendocrine tumor classification and staging systems

WHO 2010/ENETS grading			
Grade	Differentiation	Ki-67 index (%)	Mitotic count/10 HPF
G1 (low)	Well	≤ 2	< 2
G2 (intermediate)	Well	3-20	2-20
G3 (high)	Poorly	> 20	> 20
ENETS T staging[1]			
T stage	Description		
TX	Cannot be assessed		
T0	No evidence of tumor		
T1	< 2 cm, limited to pancreas		
T2	2-4 cm, limited to pancreas		
T3	> 4 cm, limited to pancreas		
T4	Involving adjacent organs or large blood vessels		
AJCC T staging[1]			
T stage	Description		
TX	Cannot be assessed		
T0	No evidence of tumor		
T1	≤ 2 cm, limited to pancreas		
T2	> 2 cm, limited to pancreas		
T3	Involves adjacent organs		
T4	Involving celiac axis or superior mesenteric artery		
Stage	ENETS staging	AJCC staging	
ⅠA	T1 N0 M0	T1 N0 M0	
ⅠB		T2 N0 M0	
ⅡA	T2 N0 M0	T3 N0 M0	
ⅡB	T3 N0 M0	T1-3 N1 M0	
ⅢA	T4 N0 M0	T4 N1 M0	
ⅢB	T1-4 N1 M0		
Ⅳ	T1-4 N0-1 M1	T1-4 N0-1 M0	

[1]Both AJCC and ENETS share common N and M staging: N0, no regional lymph node metastatic; N1, regional lymph node metastasis; M0, no distant metastasis; M1, distant metastasis. WHO: World Health Organization; ENETS: European neuroendocrine tumor society; AJCC: 7th edition American joint committee on cancer.

Table 2 Clinical features of functional pancreatic neuroendocrine tumors

Tumor	Percentage	Secreted hormone	Malignant	Clinical features	Biochemical evaluation
Insulinoma	40%-60%	Insulin	< 10%	Hypoglycemia	Insulin, pro-insulin, C-peptide, 72 h fasting insulin/glucose ratio
Gastrinoma	20%-50%	Gastrin	60%-90%	PUD, GERD, diarrhea	Fasting gastrin (off PPI), secretin stimulation test
Glucagonoma	Rare	Glucagon	50%-80%	Necrolytic migratory erythema, diabetes, venous thrombosis, depression	Glucagon
Somatostatinoma	Rare	Somatostatin	> 70%	Diabetes, hypochlorhydria, cholelithiasis, diarrhea	Somatostatin (not widely available)
VIPoma	Rare	Vasoactive Intestinal Peptide	40%-70%	Watery diarrhea, hypokalemia, achlorhydria	VIP

PUD: Peptic ulcer disease; GERD: Gastroesophageal reflux disease; PPI: Proton pump inhibitor.

a significant tumor burden. When they become symptomatic, their symptomatology is typically related to mass effect from the primary tumor or the metastasis. Many PNETs occur in the head of the pancreas where symptoms may include jaundice, abdominal pain, or weight loss. Other less frequent symptoms may include anorexia, nausea, intra-abdominal bleeding, or a palpable mass. Many will be asymptomatic and found incidentally on cross-sectional imaging performed for other indications. The vast majority of metastases occur in the liver, though other sites including bone, peritoneum, adrenal, brain and spleen have been reported[17]. Liver metastases more frequently occur with non-functional tumors and patients with symptoms. When liver metastases occur, most are multifocal and bilobar[17].

F-PNETs present with symptoms caused by the specific hormone produced. Common F-NETs include insulinoma, which presents with hypoglycemia, and gastrinoma, which presents with peptic ulcer disease, gastroesophageal reflux disease or secretory diarrhea. Less common F-NETs include VIPomas, glucagonomas, and somatostainomas. These tumors are summarized in Table 2 but are not discussed further in this review. NF-PNETs either do not produce hormones, produce hormones at a low enough level to not cause

symptoms, or are associated with hormones that do not cause symptoms, such as pancreatic polypeptide, chromogranin A, ghrelin, calcitonin or neurotensin.

While most NF-NETs are sporadic, approximately 10% of NETs will be associated with an inherited genetic syndrome[18]. Multiple endocrine neoplasia type 1 (MEN1) is an inherited autosomal dominant disease characterized by hyperparathyroidism (nearly 100%), PNETs (up to 75%) and pituitary tumors (less than 50%)[19]. NF-PNETs are the most common pancreatic neoplasms in MEN1, followed by gastrinomas, and then insulinomas. Patients with MEN1 frequently present with multiple PNETs throughout the pancreas[20]. Von Hippel-Lindau (VHL) disease is an autosomal dominant disorder that is associated with pancreatic tumors or cysts. In order of frequency, patients develop pancreatic cysts, NF-PNETs (10%-20% of patients), cystadenomas, hemangioblastomas and adenocarcinoma; F-PNETs are rare[20]. PNETs in neurofibromatosis type 1 (NF1) are relatively rare (0%-10%) but are almost exclusively duodenal somatostatinomas in the periampullary region[21]. Other functional and non-functional PNETs may rarely occur[22-24]. PNETs associated with tuberous sclerosis (TS) are relatively uncommon and may be either functional or non-functional[25].

DIAGNOSIS

Patients with PNETs require a thorough evaluation for symptoms classically associated with functional tumors as well as symptoms directly related to the primary or metastatic tumor. Past medical and family history should be carefully reviewed. A comprehensive physical examination should be undertaken. Ultimately, the diagnosis of PNETs depends on comprehensive biochemical and radiographic evaluation.

Biochemical

Neuroendocrine markers are important not only for confirming diagnosis, but also as screening tools for future surveillance. The most commonly utilized neuroendocrine markers include chromogranin A (CgA), pancreatic polypeptide (PPP), pancreastatin, and neuron-specific enolase (NSE). CgA is a glycoprotein used commonly as a tumor marker in histopathology but also has elevated circulating levels in patients with both functional and non-functional PNETs[26,27]. However, falsely elevated levels can be observed in patients with chronic renal insufficiency, liver failure and with proton-pump inhibitor (PPI) use[28,29]. Recent recommendations by the North American Neuroendocrine Tumor Society[30] and a Canadian national expert group[29] have recommended utilizing CgA in the diagnosis and surveillance of advanced PNETs. PPP may be elevated in up to 63% of PNETs[31] and has a specificity of 84% when used during surveillance[32]. Pancreastatin may provide additional diagnostic utility, especially in patients on PPIs or with normal CgA levels[33,34]. Laboratory evaluation should also include tests to rule out F-NETs, including insulinoma and gastrinoma, if suspected (Table 2). Screening for MEN1 with serum parathyroid hormone and calcium levels should be performed in appropriate patients (*e.g.*, diagnosis at young age, multifocal tumors, and/or with relevant personal or family history).

Localization

Cross-sectional imaging should be performed in all patients suspected of having a PNET. Computed tomography (CT) remains the initial imaging modality of choice given its good sensitivity, specificity and availability. PNETs typically are well-circumscribed lesions that appear hyperenhancing on contrast-enhanced scans. In fact, there is some evidence that hypoenhancement on arterial phase imaging is associated with more aggressive tumors and worse prognosis (Figure 1)[35]. Similarly, the presence of calcifications within these tumors on CT is associated with higher grade and the presence of lymph node metastases (Figure 2)[36]. Magnetic resonance imaging (MRI) is an alternative modality with the advantage of less radiation exposure. PNETs should be low signal intensity on T1 weighted images and high signal intensity on T2 weighted images. In addition, MRI may be more sensitive than CT for detecting smaller pancreatic lesions and liver metastases[37,38]. While ultrasound has a limited role in the diagnosis of PNETs, intraoperative ultrasound (IOUS) is very sensitive in identifying small PNETs[39], and endoscopic ultrasound (EUS) is a valuable technique for detection, localization and diagnosis through fine needle aspiration of identified lesions[40].

Somatostatin receptor scintigraphy (SRS), also known as an octreotide scan, is a whole body functional imaging study that uses ^{111}indium labeled pentetreotide, a somatostatin analogue. Advantages include identification of unknown metastatic sites and providing important information on functional expression of somatostatin receptors which may guide systemic therapy decisions[41]. Although less available at most institutions compared to SRS, newer functional imaging studies utilizing ^{68}Gallium labeled 1,4,7,10-tetraazacyclododecane-N,N′,N″,N‴-tetraacetic acid-d-Phe(1)-Tyr(3)-octreotide ((68)Ga-DOTA-TOC) show promising results that may be superior to conventional SRS[42,43]. Although standard positron emission technology (PET) with ^{18}flourodeoxyglucose is not typically useful in the diagnosis of NF-PNETs, PET with newer radiolabeled tracers may prove more advantageous[44,45]. In general, well differentiated tumors are positive on Octreotide scan and negative on PET scan, with the opposite being true for poorly differentiated grade 3 tumors[46].

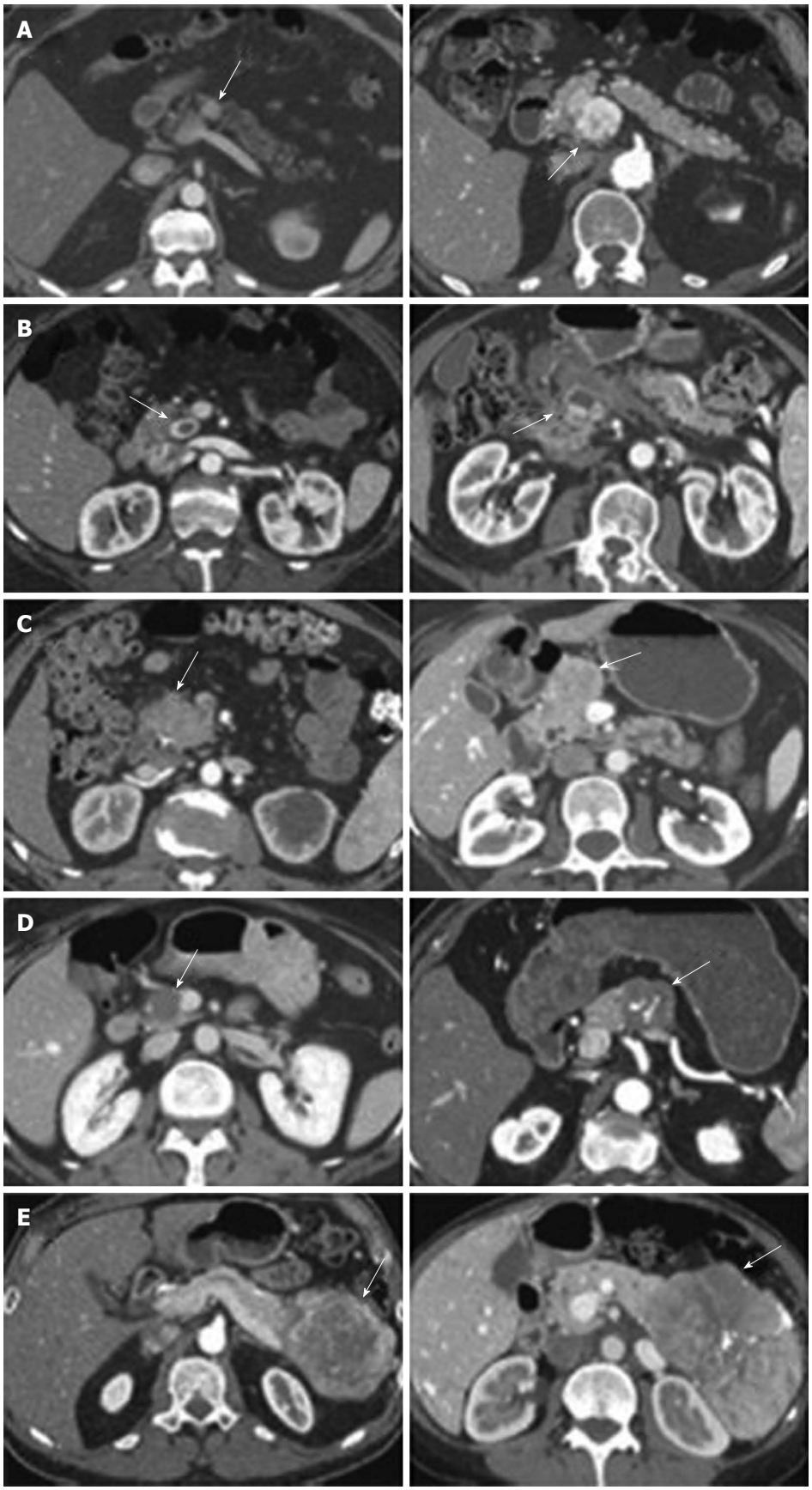


Figure 1 Representative images of the 5 types of pancreatic neuroendocrine tumor enhancement pattern on arterial phase computed tomography. Two images are shown for each type. A: Hyperenhancing, solid; B: Cystic with hyperenhancing rim; C: Isoenhancing or no mass visualized; D: Homogeneously hypoenhancing; E: Heterogeneous but mostly hypoenhancing with some peripheral enhancement. Groups D and E had worse survival after resection compared with groups A, B, and C (From Worhunsky *et al*[35]. Pancreatic neuroendocrine tumours: hypoenhancement on arterial phase computed tomography predicts biological aggressiveness. *HPB* 2014; 16: 304-311). Arrows indicate PNET.

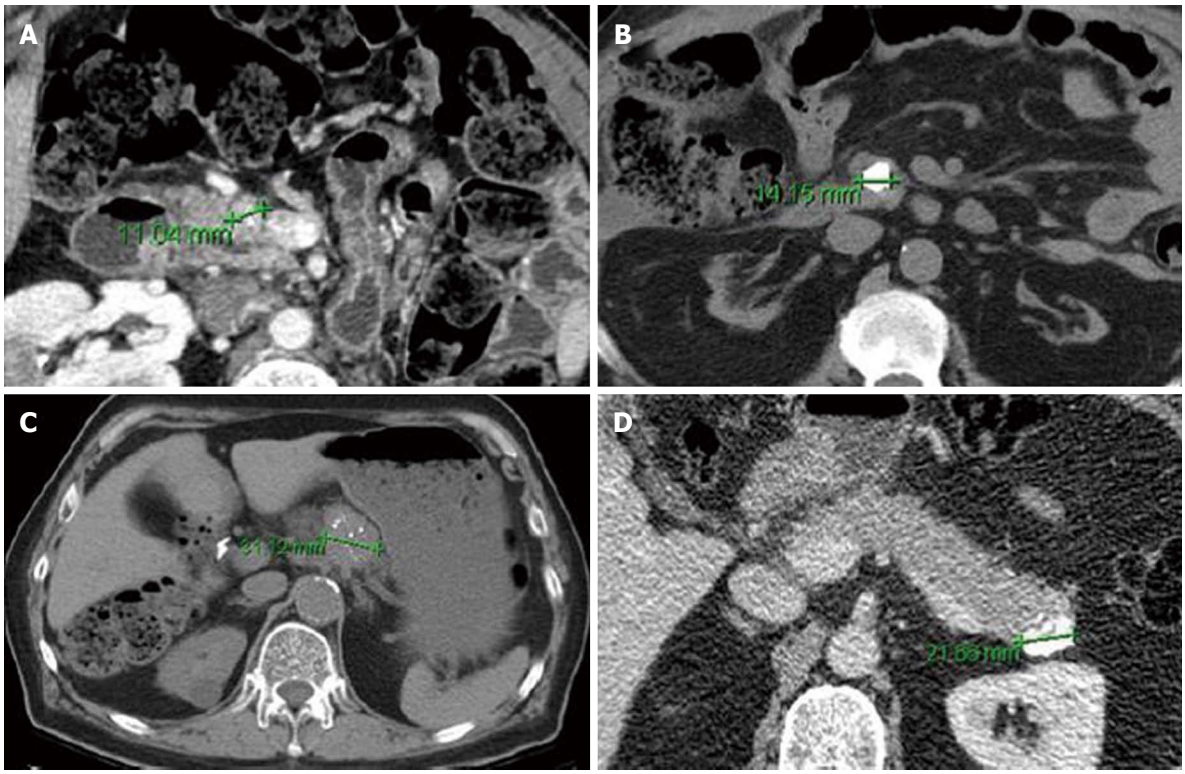


Figure 2 Axial computed tomography images of Pancreatic neuroendocrine tumor with punctate (A, C) and dense/coarse calcifications (B, D). Despite their small size, all lesions were associated with either lymph node metastasis (A-C) or intermediate (G2) grade (B-D) on pathologic evaluation (From Poultsides *et al*[36]. Pancreatic Neuroendocrine Tumors: Radiographic Calcifications Correlate with Grade and Metastasis. *Ann Surg Onc* 2012; 19: 2295-2303).

SURGICAL MANAGEMENT

Primary

Surgery remains the only curative treatment for NF-PNETs and is the mainstay of treatment in most cases. Appropriate candidates who undergo surgery have a significant survival advantage compared with those who do not. Hill *et al*[47] demonstrated a median survival difference of 114 mo *vs* 35 mo for patients who underwent resection compared to those who did not but were recommended to, across all patients with localized, regional and metastatic disease.

The exact surgical management must be individualized for each patient based on their particular tumor and staging. In general, most NF-PNETs should be resected. However, given the increase in incidentally discovered asymptomatic NF-PNETs, there is growing interest in the role of observation for patients with small indolent tumors. Lee *et al*[48] retrospectively analyzed 77 patients who underwent nonoperative observation of small, sporadic NF-PNETs without evidence of local invasion or metastasis. Median initial size was 1.0 cm and there was no documented disease specific progression or mortality during a median follow-up of 45 mo. In addition, Bettini *et al*[49] found that of 51 patients with incidentally diagnosed NF-PNETs < 2 cm, only 6% were malignant and there were no disease specific deaths on long term follow-up. Other population-based analyses have attempted to investigate this question but have been limited by methodological concerns[50-52]. Until better data are available, the ENETS guidelines states that intensive observation could be considered for NF-PNETs < 2 cm but risks and benefits must be carefully weighed in each patient[53].

Small low grade PNETs may safely undergo enucleation regardless of location in the pancreas, provided they are far away from the pancreatic duct and the integrity of this structure can be maintained during enucleation[54]. Enucleation may be performed in an open, laparoscopic or robotic fashion; the technique does not have an appreciable impact on morbidity, mortality, length of hospital stay or survival[55]. For larger or more aggressive NF-PNETs, formal resection is recommended. Tumors in the head of the pancreas typically require pancreaticoduodenectomy (PD) while body and tail lesions may be resected *via* distal pancreatectomy with or without splenic preservation. Distal pancreatectomy can often be performed *via* minimally invasive techniques which are associated with decreased morbidity, operative blood loss and hospital length of stay with similar rates of negative margins[56]. Minimally invasive PD has been slow to gain popularity given its greater learning curve and longer operating times. However, recent evidence suggests that it is a feasible option at select centers with potential benefits in morbidity and perhaps oncologic outcomes[57,58].

Several reports now have stressed the prognostic importance of lymph node involvement in patients

with NF-PNETs[59-61]. Krampitz *et al*[61] found that positive lymph nodes were associated with a shorter time interval to the development of liver metastases, and in long term follow-up, a shorter disease specific survival. Similarly, Hashim *et al*[60] found that lymph node positivity was associated with PNETs of greater size, location in the head, high Ki-67 and with lymphovascular invasion. Furthermore, positive lymph nodes were associated with decreased median disease free survival. These data support the use of routine lymphadenectomy during resection for PNETs. Controversy exists over which lesions may forego lymphadenectomy during simple enucleation. Curran *et al*[59] analyzed the SEER database and found no lymph node metastases in any low grade PNETs < 1 cm. In contrast, Gratian *et al*[50] found that among tumors < 0.5 cm in the national cancer database, 33% presented with regional lymph node metastases and 11% with distant metastases. Formal resection with adjacent lymphadenectomy, as opposed to enucleation, is the procedure of choice for PNETs greater than 2 cm, of higher grade, or with radiographic calcifications.

Several authors have described the role of aggressive extended resections for advanced PNETs[62-65]. For example, Norton *et al*[62] describe acceptable morbidity, low mortality, and excellent overall survival rates, albeit high recurrence rates, in patients with advanced PNETs. Norton *et al*[63] also described good outcomes for patients with PNETs with major vessel involvement undergoing simultaneous vascular reconstruction. Surgeons at experienced hepatopancreaticobiliary centers may follow standard oncologic principles, including multivisceral and vascular resections, in order to accomplish R0 resections.

Liver metastases

All patients with liver metastases from PNETs should be considered for surgical intervention. Although resection can be associated with high recurrence rates, it does improve progression free survival as well as symptom control[66-71]. Saxena *et al*[66] performed a meta-analysis of 1469 GEP-NETs metastatic to the liver and found 3, 5, and 10 year overall survival rates of 83%, 70.5%, and 42%, respectively, following hepatic resection. Predictors of poor outcomes included poor histologic grade, incomplete resection and extrahepatic disease[66]. When patients are not candidates for resection, alternative methods, such as thermal ablation or hepatic artery embolization, are helpful strategies that improve local control and palliate symptoms[67,69,71,72]. Insufficient data exists to recommend one liver-directed strategy over another[73]. Liver transplantation has been described for well selected patients with metastatic GEP-NET[74]. However, liver transplantation for neuroendocrine liver metastases of pancreatic origin is associated with worse overall outcomes and is not typically recommended[75].

In the setting of metastatic disease, controversy remains regarding the role of surgery for the primary tumor[53,76,77]. Capurso *et al*[77] performed a systematic review on this topic and found improved overall survival in patients undergoing resection of the primary in 2 of 3 retrospective cohort studies identified. One potential benefit of removal of the primary tumor is allowing providers to focus treatment on the liver metastatic sites with hepatic artery therapies. Primary tumors that are symptomatic should generally undergo resection for palliation of symptoms[78].

SYSTEMIC THERAPY

The goal of systemic therapy is to prolong survival in patients with recurrence or relapse as well as improve quality of life by controlling symptoms. Currently, there is no evidence to support the use of various systemic modalities in an adjuvant fashion following complete surgical resection of PNETs.

Somatostatin analogues

Nearly 80% of NF-NETs express somatostatin receptors, making them a suitable target for therapy with somatostatin analogues. In addition to their favorable safety profile and effectiveness in controlling symptoms, recent evidence has suggested improvements in oncologic outcomes as well. The PROMID trial[79] was a placebo-controlled double blinded randomized controlled trial (RCT) of long acting release (LAR) octreotide in patients with metastatic well differentiated midgut NETs. Median progression free survival was 14.3 mo in patients receiving octreotide *vs* 6 mo in the placebo group. More recently, the CLARINET trial randomized 204 patients with enteropancreatic NETs to receive long acting lanreotide or placebo and found significantly prolonged progression free survival in the lanreotide group (65.1% *vs* 33.0% at 24 mo); this finding was confirmed in a subset of patients with PNETs (Figure 3)[80].

Radionucleide therapy

Peptide receptor radionuclide therapy (PRRT) also makes use of PNETs' octreotide receptor expression by coupling radionuclides to somatostatin analogues. Typical radionuclides include ^{90}yttrium and ^{177}lutetium. Response rates range only between 10%-40% with toxicity (primarily bone marrow and renal) rates in a similar range so PRRT should be reserved for cases not responsive to less toxic therapies[81-83]. Furthermore, having been pioneered at the Erasmus Medical Center in the Netherlands, PRRT is still only available at select centers in Europe and North America and randomized data are lacking.

Chemotherapy

Indolent and well differentiated NETs are typically resistant to traditional systemic chemotherapy which

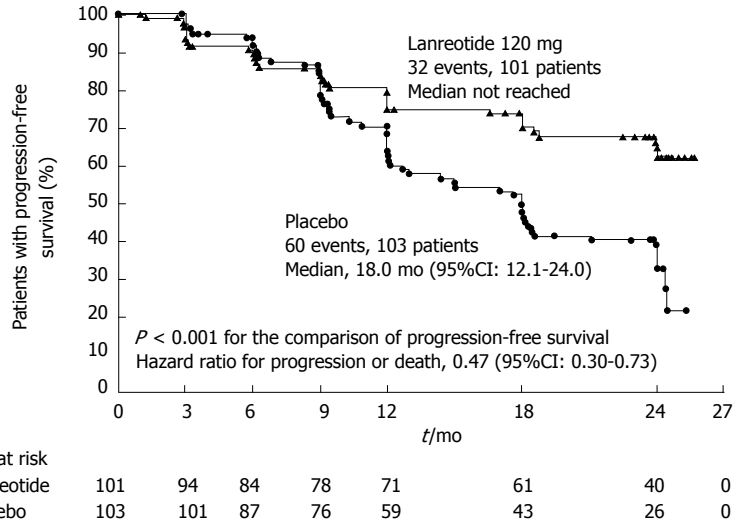


Figure 3 Results of the Clarinet trial which randomized patients with enteropancreatic neuroendocrine tumors to lanreotide *vs* placebo. From: Caplin *et al*[80]. Lanreotide in Metastatic Enteropancreatic Neuroendocrine Tumors. *N Engl J Med* 2014; 371: 224-233.

is therefore reserved for patients with high grade, poorly differentiated tumors or with rapidly progressive unresectable disease[84]. However, NETS of pancreatic origin, generally respond better to chemotherapy than other GEP-NETs. Streptozocin was one of the first agents shown to have activity patients with metastatic PNETs, either as monotherapy or in combination with doxorubicin or fluorouracil[85-87]. Currently, platinum based therapy remains the standard of care for patients with high grade metastatic PNETs. Various combinations exist but the most common regimen utilized consists of cisplatin and etoposide. Nevertheless, data supporting the use of this regimen is limited and more evidence is needed to clarify its role as aggressive first line therapy[88].

More recent research has focused on the use of oral temozolamide with or without capecitabine given its ease of administration and favorable side effect profile. A retrospective review of 30 patients with well or moderately differentiated PNETs treated with this regimen demonstrated a 70% response rate and a median PFS of 18 mo[89]. Additional research is becoming available regarding the safety and efficacy of this regimen[90,91].

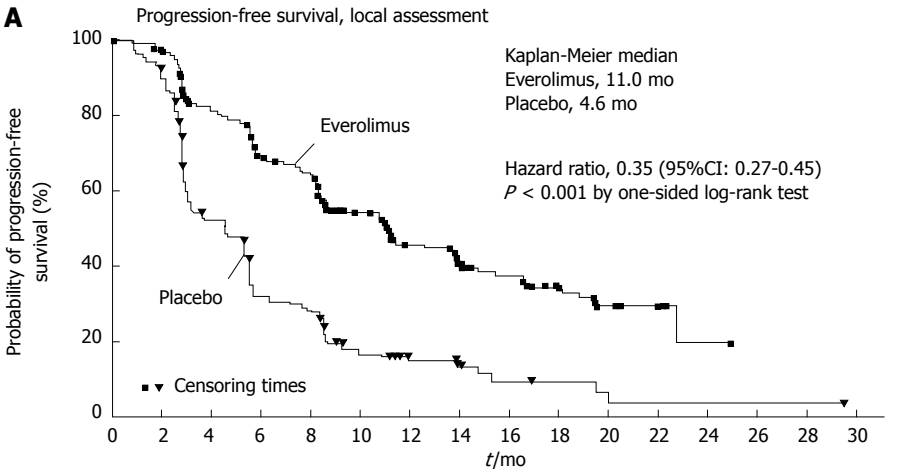
Targeted therapy

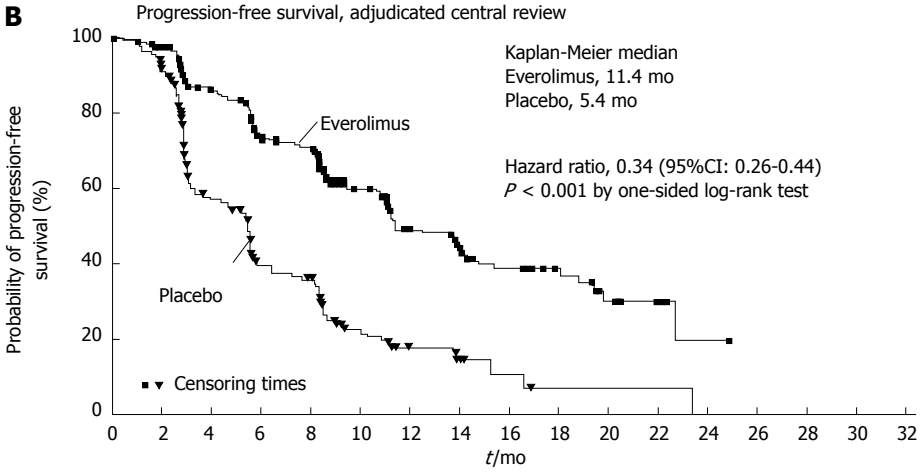
Increasingly, PNETs have been found to be responsive to targeted therapies. The purpose of these molecular agents is to stabilize disease progression in metastatic unresectable cases[92]. Much focus has been placed on Everolimus, an oral mTOR (mammalian target of rapamycin) inhibitor. Previously, Everolimus had been found to have clinical benefit in patients who progressed while on systemic cytotoxic chemotherapy[93]. The RADIANT-3 trial was an international, multisite, RCT comparing daily Everolimus to placebo in patients with low or moderate grade NF-PNETs. Although response rates were low, PFS was longer in the Everolimus group (11.0 mo *vs* 4.6 mo) (Figure 4)[94]. Similarly, the RADIANT-2 trial evaluated Everolimus in conjunction with long acting octreotide and found improved PFS in the Everolimus plus octreotide LAR group *vs* octreotide LAR alone(16.4 mo *vs* 11.3 mo)[95]. Common adverse effects included stomatitis, rash and diarrhea. Some have suggested this regimen should be first line therapy for most NETs[96].

Sunitinib is an oral, small-molecule, tyrosine kinase inhibitor with activity against vascular endothelial growth factor (VEGF) and platelet-derived growth factor (PDGF) both of which are expressed abundantly in PNETs[97]. A placebo controlled, double blind, RCT of daily sunitinib in patients with well-differentiated PNETs with documented disease progression found improved PFS in patients receiving Sunitinib (11.4 mo *vs* 5.5 mo) (Figure 5)[98]. Finally, newer therapies that target the mTOR (*e.g.*, temsirolimus) and VEGF (*e.g.*, bevacizumab) pathways are currently being investigated and hold promise both as single agents and in combination[92,99].

CONCLUSION

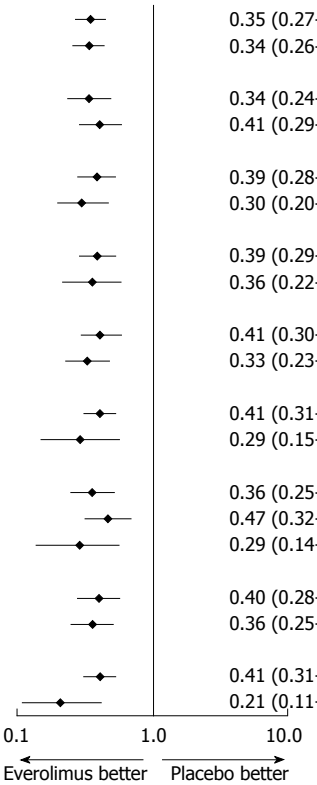
Nonfunctional neuroendocrine tumors of the pancreas are a heterogeneous group of neoplasms that are generally slow growing, however, they may become incurable when they progress to unresectable metastatic disease. Tumors of higher grade can be suspected preoperatively based on the presence of calcifications, hypoenhancement on arterial phase computed tomography, PET avidity and lack or octreotide scan uptake. Surgery is the only curative treatment and is recommended for most patients for whom complete resection is possible. Liver-directed therapies (thermal ablation, transarterial embolization) can be useful in controlling unresectable hepatic metastatic disease. In the presence of unresectable





C Progression-free survival in subgroups

Subgroups	No.	Hazard ratio (95%CI)	*P* value
Local investigator review	410	0.35 (0.27-0.45)	< 0.001
Central adjudicated review	410	0.34 (0.26-0.44)	< 0.001
Previous chemotherapy			
Yes	189	0.34 (0.24-0.49)	< 0.001
No	221	0.41 (0.29-0.58)	< 0.001
WHO performance status			
0	279	0.39 (0.28-0.53)	< 0.001
1 or 2	131	0.30 (0.20-0.47)	< 0.001
Age			
≤ 65 yr	299	0.39 (0.29-0.53)	< 0.001
> 65 yr	111	0.36 (0.22-0.58)	< 0.001
Sex			
Male	227	0.41 (0.30-0.58)	< 0.001
Female	183	0.33 (0.23-0.48)	< 0.001
Race			
White	322	0.41 (0.31-0.53)	< 0.001
Asian	74	0.29 (0.15-0.56)	< 0.001
Region			
America	185	0.36 (0.25-0.52)	< 0.001
Europe	156	0.47 (0.32-0.69)	< 0.001
Asia	69	0.29 (0.14-0.56)	< 0.001
Previous long-acting SSA			
Yes	203	0.40 (0.28-0.57)	< 0.001
No	207	0.36 (0.25-0.51)	< 0.001
Tumor grade			
Well differentiated	341	0.41 (0.31-0.53)	< 0.001
Moderately differentiated	65	0.21 (0.11-0.42)	< 0.001



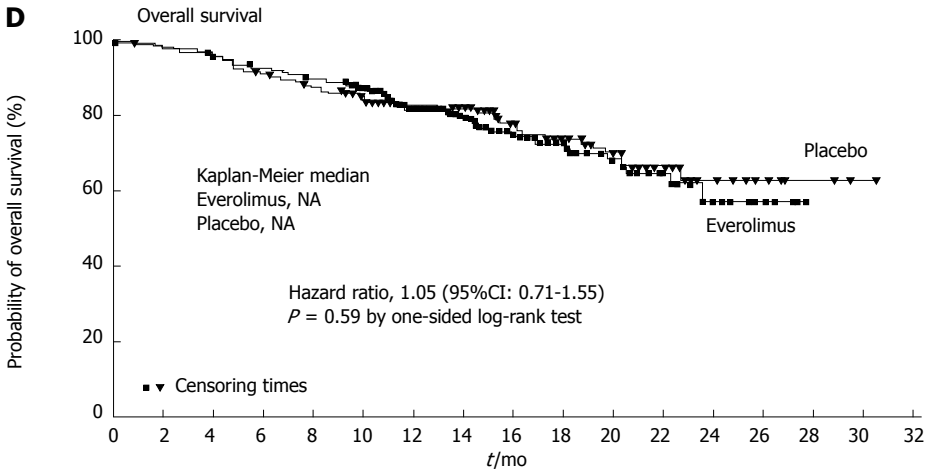
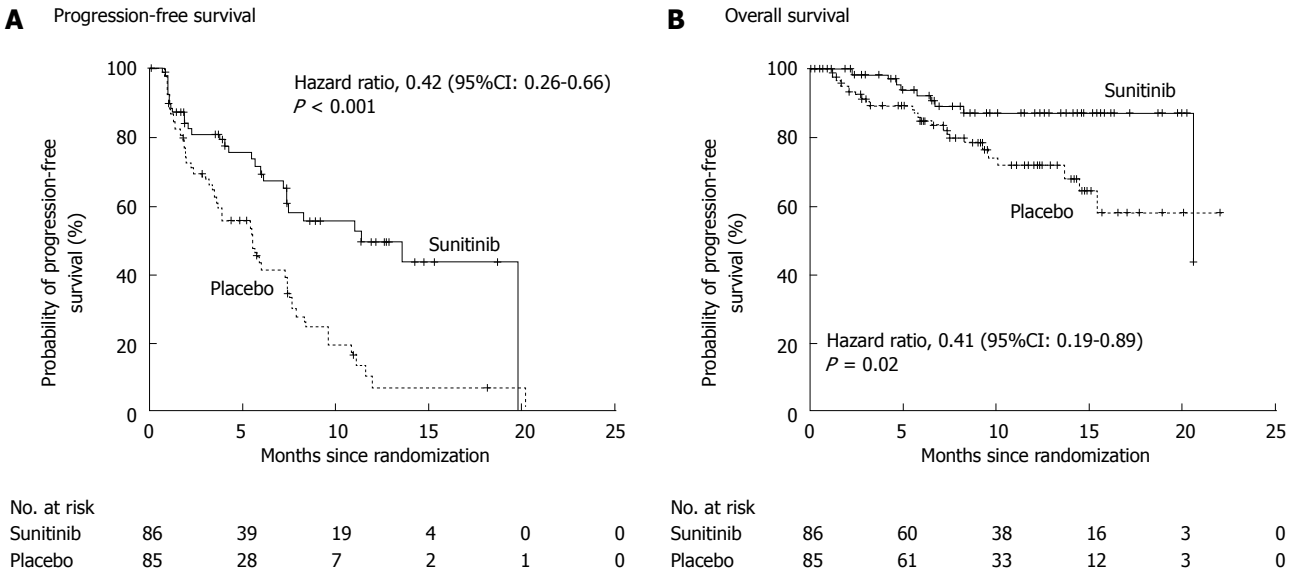


Figure 4 Results of the RADIANT-3 trial which randomized patients with nonfunctional neuroendocrine tumors of the pancreas to Everolimus *vs* placebo. From Yao *et al*[94]. Everolimus for Advanced Pancreatic Neuroendocrine Tumors. *N Engl J Med* 2011; 364: 514-523.



No. at risk						
Sunitinib	86	39	19	4	0	0
Placebo	85	28	7	2	1	0

No. at risk						
Sunitinib	86	60	38	16	3	0
Placebo	85	61	33	12	3	0

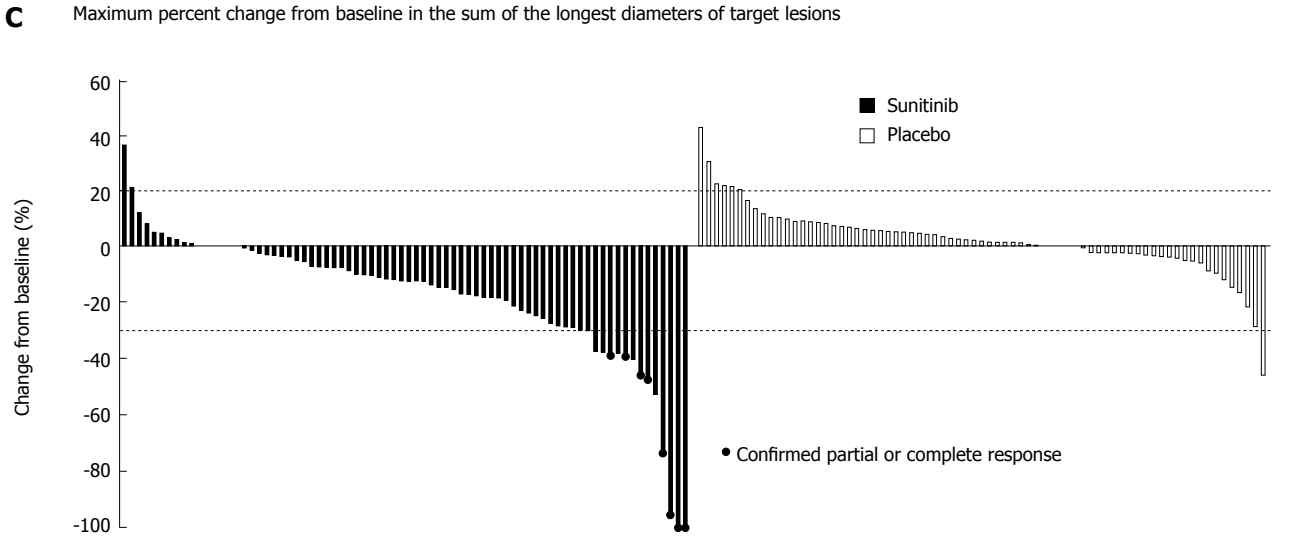


Figure 5 Results of a randomized controlled trial of Sunitinib *vs* placebo for well-differentiated pancreatic neuroendocrine tumors demonstrating (A) progression free survival and (B) overall survival. From: Raymond *et al*[98]. Sunitinib Malate for the Treatment of Pancreatic Neuroendocrine Tumors. *N Engl J Med* 2011; 364: 501-513.

progressive disease, level 1 evidence suggests that somatostatin analogues, everolimus and sunitinib can prolong progression-free survival.

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Interferon-free regimens for the treatment of hepatitis C virus in liver transplant candidates or recipients

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Abstract

The goal of therapy in chronic hepatitis C virus (HCV) infection is sustained virological response (SVR) which reflects HCV eradication. Treatment against HCV has dramatically improved with the recent availability of direct-acting antivirals (DAAs) including sofosbuvir, simeprevir, daclatasvir, ledipasvir/sofosbuvir, paritaprevir/ombitasvir and dasabuvir. Carefully selected combinations of these DAAs offer the potential for highly effective all-oral safe regimens even for patients with decompensated cirrhosis or liver transplant (LT) recipients. Like all current protease inhibitors, simeprevir and paritaprevir should not be used in patients with Child C cirrhosis, while sofosbuvir and ledipasvir/sofosbuvir should not be given in patients with severe renal impairment and glomerular filtration rate less than 30 mL/min. Drug-drug interactions may still occur with the current DAAs particularly in post-LT patients, in whom simeprevir should not be co-administered with cyclosporine and dose adjustments of calcineurin inhibitors are required in case of regimens including the ritonavir boosted paritaprevir. Phase Ⅱ clinical trials and real life cohort studies have shown that sofosbuvir based combinations are safe and can achieve improvements of clinical status, high SVR rates and even prevention of post-LT HCV recurrence in patients with decompensated cirrhosis or LT-candidates. In the post-LT setting, sofosbuvir based regimens and the combination of paritaprevir/ombitasvir and dasabuvir have been reported to be safe and achieve high SVR rates, similar to those in non-transplant

patients, being effective even in cases with cholestatic fibrosing hepatitis. Ongoing clinical trials and rapidly emerging real life data will further clarify the safety and efficacy of the new regimens in these settings.

Key words: Hepatitis C; Direct acting antiviral agents; Liver transplantation; Decompensated cirrhosis; Sofosbuvir

Core tip: Treatment against hepatitis C virus has dramatically improved with the novel direct-acting antivirals (DAAs). The currently available DAAs are sofosbuvir, simeprevir, daclatasvir, ledipasvir/sofosbuvir, paritaprevir/ombitasvir and dasabuvir. IFN-free combinations of these novel DAAs with or without ribavirin give excellent sustained virological response in patients with decompensated cirrhosis awaiting liver transplantation and those with recurrence of hepatitis C post liver transplantation. More data regarding the safety and efficacy of these new DAAs are needed, but ongoing clinical trials and real life data will clarify better these issues.

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INTRODUCTION

Chronic hepatitis C (CHC) has infected approximately 3% of the world population[1]. Patients with hepatitis C virus (HCV) infection can develop cirrhosis and hepatocellular carcinoma (HCC)[2,3], while CHC is considered the leading cause for liver transplantation (LT) in many Western countries[4]. The combination of pegylated interferon-α (pegIFN) and ribavirin (RBV) in patients with CHC had relatively low rates of sustained virological response (SVR)[5,6], but during the last years several direct acting antiviral agents (DAAs) have increased the efficacy of antiviral therapy[7].

The first approved DAAs (boceprevir and telaprevir) were associated with high rates of clinical complications, particularly among cirrhotic patients with serum albumin levels ≤ 3.5 g/dL and platelet counts ≤ 100000/mm^3[8]. Very recently, newer DAAs have been licensed by the European Medicines Agency and Food and Drug Administration to be used mainly as part of IFN-free combinations offering high SVR rates (> 95%), short treatment duration and excellent safety profiles. These agents include sofosbuvir (Sovaldi, Gilead), the first nucleotide analogue NS5B polymerase inhibitor[9], simeprevir (Olysio, Janssen), a second-wave NS3/4A protease inhibitor (achieving SVR in 77%-92% of genotype 1 CHC patients, compared to 46% under pegIFN plus RBV)[10], daclatasvir (Dankliza, Bristol-Myers Squibb)[11], a NS5A inhibitor, the co-formulation of the NS5A inhibitor ledipasvir with sofosbuvir (Harvoni, Gilead)[12], the co-formulation of a ritonavir boosted NS3/4A protease inhibitor, paritaprevir, with the NS5A inhibitor ombitasvir (Viekirax, Abbvie) and dasabuvir (Exviera, Abbvie), a non-nucleos(t)ide NS5B polymerase inhibitor[13] (Table 1). They are all given as one tablet daily, except for paritaprevir/ombitasvir (two tablets once daily) and dasabuvir (1 tablet twice daily). The purpose of this review is to summarize the recent findings concerning the use of the new IFN-free regimens in LT candidates or recipients with CHC.

TREATMENT OF HCV LT CANDIDATES

Post-transplant HCV recurrence is universal[14] and undetectable HCV RNA at the time of LT is crucial for prevention of HCV recurrence and graft loss[15]. At the same time, effective antiviral therapy could improve liver function resulting in some patients withdrawal from the transplant list, similarly to what has been observed in chronic hepatitis B patients on the list for LT. The severity of liver disease is evaluated with the MELD score or the Child-Pugh score classifying patients in class A (score 5-6), B (score 7-9) or C (score 10-15) in relation to best (A), moderate (B), or worse (C) prognosis. However, there are currently rather few data to support such an approach, especially among HCV patients with advanced decompensated cirrhosis (Table 2).

The availability of sofosbuvir started a bright era for treatment of HCV patients on the waiting list for LT. Data from studies in patients with decompensated cirrhosis exist only for sofosbuvir and ledipasvir/sofosbuvir, while real life data from the use of the combinations of sofosbuvir with simeprevir or with daclatasvir were reported recently. Although more studies with DAAs are highly needed in patients with chronic liver impairment and LT candidates, the currently results are very promising and have led in wide use of these agents in this setting.

In the first open label phase Ⅱ study[16], 61 LT candidates due to HCC with well compensated HCV cirrhosis (genotype 1-4, Child-Pugh score ≤ 7, MELD score < 15) commenced on sofosbuvir plus RBV (1000 or 1200 mg/d). Among the 46 patients who received liver graft, 43 (93%) achieved HCV-RNA < 25 IU/mL at the time of liver transplantation. Of these 43 patients, 30 (70%) achieved SVR at 12 wk post-LT, 10 (23%) had HCV recurrence and 3 (7%) died. Only one of the patients with continuously undetectable HCV-RNA for at least one month before LT had recurrence of HCV infection post-LT. Additionally, the tolerance profile was excellent, since only one patient developed severe anemia attributable to RBV administration.

A more recent randomized study[17] evaluated 25

Table 1 Main characteristics of the approved direct acting antivirals that are currently used in interferon-free regimens for the treatment of hepatitis C

DAA (commercial name), dose	Category	Dose adjustment in liver or renal impairment	Antiviral activity	CNIs co-administration	Co-administration should be avoided
Sofosbuvir (Sovaldi®), tablet 400 mg, once daily	NUC NS5B polymerase inhibitor	No change in hepatic impairment Contraindicatedin patients with GFR < 30 mL/min	Genotypes 1-6, High genetic barrier	No change	P-glycoprotein inducers (Anticonvulsants: carbamazepine, oxcarbazepine, phenobarbital, phenytoin; Antimycobacterials: rifampin, rifabutin, rifapentin; St. John's wort; HIV drugs: Tipranavir/ritonavir)
Simeprevir (Olysio®), tablet 150 mg, once daily with food	NS3/4A protease inhibitor	Contraindicated in Child class C No change in renal impairment	Genotypes 1, 4, Low genetic barrier	Contraindicated with cyclosporine	Inhibitors or inducers of CYP3A4 (Antifungals: fluconazole, itraconazole, ketoconazole, posaconazole, voriconazole; Antibiotics: clarithromycin, erythromycin, telithromycin; Dexamethasone; Cicapride; HIV drugs: cobicistat, efavirenz, delavirdine, etravirine, nevirapine, ritonavir and any HIV protease inhibitor) P-glycoprotein inducers
Daclatasvir (Daklinza®), tablet 60 mg, once daily	NS5A inhibitor	No change in liver or renal impairment	Genotypes 1,3,4, Low genetic barrier	No change	Strong inducers of CYP3A4 and/or P-glycoprotein (*e.g.*, phenytoin, carbamazepine, oxcarbazepine, phenobarbital, rifampicin, rifabutin, rifapentine, dexamethasone, St John's wort; HIV drugs: darunavir, lopinavir, etravirine)
Ledipasvir/Sofosbuvir/ (Harvoni®), tablet 90/400 mg, once daily	NUC NS5B polymerase inhibitor + NS5A Inhibitor	No change in hepatic impairment Contraindicated in patients with GFR < 30 mL/min	Genotypes 1,3,4, High genetic barrier	No change	P-glycoprotein inducers, rosuvastatin, simeprevir
Paritaprevir/Ritonavir/ Ombitasvir (Viekirax®), tablet 75/50/12.5 mg, x 2 once daily with food	Ritonavir boosted NS3/4A protease inhibitor/NS5A inhibitor	No safety and efficacy data in Child class B, Contraindicated in Child class C No change in renal dysfunction	Genotypes 1, 4, Genetic barrier depending on HCV genotype	Cyclosporine: 20% of pretreatment total daily dose; tacrolimus: 0.2 mg/72 h or 0.5 mg once weekly	P-glycoprotein inducers, gemfibrozil, lovastatin, simvastatin, oral midazolam, triazolam, pimozide, ethinyl estradiol-containing oral contraceptives, sildenafil for pulmonary hypertension
Dasabuvir (Exviera®), tablet 250 mg, every 12 h	Non-NUC NS5B polymerase inhibitor		Genotype 1, Low genetic barrier		

NUC: Nucleos(t)ide analogue; CNI: Calcineurin inhibitor.

genotype 1-4 CHC patients who received sofosbuvir plus RBV for 48 wk and 25 patients who were observed for 24 wk. All patients were cirrhotics and the Child-Pugh score was up to 10 (80% were Child class A and 20% Child class B/C). The virological response after 3 mo of antiviral treatment was 97%. Interestingly, HCV RNA undetectability appeared to occur more rapidly in patients with Child class A, compared to those with Child class B (at week 2: 56% *vs* 44%; at week 4: 100% *vs* 75%). No treatment breakthroughs were recorded; dizziness and nausea were the most common adverse events, while low rates of discontinuation were observed due to adverse event. Episodes of ascites, as well as hepatic encephalopathy, declined substantially over time in treated patients.

Gane *et al*[18] evaluated 20 patients with CHC genotype 1 and Child class B cirrhosis who received ledipasvir/sofovusvir co-formulation with or without RBV. So far, high SVR rate (89%) with good safety and tolerance and without treatment discontinuations has been reported. Finally, in a study by Flamm *et al*[19], the same combination of ledipasvir/sofosbuvir plus RBV (the latter starting from 600 mg/d) was given for 12 or 24 wk in 108 treatment naive or experienced genotype 1 and 4 patients with decompensated cirrhosis Child class B (n = 55) or C (n = 53)[19]. All patients had no HCC, well preserved renal function (glomerular filtration rate > 40 mL/min) and total serum bilirubin < 10 mg/dL. The authors found that this combination was well tolerated resulting in high SVR rates irrespectively of duration of antiviral therapy (12 wk: 87%, 24 wk: 89%) and with similar efficacy between Child B and C patients (12 wk: 87% *vs* 86%, respectively; 24 wk: 89% *vs* 90%, respectively). Only 4 (4%) patients had serious adverse events.

GUIDANCE FOR THE USE OF IFN-FREE REGIMES IN LIVER TRANSPLANT CANDIDATES

Achievement of undetectable HCV RNA can prevent HCV recurrence post LT. On the other hand, patients with portal hypertension and decompensated

Table 2 Studies of interferon-free regimens for treatment of hepatitis C virus positive liver transplant candidates

Ref.	No. of patients	Severity of liver disease based on CP score	Antiviral scheme (duration)	Virological response
Curry *et al*[16]	61	⩽ 7	SOF + RBV (for 48 wk or until LT)	69% (12 wk after LT)
Afdhal *et al*[17]	40	5-10	SOF + RBV (48 wk)	100% and 93% for Child class A and B, respectively at 24 wk under treatment
Gane *et al*[18]	20	7-9	SOF + Ledipasvir ± RBV (12 wk)	89%
Flamm *et al*[19]	108	Decompensated cirrhosis (CP score range: 7-12)	Ledipasvir/SOF + RBV (12 or 24 wk)	87% and 89% (for 12 and 24 wk, respectively)

CP: Child-Pugh; SOF: Sofosbuvir; RBV: Ribavirin.

cirrhosis may not respond equally well with patients with less advanced disease perhaps due to hepatic changes that could affect the DAAs metabolism and pharmacokinetics. It should be noted that the rates of on-therapy virological responses increase more slowly and the final SVR rates are lower in patients with decompensated cirrhosis compared to those in patients with compensated cirrhosis included in the same studies[17,19,20].

Since not only liver but also renal impairment may be present in patients with decompensated cirrhosis and LT candidates, careful selection of DAAs is required. The agents for this setting should have unchanged pharmacokinetics irrespectively of liver and/or renal dysfunction. Sofosbuvir is cleared through the kidneys and therefore its pharmacokinetic is not affected by the presence of liver impairment[21]. No dose adjustment of sofosbuvir is required in patients with creatinine clearance ⩾ 30 mL/min, but the agent is officially contraindicated in patients with creatinine clearance < 30 mL/min or under hemodialysis[22]. Recent data suggest that sofosbuvir may have acceptable pharmacokinetic in the latter patient subgroup if it is given at a daily dose of 200 mg. Similarly to sofosbuvir, the combination of ledipasvir/sofosbuvir needs no dose adjustment in patients with any liver impairment or with glomerular filtration rate ⩾ 30 mL/min, but it is also contraindicated in cases with glomerular filtration rate < 30 mL/min. Dose adjustment is not needed for any other agent in patients with any degree of liver or renal impairment. However, simeprevir and paritaprevir, as all existing NS3/4 protease inhibitors, are contraindicated in patients with severe liver impairment and Child class C cirrhosis mostly due to safety precautions[23] (Table 1).

All above data indicate that several factors should be taken into account before the choice of IFN-free regimens in the pre-LT setting. Safe combined regimens with high potency and high genetic barrier should be preferred to achieve rapid inhibition of HCV replication and eliminate the selection risk of resistant-associated viral strains. Given the high efficacy of the new regimens even in LT recipients, a key question is whether all HCV LT candidates require antiviral therapy before LT. According to reasonable clinical judgment, patients with chances of liver function improvement may be better treated and perhaps avoid the need for LT, but the treatment indication is debatable for patients who will need LT anyway. Since the latter two subgroups cannot be always differentiated, therapeutic decisions are often individualized.

TREATMENT OF HCV LT RECIPIENTS

The efficacy of previous therapeutic options was rather limited in HCV LT recipients, but the availability of the current DAAs has opened a new era in the management of these patients. The optimal timing for onset of treatment with IFN-free regimens in LT recipients has not been determined, but even the current safe DAAs should be used very cautiously in the early post-transplant period due to the potentially unstable clinical and biochemical patients' status, the possible surgical complications and the effect of heavy immunosuppression. Drug-drug interactions should be also taken into account when DAAs are going to be used by LT recipients who often receive several other medications. In particular for calcineurin inhibitors, there are no interactions with sofosbuvir, ledipasvir/sofosbuvir and daclatasvir. Simeprevir should not be given in patients receiving cyclosporine, while the combination of ritonavir boosted paritaprevir/ombitasvir increase the levels of both tacrolimus and cyclosporine which needs to be reduced to 0.5 mg weekly or 0.2 mg every 72 h for tacrolimus and to 20% of the previous dose for cyclosporine (Table 1).

Two studies have evaluated the use of sofosbuvir and RBV in LT recipients with CHC. Initially, sofosbuvir with RBV plus/minus PegIFN was given for 24-48 wk in 104 LT recipients with HCV fibrosing cholestatic hepatitis or decompensated cirrhosis within a compassionate use program[24]. Sofosbuvir plus RBV (without pegIFN) was given in 80 (77%) patients. Most of the patients had baseline decompensated liver disease with mean bilirubin 3.1 mg/dL and mean albumin 3.1 g/dL. At week 4, 54% of patients had HCV RNA < 25 IU/mL, while 54 (59%) of 92 patients achieved SVR. At the end of follow-up, the clinical condition (defined as liver function improvement and/

Table 3 Studies of sofosbuvir plus simeprevir with or without ribavirin in recipients with hepatitis C recurrence after liver transplantation

Ref.	No. of patients	Patient characteristics	Antiviral Scheme, (duration)	On treatment virological response (%)	SVR (%)
Pungpapong *et al*[28]	55	Fibrosis 3-4: 29%, Decompensated cirrhosis: 4%, Cholestatic recurrence: 15%	SOF + SMV ± RBV (12 wk)	98 (EOT)	91
Brown *et al*[29]	143	Cirrhosis: 60%, MELD score > 10: 14% Previous DAA failure: 9%	SOF + SMV ± RBV (12-24 wk)	-	90
Satokar *et al*[30]	59	Fibrosis F3/F4: 51%, Treatment experience: 71%	SOF + SMV SOF + SMV + RBV SOF + RBV	62 50 43 (4 wk)	-
Gordon *et al*[31]	17	Fibrosis: range: 0-4	SOF + SMV (12 wk)	100 (EOT)	-
Gutierrez *et al*[32]	32	-	SOF + SMV ± RBV (12 wk)	93 (EOT)	-
Punzalan *et al*[37]	10	Median Fibrosis: 2.5, Treatment experience: 40%	SOF + SMV ± RBV (12 wk)	100 (EOT)	100
Lutchman *et al*[33]	41	Treatment experience: 56%	SOF + SMV or SOF + RBV (12-24 wk)	100 (8 wk)	-
Nair *et al*[34]	22	All patients with fibrosis ⩾ 3 or decompensated cirrhosis	SOF + SMV ± RBV (12 wk)	100 (EOT)	-
Ripper *et al*[35]	25	Treatment experience: 64%	SOF + SMV ± RBV (12 wk)	100 (8 wk)	75
O'Dell *et al*[38]	16	-	SOF + SMV ± RBV	100 EOT	100
Alsabbagh *et al*[36]	17	Fibrosis F3-4: 40%, Treatment experience: 41%	SOF + SMV (n = 11) SOF + RBV (n = 6) (24 wk)	100 (4 wk)	-

EOT: End of treatment; CTP: Child-Pugh score; DAA: Direct acting antiviral.

or reduction of decompensation episodes) improved in 59 (57%) and remained stable in 23 (22%) cases. Serious adverse events including ascites, diabetes, neutropenia, hemophagocytic syndrome and bone marrow aplasia, which were usually not related to the study drugs, were noted in 5% of patients and resulted in early treatment discontinuation in 6 patients. Similarly encouraging were reported in two patients with fibrosing cholestatic hepatitis (with genotype 4 and 1a, respectively)[25,26] who were successfully treated with sofosbuvir plus RBV.

In the second open-label study[27], 40 liver transplant recipients with HCV recurrence of any genotype were enrolled 24 wk after LT. Forty percent of the patients had cirrhosis, but none liver decompensation. They were all treated for up to 6 mo with sofosbuvir plus RBV (the latter starting from 400 mg per day). All patients achieved undetectable HCV-RNA by week 4 under treatment, while the SVR rate 4 wk after the end of antiviral therapy was 77% (27/35). No graft dysfunction was recorded and no adjustments of immunosuppressive regimen were needed. Serious adverse events occurred in 15% of patients but they were all unrelated to the study drug. Common side events included fatigue, arthralgia, diarrhea and mild to moderate anemia and occurred in 18% of patients.

The combinations of sofosbuvir with other DAAs have shown excellent SVR rates without serious adverse effects in LT recipients, including prior difficult to treat subgroups such as patients with genotype 1, cirrhosis, previous intolerance or non-response to IFN therapy. The combination of sofosbuvir with simeprevir plus/minus RBV given for 3 mo showed high efficacy in 55 LT recipients with HCV recurrence (advanced fibrosis or cirrhosis: 29%, cholestatic hepatitis: 11%). SVR was observed in 96% of patients with mild to moderate fibrosis and 76% of patients with advanced fibrosis or cirrhosis[28]. Similarly, 90% SVR was observed in the multicenter HCV-TARGET study[29], in which 143 genotype 1-3 LT recipients received sofosbuvir plus simeprevir with or without RBV (60% had cirrhosis, 14% had MELD score > 10 and 9% failure to regimens with a first-generation protease inhibitor). SVR rate was higher in patients without cirrhosis (94% *vs* 86%), with MELD score < 10 (92% *vs* 77%) or with genotype 1b (95% *vs* 83%). In both studies, only mild to moderate adverse events including fatigue, anemia and headache were recorded[28,29]. Finally, sofosbuvir combination with simeprevir with or without RBV have shown good on treatment safety and efficacy in series or small cohort studies, but most of them lack SVR data yet[30-38] (Table 3).

The effectiveness of ledipasvir with sofosbuvir plus RBV for 12 or 24 wk was assessed in 223 recipients with HCV genotype 1 or 4. Of these patients, 111

Table 4 Studies of sofosbuvir plus daclatasvir with or without ribavirin in recipients with hepatitis C recurrence after liver transplantation

Authors	No. of patients	Patient characteristics	Antiviral Scheme (duration)	On treatment virological response (%)	SVR (%)
Leroy *et al*[39], (CUPILT study)	21	FCH: 100%, Treatment experience: 67%	SOF + Daclatasvir ± RBV (n = 13) SOF + RBV (n = 6) (24 wk)	95 (12 wk)	-
Conti *et al*[40]	55	Fibrosis F3-F4: 33%, FCH: 7%	SOF + Daclatasvir (24 wk)	85 (8 wk)	-

FCH: Fibrosing cholestatic hepatitis.

Table 5 Main strategies of interferon-free antiviral combinations in liver transplant candidates and recipients with chronic hepatitis C virus infection

Antiviral scheme (duration)	Antiviral activity
SOF + RBV (× 12 or 24 wk)	Genotype 2 or 3 (perhaps 5, 6)
SOF + Simeprevir ± RBV (× 12 or 24 wk)	Genotypes 1, 4
SOF + Daclatasvir ± RBV (× 12 or 24 wk)	Genotypes 1, 3, 4
Ledipasvir/SOF ± RBV (× 12-24 wk)	Genotypes 1, 3, 4, 6
Paritaprevir/Ritonavir/Ombitasvir + Dasabuvir ± RBV (× 12 or 24 wk)	Genotype 1
Paritaprevir/Ritonavir/Ombitasvir ± RBV (× 12 or 24 wk)	Genotype 4

SOF: Sofosbuvir; RBV: Ribavirin.

were non-cirrhotics, while 51, 52 and 9 cases had Child class A, B, and C cirrhosis, respectively. SVR rates were higher in non-cirrhotics (96%-98%) and patients with Child class A cirrhosis (96%) compared to patients with Child class B (83%-85%) or Child class C cirrhosis (60%-67%). However, there was no significant difference between the 12-wk and 24-wk treatment arms in each patient subgroup[20]. Similarly, the combination of sofosbuvir plus daclatasvir with or without RBV showed good safety and efficacy in LT recipients with advanced liver disease or severe fibrosing cholestatic recurrence from HCV genotype 1-4 in small cohort studies[39,40] (Table 4).

In a phase Ⅱ study, the combination of paritaprevir/ombitasvir plus dasabuvir plus RBV given for 24 wk was evaluated in 34 LT recipients with CHC genotype 1 and mild to moderate fibrosis (11% with cholestatic recurrence). All but one patient (97%) achieved SVR. Immunosuppression dosing was easily manageable over the study period by reducing cyclosporine at 20% of the pretreatment total daily dose and tacrolimus at 0.5 mg once weekly. No graft loss or episode of rejection was recorded with these modifications. In addition, the regimen was generally well tolerated with only one patient discontinuing the study due to skin rash and anxiety.

CONCLUSION

The recent progress in the management of CHC has been outstanding with the benefits being particularly evident in the treatment of patients with advanced disease[41]. In contrast to the pre-DAAs era, both LT candidates and recipients can now be safely and effectively treated (Table 5). Although the high cost of the available DAAs raises discussions and public health debates about their priorities and optimal use worldwide, there is no controversy on the use of the IFN-free regimens in LT candidates and recipients. The most important remaining clinical dilemma remains the need for therapeutic intervention in LT candidates with very advanced liver disease, mostly Child class C cirrhosis. Given the possible complications and the frequent use of other medications, the therapeutic regimens should be carefully selected in this setting. HCV genotype, liver and renal function and co-medications should be always taken into account.

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ORIGINAL ARTICLE

Basic Study

Distinctive roles of unsaturated and saturated fatty acids in hyperlipidemic pancreatitis

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Author contributions: Chang YT and Chang MC contributed to the study concept and design and conducted the experiments; Chang YT, Chang MC, Tung CC, Wei SC and Wong JM analyzed and interpreted the data; Chang YT drafted the manuscript; and Chang YT and Wong JM supervised the study.

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Abstract

AIM: To investigate how the saturated and unsaturated fatty acid composition influences the susceptibility of developing acute pancreatitis.

METHODS: Primary pancreatic acinar cells were treated with low and high concentrations of different saturated and unsaturated fatty acids, and changes in the cytosolic Ca^{2+} signal and the expression of protein kinase C (PKC) were measured after treatment.

RESULTS: Unsaturated fatty acids at high concentrations, including oleic acid, linoleic acid, palmitoleic acid, docosahexaenoic acid, and arachidonic acid, induced a persistent rise in cytosolic Ca^{2+} concentrations in acinar cells. Unsaturated fatty acids at low concentrations and saturated fatty acids, including palmitic acid, stearic acid, and triglycerides, at low and high concentrations were unable to induce a rise in Ca^{2+} concentrations in acinar cells. Unsaturated fatty acids at high concentrations but not saturated fatty acids induced intra-acinar cell trypsin activation and cell damage and increased PKC expression.

CONCLUSION: At sufficiently high concentrations, unsaturated fatty acids were able to induce acinar cells injury and promote the development of pancreatitis. Unsaturated fatty acids may play a distinctive role in the pathogenesis of pancreatitis through the activation of PKC family members.

Key words: Unsaturated fatty acid; Saturated fatty acid; Hypertriglyceridemia; Acute pancreatitis; Calcium

Core tip: The mechanism by which severe hypertriglyceridemia precipitates acute pancreatitis remains unknown. Abnormal sustained elevated cytosolic Ca^{2+} signals, which cause abnormal intracellular enzyme activation, are crucial in the initiation of acute pancreatitis. Unsaturated fatty acids at high concentrations induced a persistent rise in cytosolic Ca^{2+} concentrations in acinar cells and caused intra-acinar cell trypsin activation and cell damage. Unsaturated fatty acids at low concentrations and saturated fatty acids and triglycerides at low and high concentrations were unable to induce a rise in Ca^{2+} concentrations in acinar cells. Unsaturated fatty acids at high concentrations may play a crucial and distinctive role in the pathogenesis of hypertriglyceridemic pancreatitis.

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INTRODUCTION

Hypertriglyceridemia (HTG) is associated with acute pancreatitis (AP) and is observed in 3%-38% of patients with AP[1-6]. HTG is the third-most frequent etiology of AP in Taiwan[7]. Although the mortality rate of hyperlipidemic pancreatitis (HLP) does not differ from that of AP due to other etiologies, the disease severity and complication rates of HLP are higher than those of other forms of AP[8,9].

The mechanism by which severe HTG precipitates AP remains unknown. Havel proposed that pancreatitis results from toxic injury to the capillary endothelium and pancreatic acinar cells through the liberation of free fatty acids (FFA) by pancreatic lipase[10]. This hypothesis was supported by experimental studies by Saharia *et al*[11]; perfusion of the pancreas with FFA induced edema and hemorrhage of the pancreatic preparation. FFA has been reported to induce activation of trypsinogen and to initiate AP[12]. Furthermore, in AP animal models, HTG has been shown to contribute to and accelerate the inflammatory cascade[13]. Previous animal studies revealed that HTG can intensify the course of AP in rats[14] and impair the hemorheology in lipoprotein lipase-deficient mice[15]. However, previous studies are inconclusive with respect to predicting which patients with HTG will develop pancreatitis and why some patients with HTG seldom develop pancreatitis despite the markedly elevated TG level. Further research to understand the pathogenesis of HLP is necessary for the development of specific treatments.

Ca^{2+} signals in the pancreatic acinar cells are generated by the second messengers inositol trisphosphate (IP_3), nicotinic acid adenine dinucleotide phosphate (NAADP) and cyclic ADP-ribose (cADPR). Ca^{2+} signals are modulated in a precise spatiotemporal manner, which is necessary for normal acinar cell secretory function[16]. Abnormal, prolonged elevation of cytosolic Ca^{2+} is the critical trigger of pancreatitis[16]. Abnormal, global, and sustained elevation of cytosolic Ca^{2+} signals cause abnormal intracellular enzyme activation, vacuolization and necrosis, which are crucial in the initiation of AP[17,18]. Another pathological signal that appears to be linked to AP is the activation of protein kinase C (PKC) isoforms[19]. PKC isoforms have been linked to NF-κB expression and zymogen activation[20,21]. Although the involvement of PKC isoforms in NF-κB activation by cholecystokinin (CCK) has been addressed, their roles in the pathogenesis of HLP have not been thoroughly studied.

A previous study demonstrated that different serum FFA compositions in patients with AP were related to the severity and complications of AP[22]. Unsaturated fatty acids, mainly linoleic acid, may be involved in the development of AP complications[22]. In addition, palmitoleic acid causes a dose-dependent increase in cytosolic calcium concentrations[23], and oleic acid at high concentrations induces lactate dehydrogenase leakage from acinar cells and causes an increase in cytosolic calcium concentration[24]. We hypothesized that triglyceride composition, *i.e.*, the saturated and unsaturated fatty acid composition, may influence AP susceptibility in HTG patients. To investigate this hypothesis, we used primary pancreatic acinar cell culture to measure the change in cytosolic Ca^{2+} in acinar cells after treatment with different saturated and unsaturated fatty acids. In this study, we also investigated whether saturated or unsaturated fatty acids activate PKC isoforms in isolated pancreatic acinar cells.

MATERIALS AND METHODS

Reagents

Most of the chemicals, including cerulein, triglyceride, palmitic acid, stearic acid, oleic acid, linoleic acid, palmitoleic acid, docosahexaenoic acid (DHA), arachidonic acid, caffeine, digitonin, rhodamine 100, bis-(CBZ-L-isoleucyl-L-prolyl-L-arginine amide) dihydrochloride, and U-73122, were obtained from Sigma Co Ltd (St Louis, MO, United States). Xestospongin C was obtained from Calbiochem (EMD Millipore Corporation, Billerica, MA, United States). Fura-2 AM was obtained from Invitrogen (Molecular Probes, Eugene, Oregon, United States). The phospho-PKC antibody sampler kit was obtained from Cell Signaling (Danvers, MA, United States).

Isolation of pancreatic acinar cells

This study was conducted at the animal facility of National Taiwan University College of Medicine after approval by the National Taiwan University College of Medicine and College of Public Health Institutional Animal Care and Use Committee (IACUC) (IACUC Approval No. 20080297). Eight-week-old male BALB/c mice were used for this study. All animals were obtained from an inbred population of BALB/c mice maintained at the animal facility of National Taiwan University. Pancreatic tissue was aseptically removed from the BALB/c mice, which were sacrificed humanely by means of CO_2 asphyxiation after overnight fasting. The pancreatic tissue was dissected free of mesenteric fat, minced, washed with Hanks balanced salts solution (HBSS, pH 7.4) with 0.01% soybean trypsin inhibitor and then incubated with 10 mL 0.05% trypsin and 0.25% EDTA for 5 min at 37 ℃. After centrifugation at 200 g for 5 min, the tissue was rinsed with F-12K Nutrient Mixture (Invitrogen/Life Technologies, California, United States) with 1% antibiotics [100 U/mL penicillin-G, 100 μg/mL streptomycin and 0.25 μg/mL FUNGIZONE (Gemini Bio-Products)], 5 mg/mL bovine serum albumin (BSA) and 0.1 mg/mL soybean trypsin inhibitor. The tissue was centrifuged at 200 g for 5 min, and the pellet was resuspended in 12.5 mL of digestive solution containing 1 mg/ml collagenase V (Sigma Chemical Co.) and 0.2 mg/mL BSA in HBSS for 30 min at 37 ℃. The digested tissue was centrifuged at 200 g for 5 min and resuspended in F-12K medium with 10% fetal bovine serum (FBS) (Invitrogen/Life Technologies) twice. The obtained pancreatic acinar cells were resuspended in F-12K medium with 10% FBS and 100 U/mL penicillin-G, 100 μg/mL streptomycin and 0.25 μg/mL FUNGIZONE (Gemini Bio-Products) at 37 ℃ in an atmosphere of 5% CO_2 and 95% air.

Cytosolic Ca^{2+} concentrations in pancreatic acinar cells treated with unsaturated and saturated fatty acids

Freshly isolated mouse pancreatic acinar cells were loaded with 2.5 μmol/L fura-2/AM in loading buffer containing 140 mmol/L NaCl, 5 mmol/L KCl, 1 mmol/L $MgCl_2$, 1 mmol/L $CaCl_2$, 10 mmol/L HEPES, 0.1% BSA and 0.2% glucose (pH 7.4) for 30 min at room temperature. The cell suspension was washed twice and resuspended in loading buffer. The cells were aliquoted into cuvettes in loading buffer containing 1000 mg/dL triglyceride, 10 nmol/L cerulein, saturated fatty acids (0.1 mmol/L palmitic acid and 1.0 mmol/L stearic acid) or unsaturated fatty acids (0.1 mmol/L or 1.0 mmol/L of the following: oleic acid, linoleic acid, palmitoleic acid, DHA, or arachidonic acid). To dissolve the fatty acids, 0.25% chloroform was added to the buffer. Fluorescence was recorded in a mechanically stirred cuvette using an SLM©AMINCO-8100 spectrophotofluorometer (Spectronic Instruments, Rochester, NY). For measurements of $[Ca^{2+}]$, the excitation wavelength was alternated between 340 and 380 nm every second, and fluorescence intensity was monitored at an emission wavelength of 510 nm. The signal ratio (340:380) was determined after autofluorescence correction, and the free intracellular Ca^{2+} concentration was calculated.

Inhibition analysis

For pharmacological analysis, we added caffeine (20 mmol/L), xestospongin C (10 μmol/L), and U-73122 (10 μmol/L) to the cells for 5 min before adding different fatty acids as inhibitors; this allowed us to delineate the targets of the different fatty acids in the calcium signaling pathway of the acinar cells.

Measurement of trypsin activity

The acinar cells were loaded with 10 μmol/L rhodamine 110 bis-(CBZ-L-isoleucyl-L-prolyl-L-arginine amide) dihydrochloride (BZiPAR) at 37 ℃ for 60 min, which is a specific substrate for the serine protease trypsin that becomes fluorescent after cleavage of the two oligopeptide side chains. Activation can be observed by fluorescence of rhodamine 110 at an excitation wavelength of 485 nm. To ensure that the tryptic activity observed was from intracellular enzymes only and not from trypsin released into the extracellular fluid, the cells for these experiments were prepared in solution containing 5 μmol/L soybean trypsin inhibitor. The localization of the trypsin activity was investigated by confocal microscopy (Zeiss, LSM510, Germany).

Preparation of protein extracts and western blot analysis

After treatment with different saturated or unsaturated fatty acids for 30 min, the pancreatic acinar cells were lysed on ice in TM buffer and a cocktail of protease inhibitors (Biochain Newark, United States). The protein concentration of the extracts was determined using the Bio-Rad protein assay (Bio-Rad Laboratories, Hercules, CA). Equal amounts of protein were fractionated by 10% SDS-PAGE and electrophoretically transferred to nitrocellulose membranes. The membranes were blocked with Tris-buffered saline supplemented with 5% nonfat dry milk and antibodies against phospho-PKC-α, -δ, -ε, and -ζ (1:1,000 dilution each) for 1 h at room temperature. The membranes were incubated with secondary antibodies conjugated with horseradish peroxidase for 1 h at room temperature. The blots were developed using an enhanced chemiluminescence detection kit (Pierce, Thermo SCIENTIFIC, Rockford, United States).

RESULTS

Unsaturated fatty acids rather than saturated fatty acids induced sustained elevation of cytosolic Ca^{2+} concentrations in acinar cells

In our experimental acinar cell model, we observed that high concentrations (1 mmol/L) of unsaturated

fatty acids, including oleic acid, linoleic acid, palmitoleic acid, DHA, and arachidonic acid, induced a persistent rise in cytosolic Ca^{2+} concentrations in acinar cells. This rise was similar to that observed with supra-maximal concentrations of CCK in isolated pancreatic acinar cells (Figure 1A-C). However, neither low concentrations (0.1 mmol/L) of unsaturated fatty acids, including oleic acid, linoleic acid, palmitoleic acid, DHA, and arachidonic acid, nor low nor high concentrations of saturated fatty acids, including palmitic acid and stearic acid, were able to induce a rise in Ca^{2+} concentrations in acinar cells (Figure 1B-D). The elevation of cytosolic Ca^{2+} concentrations in acinar cells was dose-dependent on the ratio of unsaturated/saturated fatty acids (Figure 1E-G). High concentrations of triglycerides initially did not cause a rise in cytosolic Ca^{2+} concentrations in acinar cells; however, after long-term incubation with high concentrations of triglycerides, the cytosolic Ca^{2+} concentration in the acinar cells gradually increased (Figure 1H). All experiments were repeated at least three times with the same results.

Inhibition assay

Unsaturated fatty acids at high concentrations induced a persistent rise in cytosolic Ca^{2+} concentrations in acinar cells, similar to that observed with CCK, but this process was not inhibited by the IP_3R antagonists caffeine, xestospongin C or U-73122, which inhibit the hydrolysis of PPI to IP_3 (Figure 2).

Unsaturated fatty acids induced intra-acinar cell trypsin activation

High but not low concentrations of unsaturated fatty acids induced intra-acinar cell trypsin activation, as observed with CCK, whereas neither low nor high concentrations of saturated fatty acids were able to induce this activation and subsequent cell damage and death (Figure 3).

Unsaturated fatty acids induced the expression of PKC isoforms in acinar cells

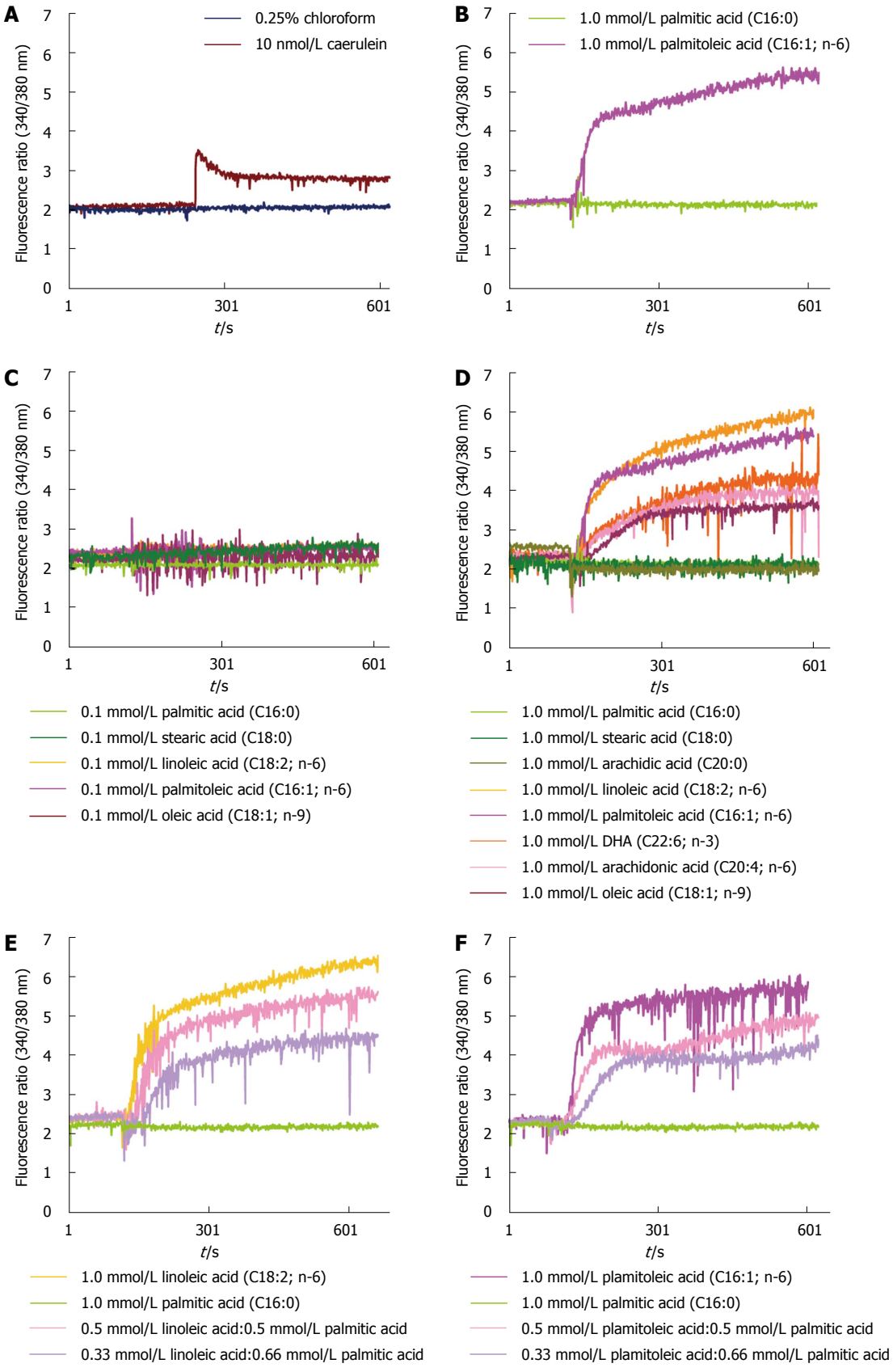
Treatment of acinar cells with high concentrations of fatty acids, induced the expression of PKC-α, -δ, and -ε. This effect was strongest upon treatment with oleic acid, linoleic acid, palmitoleic acid, DHA, and arachidonic acid and weaker with palmitic acid and stearic acid (Figure 4). PKC-ζ expression was only induced by DHA and arachidonic acid (Figure 4).

DISCUSSION

The mechanism by which HTG leads to pancreatitis is not well understood, and it is difficult to predict which hyperlipidemic patients are likely to develop an episode of pancreatitis. Hydrolysis of triglycerides by pancreatic lipase with localized release of large quantities of FFAs has been proposed to be the pathogenic mechanism by which HTG causes pancreatitis. An early feature of AP is the premature intracellular activation of trypsinogen within pancreatic acinar cells. This activation requires a rise in cytosolic Ca^{2+} from intracellular compartments[16]. A previous study reported that different serum FFA compositions in patients with AP were related to the severity and complications of AP[22,25]. Unsaturated fatty acids, mainly linoleic acid, may be involved in the development of complications of AP[22]. In addition, palmitoleic acid causes a dose-dependent increase in cytosolic calcium concentrations[23], and high concentrations of oleic acid induce lactate dehydrogenase leakage from acinar cells as well as an increase in cytosolic calcium concentrations[24]. Our data demonstrate for the first time that high but not low concentrations of unsaturated fatty acids and neither high nor low concentrations of saturated fatty acids induced an elevation of cytosolic Ca^{2+} concentrations in acinar cells as well as intra-acinar cell trypsin activation, cell damage and death. This elevation of cytosolic Ca^{2+} concentrations in acinar cells is dose-dependently related to the ratio of unsaturated/saturated fatty acids. Triglycerides are unable to induce a rise in cytosolic Ca^{2+} concentrations in acinar cells. These results indicate that triglycerides cannot induce an attack of AP. Only when triglycerides are hydrolyzed by lipase into FFAs and the concentration of unsaturated fatty acids is sufficiently high do acinar cells become injured, thereby resulting in pancreatitis. These results provide a potential explanation for why only a portion of HTG patients develop HLP clinically. High levels of unsaturated fatty acids may play a crucial role in the pathogenesis of HLP.

In pancreatic acinar cells, Ca^{2+} release from the endoplasmic reticulum (ER) is mediated by IP_3, cADPR and NAADP and primarily occurs through the activation of inositol trisphosphate receptors (IP_3Rs) and ryanodine receptors (RyRs)[16]. Caffeine and xestospongin C exhibit inhibitory effects on IP_3Rs on the ER membranes of calcium stores, and U-73122 inhibits the hydrolysis of PPI to IP_3. However, pretreatment of acinar cells with these inhibitors failed to block the persistent elevation of cytosolic Ca^{2+} observed in acinar cells exposed to high concentrations of unsaturated fatty acids. These results imply that the elevation of cytosolic Ca^{2+} concentrations in acinar cells induced by high concentrations of unsaturated fatty acids does not occur through activation of IP_3Rs. Criddle *et al*[26] reported that the unsaturated fatty acid palmitoleic acid can elicit prolonged elevation of the global cytosolic Ca^{2+} concentrations and that this elevation cannot be inhibited by IP3 receptor blockade, largely due to the inhibition of mitochondrial ATP production. The mechanism by which unsaturated fatty acids induce pathological Ca^{2+} release in acinar cells from the ER or other acidic stores requires further investigation.

Several studies have demonstrated that PKC isoforms modulate pathological secretion during AP


A
0.25% chloroform
10 nmol/L caerulein
B
1.0 mmol/L palmitic acid (C16:0)
1.0 mmol/L palmitoleic acid (C16:1; n-6)
C
0.1 mmol/L palmitic acid (C16:0)
0.1 mmol/L stearic acid (C18:0)
0.1 mmol/L linoleic acid (C18:2; n-6)
0.1 mmol/L palmitoleic acid (C16:1; n-6)
0.1 mmol/L oleic acid (C18:1; n-9)
D
1.0 mmol/L palmitic acid (C16:0)
1.0 mmol/L stearic acid (C18:0)
1.0 mmol/L arachidic acid (C20:0)
1.0 mmol/L linoleic acid (C18:2; n-6)
1.0 mmol/L palmitoleic acid (C16:1; n-6)
1.0 mmol/L DHA (C22:6; n-3)
1.0 mmol/L arachidonic acid (C20:4; n-6)
1.0 mmol/L oleic acid (C18:1; n-9)
E
1.0 mmol/L linoleic acid (C18:2; n-6)
1.0 mmol/L palmitic acid (C16:0)
0.5 mmol/L linoleic acid:0.5 mmol/L palmitic acid
0.33 mmol/L linoleic acid:0.66 mmol/L palmitic acid
F
1.0 mmol/L plamitoleic acid (C16:1; n-6)
1.0 mmol/L palmitic acid (C16:0)
0.5 mmol/L plamitoleic acid:0.5 mmol/L palmitic acid
0.33 mmol/L plamitoleic acid:0.66 mmol/L palmitic acid
Fluorescence ratio (340/380 nm)
t/s
0
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1
301
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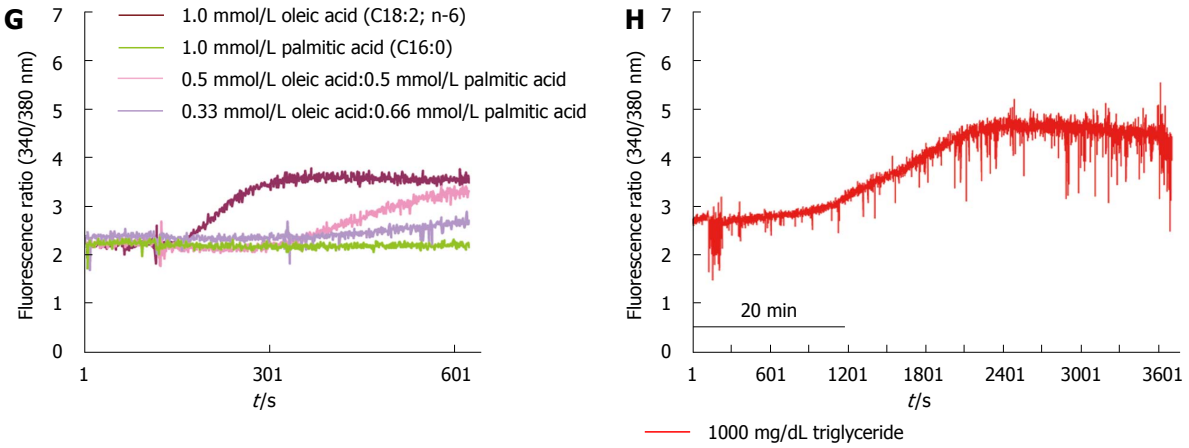


Figure 1 Effects of unsaturated and saturated fatty acids on cytosolic Ca^{2+} concentrations in acinar cells. A: A 0.25% chloroform and 10 nmol/L cerulein solution was used as the control; B: A high concentration (1.0 mmol/L) of the unsaturated fatty acid palmitoleic acid, but not of the saturated fatty acid palmitic acid, induced a sustained cytosolic Ca^{2+} elevation; C: Various unsaturated fatty acids and saturated fatty acids at low concentrations (0.1 mmol/L) did not induce sustained cytosolic Ca^{2+} elevations; D: Various unsaturated fatty acids at high concentrations (1.0 mmol/L), but not saturated fatty acids, induced sustained cytosolic Ca^{2+} elevations. The effects of different ratios of unsaturated fatty acids and saturated fatty acids on the elevation of cytosolic Ca^{2+} concentrations: linoleic acid and palmitic acid (E), palmitoleic acid and palmitic acid (F), and oleic acid and palmitic acid (G); H: High concentrations (1000 mg/dL) of triglycerides did not induce the elevation of cytosolic Ca^{2+} concentrations.

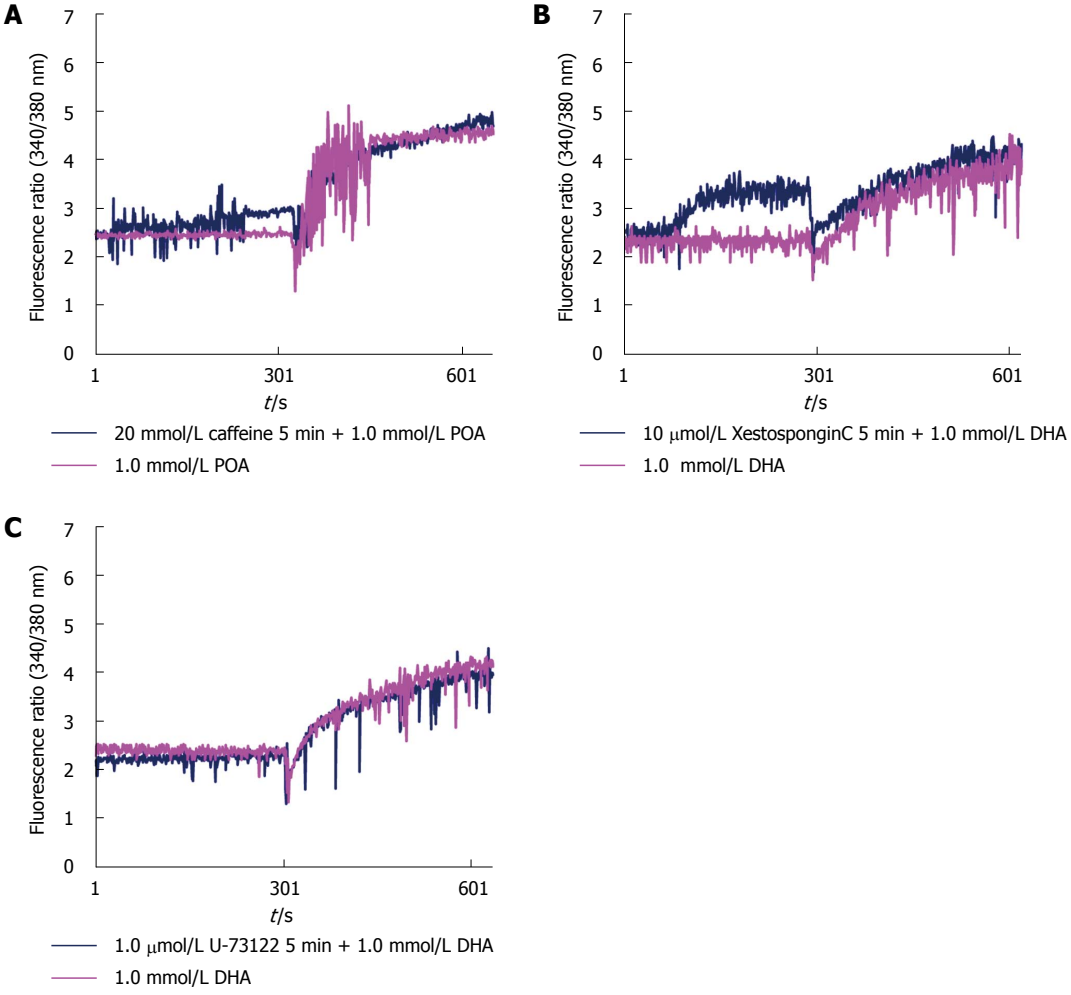
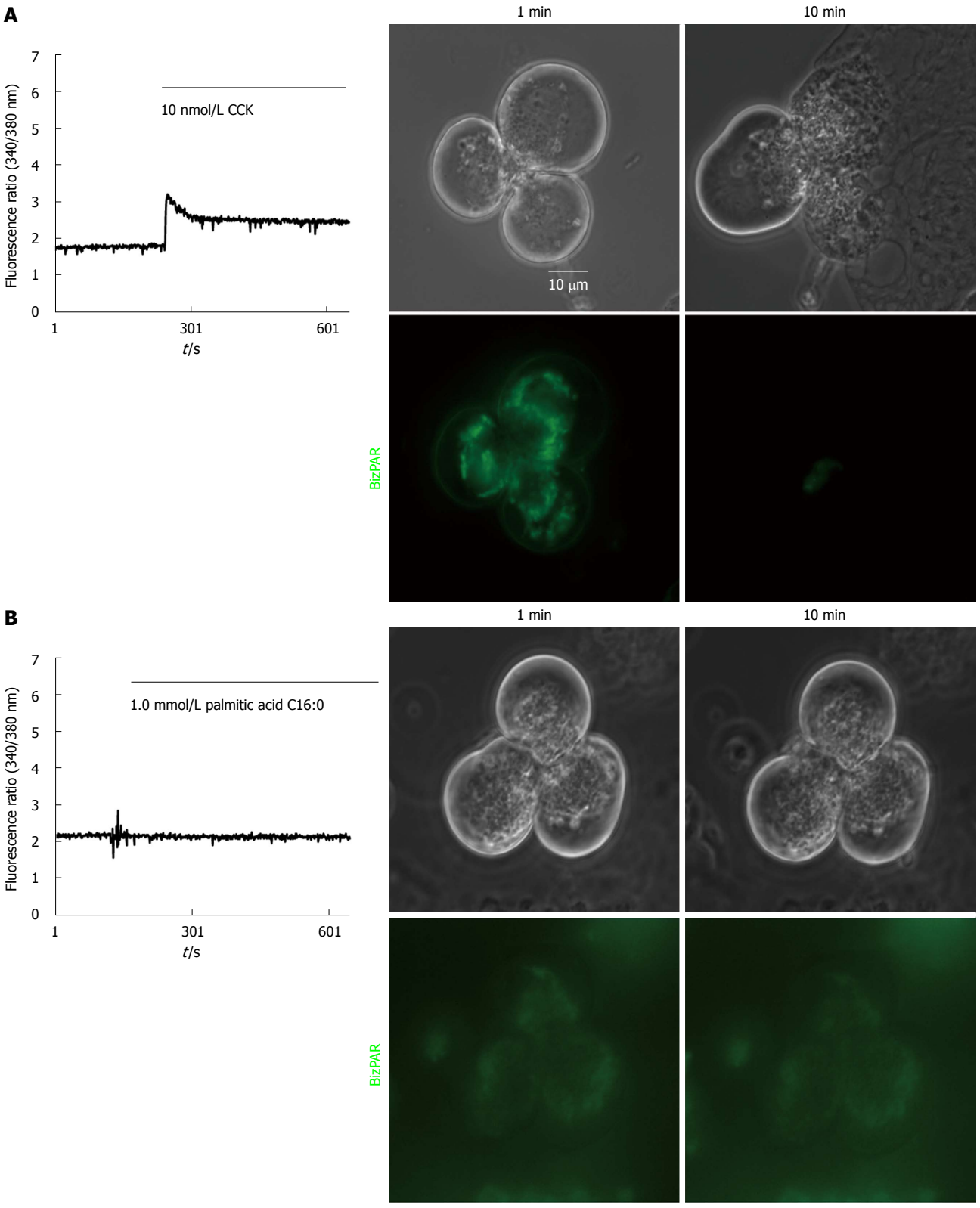


Figure 2 Inhibition of IP_3R did not prevent the elevation of cytosolic Ca^{2+} concentrations in acinar cells induced by unsaturated fatty acids: caffeine (A), xestospongin C (B), and U-73122 (C).


A
1 min
10 min
Fluorescence ratio (340/380 nm)
10 nmol/L CCK
t/s
10 μm
BizPAR
B
1 min
10 min
Fluorescence ratio (340/380 nm)
1.0 mmol/L palmitic acid C16:0
t/s
BizPAR

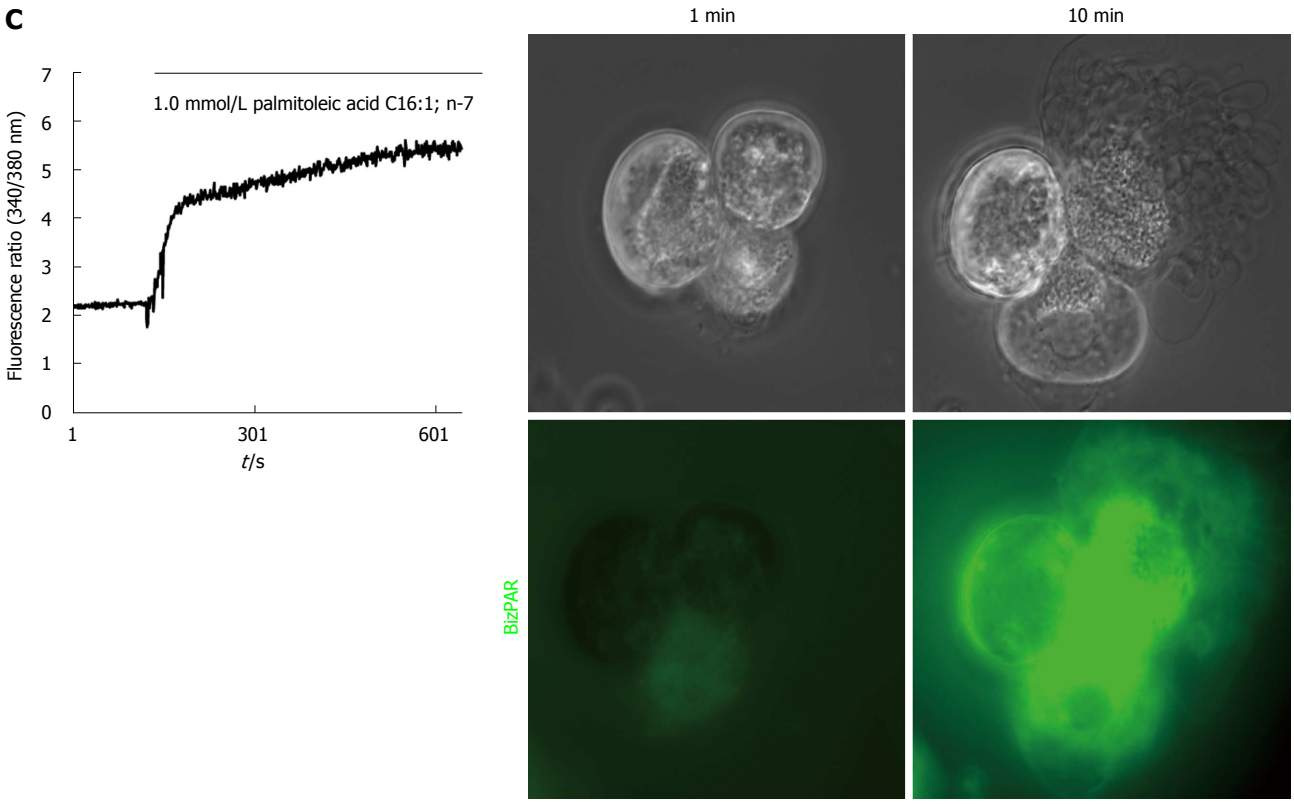


Figure 3 Unsaturated fatty acids at high concentrations (1.0 mmol/L), but not saturated fatty acids, induced intra-acinar cell trypsin activation and cell damage observed by confocal microscopy: CCK (A), palmitic acid (B), and palmitoleic acid (C).

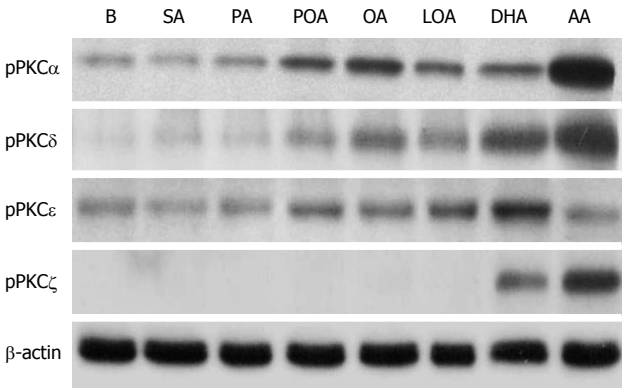


Figure 4 Effects of unsaturated fatty acids at high concentrations (1.0 mmol/L) on protein kinase C isoform expression in acinar cells as detected by western blot. B: Blank; SA: Stearic acid; PA: Palmitic acid; POA: Palmitoleic acid; OA: Oleic acid; LA: Linoleic acid; DHA: Docosahexaenoic acid; AA: Arachidonic acid.

and also regulate the expression of inflammatory mediators[19,20,27]. In pancreatic acinar cells, the conventional PKC-α, the novel PKC-δ and -ε and the atypical PKC-ζ isoforms have been identified[28]. PKC-δ has been shown to participate in premature zymogen activation within pancreatic acinar cells and in activation of the transcription factor NF-κB during experimental pancreatitis[19,29]. PKC-δ is activated and translocated to the plasma membrane and participates in amylase secretion[30], regulates protease activation[31] and modulates inflammatory molecule expression in pancreatic acinar cells[32]. The initiation of AP requires both zymogen activation and the retention of active enzymes in acinar cells. Fatty acids have been shown to increase PKC activity in HTG and AP animal models[33]. In our study, we found that unsaturated fatty acids at high concentrations upregulated the expression of the PKC isoforms PKC-α, PKC-δ, PKC-ε and atypical PKC-ζ in mouse pancreatic acinar cells. Previous studies reported that supraphysiological concentrations of CCK cause activation of the novel PKC isoforms δ and ε and the atypical PKC isoform ζ as well as the activation of NF-κB[34]. Unsaturated fatty acids at high concentrations have a similar effect to that of CCK at supraphysiological concentrations with respect to the activation of PKC-α, -δ and -ζ, implying that HLP may be triggered by unsaturated fatty acids and through the activation of specific isoforms of PKC. In a recent study, Cui *et al*[35] reported that FFAs induced ER stress by enhancing Ca^{2+} influx in pancreatic β cells, which contributed to β cell dysfunction and cell apoptosis. In addition, in an arginine model of experimental AP, ER stress sensing and signaling mechanisms were reported to be activated in acinar cells early in the development of AP[36]. Furthermore, HTG was reported to aggravate ER stress[37]. Further studies of the roles and effects of unsaturated fatty acids at high concentrations in key pathogenic cellular events, such as calcium release from acidic stores, mitochondrial dysfunction, ER stress, autophagy, impaired trafficking, and lysosomal

and secretory responses in AP are needed.

In conclusion, our *in vitro* results provide an explanation for the clinical observation that only a portion of HTG patients develop AP and that some patients with HTG seldom develop pancreatitis despite marked elevation of triglyceride level. Triglycerides were unable to induce an attack of AP. Only when triglycerides are hydrolyzed by lipase into FFAs and the concentration of unsaturated fatty acids is sufficiently high do acinar cells become injured, thereby resulting in pancreatitis. Unsaturated fatty acids may play a distinctive role in the pathogenesis of HLP through the activation of PKC family members. Further analysis of the composition of unsaturated/saturated fatty acids in acute phase sera of patients with HLP are needed. In addition, consumption of food containing different types of fat might be a strategy to reduce the risk of the development of AP in HTG patients.

COMMENTS

Background

Hypertriglyceridemia (HTG) is the third-most frequent etiology of acute pancreatitis (AP) in Taiwan. The detailed mechanism by which severe HTG precipitates AP remains unknown. Clinically, it is difficult to predict which patients with HTG will develop pancreatitis and why some patients with HTG seldom develop pancreatitis despite the markedly elevated TG level.

Research frontiers

Different serum free fatty acid (FFA) compositions in patients with AP were related to the severity and complications of AP. Unsaturated fatty acids, mainly linoleic acid, may be involved in the development of AP complications.

Innovations and breakthroughs

When triglycerides are hydrolyzed by lipase into FFAs and the concentration of unsaturated fatty acids is sufficiently high do acinar cells become injured, thereby resulting in pancreatitis. Unsaturated fatty acids may play a distinctive role in the pathogenesis of HLP.

Applications

Consumption of food containing different types of fat might be a strategy to reduce the risk of the development of AP in HTG patients.

Terminology

HTG is defined by fasting serum triglyceride level of > 150 mg/dL. HTG is considered a risk for pancreatitis when levels are > 1000 mg/dL.

Peer-review

This is a well conducted study that has resulted in a well written manuscript that helps answer a question about the aetiology of hypertryglyceride induced induced acute pancreatitis. The manuscript is worthy of publication but the introduction and discussion could both be reduced in length without any adverse effect on the paper. The authors might also wish to consider how they can demonstrate similar processes are occurring in a human population.

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ORIGINAL ARTICLE

Basic Study

Establishment of a hepatic cirrhosis and portal hypertension model by hepatic arterial perfusion with 80% alcohol

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Author contributions: Wang L and He FL contributed equally to this study; Liu FQ designed the research; Wang L performed the research; He FL contributed new reagents or analytic tools; Zhao HW analyzed the data; and Wang L wrote the paper.

Institutional review board statement: The study was reviewed and approved by the hospital Institutional Review Board.

Conflict-of-interest statement: The authors have no conflict of interest.

Data sharing statement: No additional data are available.

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Abstract

AIM: To determine the feasibility and safety of establishing a porcine hepatic cirrhosis and portal hypertension model by hepatic arterial perfusion with 80% alcohol.

METHODS: Twenty-one healthy Guizhou miniature pigs were randomly divided into three experimental groups and three control groups. The pigs in the three experimental groups were subjected to hepatic arterial perfusion with 7, 12 and 17 mL of 80% alcohol, respectively, while those in the three control groups underwent hepatic arterial perfusion with 7, 12 and 17 mL of saline, respectively. Hepatic arteriography and direct portal phlebography were performed on all animals before and after perfusion, and the portal venous pressure and diameter were measured before perfusion, immediately after perfusion, and at 2, 4 and 6 wk after perfusion. The following procedures were performed at different time points: routine blood sampling, blood biochemistry, blood coagulation and blood ammonia tests before surgery, and at 2, 4 and 6 wk after surgery; hepatic biopsy before surgery, within 6 h after surgery, and at 1, 2, 3, 4 and 5 wk after surgery; abdominal enhanced computed tomography examination before surgery and at 6 wk after surgery; autopsy and multi-point sampling of various liver lobes for histological examination at 6 wk after surgery.

RESULTS: In experimental group 1, different degrees of hepatic fibrosis were observed, and one pig developed hepatic cirrhosis. In experimental group 2, there were cases of hepatic cirrhosis, different degrees of increased portal venous pressure, and intrahepatic portal venous bypass, but neither extrahepatic portal-systemic bypass circulation nor death occurred. In experimental group 3, two animals died and three

animals developed hepatic cirrhosis, and different degrees of increased portal venous pressure and intrahepatic portal venous bypass were also observed, but there was no extrahepatic portal-systemic bypass circulation.

CONCLUSION: It is feasible to establish an animal model of hepatic cirrhosis and portal hypertension by hepatic arterial perfusion with 80% alcohol, however, the safety of this model depends on a suitable perfusion dose.

Key words: Alcohol; Hepatic arterial perfusion; Hepatic cirrhosis; Portal hypertension; Animal model

Core tip: We successfully established a porcine hepatic cirrhosis and portal hypertension model by hepatic arterial perfusion with 80% alcohol. The model accurately reproduced the pathophysiological development process similar to that occurring in humans.

Wang L, He FL, Liu FQ, Yue ZD, Zhao HW. Establishment of a hepatic cirrhosis and portal hypertension model by hepatic arterial perfusion with 80% alcohol. *World J Gastroenterol* 2015; 21(32): 9544-9553 Available from: URL: http://www.wjgnet.com/1007-9327/full/v21/i32/9544.htm DOI: http://dx.doi.org/10.3748/wjg.v21.i32.9544

INTRODUCTION

Hepatic cirrhosis is most commonly caused by viral hepatitis and alcohol abuse[1]; the 5- and 10-year survival rates of patients with compensated hepatic cirrhosis are 84% and 68%, respectively, while the 5-year survival rate of decompensated hepatic cirrhosis is only 14%[2]. According to the World Health Organization (WHO), about 800000 patients die of hepatic cirrhosis each year worldwide[3]. Thus, in order to develop new strategies for the diagnosis and treatment of hepatic cirrhosis, it is essential, despite being a major challenge, to establish large animal models similar to humans, which are applicable in all-round research, especially models of hepatic cirrhosis for application in surgery, intervention and pharmaceutical research. Currently, the following methods are widely used to establish animal models of hepatic cirrhosis or portal hypertension: carbon tetrachloride (CCl_4); thioacetamide; surgical prehepatic portal ligation; and combinations of these methods. However, no approach is capable of fully simulating the entire pathophysiological process most frequently involved in the occurrence and development of posthepatitic and alcoholic cirrhosis[4]. In the present study, we successfully established a porcine hepatic cirrhosis and portal hypertension model through hepatic arterial perfusion with alcohol, which accurately reproduced the pathophysiological development process similar to that occurring in humans. The study results are reported below.

MATERIALS AND METHODS

Materials

A total of 21 healthy Guizhou miniature pigs (male and female, 3-mo-old, 30-35 kg) were randomly assigned to six groups: experimental group 1 (n = 5), experimental group 2 (n = 5), experimental group 3 (n = 5), control group 1 (n = 2), control group 2 (n = 2) and control group 3 (n = 2). An intravenous channel was established *via* ear vein puncture, and an injection of propofol (Xi'an Libang Pharmaceuticals Co., Ltd., China) 2.5-3.5 mg/kg was used for combined anesthesia.

Surgical procedure

Percutaneous puncture of the porcine femoral artery or the external iliac artery was successfully performed using the Seldinger method under general anesthesia. A 5F or 6F arterial sheath (Avanti+, Cordis) was applied, followed by insertion of a 4F Cobra or hepatic catheter (Cordis, Miami, FL, United States) *via* the arterial sheath. Finally, one-step percutaneous portal vein puncture was successfully performed using a hepatic puncture needle (Angiotech, Vancouver, BC, Canada), followed by insertion of a 6F multi-sidehole pigtail catheter (Drainage Catheter-Locking Pigtail, Angiotech) into the portal vein, with its exposed part subcutaneously fixed into the lateral abdominal wall of the pig. Animals in the three experimental groups were perfused with 7, 12 and 17 mL of 80% alcohol (Beijing ZhenYuMinSheng Pharmaceutical Co., Ltd, Beijing, China), respectively, prepared using saline, *via* the common hepatic artery (the catheter was delivered avoiding the cystic and gastroduodenal arteries), while the animals in the three control groups were subjected to hepatic arterial perfusion with 7, 12 and 17 mL of saline, respectively. Before and after perfusion, hepatic arteriography was performed by means of DSA SimenziArtis using 9-15 mL Ultravist 370 (Bayer, Leverkusen, Germany) at 3 mL/s and 15 frames/s. Before perfusion, immediately after perfusion, and at 2, 4 and 6 wk after perfusion, portal phlebography was performed using 10-15 mL Ultravist 370 at 10 mL/s and 15 frame/s, and the portal venous pressure and diameter were then measured. If direct portal vein puncture failed, indirect portal phlebography was performed *via* the splenic artery using 10-15 mL Ultravist 370 at 2 mL/s and 15 frames/s, and the location of the portal vein was marked simultaneously, and direct portal vein puncture was performed thereafter with reference to this location. The following procedures were performed at different time points: routine blood sampling,

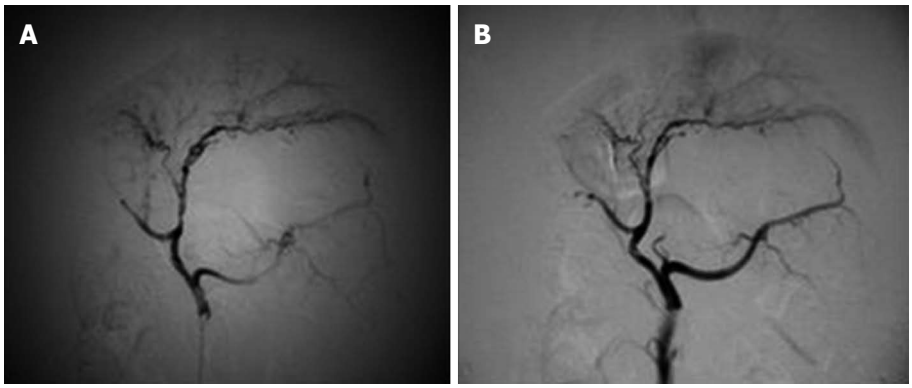

Figure 1 Hepatic artery variation. A: Hepatic arteriography of experimental group 2 before perfusion showed good distribution of hepatic arteries and their branches; B: After hepatic arterial perfusion in experimental group 2, small peripheral branches of hepatic arteries were significantly reduced compared with before perfusion.

biochemistry (liver function), blood coagulation and blood ammonia tests before surgery, and at 2, 4 and 6 wk after surgery; and multi-site, multi-point hepatic biopsies before surgery, within 6 h after surgery, and at 1, 2, 3, 4, and 5 wk after surgery (hepatic tissues collected by biopsy were subjected to pathological examination and METAVIR scoring). The METAVIR scoring criteria were as follows: 0 = no fibrosis; 1 = fibrosis without septum; 2 = fibrosis with a few septa; 3 = bridging fibrosis with numerous septa; 4 = cirrhosis. Abdominal enhanced CT examination was performed before surgery, and at 6 wk after surgery; and autopsy was conducted at 6 wk after surgery. The liver, spleen, esophagus, stomach, heart, lungs and kidneys were removed at autopsy and then fixed with 10% formalin for pathological examination. Multi-point histological samples of various liver lobes were obtained and evaluated by the following methods: firstly, samples were stained with hematoxylin, eosin and Masson's trichrome stain, followed by observation under light microscope; and secondly, the degree of hepatic fibrosis was classified into levels 0 to 4 using image analysis software (0, no fibrosis; 1, < 10% fibrosis; 2, 10%-20% fibrosis; 3, 21%-50% fibrosis; 4, ⩾ 51% fibrosis). And 1000 mg of cefminox sodium (BBCA Pharmaceutical, Hefei, China) was injected intravenously during surgery, 0.5 mg cefadroxil tablets (Qingyuan Huaneng Pharmaceutical Co., Ltd., Guangdong, China) were orally administered at a dose of 1 tablet/day for 2 wk, commencing 12 h after surgery, and enteric-coated aspirin tablets (Bayer) were orally administered at a dose of 100 mg/d for 6 wk for post-operative anticoagulation.

Statistical analysis

SPSS 17.0 software was used for statistical analyses. The *t*-test was employed for comparison of quantitative data, and either the χ^2 test or Fisher's exact test was used for comparison of qualitative data. A *P*-value < 0.05 was considered statistically significant.

RESULTS

Imaging findings

Hepatic arteriography: Before and after perfusion, there were no significant vascular changes in three pigs from experimental group 1 or in any of the pigs from the control groups. After perfusion, a slight decrease in peripheral branches of the hepatic artery (< level 4) was observed in two pigs from experimental group 1 and in one pig from experimental group 2, while there was a marked reduction in peripheral branches of the hepatic artery (< level 3) in four pigs from experimental group 2 and all pigs from experimental group 3 (Figure 1A, B).

Changes in the portal vein: Immediately after perfusion, no changes in portal venous branches were observed in three animals from experimental group 1 or in any of the pigs from the control groups, while a slight decrease in portal venous branches (< level 4) was found in two animals from experimental group 1. In all animals in experimental groups 2 and 3, a significant reduction in portal venous branches (< level 3) (Figure 2A, B) was observed. In addition, a widened portal venous diameter (t_2 = 9.679, P = 0.001; t_3 = 17.800, P = 0.0001) and an increased portal venous pressure (t_2 = 16.808, P = 0.0001; t_3 = 13.431, P = 0.0002) were found. Two weeks after perfusion, different degrees of bypass branches of the intrahepatic portal vein were found in the experimental groups during the observation period (Figure 2C). The main portal venous diameter also varied significantly, and was still greater at 6 wk after perfusion than before perfusion (t_2 = 4.007, P = 0.016; t_3 = 2.870, P = 0.028). At this time point, the portal venous pressure was higher than that before perfusion (t_2 = 3.586, P = 0.023; t_3 = 3.927, P = 0.008) (Tables 1, 2). Before and after perfusion, no significant changes in portal venous diameter or pressure were observed in experimental group 1 or the control groups (diameter:

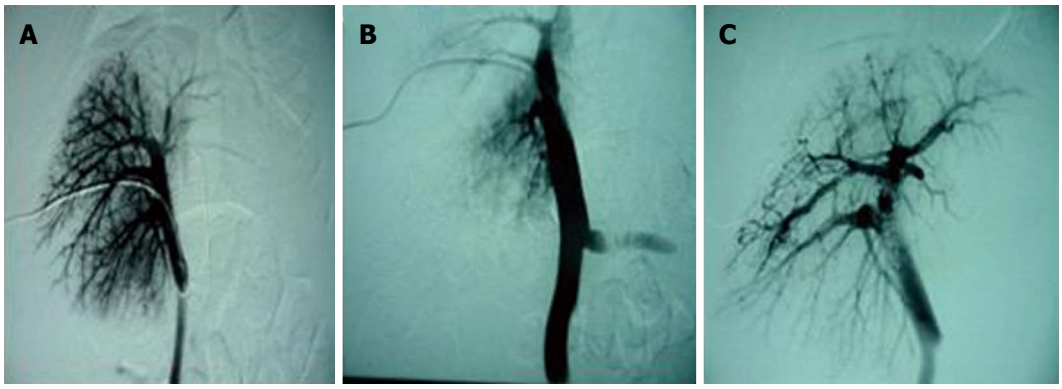

Figure 2 Portal vein variation. A: Direct portal phlebography of experimental group 2 before perfusion showed the portal venous branches clearly; B: Portal phlebography immediately after perfusion showed a significant reduction in small portal venous branches and a widened main portal vein; C: Portal phlebography at 6 wk after perfusion showed that a large number of irregular bypass branches of intrahepatic portal veins were formed, which communicated directly with hepatic veins, and the main portal vein was still wider than that before perfusion.

Table 1 Changes of portal vein in experimental group 2 before and after perfusion

Portal venous diameter (mm)	Pressure (kPa)	Extrahepatic portal venous bypass	Intrahepatic portal venous bypass
Before perfusion (n = 5)			
1	10.3	1.4	--
1	9.8	1.5	--
1	11.4	1.4	--
1	12.2	1.6	--
1	10.0	1.5	--
mean ± SD	10.74 ± 1.02	1.48 ± 0.84	
Immediately after perfusion (n = 5)			
1	18.2	2.5	--
1	17.1	2.7	--
1	20.2	2.6	--
1	16.7	2.9	--
1	18.4	2.4	--
mean ± SD	18.12 ± 1.37	2.62 ± 0.19	
2 wk after perfusion (n = 5)			
1	13.6	1.8	--
1	12.7	2.1	-+
1	14.5	2.0	-+
1	13.3	2.4	--
1	12.9	1.9	-+
mean ± SD	13.40 ± 0.71	2.04 ± 0.23	
4 wk after perfusion (n = 5)			
1	12.3	1.6	-+
1	11.8	1.9	-+
1	14.1	1.5	-+
1	13.1	2.1	-+
1	11.7	1.7	-+
mean ± SD	12.60 ± 1.00	2.04 ± 0.23	
6 wk after perfusion (n = 5)			
1	11.5	1.7	-+
1	11.9	1.8	-+
1	16.1	1.4	-+
1	16.5	2.1	-+
1	11.8	1.9	-+
mean ± SD	13.56 ± 2.51	1.78 ± 0.26	

Table 2 Changes of portal vein in experimental group 3 before and after perfusion

Portal venous diameter (mm)	Pressure (kPa)	Extrahepatic portal venous bypass	Intrahepatic portal venous bypass
Before perfusion (n = 5)			
1	9.5	1.2	--
1	10.3	1.6	--
1	11.5	1.4	--
1	9.8	1.3	--
1	12.4	1.5	--
mean ± SD	10.70 ± 1.22	1.40 ± 0.16	
Immediately after perfusion (n = 5)			
1	19.4	3.1	--
1	18.6	3.3	--
1	21.1	3.5	--
1	17.9	2.8	--
1	19.7	2.9	--
mean ± SD	19.34 ± 1.21	3.12 ± 0.29	
2 wk after perfusion (n = 4)			
1	14.2	2.4	-+
1	15.4	2.6	-+
1	16.3	2.8	--
1	16.6	2.6	-+
mean ± SD	15.80 ± 3.66	2.60 ± 0.16	
4 wk after perfusion (n = 3)			
1	11.4	2.2	-+
1	16.5	1.8	-+
1	17.9	2.1	-+
mean ± SD	15.63 ± 1.08	2.03 ± 0.21	
6 wk after perfusion (n = 3)			
1	11.6	2.1	-+
1	18.3	1.7	-+
1	17.5	1.9	-+
mean ± SD	15.27 ± 3.36	1.90 ± 0.20	

t_1 = 2.269, P = 0.086; $t_{control}$ = 2.236, P = 0.076) (pressure: t_1 = 0.885, P = 0.426; $t_{control}$ = 0, P = 1.0) (Tables 3, 4).

Changes in liver size and spleen size: A computed tomography scan revealed no significant changes in liver size and spleen size in experimental group 1 and the control groups (t_{liver} = 0.740, P = 0.468; t_{spleen} = 0.597, P = 0.557). At 6 wk after perfusion, both liver size and spleen size in all animals of experimental group 2 and the three surviving animals

Table 3 Changes of portal vein in experimental group 1 before and after perfusion

Portal venous diameter (mm)	Pressure (kPa)	Extrahepatic portal venous bypass	Intrahepatic portal venous bypass
Before perfusion (n = 5)			
1	10.5	1.5	--
1	10.3	1.7	--
1	10.6	1.7	--
1	11.4	1.6	--
1	10.2	1.6	--
mean ± SD	10.60 ± 0.47	1.62 ± 0.84	
Immediately after perfusion (n = 5)			
1	10.5	1.6	--
1	10.8	1.9	--
1	10.9	1.6	--
1	11.4	1.5	--
1	10.5	1.8	--
mean ± SD	10.82 ± 0.37	1.68 ± 0.16	
2 wk after perfusion (n = 5)			
1	11.0	1.7	--
1	10.6	1.8	--
1	11.1	1.7	--
1	10.9	1.7	--
1	11.2	1.6	--
mean ± SD	10.98 ± 0.23	1.70 ± 0.07	
4 wk after perfusion (n = 5)			
1	11.3	1.6	--
1	10.4	1.9	--
1	10.7	1.5	--
1	11.6	1.5	--
1	11.5	1.8	--
mean ± SD	11.10 ± 0.52	1.66 ± 0.18	
6 wk after perfusion (n = 5)			
1	11.7	1.6	--
1	11.1	1.8	--
1	10.3	1.4	--
1	11.0	1.6	--
1	11.4	1.8	--
mean ± SD	15.27 ± 3.36	1.64 ± 0.17	

Table 4 Changes of portal vein in control groups before and after perfusion

Portal venous diameter (mm)	Pressure (kPa)	Extrahepatic portal venous bypass	Intrahepatic portal venous bypass
Before perfusion (n = 6)			
1	10.3	1.3	--
1	10.1	1.7	--
1	10.7	1.5	--
1	10.2	1.8	--
1	9.6	1.5	--
1	9.9	1.6	--
mean ± SD	10.13 ± 0.37	1.57 ± 0.18	
Immediately after perfusion (n = 6)			
1	10.3	1.4	--
1	10. 2	1.6	--
1	10.7	1.5	--
1	10.3	1.8	--
1	9.7	1.6	--
1	10.2	1.5	--
mean ± SD	10.24 ± 0.32	1.57 ± 0.14	
2 wk after perfusion (n = 5)			
1	10.1	1.7	--
1	10.3	1.8	--
1	10.4	1.7	--
1	10.6	1. 7	--
1	9.9	1.6	--
1	10.3	1.7	--
mean ± SD	10.27 ± 0.24	1.62 ± 0.12	
4 wk after perfusion (n = 6)			
1	10.4	1.4	--
1	10.5	1.7	--
1	10.4	1.6	--
1	10.8	1.7	--
1	9.8	1. 5	--
1	10.6	1.6	--
mean ± SD	10.42 ± 0.34	1.58 ± 0.12	
6 wk after perfusion (n = 6)			
1	10.6	1.6	--
1	10.3	1.8	--
1	10.2	1.5	--
1	10.6	1.6	--
1	10.1	1.7	--
1	10.4	1.7	--
mean ± SD	10.37 ± 0.21	1.65 ± 0.10	

of experimental group 3 were greater than those before perfusion (t_{liver} = 2.312, P = 0.036; t_{spleen} = 2.209, P = 0.044), whilst maintaining a uniform density and regular edges (Figure 3A, B and Figure 4A, B) (Table 5).

Laboratory examination results

At 2 wk after perfusion, a slight increase in aminotransferase was observed in one animal from experimental group 1, all animals from experimental group 2 and the three surviving animals from experimental group 3, while other laboratory examination results were normal. Before perfusion and at 4 wk and 6 wk after perfusion, all laboratory examination results were within normal ranges in all animals from experimental group 2 and the control groups, as well as the three surviving animals from experimental group 3.

Pathological changes

Pathological changes in the liver: After perfusion with 80% alcohol, thrombi formed in miniature and small hepatic blood vessels (Figure 5A), and early pathological changes such as endothelial injury, hepatocyte degeneration and necrosis (Figure 5B), and inflammatory cell infiltration were also observed. This was followed by the development of hepatic fibrosis as time increased. In experimental group 1, hepatic tissues had different degrees of fibrosis after surgery, up to level 4 (partial liver lobes), and one animal developed cirrhosis at 6 wk after perfusion, but did not die. In experimental group 2, all animals developed fibrosis postoperatively, with an increase in fibrosis level over time (Figure 5C). Fibrosis of the various liver lobes reached level 4, and developed into cirrhosis at different time points (Figure 5D), and the animals survived. In experimental group 3, one animal died at 1 wk and one died at 3 wk after surgery, and the remaining three animals developed different levels of fibrosis at different time points, which eventually

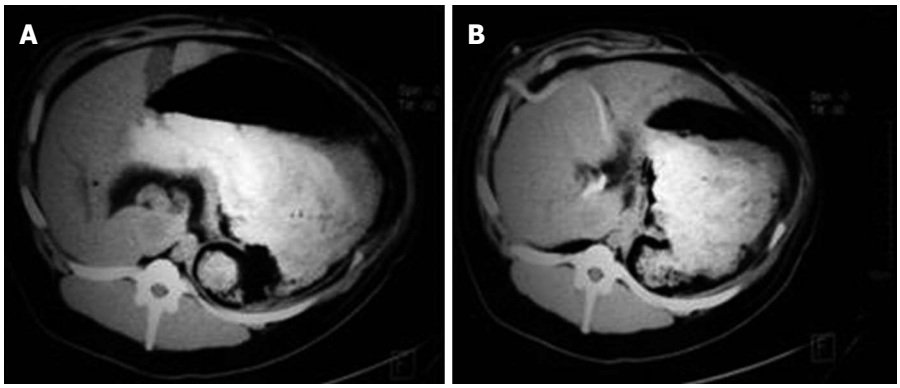

Figure 3 Liver size variation. A: Computed tomography of experimental group 2 before perfusion demonstrating normal liver size; B: At 6 wk after perfusion, liver size was markedly increased.

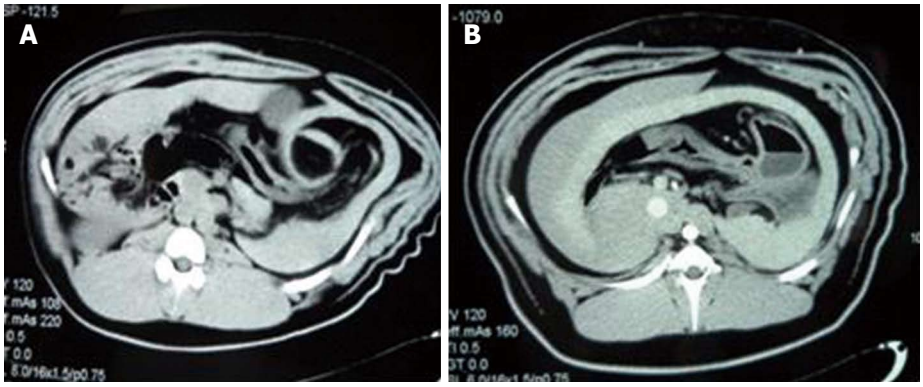

Figure 4 Spleen size variation. A: Computed tomography of experimental group 2 before perfusion demonstrating normal spleen size and the spleen was thin and curled; B: At 6 wk after perfusion, spleen size was markedly increased; the spleen was not curled and extended into the hepatic fissure.

Table 5 Changes of liver size and spleen size in experimental group 1 (5 pigs), experimental group 2 (5 pigs), experimental group 3 (3 pigs) and control groups (6 pigs) before perfusion and at 6 wk after perfusion

	Liver size (cm^3)	Spleen (cm^3)	Avoirdupois (kg)
Experimental groups 2 + 3			
Before perfusion	508 ± 67	113 ± 38	32.9 ± 3.8
6 wk after perfusion	589 ± 73	159 ± 46	40.7 ± 3.5
Experimental group 1 + control groups			
Before perfusion	479 ± 85	102 ± 47	33.8 ± 2.9
After perfusion	504 ± 73	111 ± 39	39.5 ± 3.5

reached level 4 and rapidly progressed to cirrhosis. A gross sample of cirrhotic liver was characterized by an increase in size, hardened tissues, decreased elasticity (Figure 6A, B), and small nodules uniformly distributed on the surface (Figure 6C). There was no change in fibrosis in any of the control animals (Table 6).

Pathological changes in other organs: The heart, lungs, kidneys and stomach of pigs in experimental group 1 and the control groups were all histologically normal. The spleen had different degrees of congestion in one animal from experimental group 1, all animals from experimental group 2 and the three surviving animals from experimental group 3 (Figure 7A). One animal from experimental group 2 and one animal from experimental group 3 had mild varicose veins on the stomach surface (Figure 7B).

DISCUSSION

Many methods have been used to establish a hepatic cirrhosis model, such as CCl_4[5,6], thioacetamide[7], chemical carcinogen induction[8], alcohol[9], biliary obstruction[6,10,11], dystrophy induction[12], schistosome induction[13], and immunological mediation[14]. These modeling methods are mainly applicable to the following administration modes: oral administration, inhalation, subcutaneous injection, and intraperitoneal injection. They all require drug absorption followed by entry into various body systems, which tends to damage not only the liver, but also other tissues and organs, and more importantly, may even cause animal death. The dead animals may further result in environmental pollution. In addition, rats or mice are used as experimental animals in most cases, which have a small blood volume and therefore cannot undergo several serological tests and imaging examinations. Rats or mice have a small body size and

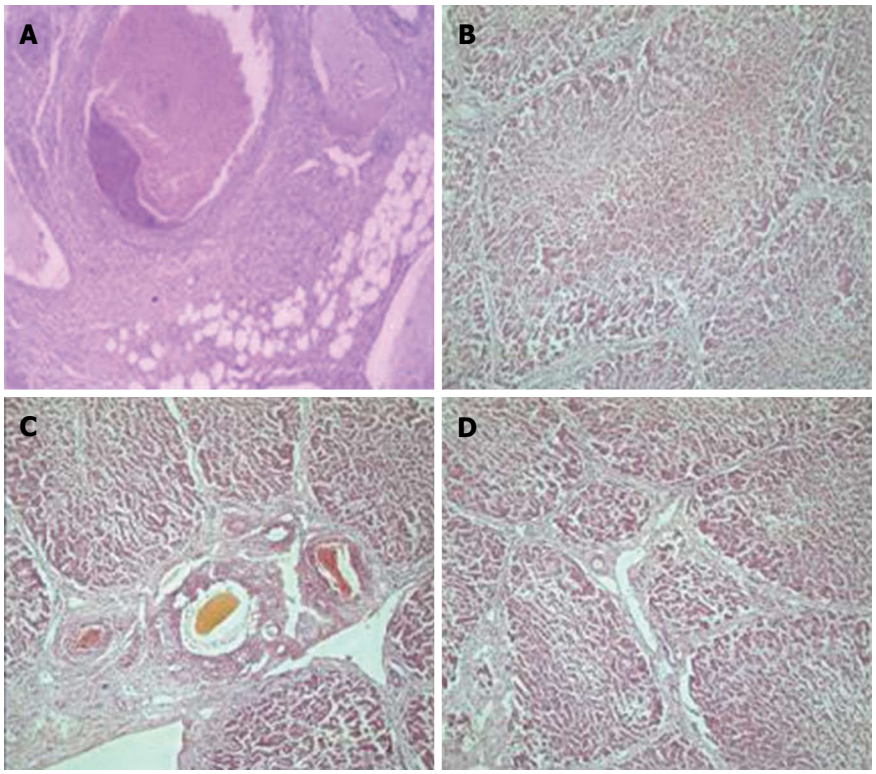

Figure 5 Pathological variation. A: Within 6 h after perfusion in experimental group 2, thrombosis was evident in interlobular veins; B: At 2 wk after perfusion in experimental group 2, irregular, massive hepatocyte degeneration and necrosis were observed; C: At 4 wk after perfusion in experimental group 2, bile duct proliferation and dilation were evident; D: At 6 wk after perfusion in experimental group 2, pseudo-lobules formed with fibrous tissue proliferation and bridging fibrosis. Hematoxylin-eosin staining, magnification × 40 (A); × 200 (B-D).

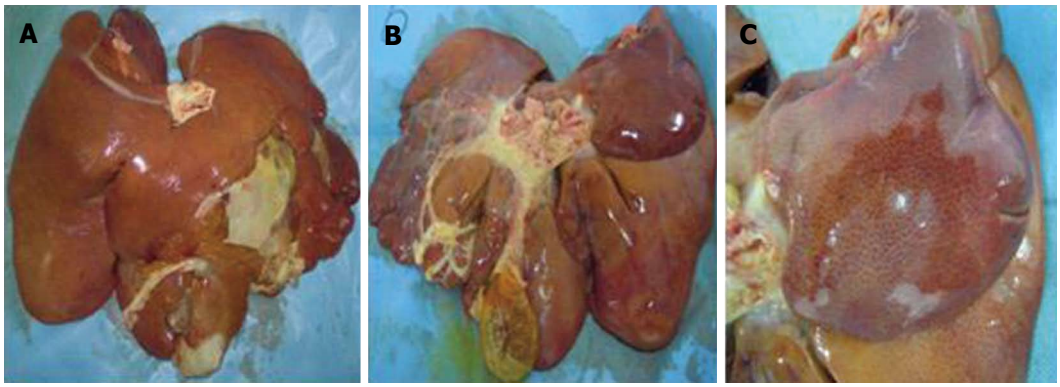

Figure 6 Gross sample of cirrhotic liver. A, B: At 6 wk after perfusion in experimental group 2, gross samples of diaphragmatic and ventral liver showed that hepatic tissues were hard and had poor elasticity, and various liver lobes were enlarged; C: Local amplification of the gross sample showed that there were relatively uniform regenerative nodules on the liver surface.

thus relatively small blood vessels, and when used as experimental animals, it is difficult to determine the changes in the portal vein. In addition, surgical procedures are complicated on such small animals, along with intervention, mini-invasive surgery, and biopsy for collecting pathological tissues. It is also not possible to observe pathophysiological changes in the liver during the modeling process. More importantly, there is a large difference in the anatomy, normal physiological and biochemical values, and in the development process of disease between rat or mouse models and humans[15]. Some researchers have also used large animals such as dogs and baboons to establish specific hepatic cirrhosis or portal hypertension models[16,17], but these models are also significantly different from humans in terms of the developmental process of common posthepatitic or alcoholic cirrhosis and portal hypertension caused by hepatic cirrhosis. In this study, using a hepatic cirrhosis model established by perfusing porcine hepatic arteries with a high concentration of alcohol (80%), we were able to perform serological and imaging examinations, evaluate changes in the portal vein at different time points, and observe physiological changes in the pigs

Table 6 Histopathological changes of hepatic biopsy samples within 6 h after perfusion with different concentrations of alcohol

Group	Venous hepatocyte thrombosis	Hepatocyte degeneration	Inflammatory cell necrosis	Endothelial cell infiltration injury
Experimental group 1 (n = 5)	3	1	0	1
Experimental group 2 (n = 5)	5	4	2	3
Experimental group 3 (n = 5)	5	5	4	5
Control group (n = 6)	0	0	0	0

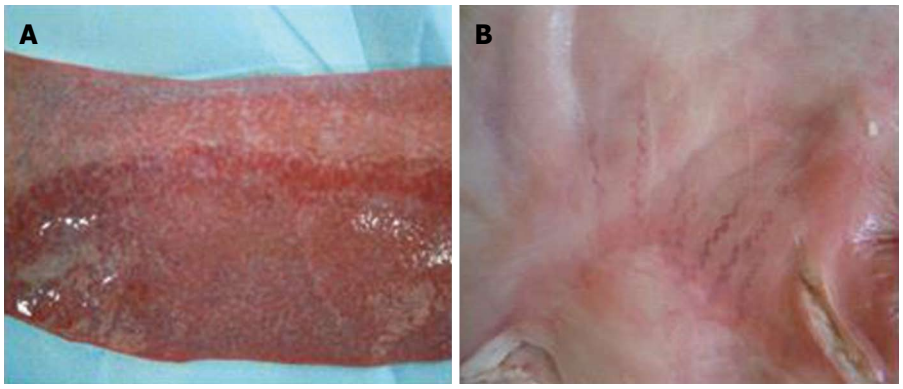

Figure 7 Gross sample of the spleen (A) and stomach (B). A: At 6 wk after perfusion in experimental group 2, the gross sample of the spleen showed that the spleen was hard and congestive, and had a non-uniform color; B: At 6 wk after perfusion in experimental group 2, the gross sample of the stomach showed that there were small varicose veins on the stomach surface.

as well. In particular, we were able to investigate the pathological developmental process of hepatic cirrhosis by means of hepatic biopsy at different sites and at different time periods.

We used 80% alcohol for porcine hepatic arterial perfusion to establish a hepatic cirrhosis and portal hypertension model, and we observed and compared liver characteristics according to different perfusion doses. Our findings showed that hepatic fibrosis and cirrhosis occurred rapidly; hepatic fibrosis was observed as early as 1 wk after perfusion, and cirrhosis was found in some animals from experimental groups 2 and 3 as early as 2-3 wk. According to the literature, the occurrence of cirrhosis is usually reported 8 wk after perfusion[9,18]. In addition, our study demonstrated only a minor influence on liver function as a result of alcohol perfusion, and no injury to other important organs and tissues was observed during the development of hepatic cirrhosis, while previous reports have shown high hepatotoxicity[4]. The pathophysiological characteristics of hepatic cirrhosis and portal hypertension in our animal model were similar to those of early human posthepatitic and alcoholic cirrhosis, and were mainly demonstrated by the following results: (1) the serological examination was nearly normal; (2) the imaging examination showed an increase in liver size and spleen size, the formation of bypass circulation in the liver, and a widened main portal venous diameter; (3) some of the animals had increased portal venous pressure; and (4) hepatic biopsies at different sites and at different time periods confirmed the typical pathological development of hepatic cirrhosis. There was extensive hepatocyte generation and necrosis, as well as collapse of the liver lobule fibrous framework. The residual hepatocytes regenerated to form irregular, nodular hepatocyte masses (regenerative nodules). A large number of fibrous connective tissues proliferated to form fiber bundles and fibrous septa. The newly-formed fibrous septa connected with each other to enclose regenerative nodules or re-divide the residual liver lobules and remodel them into pseudo-lobules. The hepatic vascular bed was reduced, occluded, or distorted. The blood vessels were compressed by regenerative nodules. The normal relationship between intrahepatic portal veins, hepatic veins and small hepatic arterial branches was no longer present and communicating anastomotic branches were formed. Relatively uniform cirrhotic particles were observed on the liver surface, and the cirrhosis affected each liver lobe. Apart from the observation of splenic congestion caused by hepatic cirrhosis and mild varicose veins on the stomach surface of a few animals, other important organs and tissues were unaffected.

In experimental group 1, there was a long duration and a low success rate of modeling by perfusion with 80% alcohol, but the animals all survived. In experimental group 2, there was a moderate duration and a high success rate of modeling, and the animals all survived. In experimental group 3, there was a short duration and a high success rate of modeling, but the rate of animal death was significantly increased.

Establishing a large animal model of hepatic cirrhosis *via* a mini-invasive intervention is still in the exploratory phase. One study[19] has shown that when a porcine hepatic cirrhosis model is established by

injecting an embolic agent containing 3:1 iodinated oil-alcohol into porcine hepatic arteries, the complex surgical procedure is challenging. Specifically, a metal spring loop is used to embolize non-target arteries, a micro-catheter is used to inject the embolic agent, and the portal venous pressure is measured indirectly by the hepatic venous pressure gradient (HVPG). As a result, total costs are markedly increased, the operation time is prolonged, tissue ischemia and necrosis within the embolization range of non-target blood vessels may occur, and large hepatic arteries may be embolized during injection of the embolic agent due to the properties of iodinated oil. In this experiment, 80% alcohol was slowly perfused into the left and right common hepatic arteries (away from the cystic and gastroduodenal arteries) *via* a 4F catheter, and then uniformly distributed into the liver by blood flow, based on the size and blood supply of the left and right hepatic arteries; and 1 mL 1% lidocaine was immediately injected after injection of 1 mL 80% alcohol until the dose of 80% alcohol was completely injected, to prevent spasm of large hepatic arteries. Only < level 3 hepatic arteries were embolized to preserve maximum blood and oxygen supply. After percutaneous hepatic portal vein puncture was successfully performed in all animals, a 6F multi-sidehole pigtail catheter was applied and lodged into the portal vein for phlebography and direct measurement of portal venous pressure and diameter. Therefore, this modeling method has many advantages, such as a simple surgical procedure, low costs, and the possibility of direct observation of changes in the portal vein and in portal venous pressure, as well as bypass branches and their distribution. Portal phlebography immediately after hepatic arterial perfusion with alcohol confirmed that alcohol passes through small arteries and enters into the hepatic sinus and subsequently small portal venous branches to produce combined hepatic arterial-portal venous embolization. Pavcinik *et al*[20] injected polyvinyl alcohol particles into porcine portal veins by percutaneous hepatic puncture to embolize portal venous branches to produce portal hypertension, however, 1 wk after the experiment they found that the portal venous pressure had decreased to the normal level, and hepatic cirrhosis did not develop. In a study of opposite hepatic decompensated hypertrophy after hepatic arterial embolization and portal venous embolization, Madoff *et al*[21] observed hepatocyte and endothelial injury, along with erythrocyte stagnation after injecting an embolic agent containing 3:1 iodinated oil-alcohol into porcine hepatic arteries, and 28 d after injecting polyvinyl alcohol particles into the portal veins and linearly embolizing the left and left-middle liver lobes, the histopathology showed atrophy of the portal vein region and interlobular septa in the embolized segments, without significant fibrosis. In the present study, hepatic biopsies were taken at different time points and pathological examination confirmed that hepatic arterial perfusion with different doses of 80% alcohol caused thrombosis in miniature and small hepatic arteries, portal venous branches and veins, as well as the hepatic sinus, and endothelial injury. This was followed by hepatocyte degeneration and necrosis, as well as inflammatory cell infiltration, which eventually developed into the typical pathophysiological process of hepatic cirrhosis. As reported in the literature, at 6-8 wk after surgery, hepatic histopathology showed fibrous septa and pseudo-lobules, but localized cirrhosis, which was not uniformly distributed, and fibrosis at different levels; and portal venous pressure increased in some animals, as measured indirectly by HVPG[18]. At 2 and 6 wk after perfusion, the local hepatic biopsy pathology and gross pathology in all animals in experimental group 2, and in the surviving animals in experimental group 3, confirmed that a hepatic cirrhosis model was successfully established, with the majority of fibrosis reaching level 4. Samples collected from each site were observed to have cirrhosis, and relatively uniform cirrhotic nodules were found on the surface of various liver lobes. These results suggest that the injuring effect of alcohol on the liver is uniform. In addition, the physiological changes in early hepatic cirrhosis and portal hypertension were also directly confirmed by portal phlebography and portal venous pressure measurement, such as the occlusion of portal venous branches, increased portal venous pressure, widening of portal venous diameters, and the formation of intrahepatic portal venous bypasses. A total of eight animals developed hepatic cirrhosis, including all animals in experimental group 2 and the surviving animals in experimental group 3, of which five animals had concomitant portal hypertension. Among the pathological samples collected at a total of 32 sites in the left and right lateral and medial liver lobes of each animal, only the samples at two sites were observed to have level 3 fibrosis and those at the remaining 30 sites had level 4 fibrosis. This also indicated that the injuring effect of alcohol on the liver was uniform. However, one study[19] using an embolic agent containing 3:1 iodinated oil-alcohol, reported that only 5 of 20 sites in the 16 mL group, and 4 of 20 sites in the 28 mL group had level 4 fibrosis, which suggests that the injuring effect on the liver is not uniform[19].

In our study, the deaths in experimental group 3 were mainly attributed to liver failure caused by extensive hepatic tissue necrosis and to postoperative infection.

In conclusion, at 2-6 wk after hepatic arterial perfusion with 80% alcohol, a porcine hepatic cirrhosis or cirrhotic portal hypertension model can be successfully established, with the typical pathophysiological development process of early hepatic cirrhosis, and with mild or no injury to other organs and tissues. The suitable dose of 80% alcohol for hepatic arterial perfusion in this experiment was the 12 mL dose used in experimental group 2, but

the most suitable alcohol concentration or dose for establishing hepatic cirrhosis or cirrhotic portal hypertension models still needs further in-depth research and investigation.

COMMENTS

Background

In order to develop new strategies for the diagnosis and treatment of hepatic cirrhosis, it is essential to establish large animal models similar to humans, which are applicable for all-round research, especially models of hepatic cirrhosis for application in surgery, intervention and pharmaceutical research.

Research frontiers

Establishing a large animal model of hepatic cirrhosis *via* a mini-invasive intervention is still in the exploratory phase and significant improvements have been made.

Innovations and breakthroughs

The authors developed a new method for establishing a porcine model of hepatic cirrhosis and portal hypertension by hepatic arterial perfusion with 80% alcohol, which accurately reproduced the pathophysiological development process similar to that occurring in humans.

Applications

The authors present a new method for establishing a porcine hepatic cirrhosis and portal hypertension model. The safety of this model depends on a suitable perfusion dose.

Terminology

Portal hypertension is hypertension (high blood pressure) in the portal venous system, which is composed of the portal vein, and its branches and tributaries.

Peer-review

This is an interesting manuscript about a porcine hepatic cirrhosis and portal hypertension model. Tables and Figures are interesting.

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ORIGINAL ARTICLE

Basic Study

Dual gRNAs guided CRISPR/Cas9 system inhibits hepatitis B virus replication

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Abstract

AIM: To screen and investigate the effective gRNAs against hepatitis B virus (HBV) of genotypes A-D.

METHODS: A total of 15 gRNAs against HBV of genotypes A-D were designed. Eleven combinations of two above gRNAs (dual-gRNAs) covering the regulatory region of HBV were chosen. The efficiency of each gRNA and 11 dual-gRNAs on the suppression of HBV (genotypes A-D) replication was examined by the measurement of HBV surface antigen (HBsAg) or e antigen (HBeAg) in the culture supernatant. The destruction of HBV-expressing vector was examined in HuH7 cells co-transfected with dual-gRNAs and HBV-expressing vector using polymerase chain reaction (PCR) and sequencing method, and the destruction of cccDNA

was examined in HepAD38 cells using KCl precipitation, plasmid-safe ATP-dependent DNase (PSAD) digestion, rolling circle amplification and quantitative PCR combined method. The cytotoxicity of these gRNAs was assessed by a mitochondrial tetrazolium assay.

RESULTS: All of gRNAs could significantly reduce HBsAg or HBeAg production in the culture supernatant, which was dependent on the region in which gRNA against. All of dual gRNAs could efficiently suppress HBsAg and/or HBeAg production for HBV of genotypes A-D, and the efficacy of dual gRNAs in suppressing HBsAg and/or HBeAg production was significantly increased when compared to the single gRNA used alone. Furthermore, by PCR direct sequencing we confirmed that these dual gRNAs could specifically destroy HBV expressing template by removing the fragment between the cleavage sites of the two used gRNAs. Most importantly, gRNA-5 and gRNA-12 combination not only could efficiently suppressing HBsAg and/or HBeAg production, but also destroy the cccDNA reservoirs in HepAD38 cells.

CONCLUSION: These results suggested that CRISPR/Cas9 system could efficiently destroy HBV expressing templates (genotypes A-D) without apparent cytotoxicity. It may be a potential approach for eradication of persistent HBV cccDNA in chronic HBV infection patients.

Key words: Dual gRNAs; CRISPR/Cas9; Hepatitis B; cccDNA; Antiviral therapy

Core tip: In this manuscript, 15 hepatitis B virus (HBV)-specific gRNAs were designed according to the HBV genome sequences of genotypes A-D. We confirmed that the CRISPR/Cas9 system with these HBV-specific gRNAs could efficiently suppress the replication of multiple HBV genotypes. Further, we demonstrated that dual gRNAs could guide the CRISPR/Cas9 system to efficiently destroy HBV cccDNA and reduce its level in HepAD38 cells. Since cccDNA, the template of HBV replication, accounts for the persistence of HBV infection, our data suggested that CRISPR/Cas9 technique may be a useful tool to eradicate HBV of multiple genotypes.

Wang J, Xu ZW, Liu S, Zhang RY, Ding SL, Xie XM, Long L, Chen XM, Zhuang H, Lu FM. Dual gRNAs guided CRISPR/Cas9 system inhibits hepatitis B virus replication. *World J Gastroenterol* 2015; 21(32): 9554-9565 Available from: URL: http://www.wjgnet.com/1007-9327/full/v21/i32/9554.htm DOI: http://dx.doi.org/10.3748/wjg.v21.i32.9554

INTRODUCTION

Despite prophylactic vaccines available for many years, hepatitis B virus (HBV) infection remains an important public health problem worldwide. Current antiviral agents, including nucleos(t)ide analogues (NAs) and interferon (IFN), can control HBV production but not eliminate HBV, due to the persistence of HBV covalently closed circular DNA (cccDNA) reservoir in the nucleus of hepatocytes. NAs show no effect on cccDNA, thus relapse of hepatitis B occurs frequently in patients who discontinued antiviral treatment[1,2]. Moreover, because the stability of HBV cccDNA was so high that it declines slowly, so life-long treatment of chronic hepatitis B (CHB) is required[1,3,4]. On the other hand, although IFN-α can degrade cccDNA followed by cytidine deamination and apurinic/apyrimidinic site formation, and can further result in virus clearance in a few patients, its efficacy is unsatisfactorily limited[5-7]. Eradication of cccDNA, the only way to reach the clinical cure of CHB, is still an unresolved problem in the treatment of CHB.

Clustered regularly interspaced short palindromic repeats/Cas9 nuclease (CRISPR/Cas9) system is a novel genome editing tool derived from the adaptive immune system of bacteria and archaea[8-10]. CRISPR/Cas9 system promotes genome editing by inducing a double-strand break (DSB) at the target genomic locus. In the absence of a repair template, DSBs are re-ligated through the non-homologous end joining (NHEJ) process, which leads to the insertion/deletion (indel) mutations[11]. CRISPR/Cas9 system has been successfully applied not only for genome editing in cells, but also for disrupting the genome of virus, including adenovirus, herpes simplex virus (HSV) and human immunodeficiency virus (HIV)[12-14]. For HBV, CRISPR/Cas9 system has been proved to efficiently cleave the expressing templates of HBV genotypes A and D[15-17]. Whereas, genotypes A, B, C, D are the predominant genotypes of HBV in East Asia and other part of the world[18-20], so it is necessary to design guide RNAs (gRNAs) specific for HBV genotypes A-D. Here, we evaluated the potential use of CRISPR/Cas9 system to clear the HBV genome of genotypes A-D.

MATERIALS AND METHODS

Plasmids

The 1.2xHBV construct (pBB4.5-HBV1.2, genotype C) was constructed using a 1.2-fold length genome of genotype C HBV DNA sequence, and was inserted into the pBB4.5-HBV1.3 (genotype D, G1896A mutation) plasmid digested with PstI and NheI enzymes. The pBB4.5-HBV1.3 (genotype D, G1896A mutation) plasmid was kindly provided by Professor Locarnini SA from the Victorian Infectious Diseases Reference Laboratory, Australia[21]. The 1.2xHBV construct (pBB4.5-HBV1.2, genotype C) has been proved to efficiently produce HBV[22]. The HBV-expression vectors pGEM-HBV1.3A (genotype A) and pGEM-HBV1.3B (genotype B) were kindly provided by Professor Ningshao Xia from School of Public Health, Xiamen

University, China.

Cas9 promotes genome editing by inducing a double-strand break (DSB) and re-ligating through the NHEJ process in the absence of a repair template. The gRNA/Cas9 dual expression vector pSpCas9(BB)-2A-GFP (PX458) was obtained from Addgene (Cambridge, MA). PX458 plasmid which expresses a nonsense gRNA (GGGTCTTCGAGAAGACCT) was used as a vector control in each experiment. HBV-specific gRNA/Cas9 dual expression vectors were constructed in our laboratory. The oligonucleotide sequences for the construction of HBV-specific gRNA/Cas9 dual expression vectors are listed in Supplementary Table S1.

Transfection of cells

Human liver cancer cell lines HuH-7[23] and HepAD38[24] (stable expression of HBV) were maintained in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum (Gibco, Carlsbad, Calif). HuH-7 and HepAD38 cells were seeded in a 12-well plate at 1.5 × 10^5 cells/well. HuH-7 cells were co-transfected with HBV expression vectors (HBV of genotype A, B or C) and HBV-specific gRNA/Cas9 dual expression vector [pSpCas9(BB)-2A-GFP] with Lipofectamain 2000 (11668019; Life Technologies). HepAD38 cells were transfected with HBV specific gRNA/Cas9 dual expression vector with Lipofectamain 3000 (L3000015; Life Technologies).

Detection of HBsAg and HBeAg

Seventy-two hours after transfection, cell culture supernatants were collected for detection of HBsAg and HBeAg by a time-resolved fluoroimmunoassay (TRFIA) according to manufacturer's instructions (SY60108A and SY60105A; PerkinElmer). In brief, culture supernatant (100 μL) was added into a microtiter plate coated with anti-HBsAg or anti-HBeAg and shaked for 40 min at room temperature, then washed for 4 times. Europium-labeled anti-HBsAg or anti-HBeAg was diluted 1:50 with HBsAg or HBeAg dilution buffer and added at 100 μL per well, shaked for 40 min in room temperature, then washed 6 times. At last, after incubation with enhancement solution (100 μL) for 5 min, the plates were read using Anytest reader (SYM-BIO), and the concentrations of HBsAg and HBeAg were calculated according to the standard curve. The relative HBsAg or HBeAg level was calculated as the ratio of HBsAg or HBeAg concentration in the cell culture supernatant of gRNA treated and vector control cells.

Detection of HBV DNA fragments cleaved by dual gRNAs

DNA was extracted from cells using QIAGEN DNA mini kit (51304; QIAGEN), according to the manufacturer's instruction. Specific primers listed below were used to PCR amplify the cleaved HBV DNA fragments: primer1F (nucleotide position: 1856-1877), 5'-CCTACTGTTCAAGCCTCCAAGC-3'; primer2F (321-342), 5'-CAACCTCCAATCACTCACCAAC-3'; primer1R (434-415), 5'-AGAAGATGAGGCATAGCAGC-3'; primer2R (2006-1986), 5'-CAGAGGCGGTGTCAAGGAGAT-3'; primer3R (1702-1682), 5'-GACTCAAGGTCGGTCGTTGAC-3'; primer4R (1285-1264), 5'-CTAGGAGTTCCGCAGTATGGAT-3'. Primer1F and 1R were used for detection of the fragment cleaved by dual gRNAs of gRNA1 + 13 or gRNA2 + 14, and primer 2F and 2R, 2F and 3R, 2F and 4R were used for detection of the fragment cleaved by dual gRNAs of gRNA3 + 5, gRNA4 + 5 and gRNA5 + 12, respectively.

PCR reaction mixture (20 μL) contained 10 μL 2 × Taq mix (Transgene), 1 μL forward primer (10 μmol/L), 1 μL reverse primer (10 μmol/L), 1 μL DNA template and 7 μL double distilled water (ddH_2O). The reaction mixture was denatured at 95 ℃ for 5 min, followed by 35 cycles of 95 ℃ for 30 s, 60 ℃ for 30 s, and 72 ℃ for 1 min, and at last 72 ℃ for 5 min. Agarose gel (1.5%) was used for separation of DNA fragments with different length. DNA markers were DL2000 and DL2000 Plus (GenStar). The DNA fragment of expected length was sequenced.

Detection of HBV cccDNA

Two gRNA/Cas9 dual expression vectors were co-transfected into HepAD38 cells at least 7 d after tetracycline removed. Cells were collected for DNA extraction at 72 h after transfection using QIAGEN DNA mini kit (51304; QIAGEN), according to the manufacturer's instruction. To obtain cccDNA, KCl precipitation and plasmid-safe™ ATP-dependent DNase (PSAD) (Epicentre, Madison, WI, United States) were used to remove HBV DNA integrated into cell genome, HBV rcDNA, replicative dsDNA and ssDNA. Afterwards, rolling circle amplification (RCA) was conducted to selectively amplify cccDNA. Finally, PCR was performed using RCA products as template, and using cccDNA specific primers which target the gap region of HBV genome[25,26].

Methyl thiazolyl tetrazolium assay

Methyl thiazolyl tetrazolium (MTT) assay is used to monitor cell viability. Cells (1500 cells per well) were plated in 96-well plates and were maintained in DMEM supplemented with 10% fetal bovine serum. 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide solution was added into the cell culture at a final concentration of 5 μg/mL and was allowed to remain in culture for 4 h before measurement. Cell viability was monitored every 24 h by measuring the absorbance in a microplate reader (Bio-Rad, Hercules, CA).

RESULTS

Construction of HBV-specific gRNAs

Since SpCas9 in our CRISPR/Cas9 system requires a 5'-NGG protospacer adjacent motif (PAM) sequence, HBV genome of genotypes A-D was searched for

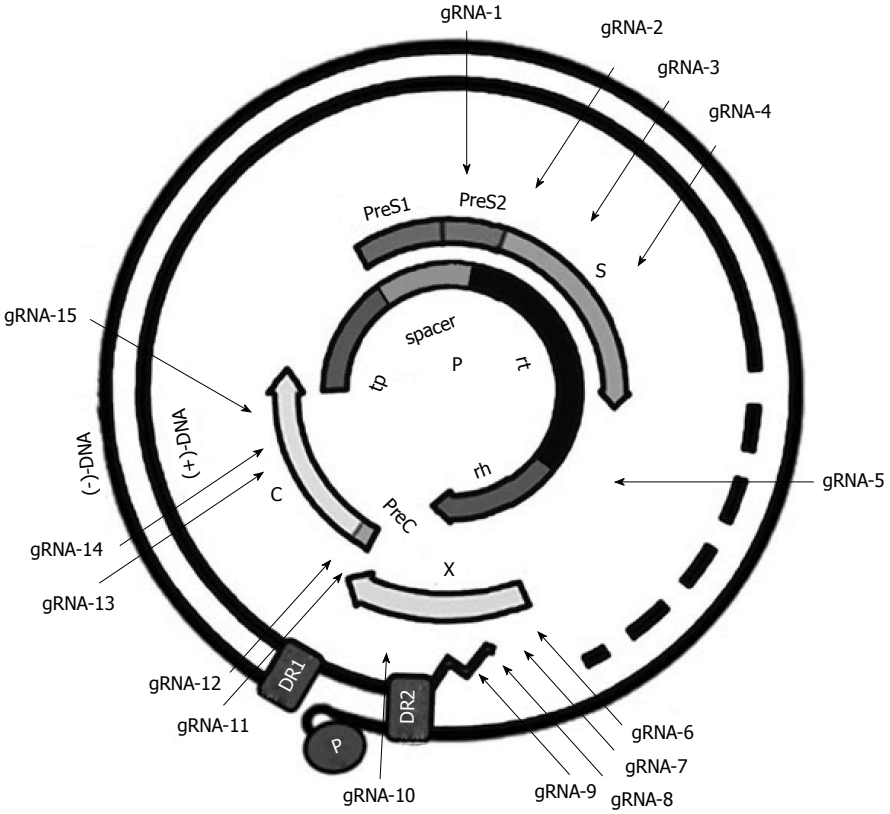


Figure 1 Illustration of the gRNA-targeted sequences located in the hepatitis B virus genome.

potential 18-20 base target sequences called the protospacer with 3'-downstream of PAM, which was shown as GN_{18-20}-NGG. Finally a panel of 15 HBV-specific gRNAs targeting different regions of HBV genome were designed (Figure 1). To avoid the off-target effect, the gRNA sequences were blasted by Nucleotide Blast search (NCBI), and the difference of more than 3 nucleotides to other sequences in human genome was assured. Finally, the gRNA expression vectors were identified by sequencing method. The sequences and locations of these HBV-specific gRNAs are listed in Table 1.

Designed HBV-specific gRNAs efficiently suppress the production of HBsAg and/or HBeAg

To examine the efficiency of each gRNA in the suppression of HBV replication, the 1.2xHBV construct (pBB4.5-HBV1.2, genotype C) was co-transfected with each gRNA/Cas9 dual expression vector in HuH-7 cells, respectively. As shown in Figure 2A, all designed gRNAs could significantly suppress HBsAg levels in culture supernatants, but with noticeable variation of efficacy, and gRNAs targeting the S region (gRNAs-1, 2, 3, and 4) and enhancer Ⅰ region (gRNA-5) of HBV genome exhibited higher HBsAg suppressing efficiency than that of the gRNAs targeting either X region (gRNAs-6, 7, 8, 9, and 10) or preC/C (gRNAs-11, 12, 13, 14, and 15) region. For HBeAg, all of gRNAs could significantly reduce HBeAg levels in culture supernatants, with the exception of gRNA-6 and gRNA-8. It is noteworthy that gRNAs targeting preC/C region exhibited higher HBeAg suppressive efficiency than that of those gRNAs targeting either S or X regions (Figure 2B). Intriguingly, gRNA-5 against enhancer Ⅰ region could efficiently suppress both HBsAg and HBeAg levels (Figure 2A and B). The off-target effect of gRNA may induce cytotoxicity, and then disturb the antiviral effect of HBV-specific gRNAs. The methods for assessing cell viability can test cytotoxicity. To exclude the possibility that the HBsAg or HBeAg suppression observed above was the result of non-specific cytotoxicity, MTT assay, which is a colorimetric assay for assessing cell viability, was conducted. The results revealed that no noticeable cytotoxicity of the gRNAs was observed in HuH-7 cells (Figure 2C).

Dual gRNAs exhibit synergistic effect on the suppression of HBV replication

Since CRISPR/Cas9 system can be efficiently used for multiple genome cleavage[11], we wondered if combination of two HBV-specific gRNAs (dual gRNAs) should be more efficient than single one in suppressing HBV replication. To confirm this, pBB4.5-HBV1.2 and combinations of two different gRNA expression vectors were co-transfected into HuH-7 cells. Firstly, we chose the region modulating the expression of HBsAg and HBeAg between two gRNAs in HBV genome, including the combinations of gRNA-1 + 13 covering the S

Table 1 Sequences and locations of hepatitis B virus-specific gRNAs in the hepatitis B virus genome of genotypes A-D

gRNA No.	Nucleotide position	Sequence ($GN_{18-20}NGG$, 5'-3')	Genotype
1	56-75	CCTGCTGGTGGCTCCAGTTC	A/B/C/D
2	182-200	GGACCCCTGCTCGTGTTAC	A/B/C/D
3	415-433	GCTGCTATGCCTCATCTTC	A/B/C/D
4	640-658	ATGGGAGTGGGCCTCAGTC	A/B/C
5	1179-1197	AGTGTTTGCTGACGCAACC	A/B/C/D
6	1393-1410	GCCAACTGGATCCTGCGC	B/C/D
7	1521-1540	GGGGCGCACCTCTCTTTACG	A/B/C/D
8	1578-1597	GAGGTGAAGCGAAGTGCACA	A/B/C/D
9	1589-1608	CTTCACCTCTGCACGTCGCA	B/C/D
10	1775-1794	AGGAGGCTGTAGGCATAAAT	A/B/C/D
11	1859-1878	AGCTTGGAGGCTTGAACAGT	A/B/C/D
12	1865-1884	CAAGCCTCCAAGCTGTGCCT	A/B/C/D
13	2336-2355	ACTACTGTTGTTAGACGACG	C/D
14	2367-2386	CGAGGGAGTTCTTCTTCTAG	A/B/C/D
15	2390-2409	GATTGAGACCTTCGTCTGCG	B/C/D

gene promoter and pre S1 region, gRNA-1 + 12 covering the S gene promoter pre S1 region and Core coding DNA sequence (CDS) region, and gRNA-8 + 12 covering Core promoter and Enhancer Ⅱ region were tested. As expected, the synergistic effect of dual gRNAs in the suppression of HBV replication was observed. Two of the tested dual gRNAs gRNA-1 + 13 (gRNA1 *vs* gRNA-1 + 13: 0.255 *vs* 0.110, $P = 0.0031$; gRNA13 *vs* gRNA-1 + 13: 0.265 *vs* 0.110, $P = 0.0017$) and gRNA-8 + 12 (gRNA8 *vs* gRNA-8 + 12: 0.319 *vs* 0.110, $P = 0.0004$; gRNA12 *vs* gRNA-8 + 12: 0.240 *vs* 0.110, $P = 0.0007$) demonstrated significantly higher suppressive efficiency in HBsAg production, when compared to that of each gRNA used alone (Figure 3A). Consistent with HBsAg, there was synergistic effect of gRNA-1 + 13 (gRNA1 *vs* gRNA-1 + 13: 0.296 *vs* 0.124, $P = 0.0034$; gRNA13 *vs* gRNA-1 + 13: 0.264 *vs* 0.124, $P = 0.0018$) and gRNA-8 + 12 (gRNA8 *vs* gRNA-8 + 12: 0.889 *vs* 0.121, $P < 0.0001$; gRNA12 *vs* gRNA-8 + 12: 0.260 *vs* 0.121, $P = 0.0018$) in the suppression of HBeAg production (Figure 3B). While for the gRNA-1 + 12, such synergistic effect could only be observed in HBeAg production (gRNA1 *vs* gRNA-1 + 12: 0.296 *vs* 0.096, $P = 0.0014$; gRNA12 *vs* gRNA-1 + 12: 0.260 *vs* 0.096, $P = 0.0006$), and an antagonistic effect for HBsAg production (gRNA1 *vs* gRNA-1 + 12: 0.265 *vs* 1.125, $P = 0.0001$; gRNA12 *vs* gRNA-1 + 12: 0.240 *vs* 1.125, $P = 0.0001$) was exhibited (Figure 3C).

Following the above observation, 11 dual gRNAs (two different gRNAs at a ratio of 1:1) were used for further study. The choice of gRNAs for combination was made according to the efficiency of each gRNA and its targeting regions (in promoter, enhancer or reverse transcriptional region of polymerase). Next, the efficiency of those 11 dual gRNAs in the suppression of HBsAg and HBeAg was examined, with different HBV genotypes taken into consideration. The HBV-expression vectors (genotype A, B or C constructs) and two different gRNAs expression vectors were co-transfected into HuH-7 cells. For HBV of genotype C, all of dual gRNAs could significantly suppress HBsAg production (Figure 3D), just as expected. However, although all dual gRNAs could also significantly suppress HBeAg production, the efficacy was lower than that of HBsAg, since higher HBeAg suppressive efficiency (≥ 80%) was detected in only 7 dual gRNAs (Figure 3E). Similar results were observed when HBV of genotypes A and B were tested (Supplementary Figure S1A and B). To exclude the possibility that excess concentration of gRNA may interfere HBV replication, we tested the concentration effect of gRNAs in HBV replication. The result revealed that the suppression efficiency of gRNA on the HBsAg level was gRNA concentration dependent (Figure S2). Similarly, in order to exclude the interference of non-specific cytotoxicity, MTT assay was conducted. The result affirmed that there was no noticeable cytotoxicity of dual gRNAs (Figure 3F).

Dual gRNAs destroy HBV genome

To confirm that the dual gRNAs mediated suppression in HBV replication was at the genome level, the HBV genome region covering the cleavage sites of dual gRNAs was amplified using PCR. If dual gRNAs worked, the fragment between the two cleavage sites would be removed, consequently a relative smaller PCR product would be detected. According to the above suppressive efficiency of dual gRNAs combinations, five dual gRNAs (1 + 13, 5 + 12, 2 + 14, 3 + 5 and 4 + 5) were chosen for further investigation. As expected, all of dual gRNAs could destroy HBV genome, especially the combination of gRNA-5 + 12, in which only the smaller fragment was detected. An up to 100% cleavage efficiency implicated the optimum combination of the two gRNAs (Figure 4A). Furthermore, direct sequencing of the PCR product showed that the smaller fragment was indeed formed by the re-ligation between the ends of two cleavage sites (Figure 4B). Then the plasmid pBB4.5-HBV1.2 was co-transfected with dual gRNA-5 + 12 expression vectors at different ratios to HuH-7 cells and the cleavage efficiency was assessed by PCR amplification. The result revealed that at the ratio of 1:3, almost all of HBV genome DNAs were cleft by gRNA-5 + 12. Surprisingly, even at the ratio of 3:1, still more than 90% of HBV genome DNAs were cleft (Figure 4C). In line with this, the efficiency in suppressing the production of HBsAg in culture supernatants was also gradually but significantly decreased from the ratio of 1:3 (reduced to 0.019% ± 0.007%) to 3:1 (reduced to 0.60% ± 0.014%), as compared to the vector control (Figure 4D). Other four dual gRNAs also showed the capability to destroy HBV genome at different extents (Figure 4A), and all were confirmed by sequencing analysis (data not shown).

As shown in Figure 4E, the target genome sequence of gRNA-13 in genotype B HBV harbors a one base

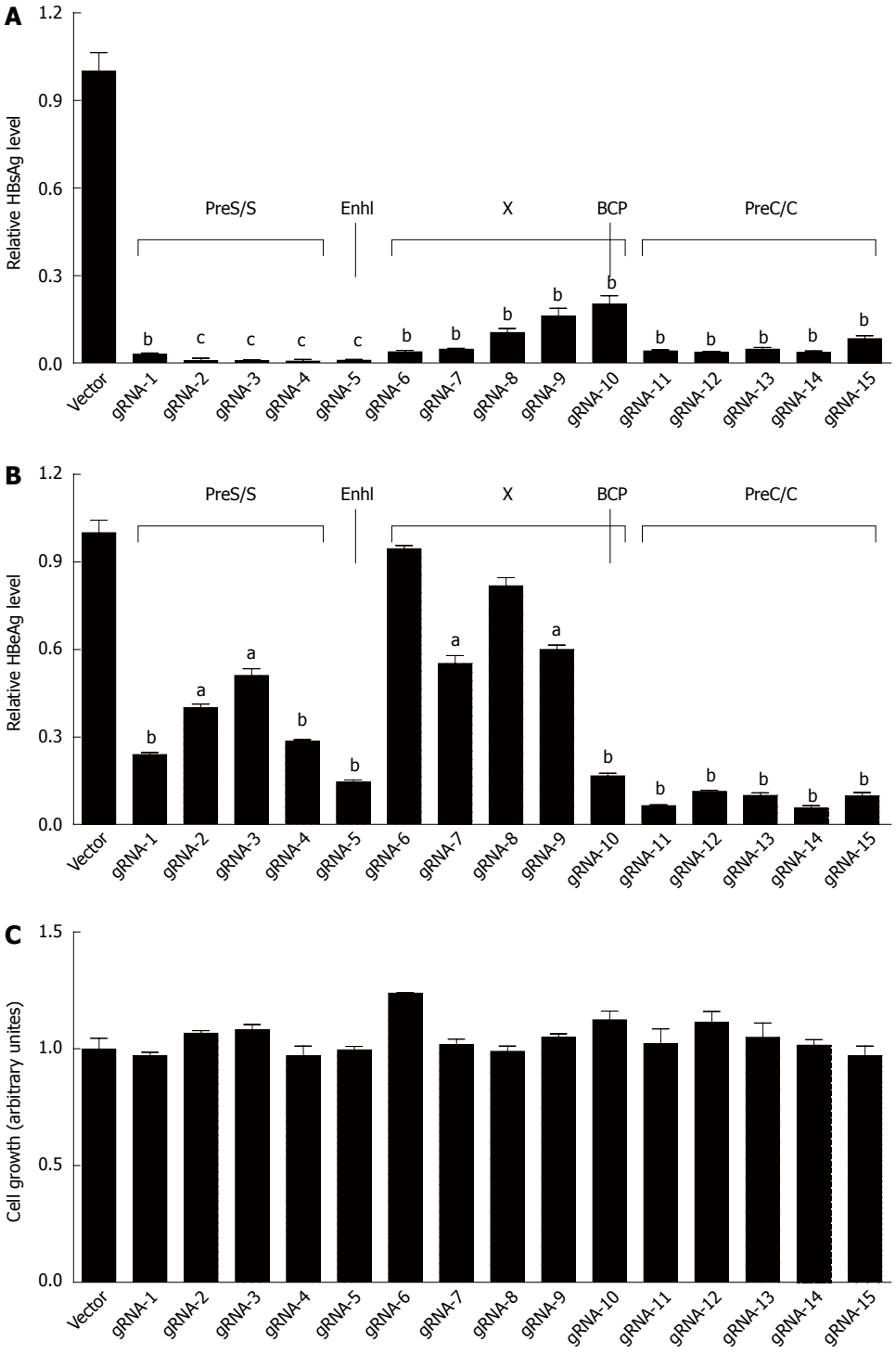
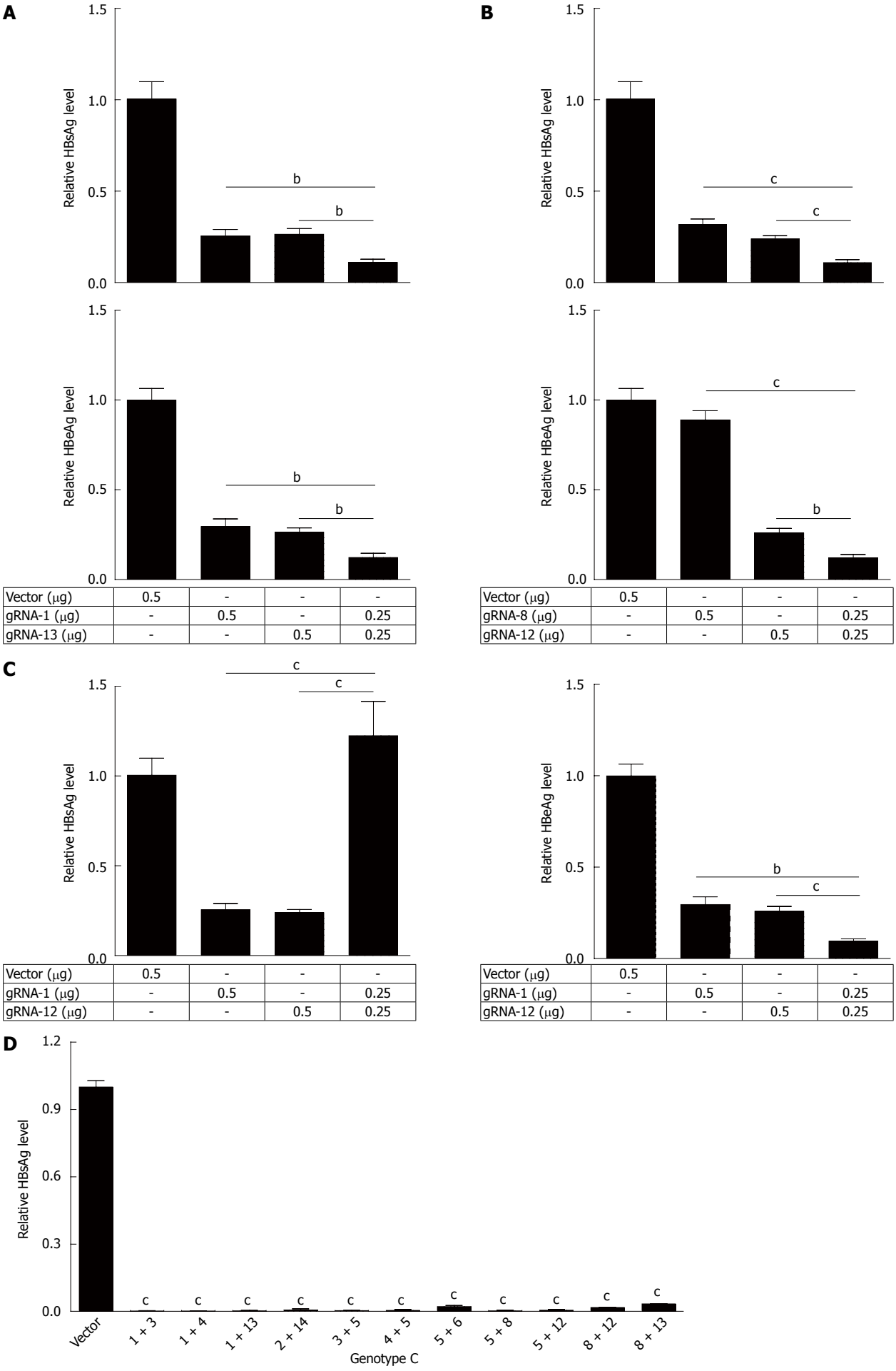


Figure 2 Fifteen hepatitis B virus-specific gRNAs could efficiently suppress the production of hepatitis B virus surface antigen or hepatitis B virus e antigen. A: The plasmid pBB4.5-HBV1.2 (0.5 μg) was co-transfected with each individual gRNA/Cas9 dual expression vector (1.5 μg) to HuH-7 cells. HBsAg level in culture supernatant was measured at 72 h post transfection using a time-resolved fluoroimmunoassay analysis; B: HBeAg level in culture supernatant was measured using a time-resolved fluoroimmunoassay analysis as above; C: The cytotoxicity of each HBV-specific gRNA was examined using an MTT assay. Data are shown as mean ± SE of 5 independent experiments. All *P*-values are from Student's *t*-test. HBV: Hepatitis B virus; HBsAg: Hepatitis B virus surface antigen; HBeAg: Hepatitis B virus e antigen; MTT: Methyl thiazolyl tetrazolium. $^{a}P < 0.05$, $^{b}P < 0.01$, $^{c}P < 0.001$.

difference as compared to genotypes A/C/D. However, dual gRNAs composed by gRNA-1 and gRNA-13 could also destroy HBV genome of genotype B (Figure 4F).

Dual gRNAs destroy HBV cccDNA in HepAD38 cells

Above data demonstrated that HBV-specific gRNAs could destroy HBV expressing template. Theoretically, such gRNAs could also destroy HBV cccDNA. To confirm this, the most powerful dual gRNAs (gRNA-5 and gRNA-12 expression vectors) were co-transfected into HepAD38 cells which stably express genotype D HBV and produce HBV cccDNA[24]. Firstly, the


A
Relative HBsAg level
1.5
1.0
0.5
0.0
b
b
Relative HBeAg level
b
b
Vector (μg) 0.5 - - -
gRNA-1 (μg) - 0.5 - 0.25
gRNA-13 (μg) - - 0.5 0.25
B
Relative HBsAg level
c
c
Relative HBeAg level
c
b
Vector (μg) 0.5 - - -
gRNA-8 (μg) - 0.5 - 0.25
gRNA-12 (μg) - - 0.5 0.25
C
Relative HBsAg level
c
c
Vector (μg) 0.5 - - -
gRNA-1 (μg) - 0.5 - 0.25
gRNA-12 (μg) - - 0.5 0.25
Relative HBeAg level
b
c
Vector (μg) 0.5 - - -
gRNA-1 (μg) - 0.5 - 0.25
gRNA-12 (μg) - - 0.5 0.25
D
Relative HBsAg level
1.2
0.9
0.6
0.3
0.0
c
Vector
1 + 3
1 + 4
1 + 13
2 + 14
3 + 5
4 + 5
5 + 6
5 + 8
5 + 12
8 + 12
8 + 13
Genotype C

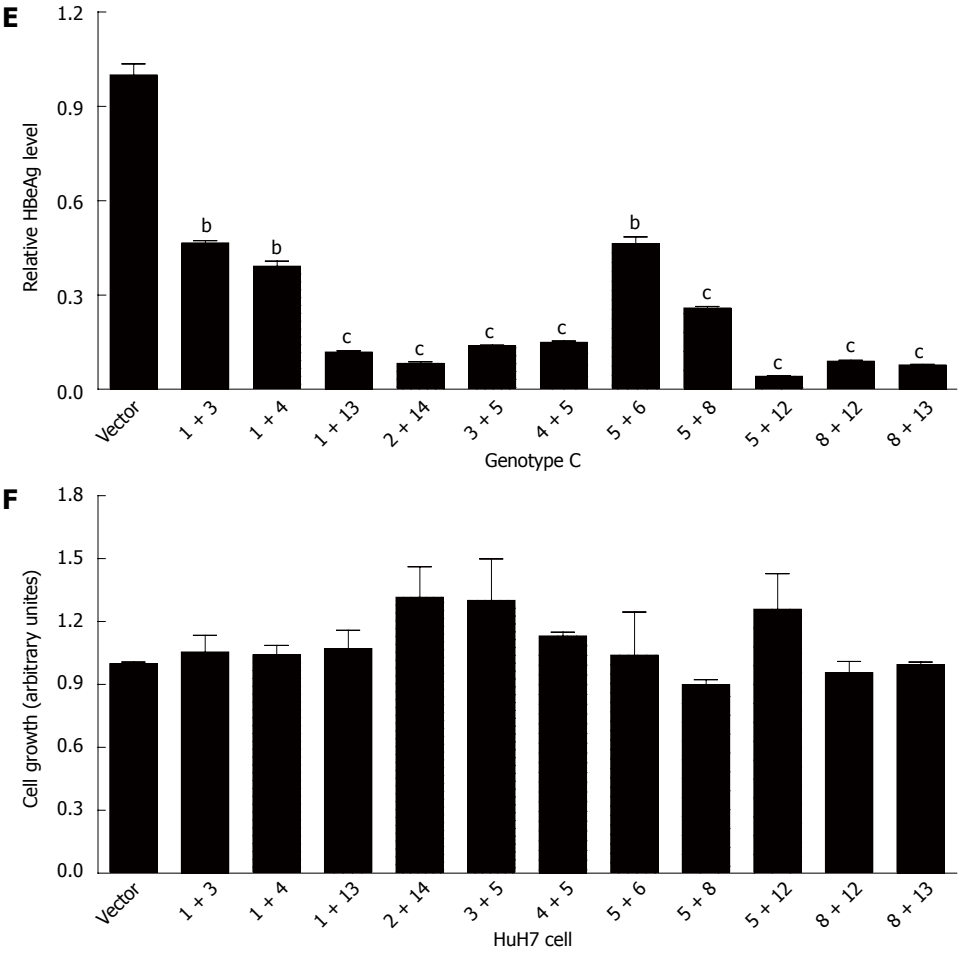


Figure 3 Eleven dual gRNAs could efficiently suppress the production of hepatitis B virus surface antigen or hepatitis B virus e antigen. The plasmid pBB4.5-HBV1.2 was co-transfected with gRNA-1 and -13 (A), gRNA-8 and -12 (B), or gRNA-1 and -13 (C) expression vectors to HuH-7 cells, alone or in combination. HBsAg and HBeAg levels in cell culture supernatant were measured at 72 h post transfection using a time-resolved fluoroimmunoassay; D: The plasmid pBB4.5-HBV1.2 (0.5 μg) was co-transfected with different combinations of two gRNAs expression vectors (each 0.75 μg) to HuH-7 cells. HBsAg level in culture supernatant was measured at 72 h post transfection using a time-resolved fluoroimmunoassay; E: HBeAg level in culture supernatant was measured using time-resolved fluoroimmunoassay as above; F: The cytotoxicity of dual gRNAs was examined using an MTT assay. Data are shown as mean ± SE of 3 independent experiments. All *P*-values are from Student's *t*-test. HBV: Hepatitis B virus; HBsAg: Hepatitis B virus surface antigen; HBeAg: Hepatitis B virus e antigen; MTT: Methyl thiazolyl tetrazolium.

HBsAg and HBeAg levels in the culture supernatant were measured. The results showed that even with low transfection efficiency, gRNA-5 and gRNA-12 together could significantly suppress both HBsAg and HBeAg levels, particularly for HBsAg which almost dropped 50% off (Figure 5A). Besides, gRNA-5 and gRNA-12 could also significantly reduce the HBV DNA level in the HepAD38 cell culture supernatant (Figure 5B). Since cccDNA reservoirs are the cause of chronic HBV infection, next, the HBV cccDNA level in HepAD38 cells was measured. To make sure that the quantitative measurement of the HBV cccDNA was reliable, KCl precipitation, plasmid-safe ATP-dependent DNase (PSAD) digestion and RCA (Isothermal PCR amplification for the circular HBV cccDNA rather than the linear rcDNA) were employed, followed by quantitative PCR with the cccDNA specific primers (sense primer covers the cleavage site of gRNA-12) that target the gap region of HBV genome. As expected, cccDNA level in HepAD38 cells was significantly suppressed by gRNA-5 and gRNA-12 (Figure 5C). PCR amplification confirmed that the fragment between two cleavage sites of dual gRNAs in HBV expressing template was removed (Figure 4A and C). Similarly, HBV-specific gRNA could also guide Cas9 to destroy HBV cccDNA (Figure 5D). Above results suggested that the downregulation of HBsAg and HBeAg levels was at least partially due to the destruction of HBV cccDNA.

DISCUSSION

Hepatitis B is an important occupational hazard for health workers. An estimated 240 million people are chronically infected with HBV[27]. Multiple studies demonstrated that without therapy, 15%-40% of HBV infected patients would eventually develop cirrhosis, liver failure, or hepatocellular carcinoma (HCC)[28]. Because of the high HBV-related morbidity and mortality, HBV infection has been a serious public

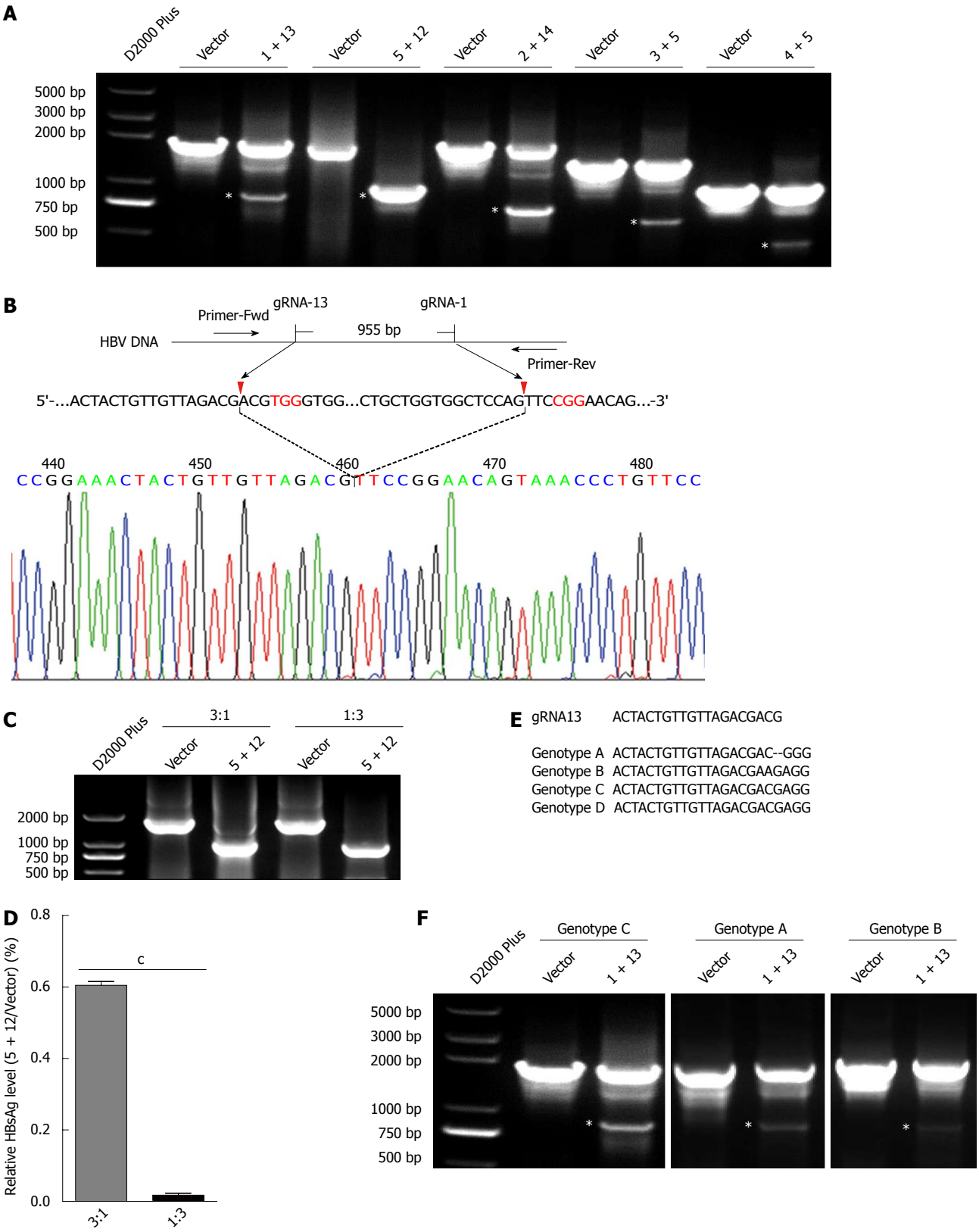


Figure 4 Dual gRNAs could destroy hepatitis B virus genome. A: The plasmid pBB4.5-HBV1.2 (0.5 μg) was co-transfected with gRNA-1 and -13, gRNA-5 and -12, gRNA-2 and -14, gRNA-3 and -5, or gRNA-4 and -5 expression vectors (each 0.75 μg) to HuH-7 cells. Cellular DNA was extracted at 72 h post transfection, and PCR amplifications were performed using the primers beyond the cleavage sites of each dual gRNAs; B: Sequencing analysis of the smaller fragment formed by gRNA-5 and -12; C: The plasmid pBB4.5-HBV1.2 was co-transfected with the gRNA-5 and -12 expression vectors at different ratios to HuH-7 cells. The smaller fragment was amplified at 72 h post transfection; D: HBsAg level in culture supernatant was measured using a time-resolved fluoroimmunoassay. Data are shown as mean ± SE of 3 independent experiments (Student's *t*-test); E: Comparative analysis for the sequences of gRNA13 and HBV genome of genotypes A-D; F: The plasmid pGEM-HBV1.3A or pGEM-HBV1.3B and gRNA-1 and -13 expression vectors were co-transfected into HuH-7 cells, and PCR amplifications were performed at 72 h post transfection as above. HBV: Hepatitis B virus; HBsAg: Hepatitis B virus surface antigen. The asterisks mean that the digested fragment of the HBV expressing templates.

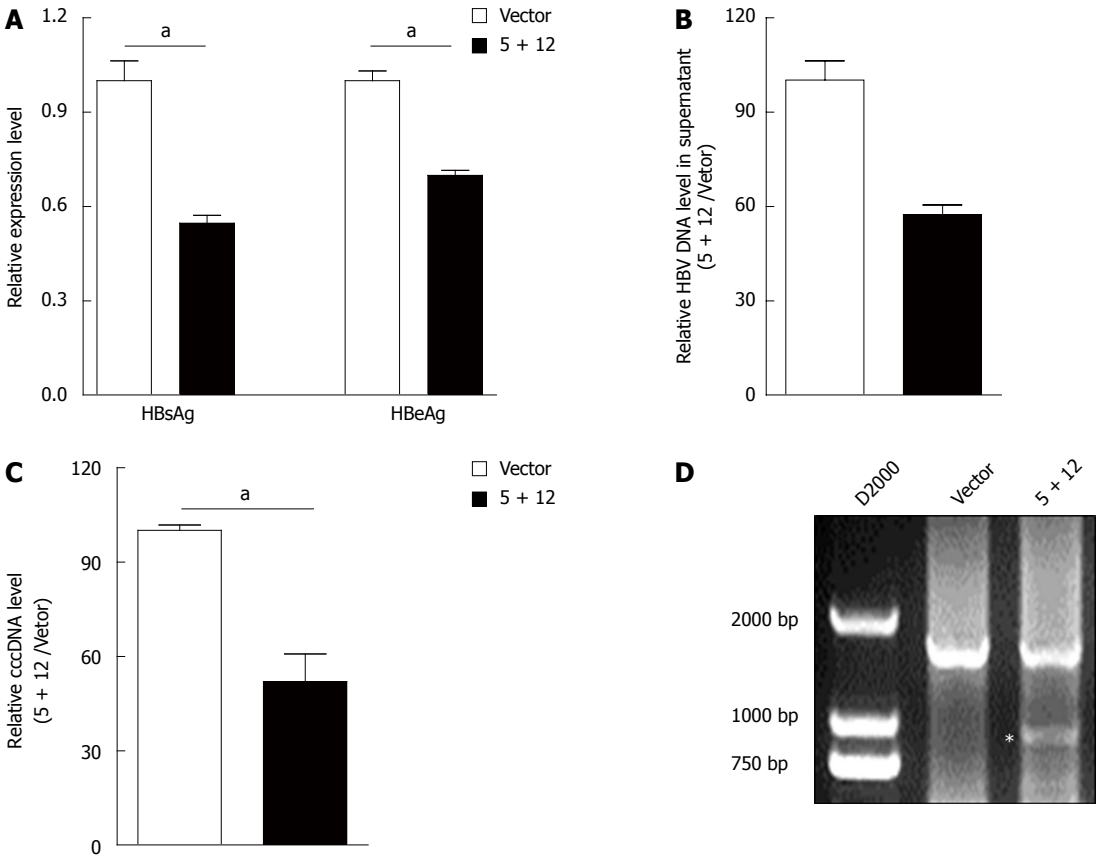


Figure 5 Hepatitis B virus-specific gRNA could suppress the genotype D hepatitis B virus replication and destroy cccDNA. A: HepAD38 cells were seeded into a 6 well plate. Then, gRNA-5 and -12 expression vectors (each 2 μg) were co-transfected into HepAD38 cells. HBsAg and HBeAg levels in the culture supernatant of HepAD38 cells were measured at 72 h post transfection using a time-resolved fluoroimmunoassay; B: HBV DNA levels in the culture supernatant of HepAD38 cells were measured at 72 h post transfection using real-time quantitative PCR; C: HBV cccDNA levels in HepAD38 cells were measured using KCl precipitation, plasmid-safe ATP-dependent DNase (PSAD) digestion, rolling circle amplification and quantitative PCR combined method; D: PCR amplification was performed using the primers beyond the cleavage sites of dual gRNAs following KCl precipitation, PSAD digestion and rolling circle amplification. HBV: Hepatitis B virus; HBsAg: Hepatitis B virus surface antigen. The asterisk means the digested fragment of cccDNA.

health problem worldwide. Unfortunately, none of the current therapy regimes could eradicate cccDNA in the nucleus of infected hepatocytes, which is the main cause of HBV persistent infection. In recent years, new strategies focused on targeting cccDNA are under intensive study. Three recent studies have suggested the potential use of CRISPR/Cas9 genome editing technique to cut HBV DNA and cccDNA *in vitro* and *in vivo*, as well as for chronic or *de novo* HBV infection[15-17]. However, those studies just focused on single genotype HBV, genotype A or D. Since genotypes B and C are also the major genotypes in the world, particularly in China, in this study, 15 gRNAs were designed according to the HBV genome sequences of genotypes A, B, C and D. These gRNAs covered different regions of HBV genome, including S, P, X, C, enhancer Ⅰ and basic core promoter (BCP) regions. Our results here indicated that most of the gRNAs could significantly suppress the production of HBsAg and HBeAg. Interestingly, the suppressive efficiency of different gRNAs varied depending on the region in which they against.

As multiple gRNAs could be efficiently used to edit genome, here different combinations of two gRNAs were used to suppress HBV replication *in vitro*. And high efficiency against genotypes A-D HBV replication was found in different dual gRNAs combinations. However, the combination of gRNA-1 and 12 exhibited an antagonistic effect in the suppression of HBsAg production. Currently we are unable to explain this phenomenon. A potential possibility is that the combination of gRNAs-1 and -12 would cut off and remove the fragment between the two cleavage sites, where C, S promoter (Sp) and preS1 regions located. As a result, the enhancer Ⅱ, the core promoter (Cp) and the basic core promoter (BCP) regions were re-ligated to the immediate upstream of S gene, and the expression of HBsAg was transcriptionally enhanced. In line with this, we found that dual gRNAs could indeed remove the fragment specifically between the two cleavage sites of gRNAs, which was confirmed by PCR sequencing. Such antagonistic effect reminded us that when multiple gRNAs were used in combination, the side effect should be taken into consideration.

In addition to genotype, due to absence of proof-reading function of the viral reverse transcriptase and a high replication rate, the HBV population in an individual was a composition of genetically distinct but

closely related variants known as quasispecies[29,30]. It is likely that such variants will cause the imperfect match between the designed gRNAs and their target viral genome sequence. The off-target effect of CRISPR/Cas system has been mentioned, and such shortage could become an advantage when gRNAs were used to guide the cleavage of the high variety HBV genome in an infected individual. Just for this, although the target sequence in HBV genotype B harbors a one base mismatch with gRNA-13, it still could be cleft (Figure 4F). Besides, we and others have proved the oncogenicity of HBV integration[31,32]. Potentially, multiple gRNAs can be used to remove the integrated HBV DNA to cure the HBV-related HCC. Most importantly, we found that wild-type HBV cccDNA level in HepAD38 cells was significantly downregulated by HBV-specific gRNA, which indicated that HBV cccDNA could be destroyed by CRISPR/Cas9 system. It is difficult to confirm the gRNA-induced destruction of cccDNA by Southern blot for the low transfection efficiency of the PX458 plasmid. We will demonstrate the gRNA-induced destruction of cccDNA by Southern blot when the CRISPR/Cas9 system was combined with the recombinant adeno-associated virus system. Above all, the indel mutation and destruction of cccDNA should be the main reason of gRNA-induced suppression of cccDNA replication.

Above all, we confirmed that CRISPR/Cas9 system had the potential to destroy cccDNA which is the template of HBV replication. Our data further demonstrated that dual gRNAs guided CRISPR/Cas9 system may be a useful tool to eradicate CHB and HBV-related disease with infection by multiple HBV genotypes. However, there is still a long way for the treatment of HBV infected patients by CRISPR/Cas9 system. Next, we will validate the effect of HBV-specific gRNA on HBV replication in HBV transgenic mice by hydrodynamic injection method or using the adenovirus or adeno-associated virus system. In the future, dual gRNAs guided CRISPR/Cas9 system will be developed by adeno-associated virus system which is a potential vector for gene therapy, and be used in combination with the current NAs and/or IFN-α based antiviral therapy for the possible clinical cure of CHB.

COMMENTS

Background

The CRISPR/Cas9 system is a novel genome editing tool which leads to genome indel mutation by inducing a double-strand break (DSB) at the target genomic locus. The CRISPR/Cas9 system has been successfully applied not only for genome editing in cells, but also for disrupting the genome of virus, including adenovirus, herpes simplex virus, human immunodeficiency virus and hepatitis B virus (HBV). It has become recognized that CRISPR/Cas9 system may be a potential tool to cure viral disease.

Research frontiers

Several recent studies have confirmed the potential use of CRISPR/Cas9 system to cleave HBV DNA and cccDNA *in vitro* and *in vivo*, as well as for chronic or *de novo* HBV infection.

Innovations and breakthroughs

Eradication of cccDNA, the only way to reach the clinical cure of chronic hepatitis B (CHB), is still an unresolved problem in the treatment of CHB. Previous studies have reported that the CRISPR/Cas9 system can efficiently cleave the expressing template of HBV. However, those studies just focus on HBV of genotype A or D. Whereas, genotypes A, B, C, D are the predominant genotypes of HBV in East Asia and other part of the world, so it is necessary to design guide RNAs (gRNAs) specific for HBV genotypes A-D. Besides, dual gRNAs can get the synergistic effect in genome editing. In this paper, we evaluated the potential use of dual gRNAs guided CRISPR/Cas9 system to clear the HBV genome of genotypes A-D.

Applications

This study provides a potential tool, the dual gRNAs guided CRISPR/Cas9 system, to eradicate CHB and HBV-related disease with infection by multiple HBV genotypes. In the future, the dual gRNAs guided CRISPR/Cas9 system will be developed by adeno-associated virus system which is a potential vector for gene therapy, and be used in combination with the current NAs and/or IFN-α based antiviral therapy for the possible clinical cure of CHB.

Terminology

The clustered regularly interspaced short palindromic repeats/Cas9 nuclease (CRISPR/Cas9) system is a novel genome editing tool derived from the adaptive immune system of bacteria and archaea. The CRISPR/Cas9 system promotes genome editing by inducing a double-strand break (DSB) at the target genomic locus. In the absence of a repair template, DSBs are re-ligated through the non-homologous end joining process, which leads to the insertion/deletion (indel) mutations.

Peer-review

The authors present a highly interesting functional study in which they showed that dual gRNA guided CRISPR/Cas9 system can suppress replication of multiple HBV genotypes as well as promote clearance of HBV cccDNA in the cell culture. This paper confirms that the presented dual gRNA/CRISPR/Cas9 system might be a potential approach for eradication of HBV cccDNA and thus considered a new and additional antiviral treatment option for CHB.

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ORIGINAL ARTICLE

Basic Study

Carvedilol may attenuate liver cirrhosis by inhibiting angiogenesis through the VEGF-Src-ERK signaling pathway

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Author contributions: Ding Q performed the majority of the experiments; Tian XG, Li Y and Wang QZ provided vital reagents and analytical tools and edited the manuscript; Ding Q and Zhang CQ designed the study and wrote the manuscript.

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Abstract

AIM: To investigate the effect of carvedilol on angiogenesis and the underlying signaling pathways.

METHODS: The effect of carvedilol on angiogenesis was examined using a human umbilical vascular endothelial cell (HUVEC) model. The effect of carvedilol on cell viability was measured by CCK8 assay. Flow cytometry was used to assess the effect of carvedilol on cell cycle progression. Cell migration, transwell migration and tube formation assays were performed to analyze the effect of carvedilol on HUVEC function. Vascular endothelial growth factor (VEGF) induced activation of HUVECs, which were pretreated with different carvedilol concentrations or none. Western blot analysis detected the phosphorylation levels of three cell signaling pathway proteins, VEGFR-2, Src, and extracellular signal-regulated kinase (ERK). The specific Src inhibitor PP2 was used to assess the role of Src in the VEGF-induced angiogenic pathway.

RESULTS: Carvedilol inhibited HUVEC proliferation in a dose-dependent manner (IC50 = 38.5 mmol/L). The distribution of cells in the S phase decreased from 43.6% to 37.2%, 35.6% and 17.8% by 1, 5 and 10 μmol/L carvedilol for 24 h, respectively. Carvedilol (10 μmol/L) reduced VEGF-induced HUVEC migration from 67.54 ± 7.83 to 37.11 ± 3.533 ($P < 0.001$). Carvedilol concentrations of 5 μmol/L and 10 μmol/L reduced cell invasion from 196.3% ± 18.76% to 114.0% ± 12.20% and 51.68% ± 8.28%, respectively. VEGF-induced tube formation was also reduced significantly by 5 μmol/L and 10 μmol/L carvedilol from 286.0 ± 36.72 to 135.7 ± 18.13 ($P < 0.05$) and 80.27 ± 11.16 ($P < 0.01$) respectively. We investigated several intracellular protein levels to determine the reason for these reductions. Treatment with 10 μmol/L carvedilol reduced VEGF-induced tyrosine phosphorylation of VEGFR-2 from 175.5% ± 8.54% to 52.67% ± 5.33%

(P < 0.01). Additionally, 10 μmol/L carvedilol reduced VEGF-induced ERK 1/2 phosphorylation from 181.9% ± 18.61% to 56.45% ± 7.64% (P < 0.01). The VEGF-induced increase in Src kinase activity was alleviated by carvedilol [decreased from 141.8% ± 15.37% to 53.57 ± 7.18% (P < 0.01) and 47.04% ± 9.74% (P < 0.01) at concentrations of 5 and 10 μmol/L, respectively]. Pretreatment of HUVECs with Src kinase inhibitor almost completely prevented the VEGF-induced ERK upregulation [decreased from 213.2% ± 27.68% to 90.96% ± 17.16% (P < 0.01)].

CONCLUSION: Carvedilol has an anti-angiogenic effect on HUVECs. This inhibitory effect is mediated by VEGF-induced Src-ERK signaling pathways.

Key words: Carvedilol; Adrenergic β-antagonists; Angiogenesis; Liver cirrhosis; Drug utilization

Core tip: Carvedilol has been used for the treatment of portal hypertension for many years. In this study, carvedilol directly inhibited the proliferation and tube formation of cultured human umbilical vascular endothelial cells. Moreover, this study is the first to investigate the mechanism of the anti-angiogenic effect of carvedilol, which functions by inhibiting vascular endothelial growth factor-induced mitogen-activated protein kinase signaling pathways. These novel activities of carvedilol provide insight into the anti-angiogenic mechanisms involved, and highlight its potential therapeutic application against angiogenesis-dependent liver fibrosis.

Ding Q, Tian XG, Li Y, Wang QZ, Zhang CQ. Carvedilol may attenuate liver cirrhosis by inhibiting angiogenesis through the VEGF-Src-ERK signaling pathway. *World J Gastroenterol* 2015; 21(32): 9566-9576 Available from: URL: http://www.wjgnet.com/1007-9327/full/v21/i32/9566.htm DOI: http://dx.doi.org/10.3748/wjg.v21.i32.9566

INTRODUCTION

Angiogenesis, the growth of new blood vessels from pre-existing ones, is an essential process during embryonic and postnatal development[1]. Although angiogenesis may be considered beneficial for tissue growth and regeneration, as well as for the growth and repair of injured tissues, the same process is also currently believed to promote inflammatory and fibro-proliferative diseases and malignancies in different organs, including the chronically injured liver. Data from the literature during the last decade have unequivocally linked angiogenesis with liver fibrogenesis and chronic liver disease progression, suggesting that angiogenesis may favor fibrogenesis[2,3]. Experimental anti-angiogenic therapies, regardless of the specific drug or therapeutic strategy employed, have resulted in significant inhibition of fibrogenic progression, reductions in inflammatory infiltrates and α-smooth muscle actin-positive myofibroblasts, a decrease in portal pressure, and, with some drugs, a reduction in porto-systemic collateral vessel formation and splanchnic vascularization in portal hypertensive animal models or in cirrhotic animals[2-4].

During angiogenesis, multiple signaling pathways are activated by various factors such as vascular endothelial growth factor (VEGF), platelet-derived growth factor, angiopoietin, Notch, Wnt, transforming growth factor-β, and guanine nucleotide binding regulatory protein-coupled receptor agonists, with VEGF as a dominating player. VEGF-A is the primary mediator of angiogenesis and primarily activates the tyrosine kinase receptor VEGF receptor (VEGFR)-2[5]. The activity of Src, a membrane-associated nonreceptor tyrosine kinase, is central in signal transduction downstream of several growth factor receptors including VEGFR[6]. Its downstream transduction pathways include phosphatidylinositol-3-kinase, focal adhesion kinase, and mitogen-activated protein kinase (MAPK)[6]. The Ras/Raf/MEK/ERK signaling pathway, also known as the extracellular signal-regulated kinase (ERK) pathway, is the most classical pathway of MAPK pathways[7], and plays an important role in angiogenesis[8,9]

The β-adrenergic system interferes with several essential steps of neovascularization, thus providing novel therapeutic opportunities for the use of β-blockers in the treatment of angiogenesis-dependent diseases. For instance, propranolol was reported to inhibit the growth of capillary hemangiomas[10] and could inhibit human umbilical vascular endothelial cell (HUVEC) proliferation and migration, as well as the VEGF-induced activation of VEGFR-2 in these cells[11]. Alpha-blockers also exhibited anti-angiogenic effects. Doxazosin and terazosin inhibited the growth and tube formation of human vascular endothelium cells and suppressed angiogenesis in mice[12,13]. Prazosin has also been reported to exhibit anti-angiogenic activity and to differentially modulate apoptotic pathways depending on the cell type. Patients with cirrhosis exhibited increased levels of catecholamines with increasing liver disease severity[14]. Therefore, non-selective beta blockers (NSBBs) may be particularly potent for the inhibition of angiogenesis in cirrhosis. Carvedilol is a third-generation, nonselective-blocker that also possesses α1-adrenergic blocking[15], antioxidant[16], and calcium antagonist properties[17]. Similar to propranolol and nadolol, the current standard NSBBs for treating portal hypertension, carvedilol has been proposed for use in decreasing portal pressure. Because of its α1- and β-blocking effects, carvedilol has the potential to induce a more pronounced decrease in portal pressure.

The present study investigated whether carvedilol can modulate HUVEC functions that are essential for angiogenesis. We observed that carvedilol inhibited growth factor-induced proliferation, migration, and tube formation *in vitro*. We also found that carvedilol caused G1 cell cycle arrest of HUVECs and inhibited VEGF-induced MAPK signaling pathways. Taken together, these novel activities of carvedilol provided insight regarding the anti-angiogenic mechanisms involved, and highlighted its potential therapeutic application against angiogenesis-dependent liver fibrosis.

MATERIALS AND METHODS

Cell culture

HUVECs were obtained from ATCC (Manassas, VA, United States) and cultured in RPMI 1640 medium containing 10% fetal calf serum, 100 U/mL penicillin, and 100 mg/mL streptomycin at 37 ℃ in a humidified atmosphere of 5% CO_2.

Recombinant human VEGF 165 was obtained from PeproTech (Rocky Hill, NJ, United States), and Matrigel was obtained from BD Biosciences (Bedford, MA, United States). Anti-phospho-VEGFR2 (Tyr1175), anti-total VEGFR, anti-phospho-p44/p42 MAPK (ERK 1/2), anti-total ERK 1/2, anti-phospho-Src family (Thr416), and anti-total Src family rabbit monoclonal antibodies were obtained from Cell Signaling Technology (Bedford, MA, United States). PP2 (Src family kinase inhibitor) was obtained from Selleckchem (United States).

Cell counting Kit-8 assay

HUVECs were seeded in 96-well plates at 5000 cells/well and allowed to adhere overnight. After the cells were treated with various concentrations of carvedilol dissolved in 1% FBS medium for 24 h, 10 μL of cholecystokinin-8 (Beyotime, Haimen, Jiangsu, China) was added to each well. The plates were incubated at 37 ℃ for 2 h, and then the optical density (OD) was measured at 450 nm using a scanning multi-well spectrophotometer (Bio-Rad Model 550, CA, United States). The cell inhibitory rate was calculated using the following equation: cell inhibitory rate = [(OD control group-OD experiment group)/OD control group] × 100%. All experiments were performed in triplicate and repeated three times.

Cell cycle analysis

HUVECs were seeded at an initial density of 5 × 10^4 cells/mL and incubated with or without the indicated concentration of carvedilol supplemented with 5% FBS for 24 h. At the end of treatment, the cells were collected by mild trypsin digestion, washed with ice-cold PBS and fixed in 70% (v/v) ethanol overnight at 4 ℃. Then, the cells were stained with propidium iodide (PI) working solution with 20 μg/mL RNase A and 50 μg/mL PI for cellular DNA staining at room temperature for 1 h in the dark before analysis. The cell population fraction in each phase of the cell cycle was determined as a function of the DNA content using a BD Biosciences FACSCalibur flow cytometer equipped with FACSDiva 7.0 software.

Endothelial cell migration assay

HUVECs were allowed to grow to full confluence in 6-well plates and then starved with cell medium containing 1% FBS overnight to inactivate cell proliferation. The next day, scrape wounds were made, and the cells were treated with or without VEGF (50 ng/mL) and various concentrations of carvedilol for 24 h. Images were acquired at time 0 and 24 h after scraping. ImageJ software was used to analyze the areas of cell migration into the scrape wound.

Endothelial cell transwell migration assay

Transwells (8-μm pore size; Costar) were precoated with 0.1% gelatin for 30 min in a cell incubator. Then, the transwells were washed with PBS and assembled into 24-well plates. HUVECs (5 × 10^4 cells) suspended in 100 μL of serum-free medium plus various concentrations of carvedilol were seeded in the top chambers, while 600 μL of serum-free medium was added to the lower chamber. After 2 h, cell migration was initiated by adding VEGF-enriched medium to the lower chamber. The plate was incubated at 37 ℃ in 5% CO_2/95% air for another 12 h. At the end of the incubation period, the cells were fixed in 4% (v/v) paraformaldehyde and stained with hematoxylin and eosin. The migrated cells in the lower surface were imaged and counted under a light microscope.

Tube formation assay

Matrigel was thawed at 4 ℃ overnight before the experiment. A 96-well plate was coated with cold Matrigel and incubated for 0.5-1 h at 37 ℃. Carvedilol and the VEGF-treated cell suspensions (100 mmol/L) were added to the wells and were incubated at 37 ℃ with 5% CO_2. The cells were monitored every 2 h under a microscope for 6-12 h, and tube formation was imaged at 8 h.

Western blot analysis

HUVECs were treated as described for the cell cycle analysis. Then, the cells were harvested with trypsin/EDTA (Clonetics) and lysed with RIPA buffer, which contains 1% PMSF and 1% protein phosphatase inhibitor mixture (Applygen, Beijing, China). The protein concentration of the samples was determined by the BCA assay (Biocolor Bioscience and Technology Company) according to the manufacturer's protocol. Equal amounts of protein extracts (50 mg) were subjected to electrophoretic analysis using a 10% sodium dodecyl sulfate (SDS)/polyacrylamide gel and transferred to PVDF membranes (Millipore, MA, United States). The membranes were incubated

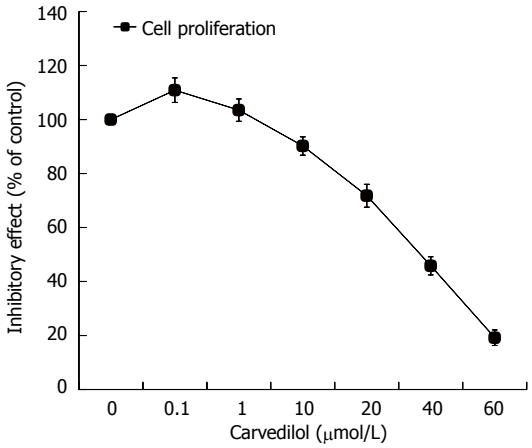

Figure 1 Effect of carvedilol on human umbilical vascular endothelial cell proliferation. Subconfluent cultures of human umbilical vascular endothelial cells were exposed to increasing concentrations of carvedilol (0.01-40 μmol/L), and the extent of cell proliferation was measured by cholecystokinin-8 assay. The values are the means of three independent experiments.

overnight with phospho-VEGFR, VEGFR, phospho-Src, Src, phospho-ERK and ERK monoclonal antibodies. Then, the membranes were washed and incubated with species-specific horseradish peroxidase-labeled secondary antibodies (Zhongshan Golden Bridge Biotechnology, Beijing, China). Signal detection was achieved using an enhanced chemiluminescence system (ECL; Millipore, MA, United States) and autoradiographed using X-ray film. Western blot results were analyzed using MultiGauge Ver. 3.2 software (Fujifilm Life Science, Japan) and all bands were normalized to β-actin expression (AA128, Beyotime, Nantong, China).

Statistical analysis

To compare two or more groups with the control group, one way analysis of variance followed by Dunnett's test was used. All statistical analyses were performed using SPSS version 17.0 (SPSS Inc., Chicago, IL, United States). The data are presented as the mean ± SE. *P* values < 0.05 were considered statistically significant.

RESULTS

Carvedilol inhibits HUVEC proliferation

HUVECs were treated with carvedilol for 24 h in a concentration range of 0.01 to 40 μmol/L. As shown in Figure 1, HUVEC proliferation was strongly inhibited by carvedilol in a dose-dependent manner. The half-maximal inhibition (IC50) was 38.5 μmol/L, which suggested a specific inhibitory effect of carvedilol on EC proliferation.

Carvedilol induces G1 phase arrest of ECs

To study the mechanism of carvedilol inhibition on cell proliferation, we performed cell cycle analysis of the carvedilol-treated HUVECs as shown in Figure 2. Exposing HUVECs to carvedilol resulted in prolongation of the G0/G1 phase ($P < 0.05$ *vs* control), which was associated with a reduction in S and M phases. These data suggested that carvedilol inhibited HUVEC proliferation by inhibiting cell cycle progression. Treatment with 1, 5 and 10 μmol/L carvedilol for 20 h effectively decreased the distribution of cells in the S phase from 43.6% to 37.2%, 35.6% and 17.8%, respectively (Figure 2B).

Inhibitory effects of Carvedilol on chemotactic motility in HUVECs

To assess the anti-angiogenic action of carvedilol *in vitro*, we investigated the inhibitory effects of carvedilol on the chemotactic motility of HUVECs by wound-healing migration assay (Figure 3) and Transwell assay (Figure 4). We found that 10 μmol/L carvedilol could reduce VEGF-induced HUVEC migration from 67.54% ± 7.831% to 37.11% ± 3.53% ($P < 0.01$) (Figure 3). Cell invasion was also reduced significantly by 5 μmol/L and 10 μmol/L carvedilol [from 196.3% ± 18.76% to 114.0% ± 12.20% ($P < 0.05$) and 51.68% ± 8.28% ($P < 0.01$), respectively] (Figure 5). These data showed that carvedilol can suppress HUVEC chemotactic motility in a dose-dependent manner.

Inhibitory effects of carvedilol on tube formation in HUVECs

Tube formation in HUVECs incubated on Matrigel-coated plates represents the initial step in angiogenesis. To examine whether carvedilol can directly inhibit angiogenesis, the effect of carvedilol on tube formation was investigated using an *in vitro* angiogenesis assay. When HUVECs were seeded on growth factor-reduced Matrigel, robust tubular-like structures were formed in the presence of VEGF. However, treatment with 5 and 10 μmol/L carvedilol significantly suppressed VEGF-induced tube formation from 286.0 ± 36.72 to 135.7 ± 18.13 ($P < 0.05$) and 80.27 ± 11.16 ($P < 0.01$). These results indicated that carvedilol could block VEGF-induced angiogenesis *in vitro* by inhibiting EC motility and tube formation.

Carvedilol suppresses VEGF-induced VEGFR2 and ERK phosphorylation

The biological action of VEGF in ECs is mediated primarily *via* VEGFR-2. VEGFR-2 is dimerized and auto-phosphorylated after binding with VEGF. Therefore, we examined whether carvedilol could have an effect on VEGF-induced tyrosine phosphorylation of VEGFR-2. Quiescent HUVECs were pretreated with various concentrations of carvedilol for 24 h and then stimulated with 50 ng/mL VEGF for 2 h. We observed that VEGF-induced tyrosine phosphorylation of VEGFR-2 was reduced by carvedilol in a dose-dependent manner [from 175.5% ± 8.54% to 129.6% ± 14.83% ($P < 0.05$), 103.5% ± 14.32% ($P < 0.01$) and 52.67% ± 5.33% ($P < 0.01$) at concentrations of 1, 5 and 10 μmol/L, respectively] (Figure 6A).

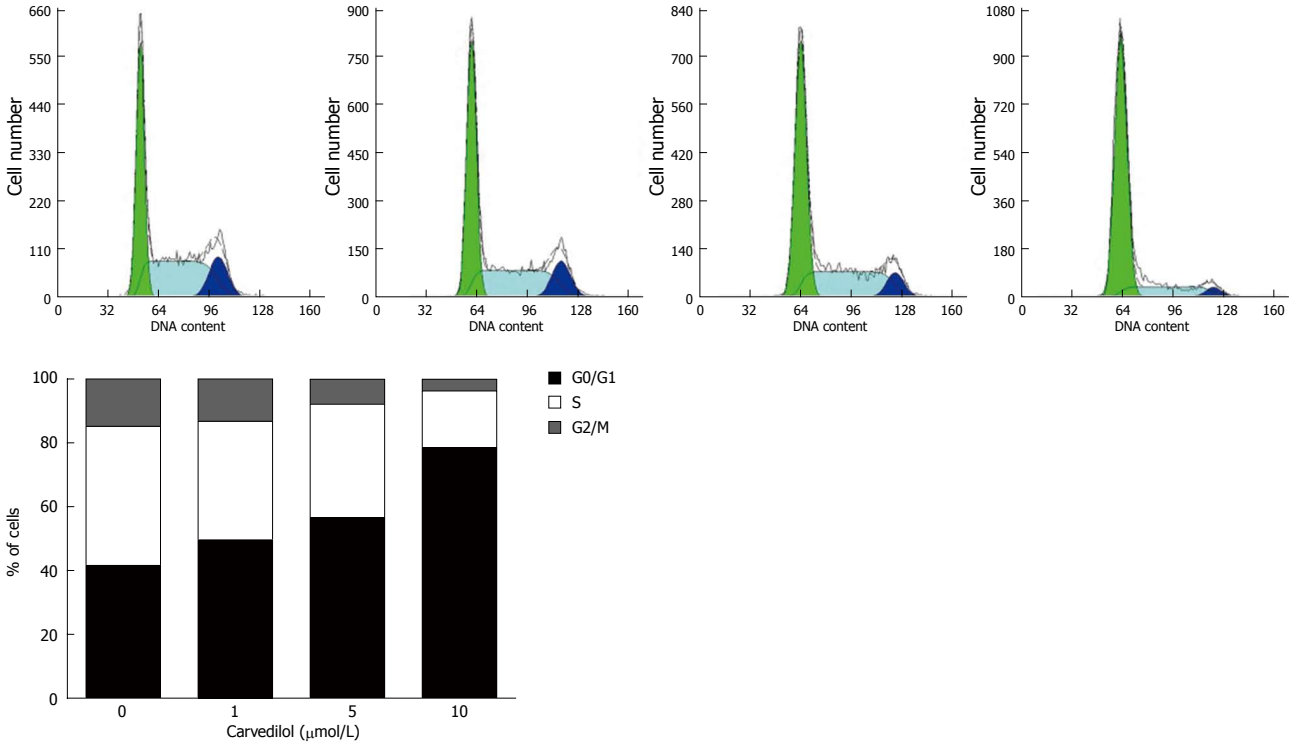

Figure 2 Effects of carvedilol on cell cycle progression in human umbilical vascular endothelial cells. Cells were exposed to either control medium (containing 5% FBS) or medium containing the indicated concentrations of carvedilol and were treated as indicated in the Materials and Methods section. Representatives cytometric profiles (upper) and the percentage of cells at G0/G1, S and G2/M phases (%) (lower). The DNA content was determined by measuring the fluorescence intensity of incorporated propidium iodide.

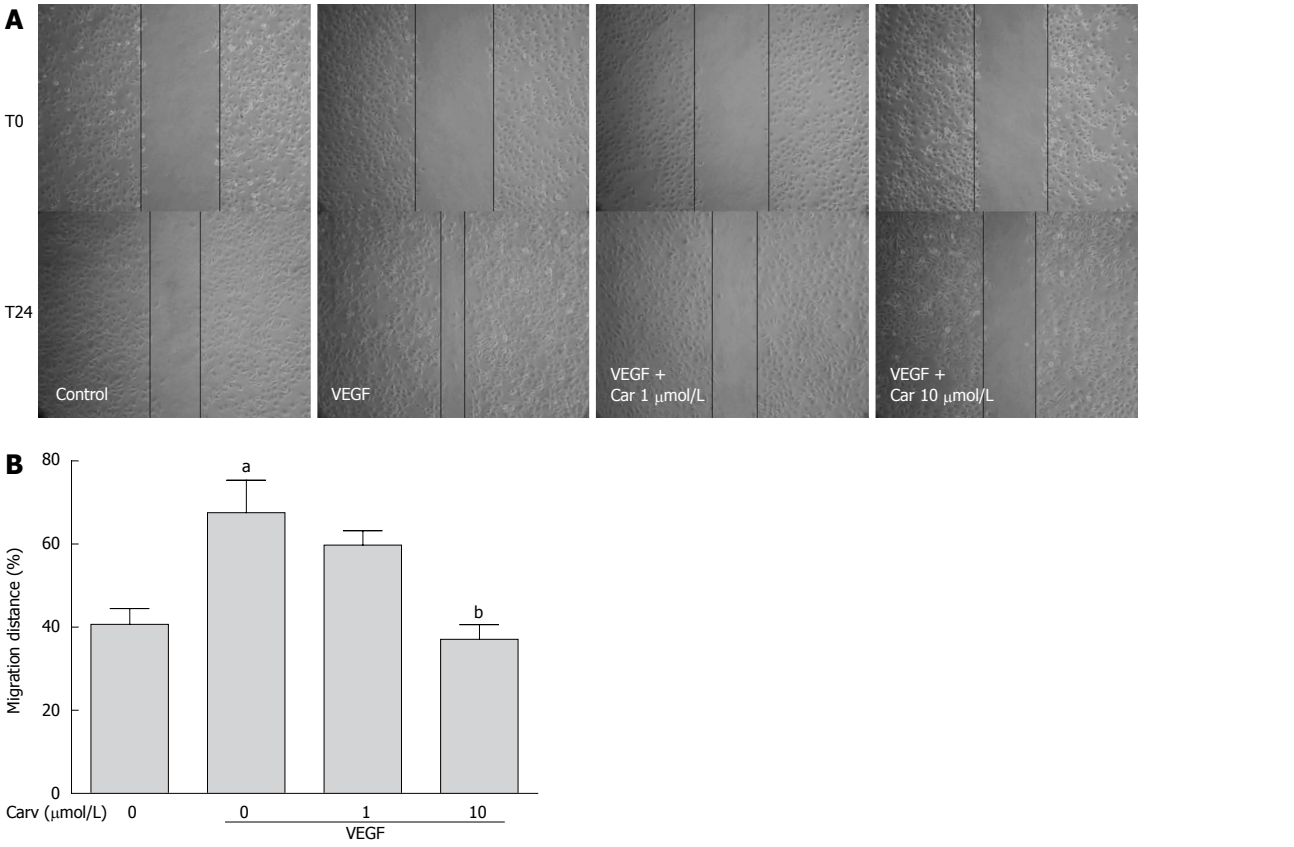

Figure 3 Effect of carvedilol on cell migration. A: HUVECs were scraped and then treated with or without VEGF (50 ng/mL) and various concentrations of carvedilol. Images were taken at 0 and 24 h after scraping; B: Cell migration across the scraped area was determined using ImageJ software. (n = 12, $^{a}P < 0.05$ *vs* control, $^{b}P < 0.01$ *vs* VEGF alone). HUVEC: Human umbilical vascular endothelial cell; VEGF: Vascular endothelial growth factor.

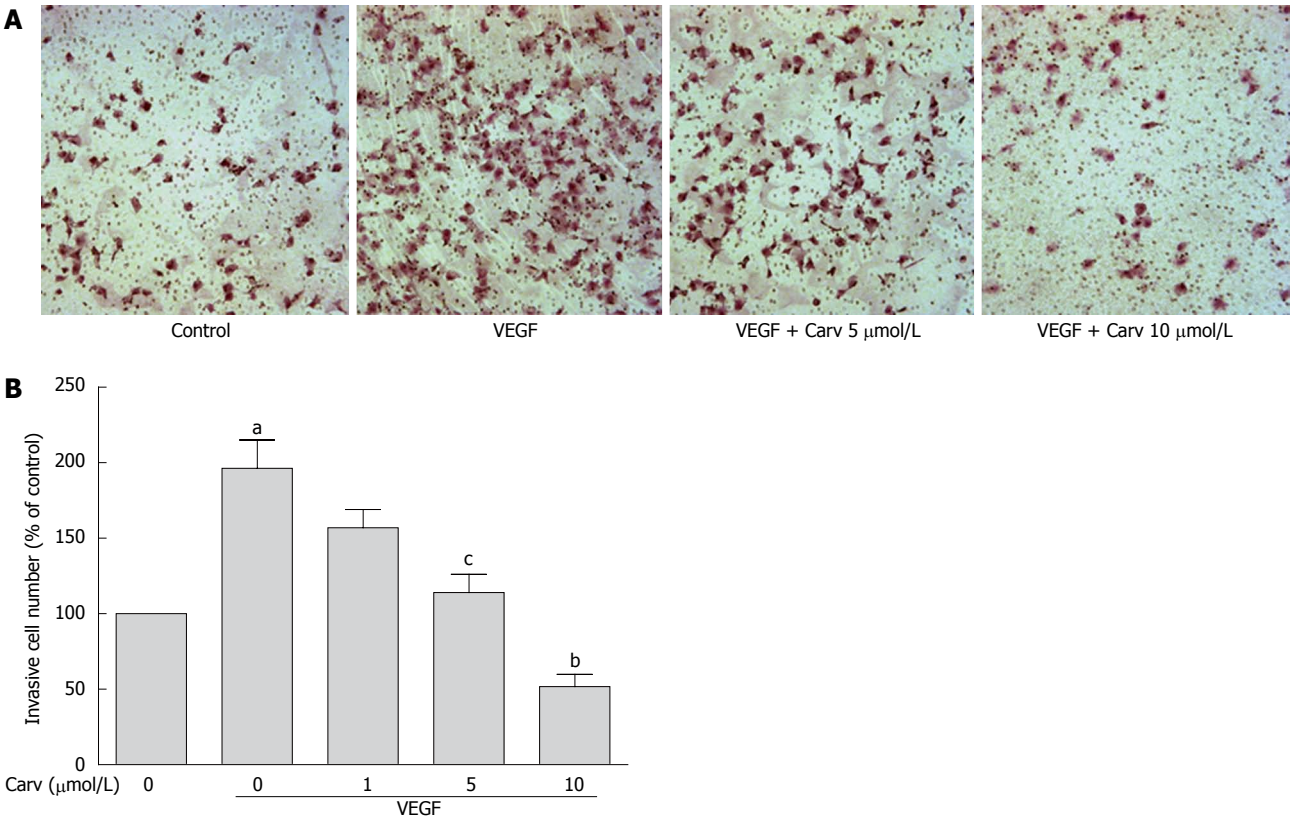

Figure 4 Effect of carvedilol on cell invasion. A: HUVECs were seeded in the upper chamber of a Transwell and treated with various concentrations of carvedilol. The bottom chamber was filled with VEGF-enriched media. After 12 h, the HUVECs that migrated through the membrane were stained by hematoxylin and eosin; B: Cell numbers were quantified (n = 12, $^{a}P < 0.05$ *vs* control, $^{c}P < 0.05$, $^{b}P < 0.01$ *vs* VEGF alone).

Quiescent HUVECs were treated as mentioned above. Compared with the control group, the relative protein expression of phospho-ERK increased in the VEGF-treated group. Treatment with 1, 5, and 10 μmol/L carvedilol also reduced VEGF-induced ERK 1/2 phosphorylation (Figure 6B) in a dose-dependent manner [from 181.9% ± 18.61% to 159.8% ± 20.52%, 124.3% ± 11.48% and 56.45% ± 7.64% ($P < 0.01$), respectively].

Role of Src kinase in carvedilol-induced inhibition of tyrosine phosphorylation of ERK1/2

Because VEGFR-2 activation results in the activation of the Src family kinase[18], we studied whether Src kinase mediates tyrosine phosphorylation signaling from VEGFR to ERK. First, we tested whether carvedilol had an effect on VEGF-induced tyrosine phosphorylation of Src. We observed an increase in Src kinase activity in cells treated with VEGF for 30 min, and this increase was attenuated by carvedilol [decreased from 141.8% ± 15.37% to 110.3% ± 10.40%, 53.57% ± 7.18% ($P < 0.01$) and 47.04% ± 9.74% ($P < 0.01$) at concentrations of 1, 5, and 10 μmol/L, respectively] (Figure 7A). Then, the experiments illustrated in Figure 7B were conducted. Pretreating HUVECs with PP2 (10 μmol/L), a Src kinase inhibitor, almost completely prevented the VEGF-induced upregulation of ERK levels [decreased from 213.2% ± 27.68% to 90.96% ± 17.16% ($P < 0.01$)]. Evidently, Src activation may be a preceding step in the VEGF-induced upregulation of ERK and may play an important role in angiogenesis. These findings suggest that Src may serve as a mediating factor for signaling from VEGFR to ERK during carvedilol-induced inhibition of angiogenesis.

DISCUSSION

Our present study is the first to demonstrate that carvedilol inhibits VEGF-induced angiogenesis by blocking the VEGF/VEGFR-Src-ERK mediated pathway. The data obtained here provide a novel mechanism to account for the inhibitory effect of carvedilol on VEGF-mediated angiogenesis.

VEGF is a focus of applications of current concepts in the development of new anti-cancer and anti-angiogenetic therapies[19,20], and is capable of provoking angiogenesis by interacting with its two receptor tyrosine kinases [VEGFR-1; Flt-1) and VEGFR-2 (Flk-1)] expressed on ECs[21,22]. This study demonstrated that carvedilol significantly suppressed the VEGF-induced phosphorylation of Tyr1175 of VEGFR-2, suggesting that carvedilol may exert anti-angiogenic behavior by targeting VEGFR-2, and could impair its associated downstream signal transduction cascade. VEGFR-2 appears to mediate the majority

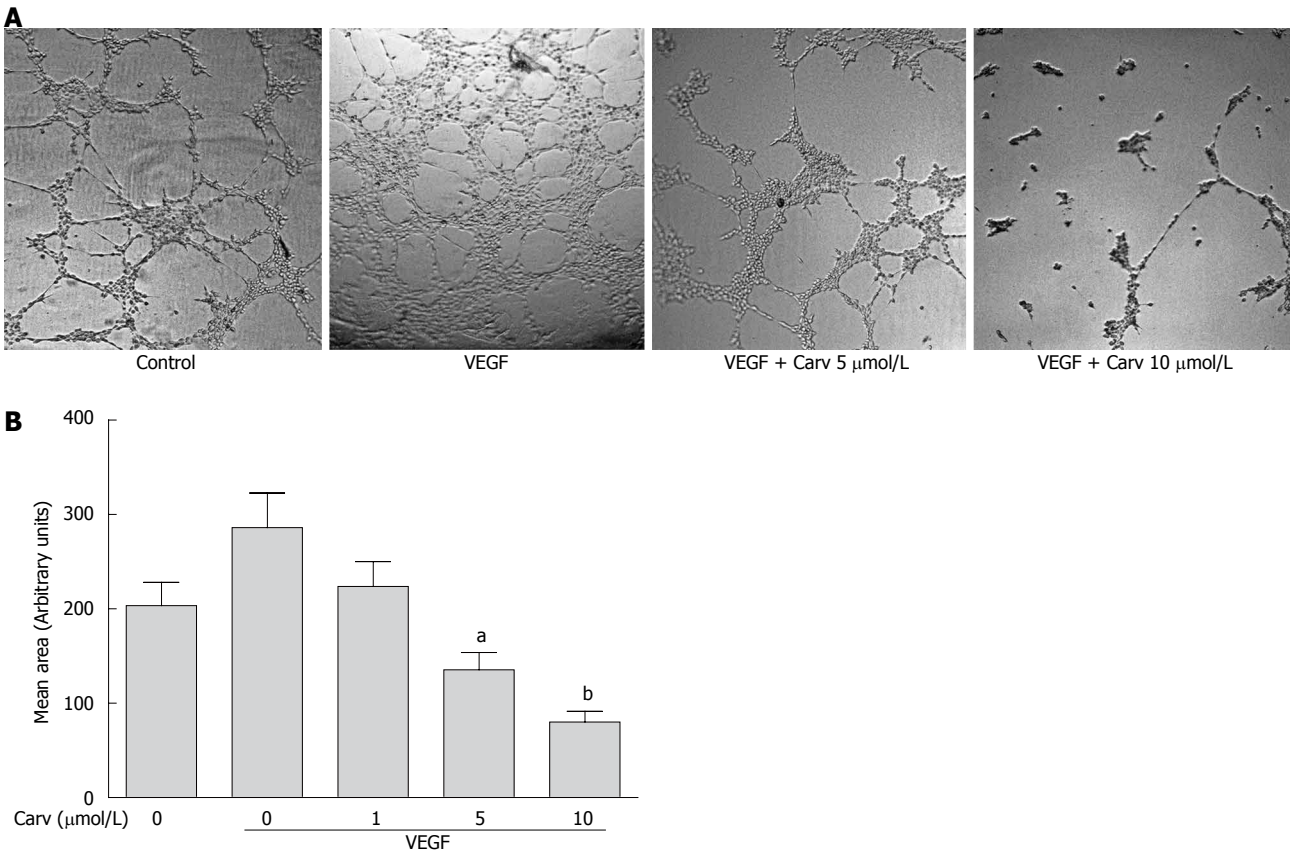

Figure 5 Carvedilol inhibited the vascular endothelial growth factor-induced tube formation of human umbilical vascular endothelial cells. A: HUVECs were placed in Matrigel with or without VEGF (50 ng/mL) in the presence or absence of different concentrations of carvedilol. After 8 h, tubular structures were imaged (magnification, × 100); B: Quantification of the area of cell alignment was determined using ImageJ software (n = 3, ^{a}P < 0.05, ^{b}P < 0.01 *vs* VEGF alone).

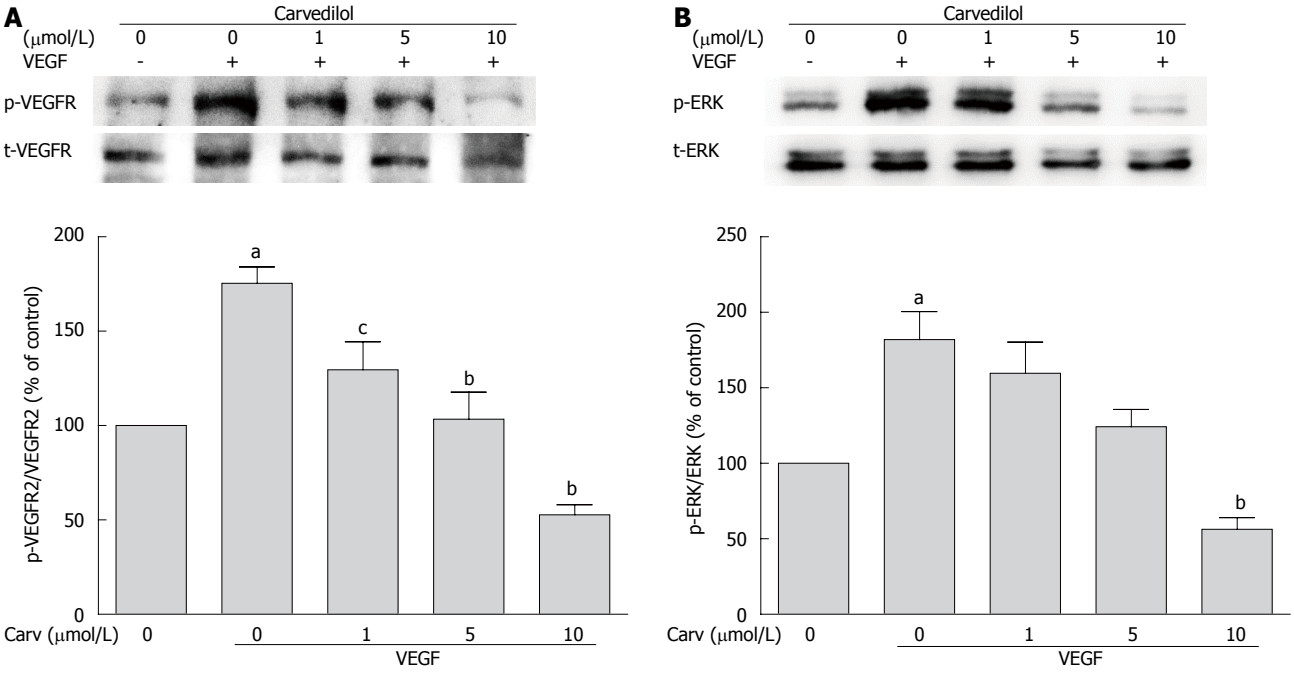

Figure 6 Effect of carvedilol on vascular endothelial growth factor angiogenic signaling. HUVECs were treated with VEGF (50 ng/mL) for 2 h in the presence or absence of different concentrations of carvedilol. Cell lysates were processed as indicated in the Materials and Methods. p-VEGFR2/VEGFR2 (A) and p-ERK/ERK (B) levels (n = 3, ^{a}P < 0.05 *vs* control, ^{c}P < 0.05, ^{b}P < 0.01 *vs* VEGF alone). ERK: Extracellular signal-regulated kinase.

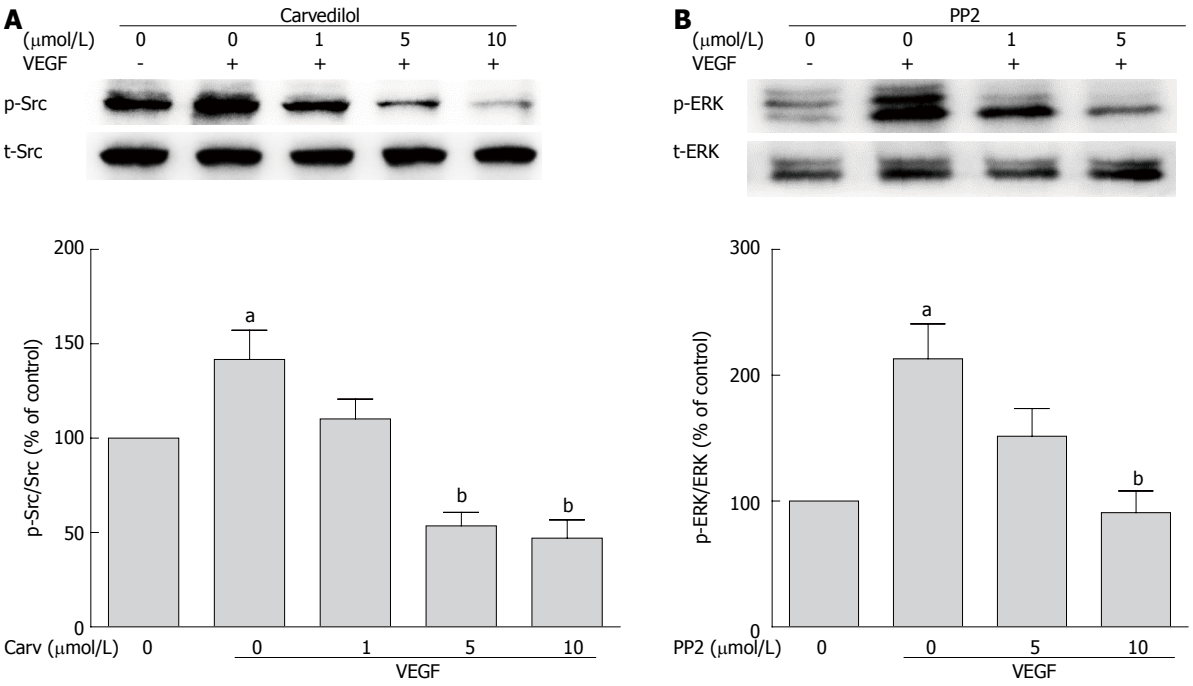

Figure 7 Role of Src in angiogenic signaling. A: HUVECs were treated with VEGF (50 ng/mL) for 2 h in the presence or absence of different concentrations of PP2. Cell lysates were subjected to SDS-PAGE for Src phosphorylation; B: HUVECs were treated with VEGF (50 ng/mL) for 2 h in the presence or absence of different concentrations of PP2. Cell lysates were processed as indicated in the Materials and Methods (n = 3, $^{a}P < 0.05$ *vs* control, $^{b}P < 0.01$ *vs* VEGF alone).

of the known cellular responses to VEGFA, and the largest class of neoangiogenesis blocking drugs are the multiple receptor tyrosine kinase inhibitors that target VEGFR-2, such as sorafenib, vatalanib, and TSU-68[23-25]. The present results showed that carvedilol inhibited the phosphorylation of VEGFR-2, and thus blocked the subsequent activation of downstream signals. However, carvedilol did not inhibit VEGFR-2 activation completely. Thus, other signaling pathways may be involved in VEGFR-2 and ERK activation in response to carvedilol; however, this possibility requires further investigation. The reduction in phosphorylated VEGFR-2 may explain the reason for the observed decrease in the VEGF-induced phosphorylation of ERK1/2, the major downstream target of VEGFR-2, following treatment with carvedilol. Various agents have been shown to block VEGFA-induced angiogenesis by inhibiting the activation of the MAPK signal cascade[26-28]. VEGF-mediated survival[29,30] and protection against receptor-mediated apoptosis were Src-dependent[31]. Thus, we further investigated the effect of carvedilol on Src, and showed that Src plays an important role in the signaling process. However, the mechanism by which carvedilol decreases VEGFR-2 phosphorylation requires further investigation.

Historically, an interest in angiogenesis has been associated with studies regarding cancer and factors involved in tumor progression[32]. Recent data regarding chronic liver diseases have demonstrated that angiogenesis may contribute to the progression of fibrosis during the wound healing process in chronic liver damage[2,3,33-35]. Anti-angiogenic therapy has been found to be efficient in the prevention of fibrosis in experimental models of chronic liver diseases; blocking angiogenesis could be a promising therapeutic option in patients with advanced fibrosis[36-38]. Carvedilol is a cardiovascular drug that is licensed for the treatment of chronic heart failure and is widely used to reduce portal pressure in patients with cirrhosis. In the present study, we extended the current knowledge regarding the role of carvedilol in anti-angiogenesis. Catecholamines exhibited pro-carcinogenic effects[39], and NSBBs antagonized catecholamine-driven cell migration and tumor angiogenesis, invasiveness and proliferation in gastric, breast and pancreatic cancers[40-42]. A number of studies have recently highlighted the potential anti-angiogenic properties of β-blockers[10,43]. Carvedilol has also been shown to inhibit the proliferation of various human cell lines such as osteosarcoma MG63[44] and rat C6 glioma cells[45]. One report indicated that carvedilol could inhibit tube formation similar to other β-blockers[40]; however, no detailed mechanism had been explored. Here, we demonstrated that carvedilol directly inhibited the proliferation and formation of capillary-like tube structures in cultured HUVECs. Importantly, we provided compelling evidence that activation of the VEGFR/Src/ERK1/2 signaling pathway was involved in the carvedilol-induced inhibition of angiogenesis in HUVECs. Interestingly, carvedilol was reported previously to have anti-inflammatory and pro-angiogenic effects in a canine model of chronic ischemic cardiomyopathy[40]. Our study did not

contradict the results of that study because the model and inducing factors are completely different. Taken together, these studies suggest that carvedilol may have a two-way regulatory effect on angiogenesis.

In addition to the effect of carvedilol on angiogenesis, carvedilol can induce human hepatoma cell death[46]. Because most cases of hepatocellular carcinoma (HCC) develop in patients with chronic liver disease (70%-90%)[47,48], finding a drug that would ameliorate either liver cirrhosis or HCC may be beneficial for the other disease. One limitation of our study was the lack of *in vivo* experiments; however, carvedilol was reported previously to have antifibrotic effects on chronic carbon tetrachloride-induced liver damage[49], suggesting that carvedilol may ameliorate liver fibrosis-related angiogenesis. We are currently examining this possibility. If carvedilol can be demonstrated to have anti-angiogenic and anti-neoplastic effects, then patients with chronic liver disease could benefit greatly from this treatment.

In conclusion, the results from the present study indicate that carvedilol can exert an anti-angiogenic effect by inhibiting the activation of the cSrc/ERK 1/2 signaling pathway. The findings from the present *in vitro* studies strongly suggest the potential application of carvedilol in the treatment of disorders associated with localized angiogenesis. If this hypothesis holds true, then the use of carvedilol in chronic liver diseases could be extended beyond the prevention of variceal bleeding; its multiple effects including anti-angiogenesis, anti-fibrosis, and even anti-cancer would reinforce the recommendation of this drug as first-line treatment in chronic liver disease patients.

COMMENTS

Background

Angiogenesis is an important process during embryonic and postnatal development. Data from the literature in the last decade have suggested that angiogenesis may favor fibrogenesis. Beta-blockers were reported to suppress angiogenesis. Carvedilol is a third-generation nonselective β-blocker that has been used for treating portal hypertension. This study investigated whether carvedilol could modulate human umbilical vascular endothelial cell functions that are essential for angiogenesis.

Research frontiers

Blocking angiogenesis could be a promising therapeutic option in patients with advanced fibrosis. Carvedilol is widely used to reduce portal pressure in patients with cirrhosis. In this study, the authors extended the current knowledge regarding the role of carvedilol in anti-angiogenesis.

Innovations and breakthroughs

This study is the first to demonstrate that carvedilol inhibits vascular endothelial growth factor (VEGF)-induced angiogenesis by blocking the VEGF/VEGFR-Src-ERK-mediated pathway. The data obtained here provide a novel mechanism to account for the inhibitory effect of carvedilol on VEGF-mediated angiogenesis.

Terminology

Angiogenesis is the growth of new blood vessels from pre-existing ones, it may fuel inflammatory and fibro-proliferative diseases including chronically injured liver. VEGF-A is the primary mediator of angiogenesis and primarily activates the tyrosine kinase receptor VEGF receptor (VEGFR)-2. Beta-blockers are a class of drugs that could block the action of endogenous catecholamines, epinephrine (adrenaline) and norepinephrine (noradrenaline), in particular on adrenergic β-receptors.

Applications

These results strongly suggest the potential application of carvedilol in the treatment of disorders associated with localized angiogenesis. The use of carvedilol in chronic liver diseases could be extended beyond the prevention of variceal bleeding; its multiple effects, including anti-angiogenesis, anti-fibrosis, and even anti-neoplastic, would reinforce the recommendation of this drug as first-line treatment in chronic liver disease patients.

Peer-review

This study investigated the effect of carvedilol on angiogenesis *in vitro*. The results suggest that carvedilol could inhibit angiogenesis by blocking the VEGF-induced VEGFR-Src-Erk pathway. Thus, these findings suggest potential new uses for carvedilol.

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ORIGINAL ARTICLE

Case Control Study

Reduction in duodenal endocrine cells in irritable bowel syndrome is associated with stem cell abnormalities

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Author contributions: El-Salhy M planned the study, recruited the patients, performed gastroscopies, analyzed the data and drafted the manuscript; Hatlebakk JG and Hausken T checked the study plan, read the manuscript and contributed to interpretation and analysis of the results; all authors approved the version of the article to be published.

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Data sharing statement: Technical appendix, statistical code, and dataset available from the corresponding author at magdy.el-salhy@helse-fonna.no.

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Abstract

AIM: To determine whether the decreased density of duodenal endocrine cells in irritable bowel syndrome (IBS) is associated with abnormalities in stem cell differentiation.

METHODS: The study sample comprised 203 patients with IBS (180 females and 23 males with a mean age of 36 years) and a control group of 86 healthy subjects without gastrointestinal complaints (77 females and 9 males with a mean age of 38 years). The patients included 80 with mostly diarrhoea (IBS-D), 47 with both diarrhoea and constipation (IBS-M), and 76 with mostly constipation (IBS-C). Both the patients and controls underwent gastroscopy and four biopsy samples were taken from the descending part of the duodenum, proximal to the papilla of Vater. The biopsy samples were sectioned and immunostained for Musashi 1 (Msi-1), neurogenin 3 (NEUROG3), secretin, cholecystokinin (CCK), gastric inhibitory peptide (GIP), somatostatin and serotonin. Immunostaining

was performed with an ultraView Universal DAB Detection Kit (v1.02.0018, Venata Medical Systems, Basal, Switzerland) using the BenchMark Ultra immunohistochemistry/*in situ* hybridization staining module (Venata Medical Systems). Endocrine cell densities were quantified by computerized image analysis using the Olympus cellSens imaging program.

RESULTS: The densities of Msi-1 and NEUROG3 cells were significantly lower in IBS patients, regardless of the subtype, than in the controls (77 ± 17 *vs* 8 ± 2; $P = 0.0001$, and 351 ± 33 *vs* 103 ± 22; $P = 0.00002$, respectively). Furthermore, the densities of secretin, and CCK cells were significantly lower in patients with diarrhoea as the predominant IBS symptom (IBS-D) than in the controls (161 ± 11 *vs* 88 ± 8; $P = 0.00007$, and 325 ± 41 *vs* 118 ± 10; $P = 0.00006$, respectively), but not in patients with constipation as the predominant IBS symptom (IBS-C) or those with both diarrhoea and constipation (IBS-M). The GIP cell density was significantly reduced in both IBS-D (152 ± 12 *vs* 82 ± 7; $P = 0.00003$), and IBS-C (152 ± 12 *vs* 107 ± 8; $P = 0.01$), but not in IBS-M. The densities of somatostatin cells in the controls and the IBS-total, IBS-D, IBS-M and IBS-C patients were 81 ± 8, 28 ± 3, 20 ± 4, 37 ± 5 and 28 ± 4 cells/mm^2 epithelium, respectively. The density of somatostatin cells was lower in IBS-total, IBS-D, IBS-M and IBS-C patients than in the controls ($P = 0.00009$, 0.00006, 0.009 and 0.00008, respectively). The density of serotonin cells did not differ between IBS patients and controls.

CONCLUSION: The reduction in duodenal endocrine cells in IBS patients found in this study is probably attributable to the reduction in cells expressing Msi-1 and NEUROG3.

Key words: Cholecystokinin; Irritable bowel syndrome; Musashi-1; Neurogenin 3; Secretin; Somatostatin

Core tip: Musashi 1 (Msi-1) is a marker for both intestinal stem cells and their early progeny, and neurogenin 3 (NEUROG3) is a marker for early intestinal endocrine cell progenitors. The densities of Msi-1 and NEUROG3 cells were reduced in the duodenum of patients with irritable bowel syndrome (IBS), regardless of the subtype, indicating disturbances in both the clonogenic renewal of small intestine stem cells and their proliferation toward endocrine cells. It is most likely that the reduction in the duodenal endocrine cells in patients with IBS is caused by an abnormality in the stem cell clonogenic and proliferation activities.

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INTRODUCTION

Irritable bowel syndrome (IBS) is considered to be a functional disorder of the colon[1,2]. There is no test or examination for the diagnosis of IBS, which is therefore a diagnosis of exclusion, whereby examinations and tests are conducted to exclude organic diseases that could explain the patient's symptoms[3-5]. Although attempts have been made to achieve a positive diagnosis based on symptom assessments[6-10], this system is not widely used in everyday clinical practice[4,11,12]. IBS does not develop into a serious disease or cause death, but it does significantly decrease the quality of life in patients[1].

Several abnormalities have been described in the endocrine cells of the stomach, duodenum, ileum and large intestine of IBS patients[13-29]. These abnormalities are believed to play a major role in the pathophysiology of the disorder and could represent a potential tool for its treatment[2,30-33]. The duodenum contains five endocrine cell types secreting secretin, cholecystokinin (CCK), gastric inhibitory polypeptide (GIP), somatostatin, and serotonin[34]. The functions and mode of actions of these hormones are described in detail elsewhere[32-34]. Briefly, secretin, CCK, GIP and serotonin inhibit gastric emptying. Whereas CCK and serotonin stimulate intestinal motility, and secretin, GIP and somatostatin inhibit its motility. Secretin stimulates pancreatic bicarbonate and fluid secretion and CCK stimulates pancreatic exocrine secretion. CCK also stimulates gallbladder contraction and regulates food intake. Somatostatin inhibits gut exocrine and neuroendocrine secretion and serotonin conveys sensation from the gut by activating submucosal sensory neurons.

In congenital malabsorptive diarrhoea, a paucity of intestinal endocrine cells is caused by a loss-of-function mutation in the gene encoding the protein neurogenin 3 (NEUROG3), which is expressed in the endocrine progenitor cells required for intestinal endocrine development[35]. Furthermore, a decrease in the number of intestinal endocrine cells following small intestine allograft rejection is associated with a reduction in the progenitors of intestinal endocrine cells that express NEUROG3 and NeuroD[36]. It is therefore logical to assume that the abnormalities encountered in the small intestine of IBS patients are associated with disturbance(s) in the small intestine stem cells and/or their progenitors. In order to test this assumption, cells expressing Musashi 1 (Msi-1, expressed in both stem cells and in their early progeny) and NEUROG3 (expressed in early endocrine cell progenitors)[37] were investigated in the duodenum of IBS patients

and were compared with those in healthy volunteers. Furthermore, the endocrine cell types known to occur in the duodenum were investigated.

MATERIALS AND METHODS

Patients

Patients with IBS according to Rome Ⅲ criteria were recruited from those referred to Stord Hospital[9,10]. Thus, 203 patients with IBS were included in the study. They were 180 females and 23 males with a mean age of 36 years (range: 18-66 years). These patients included 80 with mostly diarrhoea (IBS-D), 47 with diarrhoea and constipation (IBS-M), and 76 with mostly constipation (IBS-C). All of the patients had a long duration of IBS symptoms and a symptom onset that was not associated with any gastrointestinal infections. The patients were examined physically, and blood tests were taken to exclude inflammation, and liver, kidney and thyroid diseases. Microscopic colitis was excluded by examining tissue obtained by colonoscopy with segmental biopsy sampling.

Eighty sex-matched healthy subjects were included as controls. They were 77 females and 9 males (mean age, 38 years; age range: 18-67 years). Of these subjects, 59 were healthy volunteers recruited at Stord Hospital, Haukeland University Hospital, and the University of Bergen. Fifteen were recruited from the population of Stord city and 44 were university students or hospital employees. A further 27 were healthy subjects who underwent gastroscopy because of health worries due to a relative being diagnosed with cancer.

The local Committee for Medical and Health Research Ethics West, Norway approved the study. Both patients and healthy volunteers gave oral and written consent.

Gastroscopy, histopathology and immunohistochemistry

Both the patients and controls underwent standard gastroscopy after an overnight fast, during which four biopsy samples were taken from the descending part of the duodenum, proximal to the papilla of Vater. Biopsy samples were also taken from the antral part of the stomach and used to identify the presence of *Helicobacter pylori* (*H. pylori*) (HelicotecUT Plus, Strong Biotech, Taipei, Taiwan).

After fixation in 4% buffered paraformaldehyde, paraffin-embedded biopsies were cut into sections 5 μm thick. The sections were stained with hematoxylin-eosin and immunostained with an ultraView Universal DAB Detection Kit (v1.02.0018, Venata Medical Systems, Basal, Switzerland) using the BenchMark Ultra immunohistochemistry/*in situ* hybridization staining module (Venata Medical Systems). The sections were incubated with primary antibodies for 32 min at 37 ℃. The primary antibodies, which were diluted as per the specific suppliers' instructions, were polyclonal rabbit anti-synthetic peptide conjugated to keyhole limpet haemocyanin derived from within residues 1-100 of human Msi-1 (code ab21628, Abcam, Cambridge, United Kingdom), monoclonal mouse-anti-protein expressed in 293T cells transfected with human NEUROG3 expression vector (code ab87108, Abcam), polyclonal rabbit anti-human secretin (code sc-20938, Santa Cruz Biotechnology, Santa Cruz, CA, United States), rabbit antibodies against human synthetic gastrin-17, which cross reacts with CCK (code A0568, Dako, Glostrup, Denmark), mouse antibodies against human synthetic GIP (code Sc-57162, Santa Cruz Biotechnology), rabbit antibodies against synthetic cyclic somatostatin (code A0566, Dako) and mouse antibodies against serotonin (code R87104, Dako).

Quantification

Cell densities were quantified using the Olympus cellSens imaging program (version 1.7). A microscope (BX 43, Olympus, Tokyo, Japan) equipped with a digital camera (DP 26, Olympus) was used. The number of immunoreactive cells, the number of crypts and the area containing epithelial cells were measured. A × 40 objective was used, and each frame (field) represented a tissue area of 0.035 mm^2. Immunoreactive cells were measured in ten fields, which were chosen randomly. Immunostained sections from the IBS patients and controls were coded, and measurements were made by the same person (M.E.), who was not aware of the identity of the sections. Cell density is expressed as the number of cells per 100 crypts (for Msi-1 and NEUROG3) or the number of cells per square millimetre of epithelium (for endocrine cells).

Statistical analysis

Differences in gender between the patients and controls were determined using the χ^2 test, and the incidence of *H. pylori* infection with Fisher's exact test. The Mann-Whitney non-parametric test was used to establish the difference in age between the patients and controls. The Kruskal-Wallis non-parametric test with Dunn's post-test was used to identify the differences between controls, all IBS patients (IBS-total), and IBS-D, IBS-M, and IBS-C patients. The data are given as mean ± SE values, and $P < 0.05$ was considered statistically significant.

RESULTS

Patients

Neither gender nor age distribution differed significantly between the patients and the controls ($P = 1.0$ and 0.6, respectively). *H. pylori* was found in 12 patients and in 8 healthy subjects, which was not

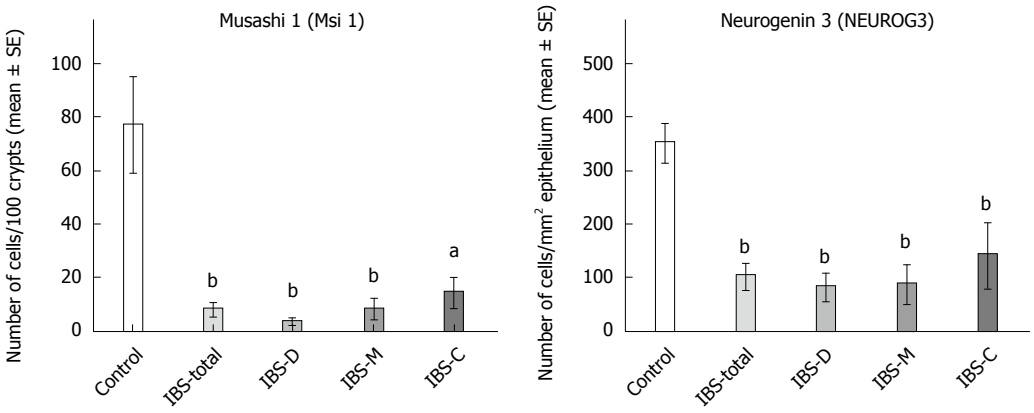

Figure 1 Densities of musashi 1 and neurogenin 3 cells in controls and irritable bowel syndrome-total, irritable bowel syndrome-C, irritable bowel syndrome-D and irritable bowel syndrome-M patients. $^{a}P < 0.05$, $^{b}P < 0.001$ *vs* the control group.

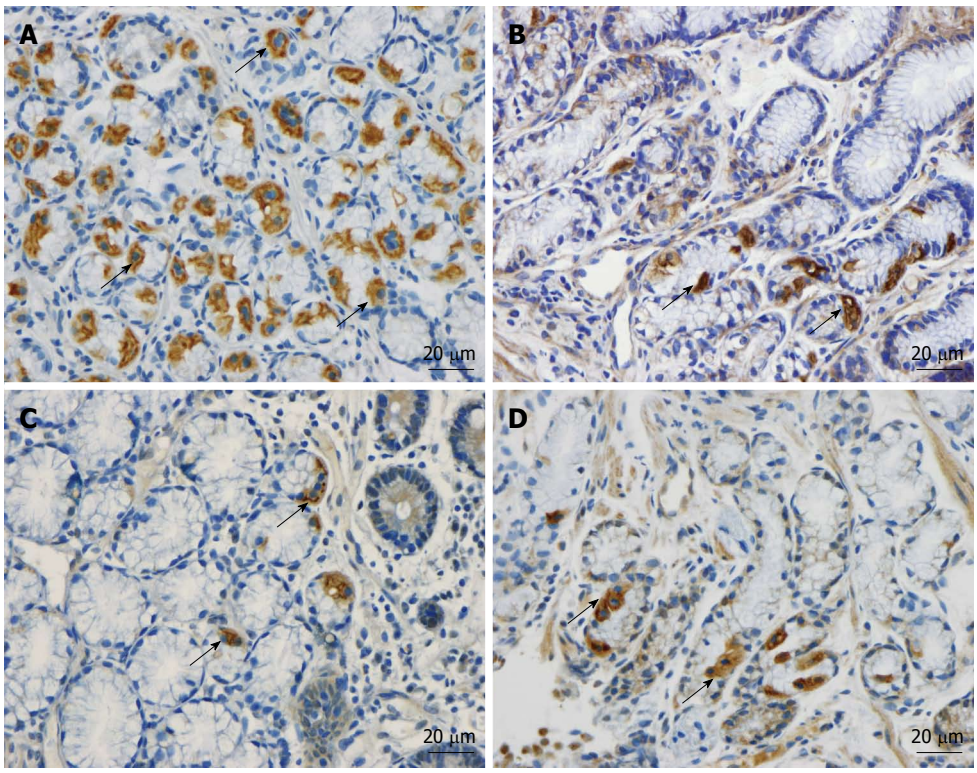

Figure 2 Musashi-1-immunoreactive cells (arrows) in representative subjects from the (A) control, (B) irritable bowel syndrome-D, (C) irritable bowel syndrome-M and (D) irritable bowel syndrome-C patients.

statistically significant ($P = 0.6$).

Gastroscopy, histopathology and immunohistochemistry

The duodenum of both the patients and controls was normal both endoscopically and microscopically. Msi-1 immunoreactivity was observed in both the cytoplasm and nucleus, and immunoreactive cells were found in the crypts of the duodenum of both the patients and the controls. NEUROG3 immunoreactivity was found exclusively in the nuclei of cells, which were observed in both the crypts and alongside the villi; secretin, CCK, GIP, somatostatin and serotonin cells were localized mostly in the crypts.

Quantification

Msi-1: The numbers of Msi-1 cells were 77 ± 17, 8 ± 2, 4 ± 0.7, 8 ± 3 and 15 ± 5 cells/100 crypts in the controls, IBS-total, IBS-D, IBS-M, and IBS-C patients, respectively (Figures 1 and 2). The Kruskal-Wallis test showed that these results were significant ($P = 0.002$). Dunn's post-test revealed that the density of Msi-1 cells was lower in IBS-total, IBS-D, IBS-M, and IBS-C than in the controls ($P = 0.0001$, 0.0005, 0.002, and

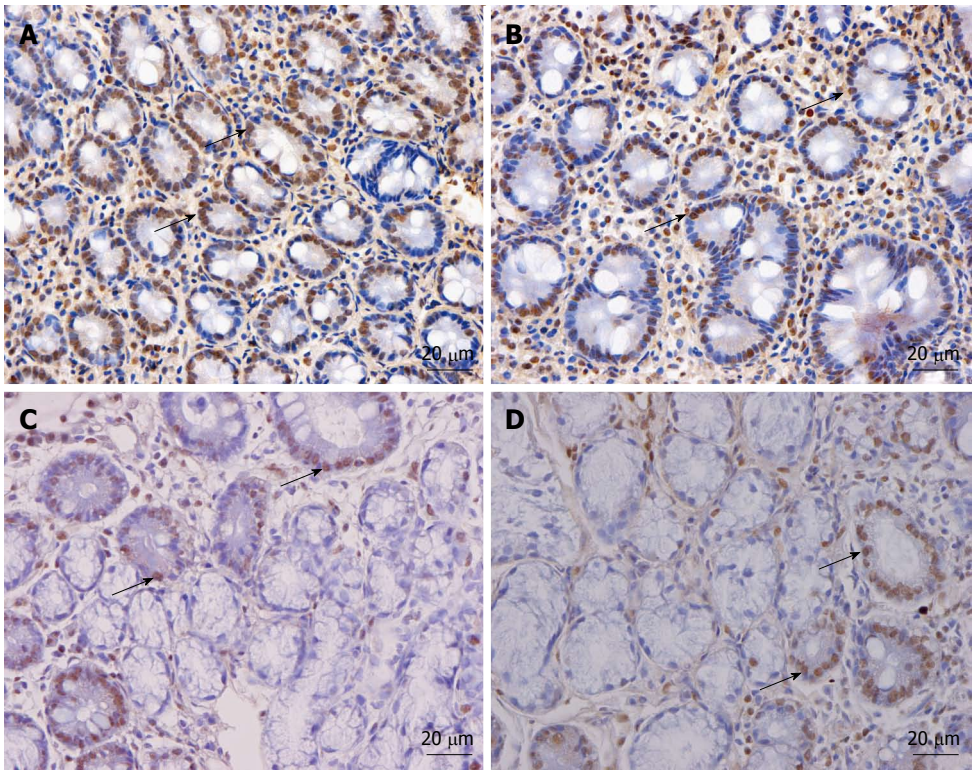

Figure 3 Neurogenin 3-immunoreactive cell nuclei (arrows) in (A) a control subject, (B) irritable bowel syndrome-D, (C) irritable bowel syndrome-M and (D) irritable bowel syndrome-C patients.

0.04, respectively).

NEUROG3: The densities of NEUROG3 cells in the controls and IBS-total, IBS-D, IBS-M and IBS-C patients were 351 ± 33, 103 ± 22, 83 ± 24, 87 ± 34,142 ± 58 and 149 ± 17 cells/100 crypts, respectively (Figure 1 and 3). The Kruskal-Wallis test showed that these results were significant (P = 0.00004). The density of NEUROG3 cells was lower in IBS-total, IBS-D, IBS-M and IBS-C than in the controls (P = 0.00002, 0.00003, 0.001 and 0.0009, respectively).

Secretin: The densities of secretin cells were 161 ± 11, 121 ± 7, 88 ± 8, 56 ± 13 and 133 ± 10 cells/mm^2 epithelium in the controls and the IBS-total, IBS-D, IBS-M and IBS-C patients, respectively (Figures 4 and 5). A comparison between the controls, IBS-total and IBS subgroups using the Kruskal-Wallis test revealed significant differences (P = 0.00003). The density of secretin cells was lower in both the IBS-total and IBS-D patients than in the controls (P = 0.001 and 0.00007, respectively).

CCK: The densities of CCK cells were 325 ± 41, 186 ± 14, 118 ± 10, 242 ± 37 and 224 ± 20 cells/mm^2 epithelium in the controls and the IBS-total, IBS-D, IBS-M and IBS-C patients, respectively (Figures 4 and 6). The Kruskal-Wallis test showed that these results were significant (P < 0.00008). Post-testing revealed that the density of CCK cells was significantly lower in IBS-total and IBS-D patients than in the controls (P = 0.00007 and 0.00006).

GIP: The densities of GIP cells in the controls and IBS-total, IBS-D, IBS-M and IBS-C patients were 152 ± 12, 103 ± 5, 82 ± 7, 116 ± 6, and 107 ± 8 cells/mm^2 epithelium, respectively (Figures 4 and 7). The Kruskal-Wallis test showed that these results were significant (P = 0.00008). The GIP cell density was significantly lower in IBS-total, IBS-D and IBS-C patients than in the controls (P = 0.0006, 0.00003 and 0.01, respectively).

Somatostatin: The densities of somatostatin cells in the controls and the IBS-total, IBS-D, IBS-M and IBS-C patients were 81 ± 8, 28 ± 3, 20 ± 4, 37 ± 5 and 28 ± 4 cells/mm^2 epithelium, respectively (Figure 4). The Kruskal-Wallis test showed that these results were significant (P = 0.00004). The density of somatostatin cells was lower in IBS-total, IBS-D, IBS-M and IBS-C patients than in the controls (P = 0.00009, 0.00006, 0.009 and 0.00008, respectively).

Serotonin: The densities of serotonin cells were 117 ± 15, 160 ± 10, 169 ± 18, 167 ± 16 and 149 ± 17 cells/mm^2 epithelium in the controls and the IBS-total, IBS-D, IBS-M and IBS-C patients, respectively (P = 0.06, Kruskal-Wallis test). There were no significant differences in the densities of serotonin cells between

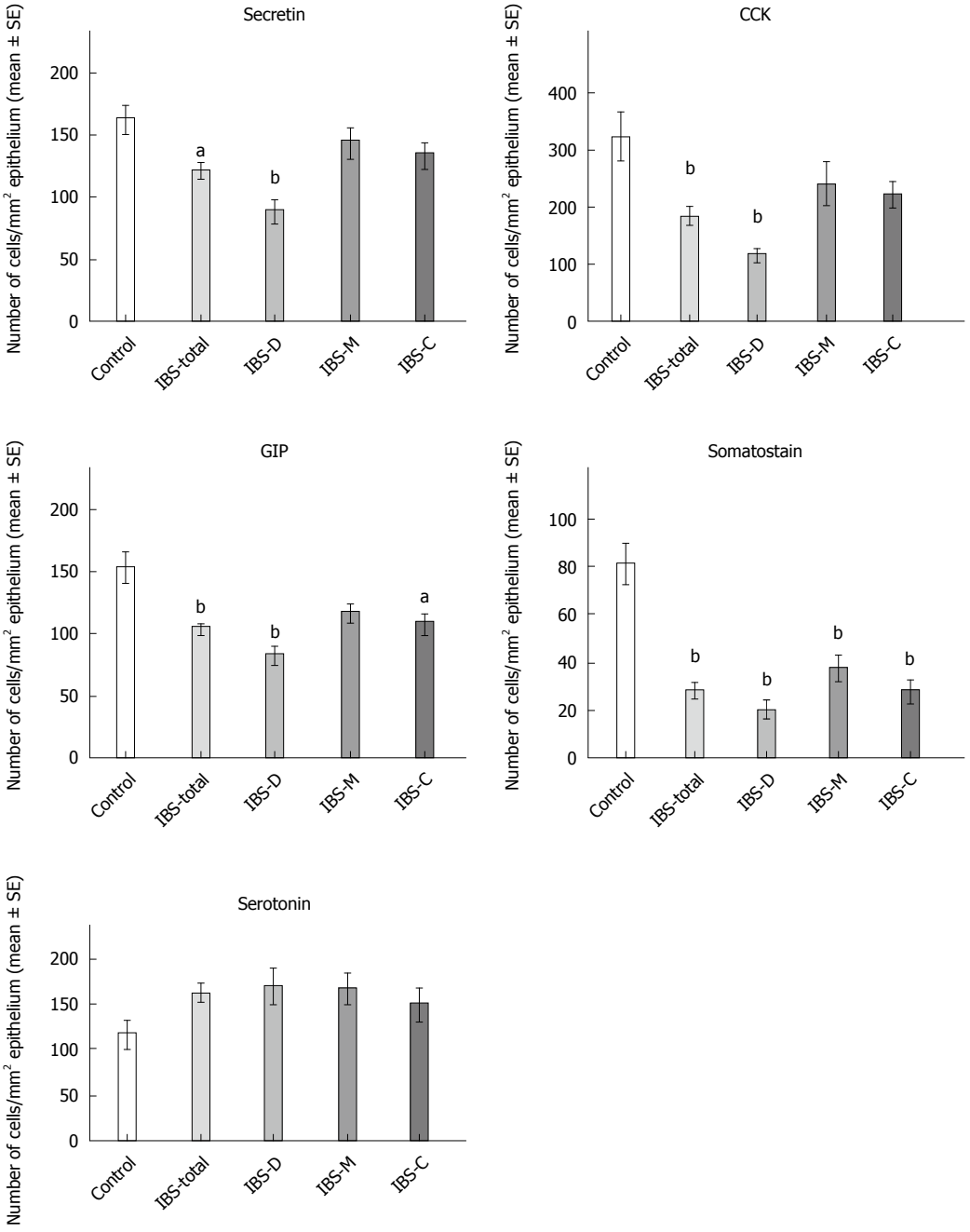

Figure 4 Densities of secretin, cholecystokinin, gastric inhibitory peptide, somatostatin and serotonin cells in controls and irritable bowel syndrome-total, irritable bowel syndrome-D, irritable bowel syndrome-M and irritable bowel syndrome-C patients. $^{a}P < 0.05$, $^{b}P < 0.001$ *vs* the control group.

the controls and the IBS-total and IBS subtypes (Figure 4).

DISCUSSION

Msi-1 is a marker for both intestinal stem cells and their early progeny[37-40]. The present study found that the Msi-1 cell density was reduced in the duodenum of all IBS patients, regardless of the subtype. This finding indicates that the clonogenic renewal of small intestine stem cells is reduced in patients with IBS. Furthermore, cells expressing NEUROG3, which is a marker for early intestinal cell progenitors[36,41,42], was reduced in the duodenum of all patients with IBS, again regardless of the subtype. A reduction in NEUROG3-expressing cells has been found in congenital malabsorptive diarrhoea[35], and a reduction in intestinal endocrine cells has been noted in small intestine allograft rejection[36]. Moreover, NEUROG3-knockout mice failed to develop any intestinal endocrine cells[43]. It is therefore logical to assume that the reduction in duodenal endocrine cells in IBS patients observed herein is attributable to the reduction in cells expressing Msi-1 and NEUROG3.

The abnormalities in duodenal endocrine cells observed in this study are in line with those reported

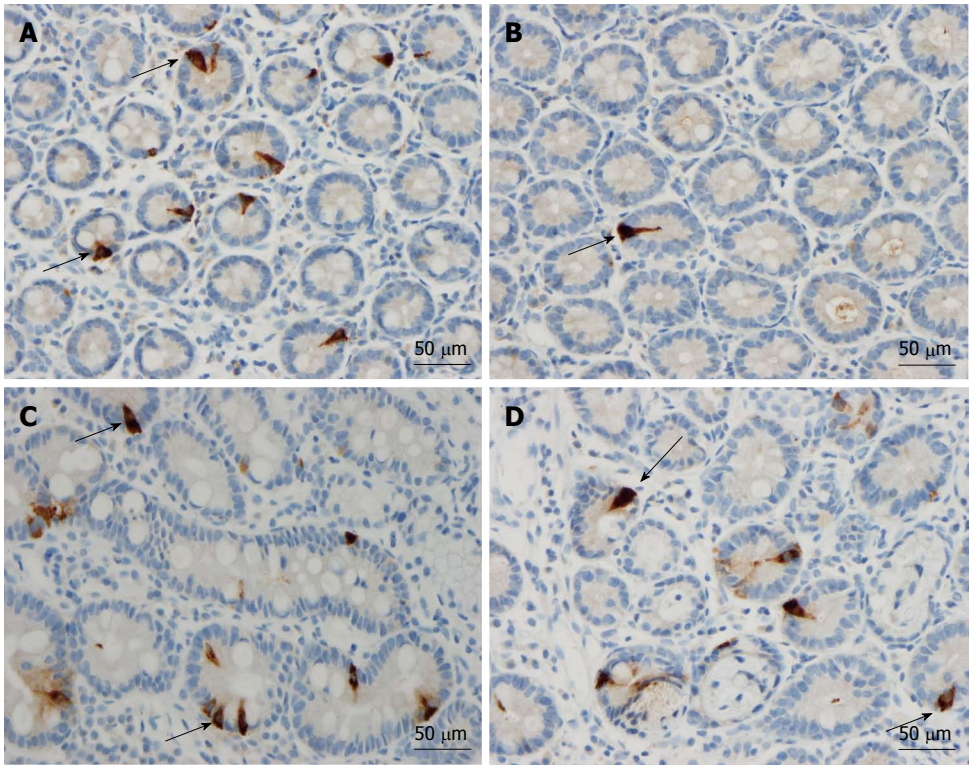

Figure 5 Secretin cells (arrows) in (A) a control subject (B) irritable bowel syndrome-D, (C) irritable bowel syndrome-M and (D) irritable bowel syndrome-C patients.

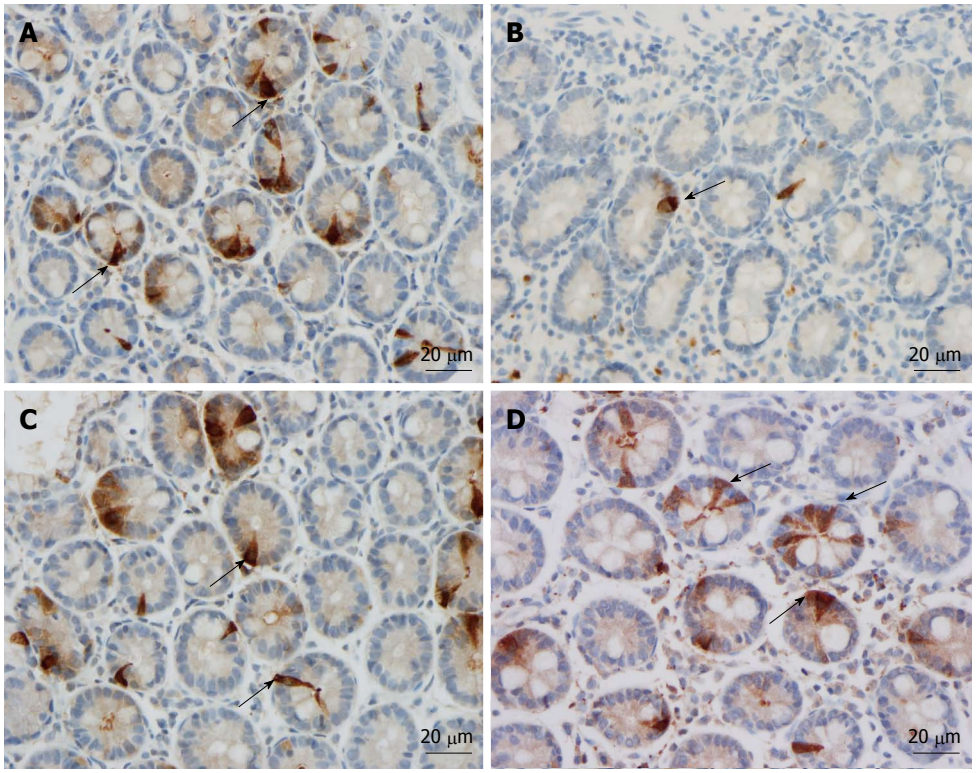

Figure 6 Cholecystokinin-immunoreactive cells (arrows) in (A) a control subject (B) irritable bowel syndrome-D, (C) irritable bowel syndrome-M and (D) irritable bowel syndrome-C patients.

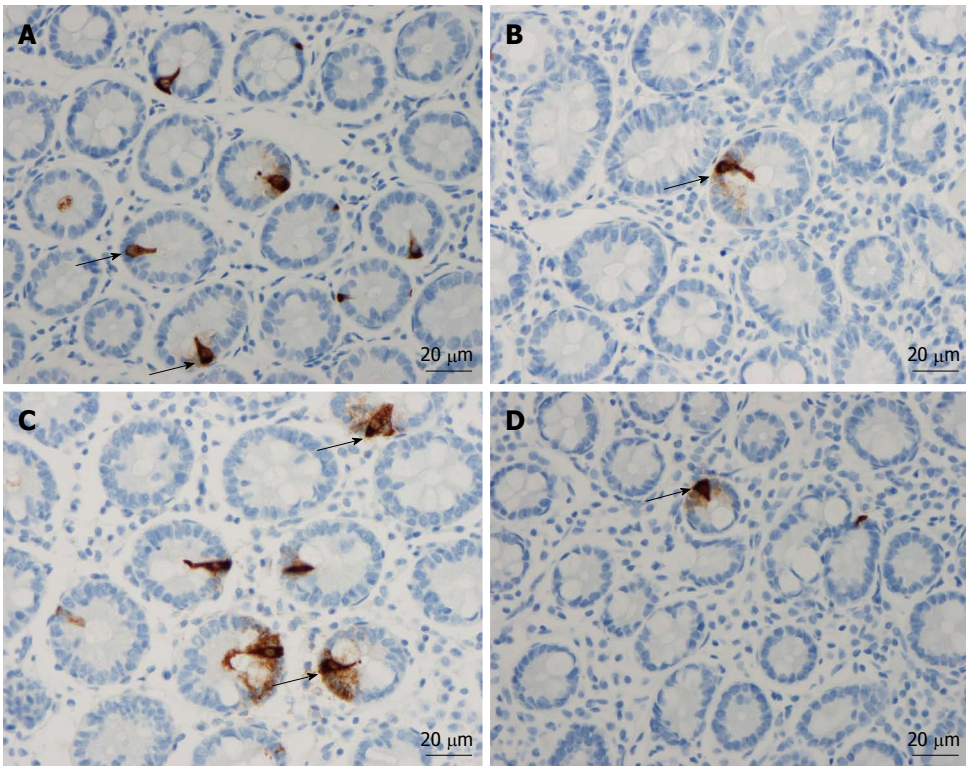

Figure 7 Gastric inhibitory peptide cells (arrows) in (A) a control subject (B) irritable bowel syndrome-D, (C) irritable bowel syndrome-M and (D) irritable bowel syndrome-C patients.

elsewhere[22]. Thus, the densities of secretin and CCK cells were reduced in IBS-D, and those of GIP and somatostatin cells were reduced in IBS-D and IBS-C. The present study also showed that the only cell type in which the density was reduced in IBS-M, an IBS subtype that has not been investigated previously in this regard, was somatostatin cells. The present finding of unaffected serotonin cells in the small intestine of IBS patients is in agreement with previously reported observations[22,27,29].

It is interesting that changes in the density of duodenal endocrine cells differ with IBS subtype, being highest in IBS-D and lowest in IBS-M. The impact of the reduction in endocrine cell types in each IBS subtype on symptom development has been discussed previously[22]. It is believed that the reduction in secretin- and CCK-cell densities in IBS-D may lead to low levels of secretion of bicarbonate, pancreatic enzymes and bile salts, resulting in diarrhoea. Furthermore, as secretin inhibits intestinal motility and both secretin and CCK inhibit gastric emptying[33], any reduction in the population of these cells would contribute to the development of diarrhoea. Secretin, GIP and somatostatin inhibit gastric acid secretion[33], and as all of the IBS subtypes are associated with a reduction in the density of one or more of the cells secreting these hormones, they may also exhibit a high level of gastric acid secretion. It has been demonstrated that the antral gastrin cell density is increased and somatostatin cell density decreased in the stomach of IBS patients[17]. Given that gastric acid secretion is stimulated by gastrin and inhibited by somatostatin, it has been suggested that gastric acid secretion is increased in IBS. The present findings, together with those reported previously[17], may explain the high incidence of dyspepsia and gastro-oesophageal reflux found in IBS patients[44-52].

Intestinal stem cell self-renewal (clonogeny) and proliferation are regulated by several signalling pathways[37]. Several factors, such as hereditary, diet, intestinal bacterial flora and low-grade inflammation, have been demonstrated to play an important role in the pathophysiology of IBS. Changes in diet, intestinal bacterial flora and low-grade inflammation have been reported to affect the density of gut endocrine cells[34,53,54]. It is tempting to speculate that the factors that have been demonstrated to play a major role in the pathophysiology of IBS can affect the signalling pathways for stem cell clonogenic renewal and proliferation, resulting in abnormalities in gastrointestinal endocrine cells with the development of IBS symptoms.

COMMENTS

Background

Irritable bowel syndrome (IBS) is a common gastrointestinal disorder in which a reduced density of small intestinal endocrine cells has been reported. In some pathological conditions such as congenital malabsorptive diarrhoea, a decreased number of stem cell endocrine progenitors and a decreased number of intestinal endocrine cells have been reported. The present study was

conducted in order to determine whether the decreased density of duodenal endocrine cells in IBS patients is associated with abnormalities in stem cell differentiation.

Research frontiers

This study showed, for the first time, abnormal intestinal stem cell clonogenic and proliferation activities in IBS. These abnormalities in duodenal stem cells appear to account for the reduction in duodenal endocrine cell density reported in IBS patients.

Innovations and breakthroughs

The pathogenesis of IBS is not completely understood, but abnormalities in the gastrointestinal endocrine cells are believed to play a major role in the pathophysiology of IBS. The cause of the reduction in intestinal endocrine cells in patients with IBS is unknown. In the present study, Musashi 1 (Msi 1), which is a marker for both intestinal stem cells and their early progeny, and neurogenin 3 (NEUROG3), which is a marker for early intestinal endocrine cell progenitors were investigated in the duodenum of IBS patients. The densities of Msi-1 and NEUROG3 cells were reduced in the duodenum of patients with IBS, regardless of the subtype, indicating disturbances in both clonogenic renewal and proliferation activities of stem cells. These disturbances in duodenal stem cells were accompanied by a reduction in endocrine cells indicating an association between the abnormalities in stem cells and the reduction in duodenal endocrine cells in IBS patients.

Applications

The identification of abnormalities in intestinal stem cells offers a new approach in the research of IBS pathogenesis and may provide an effective tool for the treatment of IBS. Thus, research concerning the cause of the abnormalities in stem cells in IBS should be carried out, and stem cell stimulation/transplantation may be an option for the treatment of IBS in the near future.

Terminology

Intestinal stem cells: each intestinal crypt contains 4 to 6 stem cells; stem cell clonogeny: represents self-renewal, in which stem cells divide into a new identical cells; stem cell differentiation progeny activity: stem cells differentiate into two lineages: the secretory lineage and absorptive lineage. The secretory lineage gives rise to goblet, endocrine and Paneth cells and the absorptive lineage to absorptive enterocytes.

Peer-review

In this article, the authors found that the reduction in the duodenal endocrine cells in patients with IBS is caused by an abnormality in the stem-cell clonogenic and proliferation activities. This is a well-written paper containing interesting results.

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ORIGINAL ARTICLE

Retrospective Cohort Study

Impact of partial reimbursement on hepatitis B antiviral utilization and adherence

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Author contributions: Qiu Q and Duan XW contributed equally to this work; Wang L, Duan ZP and Li H designed the study; Qiu Q completed the data analysis and drafted the manuscript; Duan XW conducted the questionnaire survey; Li Y, Yang LK and Chen Y retrieved the electronic data; all authors reviewed the manuscript.

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Institutional review board statement: The study protocol was approved by the Ethical Review Committee of Beijing You' an Hospital of Capital Medical University and Institute of Basic Medical Sciences Chinese Academy of Medical Sciences.

Informed consent statement: Given that the study poses no more than the minimal risk, and it would not be practicable to contact all the 30451 CHB patients in the two cohorts, a waiver of the informed consent was allowed by the Ethical Review Committee for the first part of study, involving secondary analysis of data of the two cohorts for a total of 30451 CHB patients. But for the validation study part, written or oral informed consent was obtained from the participants dependent on questionnaire survey by face-to-face interview or telephone interview. Deidentification was done to assure confidentiality of the study data for the two parts.

Conflict-of-interest statement: None declared.

Data sharing statement: Technical appendix and output files of the statistical analysis available from the corresponding author at liwang@ibms.pumc.edu.cn.

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Abstract

AIM: To determine the impact of partial reimbursement for antivirals on antiviral utilization and adherence for chronic hepatitis B patients.

METHODS: This was a retrospective cohort study. Two separate cohorts were enrolled, including 14163 and 16288 chronic hepatitis B outpatients, respectively. These patients were referred to Beijing You'an Hospital before and after the new partial reimbursement for antivirals, which was implemented on July 1, 2011. Demographic characteristics (including medical insurance status), routine biochemical, virological and serology laboratory test results, and antiviral agents' prescription

information were collected from an electronic database. Patients were also defined as new and existing patients according to treatment history. Antiviral utilization, medication possession ratio and persistence rate were calculated and compared among the patients with different characteristics. A questionnaire survey was conducted among 212 randomly sampled outpatients from the same hospital to confirm the validity of the electronic database. Propensity score matching was used to adjust the distribution of patient's characteristics which may influence the antiviral utilization. χ^2 test or ANOVA was adopted and multivariate logistic regression was used to determine the factors associated with antiviral utilization and good adherence.

RESULTS: A total of 13364 outpatients from each cohort were enrolled after the propensity score matching. The antiviral utilization rate for the insured patients increased from 57.4% to 75.9% ($P < 0.0001$) after the reimbursement, and the rate among those who paid out-of-pocket increased from 54.9% to 56.7% ($P = 0.028$). Approximately 71% of the patients had a medication possession ratio of more than 80% in each cohort before reimbursement. This increased to 79.2% and 73.1% for insured patients and those who paid out-of-pocket, respectively ($P < 0.0001$). Insured patients and those who paid out-of-pocket had the similar persistence rates before reimbursement. But after reimbursement, insured patients had higher persistence rates than those who paid out-of-pocket at 6 (86.5% *vs* 81.5%, $P < 0.0001$), 9 (79.7% *vs* 69.9%, $P < 0.0001$), 12 (73.4% *vs* 61.9%, $P < 0.0001$), and 15 mo (66.6% *vs* 53.1%, $P < 0.0001$). The reimbursement could significantly improve adherence for the insured patients than those who paid out-of-pocket even after adjusting other covariates, with an interaction odds ratio of 1.422 (95%CI: 1.220-1.657, $P < 0.0001$). The questionnaire survey supported the validity of the electronic database.

CONCLUSION: The reimbursement policy shows a positive impact on antiviral utilization as well as adherence for insured chronic hepatitis B patients.

Key words: Antiviral therapy; Adherence; Chronic hepatitis B; Compliance; Reimbursement

Core tip: High adherence is the key to ensuring the effectiveness of antiviral therapy and adherence can be influenced by cost and affordability which can be strongly influenced by reimbursement scheme. This study uniquely analyzed the impact of medication reimbursement on hepatitis B antiviral usage as well as treatment adherence in Beijing, China, where chronic hepatitis B infection is endemic. The results showed a positive impact of partial reimbursement on antiviral utilization as well as adherence for insured chronic hepatitis B patients. The results of this study could address a more global overall question rather than something at the patient level.

Qiu Q, Duan XW, Li Y, Yang LK, Chen Y, Li H, Duan ZP, Wang L. Impact of partial reimbursement on hepatitis B antiviral utilization and adherence. *World J Gastroenterol* 2015; 21(32): 9588-9597 Available from: URL: http://www.wjgnet.com/1007-9327/full/v21/i32/9588.htm DOI: http://dx.doi.org/10.3748/wjg.v21.i32.9588

INTRODUCTION

Chronic hepatitis B (CHB) is one of the most common chronic liver diseases worldwide, especially in China. Individuals with chronic hepatitis B virus (HBV) infections have a 15%-40% probability of developing compensated cirrhosis (CC), decompensated cirrhosis (DCC) or hepatocellular carcinoma (HCC)[1,2]. Therefore, the ultimate goal of therapy is to prevent these complications by suppressing HBV replication[3]. Over the past decades, there has been considerable improvement in the treatment of CHB, including two interferon (IFN)-α formulations and four nucleos(t)ide analogs (NAs) including lamivudine (LAM), adefovir (ADV), entecavir (ETV) and telbivudine (LdT), which have been approved in China[4].

Current guidelines highlight the optimal adherence of antiviral treatment to achieve the best results[3]. Medication adherence usually refers to whether patients take their medications as scheduled and continue the therapy. This has been studied in various chronic diseases, including CHB[5-10]. Several methods have been used to assess medication adherence, including measurement of drug levels in blood or urine, patient self-reporting, pill counts, electronic monitoring devices, and prescription record review[11]. Each method has its advantages and disadvantages. In recent years, pharmacy databases have been increasingly used to evaluate medication adherence. Although using the databases has a number of limitations including the inability to determine whether the patient actually consumed the dispensed medication, the relative efficiency in large populations in a "real-world" setting in a timely and efficient manner is highly advantageous if data are deemed complete and patients are unlikely to obtain the medications from other sources not captured by the database[12].

Several factors have been identified to influence the medication adherence, including age, education, marital status, social medical support; disease severity; therapy effectiveness; and cost and affordability which can be strongly influenced by reimbursement scheme[1,13]. In China, both generic and branded drugs are currently available for antivirals and annual cost differs greatly across modalities. The branded drugs

cost more than the generics. Annual cost ranges from $600 to $900 for LAM, $600 to $1000 for ADV, $1500 to $2000 for ETV, and $1400 for the only branded LdT approved in China. The conventional IFN costs as much as $1500-$2500 annually, while the pegylated IFN-α costs $7000-$8000. In Beijing, no reimbursement for all anti-HBV agents had been implemented before July 1, 2011 and all expenses were covered by the patients. Patients bear out of pockets no matter whether they were with or without medical insurance, which may have led to a poor adherence of antiviral therapy. Currently, all antiviral agents including IFN and NAs are on the list of National Reimbursement Catalogs of Drugs for Basic Medical Insurance and partial reimbursement has been implemented since July 1, 2011 in Beijing[14]. Patients with medical insurance could receive a 75%-85% reimbursement of the cost between a deductible of $300 and a ceiling of $3300. While for those without medical insurance, they still need to bear out of pockets themselves. It was estimated that the disposable personal income in Beijing was $5353 in 2012. So, annual cost for CHB patients with antivirals was a great burden for patients and their families. Whether the partial reimbursement policy can increase the antiviral medication adherence by increasing the acceptance and affordability of anti-HBV therapy needs to be explored.

In this study, we used the outpatient electronic data from Beijing You'an Hospital, one of the two biggest infectious and liver disease hospitals in Beijing, China, to estimate the antiviral treatment pattern and treatment adherence in CHB outpatients and explore the impact of the new partial reimbursement policy on the antiviral treatment adherence.

MATERIALS AND METHODS

Study population

Two cohorts were employed in this study. Cohort 1 consisted of outpatients with CHB who had been referred to Beijing You'an Hospital between January 1, 2010 and December 30, 2010, and cohort 2 referred between July 1, 2011 and June 30, 2012. Follow-up ended on June 30, 2011 and December 31, 2012 for cohort 1 and cohort 2, respectively. The inclusion criteria were as follows: (1) Beijing residents; and (2) diagnosed according to the criteria by "Asian-Pacific Consensus Statement on management of CHB"[3]. Patients co-infected with hepatitis A, C, D or E virus, human immunodeficiency virus, cytomegalovirus, or who had been admitted to hospital due to other diseases or conditions, including pregnancy, glomerulonephritis, uremia, metabolic syndrome, tumor, and severe cardiovascular diseases were excluded. A total of 14163 outpatients in cohort 1 and 16228 outpatients in cohort 2 were enrolled.

All the patients in the two cohorts were categorized into new patients and existing patients. Patients who had not undergone antiviral treatment during an 18-mo period prior to the enrollment were defined as new patients; otherwise were defined as existing patients.

Data collection

Clinical information, antiviral utilization and on-study laboratory tests for each visit of each patient were retrieved from electronic medical records. Clinical information included patients' ID number, gender, age, visit date, health insurance status, signs and symptoms of illness. Antiviral utilization included drug names, dosage and prescription date. Laboratory tests were retrieved to identify the disease severity, including routine biochemical tests [serum alanine aminotransferase (ALT)], serum HBV DNA and hepatitis B e antigen (HBeAg) status.

Outcome measurements

Antiviral agent utilization: Antiviral utilization rate was calculated as the number of patients who had received antiviral agents during their follow-up period divided by the total number of patients during the study period. The proportion of specific antiviral agent usage was also calculated as the number of patients who had received the specific antiviral agent during their follow-up period divided by the total number of patients with antiviral treatment by all the antiviral agents during the study period.

Adherence measurement: For patients with antiviral treatment, the medication possession ratio (MPR) was used to evaluate primary measurement of adherence, defined as the proportion of days within an observation period for which antivirals were supplied[15,16]. It was calculated for each patient as the total days prescribed during the treatment period divided by the days the antivirals should have been supplied (defined as the days between the first and the last antiviral agent prescription date during the study period for each patient). Also, the proportion of patients with an MPR of no less than 80% which was defined as good adherence was calculated at the same time.

The persistence rate was alternatively used as the secondary adherence measurement[15,16]. It was measured as percent of patients who were still receiving antiviral therapy at different study time points. Patients who had a gap greater than 28 d (a maximum of 28 d' worth of pills was allowed to be prescribed during a 4 wk-period in Beijing) between the prescription month and the following month without resumed treatment were considered to be off therapy. Persistence rates for all antivirals and specific NAs were calculated. In addition, because each patient was followed for a different length of time, persistence rates at 3, 6, 9, 12, and 15 mo were calculated.

Questionnaire survey: Questionnaire surveys by face-to-face or telephone interview were conducted among 212 randomly sampled outpatients to confirm

Table 1 Patient characteristics before and after propensity score matching

	Before matching		After matching	
	Cohort 1 (n = 14163)	Cohort 2 (n = 16228)	Cohort 1 (n = 13364)	Cohort 2 (n = 13364)
Age (mean ± SD)[b]	38.2 ± 12.6	39.6 ± 12.6	38.7 ± 12.6	38.9 ± 12.4
Male (%)[b]	67.7	64.7	66.7	66.5
Insurance type (%)[b]				
Medical insurance	40.6	52.4	42.9	42.6
Out-of-pocket	58.2	47.0	56.3	56.6
Others	1.3	0.6	0.7	0.8
Disease status[b]				
Yes[1]	29.3%	23.6%	28.2%	27.9%

[1]Having one of the following characteristics: (1) serum alanine aminotransferase (ALT) over two times upper limit of normal (ULN); (2) hepatitis B virus (HBV) DNA ⩾ 20000 IU/mL for hepatitis B e antigen (HBeAg) (+) patients; or (3) HBV DNA ⩾ 2000 IU/mL for HBeAg (-) patients. ANOVA was performed to compare age difference between cohort 1 *vs* cohort 2; χ^2 test was conducted to compare male (%), insurance type (%) and disease status between cohort 1 *vs* cohort 2. [b]$P < 0.01$, cohort 1 *vs* cohort 2.

the validity of the information from the electronic database. Age, gender, education, income, insurance type, and antiviral treatment including drug names, dosage, amount, prescription date and the place where they took the agents before and after reimbursement were collected. For patients with medical insurance (PMI) and patients who paid out-of-pocket (PPO) before and after reimbursement, the proportion of patients who took antiviral agents outside You'an Hospital only was calculated, respectively, to infer the validity of influence of reimbursement on antiviral utilization rate.

Statistical analysis

A de-identified dataset was used to do analysis. The statistical methods of this study were reviewed by Tao Xu from Department of Epidemiology and Biostatistics, Peking Union Medical College.

Propensity score matching: Propensity score (PS) matching[17,18] was used to adjust the distribution of patient's age, gender, medical insurance type and disease severity in the two cohorts, which may influence the antiviral utilization. The PS was calculated by logistic regression, where the dependent variable was cohort classification and the independent variables were above confounders. Disease severity status was defined as whether the patients had one of the following characteristics: (1) serum ALT over two times upper limit of normal; (2) HBV DNA ⩾ 20000 IU/mL for HBeAg (+) patients; or (3) HBV DNA ⩾ 2000 IU/mL for HBeAg (-) patients[11]. Cohort 2 was matched to cohort 1 within a range of 0.1 standard deviation of PS.

Patients were divided into four subgroups: PMI and PPO before and after reimbursement. Antiviral utilization, utilization of different antiviral agents, MPR, good adherence and persistence rate were calculated among the different groups. χ^2 test or ANOVA was adopted to compare the difference in above indexes among the groups when appropriate. Multivariate logistic regression was used to determine the factors associated with antiviral utilization and good adherence. P-values < 0.05 were considered significant.

All analyses were performed with SAS software, version 9.2 (SAS Institute, Cary, NC).

RESULTS

Subject characteristics

A total of 14163 outpatients in cohort 1 and 16228 outpatients in cohort 2 were enrolled. The distribution of age, gender, health insurance type and HBV-related disease status was significantly different between the two cohorts (Table 1). More male (67.7% *vs* 64.7%, $P < 0.001$) and younger patients were involved in cohort 1. The proportion of PMI in cohort 1 was lower than that in cohort 2 (40.6% *vs* 52.4%, $P < 0.001$). After PS matching, a matched sample size of 13,364 outpatients for each cohort was acquired, with the same PS (0.4 ± 0.1) for each cohort. The distribution of the key confounders was similar between the two cohorts (Table 1).

Further analysis of cohort 1 showed that PMI were older than PPO (42.9 ± 13.1 *vs* 35.6 ± 11.2, $P < 0.0001$) and had less male patients (65.7 *vs* 67.7, $P = 0.0126$). The proportion of PMI with severe disease status was also higher than that of PPO (29.9% *vs* 25.3%, $P < 0.0001$). A similar tendency was observed between PMI and PPO in cohort 2 (data not shown).

Antiviral agent utilization

Figure 1 shows the change of antiviral utilization among patients with different characteristics. Before the reimbursement, antiviral utilization and the rate of specific NA utilization was almost equal between PMI and PPO (Figure 1A). ADV was predominantly used, followed by LAM and ETV. The utilization of LdT was the lowest.

After the reimbursement, a 19% increase of antiviral utilization was observed among PMI, and only a 2% increase was observed among PPO (Figure 1A). As the characteristics were differently

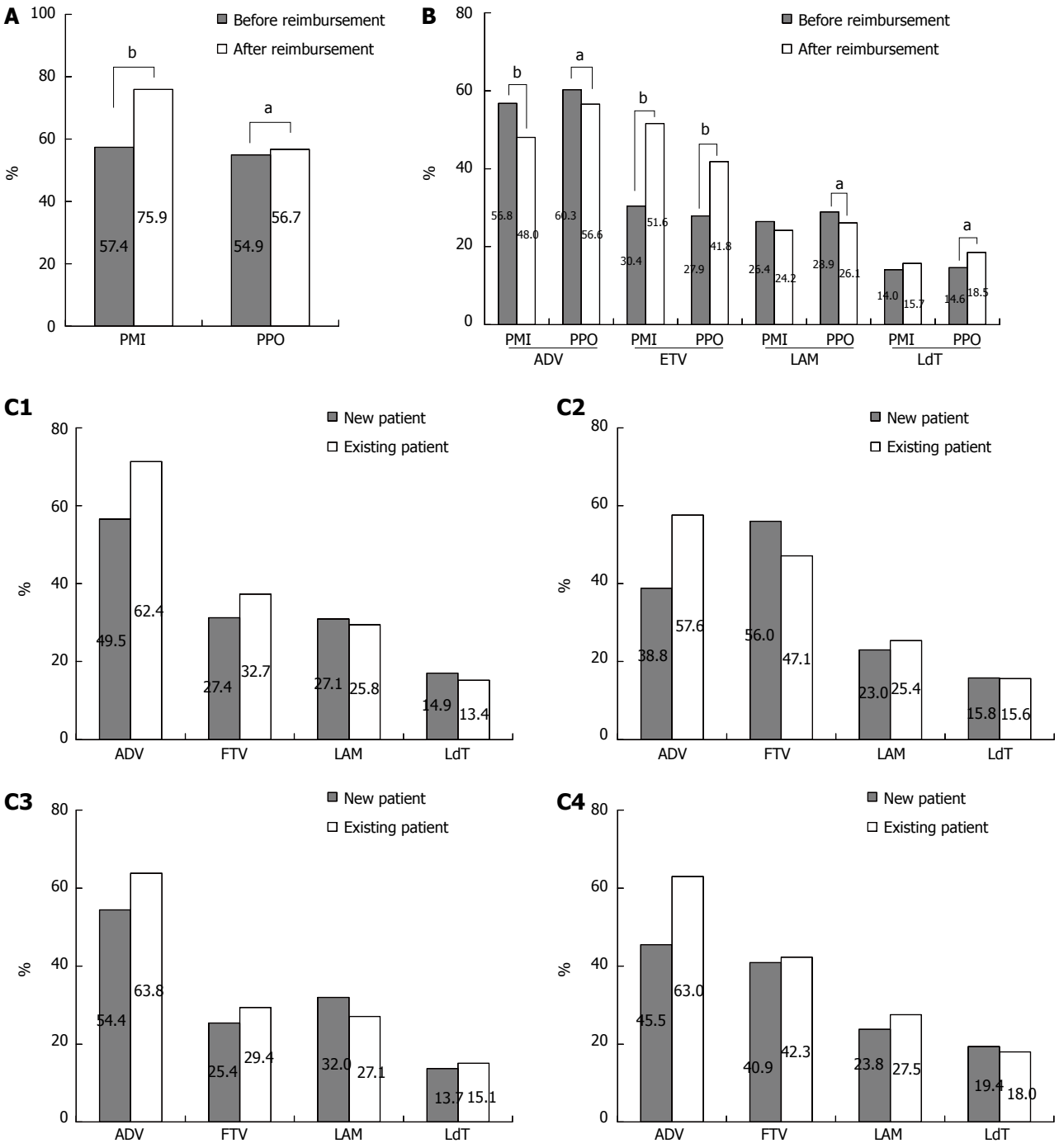

Figure 1 Antiviral agent utilization before and after reimbursement for patients with different characteristics. A: Antiviral agent utilization before and after reimbursement for patients with different insurance types; B: Utilization of different NAs for PMI *vs* PPO before and after reimbursement C1: Utilization of specific antiviral among new and existing PMI before reimbursement; C2: Utilization of specific antiviral among new and existing PMI after reimbursement; C3: Utilization of specific antiviral among new and existing PPO before reimbursement; C4: Utilization of specific antiviral among new and existing PPO after reimbursement. [a]$P < 0.05$, [b]$P < 0.01$, before reimbursement *vs* after reimbursement. PMI: Patients with medical insurance; PPO: Patients paid out-of-pocket.

distributed between PMI and PPO either before or after reimbursement, multiple logistic regression was adopted to adjust the above covariates. Our results showed that the reimbursement could significantly improve the antiviral utilization for PMI than PPO even after adjusting the covariates, with an interaction odds ratio (OR) of 2.194 (95%CI: 1.979-2.432, $P < 0.0001$) (data not shown).

For the specific NAs, a great increase rate of 21.2% for ETV utilization was observed among PMI (from 30.4% to 51.6%) and 13.9% (from 27.9% to 41.8%) among PPO. However, an 8.8% (from 56.8% to 48.0%) and 3.7% decrease of ADV utilization was observed among PMI and PPO, respectively. ETV was predominantly used among PMI while ADV was still preferred for PPO. The proportion of LdT or LAM had changed slightly after reimbursement (Figure 1B).

Further analysis showed that the utilization rate

Table 2 Medication possession ratio and good adherence rate among patients with different insurance types before and after reimbursement

			Adherence (%)			Good adherence rate		
		n	mean ± SD	New patients	Existing patients	Percent	New patients	Existing patients
Before	PMI	2825	83.4 ± 24.3[a]	82.3 ± 25.6[d]	84.5 ± 22.9[d]	70.6%	69.2	71.8
	PPO	3373	83.6 ± 24.2[b]	83.6 ± 24.8	83.6 ± 23.7	70.2%	70.5	69.9
After	PMI	4102	88.5 ± 19.7[a,c]	87.1 ± 20.8[e]	90.2 ± 18.1[e]	79.2%[g]	77.0[h]	82.1[h]
	PPO	3619	85.2 ± 23.0[b,c]	84.0 ± 24.1[f]	86.1 ± 22.1[f]	73.1%[g]	71.7	74.2

[a]$P < 0.01$ among PMI before reimbursement *vs* after reimbursement; [b]$P < 0.01$ among PPO before reimbursement *vs* after reimbursement; [c]$P < 0.01$ between PMI and PPO after reimbursement; [d]$P < 0.05$ between new and existing PMI before reimbursement; [e]$P < 0.01$ between new and existing PMI after reimbursement; [f]$P < 0.01$ between new and existing PPO after reimbursement; [g]$P < 0.01$ between PMI and PPO after reimbursement; [h]$P < 0.01$ between new and existing PMI after reimbursement. PMI: Patients with medical insurance; PPO: Patients paid out-of-pocket.

of ADV was significantly higher among existing patients than new patients either before or after the reimbursement. But for the PMI with ETV, a 10% higher rate was observed among new patients than the existing patients (56.0% *vs* 47.1%, $P < 0.0001$). For PPO with LAM, a 4.9% higher rate was observed among new patients than existing patients before the reimbursement while afterwards the rate was 3.7% lower (32.0% *vs* 27.1%, $P = 0.0044$; 23.8% *vs* 27.5%, $P = 0.0250$, respectively). No significant difference in LAM and LdT utilization was observed between new and existing patients for PMI (Figure 1C1-C4).

Adherence evaluation among patients with antiviral utilization

For the 6198 and 7721 patients with antivirals in cohort 1 and cohort 2, respectively, adherence was further evaluated, including MPR and the persistence rate. The mean follow-up period was 309.6 ± 155.5 and 375.2 ± 156.8 d for PMI before and after the reimbursement, and was 300.1 ± 155.2 and 305.5 ± 156.9 d for PPO, respectively.

MPR measurement: Before reimbursement, MPR for both PMI and PPO were more than 0.80 and no significant difference was observed ($P = 0.8042$). After reimbursement, a 5% increase was observed among PMI (83.4% ± 24.3% *vs* 88.5% ± 19.7%, $P < 0.0001$) and a less than 2% increase was observed among PPO (83.6% ± 24.2% *vs* 85.5% ± 23.0%, $P = 0.0055$) (Table 2).

We also observed that PMI and PPO had a similar proportion of good adherence before reimbursement. However, after the reimbursement, the PMI had a higher proportion of patients with good adherence than PPO (79.2% *vs* 73.1%, $P <0.0001$) (Table 2).

No difference in the proportion of patients with good adherence was observed between existing and new patients before the reimbursement. However, a 5.1% higher proportion of existing PMI than new PMI (82.1% *vs* 77.0%, $P < 0.0001$) was subsequently observed after reimbursement (Table 2).

Persistence rate: Persistence rates declined within 15 mo, more rapidly during the first 6 mo and in new patients. PMI and PPO had the similar persistence rates before reimbursement. But after reimbursement, PMI had higher persistence rates than the PPO at 6 (86.5% *vs* 81.5%, $P < 0.0001$), 9 (79.7% *vs* 69.9%, $P < 0.0001$), 12 (73.4% *vs* 61.9%, $P < 0.0001$), and 15 mo (66.6% *vs* 53.1%, $P < 0.0001$) (Figure 2A). The similar tendency were observed in the new patients and existing patients, although the new patients had a lower persistence rate than the existing patients for each specific month (Figure 2B).

Factors associated with good adherence

Age, gender, insurance type, patient status (new *vs* existing patients), reimbursement implementation, disease severity, interactions between insurance type and policy implementation, as well as interactions between insurance type and patients characteristics were included in the logistic regression model to test the factors associated with good adherence. Results showed that compared to patients 18 years or younger, patients between the age of 18-45 and aged > 45 years had a lower probability to have good adherence, with an OR of 0.719 (95%CI: 0.559-0.926, $P = 0.0105$) and 0.667 (95%CI: 0.513-0.867, $P = 0.0024$). A significant interaction was observed between insurance type and patient status (OR = 0.820, 95%CI: 0.703-0.957; $P = 0.0117$) and patients' insurance type and reimbursement implementation (OR = 1.422, 95%CI: 1.220-1.657; $P < 0.0001$). These interactions suggested that the reimbursement implementation can significantly improve more adherence for PMI than PPO, especially for existing patients (Table 3).

Questionnaire

Questionnaire surveys were conducted in 212 outpatients, including 152 PMI and 60 PPO (Table 4). There was no difference in age, gender distribution, or household income per person per month between PMI and PPO. Before reimbursement, approximately 10% of patients had ever taken antivirals outside You'an Hospital regardless of medical insurance. After reimbursement,

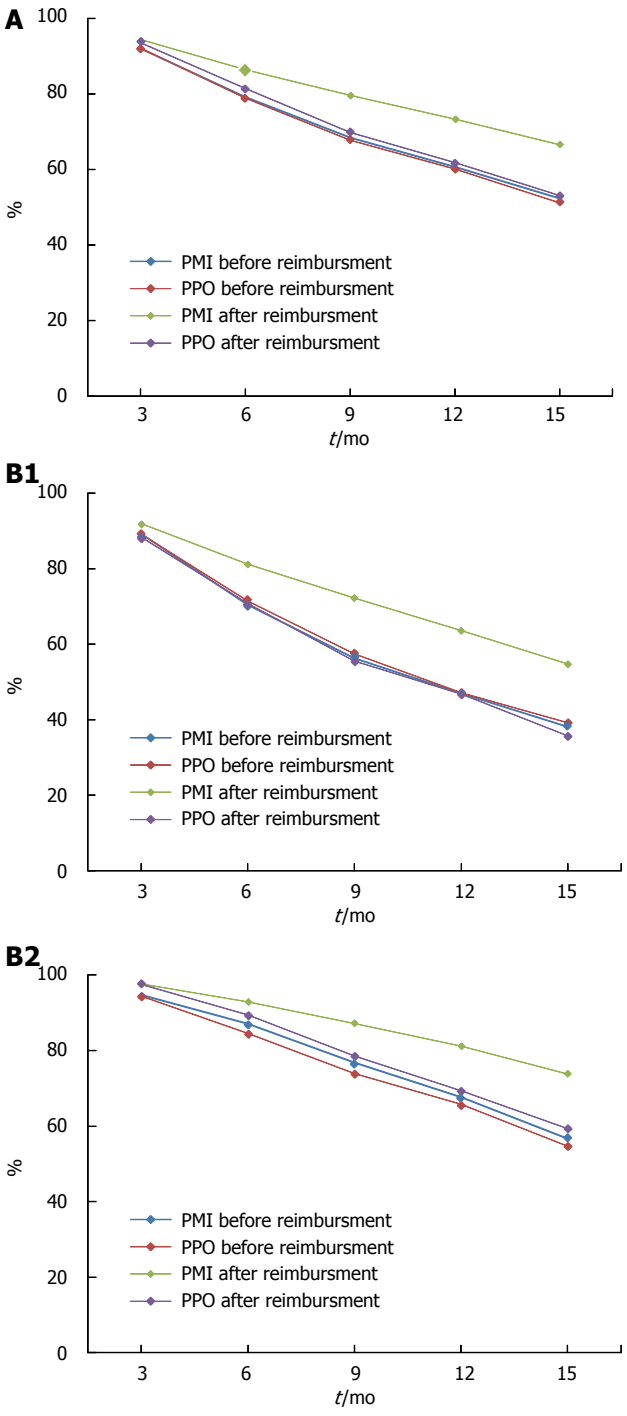

Figure 2 Persistence rate before and after reimbursement for patients with different characteristics. A: Persistence rate before and after reimbursement for patients with different insurance types; B1: Persistence rate for new patients with different insurance types; B2: Persistence rate for existing patients with different insurance types. PMI: Patients with medical insurance; PPO: Patients who paid out-of-pocket.

the proportion had decreased to 5.9% for PMI. We could still observe a higher antiviral utilization rate among PMI than PPO after reimbursement even adjusting the proportion of patients with different medical insurance status who took antiviral agents outside You'an Hospital only.

Table 3 Factors associated with good adherence

Parameter	β	OR (95%CI)
Age (yr)		
18-45 *vs* ≤ 18[a]	-0.3297	0.719 (0.559-0.926)
> 45 *vs* ≤ 18[b]	-0.4048	0.667 (0.513-0.867)
Gender (1 = male; 0 = female)	-0.0382	0.963 (0.886-1.046)
Insurance type (1 = medical insurance; 0 = paid out-of-pocket)	0.1200	1.128 (0.987-1.288)
Disease status (1 =; 0 = reference;)[b]	0.2516	1.286 (1.164-1.422)
Patient status (1 = new patients; 0 = existing patients)	-0.0341	0.966 (0.860-1.086)
Reimbursement implementation (1 = Yes; 0 = No)[b]	0.1502	1.162 (1.047-1.290)
Insurance type patient status[a]	-0.1980	0.820 (0.703-0.957)
Insurance type reimbursement implementation[b]	0.3518	1.422 (1.220-1.657)

[a]$P < 0.05$; [b]$P < 0.01$, new *vs* existing patients. 1 = having one of the following characteristics: (1) serum alanine aminotransferase over two times upper limit of normal; (2) alanine aminotransferase DNA ≥ 20000 IU/mL for hepatitis B e antigen (HBeAg) (+) patients; or (3) HBV DNA ≥ 2000 IU/mL for HBeAg (-) patients.

Table 4 Characteristics of patients receiving questionnaire survey

	Insurance (*n* = 152)	Out-of-pocket (*n* = 60)
Age (yr)	38.8 ± 8.3	39.7 ± 11.6
Male (%)	99 (65.1)	38 (64.4)[1]
Income (Yuan)		
Median (Q25-Q75)	4000 (2500, 6300)	3000 (1500, 5000)
Antivirals obtaining outside You'an Hospital only (%)		
Before reimbursement	9.8	10.8
After reimbursement	5.9	10.2

[1]The number is 59.

DISCUSSION

Studies have shown that good adherence helps to maintain virologic response and prevent virologic resistance[19]. The reimbursement scheme may increase the adherence to antivirals by increasing their affordability. In this study, electronic data of 30391 outpatients from a university affiliated infection specialty hospital in Beijing, China, from 2010 to 2012 was used to determine the effect of partial reimbursement, which was firstly implemented for the treatment of CHB patients on July 1, 2011, on antiviral utilization and adherence. We found that partial reimbursement increased the antiviral utilization, although slightly for medical insured CHB patients.

Our study from electronic dataset showed that the antiviral utilization was almost the same (50%-60%) between PMI and PPO before reimbursement. After reimbursement, the rate increased to 75.9% for PMI while kept constant for PPO. Although questionnaire surveys found about 8.5% of PMI and 10% of PPO had taken medication outside You'an Hospital, which

inferred that our results from electronic dataset might underestimate the antiviral utilization, the utilization was still much higher among PMI than PPO, indicating that reimbursement could improve antiviral utilization by reducing the economic burden. We also found that ADV was predominantly used for both PMI and PPO before reimbursement. But after reimbursement, ETV replaced ADV as the first choice NA for PMI. Monotherapy with ETV has been recommended as the first-line oral antiviral treatment for CHB by the Asian Pacific Association for the Study of the Liver, the American Association for the Study of Liver Diseases and the European Association for the Study of the Liver[3,20,21]. This could explain that the reimbursement policy had mitigated the economic burden for PMI, thus treatment effectiveness was more of a concern than cost when choosing an antiviral agent. However, PPO may have chosen less costly and more common antivirals of ADV. A higher ADV utilization among existing patients was also observed, which may be explained by the fact that most of the patients who had received antiviral therapy for a long time before our enrollment might continue to select ADV.

MPR analysis showed that the mean MPR for both PMI and PPO was more than 80% before reimbursement, which had already reached an optimistic level, similar to the 80%-99% mean adherence rate in the other studies regarding oral antiviral adherence for CHB patients[22-24]. After the reimbursement the MPR had increased 5% for PMI and 2% for PPO. Even after adjusting other covariates, the reimbursement could still significantly improve adherence for PMI than PPO, with an interaction OR of 1.422 (1.220-1.657).

The persistence rate was used to assess whether a patient stays on therapy consistently. We found that persistence rates declined during the study period, especially more rapidly during the first 6 mo, indicating that patients tend to miss or stop the medication at the beginning of the treatment, probably due to side effects or lack of treatment effectiveness[12]. Similar to MPR, the persistence rate was observed higher among PMI. This was supported by a report by Liaw *et al*[1] that lack of adequate reimbursement was correlated with inadequate anti-HBV therapy according to treatment guidelines.

Our study also observed higher MPR and persistence rates and a more gradually decline of persistence rate for existing patients compared to new patients. Poorer adherence for new patients who have received antiviral therapy for a short duration might be due to inability to endure the side effects of antiviral agents, which was also suggested in other studies that a greater decline in adherence for chronic medications was often observed among new users[12,22].

Our study has several limitations. First, our patients might not represent the general CHB population in Beijing. However, the demographic characteristics in our study population showed that the proportion of male patients was 64%-67% and the mean age was 38-40 years, which are consistent with the gender and age distribution of CHB infection in other studies[25-29]. Second, the difference in patients' characteristics which may result in different antiviral utilization may exist between the patients referred to You'an Hospital before and after reimbursement based on real-world electronic datasets. Although PS matching was adopted to try to balance the baseline characteristics, the bias generated by unmeasured confounding factors cannot be eliminated. Third, pharmacy database was used in our study to evaluate medication adherence which may not capture the exact amount of the agents used. But after the validation study by questionnaire surveys, we can still conclude that the reimbursement can improve the utilization for patients with medical insurance.

In conclusion, the utilization of antivirals and adherence for insured CHB patients had significantly increased after the new partial reimbursement implementation, especially for patients receiving ETV and ADV. Thus, the new policy had a positive impact on antiviral treatment pattern, thereby offering improved outcomes.

COMMENTS

Background

Chronic hepatitis B (CHB) is one of the most common chronic liver diseases worldwide, especially in China, leading to a high rate of incidence and mortality from development of cirrhosis and hepatocellular cancer (HCC). Effective antiviral treatment is the only way to prevent the development of cirrhosis and HCC after infection and the essential prerequisite is long-term adherence. There are many factors which influence the therapy adherence for CHB patients, and one of the most important factors is the cost and affordability for antiviral drugs, which can be strongly influenced by reimbursement scheme. In Beijing, no reimbursement for all antivirus agents for CHB patients had been implemented before July 1, 2011 and all expenses of drugs are borne by the patients, which may lead to a low compliance rate of antivirus therapy. However, all antivirus agents including IFN and NAs, have been in the list of National Reimbursement Catalogs of Drugs for Basic Medical Insurance and partial reimbursement has been implemented since July 1, 2011 in Beijing. Whether or not the compliance with NAs can be increased under the new partial reimbursement policy needs to be explored.

Research frontiers

Medication adherence has been studied in different chronic diseases and several methods have been used to assess medication adherence. In recent years, pharmacy databases have been increasingly used to evaluate medication adherence in large populations in a "real-world" setting and the relative efficiency in a timely and efficient manner is more advantageous than the other ways. Moreover, as the new reimbursement policy has just been implemented for a short period, this is the first study to evaluate its impact on antiviral therapy adherence in Beijing, China.

Innovations and breakthroughs

This study uniquely analyzed the impact of medication reimbursement on hepatitis B antiviral usage as well as treatment adherence in Beijing, China, where chronic hepatitis B infection is endemic. Two cohorts were contrasted, one before implementation of the new partial reimbursement policy, and the other after implementation. Propensity score matching was used to control the effects from confounding. Antiviral usage rates and adherence rates were compared between the two groups as well as for those with insurance and those paying out-of-pocket. Also, a questionnaire survey was conducted to infer the validity of reimbursement on antiviral usage. Results have shown that the antiviral utilization rate for the insured patients increased from 57.4% to 75.9% ($P < 0.0001$) after the reimbursement. The rate among those who paid

out-of-pocket increased only from 54.9% to 56.7% (P = 0.028). Approximately 71% of the patients had an MPI of more than 80% in each cohort before reimbursement. This increased to 79.2% and 73.1% for insured patients and those who paid out-of-pocket, respectively (P < 0.0001). The reimbursement could significantly improve adherence for the insured patients than those who paid out-of-pocket even after adjusting other covariates, with an interaction odds ratio of 1.422 (95%CI: 1.220-1.657, P < 0.0001).

Applications

The results suggested a positive impact of partial reimbursement on antiviral utilization as well as adherence for insured chronic hepatitis B patients. The results of this study could address a more global overall question rather than something at the patient level.

Terminology

Reimbursement is an act of compensating someone for an expense. Often, a person is reimbursed for out-of-pocket expenses when the person incurs those expenses through government, employment or in an account of carrying out the duties for another party or member. Medication adherence usually refers to whether patients take their medications as scheduled and continue the therapy.

Peer-review

This study appears as a well-designed study that for the first time analyzed the impact of medication reimbursement on adherence to hepatitis B antiviral treatment in Beijing, China, where chronic hepatitis B infection is endemic. The study is interesting and results showed that partial reimbursement, implemented in 2011, improved adherence as well as influenced the choice of NAs selected by the patients which should improve the overall outcome. These types of studies are highly warranted not only in China but also from other parts of the world to design antiviral treatment for chronic hepatitis B.

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ORIGINAL ARTICLE

Retrospective Cohort Study

Histological outcome for chronic hepatitis B patients treated with entecavir *vs* lamivudine-based therapy

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Author contributions: Wang JL and Du XF contributed equally to this work; Wang JL performed the analysis, interpreted the data, and drafted the article; Du XF collected all of the human material and clinical data; Chen SL, Yu YQ and Wang J were involved in editing the manuscript; Hu XQ evaluated all of the biopsy specimens as a pathologist; Shao LY coordinated the collection of data and was involved in data analysis; Chen JZ performed the experiments; Weng XH and Zhang WH designed the study and revised the manuscript.

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Informed consent statement: All study participants or their legal guardian provided informed written consent to liver biopsy. Informed consent was not obtained prior to study enrollment because this is a retrospective study and all study participants were de-identified.

Conflict-of-interest statement: There is no conflict of interest for any of the authors.

Data sharing statement: No additional data are available.

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Abstract

AIM: To compare the histological outcome of chronic hepatitis B (CHB) patients treated with entecavir (ETV) or lamivudine (LAM)-based therapy.

METHODS: We conducted a retrospective analysis of data from 42 CHB patients with advanced fibrosis (baseline Ishak score $\geq$ 2) or cirrhosis who were treated with ETV or LAM-based therapy in Beilun People's Hospital, Ningbo between January 2005 and May 2012. The patients enrolled were more than 16 years of age and underwent a minimum of 12 mo of antiviral therapy. We collected data on the baseline

characteristics of each patient and obtained paired liver biopsies pre- and post-treatment. The Knodell scoring system and Ishak fibrosis scores were used to evaluate each example. An improvement or worsening of necroinflammation was defined as ⩾ 2-point change in the Knodell inflammatory score. The progression or regression of fibrosis was defined as ⩾ 1-point change in the Ishak fibrosis score. The continuous variables were compared using *t*-test or Mann-Whitney test, and the binary variables were compared using χ^2 test or Fisher's exact test. The results of paired liver biopsies were compared with a Wilcoxon signed rank test.

RESULTS: Nineteen patients were treated with ETV and 23 patients were treated with LAM therapy for a mean duration of 39 and 42 mo, respectively. After long-term antiviral treatment, 94.74% (18/19) of the patients in the ETV arm and 95.65% (22/23) in the LAM arm achieved an HBV DNA level less than 1000 IU/mL. The majority of the patients (94.74% in the ETV arm and 73.91% in the LAM arm) had normalized ALT levels. The median Knodell necroinflammatory score decreased from 11 to 0 in the patients receiving ETV, and the median Knodell score decreased from 9 to 3 in the patients receiving LAM (P = 0.0002 and < 0.0001, respectively). The median Ishak fibrosis score showed a 1-point reduction in ETV-treated patients and a 2-point reduction in LAM-treated patients (P = 0.0019 and 0.0205, respectively). The patients receiving ETV showed a more significant improvement in necroinflammation than the LAM-treated patients (P = 0.0003). However, there was no significant difference in fibrotic improvement between the two arms. Furthermore, two patients in each arm achieved a fibrosis score of 0 post-treatment, which indicates a full reversion of fibrosis after antiviral therapy.

CONCLUSION: CHB patients with advanced fibrosis or cirrhosis benefit from antiviral treatment. ETV is superior to LAM therapy in improving necroinflammatory but not fibrotic outcome.

Key words: Advanced fibrosis; Chronic hepatitis B; Cirrhosis; Entecavir; Histological outcome; Lamivudine

Core tip: This retrospective cohort study compared the histological outcomes of long-term antiviral treatment for an average of more than 3 years with entecavir monotherapy or lamivudine-based combination therapy in chronic hepatitis B patients with significant fibrosis or cirrhosis. There was a histological improvement observed in the majority of patients in both arms. There were also improved virological responses, alanine aminotransferase normalization, and serological responses. Additionally, 4 of 48 patients achieved a full reversion of fibrosis or cirrhosis. Entecavir was superior to lamivudine-based therapy in improving necroinflammatory but not fibrotic outcome.

Wang JL, Du XF, Chen SL, Yu YQ, Wang J, Hu XQ, Shao LY, Chen JZ, Weng XH, Zhang WH. Histological outcome for chronic hepatitis B patients treated with entecavir *vs* lamivudine-based therapy. *World J Gastroenterol* 2015; 21(32): 9598-9606 Available from: URL: http://www.wjgnet.com/1007-9327/full/v21/i32/9598.htm DOI: http://dx.doi.org/10.3748/wjg.v21.i32.9598

INTRODUCTION

Chronic hepatitis B (CHB) affects approximately 350 million people worldwide and hepatitis B virus (HBV)-related end stage liver diseases are responsible for 1 million deaths annually[1-3]. In China, the prevalence of hepatitis B surface antigen (HBsAg) is found in approximately 7% of the general population[4]. Liver fibrosis is one of the common complications of CHB and is a result of sustained viral replication and chronic inflammation. Liver fibrosis may also lead to cirrhosis. Hepatic cirrhosis is the end stage of fibrosis and is characterized by distinguishing histological features such as the formation of regenerative nodules and diffuse fibrosis. Patients with cirrhosis have high mortality and morbidity due to poor liver function and the development of portal hypertension[5]. Another major complication is the development of hepatocellular carcinoma (HCC)[6]. Thus, effective therapies that prevent the progression of HBV-related liver diseases are urgently needed in clinical practice.

The current therapy regimens available for CHB patients include nucleos(t)ide analogues (NAs) and interferon-α (INF-α). These therapy regimens differ with respect to antiviral mechanism and treatment responses[7,8]. The drugs lamivudine (LAM), adefovir (ADV), telbivudine (LdT), entecavir (ETV) and tenofovir disoproxil fumarate (TDF) are approved NAs for the treatment of CHB. NA therapy directly inhibits viral replication by targeting HBV DNA polymerases and can achieve rapid viral suppression and normalization of serum transaminases[9]. LAM was the first approved oral antiviral agent and was initially considered the best choice for CHB treatment because of its good potency and safety profile[10,11]. However, the major limitation of LAM is the high rate of virologcial resistance, and ADV add-on is a rescue therapy for LAM resistance[12,13]. The use of LAM has been gradually reduced since the introduction of novel antiviral agents with a higher genetic barrier of resistance such as ETV and TDF. However, LAM is still widely used in developing countries due to its low cost.

Two early global clinical trials found that 48 wk of ETV therapy was superior to LAM in treatment-naïve CHB patients with respect to virological response, alanine aminotransferase (ALT) normalization, and histological improvement[9,10]. However, it is unclear whether patients with advanced fibrosis or cirrhosis benefit more from ETV therapy than LAM therapy.

Thus, the aim of this study was to compare the histological outcome of CHB patients with advanced fibrosis or cirrhosis after long-term treatment with ETV or LAM-based therapy.

MATERIALS AND METHODS

Study population

The patients eligible for this retrospective study were CHB cases with advanced fibrosis or cirrhosis who had received ETV or LAM-based combination therapy (ADV was added when patients had LAM resistance) at the clinic or were hospitalized in Beilun People's Hospital, Ningbo between January 2005 and May 2012. The inclusion criteria of our study were the following: age $\geqslant$ 16 years, HBsAg-positivity for more than 6 mo, a minimum of 12 mo of antiviral therapy with ETV or LAM-based combination therapy (LAM + ADV), adequate pre- and post-treatment biopsy samples, and a diagnosis of advanced fibrosis or compensated cirrhosis at baseline (Ishak score $\geqslant$ 2). The patient exclusion criteria included the following: co-infection with hepatitis C virus, hepatitis D virus, or human immunodeficiency virus, hepatic decompensation, or prior exposure to either IFN-α or other NAs including LdT or ADV monotherapy. There were 42 patients enrolled in the current study including 19 in the ETV-treated arm and 23 in the LAM-based combination arm. Paired liver biopsies and clinical data were collected and analyzed from each patient. The study protocol was reviewed by the local ethics committees, and the ethical approval documents were waived. Written informed consent for the liver biopsy was obtained from each participant. This is a retrospective study and all study participants were de-identified. Therefore, informed consent was not obtained prior to study enrollment.

Analysis of necroinflammatory level and fibrosis

Both the Knodell scoring system (0 to 18) and Ishak fibrosis scores (0 to 6) were used to evaluate the necroinflammatory level and fibrotic extent in the liver biopsy specimens. A pathologist who was blinded to the patient clinical data and the sequences of biopsy samples evaluated the liver histology. An improvement or worsening of necroinflammation was defined as $\geqslant$ 2-point change in the Knodell inflammatory score. The progression or regression of fibrosis was defined as $\geqslant$ 1-point change in Ishak fibrosis score.

Statistical analysis

The continuous variables are expressed as mean ± SD and were compared by *t*-test or Mann-Whitney test. All binary variables were summarized as counts and percentages and were compared by χ^2 test or Fisher's exact test. The results of paired liver biopsies were expressed as the median and were compared by Wilcoxon signed rank test. The statistical analyses were performed using SPSS version 19.0. A two-sided *P*-value < 0.05 was considered statistically significant.

The statistical methods of this study were reviewed by Xiao-Qin Wang from the Department of Clinical Epidemiology of Huashan Hospital, Fudan University, Shanghai, China.

RESULTS

Baseline characteristics

This retrospective cohort study enrolled 42 CHB patients with advanced fibrosis or cirrhosis. There were 19 cases treated with ETV and 23 cases treated with LAM-based combination therapy for a mean duration of 39 and 42 mo, respectively. The baseline characteristics of the two arms are summarized in Table 1.

Virological, biochemical and serological responses after long-term therapy

The outcomes of the two arms after long-term therapy are outlined in Table 2. The results include virological, biochemical, and serological responses. The data indicated that 94.74% (18/19) of patients in the ETV arm and 95.65% (22/23) in the LAM arm achieved an HBV DNA level less than 1000 IU/mL after long-term antiviral therapy. Furthermore, 76.92% (10/13) of patients in the ETV arm and 68.75% (11/16) in the LAM arm achieved an HBV DNA level less than 60 IU/mL based on the results of the COBAS AmpliPrep/COBAS TaqMan HBV Test. The majority of patients (94.74% in the ETV arm and 73.91% in the LAM arm) achieved ALT normalization after long-term treatment. However, there were no significant differences between the two arms (P = 0.0715). Hepatitis B e antigen (HBeAg) loss and seroconversion occurred in 46.15% (6/13) and 38.46% (5/13) of HBeAg positive patients in the ETV arm, respectively. Additionally, 71.43% (10/14) and 42.86% (6/14) of patients in the LAM arm experienced HBeAg loss and seroconversion, respectively. HBsAg loss was not observed in any patient.

Histological outcome after long-term therapy

There was a significant improvement of histological outcome observed in both treatment arms compared to baseline (Table 3). The median Knodell necroinflammatory score decreased from 11 to 0 in the ETV arm and from 9 to 3 in the LAM arm (P = 0.0002 and < 0.0001, respectively). The median Ishak fibrosis score showed a 1-point reduction in the ETV arm and a 2-point reduction in the LAM arm (P = 0.0019 and 0.0205, respectively). Eighteen of the 19 patients in the ETV arm showed improvement of necroinflammatory scores. Additionally, 11 patients achieved a score of 0 post-treatment. This result indicates full reversion of the necroinflammatory condition. Furthermore, 68.42% of patients in the ETV

Table 1 Demographic data and liver biopsy results for chronic hepatitis B patients in both entecavir and lamivudine arms at baseline

	ETV	LAM ± ADV	*P*-value
n	19	23	
Male gender, *n* (%)	14 (73.7)	16 (69.6)	0.7687
Age (mean ± SD), yr	43.2 ± 9.8	43.7 ± 9.9	0.8721
log_{10} HBV DNA (mean ± SD), IU/mL	6.6 ± 1.5	6.2 ± 1.4	0.4019
ALT (mean ± SD), U/L	107 ± 84	96 ± 112	0.2450
HBeAg positive, *n* (%)	13 (68.42)	14 (60.87)	0.6112
Median grade (range)	11 (0-16)	9 (3-12)	0.0749
Median stage (range)	4 (2-6)	5 (2-6)	0.7381
Duration of treatment (mean ± SD), mo	39 ± 11	42 ± 15	0.6951

ETV: Entecavir; LAM: Lamivudine; ADV: Adefovir; HBV: Hepatitis B virus; ALT: Alanine aminotransferase; HBeAg: Hepatitis B e antigen.

Table 2 Virological, biochemical and serologic outcomes after long-term treatment in both entecavir and lamivudine arms *n* (%)

	ETV	LAM ± ADV	*P*-value
n	19	23	
HBV DNA < 1000 IU/mL	18 (94.74)	22 (95.65)	0.8897
HBV DNA < 60 IU/mL	10/13 (76.92)	11/16 (68.75)	0.6243
ALT ≤ 1 × upper limit of normal	18 (94.74)	17 (73.91)	0.0715
HBeAg loss/HBeAg positive at baseline	6/13 (46.15)	10/14 (71.43)	0.1817
HBe seroconversion/HBeAg positivity at baseline	5/13 (38.46)	6/14 (42.86)	0.8163
HBsAg loss	0 (0)	0 (0)	-

ETV: Entecavir; LAM: Lamivudine; ADV: Adefovir; HBV: Hepatitis B virus; ALT: Alanine aminotransferase; HBeAg: Hepatitis B e antigen; HBsAg: Hepatitis B surface antigen.

arm and 47.83% in the LAM arm showed improvement in fibrosis. The results indicate 26.32% of patients in the ETV arm and 39.13% in the LAM arm remained stable. There was 1 patient from the ETV arm and 3 patients from the LAM arm who had worsening Ishak fibrosis scores.

There was a significant improvement in necro-inflammatory score in the ETV arm compared to the LAM arm after long-term therapy ($P = 0.0003$) (Figure 1A). However, there was no significant difference in Ishak score improvement observed between the two arms ($P = 0.0810$) (Figure 1B). There were more patients in the ETV arm who achieved regression and fewer patients suffered Ishak score progression (Figure 2).

We further analyzed the distribution of the Ishak score pre- and post-treatment in the two arms and found that two patients from each arm had achieved a score of 0, which indicates a full reversion of advanced fibrosis or cirrhosis (Figure 3). The two patients from the ETV arm had baseline Ishak scores of 4 and 6. The scores for both cases decreased to 0 after 44 and

Table 3 Comparison of liver histology at baseline and after long-term treatment in both entecavir and lamivudine arms *n* (%)

	ETV (*n* = 19)	LAM ± ADV (*n* = 23)
Change in necroinflammation		
Median grade (range)	11 (0-16)[1] to 0 (0-4)[1]	9 (3-12)[2] to 3 (0-12)[2]
Improved	18 (94.74)	19 (82.61)
No change	1 (5.26)	4 (17.39)
Worsened	0 (0)	0 (0)
Change in fibrosis		
Median stage (range)	4 (2-6)[3] to 3 (0-5)[3]	5 (2-6)[4] to 3 (0-6)[4]
Improved	13 (68.42)	11 (47.83)
No change	5 (26.32)	9 (39.13)
Worsened	1 (5.26)	3 (13.04)

[1]Comparison of change in necroinflammation at baseline and after long-term treatment in ETV arm, $P = 0.0002$; [2]Comparison of change in necroinflammation at baseline and after long-term treatment in LAM arm, $P < 0.0001$; [3]Comparison of change in fibrosis at baseline and after long-term treatment in ETV arm, $P = 0.0019$; [4]Comparison of change in fibrosis at baseline and after long-term treatment in LAM arm, $P = 0.0205$. ETV: Entecavir; LAM: Lamivudine; ADV: Adefovir.

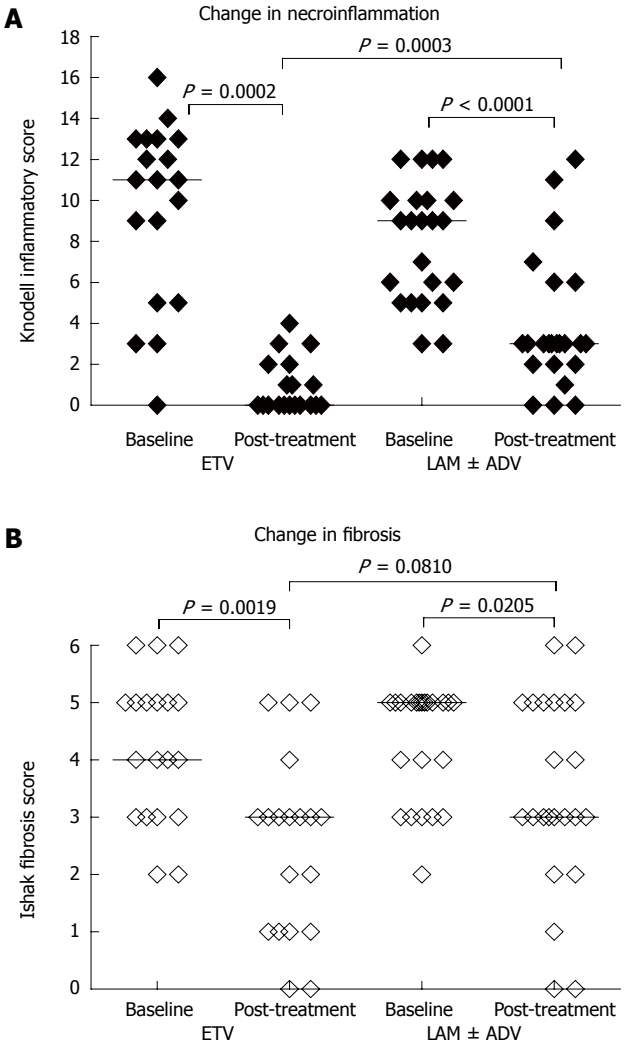


Figure 1 Plots of Knodell inflammatory scores (A) and Ishak fibrosis scores (B) of both entecavir and lamivudine arms at baseline and post-treatment. ETV: Entecavir; LAM: Lamivudine; ADV: Adefovir.

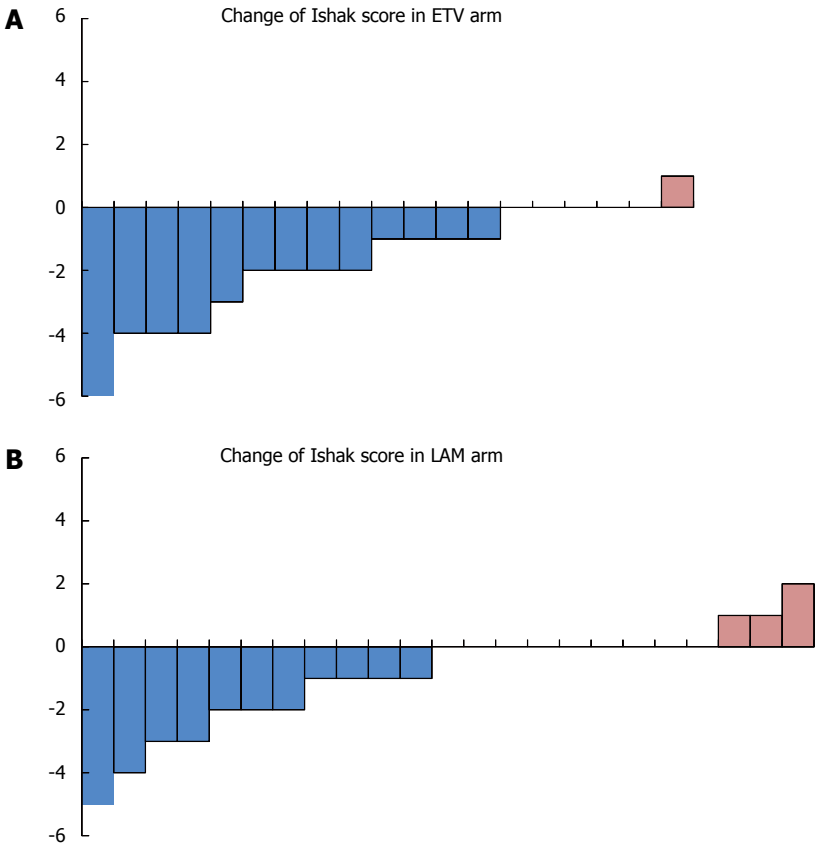


Figure 2 Changes between post- and pre-treatment Ishak fibrosis scores for all patients in the entecavir and lamivudine arms. The blue columns indicate patients with improvement, and pink columns indicate progression. The scales indicate no change. ETV: Entecavir; LAM: Lamivudine.

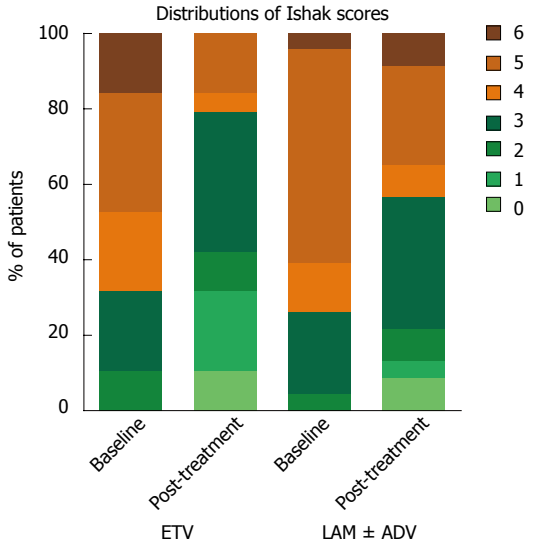


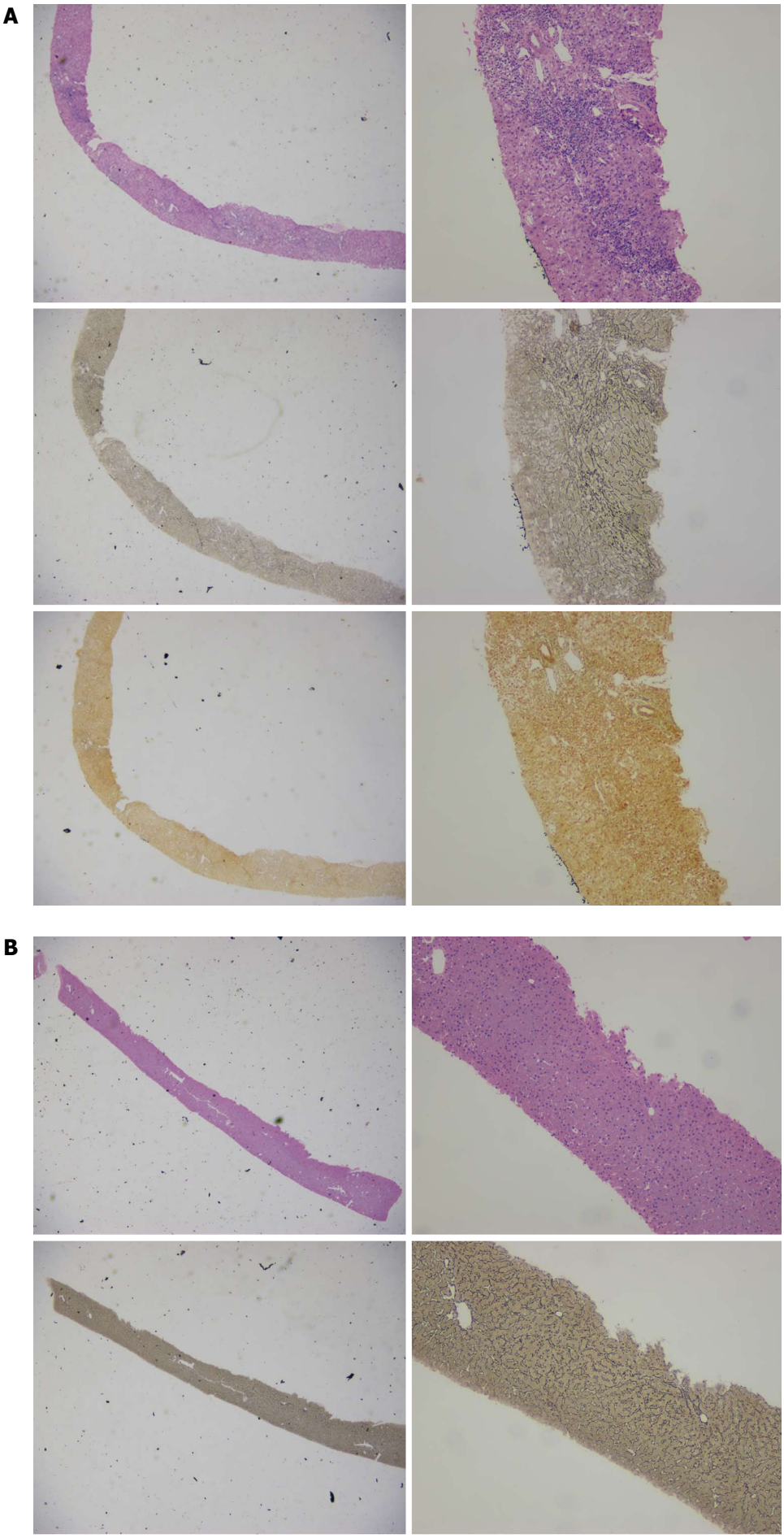
Figure 3 Distribution of Ishak fibrosis scores for the entecavir and lamivudine arms at baseline and post-treatment. The green areas represent the percentage of patients who had an Ishak fibrosis score of 0 post-treatment. ETV: Entecavir; LAM: Lamivudine; ADV: Adefovir.

30 mo of ETV monotherapy, respectively. The two patients in the LAM arm had baseline fibrosis scores of 3 and 5. These patients achieved a score of 0 after 43 mo of LAM monotherapy and 47 mo of LAM and ADV combination therapy (ADV was added when LAM resistance emerged), respectively. Figure 4 shows the favorable histological outcome of a 35-year old female patient with a Knodell score of 14 and an Ishak score of 4 at baseline. This patient achieved a score of 0 for both necroinflammatory and fibrotic score after 44 mo of ETV monotherapy.

We further investigated which patients benefited most from long-term NA therapy in fibrotic regression or reversion. The results showed that patients with higher Ishak score at baseline and longer duration of treatment benefit more in fibrotic regression (Table 4). Conversely, patients who experienced significant improvement in fibrosis after long-term NAs therapy had higher HBV DNA and ALT levels at baseline (Table 5).

DISCUSSION

This was a retrospective study that investigated the efficacy of long-term treatment with two different NAs in CHB patients with advanced fibrosis or cirrhosis. LAM was the first approved NA for treatment of CHB in 1998 and was effective in suppressing HBV DNA, normalizing ALT, and improving histological outcome. However, the use of LAM was limited by the high rate of LAM resistance[14]. It was reported that 5 years of LAM treatment resulted in 70% drug relevant resistance[3,15]. The addition of ADV is one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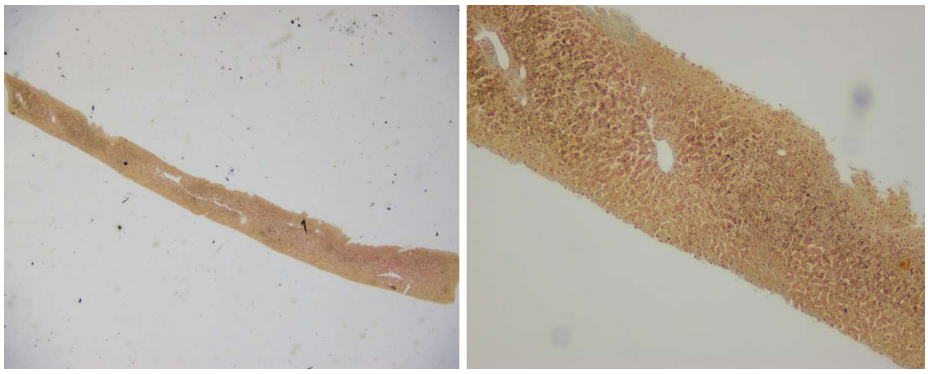

Figure 4 Pre- (A) and post-treatment (B) micrographs for a typical 35-year-old female patient with a favorable treatment outcome. Hematoxylin-eosin, reticular fiber and Masson's trichrome staining was used in each row of the photos, respectively; × 20 for the left column and × 100 for the right column, respectively.

Table 4 Comparison of baseline characteristics between patients with and without improvement in fibrosis after long-term antiviral treatment

	With improvement in fibrosis	Without improvement in fibrosis	*P*-value
n	24	18	
Male gender, *n* (%)	17 (70.83)	13 (72.22)	0.9215
Age (mean ± SD), yr	43.8 ± 9.9	42.9 ± 9.8	0.7596
$\log_{10}$ HBV DNA (mean ± SD), IU/mL	6.7 ± 1.2	5.9 ± 1.5	0.0602
ALT (mean ± SD), U/L	102 ± 82	99 ± 121	0.6473
HBeAg positivity, *n* (%)	17 (70.83)	10 (55.56)	0.3065
Median grade (range)	10 (3-16)	9 (0-13)	0.1713
Median stage (range)	5 (3-6)	3.5 (2-5)	0.0230
Treated with ETV, *n* (%)	13 (54.17)	6 (33.33)	0.1795
Treatment duration ≥ 24 mo, *n* (%)	24 (100)	13 (72.22)	0.0101

HBV: Hepatitis B virus; ALT: Alanine aminotransferase; HBeAg: Hepatitis B e antigen; ETV: Entecavir.

Table 5 Comparison of baseline characteristics between patients with significant or without improvement in fibrosis after long-term antiviral therapy

	With significant improvement[1]	Without improvement	*P*-value
n	9	18	
Male gender, *n* (%)	6 (66.7)	13 (72.2)	0.7657
Age (mean ± SD), yr	42.1 ± 11.6	42.9 ± 9.8	0.8557
$\log_{10}$ HBV DNA (mean ± SD), IU/mL	7.3 ± 1.0	5.9 ± 1.5	0.0175
ALT (mean ± SD), U/L	140 ± 72	99 ± 121	0.0270
HBeAg positivity, *n* (%)	7 (77.8)	10 (55.6)	0.2597
Median grade (range)	11(3-14)	9 (0-13)	0.2778
Median stage (range)	5 (3-6)	3.5 (2-5)	0.2717
Duration of treatment (mean ± SD), mo	44 ± 11	41 ± 18	0.3956

[1]Significant improvement in fibrosis was defined as an Ishak fibrosis score of 0 or 1 post-treatment. HBV: Hepatitis B virus; ALT: Alanine aminotransferase; HBeAg: Hepatitis B e antigen.

of the rescue therapies for patients who experienced LAM resistance. In 2005, ETV was approved for use in treatment-naïve and LAM-resistant patients at a dose of 0.5 mg/d and 1 mg/d, respectively[16]. In 2006, two global multicenter clinical trials demonstrated that both HBeAg negative and positive patients benefit more from 48 wk of ETV treatment than LAM treatment in virological, biochemical, and histological improvements[17,18]. Moreover, a study in 2008 found a trend of histological improvement after 48 wk of LAM and ETV treatment in patients with advanced fibrosis or cirrhosis was similar[19]. The respective histological improvements after long-term treatment of the two NAs were significant. However, whether ETV is superior to LAM after long-term treatment remains unclear[20-23]. In this study, we enrolled patients with advanced fibrosis or cirrhosis receiving more than three years of treatment with the two agents. Several patients in the LAM-treated arm were also treated with ADV when LAM resistance was detected. We found that patients all benefited from long-term treatment of the two agents and most patients in both arms achieved undetectable HBV DNA and normalization of ALT levels after 3 years of NA treatment. There was

no significant difference in HBV DNA suppression, ALT normalization, HBeAg loss, and seroconversion in the LAM-treated and ETV-treated arms. Thus, it raised the question of whether the superior efficacy of 48 wk ETV and LAM therapy proven by the global clinical trials was caused by LAM resistance. We suggest that low genetic barrier NAs such as LAM remain a suitable candidate for management of CHB if viral resistance is well controlled.

Previous studies demonstrated that prolonged treatment with the currently approved NAs (including LAM, ADV, LdT, ETV, TDF) could reduce inflammation grades and fibrosis stages, which results in the prevention of cirrhosis and subsequent hepatic decompensation[24]. It is unclear whether there was any difference among the 5 NAs in the improvement of inflammation and regression of fibrosis. In the current study, we found that long-term treatment with NAs resulted in significant histological improvement. This result indicates that long-term NA therapy improves histological outcome. Moreover, a more significant decline of median Knodell necroinflammatory score was observed in the ETV arm than in the LAM arm. However, the median Ishak fibrosis score was not different between groups. Interestingly, the same conclusion was drawn in a 48-wk study comparing LAM and ETV in the treatment of naïve patients. Our data suggest that patients may benefit more from ETV than LAM therapy in the improvement of inflammation but not in the regression of fibrosis.

Accumulating clinical data have revealed that hepatic fibrosis or cirrhosis can be reversed when active treatment was performed. This result was further proven in our study showing hepatitis B-associated advanced fibrosis or cirrhosis can be regressed or reversed by long-term treatment with NAs. We found that the regression of hepatitis B-associated advanced fibrosis was closely related with baseline fibrotic stage and treatment duration. Interestingly, patients with higher ALT and HBV DNA levels at baseline achieved significant improvement in fibrosis. However, activated hepatic stellate cells were key players in hepatic fibrogenesis and its resolution is closely associated with fibrotic regression[25]. Taken together, we suggest that the regression of hepatitis B associated fibrosis might not simply result from the suppression of HBV DNA and there are probably other benefits caused by long-term NA therapy. It has been proven that long-term therapy with NAs can restore the function of HBV specific T cells[26,27]. Previous studies demonstrated that NA therapy can regulate the immune system[28-30]. Thus, immunomodulation effects of long-term NA treatment may also play a role in the regression of hepatic fibrosis. Hepatic stellate cells play a key role in fibrogenesis and it remains unclear whether NA therapy can directly or indirectly regulate activated hepatic stellate cells. Finally, we believe that significant progress in understanding mechanism of hepatic fibrogenesis and reversion of cirrhosis can eventually translate into more effective treatment strategies for hepatitis B associated fibrosis or cirrhosis.

COMMENTS

Background

Chronic hepatitis B (CHB) is caused by hepatits B virus (HBV) infection and can lead to long-term consequences that include liver cirrhosis and hepatocellular carcinoma. Both lamivudine (LAM) and entecavir (ETV) are nucleos(t)ide analogues (NAs) that are effective in treating CHB by directly suppressing viral replication. Studies have demonstrated that 48 wk of ETV therapy is superior to LAM in terms of viral suppression, alanine aminotransferase (ALT) normalization and histological improvement. However, there is no comparison of the histological efficacy after long-term treatment of the two agents, especially in CHB patients with significant fibrosis or cirrhosis.

Research frontiers

In the treatment for CHB, histological outcome remains an important aspect of efficacy evaluation and contributes to decision making in terms of treatment duration and choice of medication.

Innovations and breakthroughs

Previous studies showed that the histological outcome after 48 wk of ETV therapy was superior to LAM. The authors compared the histological outcome after long-term treatment with ETV monotherapy and LAM-based combination therapy in CHB patients with significant fibrosis or cirrhosis. The results showed that the majority of patients experienced histological improvement in both arms and some patients achieved full reversion of fibrosis or cirrhosis. ETV was superior to LAM-based therapy in improvement of necroinflammatory but not fibrotic outcome.

Applications

The results of this study suggest that ETV is superior to LAM-based therapy in improving liver necroinflammation but not fibrosis in CHB patients. Significant fibrosis or cirrhosis can be fully reversed in some patients after long-term treatment.

Terminology

Liver fibrosis is the excessive accumulation of extracellular matrix proteins that occurs as a result of sustained viral replication and chronic inflammation in CHB and may eventually result in cirrhosis, which is the end stage of fibrosis characterized by replacement of liver tissue with diffuse fibrosis and regenerative nodules.

Peer-review

This is a good retrospective study in which the authors compared the histological outcome of long-term ETV and LAM therapy in CHB patients with significant fibrosis or cirrhosis. The results suggest that ETV is superior to LAM-based therapy in improvement of necroinflammatory but not fibrotic outcome. It is also inspiring that some patients even achieved full reversion of fibrosis or cirrhosis.

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ORIGINAL ARTICLE

Retrospective Cohort Study

Comparison of effects of obesity and non-alcoholic fatty liver disease on incidence of type 2 diabetes mellitus

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Data sharing statement: Technical appendix, statistical code, and dataset available from the corresponding author at xjr049540@163.com.

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Abstract

AIM: To compare and analyze the effects of obesity and non-alcoholic fatty liver disease (NAFLD) on the incidence of type 2 diabetes mellitus (T2DM) in Chinese subjects.

METHODS: In 2008, a population of 4847 subjects was randomly sampled from 17 medical units for enrollment in this cohort study. Baseline information was obtained *via* a questionnaire on general information, physical examination (height, weight, and blood pressure), laboratory tests (triglycerides, total cholesterol, fasting blood glucose, alanine aminotransferase (ALT), uric acid, and creatinine), B-mode ultrasound, and ECG screening. The incidence of T2DM after four years of follow-up was calculated. Numeric variable data was tested for normality, with the data expressed as mean ± SD. Kaplan-Meier analysis was performed to calculate the cumulative incidence. The Cox proportional hazards model was used to analyze the relative risk (RR) of different body mass index (BMI) levels and NAFLD on T2DM, as well as analyzing

the RR adjusted for age, sex, blood pressure, lipids, transaminases, uric acid, and creatinine.

RESULTS: A total of 4736 (97.71%) subjects completed 4-year follow-up, with a median follow-up time of 3.85 years, totaling 17223 person-years. 380 subjects were diagnosed with T2DM, with a cumulative incidence of 8.0%. The cumulative incidence of T2DM in the NAFLD and control groups was 17.4% *vs* 4.1% ($P < 0.001$), respectively, while the incidence in overweight and obese subjects was 11.0% *vs* 15.8% ($P < 0.001$), respectively. The incidence of T2DM increased with an increase in baseline BMI. Cox regression analysis showed that the risk of T2DM in the NAFLD group (RR = 4.492, 95%CI: 3.640-5.542) after adjustment for age, sex, blood pressure, lipids, ALT, uric acid, and creatinine was 3.367 (2.367-4.266), while the value (RR, 95%CI) in overweight and obese subjects after adjustment for age, sex, BMI, blood pressure, lipids and other factors was 1.274 (0.997-1.629) and 1.554 (1.140-2.091), respectively. Stratification of three BMI levels (BMI < 24 kg/m^2, 24 kg/m^2 ≤ BMI < 28 kg/m^2, BMI ≥ 28 kg/m^2) showed that the risk of T2DM in the NAFLD group was significantly higher than that in the control group (RR = 3.860, 4.049 and 3.823, respectively).

CONCLUSION: Compared with BMI, NAFLD could be better at forecasting the risk of T2DM in Chinese subjects, and may be a high risk factor for T2DM, independent of overweight/obesity.

Key words: Non-alcoholic fatty liver disease; Type 2 diabetes; Cohort study; Incidence

Core tip: A population of 4847 Chinese subjects was randomly sampled from 17 medical units for enrollment in this cohort study with a 4-year follow-up. The effects of obesity and non-alcoholic fatty liver disease (NAFLD) were compared and analyzed on the incidence of type 2 diabetes mellitus (T2DM). Compared with body mass index, NAFLD could be better at forecasting the risk of T2DM in Chinese subjects, and may be a high risk factor for T2DM, independent of overweight/obesity.

Li WD, Fu KF, Li GM, Lian YS, Ren AM, Chen YJ, Xia JR. Comparison of effects of obesity and non-alcoholic fatty liver disease on incidence of type 2 diabetes mellitus. *World J Gastroenterol* 2015; 21(32): 9607-9613 Available from: URL: http://www.wjgnet.com/1007-9327/full/v21/i32/9607.htm DOI: http://dx.doi.org/10.3748/wjg.v21.i32.9607

INTRODUCTION

As addition to being a chronic disease, obesity is an important risk factor for type 2 diabetes mellitus (T2DM), cardiovascular disease, hypertension, respiratory disease, hepatobiliary disease, certain cancers, and other chronic non-infectious diseases and psychosocial disorders, and is an important global public health problem that leads to disability (which adversely affects the individual's quality of life and increases their financial burden on the state) and premature death[1-3]. Although the prevalence of obesity in China is not as high as that in developed countries, in recent years it has shown an epidemic trend[4], with a number of obese people second only to that in the United States, and obesity-related metabolic syndrome in China has received widespread attention.

Body mass index (BMI) is obtained by dividing body weight in kilograms by height in meters squared. In developed countries, subjects with a BMI ≥ 25 kg/m^2 are defined as overweight and those with a BMI ≥ 30 kg/m^2 are defined as obese, and there are good associations and positive predictive effects between BMI and obesity-related chronic diseases[5,6].

However, BMI values and the number of obese subjects in the Asia-Pacific region are generally lower than those in Western countries due to ethnic differences and dietary habits[7,8]. Research in China showed that central obesity and the waist/hip ratio correlate with metabolic syndrome[9,10]. It may be more meaningful to study body fat deposition in Asia-Pacific populations.

Non-alcoholic fatty liver disease (NAFLD) was first proposed by Ludwig *et al*[11], and refers to the pathological features of alcoholic fatty liver disease. It is now recognized that NAFLD results in hepatic metabolic stress damage, and is closely related to insulin resistance (IR) and genetic susceptibility. Although the pathological changes in NAFLD are similar to those in alcoholic liver disease, patients have no history of excessive alcohol consumption, non-alcoholic fatty liver (NAFL), non-alcoholic steatohepatitis (NASH), or related liver cirrhosis and hepatocellular carcinoma[12-14]. NAFLD is the most common chronic liver disease in developed countries[15,16].

Both obesity and NAFLD are closely related to T2DM and share a common pathogenesis associated with "insulin resistance". However, although studies have shown that NAFLD is a predictor of pre-diabetes or T2DM[17,18], and incurs a higher incidence of T2DM compared with obesity[19,20], it is not widely accepted that NAFLD is a risk factor for T2DM, and thus this issue requires further research, especially in China[21,22].

MATERIALS AND METHODS

Subjects

The study cohort was established in 2008, with subjects selected from physical examination centers at three hospitals in Nanjing (Nanjing Provincial Units

Table 1 Study subjects' general characteristics at baseline

Variable	Value
Gender (male/female)	3149/1587
Age (yr)	52.70 ± 14.98
Body mass index (kg/m^2)	24.04 ± 3.13
Fasting plasma glucose (mmol/L)	5.29 ± 0.62
Systolic blood pressure (mmHg)	127.65 ± 17.28
Diastolic blood pressure (mmHg)	81.87 ± 10.46
Cholesterol (mmol/L)	4.94 ± 0.95
Triglycerides (mmol/L)	1.56 ± 1.10
Alanine aminotransferase (U/L)	26.42 ± 17.16
Creatinine (μmol/L)	76.77 ± 21.71
Uric acid (μmol/L)	328.26 ± 82.35
Non-alcoholic fatty liver disease, *n* (%)	1412 (29.81)

Hospital, Nanjing Armed Police Hospital, and Nanjing Disease Prevention and Control Center). Using the cluster sampling method, the units which carried out staff physical examinations in the three medical centers were numbered and 17 units were randomly selected. From January 2008 to December 2008, baseline information on all employees in each unit was obtained, and included a questionnaire on general information, physical examination (height, weight, and blood pressure), laboratory tests (triglycerides, total cholesterol, fasting blood glucose, alanine aminotransferase (ALT), uric acid, and creatinine), B-mode ultrasound, and ECG screening. The assessments were carried out using unified research programs, a unified questionnaire, and standardized methods. Participating personnel were trained and assessed, and informed consent forms were signed by all participants and the study was approved by the Ethics Committee of the Nanjing Branch of Jiangsu Armed Police General Hospital, Nanjing, Jiangsu Province, China.

Follow-up

T2DM patients or those using insulin (1881), patients with hepatitis B surface antigen or who were positive for hepatitis C antibody positive (1043), patients with other chronic liver diseases (67), inflammatory bowel disease (46), celiac diseases (ileus, appendix, small intestine, or colon resection) (194), and alcoholics (male > 20 g/d, and women > 10 g/d) (314) were excluded according to serum antibody levels and questionnaires. Additional tests, such as 2-h postprandial plasma glucose, oral glucose tolerance test (OGTT) and a C-peptide release test, were performed when the fasting blood glucose level of subjects were greater than or equal to 6.1 mmol/L.

A total of 4847 subjects without T2DM during the baseline assessment were followed up annually from 2008 to 2012. During this period, 111 subjects died or were moved, transferred, or had just one set of data, thus 4736 (97.71%) subjects completed the 4-year follow-up, with a median follow-up time of 3.85 years.

Diagnostic criteria for NAFLD and T2DM

The diagnosis of NAFLD was in accordance with the Assessment and Management Guidelines of Non-alcoholic Fatty Liver Disease in Asia and the Pacific Region[23]: (1) Diffuse fatty liver could be defined by B-mode ultrasound *via* diffusely increased liver near the field ultrasound echo, a liver echo greater than the kidney, vascular blurring, and the gradual attenuation of the far field ultrasound echo; (2) There was no history of alcohol consumption, or ethanol intake was less than 140 g in men and 70 g in women per week in the past 12 mo; and (3) Specific diseases that could lead to steatosis, such as viral hepatitis, drug-induced liver disease, total parenteral nutrition, Wilson's disease, and autoimmune liver disease, were excluded. The diagnosis of T2DM patients was in line with the 1999 WHO diagnostic criteria for T2DM, and excluded gestational diabetes, type 1 diabetes, and special types of diabetes. A BMI $\geqslant$ 24 kg/m^2 was defined as overweight and a BMI $\geqslant$ 28 kg/m^2 was defined as obese; serum triglyceride (TG) $\geqslant$ 1.70 mmol/L was defined as high TG; serum total cholesterol (TC) $\geqslant$ 5.7 mmol/L was defined as high TC; serum aspartate aminotransferase (AST) or ALT $\geqslant$ 40 U/L was defined as high AST or high ALT.

Statistical analysis

EpiData 3.02 double-track entry and error correction software was used to establish a database, and SPSS17.0 software was used for statistical analysis. The numeric variable data were tested for normality and, if present, the data were expressed as mean ± SD. Kaplan-Meier analysis was performed to calculate the cumulative incidence and compare the groups. The Cox proportional hazards model was used to analyze the relative risk (RR) of different BMI levels and NAFLD on T2DM, and to analyze the RR adjusted for age, sex, blood pressure, lipids, transaminases, uric acid, and creatinine.

RESULTS

Baseline characteristics

Of the 4736 subjects, 3149 were male (66.5%) and 1587 were female (33.5%). The median follow-up time was 3.85 years, totaling 17223 person-years. A total of 380 subjects were diagnosed with T2DM during follow-up, with a cumulative incidence of 8.0%. The baseline characteristics of the study subjects in 2008 are shown in Table 1.

Influence of NAFLD and baseline BMI on incidence of T2DM

Subjects were divided into the NAFLD or control groups according to NAFLD diagnosis using B-mode ultrasound in the 2008 baseline assessment. Kaplan-Meier analysis was used to calculate and compare

Table 2 Incidence of type 2 diabetes mellitus and Cox hazards regression analysis in subjects with different baseline non-alcoholic fatty liver disease and body mass index levels

Group	*n*	T2DM	Incidence rate (%)[1]	RR (95%CI)	RR[2] (95%CI)
Non-alcoholic fatty liver disease					
No	3379	135	4.1	1	1
Yes	1412	245	17.4	4.492 (3.640-5.542)	3.367 (2.367-4.266)
Body mass index (kg/m^2)					
< 24	2999	166	5.5	1	1
About 24	1249	137	11	2.023 (1.614-2.537)	1.274 (0.997-1.629)
About 28	488	77	15.8	2.954 (2.254-3.870)	1.554 (1.140-2.091)
Age (yr)					
< 30	291	5	1.7	1	1
About 30	722	17	2.4	1.350 (0.498-3.658)	1.044 (0.379-2.875)
About 40	1033	54	5.2	3.043 (1.217-7.607)	1.853 (0.736-4.665)
About 50	1135	114	10	6.021 (2.459-14.743)	3.136 (1.270-7.747)
About 60	1555	190	12.2	7.469 (3.074-18.152)	4.344 (1.772-10.651)
Gender					
F	3149	86	5.4	1	1
M	1587	294	9.3	1.748 (1.374-2.222)	1.327 (1.025-1.720)
SBp					
< 140	3672	237	6.5	1	1
⩾ 140	1064	143	13.4	2.164 (1.759-2.664)	1.462 (1.139-1.877)
Alanine aminotransferase					
< 40	4094	303	7.4	1	1
⩾ 40	642	77	12	1.628 (1.268-2.091)	1.522 (1.165-1.988)
Total	4736	380	8		

[1]P < 0.001 by Log Rank (Mantel-Cox) test, showing differences in the incidence rate between the groups; [2]RR adjusted for age, sex, blood pressure, lipids, alanine aminotransferase, uric acid, and creatinine. T2DM: Type 2 diabetes mellitus.

Table 3 Cox regression analysis of the relationship between non-alcoholic fatty liver disease and type 2 diabetes mellitus at different body mass index levels

BMI	NAFLD	*n*	T2DM	Incidence rate[1] (%)	RR (95%CI)	RR[2] (95%CI)
< 24	Control	2383	85	3.6	1.0	1.0
	NAFLD	616	81	13.1	3.860 (2.847-5.233)	3.407 (2.461-4.717)
About 24	Control	712	35	4.9	1.0	1.0
	NAFLD	537	102	19.0	4.049 (2.758-5.944)	3.455 (2.269-5.262)
About 28	Control	229	15	6.6	1.0	1.0
	NAFLD	259	62	23.9	3.823 (2.175-6.719)	3.438 (1.841-6.420)

[1]P < 0.001 by Log Rank (Mantel-Cox) test, indicating a significant difference in T2DM incidence between the two groups; [2]RR adjusted for age, sex, blood pressure, lipids, alanine aminotransferase, uric acid, and creatinine. NAFLD: Non-alcoholic fatty liver disease; T2DM: Type 2 diabetes mellitus.

the cumulative incidence of T2DM in the two groups (Table 2), and showed that the incidence of T2DM in the NAFLD group was significantly higher than that in the control group. Cox regression analysis showed that the risk of T2DM in the NAFLD group was significantly higher than that in the control group (RR = 4.492; RR = 3.367 after adjustment for age, sex, BMI, blood pressure, lipids, and other factors).

The subjects were divided into three groups according to their baseline BMI. Kaplan-Meier analysis was used to calculate and compare the cumulative incidence of T2DM in the groups (Table 2), and showed that the incidence of T2DM in overweight (BMI ⩾ 24) and obese (BMI ⩾ 28) subjects was significantly higher than that in subjects with a BMI < 24. Cox regression analysis showed that the risk of T2DM in overweight and obese subjects was significantly higher than that in subjects of normal weight (RR = 2.023 and 2.954, respectively; RR = 1.274 and 1.554, respectively, after adjustment for age, sex, NAFLD, blood pressure, cholesterol and other factors).

Influence of NAFLD and different BMI levels on the risk of T2DM

BMI was stratified into three levels, and the effect of obesity and NAFLD on the risk of T2DM was evaluated and compared (Table 3). All three levels of BMI showed that the risk of T2DM in the NAFLD group was significantly higher than that in the control group, RR = 3.860, 4.049 and 3.823, respectively (almost 4.492

when not stratified; Table 2).

DISCUSSION

Prediction of the risk of T2DM according to different BMI levels

Although the standards for the definition of obesity using BMI in Western countries and in the Asia-Pacific region are not the same, a meta-analysis[24] showed that the RR of T2DM predicted by BMI was 1.18 (95%CI: 1.16-1.20), which increased with increasing BMI[25]. Although BMI values were lower in the Asia-Pacific region, BMI was still associated with T2DM risk[26]. In the present study, in accordance with the provisions of the Chinese Adult Overweight and Obesity Prevention and Control Guidelines[27], a BMI $\geqslant$ 24 was defined as overweight and a BMI $\geqslant$ 28 was defined as obese. The results showed that, after adjustment for age, sex, blood pressure, lipids, ALT, uric acid, and creatinine, the risk of T2DM in overweight or obese subjects was still significantly higher than that in normal weight subjects [RR = 1.274 (95%CI: 0.997-1.629) and 1.554 (95%CI: 1.140-2.091), respectively], and the incidence of T2DM increased with increasing BMI, indicating that BMI can predict the risk of T2DM in Chinese subjects.

Prediction of the effect of NAFLD on T2DM risk

As a characteristic of visceral fat accumulation, NAFLD is closely associated with insulin resistance and T2DM[28]. Studies from Japan showed that pre-diabetic patients with NAFLD developed T2DM, with a hazard ratio (HR) of 6.39 (95%CI: 5.00-8.18, $P < 0.001$)[29]. NAFLD was found to be a risk factor for T2DM in non-obese and non-diabetic Korean men, with the NAFLD group having more subjects with impaired fasting glucose (IFG) and T2DM than the non-NAFLD group during a 5-year follow-up period (32.7% *vs* 17.6%, 1.9% *vs* 0.3%, respectively; $P < 0.05$) [30]. Moreover, a five-year cohort study from China confirmed that NAFLD predicts T2DM, but not pre-diabetes. The adjusted RR (95%CI) of T2DM and pre-diabetes in the NAFLD group of said study were 4.462 (1.855-10.734, $P < 0.001$) and 1.642 (0.965-2.793, $P = 0.067$), respectively, compared with a non-NAFLD group[31].

The results of our study showed that the RR (95%CI) of T2DM in the NAFLD group was 3.367 (2.367-4.266), which was significantly higher than that in the control group. Thus, NAFLD is better than BMI in forecasting the risk of T2DM in Chinese subjects, and NAFLD may be an unrecognized risk factor in China's recent increased incidence of T2DM.

NAFLD is a risk factor for T2DM independent of overweight/obesity

In order to evaluate and compare the impact of BMI and NAFLD on the incidence of T2DM in China, BMI was classified as either normal, overweight, or obese. The analytical results showed that NAFLD groups with different BMI levels had a significantly higher risk of T2DM than the control group, similar to the risk without stratification. The risk of T2DM in NAFLD patients with normal or abnormal BMI showed little difference, suggesting that irrespective of BMI, NAFLD increased the risk of T2DM and is thus a BMI-independent risk factor affecting T2DM incidence in China.

Relationship between NAFLD and T2DM incidence is closer than that between overweight/obesity and T2DM

It is generally considered that high BMI (overweight/obesity) is part of the metabolic syndrome and is a risk factor for T2DM. This study showed that the four-year cumulative incidence rate of T2DM in the NAFLD group was 17.4% and the RR (95%CI) after adjustment was 3.367 (2.367-4.266), while these values in overweight and obese subjects were 11.0% and 15.8%, respectively, and the RR (95%CI) after adjustment were 1.274 (0.997-1.629) and 1.554 (1.140-2.091), respectively. These results indicate that the risk of T2DM in NAFLD subjects is significantly higher than that in overweight and obese subjects. Although NAFLD is not widely recognized as a high risk factor for T2DM[17,19,28], our study results show that the relationship between NAFLD and the incidence of T2DM could be closer than that between overweight/obesity and T2DM in Chinese subjects. In addition, abnormal BMI and NAFLD together increased the incidence of T2DM by 6.6 fold, suggesting the presence of an additive effect on T2DM risk. However, since the observed objects were only from Nanjing district, the limited sample size and observation time were limitations of this study. More studies are needed to confirm our findings.

Tissues and organs which lower blood glucose include the liver, muscle, and adipose tissue, and the liver is a vital organ in substance, energy, and hormone metabolism. In addition to lowering blood glucose, the liver can also raise blood glucose by breaking down glycogen and through gluconeogenesis, thus the liver plays a pivotal role in blood glucose regulation. Due to the huge compensatory ability of the liver, it can be speculated that it is only when damage or loss of liver cell function due to hepatic steatosis reaches a certain level[32] does a reduction in the regulatory ability of the liver on blood glucose and the metabolism of hormones potentially occur, thus leading to insulin resistance and T2DM, which allows time for the early prevention of T2DM.

In this study, NAFLD was screened using B-ultrasound, which is a routine method used in clinical diagnosis and physical examination, and has the advantages of convenience, quickness, and reduced financial cost, while CT examination and liver biopsy are unsuitable for population screening. Subjects undergoing physical examination are screened for

NAFLD using B-ultrasound and guided by health and lifestyle education for the intervention and treatment of NAFLD. *Via* these methods, liver fat accumulation should decrease to the normal range[33] and the liver should recover the ability to regulate blood glucose and hormone metabolism, which may reduce the incidence of T2DM. Clinicians and patients should be suitably educated on the dangers of NAFLD.

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COMMENTS

Background

Although the prevalence of obesity in China is not as high as that in developed countries, it is generally accepted that obesity is a major risk factor for type 2 diabetes mellitus (T2DM) and is involved in the primary prevention of T2DM in China. Non-alcoholic fatty liver disease (NAFLD) has shown an epidemic trend in China in recent years, but in-depth studies on the long-term harm of NAFLD and its relationship with T2DM are rare. In addition, NAFLD presents almost no obvious clinical symptoms, resulting in delayed diagnosis and treatment in most Chinese patients.

Research frontiers

A previous study showed that sustained NAFLD was associated with an increased risk of type 2 diabetes in non-obese and non-diabetic Korean men. The latest research shows that NAFLD is a significant predictor for future diabetes, but not pre-diabetes, in Chinese subjects.

Innovations and breakthroughs

Using a cohort study design, this research included NAFLD as a risk factor for T2DM and analyzed whether there was a causal association between NAFLD and the incidence of T2DM in China, compared the risk of obesity and NAFLD on the incidence of T2DM, and looked for possible reasons for the increased incidence of T2DM in China in recent years.

Applications

The study results suggest that compared with body mass index, NAFLD is better at forecasting the risk of T2DM in Chinese subjects and is a high risk factor for T2DM, independent of overweight/obesity.

Peer-review

This study has value in confirming this finding in other Asian populations.

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ORIGINAL ARTICLE

Retrospective Study

Prognosis of acute-on-chronic liver failure patients treated with artificial liver support system

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Abstract

AIM: To establish a new model for predicting survival in acute-on-chronic liver failure (ACLF) patients treated with an artificial liver support system.

METHODS: One hundred and eighty-one ACLF patients who were admitted to the hospital from January 1, 2012 to December 31, 2014 and were treated with an artificial liver support system were enrolled in this retrospective study, including a derivation cohort (n = 113) and a validation cohort (n = 68). Laboratory parameters at baseline were analyzed and correlated

with clinical outcome. In addition to standard medical therapy, ACLF patients underwent plasma exchange (PE) or plasma bilirubin adsorption (PBA) combined with plasma exchange. For the derivation cohort, Kaplan-Meier methods were used to estimate survival curves, and Cox regression was used in survival analysis to generate a prognostic model. The performance of the new model was tested in the validation cohort using a receiver-operator curve.

RESULTS: The mean overall survival for the derivation cohort was 441 d (95%CI: 379-504 d), and the 90- and 270-d survival probabilities were 70.3% and 58.3%, respectively. The mean survival times of patients treated with PBA plus PE and patients treated with PE were 531 d (95%CI: 455-605 d) and 343 d (95%CI: 254-432 d), respectively, which were significantly different (P = 0.012). When variables with bivariate significance were selected for inclusion into the multivariate Cox regression model, number of complications, age, scores of the model for end-stage liver disease (MELD) and type of artificial liver support system were defined as independent risk factors for survival in ACLF patients. This new prognostic model could accurately discriminate the outcome of patients with different scores in this cohort (P < 0.001). The model also had the ability to assign a predicted survival probability for individual patients. In the validation cohort, the new model remained better than the MELD.

CONCLUSION: A novel model was constructed to predict prognosis and accurately discriminate survival in ACLF patients treated with an artificial liver support system.

Key words: Acute-on-chronic liver failure; Artificial liver support system; Model for end-stage liver disease; Plasma exchange; Plasma bilirubin adsorption

Core tip: Liver failure has a high mortality. The current prognostic model to estimate the survival in acute-on-chronic liver failure (ACLF) patients treated with an artificial liver support system (ALSS) is not fully characterized. The aim of this study was to establish a new scoring model and to test its ability to predict the survival of ACLF patients treated with ALSS. This prognostic model accurately differentiated the outcome of ACLF patients with different risk scores and also had the ability to assign a predicted survival probability for individual patients.

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INTRODUCTION

Liver failure (LF) can be classified as acute LF occurring without any potential liver diseases, acute-on-chronic liver failure (ACLF), which is caused by an acute exacerbation of chronic liver diseases resulting from virus, alcohol or drugs, and chronic decompensation in any type of end-stage liver disease. ACLF, the most common type of LF, constitutes a serious condition with a sophisticated etiology, diversified manifestations and a high short-term mortality[1]. So far, liver transplantation is identified as the most useful approach for ACLF; however, few patients benefit from this treatment due to the extreme lack of healthy livers and/or the costly operation[2].

In the past five decades, a variety of different types of artificial liver support systems (ALSS) have been developed to bridge patients with LF to liver transplantation or to support the failing liver temporarily, until it is able to regenerate. Although ALSS cannot substitute for the whole spectrum of liver functions, these methods can take the place of a few basic hepatic functions[3-7]. It has been demonstrated that some types of ALSS, such as plasma exchange (PE) and plasma bilirubin adsorption (PBA), are able to remove toxic substances, improve coagulopathy, and prevent bleeding. Although some studies have reported success in prolonging survival, the survival of patients with LF is variable, approximately 21%-60%[8-11].

Prognostic models have been used for estimating disease severity and survival and are of great importance for doctors to make therapeutic decisions. The current prognosis models for ACLF were only analyzing ACLF caused by HBV and might not be ideal for clinical practice because of the diversified etiologies of ACLF in Asia[12-14]. Moreover, these models were established based on data from standard medical treatment (SMT) and without considering the impact of ALSS on the prognosis of patients. Therefore, a feasible prognostic model is urgently needed to estimate the outcomes of patients with ACLF.

We have assessed the overall survival and the possible prognostic predictors in a cohort of ACLF patients treated with SMT together with ALSS. The aim of this study was to develop a novel model to supply reliable predictive information about these patients.

MATERIALS AND METHODS

Study design

The present study was based on a retrospective cohort, including 181 patients with ACLF hospitalized between January 1, 2012 and December 31, 2014 at the Department of Infectious Diseases, Union Hospital, Wuhan, China. The ACLF patients hospitalized from January 1, 2012 to December 31, 2013 were included in a derivation cohort (n = 113). This dataset was used to create the prognostic model. Then, the new model was validated in another 68 patients hospitalized from

January 1, 2014 to December 31, 2014.

Patients who met the diagnostic criteria for ACLF were hospitalized, and besides SMT, they were treated with PE or PBA combined with PE. In this study, liver transplantation was not available for ACLF patients owing to the extreme deficiency of healthy livers and/or the costly operation. ACLF patients with persistent bleeding, circulatory shock, severe bacterial infection, pregnancy, international normalized ratio (INR) $\geqslant$ 3.0 or platelet count $\leqslant$ 30000/μL were excluded for ALSS.

ACLF patients were randomly divided into either a PBA plus PE group or a PE only group. The follow-up began at the date of initial treatment of ALSS. In the derivation cohort, patients were followed until death or censored at the end point of January 31, 2014. While in the validation cohort, patients were followed until the end point of January 31, 2015. Medical history, physical examination, and auxiliary investigations, such as laboratory test, abdominal ultrasound or computed tomography (CT) scan, were finished at admission. Laboratory parameters included serum total bilirubin (TB), aspartate aminotransferase (AST), alanine aminotransferase (ALT), creatinine, and INR, and others. Adverse events and drugs received were documented during the whole study period.

The data of ACLF patients were analyzed anonymously based on the Declaration of Helsinki. This study was approved by the Ethics Committee of the Union Hospital, Tongji Medical College, Huazhong University of Science and Technology, and all enrolled patients gave their written consent, which was collected in the hospital and could be used for research.

Disease definition

According to the recommendations generated by the Asian Pacific Association for the Study of the Liver, ACLF was defined as acute liver injury emerging as jaundice and coagulopathy, complicated by ascites and/or encephalopathy within 4 wk in a patient with known or unknown chronic liver disease[15]. The definition of LF in ACLF was as follows: severe jaundice (total serum bilirubin $\geqslant$ 5 mg/dL) and coagulopathy (INR $\geqslant$ 1.5 or prothrombin activity < 40%), which were indispensable, and ascites and/or encephalopathy, which were diagnosed by physical examination[15].

The diagnosis of hepatitis E virus (HEV) infection was made according to the detection of anti-HEV IgM and/or HEV RNA in serum[16]. Liver cirrhosis (LC) was diagnosed by the medical history, physical examination, and laboratory tests, together with ultrasonography or CT[17]. Hepatic encephalopathy (HE) was defined as neuropsychiatric abnormalities including the cognitive, affective, behavior and consciousness. It was diagnosed by clinical manifestations and brain edema identified by magnetic resonance imaging (MRI) or CT[18]. Hepatorenal syndrome (HRS) was defined as a functional renal failure according to the criteria created by the International Ascites Club[19]. Spontaneous bacterial peritonitis (SBP) was diagnosed by the examination of ascites[20]. Upper gastrointestinal bleeding commonly arose from the esophagus, stomach, or duodenum. In some cases, blood could be observed in vomit or in tarry stool[21].

Treatments

SMT: SMT was intended to support the liver and to prevent and treat complications of ACLF. All patients were administered according to the following recommendations: absolute bed rest, the use of hepatocyte growth factor and adenosylmethionine to regenerate liver cells, the infusion of albumin, maintaining electrolyte or acid-base equilibrium and preventing and curing complications. Oral antiviral drugs including lamivudine, telbivudine, or entecavir were ordered for the patients with activated replication of hepatitis B virus.

PE

In addition to SMT, ACLF patients in this group underwent treatment of PE, which was performed with a membrane plasma filter (Plasmaflo OP-08W, Asahi Kasei Kuraray Medical Co., Ltd., Tokyo, Japan). A double-lumen catheter was inserted into the right femoral vein of ACLF patients, and approximately 3000 mL of plasma was exchanged per time at a blood flow rate of 20 to 25 mL/min. Each patient in the derivation cohort received PE 1 to 4 times, and 93 times in total were performed in 54 patients. In the validation cohort, each patient received PE 1 to 4 times, and 62 times in total were performed in 33 patients.

PBA plus PE

Blood was separated by Plasmaflo OP-08W. Then, the plasma was passed through an adsorbent column (Plasorba BR-350, Asahi Kasei Kuraray Medical Co., Ltd., Tokyo, Japan). Approximately 2000-2500 mL of plasma was separated per time at a blood flow rate of 20 mL/min. Perfused over the adsorbent column, the plasma was returned to the patient after being merged with hemocytes coming from the plasma filter. After the PBA process, the patient was treated with PE using a total of 1500-2000 mL of fresh, frozen plasma. In the derivation cohort, each patient received PBA plus PE 1 to 4 times, and 135 times in total were performed in 59 patients. In the validation cohort, each patient received PBA plus PE 1 to 4 times, and 76 times in total were performed in 35 patients.

Statistical analysis

Continuous variables are shown as mean ± SD or median, and categorical variables are expressed by count. The period from the date of the initial ALSS to the date of death or loss to follow-up was defined as survival time. The comparison of the survival distributions in different groups of patients was determined by the Kaplan-Meier analysis. The model for

Table 1 Patient baseline demographics and the treatment *n* (%)

Variable	Derivation cohort (*n* = 113)	Validation cohort (*n* = 68)	*P* value
Age (yr)	44.6 (22-81)	46.1 (20-79)	0.529
Men	94 (83)	52 (76)	0.268
Etiology of liver failure			
HBV	64 (56.6)	40 (59)	0.773
HBV + HEV	36 (31.9)	18 (26)	0.443
Other causes[1]	13 (11.5)	10 (15)	0.531
MELD score	24 (12-44)	26 (13-46)	0.659
Total bilirubin (mg/dL)	24.8 (5.9-51.8)	23.1 (5.7-50.1)	0.841
ALT (IU/mL)	262 (12-1757)	211 (23-1519)	0.169
AST (IU/mL)	232 (17-1634)	206 (20-1301)	0.326
Creatinine (mg/dL)	0.9 (0.3-6.8)	1.0 (0.3-7.1)	0.533
INR	2.4 (0.8-11.4)	2.6 (1.0-16.1)	0.285
Cirrhosis	58 (51.3)	45 (66.2)	0.051
Number of complications			
1	57 (50.4)	39 (57.4)	0.367
2	27 (23.9)	13 (19.1)	0.453
3	20 (17.7)	10 (14.7)	0.600
4	8 (7.1)	4 (5.9)	0.996
5	1 (0.9)	2 (2.9)	0.654
Type of ALSS			
PE	54 (47.8)	33 (48.5)	0.923
PE + PBA	59 (52.2)	35 (51.5)	0.923

[1]Includes alcohol combined with drugs; autoimmune combined with drugs; schistosome combined with alcohol. Number (proportion) or median (interquartile range) are shown. HBV: Hepatitis B virus; HEV: Hepatitis E virus; MELD: The model for end stage liver disease; ALT: Alanine aminotransferase; AST: Aspartate aminotransferase; INR: International normalized ratio; ALSS: Artificial liver support system; PE: Plasma exchange; PBA: Plasma bilirubin adsorption.

end-stage liver disease (MELD) scores were calculated by the formula: 9.57 × ln[creatinine (mg/dL)] + 3.78 × ln[total bilirubin (mg/dL)] + 11.2 × ln(INR) + 6.43 (etiology: 0 if cholestatic or alcoholic, 1 otherwise)[22].

The variables acquired at baseline before the initial ALSS with $P < 0.05$ in the bivariate analysis were introduced to create a multivariate Cox regression analysis with a P value < 0.1 (using the backward conditional stepwise regression manner). $P < 0.05$ was considered significant with a CI of 95%. The receiver-operating characteristic (ROC) curve was used to describe the MELD and the Cox regression model. The performance of the model was determined by the concordance statistic (c-statistic), which was equal to the area under the ROC curve (AUC). A c-statistic > 0.7 was considered useful[23]. The predictive accuracy of the new model was examined in the validation cohort by calculating the c-statistic[23]. The AUCs were compared by the *z*-test. SPSS 18.0 (SPSS, Chicago, IL, United States) and MedCalc 11.4 (Mariakerke, Belgium) software programs were used for data analyses.

RESULTS

Model derivation cohort

In the derivation cohort, a total of 113 ACLF patients were reviewed and registered into this study. Table 1 shows the demographics at baseline. The median age of these patients was 44.6 years, and 83% of patients were male. In this Asian cohort, hepatitis B virus was the prevailing etiology of liver disease. The average level of HBV DNA of patients infected with hepatitis B virus was (5.03 ± 2.11) $\log_{10}$ IU/mL. More than half of the patients (50.4%) had one complication, such as HE, SBP, HRS, upper gastrointestinal hemorrhage, or electrolyte disturbances, while others suffered with two or more complications. The median MELD score of these patients was 24.

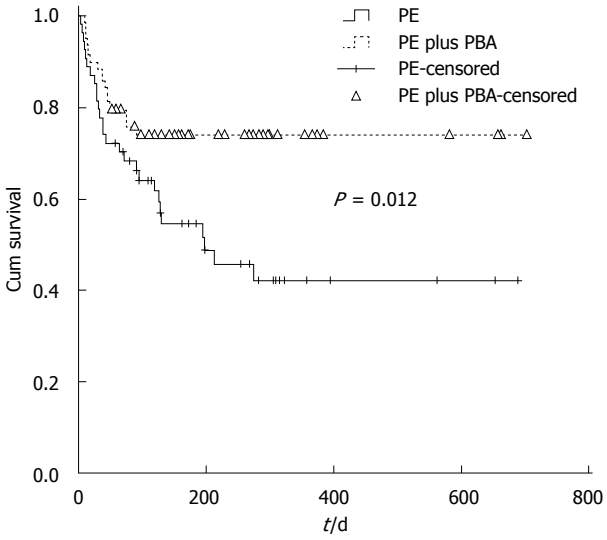

Figure 1 Comparing survival in patients with acute-on-chronic liver failure treated with different types of artificial liver support systems. The Kaplan-Meier curves were calculated to compare the overall survival probability between acute-on-chronic liver failure patients who underwent plasma exchange (PE) *vs* those treated with PE plus plasma bilirubin adsorption (PBA).

One hundred and thirteen ACLF patients were treated with SMT plus PBA and/or PE therapy. No therapy-related adverse events, including severe hemorrhage, shock, or hypersensitivity, occurred in the PBA process, but a few allergies such as rash occurred in cases during PE. At the end point of this study, 65 patients were alive, 42 (37.2%) patients died, and 5 were lost to follow-up.

For the derivation cohort, the mean overall survival was 441 d (95%CI: 379-504 d), and the 90- and 270-d survival probabilities were 70.3% and 58.3%, respectively. The mean survival times of patients treated with SMT together with PBA plus PE and patients treated with SMT plus PE were 531 d (95%CI: 455-605 d) and 343 d (95%CI: 254-432 d), respectively, which were significantly different (P = 0.012, Figure 1).

Predictors for survival

Moreover, we investigated the correlation between survival time and clinical data, such as gender, age, etiology, numbers of complications, type of ALSS, and serum biomarkers tested at baseline, including TB, ALT, AST, INR and creatinine. In the bivariate analysis,

Table 2 Univariate and multivariate analyses of risk factors for survival

Variable	Univariate		Multivariate		
	HR (95%CI)	*P* value	HR (95%CI)	*P* value	Coefficient
Age	1.045 (1.024-1.067)	< 0.001	1.031 (1.008-1.054)	0.008	0.030
Gender[1]	0.704 (0.296-1.676)	0.428			
Etiology[2]	0.736 (0.178-3.049)	0.673			
MELD score	1.120 (1.080-1.161)	< 0.001	1.102 (1.056-1.150)	< 0.001	0.097
Total bilirubin	1.002 (1.000-1.003)	0.047			
ALT	1.001 (1.000-1.002)	0.022			
AST	1.001 (1.000-1.002)	0.011			
Creatinine	1.003 (1.000-1.005)	0.055			
BUN	1.083 (1.015-1.157)	0.017			
INR	1.369 (1.209-1.550)	< 0.001			
Times[3]	0.753 (0.549-1.034)	0.080			
Complication[4]	2.246 (1.721-2.933)	< 0.001	1.694 (1.224-2.344)	0.001	0.527
ALSS[5]	0.455 (0.242-0.856)	0.015	0.454 (0.232-0.889)	0.021	-0.790

[1]Gender: 0 = male, 1 = female; [2]Etiology: 0 = nonviral, 1 = viral; [3]Times of ALSS: 1 = 1, 1 = 1, 2 = 2, 3 = 3, 4 = 4, 5 = 5, 6 = 6; [4]Number of complications: 1 = 1, 2 = 2, 3 = 3, 4 = 4, 5 = 5; [5]Type of ALSS: 0 = PE, 1 = PE plus PBA. MELD: Model for end-stage liver disease; AST: Aspartate aminotransferase; ALT: Alanine aminotransferase; INR: International normalized ratio; ALSS: Artificial liver support system.

Table 3 Calculation of probability of survival according to the risk score

Days	30	60	90	120	180	270
S0 (t)	92.50%	84.90%	79.70%	77.40%	71.80%	65.60%

S0(t) gives the estimated survival probabilities for a patient with a risk score of 3.7 which is the mean risk score of acute-on-chronic liver failure patients in the derivation cohort. To calculate the probability of survival at t days of a given patient use the following equation: $S(t) = S0(t)^{exp(score-3.7)}$.

some factors evaluated at baseline showed a predictive impact on overall survival, comprising the type of ALSS, age, number of complications, MELD score, TB, ALT, AST, and INR (Table 2).

Multivariate model

Significant variables in the bivariate analysis were selected into a multivariate Cox regression analysis, such as type of ALSS, age, number of complications and MELD score, to define independent predictive factors for survival (Table 2). According to the multivariate Cox regression analysis, a risk score (R) can be calculated by the equation: R = 0.03*(age) + 0.097*(MELD score) + 0.527*(the number of complications) - 0.79*(the type of ALSS).

Further, using the means of covariates for (age, type of ALSS, MELD and number of complications) all of the patients in the derivation cohort, a mean R value of 3.7 could be calculated based on the formula. Simultaneously, the survival probabilities for an individual with an R value of 3.7 were estimated by the survival table created by SPSS (Table 3). If $S_0(t)$ was assigned to the estimated survival probabilities for a patient with an R value of 3.7, we can calculate the survival probabilities at t days for any patient by using the formula: $S(t) = S_0(t)^{exp(score-3.7)}$, which was previously described[24]. Particularly, based on the score, the expected survival probability for an individual patient can be calculated. For instance, the 90- and 270-d survival probabilities for ACLF patients in the lowest quartile (R = 1.9) were 96.3% and 93.3%, respectively. However, in the highest quartile (R = 5.6), the survival probabilities sharply decreased to 21.9% and 6.0% at 90 and 270 d, respectively (Figure 2).

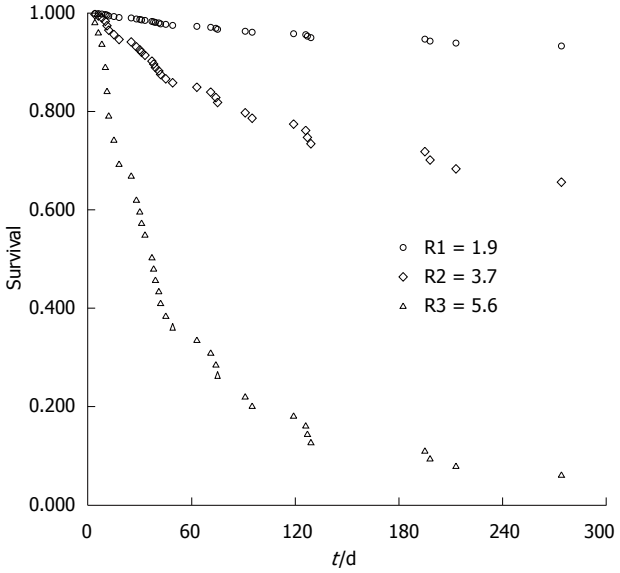

Figure 2 Prospective survival probabilities of three assumed acute-on-chronic liver failure patients. According to the equation in Table 3, the prospective survival probabilities can be computed in individual acute-on-chronic liver failure patients with scores of 1.9, 3.7, and 5.6, respectively.

The new prognostic model also illustrated that ACLF patients who had lower R values might have a better survival probability. If the outcomes of patients with R values less than 3.0 were assigned as "Good", patients with an R value more than 5.0 were "Poor", and the rest with an R value between 3.0 and 5.0 were defined as "Fair". There were significant differences in

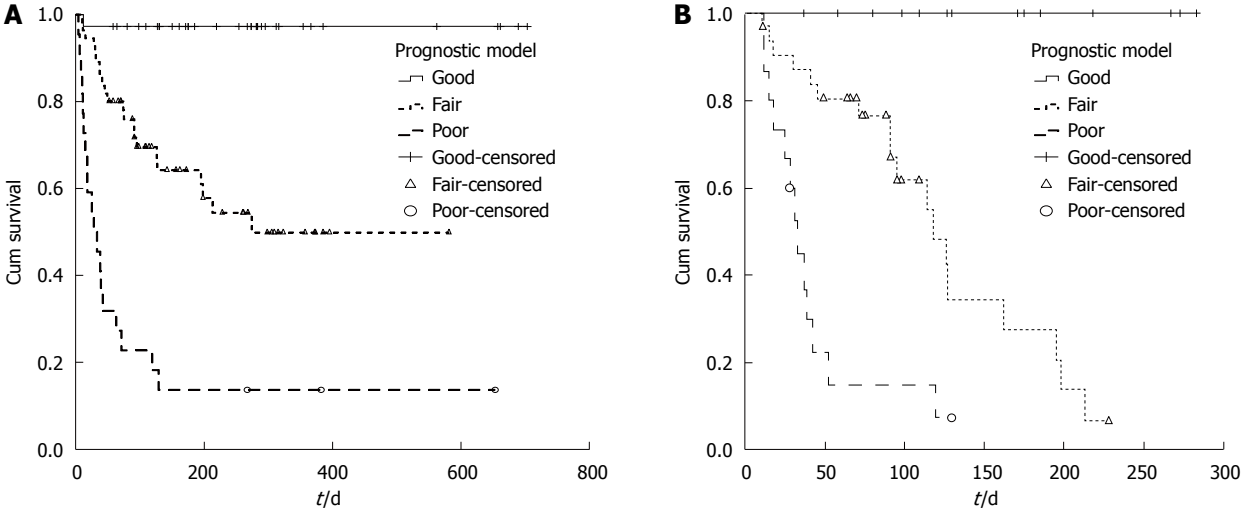

Figure 3 Survival probabilities of acute-on-chronic liver failure patients with different prognoses. The Kaplan-Meier curves were drawn to contrast survival probabilities among acute-on-chronic liver failure patients whose prognoses were estimated to be good, fair, and poor, according to the risk scores calculated by the new model in the derivation cohort (A) and validation cohort (B).

Table 4 Prognosis in the derivation and validation cohorts according to risk score *n* (%)

Prognosis	Derivation cohort (*n* = 113)	Validation cohort (*n* = 68)	*P* value
Good	36 (31.9)	21 (30.9)	0.891
Fair	55 (48.7)	32 (47.1)	0.833
Poor	22 (19.4)	15 (22.0)	0.676

the survival probabilities of patients with "Good", "Fair", and "Poor" prognoses ($P < 0.001$, Figure 3A), and the mean survival times of these patients were 684, 344 and 120 d, respectively ($P < 0.001$).

Model validation

The predictive accuracy of the new model was validated in another cohort of 68 ACLF patients. The clinical characteristics of the patients in the validation cohort are shown in Table 1. For the validation cohort, the c-statistic for the new prognostic model was 0.879 (95%CI: 0.799-0.959) (Figure 3B).

The classification of the outcome of patients in the derivation cohort based on the R value resulted in the assignment of 31.9% of the patients to the "Good" group ($R < 3.0$), 48.7% to the "Fair" group ($3.0 \leqslant R \leqslant 5.0$), and 19.4% to the "Poor" group ($R > 5.0$) (Table 4). Similar results were also found in the validation cohort: 30.9% of the patients were in the "Good" group, 47.1% in the "Fair" group, and 22.0% in the "Poor" group.

Comparison with MELD model

We further evaluated the predictive values of MELD scores and the new Cox model. The results showed that the c-statistics were 0.799 (95%CI: 0.711-0.887) and 0.882 (95%CI: 0.818-0.945), respectively, for these two scoring systems used in the derivation cohort. Furthermore, the AUC was obviously greater in the new prognostic model than in the MELD ($z = 2.330$, $P = 0.0198$) (Figure 4A). In the validation cohort, the c-statistics were 0.752 (95%CI: 0.636-0.867) and 0.879 (95%CI: 0.799-0.959), respectively, and the AUC was also greater than in the MELD ($z = 2.794$, $P = 0.0052$) (Figure 4B).

DISCUSSION

ACLF is distinguished by the acute exacerbation of liver function in patients with pre-existing chronic liver disease that occurs due to acute episodes, including both infectious and noninfectious causes. Alcohol and drugs make up the main acute events in the West; however, infectious causes are common in Asia. The reactivation of HBV infection is the major etiology of ACLF in Asia[25,26]. Another significant infectious cause of the acute episode is superinfection with HEV[27,28]. Because there are still no effective therapies available, except liver transplantation, ACLF is correlated with a poor prognosis[29]. Thus, ALSS had been developed to bridge patients with LF to liver transplantation. Early estimation of outcome is significant for differentiating ACLF patients who need liver transplantation from patients who would survive followed by ALSS. Current prognostic models based on parameters of clinical characteristics and the degree of liver dysfunction had been generated to evaluate the short-term survival probability of ACLF patients[12-14]. However, these models were usually created on the basis of the data at baseline from patients without ALSS treatment. Specifically, in the present study, we had screened the prognostic factors in a cohort of ACLF patients treated with ALSS besides SMT and established a scoring model that could accurately predict the prognosis in

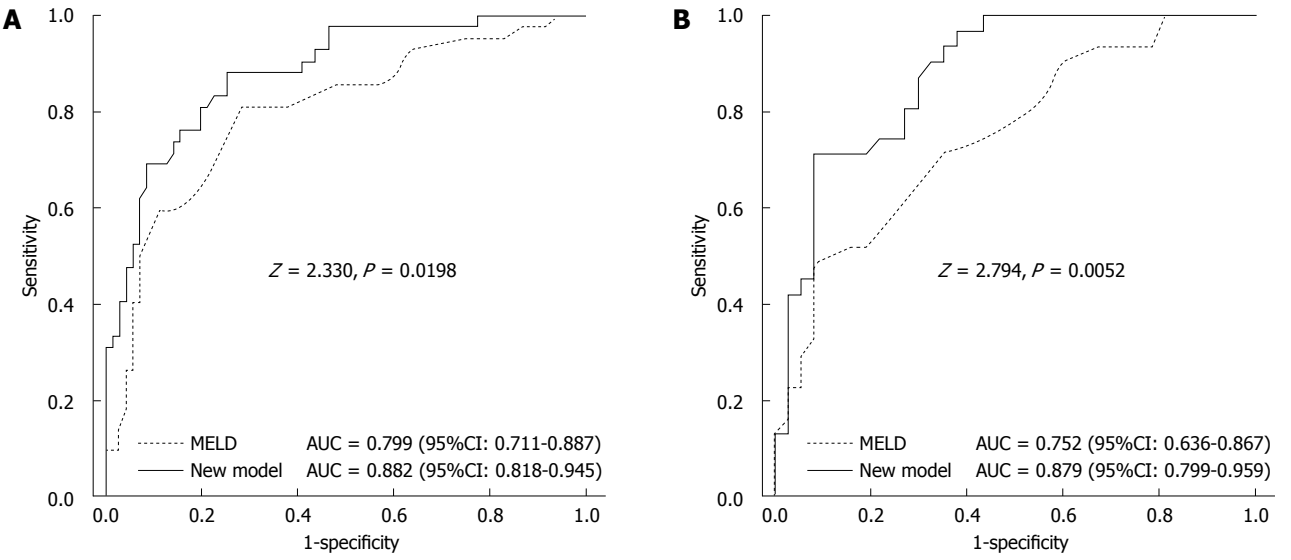

Figure 4 Comparison of the predictive accuracy for survival between the new model and the model for end-stage liver disease. The area under the receiver-operating characteristic curve (AUC) with 95%CI of the new model and model for end-stage liver disease was 0.882 (0.818-0.945) and 0.799 (0.711-0.887), respectively, in the derivation cohort (A) and 0.879 (0.799-0.959) and 0.752 (0.636-0.867), respectively, in the validation cohort (B).

different groups of these patients.

It was reported that some types of ALSS such as PE or PBA could supply a beneficial internal environment for liver cells to restore liver functions in ACLF patients[30-33]. PE can separate and discard the plasma of LF patients to get rid of toxic substances and replenish it with normal plasma to supply several essential substances, such as coagulation factors and immunoglobulin[34,35]. However, PE requires exchanging plenty of fresh plasma and bears the risk of some potential infections. PBA could absorb conjugated and albumin-bound bilirubin from the plasma and had proved to be an effective treatment for LF patients[36,37]. PE and PBA have similar effects in lowering bilirubin and inflammatory cytokines, but PBA has the advantages of no risk of blood-transmissible diseases and rare side effects[32,33]. Recently, due to a shortage of plasma, the combination therapy of PBA plus PE, which needs less plasma than PE, is only widely used for ACLF patients in China. Our results showed that ACLF patients treated with PBA plus PE had better outcomes than patients treated with PE alone.

In this study, the MELD score served as an independent predictor of survival in accordance with previous reports[12,13]. In addition to MELD, we encompassed the clinical and biochemical variables into a Cox regression analysis to identify which were potential predictors of survival. As previously described, age was significantly associated with 3-mo mortality in ACLF patients[12,13]. Here, older age had an unfavorable prognostic relevance. Complications such as HE[12] and HRS[12,13] were also determined to be significantly associated with mortality in ACLF patients. The multivariate analysis in the present study showed that age, number of complications, MELD score and type of ALSS independently determined the outcomes of patients suffering with ACLF. Then, a predictive scoring system was created based on the above variables. As a successive score, the scoring model could precisely distinguish the prognosis of ACLF patients with different scores. Furthermore, high-score patients who are estimated to have a poor outcome probability could be recognized at baseline and considered for early alternative treatments, such as liver transplantation. Specifically, the model could estimate a forecasted survival probability for each individual by calculating the risk score based on the model.

As the prognostic ability of the MELD scoring system had been reported in many studies[38], we further verified the validity of MELD in patients with ACLF. The results showed that MELD did well in categorizing patients based on their risk scores. The c-statistic was 0.799, indicating that the MELD scoring system was useful in forecasting survival in ACLF patients. However, the c-statistic in the new model created in our study was 0.882. By statistical analysis, we found that the new scoring model had a higher predictive capability than MELD. MELD scoring system was originally developed to determine the priority of liver transplantation objectively and was built with only subjective parameters. Therefore, this new prognostic model including some other clinical variables besides MELD had a better performance than MELD scoring system.

However, there are several limitations in this study. First, it was a retrospective study, and the patients only came from a single medical center. Second, the clinical characteristics of the derivation cohort may limit the new model to be applied in other populations, such as patients in Western countries, where alcohol and drugs are the main cause of ACLF. Studies of more heterogeneous groups of patients from geographically

diverse areas are needed. Third, several widely accepted prognostic models for LF were not selected, and only MELD was compared in this study. ALSS is not actually proven to be effective in prolonging the patient's survival by a well-designed randomized controlled trial. Though the new model could predict the patient's survival better than MELD, it can only be applied to ACLF patients who undergo ALSS besides SMT.

In summary, based on a cohort of patients with ACLF, we have established and validated a new prognostic model for ACLF patients. It was the first to explore an approach to estimate the prognosis of patients treated with ALSS in Asia, and the feasibility of this novel scoring system should be validated by additional larger prospective studies.

COMMENTS

Background

Liver failure has a high mortality. The current prognostic model to estimate the survival in acute-on-chronic liver failure (ACLF) patients treated with artificial liver support system (ALSS) is not fully characterized.

Research frontiers

Current prognostic models based on parameters of clinical characteristics and the degree of liver dysfunction had been generated to evaluate the short-term survival probability of ACLF patients. However, these models were usually created on the basis of the data at baseline from patients without ALSS treatment.

Innovations and breakthroughs

In the present study, the authors had screened for the predictive factors in a cohort of ACLF patients treated with ALSS, besides standard medical therapy, and established a scoring model which could accurately predict the prognosis in different groups of these patients.

Applications

This prognostic model could accurately differentiate the outcome of patients with different risk scores for ACLF patients and also had the ability to assign a predicted survival probability for individual patients.

Terminology

ACLF was defined as acute liver injury emerging as jaundice and coagulopathy, complicated by ascites and/or encephalopathy within 4 wk in a patient with known or unknown chronic liver disease.

Peer-review

The authors of this paper established a model to predict the prognosis of patients with ACLF treated with artificial liver support system (plasma exchange or plasma bilirubin adsorption combined with plasma exchange). The model seems to be accurate in predicting patients' survival.

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ORIGINAL ARTICLE

Retrospective Study

Contrast enhanced computed tomography and reconstruction of hepatic vascular system for transjugular intrahepatic portal systemic shunt puncture path planning

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Author contributions: Qin JP performed the surgical procedures, wrote the paper and analyzed the data; Tang SH collected the data and wrote the paper; Jiang MD provided clinical treatments, analyzed the data and revised the paper; He QW performed the surgical procedures and reconstructed three-dimensional images; Chen HB and Yao X participated in surgical operation and collected the data; Zeng WZ managed the patients, collected the data and revised the paper; Gu M performed the surgical procedures and reconstructed three-dimensional images; Qin JP and Tang SH contributed equally to this work.

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Abstract

AIM: To describe a method for the transjugular intrahepatic portal systemic shunt (TIPS) placement performed with the aid of contrast-enhanced computed tomography (CECT) and three-dimensional reconstructed vascular images (3D RVIs), and to assess its safety and effectiveness.

METHODS: Four hundred and ninety patients were treated with TIPS between January 2005 and December 2012. All patients underwent liver CECT and reconstruction of 3D RVIs of the right hepatic vein to portal vein (PV) prior to the operation. The 3D RVIs were carefully reviewed to plan the puncture path from

the start to target points for needle pass through the PV in the TIPS procedure.

RESULTS: The improved TIPS procedure was successful in 483 (98.6%) of the 490 patients. The number of punctures attempted was one in 294 (60%) patients, 2 to 3 in 147 (30%) patients, 4 to 6 in 25 (5.1%) patients and more than 6 in 17 (3.5%) patients. Seven patients failed. Of the 490 patients, 12 had punctures into the artery, 15 into the bile duct, eight into the gallbladder, and 18 through the liver capsule. Analysis of the portograms from the 483 successful cases indicated that the puncture points were all located distally to the PV bifurcation on anteroposterior images, while the points were located proximally to the bifurcation in the three cases with intraabdominal bleeding. The complications included three cases of bleeding, of whom one died and two needed surgery.

CONCLUSION: Use of CECT and 3D RVIs to plan the puncture path for TIPS procedure is safe, simple and effective for clinical use.

Key words: Transjugular intrahepatic portal systemic shunt; Contrast-enhanced computed tomography; 3D vascular reconstruction; Interventional radiology

Core tip: Precise planning of puncture path is crucial for safe and effective transjugular intrahepatic portal systemic shunt (TIPS) placement. We have developed and applied an approach that combines contrast-enhanced computed tomography and reconstruction of hepatic vascular system in TIPS placement. Our retrospective study with 490 patients over a period of seven years shows that the improved TIPS procedure was successful in 98.6% of the patients, indicating that this method is safe, simple and effective for clinical use.

Qin JP, Tang SH, Jiang MD, He QW, Chen HB, Yao X, Zeng WZ, Gu M. Contrast enhanced computed tomography and reconstruction of hepatic vascular system for transjugular intrahepatic portal systemic shunt puncture path planning. *World J Gastroenterol* 2015; 21(32): 9623-9629 Available from: URL: http://www.wjgnet.com/1007-9327/full/v21/i32/9623.htm DOI: http://dx.doi.org/10.3748/wjg.v21.i32.9623

INTRODUCTION

China has a high prevalence rate of hepatitis B, and chronic hepatitis often results in hepatic cirrhosis, portal hypertension and other related complications. These diseases impose a heavy burden on the patient's family and the country[1,2]. Currently, liver transplantation is still limited in China, and the common therapeutic option is to manage the complications of hepatic cirrhosis, particularly to treat the rupture and bleeding due to esophageal varices resulting from portal hypertension and refractory ascites. Since the late 1980s when Rösch *et al*[3] and Rössle *et al*[4] first reported the clinical use of self-expandable metallic stents, transjugular intrahepatic portosystemic shunt (TIPS) has been increasingly used clinically with satisfactory results to treat cirrhotic portal hypertension and associated complications[4-8]. However, TIPS procedures are operationally complicated with many steps and technical challenges. One of the most important and critical steps is to precisely puncture the portal vein (PV) branch and to ensure that the target site is safe to puncture. Precise puncture into the PV branch is a prerequisite for successful operation, while the safety of the puncture point is critical to the safety of patients. For this reason, a large number of studies have been done to explore various methods that can improve the puncture accuracy. Although those studies have helped improve the TIPS techniques, continued improvement of puncture method is being pursued for better, simpler, and safer TIPS procedures with minimally invasion and reduced radiation exposure. Since 2005, our center has been using preoperative contrast-enhanced computed tomography (CECT) and three-dimensional reconstructed vascular images (3D RVIs) to plan and guide the puncture of the PV branch. The clinical outcomes have been satisfactory[9,10]. In this report, the technical details of the method are described and their safety and efficacy were analyzed retrospectively.

MATERIALS AND METHODS

Subjects

Between January 2005 and December 2012, 490 patients diagnosed with portal hypertension and hepatic cirrhosis underwent TIPS placement in our departments[4]. These patients were analyzed retrospectively. All patients for TIPS were selected according to the criteria of the American Hepatological Association for the clinical application of TIPS[11,12]. The study protocol was approved by the institutional ethics committee, and informed consent was obtained from all patients.

TIPS procedure

Before operation, the patients underwent CECT with an Aquilion One scanner (Toshiba, Japan) and the obtained imaging data were used to reconstruct three-dimensional (3D) right hepatic vein to PV images at the Vitrea workstation. The anatomical relationship between the right vein and PV was carefully reviewed to define the spatial relationship between the two puncture points to plan the PV puncture. In typical TIPS procedure, the point of origin is generally started from the right hepatic vein about 1.5 cm away from the confluence of the right hepatic vein to the inferior vena cava (IVC), while the target point is on the

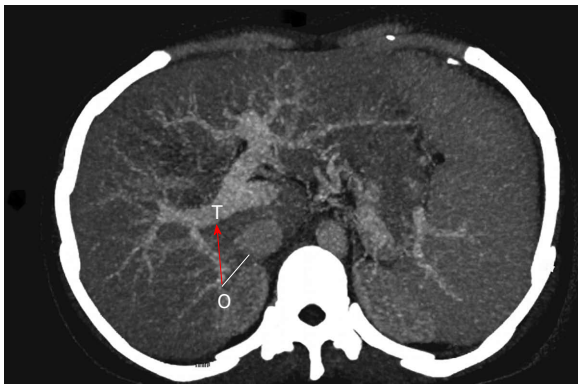

Figure 1 Axial distance between puncture point of origin (o) and target point (t) of the portal vein. Point of origin (o) is located about 1.5 cm (white line) from the confluence of the right hepatic vein to inferior vena cava, and the red arrow between the two points is the anterior-posterior distance of the puncture.

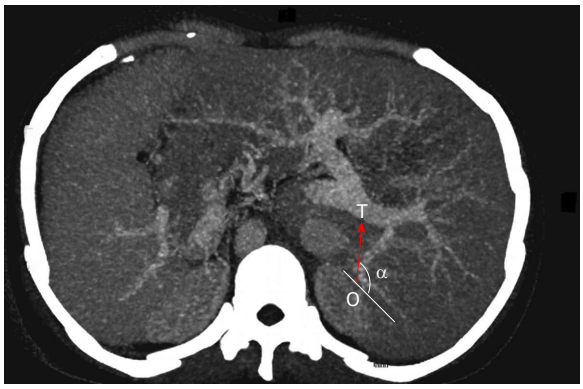

Figure 2 Determination of rotation angle for puncture set. Contrast-enhanced computed tomography image in Figure 1 is rotated 180 degrees, and the angle between the direction given by the extension line that links the two puncture points and direction of the right vein (red arrow) is the rotation angle (α) for the puncture set.

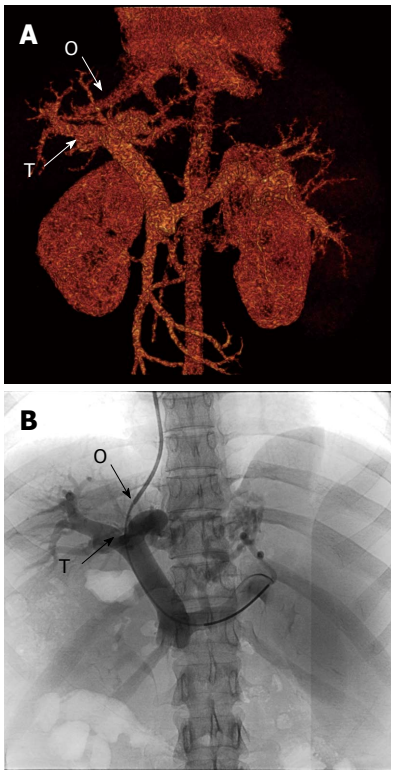

Figure 3 Comparison of preoperative three-dimensional reconstructed image of the portal vein system and direct portogram obtained with non-iodinated contrast medium (Iopamiro 370) after a successful portal vein puncture. A: Preoperative three-dimensional reconstructed vascular image; B: Direct portogram of the portal vein (PV) during a successful puncture. O: Puncture point of the right hepatic vein; T: Target point of the right PV branch.

trunk of the right PV. Thus, once the relationship between the desired start point and target point is adequately defined, the puncture can be achieved precisely. Therefore, the cephalad-caudal and anterior-posterior distances between the two puncture points and left or right position need to be determined. In our TIPS procedure, these distances were adequately determined based on the CECT and 3D RVIs. For the cephalad-caudal distance, we first located the slices of the axial CECT image with the intended puncture start and target points, and then calculated the distance by multiplying the number of slices between the two points and the thickness of the slice. For the anterior-posterior distance, a line was drawn between the desired point of origin and target point on the axial CECT image, and its length was measured (Figure 1). In addition to these distances, the rotation angle (α) of the puncture set was determined as follows: On the axial slice of the desired puncture point, a line was drawn between the point of origin and target point of the right PV trunk, and the direction of the line extending toward the abdomen was the direction of puncture (Figure 2). The image in Figure 1 was flipped horizontally by 180 degrees, and the angle between the direction given by the extension line that links the two puncture points and direction of the right hepatic vein was measured as rotation angle (α). Once these distances were determined, additional information regarding the spatial relationship between the two puncture points was obtained from the reconstructed venograms from coronal CECT (Figure 3A). We used all these parameters and the angle at which the right hepatic vein joins the IVC to adjust the tip angle of the puncture set and to set the counterclockwise rotation angle, as well as to plan puncture path for individual patients. Generally, the directions of the punctures were from left to right, anterior to posterior and cephalad to caudal. All angiographic studies and punctures were performed with the aid of an image-guided system (Innova 3100-IQ, GE, United States). To create the stunt, the Rösch-Uchida set (RUPS-100, COOK Company, United States) *via* the transjugular approach was inserted into IVC with the aid of a guidewire and advanced to the right hepatic vein. The puncture start point selected based on the above parameters was visualized by hand venography and the catheter was advanced to the target point of the PV branch. After entering the hepatic parenchyma, the

Table 1 Clinical data for 490 patients who underwent transjugular intrahepatic portal systemic shunt placement

Clinical factor	Number of patient
Gender	
Male/female	388/102
Age (mean ± SD)	48.2 ± 13.7
TIPS indication	
Upper gastrointestinal bleeding	440
Refractory ascites	22
Hepatorenal syndrome	20
Intractable pleural effusion	8
Cause of disease	
Hepatitis B virus associated cirrhosis	307
Hepatitis C virus associated cirrhosis	22
Alcoholic hepatic disease	52
Autoimmune hepatic disease	17
Hepatitis B virus infection combined with other factors[1]	78
Cirrhosis of unknown cause	14

[1]Other factors include alcohol consumption and schistosome infection.

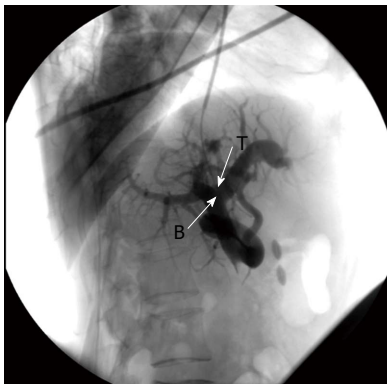

Figure 4 Portogram showing target point in transjugular intrahepatic portal systemic shunt puncture. The points were distal to the image bifurcation (T: Target point of the portal vein; B: The bifurcation).

needle was retrieved, leaving the 5F cannula in place. The cannula was then connected to a 5 mL syringe with 2 mL of heparin saline to aspire the blood. The color and pressure of the blood were used to judge if it was from the portal vein. Five milliliters of non-iodinated contrast medium (Iopamiro 370) was then slowly injected to visualize the PVs distal to the target point. If only one branch was seen, it indicated that the target point would be in the PV branch. If both right and left branches or/and the main trunk were seen, the puncture target point was expected to be near the PV bifurcation or in the PV trunk. Once the safety of the puncture target point was confirmed, the catheter was advanced to the trunk with the aid of the super tiff guidewire, leaving the 10F sheath in the trunk. Pressure measurements were then preformed and direct portography was made at lateral and anteroposterior positions to further confirm the safety. The collateral vessels that cause esophageal varices could be identified by direct PV angiography and embolized with steel spring and gelatin sponge. A balloon (measuring 8 mm in diameter) was inserted into the puncture tract to place a self-expandable metallic stent (measuring 8 mm in diameter). The patient was again measured for pressure and imaged at lateral and axial positions (Figure 3B).

RESULTS

Clinical data

A total of 490 patients with hepatic cirrhosis and portal hypertension underwent TIPS placement in our departments between January 2005 and December 2012[9]. All patients underwent preoperative CECT of the liver and the imaging data were used to reconstruct the 3D right hepatic to portal venograms. Their clinical data are shown in Table 1.

Outcomes of TIPS procedure

Out of the 490 patients, 483 (98.6%) had successful portosystemic shunt creation. The TIPS procedure failed in seven patients. In the seven patients, the punctures caused hepatic subcapsular hematoma, as revealed by intraoperative angiography, and stopping of the TIPS procedure in two patients, portal vein thrombosis blocked the guidewire to enter the PV trunk in two patients, and abdominal bleeding developed in three patients (one died, and two survived after surgical repair or placement of covered stent to stop bleeding). With regards to the number of punctures attempted, the number was one in 294 (60%) patients, 2 to 3 in 147 (30%) patients, 4 to 6 in 25 (5.1%), and more than 6 in 17 (3.5%) patients. During the procedure, all collateral vessels that caused esophageal varices were embolized.

Puncture safety

In 455 (92.8%) patients, the points of origin were selected near the confluence of the right hepatic vein where it joins the IVC and the target points were the right hepatic vein trunk. In the remaining 35 (7.2%) patients, the target points were the left branch of PV; some of these patients had very small right portal vein branches that were not suitable for puncture and the other had thrombus in the right portal veins. In all 490 patients, the target points were all distal to the imaged bifurcation of the right veins. For the three patients with intraabdominal bleeding, their target points were in the left vein branches, and were all near the bifurcation of the PV (Figure 4). When the tracts were dilated with a balloon, they bled.

Puncture-related complications

Three patients had intraoperative intraabdominal bleeding and two had hepatic subcapsular hematoma. Other complications included puncture into the hepatic artery, biliary tract, gallbladder, and through liver capsule, as revealed by leaking of contrast agents (Table 2). The patients were asymptomatic following

Table 2 Incidence of complications related to the transjugular intrahepatic portal systemic shunt procedure *n* (%)

Complication	Number
Intraabdominal bleeding	3 (0.6)
Hepatic subcapsular hematoma	2 (0.4)
Puncture into	
biliary tract	15 (3.1)
gallbladder	8 (1.6)
hepatic artery	12 (2.4)
Puncture through liver capsule	18 (3.7)

rapid needle removal.

DISCUSSION

In TIPS procedures, various methods have been attempted to improve the accuracy and safety of PV puncture. Warner *et al*[13] used a radiopaque marker in the hepatic artery to localize the PV during TIPS for better needle guidance. Yamagami *et al*[14] and Matsui *et al*[15] reported the use of artery-targeting guidewires to increase safety and efficacy of TIPS. The fluoroscopic landmarks in the hepatic artery or vein can be beneficial for the catheterization, guidance of the guidewire, tract balloon dilation, and stent placement. However, the blind passage of puncture needle from the hepatic vein to portal venous system can be difficult, especially for hepatic cirrhosis patients with disordered hepatic anatomy. In addition, ultrasound- and intravascular ultrasound-guided punctures have been reported[16-20]. These methods are noninvasive, real-time, readily available, relatively fast, and have less expense, and less radiation exposure. However, sonographic imaging might be obscured by the ascites and bowel gas in the peritoneal cavity. Magnetic resonance imaging (MRI) is believed to be a relatively ideal method for hepatic and portal vascular imaging, with the advantages of cross-sectional nature, no radioactivity, high sensitivity to vascular flow, and soft-tissue visualization, especially for complex interventions[21]. However, MRI imaging needs relatively long acquisition and display time, which can be influenced by the respiratory motion and surgical metallic devices in patients. The hepatic wedged venography is one of the most frequently used methods for portography, with CO_2 used as the contrast medium[22-24]. This method, without additional invasive access, can produce low-cost, real-time, fast and good images with minimal extra equipment. However, the imaging resolution of CO_2 is lower compared with those obtained with iodinated agents. Due to the low viscosity, CO_2 mainly diffuses to the hepatic veins and branches of the PV, not to the portal vein trunks, resulting in poor ability to image the PV and trunks. The guided methods reported so far have significantly contributed to the improvement of accuracy of TIPS puncture. However, in some of the methods, extra equipment is needed, such as ultrasound, and magnetic resonance imaging machine, while others are invasive, such as placement of intravascular markers and indirect portal venography, and may lead to complications such as bleeding, local and abdominal hematoma, femoral artery pseudoaneurysm, femoral arterial to venous fistula and high vagal reflex, or even death caused by retroperitoneal hematoma[25-27]. Indirect angiography *via* the superior mesenteric artery increases the amount of contrast medium used in the surgery. This not only increases the risk of traumatic injury to organs, particularly the kidney, but also radiation exposure. Patients need to stay supine after angiography, which is inconvenient for patients. As an effort to improve the effectiveness and safety of TIPS procedure, we started to use CECT imaging to plan and guide the puncture path in 2005, and have since been satisfactory with this method[9]. Between 2005 and 2012, the method has been applied to 490 patients. In 98.6% of the patients the stunts were satisfactorily created between the hepatic vein and portal vein. Among the seven who failed, three developed intraabdominal bleeding and two had subcapsular hematoma. The two patients completed the surgery in later times. The remaining two had portal vein thrombosis that prevented the guidewire from entering the PV trunk. Although the reported success rates of indirect portography-, CT- and ultrasound-guided TIPS are nearly 100%[13,24], the number of cases in those studies is much less than that of our study. Furthermore, there are also differences in the case selection in those studies. On the other hand, the seven patients who failed in our study were highly difficult patients with significantly reduced sized liver and shortened cephalad-caudal distance. Therefore the failure is not due to the puncture planning method. In 3D ultrasound-guided needle puncture, the single-passage success rate was shown to be 40% and the rate of over 10 passages was 10%[28]. Using our method, the successful rates for single, 2 to 3, 4 to 6 and more than six needle passages were 60.0%, 29.2% 4.9% and 3.5%, respectively. Therefore, compared with earlier methods, our method has certain advantages and is able to reduce the risk of related complications. In our operation, a few of patients were attempted for more than three needle passages before success. This is partially because some of these patients had relatively large cephalad-caudal and anterior-posterior distances between the right veins and PV branches. This resulted in a puncture distance that is too large for the puncture set (the Rösch-Uchida set RUPS-100) used in the procedure, whose maximal puncture distance is 5.2 cm. For patients with a significantly reduced sized liver, the cephalad-caudal distance between the right vein and PV branch is very short. This would require a greater needle rotating angle, making the puncture

more difficult. Our experience also shows that when the left to right distance between the start and intended target point is too large, it will also make the puncture difficult. Since the spatial relationships between the right vein and PV branch in individual patients with different degrees of hepatic cirrhosis are highly variable, careful review of the CECT images and 3D RVIs can effectively help improve the puncture accuracy. As shown in an earlier study[9], the spatial relationship between the right hepatic vein and the PV branch is not affected by sex, age, ascites and the Child score. Therefore, the PV puncture can be planned with a full understanding of the patient's anatomic structures between the right hepatic and PV. The tip curve and rotating angle can be adjusted based on the spatial parameters that measure the cephalad-caudal and anterior-posterior distances between the start point and intended target point. Our clinical data show that CECT and 3D RVIs can provide accurate 3D relationship between the two puncture points and can be used to successfully plan the puncture path. In fact, the direct portogram in successful TIPS patients and preoperatively reconstructed 3D right hepatic vein to PV image are fully consistent (Figure 3), indicating that the CECT and 3D RVIs can be used effectively to plan the puncture. Using this method, fewer equipment is needed during the procedure for the needle guidance and the use of indirect portography is reduced. Therefore, TIPS procedure can be performed *via* the jugular vein approach with fewer steps, thus reducing complications and patient costs. Access to the PV is important for portosystemic shunt creation. The safety of the target point is the key to the success of operation. If wrongly placed, the puncture would result in intraabdominal bleeding, which often endangers the patient's life. So far, the data used to determine the anatomical bifurcation of the PV and puncture point safety are based on autopsy studies[29] and the puncture point that is 2 cm above the bifurcation is considered safe. Our retrospective analysis of 490 TIPS patients based on their direct portograms showed that all successful puncture points were distal to the bifurcation on the portograms (Figure 3). For the three patients with intraabdominal bleeding after the balloon inflation, their livers were remarkably atrophied with increased hepatic fissure and the target points were located on the left branch of the PV, close to the bifurcation[30]. Due to liver atrophy, the target points were in hepatic fissure. Once the balloon was inflated, it resulted in bleeding. In TIPS procedures, no matter which guiding method is used, it is not possible to completely avoid misplaced puncture into the bile duct, gallbladder, liver and hepatic artery. This is because the guidance technique is still not precise enough, and because of the anatomical concomitance of PV branches with the bile duct and hepatic artery. Furthermore, most of the TIPS patients in China have hepatitis B virus associated cirrhosis. They often have remarkable parenchymal atrophy with increased liver hardness. Therefore, the puncture is more difficult to preform by using the most commonly used Rösch-Uchida transjugular liver access set RUPS-100, which uses a 5-Fr catheter, a trocar stylet (diameter 0.038 inch) and a spring tip. In puncture operation, the front part of the stylet assembly is inserted into the hepatic parenchyma toward the portal system, the stylet is then removed from the catheter and the cannula is retrieved to the PV branch. If puncture is misplaced, the catheter can be removed immediately without traumatic injury to the patient. However, if the stylet is advanced too deep into the right hepatic vein, it is more likely to puncture the gallbladder or puncture through the liver capsule. Of course, these complications can be reduced with improvement of the operator's skill.

In summary, we describe here a method for puncture planning and guidance in TIPS procedure using CECT and 3D reconstruction of the hepatic vascular system. Our analysis is based on the stunt creation in a large sample of patients and shows that this method is effective, safe, simple, and conducive to clinical use.

COMMENTS

Background

The transjugular intrahepatic portosystemic shunt (TIPS) has been increasingly used clinically with satisfactory results to treat cirrhotic portal hypertension and associated complications. However, TIPS procedures are operationally complicated with many steps and technical challenges. One of the most important and critical steps is to precisely puncture the portal vein (PV) branch and to ensure that the target site is safe to puncture. Precise puncture into the PV branch is a prerequisite for successful operation.

Research frontiers

The center has been using preoperative contrast-enhanced computed tomography (CECT) and three-dimensional reconstructed vascular images (3D RVIs) to plan and guide the puncture of PV branch.

Innovations and breakthroughs

Compared with earlier methods, our method has certain advantages and is able to reduce the risk of related complications. Careful review of the CECT images and 3D RVIs can effectively help improve the puncture accuracy. Using this method, fewer equipment is needed during the procedure for the needle guidance and the use of indirect portography is reduced.

Applications

The authors describe here a method for puncture planning and guidance which can be used in TIPS procedures using CECT and three-dimensional reconstruction of the hepatic vascular system.

Peer-review

In this manuscript, the authors report the results of their work in which the authors have developed and applied an approach that combines CECT and reconstructed vascular systems in the TIPS placement. The authors report that the application of the technique over a period of seven years was safe, simple and effective for clinical use. Obviously, the results would be of interest to medical researchers and physicians conducting TIPS placements.

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ORIGINAL ARTICLE

Retrospective Study

Efficacy of hepatic resection *vs* transarterial chemoembolization for solitary huge hepatocellular carcinoma

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Informed consent statement: All study participants or their legal guardian provided informed written consent prior to enrollment.

Conflict-of-interest statement: The authors disclose no conflicts.

Data sharing statement: No additional data are available.

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Abstract

AIM: To compare the efficacy of hepatic resection (HR) and transarterial chemoembolization (TACE) for patients with solitary huge (≥ 10 cm) hepatocellular carcinoma (HCC).

METHODS: Records were retrospectively analyzed of 247 patients with solitary huge HCC, comprising 180 treated by HR and 67 by TACE. Long-term overall survival (OS) was compared between the two groups using the Kaplan-Meier method, and independent predictors of survival were identified by multivariate analysis. These analyses were performed using all patients in both groups and/or 61 pairs of propensity score-matched patients from the two groups.

RESULTS: OS at 5 years was significantly higher in the HR group than the TACE group, across all patients (P = 0.002) and across propensity score-matched

pairs (36.4% *vs* 18.2%, P = 0.039). The two groups showed similar postoperative mortality and morbidity. Multivariate analysis identified alpha-fetoprotein ⩾ 400 ng/mL, presence of vascular invasion and TACE treatment as independent predictors of poor OS.

CONCLUSION: Our findings suggest that HR can be safe and more effective than TACE for patients with solitary huge HCC.

Key words: Hepatic resection; Transarterial chemoembolization; Solitary huge hepatocellular carcinoma; Overall survival; Propensity score matching

Core tip: Hepatic resection (HR) and transarterial chemoembolization (TACE) are the generally accepted treatment options for huge hepatocellular carcinoma (HCC) (⩾ 10 cm), but the most appropriate treatment option for treating solitary huge HCC (⩾ 10 cm) is controversial. This subtype of huge HCC involves similar clinicopathology and prognosis as small HCC after HR. Since reports of TACE to treat solitary huge HCC are limited, we compared the efficacy of HR and TACE in a retrospective analysis with and without propensity score matching.

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INTRODUCTION

Hepatocellular carcinoma (HCC) is the sixth most common malignant tumor and the third most common cause of cancer-related death worldwide. More than 660000 new cases of HCC are registered every year, and incidence in most countries appears to be increasing[1,2]. Huge HCC (⩾ 10 cm) is common in clinical practice, and hepatic resection (HR) and transarterial chemoembolization (TACE) are the generally accepted treatment options. The most appropriate treatment option for huge HCC remains controversial[3]. HR is technically difficult for treating huge HCC because extensive resection is usually required, which may be associated with high risk of mortality and poor prognosis. While TACE should provide reasonable efficacy and low procedure-related mortality based on comparisons of HR and TACE in patients with other types of HCC, studies suggest 5-year overall survival (OS) is < 10% in patients with huge HCC[4,5].

Even less clear is the most appropriate treatment for patients with a subtype of huge HCC known as solitary huge HCC. Several large case series suggest that the large tumor size does not affect prognosis, such that patients with this subtype generally have similar clinicopathological characteristics and prognosis as those with small HCC after HR[6,7]. Moreover, one large case series concluded that HR should be more effective than TACE as initial treatment for huge HCC[3]. The clinical reality is unknown, since we are unaware of direct comparisons of HR and TACE in patients with solitary huge HCC, and few studies have even looked at TACE in these patients.

Therefore we investigated the long-term OS of patients with solitary huge HCC who received HR or TACE. Post-treatment complications and mortality were analyzed, and independent factors associated with prognosis were identified. To reduce patient selection bias inherent in this non-randomized comparison of interventions, we performed propensity score matching to generate pairs of patients from both treatment arms.

MATERIALS AND METHODS

Patients

This retrospective analysis examined patients newly diagnosed with solitary huge HCC (⩾ 10 cm) at our hospital between April 2008 and April 2010. Patients were excluded if they showed metastasis at the time of diagnosis or had received any initial HCC treatment, such as chemotherapy, radiotherapy, supportive care, or sorafenib. Patients were also excluded if they had Child-Pugh C liver function or if medical records were incomplete, such that 5-year OS could not be determined.

HCC diagnosis was confirmed in TACE patients by needle biopsy or by analysis using two image methods [ultrasonography, computed tomography (CT), or magnetic resonance imaging (MRI)] in conjunction with serum level of α-fetoprotein (AFP) > 400 ng/mL. Needle biopsy was performed in patients with uncertain diagnosis based on imaging and AFP level.

Patients enrolled in the study were assigned to groups based on whether they were treated initially with HR or TACE. Indications for surgery were lack of ascites, hepatic encephalopathy, and hypersplenism, as well as the presence of appropriate residual liver volume, as determined by volumetric computed tomography[8,9]. Indications for TACE were lack of ascites, Child-Pugh A liver function or Child-Pugh B liver function with a score of 7, and insufficient estimated residual liver volume for HR[9]. Patients who satisfied the indications for both HR and TACE were treated with HR unless they requested TACE.

Interventions

HR was performed as described[9-11], while TACE was performed as follows. With the patient under local anesthesia, a 4F-to-5F French catheter was introduced

into the abdominal aorta *via* the superficial femoral artery using the Seldinger technique. Hepatic arterial angiography was performed using fluoroscopy to guide the catheter into the celiac and superior mesenteric artery. Then the feeding arteries, tumor, and vascular anatomy surrounding the tumor were identified. A microcatheter was introduced through the 4F-to-5F catheter into the feeding arteries. An emulsion of 5-15 mL lipiodol (Andre Guerbet, Aulnay-sous-Bois, France) and 5-fluorouracil (500 mg/m^2) with or without adriamycin (30 mg/m^2) was infused into the feeding arteries until blood flow nearly stopped[12]. Follow-up CT scanning was performed one month later to evaluate the effects of TACE. The course was repeated once every 1-2 mo for 2-6 cycles.

Follow-up

Every 2-3 mo after HR or TACE, especially during the first 2 years, patients underwent follow-up liver function testing, serum AFP determination, chest radiography and liver imaging by CT, MRI, and ultrasonography.

Outcome

OS was calculated from the day of surgery until the date of the last follow-up, and survival was calculated using the Kaplan-Meier method. Since residual viable tumor cells remained after TACE, disease-free survival (DFS) was not used as an outcome to compare the two interventions.

Propensity score matching

We used propensity score matching to reduce potential effects of patient selection bias and baseline differences in this non-randomized comparison of interventions[13]. Matching was performed using the PSM module developed by Felix Thoemmes for SPSS[9]. Propensity scores were estimated for each patient using a logistic regression model based on age, gender, tumor size, hepatitis B virus (HBV) infection status, Child-Pugh class, total bilirubin, serum AFP level, alanine aminotransferase (ALT), aspartate aminotransferase (AST), prothrombin time, albumin and platelet count. One-to-one matching without replacement was performed using a 0.1 caliper width. Then we assessed whether the two groups showed sufficient overlap in their propensity scores to ensure that propensity score matching was feasible in our cohort (data not shown). Balance in the matched cohort was assessed by calculating standardized differences, with differences of < 10% indicating good balance[14].

OS was compared between all patients and between propensity score-matched patients in the HR and TACE groups.

Statistical analysis

Results for continuous variables are expressed as mean ± SD and compared between the HR and TACE groups using the *t*-test. Results for categorical variables were compared using the chi-squared or Fisher's exact test as appropriate. Differences in OS were assessed for significance using the log-rank test. Multivariate analysis was carried out using the Cox proportional hazards model to identify independent prognostic factors. All statistical analyses were performed with SPSS 19.0 (Chicago, IL, United States) using a significance threshold of $P < 0.05$.

RESULTS

Medical records for 1218 patients newly diagnosed with HCC at our hospital between April 2008 and April 2010 were retrospectively analyzed (Figure 1). Of these patients, 245 were excluded because they had metastasis at the time of diagnosis or had received initial HCC treatment at other centers. Among the remaining 973 patients, 302 had solitary huge HCC (⩾ 10 cm). Of these patients, 38 were excluded because they had received other treatments, including chemotherapy, radiotherapy, supportive care, or sorafenib; another 17 were excluded because they had Child-Pugh C liver function or medical records were incomplete. The remaining 247 patients were assigned to either a group that received HR (n = 180) or a group that received TACE (n = 67). Patients in the TACE group received a mean of 2.04 ± 0.99 cycles of chemoembolization (range: 1-5).

The clinicopathological characteristics of the two groups were compared (Table 1). The two groups were similar for all parameters analyzed, except that the HR group contained a significantly greater proportion of HBsAg-positive patients, as well as significantly higher levels of total bilirubin and albumin. The standardized difference of most variables between the two groups was > 10%, indicating that the two groups were not well matched for most baseline characteristics.

Propensity score matching

Propensity score matching generated 61 pairs of patients, for which baseline characteristics showed no significant differences ($P > 0.05$) and for which the standardized difference was < 10% for all parameters (Table 2).

OS

Median follow-up across all patients (without propensity score matching) was 47.1 mo in the HR group and 33.4 mo in the TACE group. OS was significantly higher in the HR group at 1 year (87.4% *vs* 80.6%), 3 years (52.7% *vs* 33.4%), and 5 years (38.7% *vs* 20.8%) (P = 0.002; Figure 2).

Median follow-up among the propensity score-matched pairs was 49.7 mo in the HR group and 32.6 mo in the TACE group. OS was significantly higher in the HR group at 1 year (89.1% *vs* 76.9%), 3 years (55.4% *vs* 36.1%), and 5 years (36.4% *vs* 18.2%) (P

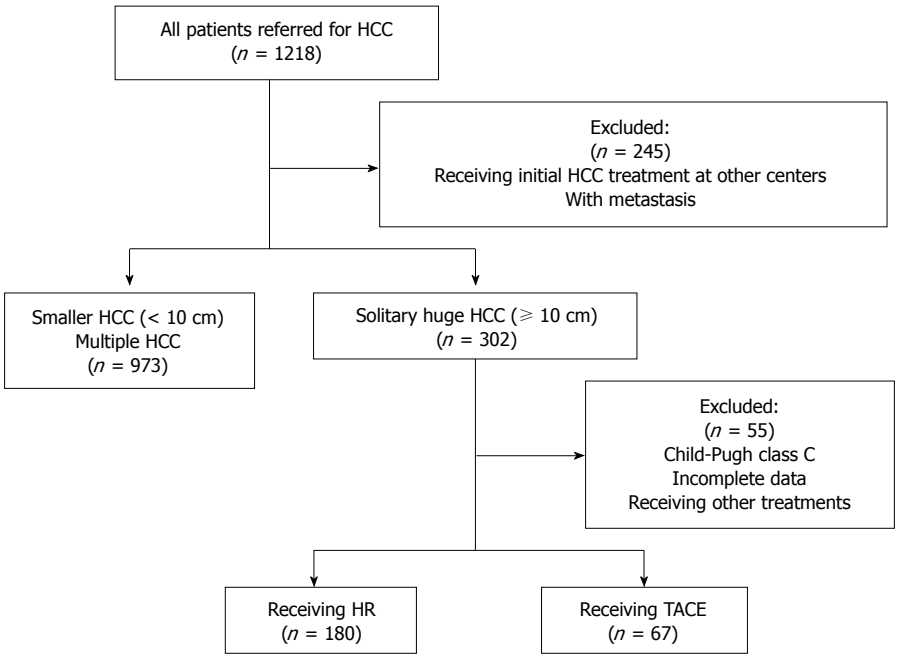

Figure 1 Flowchart of patient selection. HCC: Hepatocellular carcinoma; HR: Hepatic resection; TACE: Transarterial chemoembolization.

Table 1 Clinicopathologic features of all study participants with solitary huge hepatocellular carcinoma (⩾ 10 cm) receiving hepatic resection or transarterial chemoembolization *n* (%)

Variable	HR (*n* = 180)	TACE (*n* = 67)	Standardized difference, %	*P*-value
Age, yr	46.3 ± 11.9	48.1 ± 12.4	14.2	0.307
M/F	158 (87.8)/22 (12.2)	64 (95.5)/3 (4.5)	37.2	0.073
Tumor size, cm	11.3 ± 2.2	11.9 ± 2.2	26.7	0.059
HBsAg (+)	153 (85)	65 (97.0)	70.1	0.009
Child-Pugh class				
A	175	64	8.2	0.790
B	5	3		
Cirrhosis	133 (73.9)	57 (85.1)	31.2	0.064
AFP				
⩾ 400 ng/mL	75 (41.7)	37 (55.2)	27.1	0.057
⩽ 400 ng/mL	105 (58.3)	30 (44.8)		
Total bilirubin, μmol/L	13.4 ± 5.9	16.1 ± 8.4	37.6	0.004
ALT, U/L	50.7 ± 52.4	63.6 ± 44.5	29.1	0.074
AST, U/L	60.6 ± 40.9	56.0 ± 35.4	13.0	0.418
Prothrombin time, s	12.8 ± 1.4	13.1 ± 2.3	9.7	0.355
Albumin, g/L	39.4 ± 4.6	37.5 ± 6.5	29.0	0.012
Platelet count, 10^9/L	210.0 ± 77.6	213.4 ± 89.4	3.8	0.771
Vascular invasion	26 (14.4)	6 (9.0)	19.1	0.253

Values with ''±'' are written as mean ± SD or number (%) of patients. ALT: Alanine aminotransferase; AST: Aspartate aminotransferase; AFP: Alpha-fetoprotein; HR: Hepatic resection; TACE: Transarterial chemoembolization.

= 0.039; Figure 3).

Tumor recurrence

Among the 61 propensity score-matched patients in the HR group, recurrence occurred in 20 (32.8%), 12 of whom suffered intrahepatic recurrence, 2 extrahepatic recurrence and 6 concurrent intra- and extrahepatic recurrence. Nine of the 20 patients received additional treatment, including re-resection (*n* = 4), TACE (*n* = 3) and ablation (*n* = 2). DFS for propensity score-matched patients who received HR was 61.2% at 1 year, 27.1% at 3 years and 21.3% at 5 years (Figure 4).

Safety

Across all patients in the study, two patients in the HR group and one patient in the TACE group died within 30 d of treatment (*P* = 1.000). Mortality at 90 d was also similar in both groups (3.3% *vs* 3.0%; *P* = 1.000), as was the incidence of postoperative complications. The most common complication was hydrothorax in the HR group (3.9) and liver failure in the TACE group. Across the 61 pairs of propensity score-matched patients, the HR and TACE groups again showed

Table 2 Clinicopathologic features of propensity score-matched study participants with solitary huge hepatocellular carcinoma (≥ 10 cm) receiving hepatic resection or transarterial chemoembolization *n* (%)

Variables	HR (*n* = 61)	TACE (*n* = 61)	Standardized difference, %	*P*-value
Age (yr)	46.3 ± 11.9	48.1 ± 12.4	4.3	0.808
Gender (M/F), *n* (%)	58 (95.1)/3 (4.9)	58 (95.1)/3 (4.9)	0	1.000
Tumor size (cm)	11.9 ± 3.0	11.8 ± 2.3	2.3	0.915
HBsAg (+)	60 (98.4)	60 (98.4)	0	1.000
Child-Pugh class				
A	58	58	0	1.000
B	3	3		
Cirrhosis	52 (85.2)	51 (83.6)	4.4	0.803
AFP (ng/mL)				
⩾ 400	31 (50.8)	32 (52.5)	3.3	0.856
⩽ 400	30 (49.2)	29 (47.5)		
Total bilirubin (μmol/L)	13.6 ± 6.8	15.1 ± 8.1	8.9	0.261
ALT (U/L)	59.0 ± 56.5	60.2 ± 42.6	3.0	0.888
AST (U/L)	57.6 ± 30.3	57.4 ± 36.3	0.4	0.983
Prothrombin time (s)	12.7 ± 1.4	13.0 ± 2.3	9.7	0.504
Albumin (g/L)	37.7 ± 4.6	37.7 ± 6.6	0.5	0.972
Platelet count (10^9/L)	218.1 ± 86.9	213.0 ± 88.6	3.8	0.750
Vascular invasion	5 (8.2)	6 (9.8)	5.5	0.752

Values with "±" are written as mean ± SD. ALT: Alanine aminotransferase; AST: Aspartate aminotransferase; AFP: Alpha-fetoprotein; HR: Hepatic resection; TACE: Transarterial chemoembolization.

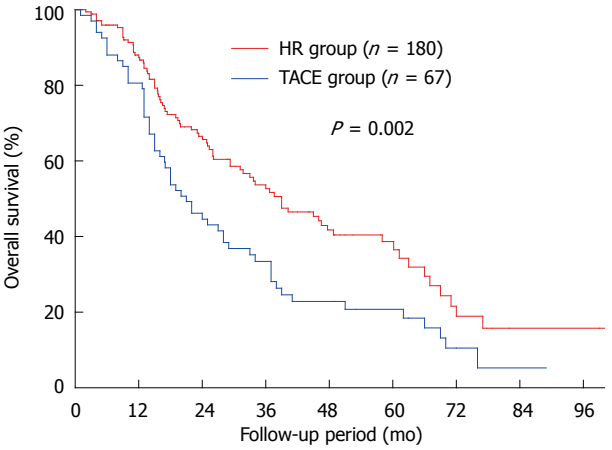

Figure 2 Comparison of overall survival across all study participants with solitary huge hepatocellular carcinoma undergoing hepatic resection or transarterial chemoembolization. HR: Hepatic resection; TACE: Transarterial chemoembolization.

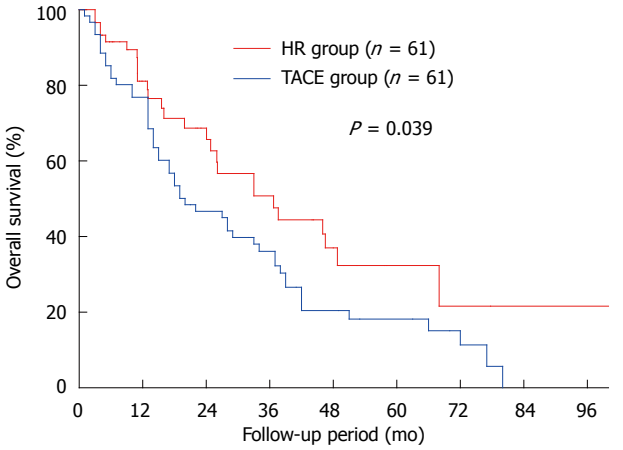

Figure 3 Comparison of overall survival across propensity score-matched study participants with solitary huge hepatocellular carcinoma undergoing hepatic resection or transarterial chemoembolization. HR: Hepatic resection; TACE: Transarterial chemoembolization.

similar mortality at 30 and 90 d (P = 1.000 for both). Liver failure was the most common complication in both groups. Specific complications in the two groups are summarized in Table 3.

Identification of prognostic factors for OS

Cox proportional hazards regression of data from the 61 pairs of propensity score-matched patients identified several predictors of OS (Table 4). Univariate analysis identified three predictors of increased risk of poor OS, all of which were confirmed by multivariate analysis: AFP ⩾ 400 ng/mL (HR = 1.997, 95%CI: 1.259 to 3.166, P = 0.003), vascular invasion (HR = 2.347, 95%CI: 1.051 to 5.242, P = 0.037) and TACE treatment (HR = 2.492, 95%CI: 1.550 to 4.006, P < 0.001).

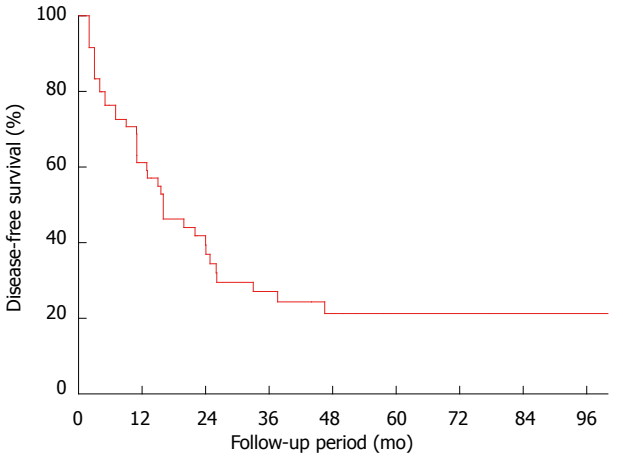

Figure 4 Disease-free survival in propensity score-matched patients with solitary huge hepatocellular carcinoma undergoing hepatic resection.

Table 3 Treatment outcomes in patients with solitary huge hepatocellular carcinoma receiving hepatic resection or transarterial chemoembolization, before and after propensity score matching *n* (%)

	Before matching			After matching		
	HR (*n* = 180)	TACE (*n* = 67)	*P*-value	HR (*n* = 61)	TACE (*n* = 61)	*P*-value
30-d mortality	2 (1.1)	1 (1.5)	1.000	1 (1.6)	1 (1.6)	1.000
90-d mortality	6 (3.3)	2 (3.0)	1.000	3 (4.9)	2 (3.3)	1.000
Postoperative complications	36 (20.0)	11 (16.4)	0.524	14 (23.0)	10 (16.4)	0.362
Liver failure	5 (2.8)	5 (7.5)	0.194	4 (6.6)	4 (6.6)	1.000
Bleeding	4 (2.2)	0 (0)	0.507	1 (1.6)	0 (0)	1.000
Wound infection	5 (2.8)	0 (0)	0.384	2 (3.3)	0 (0)	0.476
Puncture hematoma	0 (0)	3 (4.5)	0.019	0 (0)	3 (4.9)	0.242
Bile fistula	2 (1.1)	0 (0)	1.000	0 (0)	0 (0)	1.000
Pulmonary infection	6 (3.3)	3 (4.5)	0.964	2 (3.3)	3 (4.9)	1.000
Incision dehiscence	2 (1.1)	0 (0)	1.000	0 (0)	0 (0)	1.000
Abdominal infection	3 (1.7)	0 (0)	0.565	1 (1.6)	0 (0)	1.000
Hydrothorax	7 (3.9)	0 (0)	0.228	4 (6.6)	0 (0)	0.127
Intestinal obstruction	2 (1.1)	0 (0)	1.000	0 (0)	0 (0)	1.000

HR: Hepatic resection; TACE: Transarterial chemoembolization.

Table 4 Prognostic factors predicting overall survival in propensity score-matched patients with solitary huge hepatocellular carcinoma undergoing hepatic resection or transarterial chemoembolization

Variable	Univariate analysis			Multivariate analysis		
	HR	95%CI	*P*-value	HR	95%CI	*P*-value
Age (yr)	0.988	0.970-1.007	0.220			
Gender (M/F)	1.459	0.357-5.962	0.599			
Tumor size (cm)	1.054	0.973-1.141	0.197			
HBsAg (+/-)	1.391	0.340-5.685	0.646			
Child-Pugh class (A/B)	0.919	0.289-2.921	0.887			
Cirrhosis (present/absent)	1.207	0.621-1.611	0.579			
AFP (≥ 400/< 400 ng/mL)	1.721	1.097-2.347	0.018	1.997	1.259-3.166	0.003
Total bilirubin (μmol/L)	1.025	0.994-1.057	0.116			
ALT (U/L)	1.004	1.000-1.009	0.052			
AST (U/L)	1.003	0.997-1.008	0.322			
Prothrombin time (s)	1.052	0.922-1.201	0.453			
Albumin (g/L)	1.010	0.969-1.053	0.653			
Platelet count (10^9/L)	0.998	0.995-1.001	0.190			
Vascular invasion (present/absent)	2.335	1.057-5.159	0.036	2.347	1.051-5.242	0.037
Treatment modality (TACE/hepatic resection)	2.343	1.468-3.741	< 0.001	2.492	1.550-4.006	< 0.001

ALT: Alanine aminotransferase; AST: Aspartate aminotransferase; AFP: Alpha-fetoprotein; HR: Hazard ratio; TACE: Transarterial chemoembolization.

DISCUSSION

The present study provides some of the few data available on efficacy and safety of TACE in patients with solitary huge HCC, and we believe it to be the most rigorous direct comparison of HR and TACE in such patients. Our results suggest that HR is safe and effective in these patients and is associated with significantly higher long-term OS than TACE.

One previous study comparing HR and various nonsurgical therapies (hepatic arterial infusion, transcatheter arterial embolization, and percutaneous acetic acid injection) to treat patients with solitary huge HCC found that HR provided longer 5-year OS (24.5% *vs* 8.2%, $P < 0.001$)[4]. Consistently, another study reported 5-year OS in such patients to be 7% when not treated by HR[5]. The present study significantly extends that previous work because it minimizes the effects of confounding factors using propensity score matching. In the end, our key finding of longer OS with HR was obtained both across all patients and across propensity score-matched pairs.

The large tumors in solitary huge HCC are surgically challenging because of the increased bleeding, higher risk of liver failure and other complications, and higher postoperative mortality. Nevertheless, surgical techniques have improved substantially in recent years. Mortality in our propensity score-matched patients, regardless of treatment, was 1.6% at 30 d and approximately 3% at 90 d; this is at the low end of the range of 0%-6.9% reported for postoperative 30-d mortality for huge HCC[15]. In addition, both treatments in the propensity score-matched patients showed a 16%-23% rate of complications. These favorable outcomes may reflect the skill and experience of surgeons at our medical center, which annually performs more than 400 HRs on patients with HCC, as well as rigorous patient selection procedures.

As a result, liver failure, a well-established complication of HR, occurred with the same frequency (6.6%) in propensity score-matched patients treated by either procedure.

Various prognostic factors for patients with huge HCC have been reported[5-8,15-17], but those studies aggregated data for patients with solitary or multinodular huge HCC. The present study focused on propensity score-matched patients with solitary huge HCC and identified three independent predictors of poor OS: AFP ⩾ 400 ng/mL, vascular invasion and TACE treatment. Several European and Japanese reports have stressed the importance of preoperative AFP levels in prognosis, integrating them in prognostic scoring systems[18,19]. Vascular invasion has already been shown to be a risk factor for poor prognosis in HCC[3,7]. Even though our data implicate TACE as a predictor of poor prognosis, several studies, including from our own research group, have suggested that adjuvant TACE after HR can improve survival and reduce risk of recurrence[20-22]. Therefore, the present findings and previous work suggest that combining HR with adjuvant TACE may prove the most effective for treating solitary huge HCC. Future studies should examine this possibility.

Despite its insights, the present study has several important limitations. First, it was a single-center study performed in the Asia-Pacific region, where > 80% of HCC patients have chronic hepatitis B virus infection; this incidence is significantly higher than that in Western countries. Therefore our results may not be representative of all patients with solitary huge HCC. Second, the cohort in our study was enrolled between 2008 and 2010, when the chemotherapeutic agent 5-fluorouracil was routinely used. Current chemotherapeutics may be more effective and less toxic, suggesting that our results may overestimate the clinical advantage of HR over TACE. Third, our study involved relatively few patients and examined them using a non-randomized, retrospective design.

In conclusion, the present work suggests that HR may offer significantly better long-term OS than TACE to patients with solitary huge HCC, with no increase in mortality or morbidity. Large prospective studies are needed to verify and extend these findings.

COMMENTS

Background

Hepatic resection (HR) and transarterial chemoembolization (TACE) are the generally accepted treatment options for huge hepatocellular carcinoma (HCC) (⩾ 10 cm). Few studies have examined the safety and efficacy of TACE for a subtype of huge HCC known as solitary huge HCC (⩾ 10 cm), and we are unaware of direct comparisons of HR and TACE for such patients.

Research frontiers

This study provides the first direct comparison of HR and TACE in patients with solitary huge HCC, and it provides the most rigorous data so far on the safety and efficacy of TACE in such patients. In addition, the authors use propensity score matching to reduce the potential bias in our non-randomized comparison of interventions.

Innovations and breakthroughs

This study provides the first direct evidence that, after controlling for confounders, HR provides better long-term overall survival than TACE for solitary huge HCC.

Applications

This study may help guide clinicians in choosing the optimal treatment for their patients with solitary huge HCC. It also lays the groundwork for future research, particularly large, prospective studies comparing HR and TACE in patients inside and outside the Asia-Pacific region, where chronic hepatitis B infection often co-occurs with HCC.

Terminology

HR and TACE are the generally accepted treatment options for huge HCC (⩾ 10 cm). Solitary huge HCC (⩾ 10 cm) was reported a specific subtype of huge HCC which has clinicopathological characteristics and prognosis similar to that of small HCC after HR. No direct comparative study of the treatment outcomes of HR and TACE in solitary huge HCC patients has been performed to date.

Peer-review

This article includes important data. The authors collected a consecutive series of 247 huge HCCs. Among them 67 HCCs received TACE and the other 180 HCCs received HR. Sixty-one pairs of matched patients were selected from each treatment arm by conducting propensity score matching. They found that survival rate was better in the HR group than in the TACE group.

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ORIGINAL ARTICLE

Observational Study

Liver transplantation for biliary atresia: A single-center study from mainland China

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Abstract

AIM: To summarize our single-center experience with liver transplantation (LT) for biliary atresia (BA).

METHODS: From October 2006 to December 2012, 188 children with BA were analyzed retrospectively. The stage Ⅰ group (from October 2006 to December 2010) comprised the first 74 patients, and the stage Ⅱ group (from January 2011 to December 2012) comprised the remaining 114 patients. Finally, 123 liver transplants were performed in 122 (64.9%) patients, whereas 66 patients did not undergo LT due to denial by their parents or lack of suitable liver grafts. The selection of graft types depended on the patients' clinical status and whether a suitable living donor was available. The characteristics of patients in stages Ⅰ and Ⅱ were described, and the surgical outcomes of LT recipients were compared between the two stages. The Kaplan-Meier method was used to estimate the cumulative patient and graft survival rates, and the equality of survival distributions was evaluated using the log-rank test.

RESULTS: The 188 children consisted of 102 boys

and 86 girls. Their ages ranged from 3 to 144 mo with a median of 8 mo. One hundred and fifteen (61.2%) patients were born in rural areas. Comparing stage Ⅰ and stage Ⅱ patients, the proportion of patients referred by pediatricians (43.2% *vs* 71.1%, respectively; $P < 0.001$) and the proportion of patients who previously received a Kasai procedure (KP) (32.4% *vs* 44.7%, respectively; $P = 0.092$) obviously increased, and significantly more parents were willing to treat their children with LT (73% *vs* 86%, respectively; $P = 0.027$). Grafts from living donors (102/122, 83.6%) were the most commonly used graft type. Surgical complications (16/25, 64.0%) were the main reason for posttransplant mortality. Among the living donor liver transplantation recipients ($n = 102$), the incidence of surgical complications was significantly reduced (34.1% *vs* 15.5%, respectively; $P = 0.029$) and survival rates of patients and grafts were greatly improved (81.8% *vs* 89.7%, respectively, at 1 year; 75.0% *vs* 87.8%, respectively, at 3 years; $P = 0.107$) from stage Ⅰ to stage Ⅱ.

CONCLUSION: The status of surgical treatments for BA has been changing in mainland China. Favorable midterm outcomes after LT were achieved as centers gained greater technical experience.

Key words: Biliary atresia; Liver transplantation; Kasai; Living donor; Pediatric; Survival

Core tip: Biliary atresia (BA) accounts for at least 50% of the liver transplants performed in pediatric patients. However, in mainland China, various social, cultural and financial factors are responsible for a low diagnostic rate or a delayed Kasai procedure for children with BA. Pediatric liver transplantation has been progressing immensely in mainland China. In this study, we analyzed our single-center data of children with BA between 2006 and 2012, representing the largest series of BA patients in mainland China ever reported. Based on these data, socioeconomic backgrounds that impact the current status of surgical treatments for BA in mainland China were introduced.

Li QG, Wan P, Zhang JJ, Chen QM, Chen XS, Han LZ, Xia Q. Liver transplantation for biliary atresia: A single-center study from mainland China. *World J Gastroenterol* 2015; 21(32): 9638-9647 Available from: URL: http://www.wjgnet.com/1007-9327/full/v21/i32/9638.htm DOI: http://dx.doi.org/10.3748/wjg.v21.i32.9638

INTRODUCTION

Biliary atresia (BA) is the most common cause of chronic cholestasis in infants, accounting for at least 50% of the liver transplants performed in pediatric patients. BA occurs in approximately 1:5000 to 1:19000 live births[1-6]. In mainland China, large-scale epidemiological data are still not available; however, a huge population of children with BA can be presumed based on the 20 million newborns per year. Although the study by Alexopoulos *et al*[7] suggested that patients who underwent salvage liver transplantation (LT) following a Kasai procedure (KP) might have a lower survival rate than those who directly proceeded to a transplant, the sequential surgical treatment with a KP followed by LT is currently accepted to be a conventional treatment strategy for most cases[8,9]. However, in mainland China, various social, cultural and financial factors are responsible for a low diagnostic rate or a delayed KP for children with BA. Thus, many pediatric BA patients without a prior KP or with a failed KP are potential LT candidates in our country.

In recent years, pediatric LT has been progressing immensely in mainland China. However, to our knowledge, there are very few English language literature sources from mainland China concerning the diagnosis and surgical treatment of BA. It is essential to introduce these real things happening to this population and to help other peers understand these backgrounds and trends in China. Ren Ji Hospital (Shanghai) has been a pioneer for pediatric LT in mainland China since the first liver transplant for infantile patients was performed in October 2006 with the assistance of Professor Chao-Long Chen from Chang Gung Memorial Hospital (Taiwan). Here, we will retrospectively analyze our single-center data of children with BA between 2006 and 2012 and introduce the overall profile of surgical treatments for BA in mainland China.

MATERIALS AND METHODS

Study population and data collection

All pediatric transplant candidates at the Department of Liver Surgery, Ren Ji Hospital (Shanghai) have been enrolled in our prospective database under the supervision of a full-time coordinator. The data of those who underwent LT were synchronized with the China Liver Transplant Registry (CLTR) online (http://www.cltr.org/), and those who did not receive a transplant were kept in our database. The clinical characteristics and surgical data of the patients were retrospectively reviewed from the database. From October 2006 to December 2012, 220 children with BA visited our clinic. Thirty-two cases were considered ineligible due to transfers to other centers or incomplete data. The remaining 188 cases were included in the analysis of this study, most of whom were confirmed with BA through liver biopsy or intraoperative cholangiography. Stage Ⅰ (from October 2006 to December 2010) comprised the first 74 patients, while stage Ⅱ

(from January 2011 to December 2012) comprised the remaining 114 patients. The following patient information was described according to the different transplant stages: demographic characteristics, geographical location, place of birth (urban or rural), hospital class for the initial diagnosis, previous surgical history, *et al*. Finally, 123 liver transplants were performed in 122 (64.9%) patients, and the patient characteristics and follow-up results were analyzed. Moreover, the annual caseloads were calculated to describe the time trends of transplants from 2006 to 2012.

Preoperative assessment and LT criteria

Patients received a series of laboratory and imaging tests for the assessment of the clinical status, and pediatric end-stage liver disease (PELD) scores and Child-Pugh scores were calculated to measure the illness severity. Selection of graft types depended on the patients' clinical status and whether a suitable living donor was available. The suitable age for a living donor ranged from 18 to 55 years. The quality, volume and anatomy of the donor liver were carefully evaluated through computed tomography (CT) angiography, and the liver-to-spleen ratio of CT values was used to evaluate the fatty degeneration degree of the liver. If both the donor and recipient were considered to be in suitable conditions for living donor liver transplantation (LDLT), an ethical review would be arranged.

Operative techniques and immunosuppression

All of the surgical procedures were performed by specialists with experience in the pediatric LT technique at the Department of Liver Surgery, Ren Ji Hospital. For LDLT, intraoperative real-time cholangiography was indispensable and the cut-ultrasound aspiration was used in the donor operation, and then the graft was implanted into the recipient's abdominal cavity using the piggyback technique. The *ex situ* splitting technique was used for split liver transplantation (SLT), and classic orthotopic LT was performed for whole liver recipients. All patients underwent Roux-en-Y hepaticojejunostomy for bile duct reconstruction. Intraoperative color Doppler ultrasonography was performed to measure the blood flow velocity and pattern after the vascular anastomosis and abdominal wall closure. The severity of postoperative complications of the living donors was evaluated using the modified Clavien-Dindo classification[10]. Organ donation or transplantation in the study was strictly implemented under the regulation of the Shanghai Organ Transplant Committee and Declaration of Helsinki. All of the living organs were donated with informed consent. Deceased donors involved in the study were obtained from brain-dead or non-heartbeating donors. The postoperative immunosuppression regimen was described previously[11]. Briefly, the initial immunosuppressive therapy after LT consisted of a dual drug regimen of tacrolimus (0.15 mg/kg per day)/cyclosporine (8 mg/kg per day) combined with methylprednisolone (4 mg/kg per day). The target trough level for tacrolimus was 8-12 ng/mL during the first 30 d. The target C0 and C2 levels for cyclosporine were 150-200 ng/mL and 800-1200 ng/mL, respectively. The dose of methylprednisolone was gradually tapered by 4 mg per day and maintained with prednisone 2.5 mg daily taken orally. Prednisone was withdrawn within 3-6 mo after LT. Additional mycophenolate mofetil was used when necessary.

Posttransplant follow-up

After discharge of the initial hospital stay, the patients were regularly followed in the clinic weekly during the first 3 mo after LT, biweekly from the 4th to the 6th mo, monthly from the 7th to the 12th mo, and every 3 mo thereafter. The following tests were performed at each follow-up visit: measurements of the height and body weight, serum liver and renal function tests, serological viral tests (cytomegalovirus and Epstein-Barr virus) and measurements of serum levels of tacrolimus/cyclosporine. Abdominal sonography was performed every 3 mo during the first 2 years and annually thereafter. Serum tests for hepatitis B virus were performed for patients who received a hepatitis B core antibody (anti-HBc)-positive graft[11]. Because children came from various parts of the country, some children had these tests performed at the local hospitals; however, the reports were regularly sent to us by facsimile or electronic mail, and any medication adjustments were made after a consultation with physicians at our department. The duration of survival was calculated from the time of LT until death or the last follow-up contact, and the cut-off date of follow-up was April 30, 2014. The follow-up period ranged from 0.1 to 90.3 mo, with a median of 28.6 mo.

Statistical analysis

Statistical analyses were performed using SPSS 18.0 for Windows (SPSS Inc. Chicago, IL, United States). Categorical data are expressed as numbers with percentages, and continuous data are expressed as medians with a range. The survival rates of patients and grafts were plotted using Kaplan-Meier curves, and the differences in selected factors were evaluated using the log-rank test. *P*-values less than 0.05 were considered to indicate statistical significance.

RESULTS

Overall characteristics of patients

The 188 BA patients comprised 102 boys and 86 girls, with a median age of 8 mo (range: 3 to 144 mo) at the initial consultation at our hospital. Patients were distributed throughout the mainland, and most of them (45.2%) came from provinces close to Shanghai (East China) (Figure 1). One hundred and

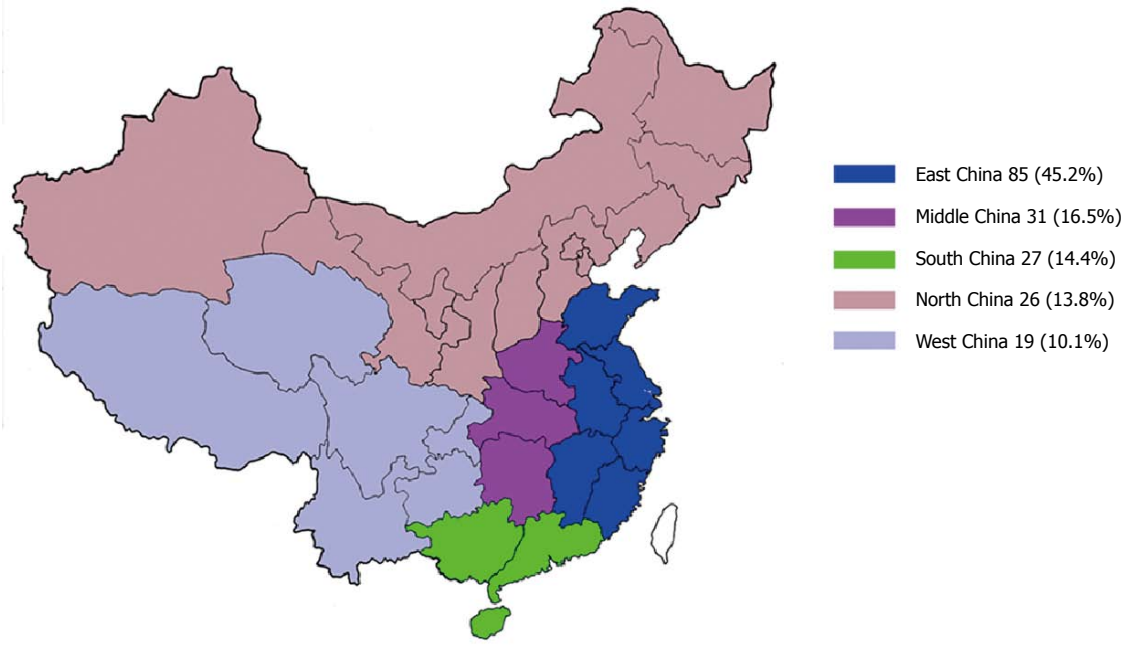

Figure 1 Geographical distribution of children with biliary atresia (*n* = 188).

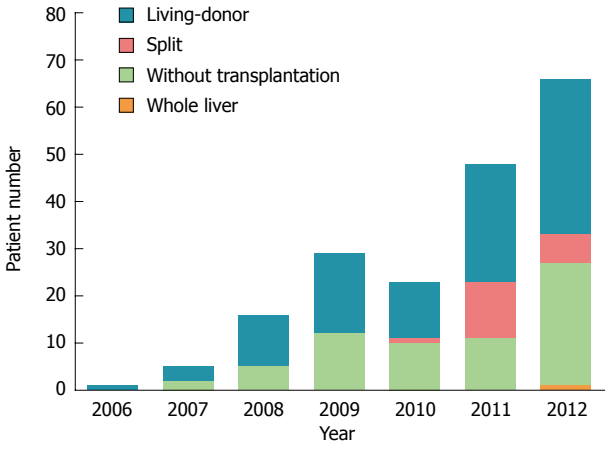

Figure 2 Caseload of children with biliary atresia from 2006 to 2012 (*n* = 188).

fifteen (61.2%) patients were born in rural areas, and 73 (38.8%) patients came from urban areas. The caseload of LT for BA showed a trend of an annual increase. During the last two years, the rapid growth of case numbers and the increase in donation types were obvious (Figure 2). The first SLT and whole liver transplantation (WLT) for BA patients were performed in December 2010 and January 2012, respectively. In this cohort, grafts from living donors were the only graft type used before 2010, and LDLT accounted for 93.2% (12 cases), 67.6% (25 cases), and 82.5% (33 cases) of liver transplants for BA in 2010, 2011, and 2012, respectively.

Previous treatment and changes between stages Ⅰ and Ⅱ

One hundred and thirty-eight (73.4%) patients were initially seen at a class Ⅰ or Ⅱ hospital, and 34.8% (48/138) of them were transferred to class Ⅲ hospitals to receive KP; on the other hand, 50 (26.6%) patients were initially treated at a class Ⅲ hospital, with 54.0% (27/50) of them directly proceeding to a KP. Thus, a total of 75 (39.9%) patients in this study had a history of a prior KP, and patients who initially visited a class Ⅲ hospital showed a significantly higher proportion undergoing KP than those initially seen at a class Ⅰ or Ⅱ hospital ($P = 0.017$). Comparing patients from rural areas with those from urban areas, urban patients were more inclined to directly go to a class Ⅲ hospital for medical support (13.9% *vs* 46.6%, respectively; $P < 0.001$), and KP was more frequently performed in urban children than in rural children (43.8% *vs* 37.4%, respectively; $P = 0.379$). Additionally, rural parents were more likely to refuse LT treatment than urban parents (62.8% *vs* 39.1%, respectively; $P = 0.066$).

The changes and trends of the patient characteristics between stages Ⅰ and Ⅱ are summarized in Table 1. In stage Ⅱ, the proportion of BA patients referred by pediatricians was significantly higher than that in stage Ⅰ (71.1% *vs* 43.2%, respectively; $P < 0.001$). Parents who refused LT treatment for their children decreased significantly from stage Ⅰ to stage Ⅱ (27.0% *vs* 14.0%, respectively; $P = 0.027$), and the lack of organ sources became the predominant cause for pretransplant deaths of patients in stage Ⅱ. Furthermore, patients who had a prior history of KP showed a slightly higher proportion in stage Ⅱ than in stage Ⅰ (44.7% *vs* 32.4%, respectively; $P = 0.092$). Figure 3 shows the flowchart providing outcomes of the 188 BA patients.

Characteristics of LT recipients and living donors

A total of 122 patients finally underwent LT, including 102 (83.6%) LDLT recipients, 19 (15.6%) SLT

Table 1 Characteristics of children with biliary atresia in stages Ⅰ and Ⅱ (*n* = 188) *n* (%)

Variable	Stage Ⅰ (*n* = 74)	Stage Ⅱ (*n* = 114)	*P*-value
Gender			0.146
Boys	45 (60.8)	57 (50.0)	
Girls	29 (39.2)	57 (50.0)	
Age			0.315
≤ 12 mo	53 (71.6)	89 (78.1)	
> 12 mo	21 (28.4)	25 (21.9)	
Place of birth			0.698
Rural	44 (59.5)	71 (62.3)	
Urban	30 (40.5)	43 (37.7)	
Hospital class for initial treatments			0.072
Ⅰ or Ⅱ	49 (66.2)	89 (78.1)	
Ⅲ	25 (33.8)	25 (21.9)	
Previous surgical intervention			
None	28 (37.9)	39 (34.2)	0.612
Laparotomy	22 (29.7)	24 (21.1)	0.176
KP	24 (32.4)	51 (44.7)	0.092
Referral for transplantation			< 0.001
Referred	32 (43.2)	81 (71.1)	
Non-referred	42 (56.8)	33 (28.9)	
Reason for no transplantation			
Refusal by the parents	20 (27.0)	16 (14.0)	0.027
Lack of a suitable graft	9 (12.2)	21 (18.4)	0.252

KP: Kasai procedure.

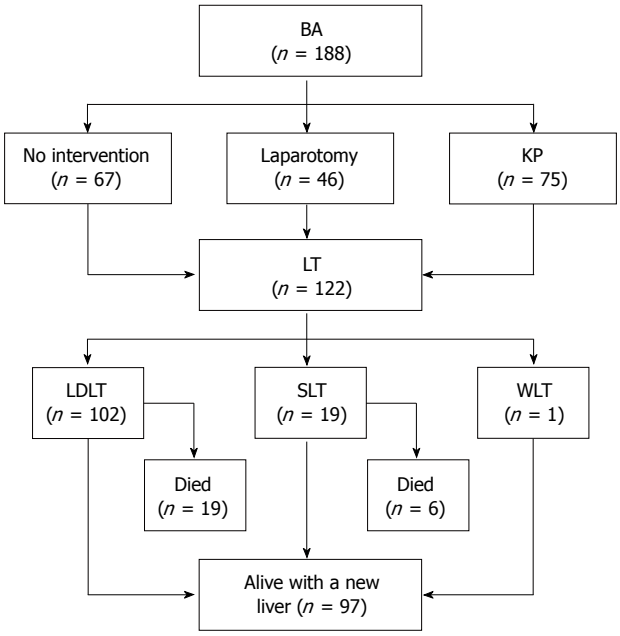

Figure 3 Flowchart providing outcomes of children with biliary atresia. BA: Biliary atresia; KP: Kasai procedure; LDLT: Living donor liver transplantation; LT: Liver transplantation; SLT: Split liver transplantation; WLT: Whole liver transplantation.

recipients and 1 (0.8%) WLT recipient. The median age when the transplant occurred was 9.4 mo (range: 4.5 to 118.4 mo). Seventy-four (60.7%) recipients did not undergo KP before LT, and their median age at the consultation at our hospital was 8.2 mo (range: 4.1 to 31.0 mo). For the 48 recipients with a prior KP (39.3%), the median age at KP was 73.5 d (range: 27 to 845 d),

Table 2 Baseline characteristics of liver transplantation recipients with biliary atresia (*n* = 122)

Variable	Living-donor (*n* = 102)	Split (*n* = 19)	Whole liver (*n* = 1)
Gender			
Boys	57 (55.9)	7 (36.8)	1
Girls	45 (44.1)	12 (63.2)	0
Age (mo)	9.3 (4.5-70.1)	10.5 (5.6-118.4)	7.8
Body weight (kg)	8.0 (5-19)	8.0 (6-28)	10.0
Height (cm)	67 (56-108)	67 (62-115)	70
PELD score	17 (-9-36)	16 (-7-36)	21
Surgical history			
KP	42 (41.2)	6 (31.6)	0
Laparotomy	29 (28.4)	6 (31.6)	0
ABO blood group			
A	34 (33.3)	4 (21.1)	0
B	27 (26.5)	4 (21.1)	0
AB	11 (10.8)	5 (26.3)	1
O	30 (29.4)	6 (31.5)	0
Graft type			
Whole liver	0	0	1
LLS	99	17	0
Left lobe without MHV	2	1	0
Left lobe with MHV	1	0	0
Extended right lobe	0	1	0
GRWR (%)	2.7 (1.5-5.4)	2.7 (1.1-4.2)	4.0

The values are expressed as numbers (%) or medians (range). GRWR: Graft-to-recipient body weight ratio; KP: Kasai procedure; LLS: Left lateral segment; MHV: Middle hepatic vein; PELD: Pediatric end-stage liver disease.

and only 13 (10.7%) patients underwent KP within 60 d of age. The median ages at LT for patients with a KP before 60 d (*n* = 13), patients with a KP after 60 d (*n* = 35) and patients without a prior KP (*n* = 74) were 20.4 mo (range: 6.3 to 118.4 mo), 14.2 mo (range: 5.7 to 82.0 mo), and 8.5 mo (range: 4.5 to 31.1 mo), respectively ($P < 0.001$).

The baseline characteristics of the recipients and grafts are shown in Table 2. In the SLT group, 17 infants shared 17 whole livers with adult recipients, and a 920-g whole liver was shared by a 6-year-old girl and a 2-year-old boy. The characteristics and outcomes of the 102 living donors are summarized in Table 3. No deaths or major complications occurred in the living donors after surgery, but 4 (3.9%) donors experienced minor complications, including wound infections and pulmonary infections.

Postoperative complications after LT

The management and outcomes of the posttransplant complications are listed in Table 4. By the last follow-up contact, 12 (26.7%) stage Ⅰ recipients and 13 (16.9%) stage Ⅱ recipients died of postoperative complications, and 1 stage Ⅰ patient underwent retransplantation 61 mo after the primary LT due to a severe biliary complication. Among them, 16 (16/25, 64.0%) deaths were caused by surgical complications. Therefore, surgical complications were the main reason for posttransplant mortality, but the incidence of surgical complications was greatly reduced with

Table 3 Characteristics of living donors (n = 102)

Variable	
Age (yr)	30 (20-56)
Gender	
Male	43 (42.2)
Female	59 (57.8)
BMI (kg/m^2)	21.4 (16.9-27.5)
D/R ABO compatibility	
Identical	78 (76.5)
Compatible	24 (23.5)
D/R relationship	
Parent	94 (92.1)
Grandparent	6 (5.9)
Uncle/Aunt	2 (2.0)
Graft weight (g)	247.5 (145-420)
Postoperative hospital stay (d)	7 (4-19)
Postoperative complications	
Wound infection (grade Ⅰ)	2
Pulmonary infection (grade Ⅱ)	2

The values are expressed as numbers (%) or medians (range). BMI: Body mass index; D/R: Donor/recipient.

greater technical experience. In this cohort, 15 (33.3%) stage Ⅰ patients and 14 (18.2%) stage Ⅱ patients experienced one or more surgical complications (P = 0.058). Furthermore, the incidence of surgical complications was significantly decreased from 34.1% (15/44) in stage Ⅰ to 15.5% (9/58) in stage Ⅱ within the LDLT group (n = 102; P = 0.029). Regarding non-surgical complications, stage Ⅱ recipients also had greatly improved outcomes compared with stage Ⅰ recipients.

Posttransplant survival

The 1-, 3-, and 5-year patient and graft survival rates of the 122 LT recipients were 83.6%, 80.0%, and 76.9%, respectively. Although the difference between the survival rates after LDLT and SLT did not reach statistical significance (P = 0.133), LDLT conferred a 14.4% survival benefit in the 3-year survival rate compared with SLT (82.1% *vs* 67.7%); thus, the LDLT recipients were expected to achieve a more favorable prognosis than those who underwent SLT (Figure 4A). The survival rates of patients who proceeded directly to LT (n = 74) were comparable to those with a prior KP (n = 48) (Figure 4B; 82.4% *vs* 85.4% at 1 year, respectively; 80.8% *vs* 71.2% at 5 years, respectively; P = 0.701). Because most cases of SLT (18 of 19) were in stage Ⅱ, the survival benefit from stage Ⅱ was impaired (Figure 4C; P = 0.358). However, the patient and graft survival rates after LDLT were greatly improved from stage Ⅰ to stage Ⅱ because our center gained greater experience with LDLT (Figure 4D; 81.8% *vs* 89.7% at 1 year, respectively; 75.0% *vs* 87.8% at 3 years, respectively; P = 0.107).

DISCUSSION

China is a vast country consisting of 28 provinces and 4 municipalities. There are large discrepancies in the socioeconomic development between coastal areas in the east and inland areas in the west. Presently, more than half of the Chinese population live in rural areas[12]. On the other hand, pediatric congenital diseases occur much more commonly in rural populations because neonatal screening for congenital diseases is not conducted in most rural areas[13-15]. Medical services provided by the hospitals in different areas are unequal, and well-equipped healthcare facilities are usually not available in rural areas. Moreover, the Chinese household registration system (known as "huji") officially identifies a person as a resident of a certain area, and residents from different areas are enrolled in different medical insurance coverage, which depends on the financial condition of the local area. For most rural families, the parents are financially responsible for their children's medical expenses. When facing a high medical expense, very few of these parents can afford the treatment cost in the hospital. As a result, a large proportion of children with BA could not get timely diagnoses and surgical interventions when necessary medical care is required. In mainland China, at least 1500 new cases of BA occur every year as calculated by the recognized incidence of BA. However, the recognition rate of BA is less than 50%, and most children with BA in mainland China die without any surgical interventions. These factors have led to a low rate of BA diagnoses, a low rate of the KP performance and a low rate of post-KP jaundice clearance.

Currently, Kasai's portoenterostomy has gained worldwide acceptance as the initial surgical therapy for BA infants[16]. However, it was reported that only 17% of BA patients who were treated with KP could achieve long-term transplant-free survival, and even these patients require assiduous lifelong care[17]. Therefore, KP is considered a transitional treatment for BA before LT because the transplant operation is not well-tolerated for most infants aged less than 6 mo. Data from the Netherlands Study Group of Biliary Atresia and Registry (NeSBAR) indicated that KP should be performed before 60 d of age to obtain an acceptable transplant-free survival[18], and a late referral for KP was associated with poor outcomes. However, in mainland China, specialized children's hospitals that are qualified to perform KP are available in only several well-developed cities such as Beijing, Shanghai, Guangzhou, Hangzhou, and Chongqing. Delayed referral for a KP produced a phenomenon that BA patients in mainland China had their transplantations fairly early. Our data showed that only 10.7% of children with BA were treated with KP before 60 d of age and that 60.7% of children had their liver functions irreversibly deteriorated and lost the chance to receive a KP before LT.

In developed countries and regions, the pre-transplant conditions of BA patients are completely different, and more than 80% of LT recipients had a prior KP before LT. Nonetheless, the patient age at

Table 4 Postoperative complications after liver transplantation

Complications	Patient number[1]		Managements	Outcomes[2]
	Stage Ⅰ (*n* = 45)	Stage Ⅱ (*n* = 77)		
Surgical complications				
HAT	4	2	DSA (3 pts); thrombectomy and reconstruction (6 pts)	3 pts died
PVT	5	5	Reoperation (5 pts); metal stent placement (1 pt)	7 pts died
Biliary leakage	3	2	Drainage or reoperation	3 pts died
Biliary stricture	2	1	PTCD (1 pt)	2 pts died
Biliary sludge	1	0	Retransplantation	Alive
Wound dehiscence	3	1	Debridement and re-closure	2 pts died
Digestive tract perforation	2	2	Reoperation	4 pts died
Intra-abdominal bleeding	0	1	Reoperation	Alive
Wound infection	1	0	Regular wound dressing	1 pt died
Small-for-size syndrome	0	1	-	1 pt died
Large-for-size syndrome	0	1	-	1 pt died
Non-surgical complications				
Pulmonary infection	14	16	Antibiotics (30 pts); mechanical ventilation (5 pts)	11 pts died
CMV infection	17	13	Antivirus therapy	7 pts died
EBV infection	3	10	Antivirus therapy	1 pt died
De novo HBV infection	7	6	Antivirus therapy	2 pts died
Drug-induced liver injury	0	1	Withdrawal of the drug	1 pt died
Tuberculous pleurisy	1	0	Anti-tuberculosis	Alive
Acute rejection	15	13	Increased the dosage of the immunosuppressant; bolus doses of steroids	5 pts died
PTLD	0	1	-	1 pt died
Hirsutism	7	0	Replacement of cyclosporine with tacrolimus	1 pt died
Intravascular hemolysis	1	1	Steroid therapy; withdrawal of blood transfusion	1 pt died

[1]Multiple complications might occur in a single patient; [2]Patients who experienced the complication but died from other reasons before the last follow-up contact were also included. CMV: Cytomegalovirus; DSA: Digital subtraction angiography; EBV: Epstein-Barr virus; HAT: Hepatic artery thrombosis; HBV: Hepatitis B virus; pt: Patient; PTCD: Percutaneous transhepatic cholangial drainage; PTLD: Posttransplant lymphoproliferative disease; pts: Patients; PVT: Portal venous thrombosis.

KP is considered the key determinant for the post-KP patient survival with their native liver[9,19-21]. In Taiwan, a universal stool color screening system was established for the neonatal population since 2004, which has greatly reduced the proportion of late referrals for infants with BA[22-24]. The success rate of KP could be improved by enhancing the early referral, and better postoperative outcomes of children with BA could be obtained by the timely performance of KP[25]. In the United Kingdom, surgical outcomes have been improved by the centralization of care to supra-regional centers[26]. Moreover, a French study reported that the caseload experience of KP influenced the patient prognosis with centers managing more than 20 cases per year associated with better outcomes[27].

In mainland China, the development of pediatric LT has lagged behind that of adult LT during the past two decades[28]. Pediatric transplants are performed at large transplant centers which mainly engage in adult transplantation, and most children's hospitals are not authorized to perform LT. There is little communication between pediatricians and transplant surgeons. Additionally, most families prefer to bear another child rather than choose transplantation when they are confronted with the high cost and the "one child policy". However, recent changes in these situations are encouraging and gratifying with increasingly more attention from society being paid to this group of patients. Pediatric LT in our country has undergone immense progress in recent years. Our hospital is currently the largest transplant center for pediatric LT in mainland China. We work in close collaboration with Shanghai Children's Medical Center to enable children to maximize the benefit gained from surgery. In this study, the annual caseload was hugely increased, the postoperative outcomes were greatly improved in stage Ⅱ patients, particularly for LDLT recipients, and the 3-year patient and graft survival rates after LDLT reached 87.8%, which was comparable to those of developed countries[29,30]. This progress was mainly attributed to the following factors: (1) our results in stage Ⅰ enhanced the understanding of transplantation by parents and pediatricians and promoted their willingness for referral or acceptance; (2) some charitable organizations voluntarily provided financial support during stage Ⅱ; (3) grafts from deceased donors have been used since December 2010 to expand the donor pool for recipients without a suitable living donor; and (4) improvements in surgical techniques and posttransplant management with feedback on the long-term outcomes significantly decreased the incidence of posttransplant complications.

Although the shortage of deceased donors is a universal problem, the situation is particularly serious in Asia for various social, cultural, and historical reasons. Thus, the living donor was the only graft type available for most recipients in this study, and grafts from cadaveric organ donations were mostly used

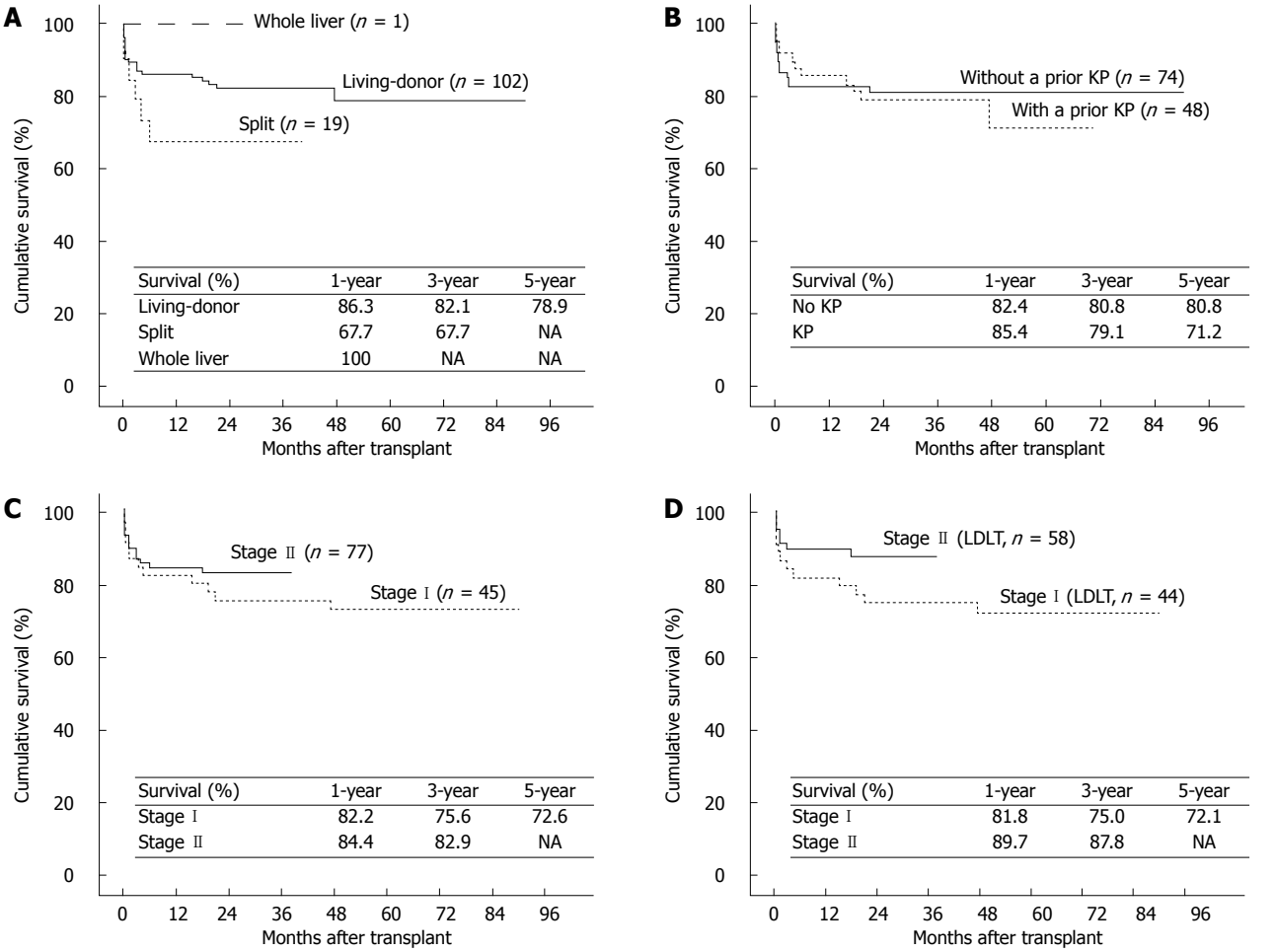

Figure 4 Patient and graft survival after liver transplantation for biliary atresia (*n* = 122). A: Comparison between patients using different donor types (P = 0.286); B: Comparison between patients with or without a prior Kasai procedure (KP) (P = 0.701); C: Comparison between patients in stages I and II (P = 0.358); D: Comparison between the two stages within the living donor liver transplantation (LDLT) group (P = 0.107). NA: Not available.

by patients without a suitable living donor. However, the LDLT recipients would have priority in acquiring financial support. It was shown in our previous work that the LDLT benefit was magnified with respect to hospital mortality, postoperative hospitalization rates, and midterm survival as centers gained greater surgical experience[31]. Thus, the postoperative outcomes in stage Ⅰ were relatively unfavorable due to the effect of the learning curve. Specifically, the retransplantation rate is extremely low in mainland China, which is also influenced by the aforementioned socioeconomic factors, and only 1 patient underwent retransplantation in this study. This report provides a general description of surgical treatments for BA in mainland China based on our single-center experience. Conceivably, some effective steps ought to be taken in the future: (1) a nationwide BA screening system should be established; (2) medical insurance should cover all children from different areas; (3) timely referrals must be executed between junior and senior hospitals; and (4) close communication and cooperation should be promoted between pediatricians and transplant surgeons.

In conclusion, many children with BA in mainland China could not receive a timely KP due to various socioeconomic factors, but the situation has been changing. LT for BA could yield favorable outcomes through the accumulation of experience. Grafts from living donors are currently the most commonly used graft type for children with BA, and the 3-year patient and graft survival rates of 87.8% could be achieved by LDLT recipients. However, efforts should be directed to enhance the disease screening and insurance coverage for children with BA.

COMMENTS

Background

Biliary atresia (BA) is the most frequent cause of chronic cholestasis in infants and accounts for at least 50% of the liver transplants performed in pediatric patients. The sequential surgical treatment comprising the Kasai procedure followed by liver transplantation (LT) is currently accepted to be the conventional treatment strategy for most cases. However, in mainland China, various social, cultural and financial factors are responsible for a low diagnostic rate or a delayed Kasai procedure for children with BA.

Research frontiers

In mainland China, pediatric LT has been progressing immensely in recent

years. However, there are very few English language literature sources from mainland China concerning the diagnosis and surgical treatment of BA. The research hotspot is to introduce these real things happening to this population and to help other peers understand these backgrounds and trends in China.

Innovations and breakthroughs

In recent years, the status of surgical treatments for BA has been changing immensely in mainland China. The present study represents the largest series of BA patients in mainland China ever reported, showing that proportions of patients referred by pediatricians and those of patients who previously received a Kasai procedure were increasing obviously and that an increasing number of parents were willing to treat their children with LT in recent years. On the other hand, the current data also suggested that the incidence of surgical complications could be significantly reduced and the survival rates of patients and grafts could greatly improve as the transplant center gained greater technical experience.

Applications

The data in this study suggested that LT for BA could yield favorable outcomes through the accumulation of technical experience. Furthermore, this study also provided readers with important information regarding the socioeconomic obstacles to BA treatment and the substantial progress of pediatric LT in mainland China.

Terminology

BA is a birth defect in newborn infants that is characterized by extrahepatic ductopenia and progressive cholestasis. The only effective treatments for BA are the Kasai procedure and LT. The Kasai procedure, also known as hepatoportoenterostomy, is a surgical technique performed in children with BA to allow bile drainage by attaching part of the small intestine to the porta hepatis.

Peer-review

Available papers concerning pediatric LT in mainland China are scarce. The authors in this study analyzed the characteristics and outcomes of children with BA based on a large single-center series. This study showed that favorable midterm outcomes after LT could be achieved as the transplant center gained greater technical experience. The results were interesting and provided important information concerning the background and trends of surgical treatments for BA in mainland China.

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ORIGINAL ARTICLE

Prospective Study

Liver reserve function assessment by acoustic radiation force impulse imaging

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Informed consent statement: All study participants, or their legal guardian, provided informed written consent prior to study enrollment.

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Data sharing statement: No additional data are available.

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Abstract

AIM: To evaluate the utility of liver reserve function by acoustic radiation force impulse (ARFI) imaging in patients with liver tumors.

METHODS: Seventy-six patients with liver tumors were enrolled in this study. Serum biochemical indexes, such as aminotransferase (ALT), aspartate aminotransferase (AST), serum albumin (ALB), total bilirubin (T-Bil), and other indicators were observed. Liver stiffness (LS) was measured by ARFI imaging, measurements were repeated 10 times, and the average value of the results was taken as the final LS value. Indocyanine green (ICG) retention was performed, and ICG-K and ICG-R15 were recorded. Child-Pugh (CP) scores were carried out based on patient's preoperative biochemical tests and physical condition. Correlations among CP scores,

ICG-R15, ICG-K and LS values were observed and analyzed using either the Pearson correlation coefficient or the Spearman rank correlation coefficient. Kruskal-Wallis test was used to compare LS values of CP scores, and the receiver-operator characteristic (ROC) curve was used to analyze liver reserve function assessment accuracy.

RESULTS: LS in the ICG-R15 10%-20% group was significantly higher than in the ICG-R15 < 10% group; and the difference was statistically significant (2.19 ± 0.27 *vs* 1.59 ± 0.32, $P < 0.01$). LS in the ICG-R15 > 20% group was significantly higher than in the ICG-R15 < 10% group; and the difference was statistically significant (2.92 ± 0.29 *vs* 1.59 ± 0.32, $P < 0.01$). The LS value in patients with CP class A was lower than in patients with CP class B (1.57 ± 0.34 *vs* 1.86 ± 0.27, $P < 0.05$), while the LS value in patients with CP class B was lower than in patients with CP class C (1.86 ± 0.27 *vs* 2.47 ± 0.33, $P < 0.01$). LS was positively correlated with ICG-R15 ($r = 0.617$, $P < 0.01$) and CP score ($r = 0.772$, $P < 0.01$). Meanwhile, LS was negatively correlated with ICG-K ($r = -0.673$, $P < 0.01$). AST, ALT and T-Bil were positively correlated with LS, while ALB was negatively correlated with LS ($P < 0.05$). The ROC curve revealed that the when the LS value was 2.34 m/s, the Youden index was at its highest point, sensitivity was 69.2% and specificity was 92.1%.

CONCLUSION: For patients with liver tumors, ARFI imaging is a useful tool for assessing liver reserve function.

Key words: Acoustic radiation force impulse; Liver reserve function; Liver tumor; Receiver-operator characteristic

Core tip: Seventy-six patients with liver tumors were assessed by acoustic radiation force impulse (ARFI) imaging. We found that liver stiffness (LS) was positively correlated with Indocyanine green (ICG)-R15 and the Child-Pugh score, but was negatively correlated with ICG-K. Aspartate aminotransferase, aminotransferase and total bilirubin were positively correlated with LS, while albumin was negatively correlated with LS. The receiver-operator characteristic curve revealed that when LS value was 2.34 m/s, the Youden index was at its highest point. For patients with liver tumors, ARFI imaging is a useful tool to assess liver reserve function.

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INTRODUCTION

Hepatocellular carcinoma (HCC) is one of the major malignant tumors in China, and it has the third highest mortality after gastric carcinoma and lung cancer. Most HCC patients have liver fibrosis and cirrhosis[1,2]. Hepatectomy is the preferred treatment for early HCC. However, the presence of cirrhosis, results in different degrees of liver injury and low liver reserve function. Poor prognosis, such as postoperative liver failure, usually occurs[3,4]. Therefore, a good liver reserve function is a key factor for a successful surgery, and preoperative liver reserve function assessment is very important[5]. The indocyanine green (ICG) excretive test[6] and Child-Pugh (CP) scores are the main methods used to assess liver reserve function in clinical practice. However, both methods have different limitations and accuracies. In recent years, because of the development of ultrasound elastography in liver fibrosis and cirrhosis in the clinic, acoustic radiation force impulse (ARFI) imaging has become an innovative ultrasound technique that has received increasing attention[7-12]. Studies have reported[13,14] that ARFI imaging could be used for the qualitative detection of liver tumors, and that it could also be used to determine the degree of liver fibrosis by detecting liver stiffness (LS). Hence, it is hoped that this technique could evaluate liver reserve function accurately. Furthermore, ARFI imaging technology is non-invasive, simple and repeatable[15,16]. However, there have been few reports on liver reserve function assessment using ARFI imaging. Therefore, this study aimed to explore the clinical value of ARFI imaging by determining its value in assessing liver reserve function in patients with liver tumors, and providing theoretical guidance for clinical treatment.

MATERIALS AND METHODS

General information

Seventy-six patients with liver tumors combined with liver fibrosis or cirrhosis, who were admitted from October 2012 to May 2014, were enrolled into this study. All patients planned to receive hepatectomy. Among them, 42 patients were male and 34 patients were female. The patient age range was 33-75 years old, and the average age was 56.71 ± 9.32. Inclusion criteria: single tumor with a tumor diameter < 7 cm. There was neither intrahepatic metastasis, nor portal vein thrombosis or ascites by imaging examination. All patients were informed of the study and agreed to participate.

Test methods

Instruments and reagents: Instruments: (1) Siemens ACUSON S2000™ ultrasound system[17,18]; and (2) DDG-3300K liver reserve function analyzer (Nihon Kohden, Tokyo, Japan)[19-22]. Reagent: Indocyanine green (ICG) reagent (Shenyang Jishi Pharmaceutical Co., Ltd.).

Detection methods: (1) Conventional serum-based liver function tests were performed including alanine aminotransferase (ALT), aspartate aminotransferase (AST), serum albumin (ALB), total bilirubin (T-Bil) and other indicators. All data were recorded; and (2) ARFI imaging detection.

Patients underwent fasting and were kept in a left lateral position while raising the right hand. Liver right lobe tissues were detected. Patients were instructed to hold their breath. The elasticity imaging sample frame was perpendicular to the liver surface and was located 2-5 cm below the surface, avoiding nearby blood vessels. The update button was pressed to launch a high-strength low-frequency pulse. Transversal shear wave velocity (Vs, m/s) was received and recorded.

Measurements were repeated 10 times, and the average value of the Vs results was used as the LS value.

ICG examination: Patients were kept in a supine position. The environment was kept quiet and other factors (such as cell phones, contrast, *etc*.) were excluded. (1) Body weight (kg) was measured and the amount of ICG (0.5 mg/kg) was calculated; (2) an indwelling needle was injected and placed through the median cubital vein on one side to collect 2 mL of blood and determine the hemoglobin (Hb) content; (3) preparation of ICG solution, sterile water for injection/ ICG = 5 mL: 25 mg; (4) data such as Hb, weight and ICG dosage were analyzed by a DDG-3300K analyzer; (5) a sensitive probe was fixed to the patient's nose, and the entire ICG solution was injected to the median cubital vein *via* the indwelling needle in 10 s; (6) data were saved, and ICG-k and ICG-R15 were calculated; and (7) Patients were kept in a quiet environment with normal respiration at all times.

CP score: CP scores were carried out based on patient's preoperative biochemical tests and physical condition. CP scoring criteria are shown in Table 1.

Research index

Biochemical index such as ALT, AST, ALB and T-Bil were observed. The correlations among CP scores, ICG-R15, ICG-K and LS values were observed and analyzed. A receiver-operator characteristic (ROC) curve was used to analyze LS indicators and assess the best diagnostic threshold of hepatic functional reserve.

Statistical analysis

All data were analyzed using SPSS 19.0 software. Data were expressed as the mean ± SD. To test the relationship between two variables, the Pearson correlation coefficient was used to analyze two variables if the data showed a normal distribution; while the Spearman rank correlation coefficient was used to analyze two variables if the data showed a non-normal distribution. Kruskal-Wallis test was used to compare LS values of CP scores. LS values of non-surgical patients were expressed by sensitivity, specificity and the Youden index. A ROC curve was used to analyze liver reserve function assessment accuracy. $P < 0.05$ means that difference was statistically significant.

RESULTS

Biochemical examination results

Seventy-six patients with liver tumors were successfully enrolled and assessed in this study. Serum biochemical indexes such as ALT, AST, γ-glutamyl transferase (γ-GT), ALB, T-Bil and prothrombin time (PT) are shown in Table 2.

LS and ICG test results

The LS values of all subjects were successfully obtained. Patients with different liver reserve functions revealed different Vs values. Representative examples are shown in Figures 1 and 2. The patient in Figure 1 had a normal liver reserve function. The Vs value was 1.07 m/s, ICG-R15 was 5.13%, and ICG-K was 0.24 L/min. The patient in Figure 2 had poor liver reserve function. The Vs value was 1.75 m/s, ICG-R15 was 16.83%, and ICG-K was 0.08 L/min.

Data from all subjects revealed that the average value of ICG-R15 was 8.13% ± 3.72% and the ICG-K average value was 0.24 ± 0.29 L/min. ICG-R15 values were divided into three groups: ICG-R15 < 10%, ICG-R15 10%-20% and ICG-R15 > 20% groups. The average LS value of the three groups is shown in Table 3. LS significantly differed among patients in the three groups ($H = 25.89$, $P = 0.000$). Kruskal-Wallis H test was further carried out. LS was significantly higher in the ICG-R15 10%-20% group than in the ICG-R15 < 10% group, and the difference was statistically significant ($P < 0.01$). LS was significantly higher in the ICG-R15 > 20% group than in the ICG-R15 < 10% group, and the difference was statistically significant ($P < 0.01$). LS was higher in the ICG-R15 >20% group than in the ICG-R15 10%-20% group, but the difference was not statistically significant ($P > 0.05$).

CP score results

Results of different average LS, ICG-R15 and ICG-K values according to different CP scores are shown in Table 4. The average LS values were significantly different among patients with CP class A, B and C; and the difference was statistically significant ($H = 33.84$, $P = 0.0000$). Pairwise comparison results of LS among patients with CP class A, B and C showed that the LS value in patients with CP class A was lower than in patients with CP Class B; and the difference was statistically significant ($P < 0.05$). The LS value was lower in patients with CP class A than in patients with CP Class C, while the LS value was lower in patients

Table 1 Child-Pugh scoring criteria

Indexes	Score		
	1 score	2 score	3 score
Hepatic encephalopathy (class)	Without	Mild	Occasional lethargy
Ascites	Without	Little/controllable by diuretics	A lot
T-Bil (μmol/L)	< 34	34-51	> 51
ALB (g/L)	> 35	28-35	< 28
Prolonged prothrombin time (s)	< 4	4-6	> 6

Child-Pugh class A: 5-6 score; Child-Pugh class B: 7-9 score; Child-Pugh class C: ⩾ 10 score. T-Bil: Total bilirubin; ALB: Albumin.

Table 2 Biochemical examination results of patients

Indexes	mean ± SD	Media, *n*	Minimum value	Maximum value
ALT (U/L)	58.77 ± 51.13	57.81	13.74	127.28
AST (U/L)	64.21 ± 60.33	60.03	15.62	153.10
ALB (g/L)	38.19 ± 10.14	37.31	10.01	67.43
T-Bil (μmol/L)	26.28 ± 20.61	22.33	4.72	126.74

ALT: Aminotransferase; AST: Aspartate aminotransferase; ALB: Serum albumin; T-Bil: Total bilirubin.

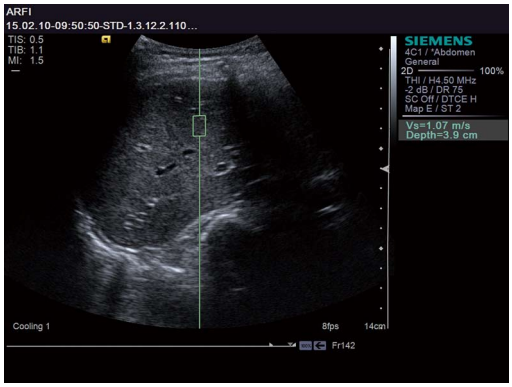

Figure 1 Patient with normal liver reserve function. The Vs value was 1.07 m/s, ICG-R15 was 5.13% and ICG-K was 0.24 L/min. ICG: Indocyanine green.

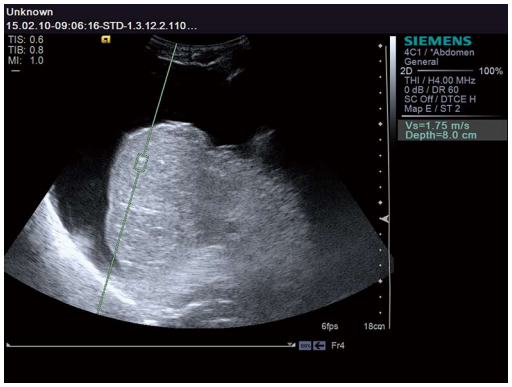

Figure 2 Patient with poor liver reserve function. The Vs value was 1.75 m/s, ICG-R15 was 16.83% and ICG-K was 0.08 L/min. ICG: Indocyanine green.

with CP class B than in patients with CP class C; and the differences were statistically significant ($P < 0.01$).

Correlation analysis of LS and serum biochemical tests, ICG test and CP scores

LS was positively correlated with ICG-R15 ($r = 0.862$, $P < 0.01$; Figure 3A) and CP scores ($r = 0.772$, $P < 0.05$). Meanwhile, LS was negatively correlated with ICG-K ($r = -0.708$, $P < 0.05$; Figure 3B). AST, ALT and T-Bil were positively correlated with LS, while ALB was negatively correlated with LS ($P < 0.05$) (Table 5, Figure 3).

Distribution of disease in patients with liver lesions, and LS comparison between surgical and non-surgical patients

After liver reserve function assessment, 13 patients with poor liver reserve function received radiofrequency ablation (RFA) or interventional embolization, while 63 patients with normal liver reserve function received hepatectomy. The average LS of non-surgical patients was 2.61 ± 0.44 m/s, while the average LS of surgical patients was 1.57 ± 0.35 m/s. The average LS of the surgical patients was lower compared with non-surgical patients, and the difference was statistically significant ($P < 0.01$). The ROC curve is shown in Figure 4. When the LS value was 2.34 m/s, the Youden index was at its highest point, sensitivity was 69.2% and specificity was 92.1%.

DISCUSSION

Liver reserve function assessment is receiving increasingly attention from hepatobiliary surgeons. Preoperative evaluation of liver reserve function is very important for selecting a reasonable surgical method and a reasonable liver resection range, leading to a successful surgery and preventing postoperative hepatic failure[23]. CP scores are the main methods used to assess liver reserve function

Table 3 Average values of liver stiffness, indocyanine green-R15 and indocyanine green-K in each group, based on indocyanine green-R15 grouping

Groups	Number of cases	LS average value (m/s)	ICG-R15 (%)	ICG-K (L/min)
< 10%	57	1.59 ± 0.32	8.99 ± 2.15	0.19 ± 0.04
10%-20%	16	2.19 ± 0.27[a]	13.54 ± 2.12	0.16 ± 0.05
> 20%	3	2.53 ± 0.29[a]	17.32 ± 0.73	0.11 ± 0.07

[a]$P < 0.05$, *vs* the < 10% group. LS: Liver stiffness; ICG: Indocyanine green.

Table 4 Different average liver stiffness, indocyanine green-R15 and indocyanine green-K values according to different Child-Pugh scores

CP class	Cases	LS average value (m/s)	ICG-R15 (%)	ICG-K (L/min)
Class A (5-6)	48	1.57 ± 0.34	8.90 ± 3.67	0.23 ± 0.04
Class B (7-9)	15	1.86 ± 0.27[a]	11.38 ± 2.71	0.19 ± 0.05
Class C (10-15)	13	2.47 ± 0.33[a,c]	14.44 ± 3.58	0.14 ± 0.07

[a]$P < 0.05$, *vs* class A; [c]$P < 0.05$, *vs* the class B. CP: Child-Pugh; LS: Liver stiffness; ICG: Indocyanine green.

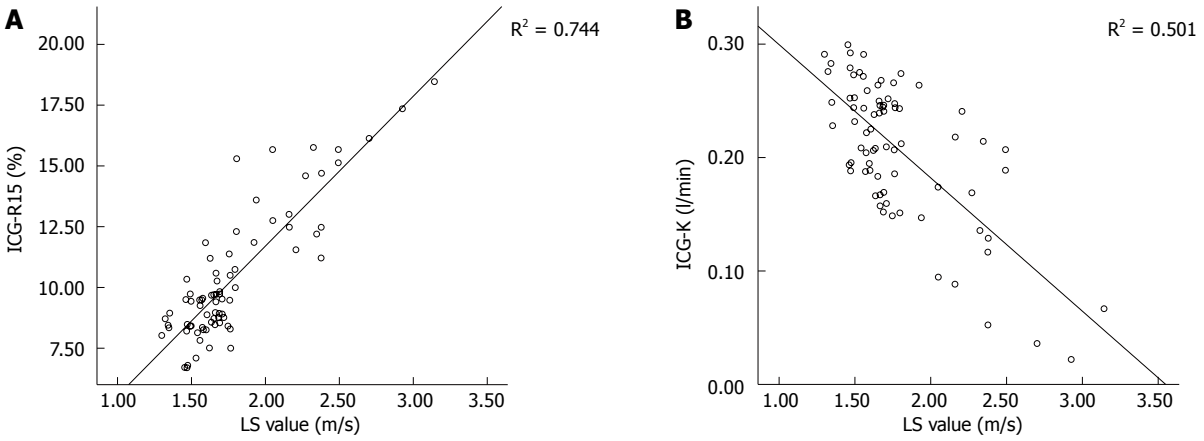

Figure 3 Correlations between liver stiffness and indocyanine green-R15 (A) or indocyanine-K (B) levels are shown. LS: Liver stiffness.

in clinical practice[24,25], and ICG excretion tests have become an important reference for surgical decision making[26]. The ICG test has been widely used to determine liver reserve function, which is an important indicator to assess surgical safety. However, ICG tests needs to be excreted through the biliary tract, which is not suitable for patients with obstructive jaundice. Hence, its application is restricted. The ARFI imaging technology applied in this study is not restricted. ARFI imaging is non-invasive, simple and repeatable, and has more extensive applications. Therefore, this study evaluated the clinical value of ARFI imaging to assess liver reserve function in patients with liver tumors, providing theoretical guidance for clinical treatment.

Correlation analysis between LS and liver reserve function index

As liver fibrosis develops, LS increases, while liver compliance and elasticity decrease[27]. Lupsor *et al*[28] reported that there was a significant correlation between LS value and liver fibrosis stage. This proved that ARFI imaging could also be used to determine the degree of liver fibrosis by detecting LS, and to correctly evaluate liver reserve function. In this study, there was a strong positive correlation between LS and ICG-R15, and a negative correlation between LS and ICG-K. ICG-R15 values were divided into three groups: ICG-R15 < 10%, ICG-R15 10%-20% and ICG-R15 > 20% groups. LS significantly differed among patients in the three groups. LS was significantly higher in the ICG-R15 10%-20% group compared with the ICG-R15 < 10% and ICG-R15 > 20% groups. LS was significantly higher in the ICG-R15 > 20% group than in the ICG-R15 < 10% group. LS was higher in the ICG-R15 > 20% group than in the ICG-R15 10%-20% group, but there was no statistical significance. This implied that the LS value increases as ICG-R15 levels increase, resulting in reduced hepatic clearance and aggravated liver damage. This also demonstrated that ARFI imaging could be used to determine the degree of liver fibrosis by detecting LS, and to evaluate liver reserve function.

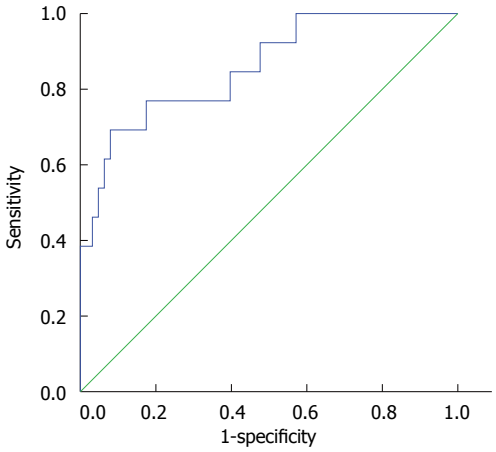

Figure 4 Receiver-operator characteristic curve used to analyze liver reserve function assessment accuracy. When the liver stiffness value was 2.34 m/s, the Youden index was at its highest point, sensitivity was 69.2% and specificity was 92.1%.

Correlation between LS values and CP scores

CP scores can reflect liver cell damage. The higher the score, the more severe the hepatic dysfunction. In this study, LS values for CP class A, B and C were 1.57 ± 0.34 m/s, 1.86 ± 0.27 m/s and 2.47 ± 0.33 m/s, respectively. Comparative results of LS values among the three CP classes revealed that: the LS value of CP class A was lower than CP class B, and the LS value of class A and B were significantly lower than CP class C. These results showed that LS values increase as CP scores increase. This outcome reflected severe liver function damage, causing LS values to increase, while the liver reserve function decreases. Therefore, this also demonstrated that LS values could reflect liver reserve function. CP scores could be used to evaluate liver reserve function based on routine liver function tests. CP scores are widely used in clinical practice because they are an easy method of detection. However, traditional biochemical indicators simply reflect the degree of liver cell damage and compensation function status; and the reserve capacity of the liver could not be accurately predicted when the body is exposed to trauma, infection, surgery and other invasions[29]. Therefore, ARFI imaging technology can supplement the CP scoring method; providing a more detailed liver reserve function assessment for predicting body endurance and permits a better choice of treatment modes.

Best diagnostic threshold of liver reserve function as assessed by LS indicators

Average LS values were lower in surgical patients compared with non-surgical patients. The ROC curve revealed that when the LS value was 2.34 m/s, the Youden index was at its highest point, sensitivity was 69.2% and specificity was 92.1%; which demonstrated that the higher the LS value, the worse the liver reserve function and prognosis after surgery. This also confirmed that LS values could react to liver reserve functions. Furthermore, an LS value greater than 2.34 m/s suggested poor liver reserve function; therefore, non-surgical treatment is recommended. Clinicians can use ARFI imaging technology to measure LS value and assess liver reserve function to determine if a patient is suitable for surgery; thereby, providing a more suitable treatment option for patients.

Table 5 Correlations between liver stiffness and liver function index

Index	*r*	*P* value
ICG-R15	0.862	0.000
ICG-K	-0.708	0.000
CP score	0.772	0.000
AST	0.318	0.024
ALT	0.493	0.001
ALB	-0.511	0.001
T-Bil	0.225	0.017

ICG: Indocyanine green; CP: Child-Pugh; ALT: Aminotransferase; AST: Aspartate aminotransferase; ALB: Serum albumin; T-Bil: Total bilirubin.

Limitations and prospects

Although ARFI imaging is expected to provide fast, simple and accurate guidance in assessing liver reserve function in clinical practice, there are also limitations for the use of ARFI imaging. We found that ARFI imaging has high requirements from the patient. It requires patients to keep holding their breath during the course of the examination, which is difficult for elderly patients or patients in poor physical condition. The ARFI sampling frame would show deviations caused by a patient's breathing problems, which would affect the accuracy of the measurement. To compensate for this limitation, we repeated the measurement 10 times to reduce errors. In addition, there were few subjects in this study. Seventy-six cases are not sufficient to demonstrate that LS > 2.34 m/s is the best demarcation point. Large multicenter studies are needed to obtain enough data for analysis and to provide convincing evidence.

In conclusion, ARFI could be used to determine liver reserve function by detecting LS. ARFI imaging has advantages, such as speed, simplicity, and non-invasiveness, and has a wide range of applications. It is important to determine a reasonable surgical method and liver resection range. ARFI imaging technology is a useful tool to assess liver reserve function in patients with liver tumors. It has an important clinical application and should be promoted.

COMMENTS

Background

Hepatocellular carcinoma (HCC) is one of the major malignant tumors in China. It has the third highest mortality after gastric carcinoma and lung cancer. Most patients with HCC have liver fibrosis and cirrhosis. Hepatectomy is the preferred treatment for early HCC. However, most HCC patients also have chronic liver diseases, such as cirrhosis, resulting in different degrees of liver

injury and low liver reserve function. Poor prognosis, such as postoperative liver failure, usually occurs. Therefore, a good liver reserve function is a key factor for a successful operation, and preoperative liver reserve function assessment is of great importance.

Research frontiers

In recent years, because of the development of ultrasound elastography in liver fibrosis and cirrhosis in clinic, acoustic radiation force impulse (ARFI) imaging has become an innovative ultrasound elastography that has received increasing attention. Studies have reported that ARFI imaging could be used for the qualitative detection of liver tumors, and could also be used to determine the degree of liver fibrosis by detecting liver stiffness (LS). Hence, it is hoped that this technique could evaluate liver reserve function accurately. Furthermore, ARFI imaging is non-invasive, simple and repeatable.

Innovations and breakthroughs

ARFI could be used to determine liver reserve function by detecting LS. ARFI imaging has advantages, such as speed, simplicity, non-invasiveness, and a wide range of applications. It is important to determine a reasonable surgical method and liver resection range. ARFI imaging technology is a useful tool to assess liver reserve function in patients with liver tumors. It has an important clinical application and should be promoted.

Applications

This study demonstrated that ARFI imaging could be used to assess liver reserve function by detecting LS, and to determine a more successful surgical option. The results showed that the higher the LS value, the worse the liver reserve function and prognosis after surgery. This also confirmed that LS values could react to liver reserve functions. Furthermore, an LS value greater than 2.34 m/s suggested poor liver reserve function; therefore, non-surgical treatment should be recommended.

Peer-review

This study demonstrated that ARFI imaging could be used to assess liver reserve function by detecting LS before surgery. This provides a means of determining a reasonable surgical method for patients with liver tumors. ARFI imaging has advantages. It is fast, simple, non-invasive and has a wide range of applications. Thus, ARFI imaging is a useful and valuable tool for clinical application and should be promoted.

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Systematic review of anastomotic complications of esophagojejunostomy after laparoscopic total gastrectomy

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Abstract

AIM: To investigate the anastomotic complications of esophagojejunostomy (EJS) after laparoscopic total gastrectomy (LTG), we reviewed retrospective studies.

METHODS: A literature search was conducted in PubMed for studies published from January 1, 1994 through January 31, 2015. The search terms included "laparoscopic," "total gastrectomy," and "gastric cancer." First, we selected 16 non-randomized controlled trials (RCTs) comparing LTG with open total gastrectomy (OTG) and conducted an updated meta-analysis of anastomotic complications after total gastrectomy. The Newcastle-Ottawa scoring system (NOS) was used to assess the quality of the non-RCTs included in this study. Next, we reviewed anastomotic complications in 46 case studies of LTG to compare the various procedures for EJS.

RESULTS: The overall incidence of anastomotic leakage associated with EJS was 3.0% (30 of 984 patients) among LTG procedures and 2.1% (31 of 1500 patients) among OTG procedures in the 16 non-RCTs. The incidence of anastomotic leakage did not differ significantly between LTG and OTG (odds OR = 1.42, 95%CI: 0.86-2.33, P = 0.17, I^2 = 0%). Anastomotic stenosis related to EJS was reported in 72 (2.9%) of 2484 patients, and the incidence was 3.2% among LTG procedures and 2.7% among OTG procedures. The incidence of anastomotic stenosis related to EJS was slightly, but not significantly, higher in LTG than in OTG (OR = 1.55, 95%CI: 0.94-2.54, P = 0.08, I^2 = 0%). The various procedures for LTG were classified into six categories in the review of case studies of LTG. The incidence of EJS leakage was similar (1.1% to 3.2%), although the incidence of EJS stenosis was relatively high when the OrVil™ device was used (8.8%) compared with other procedures (1.0% to 3.6%).

CONCLUSION: The incidence of anastomotic complications associated with EJS was not different between LTG and OTG. Anastomotic stenosis was relatively common when the OrVil™ device was used.

Key words: Gastric cancer; Laparoscopic; Gastrectomy; Anastomosis; Complication

Core tip: In this updated meta-analysis of non-randomized controlled trials comparing laparoscopic total gastrectomy (LTG) and open total gastrectomy, the incidence of anastomotic leakage was similar, and that of anastomotic stenosis was slightly, but not significantly, higher when LTG was performed. The incidence of anastomotic stenosis was relatively high for new procedures that utilize a trans-orally inserted anvil (OrVil™) in reported case series of LTG.

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INTRODUCTION

Laparoscopic distal gastrectomy (LDG) is an established minimally invasive procedure for the treatment of gastric cancer, particularly early-stage disease. Several meta-analyses of randomized controlled trials (RCTs) comparing LDG with conventional open distal gastrectomy (ODG) have reported the short-term advantages of LDG, such as less pain, less operative bleeding, and earlier recovery[1-5]. Moreover, LDG was associated with fewer minor postoperative complications, such as wound infection and medical complications, compared with ODG in several meta-analyses, including non-RCTs[6,7]. In contrast, no RCTs comparing laparoscopic total gastrectomy (LTG) with open total gastrectomy (OTG) have been reported, although one RCT compared laparoscopy-assisted gastrectomy including both distal and total gastrectomy with open gastrectomy[8]. Esophagojejunostomy (EJS) after LTG is a complicated procedure requiring extensive experience and a skilled technique, which is a major reason why LTG is not as commonly performed as LDG. However, several meta-analyses of non-RCTs that included patients with mismatched clinical factors have been reported. First, in 2012, Haverkamp *et al*[9] published a meta-analysis of 8 non-RCTs, showing that LTG was associated with a longer operative time, less blood loss, and a shorter hospital stay than OTG. Postoperative complications did not differ between LTG and OTG in their meta-analysis. Shen *et al*[10] demonstrated that LTG was associated with a slightly, but not significantly, lower incidence of postoperative complications than OTG. Regarding individual complications, there were slightly lower risks of wound infection and pneumonia with LTG. Chen *et al*[11] showed that postoperative medical complications were significantly less frequent with LTG than with OTG, but surgical complications were not. In two meta-analyses published in 2014, LTG was shown to result in a longer operative time, less blood loss, lower analgesic use, earlier passage of flatus, quicker resumption of oral intake, earlier hospital discharge, and fewer postoperative complications[12,13]. Regarding individual complications, LTG was associated with fewer wound-related problems than OTG[12].

This review focused on anastomotic complications of EJS after LTG. We conducted a meta-analysis of postoperative anastomotic complications of EJS, such as anastomotic leakage and stenosis, by analyzing the results of non-RCTs that compared LTG with OTG. In addition, we analyzed case series of EJS in conjunction with LTG and evaluated the different procedures used to perform EJS.

MATERIALS AND METHODS

Literature overview

First, to conduct this meta-analysis comparing anastomotic complications of EJS between LTG and OTG, a literature search was performed in PubMed for studies published from January 1, 1994 through January 31, 2015. The search terms included "laparoscopic," "total gastrectomy," and "gastric cancer." Reports in languages other than English, reviews, and meta-analyses were excluded. Twenty non-RCTs, but no RCTs, were found. To minimize bias in this meta-analysis of anastomotic complications, we excluded studies that included hand-assisted or robotic approaches, other diseases, and mismatched reconstruction procedures. Four studies were excluded from this meta-analysis for the following reasons. The text of a study by Du *et al*[14] was not available online; a study by Usui *et al*[15] included hand-assisted procedures; a study by Kwon *et al*[16] included robotic surgery; and a study by Mochiki *et al*[17] included jejunal pouch interposition reconstruction in OTG. The 16 selected non-RCTs are summarized in Table 1. LTG and OTG were compared with regard to anastomotic leakage or stenosis of the EJS.

The Newcastle-Ottawa scoring system (NOS) was used to assess the quality of the non-RCTs[18]. With the NOS, the maximum scores are four points for selection, two for comparability (reconstruction method and the extent of lymphadenectomy), and three for outcome assessment. The studies included in this meta-analysis were of sufficient quality according to the NOS (Table 2).

Second, to review case series reporting anastomotic complications of EJS in LTG, a search of PubMed, performed as described above, yielded 53 case series reports (including more than 10 patients) of LTG that included reconstruction procedures and a results of postoperative anastomotic complications.

Table 1 Summary of non-randomized controlled trials comparing laparoscopic total gastrectomy and open total gastrectomy

Author	Year	Nation	*n*	Extent of LND[1]	Matched factors
Kim *et al*[27]	2008	South Korea	60	D1 + 8a, 9	1, 2, 3, 5, 6, 7
Topal *et al*[28]	2008	Belgium	60	D2	1, 2, 3, 4, 5, 6, 7
Kawamura *et al*[29]	2009	Japan	81	D2-No.10	1, 2, 3, 4, 6, 7
Sakuramoto *et al*[30]	2009	Japan	74	D1 + 8a, 9/D2-No.10	1, 2, 3, 4, 6, 7
Kim *et al*[31]	2011	South Korea	190	D2-No.10	1, 2, 3, 4, 5, 6, 7
Arrington *et al*[32]	2012	United States	50	D0/D1/D2-No.10	1, 2, 5, 6, 7
Eom *et al*[33]	2012	South Korea	448	D2-No.10	4, 6, 7
Siani *et al*[34]	2012	Italy	50	D2-No.10	1, 2, 5, 6, 7
Bo *et al*[35]	2013	China	234	D2-No.10	1, 2, 3, 5, 6, 7
Guan *et al*[36]	2013	China	97	D2	2, 5, 6, 7
Jeong *et al*[37]	2013	South Korea	244	D1 + No.8a, 9/D2	1, 2, 3, 4, 5, 7
Kim *et al*[38]	2013	South Korea	346	D2-No.10	1, 2, 3, 4, 5, 6, 7
Lee *et al*[39]	2013	South Korea	348	D2	1, 2, 4, 6, 7
Shim *et al*[40]	2013	South Korea	70	D1 + 8a, 9, 11p/D2	1, 2, 5, 6, 7
Lee *et al*[41]	2014	South Korea	84	D1 + No.8a, 9, 11p	1, 2, 5, 6, 7
Matsuda *et al*[42]	2015	Japan	48	D1 + No.8a, 9, 11p	2, 3, 4, 5, 6, 7

[1]Based on Japanese gastric cancer treatment guidelines. 1, age; 2, sex; 3, body mass index; 4, ASA or comorbidity; 5, tumor stage; 6, extent of LND; 7, reconstruction method. LND: Lymph node dissection.

Table 2 Quality assessment of non-randomized controlled trials based on the Newcastle-Ottawa scoring system

Author	Selection				Comparability[1]	Exposure		
	Is the case definition adequate?	Representativeness of the cases	Selection of controls	Definition of controls	of cases and controls on the basis of the design or analysis	Ascertainment of exposure	Same method of ascertainment for cases and controls	Non-response rate
Kim *et al*[27]	*	*			**	*	*	*
Topal *et al*[28]	*	*			**	*	*	*
Kawamura *et al*[29]	*	*			**	*	*	*
Sakuramoto *et al*[30]	*	*			**	*	*	*
Kim *et al*[31]	*	*			**	*	*	*
Arrington *et al*[32]	*	*			**	*	*	*
Eom *et al*[33]	*	*			**	*	*	*
Siani *et al*[34]	*	*			**	*	*	*
Bo *et al*[35]	*	*			**	*	*	*
Guan *et al*[36]	*	*			**	*	*	*
Jeong *et al*[37]	*	*			*	*	*	*
Kim *et al*[38]	*	*			**	*	*	*
Lee *et al*[39]	*	*			**	*	*	*
Shim *et al*[40]	*	*			**	*	*	*
Lee *et al*[41]	*	*			**	*	*	*
Matsuda *et al*[42]	*	*			**	*	*	*

[1]Controls selected on the basis of the extent of lymphadenectomy and reconstruction procedure (maximum, 2 stars).

Several studies partly included comparisons, such as comparisons between LTG and LPG or between different EJS procedures. However, 4 studies were excluded because they also included proximal gastrectomy or other diseases, and 3 studies were excluded because of a suspicion of overlapping data from the same institution. The remaining 46 studies were included in this review (Table 3). We classified anastomotic procedures into the following 6 categories: (1) extracorporeal reconstruction by a single stapling technique using a circular stapler; (2) intracorporeal reconstruction by a single stapling technique using a circular stapler; (3) intracorporeal reconstruction by a double (or hemi-double) stapling technique using a circular stapler with a trans-abdominally inserted anvil (DST/HDST); (4) intracorporeal reconstruction by a double (or hemi-double) stapling technique using a circular stapler with a trans-orally inserted anvil (OrVil™) (DST/HDST by TOA); (5) intracorporeal reconstruction by side-to-side anastomosis using a linear stapler; and (6) intracorporeal reconstruction by functional end-to-end anastomosis using linear staplers.

Statistical analysis

Review Manager software, version 5.2 (Cochrane Collaboration, Oxford, United Kingdom), was used to perform this meta-analysis. For discontinuous variables, each postoperative complication was extracted from the trial report; odds ratios (ORs) were

Table 3 Summary of case series of esophagojejunostomy in laparoscopic total gastrectomy *n* (%)

Author	Year	Country	*n*	EJS leakage	EJS stenosis
Extracorporeal SST					
Hur *et al*[43]	2008	South Korea	18	0 (0)	0 (0)
Lee *et al*[44]	2009	South Korea	67	1 (1.5)	6 (9.0)
Kunisaki *et al*[45]	2011	Japan	15	1 (6.7)	0 (0)
Yoon *et al*[46]	2012	South Korea	65	3 (4.6)	3 (4.6)
Mou *et al*[47]	2013	China	12	0 (0)	0 (0)
Jung *et al*[48]	2013	South Korea	47	2 (4.3)	2 (4.3)
Li *et al*[49]	2014	China	108	1 (0.9)	0 (0)
Sahoo *et al*[50]	2014	India	47	0 (0)	0 (0)
Total			379	8 (2.1)	11 (2.9)
Intracorporeal SST with trans-abdominally inserted anvil					
Usui *et al*[51]	2008	Japan	15	0 (0)	0 (0)
Kinoshita *et al*[52]	2010	Japan	10	0 (0)	0 (0)
Lee *et al*[53]	2012	South Korea	88	3 (3.4)	0 (0)
Shim *et al*[54]	2013	South Korea	12	2 (17)	5 (42)
Kim *et al*[55]	2013	South Korea	36	0 (0)	0 (0)
Yoshikawa *et al*[56]	2013	Japan	20	0 (0)	0 (0)
Du *et al*[57]	2013	China	52	0 (0)	0 (0)
Total			233	5 (2.1)	5 (2.1)
Intracorporeal HDST/DST with trans-abdominally inserted anvil					
Omori *et al*[58]	2009	Japan	10	0 (0)	0 (0)
Nunobe *et al*[59]	2011	Japan	41	2 (4.9)	3 (7.3)
Shim *et al*[54]	2013	South Korea	14	1 (7.1)	1 (7.1)
Lafemina *et al*[60]	2013	United States	17	1 (5.9)	1 (5.9)
Muguruma *et al*[61]	2014	Japan	32	0 (0)	0 (0)
Zhao *et al*[62]	2014	China	26	0 (0)	0 (0)
Total			140	4 (2.9)	5 (3.6)
Intracorporeal HDST/DST with trans-orally inserted anvil					
Jeong *et al*[63]	2009	South Korea	16	0 (0)	0 (0)
Kachikwu *et al*[64]	2011	United States	16	0 (0)	3 (19)
Kunisaki *et al*[45]	2011	Japan	30	1 (3.3)	0 (0)
Marangoni *et al*[65]	2012	United Kingdom	13	0 (0)	0 (0)
Liao *et al*[66]	2013	China	27	1 (3.7)	1 (3.7)
Shim *et al*[54]	2013	South Korea	12	2 (17)	4 (33)
Xie *et al*[67]	2013	China	28	0 (0)	0 (0)
Zuiki *et al*[25]	2013	Japan	52	1 (1.9)	11 (21)
Hiyoshi *et al*[68]	2014	Japan	21	2 (9.5)	0 (0)
Total			215	7 (3.2)	19 (8.8)
Intracorporeal STSA					
Huscher *et al*[69]	2007	Italy	11	0 (0)	0 (0)
Inaba *et al*[70]	2010	Japan	53	2 (3.8)	0 (0)
Bracale *et al*[71]	2010	Italy	67	4 (6.0)	2 (3.0)
Tsujimoto *et al*[72]	2012	Japan	15	0 (0)	0 (0)
Nagai *et al*[73]	2013	Japan	94	2 (2.1)	0 (0)
Petersen *et al*[74]	2013	Denmark	30	3 (10)	0 (0)
Shim *et al*[54]	2013	South Korea	10	0 (0)	1 (10)
Morimoto *et al*[75]	2014	Japan	77	0 (0)	1 (1.3)
Yamamoto *et al*[76]	2014	Japan	52	1 (1.9)	0 (0)
Total			409	12 (2.9)	4 (1.0)
Intracorporeal FETEA					
Ziqiang *et al*[77]	2008	China	14	0 (0)	0 (0)
Kim *et al*[78]	2012	South Korea	124	3 (2.4)	6 (4.8)
Kim *et al*[79]	2013	South Korea	139	1 (0.7)	1 (0.7)
Ebihara *et al*[80]	2013	Japan	65	0 (0)	3 (4.6)
Hiyoshi *et al*[68]	2014	Japan	24	0 (0)	0 (0)
Tsunoda *et al*[81]	2014	Japan	97	1 (1.0)	0 (0)
Total			463	5 (1.1)	10 (2.2)

EJS: Esophagojejunostomy; SST: Single-stapling technique; DST: Double-stapling technique; HDST: Hemi-double stapling technique; STSA: Side-to-side anastomosis; FETEA: Functional end-to-end anastomosis.

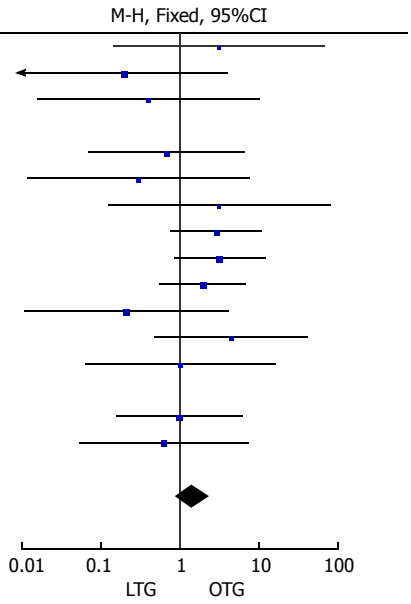
calculated from the total number of patients and the observed numbers of events of interest in all groups using a random-effects model. In the tables of our results, squares indicate point estimates of ORs, with 95% confidential intervals (CIs) indicated by horizontal bars. The diamonds represent the summary ORs with 95%CIs from the included studies. *P* values < 0.05 were considered to indicate statistical significance.

A

Study or Subgroup	LTG Events	LTG Total	OTG Events	OTG Total	Weight	M-H, Fixed, 95%CI	Year
Topal	2	38	0	22	2.3%	3.08 [0.14, 67.16]	2008
Sakuramoto	0	30	3	44	11.0%	0.19 [0.01, 3.90]	2008
Kim SG	0	27	1	33	5.2%	0.39 [0.02, 10.07]	2008
Kawamura	0	46	0	35		Not estimable	2009
Kim MG	1	63	3	127	7.7%	0.67 [0.07, 6.54]	2011
Arrington	0	26	1	24	6.0%	0.30 [0.01, 7.61]	2012
Siani	1	25	0	25	1.8%	3.12 [0.12, 80.39]	2012
Eom	4	100	5	348	8.4%	2.86 [0.75, 10.85]	2012
Jeong	9	122	3	122	10.9%	3.16 [0.83, 11.97]	2013
Lee MS	5	120	5	228	12.9%	1.94 [0.55, 6.84]	2013
Kim HS	0	139	3	207	11.0%	0.21 [0.01, 4.09]	2013
Shim	4	35	1	35	3.5%	4.39 [0.46, 41.40]	2013
Bo	1	117	1	117	3.9%	1.00 [0.06, 16.18]	2013
Guan	0	41	0	56		Not estimable	2013
Lee SR	2	34	3	50	8.9%	0.98 [0.15, 6.19]	2014
Matsuda	1	21	2	27	6.5%	0.63 [0.05, 7.40]	2015
Total (95%CI)		984		1500	100.0%	1.42 [0.86, 2.33]	
Total events	30		31				

Heterogeneity: χ^2 = 9.96, *df* = 13 (*P* = 0.70); I^2 = 0%

Test for overall effect: *Z* = 1.37 (*P* = 0.17)



B

Study or Subgroup	LTG Events	LTG Total	OTG Events	OTG Total	Weight	M-H, Fixed, 95%CI	Year
Kim SG	0	27	0	33		Not estimable	2008
Sakuramoto	2	30	1	44	3.1%	3.07 [0.27, 35.49]	2008
Topal	0	38	0	22		Not estimable	2008
Kawamura	0	46	1	35	6.9%	0.25 [0.01, 6.26]	2009
Kim MG	0	63	0	127		Not estimable	2011
Arrington	1	26	2	24	8.2%	0.44 [0.04, 5.19]	2012
Eom	9	100	26	348	43.5%	1.22 [0.55, 2.71]	2012
Siani	0	25	0	25		Not estimable	2012
Guan	0	41	0	56		Not estimable	2013
Kim HS	2	139	2	207	6.5%	1.50 [0.21, 10.75]	2013
Bo	0	117	0	117		Not estimable	2013
Jeong	2	122	2	122	8.1%	1.00 [0.14, 7.22]	2013
Lee MS	3	120	4	228	11.1%	1.44 [0.32, 6.52]	2013
Shim	9	35	3	35	9.2%	3.69 [0.91, 15.05]	2013
Lee SR	2	34	0	50	1.6%	7.77 [0.36, 167.06]	2014
Matsuda	1	21	0	27	1.7%	4.02 [0.16, 103.93]	2015
Total (95%CI)		984		1500	100.0%	1.55 [0.94, 2.54]	
Total events	31		41				

Heterogeneity: χ^2 = 5.93, *df* = 9 (*P* = 0.75); I^2 = 0%

Test for overall effect: *Z* = 1.73 (*P* = 0.08)

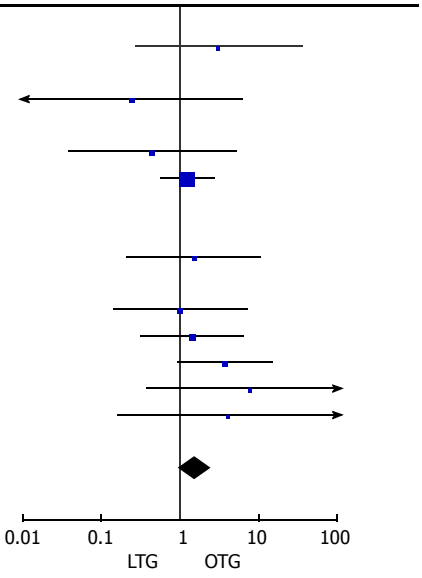


Figure 1 Outcomes of the meta-analysis comparing anastomotic leakage (A) and anastomotic stenosis (B). LTG: Laparoscopic total gastrectomy; OTG: Open total gastrectomy.

The I^2 statistic was used to quantitatively assess heterogeneity. Graphical exploration with funnel plots was used to evaluate publication bias. Publication bias was assessed on the basis of the funnel plot of the included studies.

RESULTS

This meta-analysis included a total of 2484 patients, 984 of whom underwent LTG and 1500 of whom underwent OTG. Anastomotic leakage of EJS was reported in 61 (2.5%) of 2484 patients in the 16 studies. The overall incidence of anastomotic leakage of EJS was 3.0% (30 of 984 patients) with LTG and 2.1% (31 of 1500 patients) with OTG in the 16 studies. The incidence of anastomotic leakage did not differ significantly between LTG and OTG (Figure 1A). Anastomotic stenosis of EJS was reported in 72 (2.9%) of the 2484 patients, and the incidence was 3.2% with LTG and 2.7% with OTG. The incidence of anastomotic stenosis of EJS was slightly, but not significantly, higher in LTG than in OTG (Figure 1B). Publication bias was assessed for each complication using the funnel plot of the included studies. No complications were associated with publication bias, and a symmetric distribution was maintained with all of the studies lying within the 95%CI (data not shown).

In the review of the case series, the overall incidence

of anastomotic leakage of EJS in the 46 studies was 2.2% (41 of 1839). The incidences of EJS leakage according to the anastomotic procedure are also shown in Table 3. The overall incidence of anastomotic stenosis of EJS was 2.9% (54 of 1839). The incidences of anastomotic stenosis according to the anastomotic procedure are also shown in Table 3. It was relatively common with the DST/HDST by TOA procedure.

DISCUSSION

In this updated meta-analysis, the incidence of anastomotic leakage of EJS did not differ significantly between LTG and OTG. This outcome was consistent with the findings of previous meta-analyses by Wang *et al*[12,19]. The incidence of anastomotic leakage of EJS after TG in our review was not higher than that in other studies of OTG, which have reported incidences of 1.0% to 2.1%[20-22]. The Japanese National Clinical Database (NCD) of digestive surgery reported that the incidence of anastomotic leakage after total gastrectomy was 4.4% (881 of 20011) in 2011[23]. Detailed information, specifically on LTG or OTG, was unavailable. Most of the leaks must have occurred at the EJS in that study. Diverse anastomotic procedures have been reported in studies of LTG. In our review, the incidence of anastomotic leakage of EJS was similar between the various procedures.

In our study, the incidence of anastomotic stenosis of EJS was slightly, but not significantly, higher with LTG than with OTG. One problem was that EJS stenosis was not clearly defined in many of the studies included in our analysis. EJS stenosis was not graded based on a standardized assessment, such as the Clavien-Dindo classification. Therefore, it was unclear whether endoscopic dilation or reoperation was performed in all of the patients diagnosed with EJS stenosis. Another problem was that EJS stenosis often occurred several weeks or months after LTG. Therefore, an accurate incidence of anastomotic stenosis was not shown among the short-term outcomes of LTG, and anastomotic stenosis was not mentioned in the NCD report. In our review of case-series studies, the incidence of anastomotic stenosis was higher among the procedures performed using the OrVil™ device. In a review by Umemura *et al*[24] comparing procedures used to perform EJS after LTG, the use of circular staplers was significantly associated with higher incidences of both anastomotic leakage (4.7%) and stenosis (8.3%) compared with the use of linear staplers (1.1% and 1.8%, respectively). Even in our analysis, linear stapler methods apparently reduced the risk of stenosis. An anastomotic site formed by a linear stapler could probably secure a wider diameter than one formed by a circular stapler[24]. As another investigator insisted, the high incidence of anastomotic stenosis after DST/HDST may be explained by the following causes: excessive tension at the anastomotic site and focal ischemia at the site where the two staple lines overlap[25]. In the study of the Orvil™ device, which was associated with the highest incidence of anastomotic stenosis, the use of a circular stapler with a smaller size (21 mm) significantly increased the rate of EJS anastomosis compared with the use of a normal-sized stapler (25 mm)[25]. To pass the anvil head of Orvil™ easily through the esophageal entrance, the smaller anvil was probably used in some cases in that study. In OTG, the use of a circular stapler with a small diameter (21 mm) was a significant risk factor for EJS stenosis[26]. Both the DST/HDST procedure and the use of a smaller circular stapler could increase the stenosis in the EJS when the Orvil™ device is used. However, several studies on the use of Orvil™ have shown favorable results. Anastomotic complications may be closely associated with learning curves of surgeons[25]. Therefore, they will probably decrease in any procedures as surgeons acquire more experience and improve their technical skills in performing EJS.

In addition, the value of meta-analyses of non-RCTs remains controversial, as non-RCTs often include groups of patients who are mismatched with respect to background characteristics. Our meta-analysis also had limitations despite the inclusion of studies in which the patients were matched as closely as possible. To draw definitive conclusions, prospective studies are needed to clarify the usefulness of LTG. A prospective phase Ⅱ study of LTG or laparoscopic proximal gastrectomy has begun in Japan, with anastomotic leakage as the primary endpoint. The problems currently associated with EJS after LTG are an important concern. However, the postoperative outcomes of EJS are expected to improve in the future with increased experience and enhanced surgical skills.

In conclusion, the incidences of anastomotic complications of EJS were similar in this meta-analysis comparing LTG and OTG. In case studies of LTG, the incidence of anastomotic leakage of EJS was not different between various anastomotic procedures, although anastomotic stenosis was relatively common in the DST/HDST by TOA procedure.

COMMENTS

Background

Esophagojejunostomy (EJS) after laparoscopic total gastrectomy (LTG) is a complicated procedure requiring extensive experience and a skilled technique, which is a major reason why LTG is not as commonly performed as laparoscopic distal gastrectomy. No randomized controlled trials (RCTs) comparing LTG with open total gastrectomy (OTG) has been reported yet. Several meta-analyses of non-RCTs, including patients with mismatched clinical factors, have been reported.

Research frontiers

Anastomotic complication was a major issue in LTG. Various anastomotic procedures of EJS have been attempted for EJS in LTG. Anastomotic methods were roughly categorized into two groups; circular stapler method had been usually performed in OTG, and linear stapler method developed in LTG.

Innovations and breakthroughs

This meta-analysis of non-RCT of LTG *vs* OTG was updated, and several non-

RCTs were excluded due to including hand-assisted or robotic approaches, other diseases, and mismatched reconstruction procedures. Furthermore, we reviewed case series of LTG, and categorized various anastomotic methods of EJS into the following six procedures: (1) extracorporeal reconstruction by single stapling technique using a circular stapler; (2) intracorporeal reconstruction by single stapling technique using a circular stapler; (3) intracorporeal reconstruction by double (or hemi-double) stapling technique using a circular stapler with a trans-abdominally inserted anvil (DST/HDST); (4) intracorporeal reconstruction by double (or hemi-double) stapling technique using a circular stapler with a trans-orally inserted anvil (OrVilTM) (DST/HDST by TOA); (5) intracorporeal reconstruction by side-to-side anastomosis using a linear stapler; and (6) intracorporeal reconstruction by functional end-to-end anastomosis using linear staplers.

Applications

The incidence of anastomotic leakage of EJS was similar between LTG and OTG, although that of anastomotic stenosis was slightly, but not significantly, higher with LTG than with OTG. In case series of LTG, the incidence of anastomotic leakage of EJS was not different in various anastomotic procedures, although anastomotic stenosis was slightly higher in the procedure of DST/HDST by TOA.

Terminology

Single stapling technique of EJS is the following procedure. The purse-string suture is placed in distal esophageal stump. The anvil head of a circular stapler is inserted into the esophageal lumen. The circular stapler is inserted into the distal limb of the jejunum. The circular stapler is combined with the anvil head, and EJS is performed. In double or hemi-double stapling technique, abdominal esophagus is cut by a linear stapler, and EJS is performed by a circular stapler. The anvil head is inserted trans-abdominally before esophageal transection. However, OrVilTM is a device including a trans-orally inserted anvil. The anvil head of OrVilTM connected with gastric tube is inserted through pharynx and esophageal entrance intraoperatively. Side-to-side anastomosis is performed peristaltically by a linear stapler. Functional end-to-end anastomosis is performed anti-peristaltically, and the entry hall is closed by a linear stapler.

Peer-review

This paper is an interesting article. Perhaps the only drawback is that there is not any RCT study, but it has been correctly referred.

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CASE REPORT

Radioembolisation and portal vein embolization before resection of large hepatocellular carcinoma

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Abstract

Resectability of hepatocellular carcinoma in patients with chronic liver disease is dramatically limited by the need to preserve sufficient remnant liver in order to avoid postoperative liver insufficiency. Preoperative treatments aimed at downsizing the tumor and promoting hypertrophy of the future remnant liver may improve resectability and reduce operative morbidity. Here we report the case of a patient with a large hepatocellular carcinoma arising from chronic liver disease. Preoperative treatment, including tumor downsizing with transarterial radioembolization and induction of future remnant liver hypertrophy with right portal vein embolization, resulted in a 53% reduction in tumor volume and compensatory hypertrophy in the contralateral liver. The patient subsequently underwent extended right hepatectomy with no postoperative

signs of liver decompensation. Pathological examination demonstrated a margin-free resection and major tumor response. This new therapeutic sequence, combining efficient tumor targeting and subsequent portal vein embolization, could improve the feasibility and safety of major liver resection for hepatocellular carcinoma in patients with liver injury.

Key words: Hepatocellular carcinoma; Chronic liver disease; Radioembolisation; Portal vein embolization; Hepatectomy; Cirrhosis

Core tip: Surgical treatment of hepatocellular carcinoma in patients with chronic liver disease is challenging due to the contradictory need to perform a radical tumor resection while preserving a maximal amount of tumor-free remnant liver. Preoperative treatment may be indicated for tumor downsizing and to promote hypertrophy of the future remnant liver. We report the case of a cirrhotic patient undergoing extended right hepatectomy for a large hepatocellular carcinoma after transarterial radioembolization and right portal vein embolization. Our results suggest that this approach is feasible and safe and may represent a new therapeutic option before major hepatectomy in patients with liver injury.

Bouazza F, Poncelet A, Garcia CA, Delatte P, Engelhom JL, Galdon MG, Deleporte A, Hendlisz A, Vanderlinden B, Flamen P, Donckier V. Radioembolisation and portal vein embolization before resection of large hepatocellular carcinoma. *World J Gastroenterol* 2015; 21(32): 9666-9670 Available from: URL: http://www.wjgnet.com/1007-9327/full/v21/i32/9666.htm DOI: http://dx.doi.org/10.3748/wjg.v21.i32.9666

INTRODUCTION

Surgical resection or local destruction remain the only potentially curative options in patients with hepatocellular carcinoma (HCC) and chronic liver disease (CLD) who are not candidates for liver transplantation. However, in cases of liver injury, and particularly of cirrhosis, the need to preserve sufficient liver parenchyma to minimize the risk of postoperative hepatic insufficiency dramatically limits the options for radical liver resection. Accordingly, resection of HCC in patients with CLD is usually only recommended in patients with compensated cirrhosis and small uninodular tumors[1]. A recent report indicated that extension of this strict indication might be feasible in some cases involving large tumors, leading to satisfactory long-term results in selected patients[2]. In this context, the safety and efficacy of major liver resection for large HCC could be improved by preoperative treatments designed to downsize the tumor and allow safer parenchyma-sparing resection. In the tumor-free portion of the liver, preoperative treatments aimed at promoting compensatory hypertrophy of the anticipated future remnant liver (FRL) could reduce the risk for post-hepatectomy liver failure. To target the tumor, intra-arterial therapies are well-established treatments based on the fact that HCCs mainly rely on arterial vascularization. Transarterial chemoembolization (TACE) has been shown to be an efficient palliative treatment for unresectable HCC[3], but, in a pre-operative setting, neoadjuvant TACE failed to demonstrate a clear oncological long-term benefit as compared with surgical resection alone[4]. Transarterial radioembolization (TARE), consisting of intra-arterial injection of microspheres labelled with yttrium-90 (^{90}Y), has recently gained ground in the treatment of HCC with several reports suggesting the efficacy of TARE for induction of tumor necrosis[5]. In combination with surgery, preoperative TARE has been shown to be feasible[6] while no clear clinical benefit has been demonstrated yet. Portal vein embolization (PVE) is the standard technique used to promote liver hypertrophy in patients who are not initially amenable to surgery due to anticipated insufficient FRL. In cases involving major liver resection in patients with cirrhosis, preoperative PVE significantly reduces the risk of postoperative liver failure[7]. It has also been shown that sequential TACE and PVE may improve the safety of major surgical resection through synergistic effects that increase the rate of compensatory hypertrophy of the FRL[8]. A similar sequence, but including TARE instead of TACE, followed by PVE before surgical resection has not yet been reported but could potentially represent a new therapeutic option. We describe herein the case of a patient with a large HCC who underwent an extended right hepatectomy after TARE followed by PVE and discuss the potential advantages of this new approach.

CASE REPORT

A 62 year-old man with a past medical history of mild to severe alcohol consumption presented with a liver tumor. Contrast-enhanced abdominal computed tomography (CT) scan demonstrated the presence of a 167 mm mass in segment Ⅷ of the liver with HCC characteristics as indicated by arterial wash-in and portal phase wash-out (Figure 1A). This corresponded to a LI-RADS 5 lesion and a stage B tumor in the BCLC classification. Additionally, the presence of cirrhosis was suggested on the basis of the irregular aspect of the liver surface and the relative hypertrophy of segment Ⅰ. Blood tests, including liver function and alpha-fetoprotein, were normal. Complete work-up did not demonstrate extra-hepatic tumor dissemination. Due to tumor size and location, a right hepatectomy extended to the middle hepatic vein was indicated. Analysis of liver volumes on CT scan showed a total

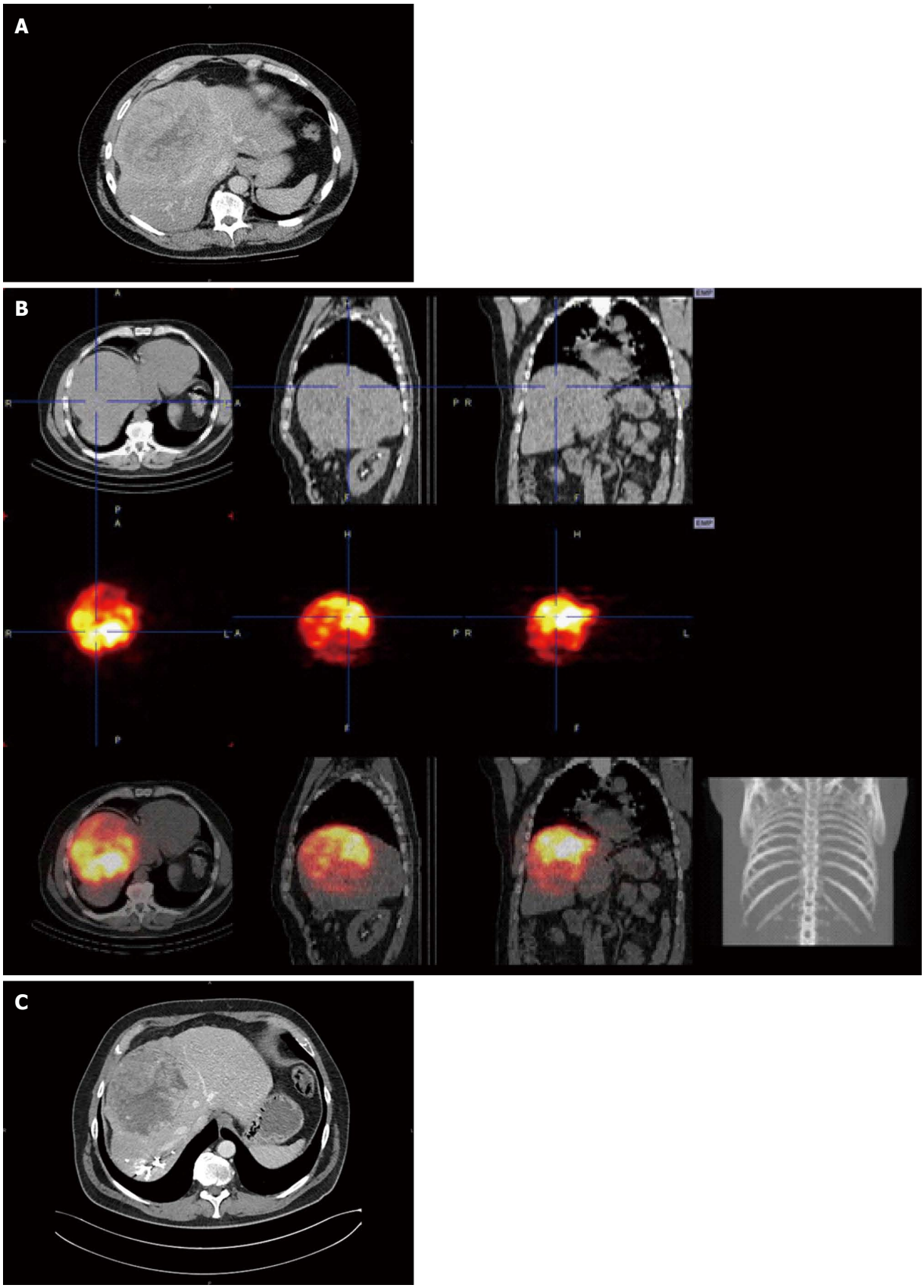

Figure 1 Tumor evolution throughout the therapeutic sequence. A: Contrast-enhanced computed tomography (CT) scan at presentation showing, in portal phase, a 167 mm tumor in segment Ⅷ with features of hepatocellular carcinoma; B: Yttrium-90 (^{90}Y) positron emission tomography (PET)-CT performed 12 hours after transarterial radioembolization (TARE). Images are displayed in orthogonal views, centered on the liver tumor. Upper row: CT scan without contrast. Middle row: ^{90}Y emission PET images showing high and selective accumulation of ^{90}Y microspheres in the tumor site. Lower row: fused ^{90}Y PET-CT images; C: Contrast-enhanced CT scan 17 wk after TARE and 6 wk after portal vein embolization showing, in portal phase, a 50% tumor volume reduction and hypertrophy of the future remnant left liver.

liver volume (TLV) of 3100 mL, a tumor volume of 1548 mL, and an FRL (segments Ⅰ, Ⅱ, Ⅲ, Ⅳ) of 470 mL, corresponding to an FRL/TLV ratio of 15%, an FRL/TLV tumor volume of 30%, and an FRL/body weight ratio of 0.48. Therefore, a 2-step preoperative strategy was proposed, consisting of tumor-targeted TARE followed by right PVE. After simulation of TARE with technetium-99 (^{99}Tc) macroaggregated albumin showed no extra-hepatic deposition and excellent tumor targeting, ^{90}Y hyperselective radioembolization of the segment Ⅷ artery to the tumor was performed allowing the delivery of 97 mCi of ^{90}Y microspheres (Figure 1B). No side effects related to this procedure were observed. A right PVE was performed 11 wk after TARE treatment, without complications. Preoperative CT scan at 6 wk after PVE, showed a tumor volume of 717 mL, a TLV of 2770 mL, and an FRL of 752 mL, corresponding to an FRL/TLV ratio of 27%, FRL/TLV tumor volume of 37%, and FRL/body weight ratio of 0.77 (Figure 1C). Accordingly, considering the fact that such compensatory hypertrophy indicated a preserved regenerative capacity of the future remnant liver, an extended right hepatectomy was judged to be feasible. Right hepatectomy, extended to the middle hepatic vein and partially to segment Ⅳ, was performed 6 wk after PVE. During surgery, no adhesions between tumor and diaphragm were found. No portal triad clamping or perioperative transfusions were required and intraoperative blood loss was 600 mL. The patient's postoperative course was unremarkable, clinically and biologically. No signs of liver decompensation were observed (minimal values of prothrombin time, peak international normalized ratio, and total bilirubin were 64%, 1.2, and 1.2 mg/dL on postoperative days 1, 1, and 2, respectively) and the patient was discharged on day 9. Pathological examination of the specimen demonstrated a margin-free resection and a major tumor response, as indicated by approximately 30% of residual cancer cells, and confirmed the presence of cirrhosis in tumor-free liver. Twelve months after surgery the patient is disease free and has achieved full rehabilitation.

DISCUSSION

The dual pathology in patients with large HCC and CLD imposes contradictory therapeutic objectives, requiring a radical oncological resection on the one hand, and the preservation of a maximal amount of liver parenchyma on the other hand. In this context, preoperative treatments could improve the feasibility and safety of surgical resection by tumor downsizing and expansion of the FRL. In addition, neoadjuvant treatments can contribute to patient selection by allowing preoperative measurement of response to treatment, evaluation of the biological behavior of the tumor, and assessment of liver functional reserve as indicated by regenerative capacity. The case reported here indicates that a therapeutic sequence combining TARE and PVE before major liver resection could be feasible and may potentially represent a new strategy for treatment of large HCC in patients with CLD. The anti-tumor efficacy of TARE was demonstrated by significant tumor downsizing, as indicated by a 53% reduction of tumor volume 17 wk after TARE and by significant tumor necrosis at pathology. After TARE, PVE led to 37.5% compensatory hypertrophy in the FRL, allowing uneventful margin-free extended right hepatectomy. As compared with the combination of TACE and PVE that was proven feasible but not associated with oncological benefit, preoperative TARE and PVE could have several advantages. From a safety point of view, it can be postulated that TARE before PVE could be less harmful to the liver than TACE, as particles used for radioembolization are smaller than those used for chemoembolization, allowing a more distal and selective distribution. Furthermore, the safety of sequential TARE and PVE is suggested by the feasibility of TARE in patients with portal vein thrombosis[5], while this condition contraindicates TACE. In addition, as compared with a strategy using preoperative PVE only, this sequence in which TARE precedes PVE could potentially limit the risk of tumor growth while waiting for liver regeneration. The capacity of TARE to have a synergistic effect on PVE-induced FRL hypertrophy is hypothetical but, interestingly in this context, TARE could also induce atrophy of the irradiated-parenchyma and promote compensatory regeneration of the non-irradiated segments[5]. The potential synergy of this effect with liver hypertrophy promoted by PVE and its influence on the safety of subsequent surgery remains to be investigated but may offer new therapeutic perspectives. Of note, in contrast with previous reports[6], preoperative TARE in the present case did not create post-irradiation adhesions which may complicate surgery. Aside from its feasibility and safety, the oncological benefit of preoperative TARE remains hypothetical. Yet, in this setting, recent studies have suggested an advantage of TARE over TACE in terms of efficacy for treatment of HCC, as indicated by increased tumor downstaging and prolonged time to progression[9]. In conclusion, the combination of TARE followed by PVE could represent a new option in patients with CLD and large HCC that was not initially considered to be resectable due to insufficient RLV. Due to the unique capacity of TARE to induce tumor necrosis and contralateral hypertrophy of the non-embolized liver at the same time, this sequence may offer new therapeutic perspectives and requires further investigation.

COMMENTS

Case characteristics

A sixty year old male patient presented with a large liver mass in the right lobe.

Clinical diagnosis
Due to the presence of alcohol-related chronic liver disease, a diagnosis of hepatocellular carcinoma was suspected.

Differential diagnosis
Differential diagnoses included other solid liver tumors, primary or secondary.

Laboratory diagnosis
Laboratory data, including alpha-fetoprotein, were not contributive.

Imaging diagnosis
Contrast-enhanced computed tomography scan revealed characteristic hepatocellular features such as arterial wash-in and portal phase wash-out.

Pathological diagnosis
On the operative specimen, pathology confirmed the diagnosis of hepatocellular carcinoma and a major response to preoperative radioembolization as indicated by tumor necrosis greater than 50%.

Treatment
Extended right hepatectomy was preceded by transarterial radioembolisation to induce tumor downsizing and portal vein embolization to promote future remnant liver hypertrophy.

Related reports
In such cases of large hepatocellular carcinoma arising from diseased liver, the resectability is dramatically limited by the need to preserve sufficient tumor-free liver to avoid postoperative liver insufficiency.

Experiences and lessons
This case indicates that new a preoperative strategy, combining radio-embolization and portal vein embolization, could be feasible and safe and allow major resection for hepatocellular carcinoma in patients with fibrotic liver.

Peer-review
The feasibility and safety of this approach should be confirmed in further series, while the oncological benefit remains to be determined.

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CASE REPORT

Hepatitis A complicated with acute renal failure and high hepatocyte growth factor: A case report

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Author contributions: Oe S, Shibata M, Miyagawa K, Honma Y, Hiura M, Abe S and Harada M designed the report; Oe S and Shibata M wrote the paper.

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Abstract

A 58-year-old man was admitted to our hospital. Laboratory data showed severe liver injury and that the patient was positive for immunoglobulin M anti-hepatitis A virus (HAV) antibodies. He was also complicated with severe renal dysfunction and had an extremely high level of serum hepatocyte growth factor (HGF). Therefore, he was diagnosed with severe acute liver failure with acute renal failure (ARF) caused by HAV infection. Prognosis was expected to be poor because of complications by ARF and high serum HGF. However, liver and renal functions both improved rapidly without intensive treatment, and he was subsequently discharged from our hospital on the 21st hospital day. Although complication with ARF and high levels of serum HGF are both important factors predicting poor prognosis in acute liver failure patients, the present case achieved a favorable outcome. Endogenous HGF might play an important role as a regenerative effector in injured livers and kidneys.

Key words: Acute hepatitis; Acute renal failure; Hepatitis A virus; Hepatocyte growth factor

Core tip: Renal involvement with hepatitis B and C is well described. However, the mechanism of hepatitis A-associated acute renal failure (ARF) is uncertain. Although the prognosis of hepatitis A is generally good, complication with ARF can have a negative impact. Hepatocyte growth factor (HGF) is a predictive factor for acute liver failure. Fulminant hepatic failure patients with high serum HGF have high mortality. By contrast, HGF is also an important factor accelerating tissue regeneration of injured organs, including the liver and kidneys. Here, we describe a patient with acute hepatitis A who achieved a favorable outcome despite complications with both ARF and high serum HGF.

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INTRODUCTION

Acute hepatitis A is usually a mild to moderate illness, but in rare cases it can lead to severe complications, such as fulminant hepatitis, acute renal failure (ARF), blood dyscrasias, including hemolytic and aplastic anemia, and autoimmune hepatitis[1]. Although ARF can develop in more than 80% of patients with fulminant hepatitis with massive hepatic necrosis[2], the development of ARF is not a common complication of nonfulminant hepatitis A. Although the prognosis of hepatitis A is generally good, complication with ARF can have a negative impact.

Hepatocyte growth factor (HGF) is predictive factor of acute liver failure. Tsubouchi *et al*[3] reported that fulminant hepatic failure patients with high serum HGF showed high mortality. By contrast, HGF is also an important factor accelerating tissue regeneration of injured organs, including the liver and kidney[4].

Here, we describe a patient with acute hepatitis A who achieved a favorable outcome despite complications with both ARF and high serum HGF.

CASE REPORT

A 58-year-old man was admitted to our hospital in 2010 with fever, malaise, loss of appetite and jaundice for 3 d. Although he had consumed about 180 g/d of alcohol for 38 years, he had been in good health and had no history of abnormality in annual medical checkups, including urinalysis.

A physical examination showed icteric skin and hepatomegaly, but no signs of dehydration. Laboratory findings revealed severe liver injury and coagulopathy; white blood cells were 10200/μL [reference value (RV); 3100-9.1/μL)], red blood cells were 503 × 10^4/μL (RV; 4.27 × 10^4-5.58 × 10^4/μL), hemoglobin was 16.2 g/dL (RV; 13.5-17.2 g/dL), platelets were 98000/μL (RV; 157000-340000/μL), total protein was 5.8 g/dL (RV; 6.7-8.3 g/dL), albumin was 3.0 g/dL (RV; 4.0-5.0 g/dL), total bilirubin was 4.7 mg/dL (RV; 0.2-1.5 mg/dL), direct bilirubin was 3.9 mg/dL (RV; 0.1-0.4 mg/dL), aspartate aminotransferase was 12217 IU/L (RV; 13-33 IU/L), alanine aminotransferase was 5725 IU/L (RV; 8-42 IU/L), gamma glutamyltranspeptidase was 878 IU/L (RV; 10-47 IU/L), lactate dehydrogenase was 9536 IU/L (RV; 119-229 IU/L), blood urea nitrogen was 51 mg/dL (RV; 8-22 mg/dL), creatinine was 5.40 mg/dL (RV; 0.6-1.1 mg/dL), prothrombin time percentage was 28.2% (RV; more than 74%), and markers of hepatitis B virus, hepatitis C virus, Epstein-Barr virus and cytomegalovirus were negative. A chemiluminescent immunoassay showed that his serum immunoglobulin M anti-hepatitis A virus (HAV) antibody was strongly positive at 11.4 Index (RV; below 0.8 Index). The serum level of hepatocyte growth factor (HGF) was extremely high at 12.28 ng/mL (RV; below 0.4 ng/mL). Moreover, laboratory data showed renal dysfunction with abnormal urinalysis, such as macroproteineuria and many granular casts. Serum level of complement (C) 3 was 25 mg/dL (RV; 78-128 mg/dL), C4 was less than 5 mg/dL (RV; 12-31 mg/dL) and C1q-binding immune complex in sera was within normal limits. Hence, he was diagnosed with HAV-related acute liver failure complicated with ARF. His laboratory findings were very severe. His general condition and appetite were not good. However, both his general condition and laboratory data rapidly improved after supportive treatment, such as administration of proton pump inhibitors to prevent gastrointestinal bleeding and lactulose for enterotoxins, such as ammonia. On the 10th hospital day, ALT, prothrombin activity and creatinine had improved to 372 IU/L, 100% and 1.79 mg/dL, respectively (Figure 1). Proteinuria had also disappeared. On the 21st hospital day, HGF decreased to 0.45 ng/mL and the patient was subsequently discharged from our hospital. One month later, his liver and renal function test had improved to within normal limits.

DISCUSSION

ARF is a common complication in patients with fulminant hepatitis, but it is also found in 1.5%-4.7% of nonfulminant hepatitis A[5]. Since the first case of ARF with hepatitis A reported by Wilkinson *et al*[6] in 1978, about 50 cases of hepatitis A-associated ARF have been described in the literature[5,7]. In an analysis of 208 hepatitis A patients, 15 of 205 non-fulminant and all three fulminant patients were complicated with ARF[8]. Moreover, hepatitis A patients with ARF had higher ALT, peak total bilirubin and more prolonged prothrombin time compared with patients without ARF. Excessive alcohol consumption and diabetes were more prevalent in the ARF patients. In these patients, development of ARF might be related to the degree of acute hepatic injury, because both long-term alcohol abuse and diabetes are associated with renal dysfunction[9,10]. His history of heavy alcohol consumption might have contributed to ARF.

Renal involvement with hepatitis B and C is well described. These mechanisms are thought to be associated with immune complex deposition[11]. The deposition of immune complexes containing hepatitis B surface antigen to glomeruli is the key pathological process of diffuse membranous glomerulonephritis. Hepatitis C virus (HCV) infection is associated with cryoglobulinemia and membranoproliferative glomerulonephritis. Deposition of immune complexes involving HCV and anti-HCV IgG is also important in HCV-related

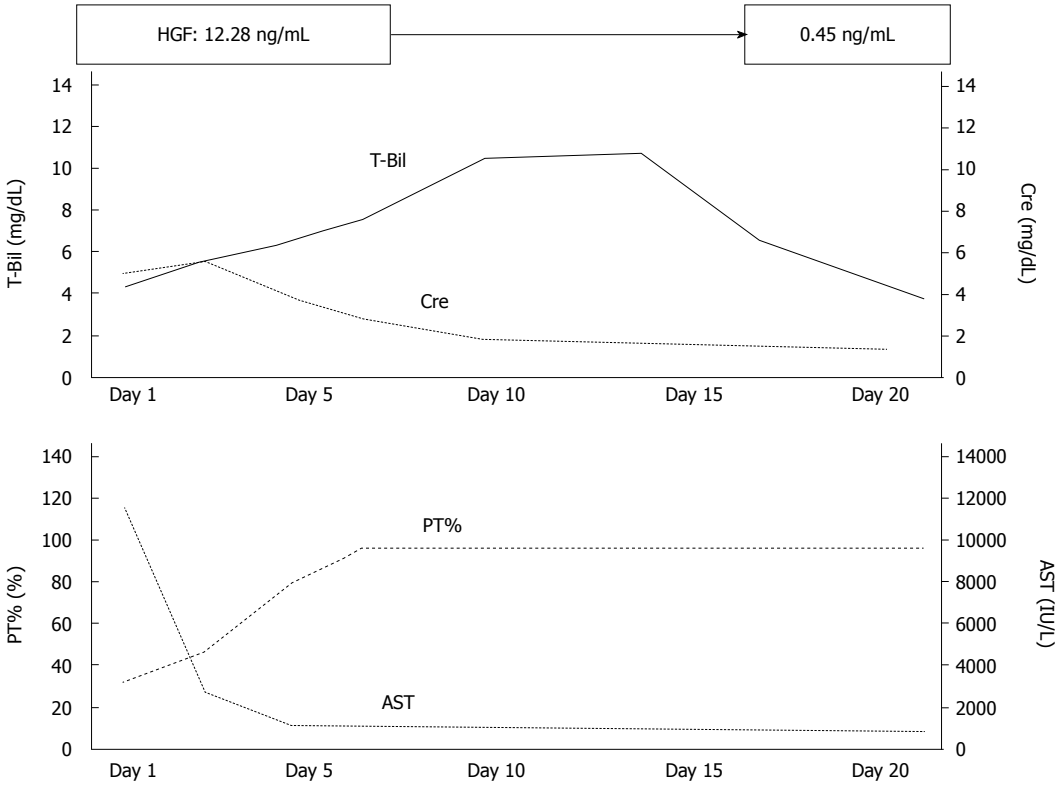

Figure 1 Clinical course of the patient. Laboratory findings revealed severe liver injury and renal failure on admission. Both the patient's general condition and laboratory data, including hepatocyte growth factor, were rapidly improved by supportive treatment. AST: Aspartate transaminase; T-Bil: Total bilirubin; PT: Prothrombin time; Cre: Creatinine.

renal disease. However, the mechanism of hepatitis A associated ARF is uncertain, and several possible mechanisms have been considered. First, because most hepatitis A patients complain of nausea, vomiting and diarrhea, circulatory insufficiency caused by dehydration may activate the renin-angiotensin system and may induce prerenal renal failure[5]. In our case, the patient had severe nausea, vomiting and poor oral intake. Second, immune complex-mediated nephritis is possible, because hepatitis A patients have been found to have several types of glomerular disorders, including membranous nephropathy, mesangial proliferative glomerulonephritis and membranoproliferative glomerulonephritis[12]. Although the present case also had proteinuria suggestive of glomerulonephritis, the level of C1q-binding immune complex in their sera was within normal limits. Third, endotoxemia induced by acute hepatitis may cause renal injury. Systemic hypotension, renal vasoconstriction, release of cytokines and activation of neutrophils, may all contribute to the development of renal injury from endotoxemia. Endotoxemia is often observed within 10 d of onset in patients with acute hepatitis A[13]. Although the present case was hospitalized at the fifth day after onset, serum endotoxin levels were within the normal range.

HGF is a Kringle-containing polypeptide growth factor originally identified as a potent mitogen for mature hepatocytes in primary culture[14]. HGF is produced and secreted as pro-HGF by stroma cells, such as fibroblasts, macrophages and renal mesangium, and predominantly acts on a variety of epithelial cells to regulate cell growth, cell motility and morphogenesis[15]. HGF is predictive factor of acute liver failure. Tsubouchi *et al*[3] reported that 14 of 15 fulminant hepatic failure patients with a maximum serum HGF over 10 ng/mL died. By contrast, in patients with acute liver failure, serum levels of both growth and growth-inhibitory factors are elevated[3,16]. It was reported that HGF and its receptor, the c-met system, was activated, but was hampered by elevated growth-inhibitory factors, such as TGF-β in patients with liver failure[17]. The reciprocal action of these factors is thought to be a result of impaired liver regeneration. The effectiveness of recombinant HGF as a therapeutic drug for acute liver failure has been reported[4]. Similarly, HGF is also effective for renal regeneration. HGF stimulates proliferation of renal epithelial cells and induces tubular formation *in vitro*[17]. HGF may function as a renotropic factor for regeneration with ARF[18]. Expression of HGF mRNA and HGF activity in the kidney increased in rat models with renal ischemia or $HgCl_2$ administration. This phenomenon suggested the possibility that HGF is a renotropic factor for renal regeneration following acute renal injury. Hence, recombinant HGF has been administered clinically as therapeutic drug for ARF.

In conclusion, although the present case with severe acute liver failure because of hepatitis A complicated with ARF and high serum HGF level, the

patient recovered rapidly without intensive treatment. Endogenous HGF may play an important role in recovery from injuries of liver and kidney.

COMMENTS

Case characteristics

A 58-year-old man with fever, malaise, loss of appetite and jaundice.

Clinical diagnosis

The patient had icteric skin and hepatomegaly, but no signs of dehydration.

Differential diagnosis

Acute hepatitis B, acute hepatitis C, biliary tract disease.

Laboratory diagnosis

Laboratory findings revealed severe liver injury, renal dysfunction and coagulopathy. The patient's serum was positive for serum immunoglobulin M anti-hepatitis A virus antibody. Serum level of hepatocyte growth factor (HGF) was extremely high (12.28 ng/mL).

Imaging diagnosis

An abdominal computed tomography scan revealed an enlarged liver and normal kidneys.

Treatment

The patient was treated with supportive therapy, such as administration of proton pump inhibitors and lactulose.

Related reports

ARF has been found in relatively few cases of nonfulminant hepatitis A. About 50 cases of hepatitis A associated ARF have been described in the literature. HGF was a predictive factor of acute liver failure. Fulminant hepatic failure patients with high serum HGF had a high mortality.

Experiences and lessons

The present case had severe acute liver failure caused by hepatitis A that was complicated with ARF and high serum HGF level. However, the patient recovered rapidly with supportive treatment. HGF might play an important role as a regenerative effector in injured livers and kidneys.

Peer-review

This article reports a rare case of severe hepatitis A complicated with ARF and high serum HGF level.

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CASE REPORT

Granulomatous reaction in hepatic inflammatory angiomyolipoma after chemoembolization and spontaneous rupture

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Institutional review board statement: We consider that ethics approval is not needed because this is a single case report without personal information.

Informed consent statement: Written informed consent was obtained.

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Abstract

A 77-year-old Japanese woman was transported to a nearby hospital due to sudden abdominal pain and transient loss of consciousness. Abdominal computed tomography (CT) suggested hemoperitoneum and hepatic nodule. She was conservatively treated. Contrast-enhanced CT two months later revealed an increased mass size, and the enhancement pattern suggested the possibility of hepatocellular carcinoma (HCC). Under a clinical diagnosis of HCC, transcatheter arterial chemoembolization (TACE) was performed. A subsequent imaging study revealed that most of the lipiodol used for the embolization was washed out. Therefore, surgical resection was performed. Histologically, the nodule contained numerous inflammatory cells including small lymphocytes, plasma cells and macrophages. Notably, epithelioid granulomatous features with multinucleated giant cells were observed in both the nodule and background liver. Some of the multinucleated giant cells contained oil lipid. Among the infiltrating inflammatory cells,

spindle-shaped, histiocytoid or myoid tumor cells with eosinophilic cytoplasm were found. The tumor cells were positive for Melan A and HMB45. The nodule contained many IgG4-positive plasma cells; these were counted and found to number 72.6 cells/HPF (range: 61-80). The calculated IgG4:IgG ratio was 33.2%. The nodule was finally diagnosed as previously ruptured inflammatory angiomyolipoma modified by granulomatous reaction after TACE.

Key words: Angiomyolipoma; Inflammatory; Rupture; Lipiodol; Liver

Core tip: Hepatic angiomyolipoma (AML) is a rare benign neoplasm. Particularly, the inflammatory variant of hepatic AML is extremely rare. Spontaneous rupture of hepatic AML is also extremely rare phenomenon. The authors herein present a case of spontaneously ruptured hepatic inflammatory AML associated with extensive granulomatous reaction induced by chemoembolization. This case is not only rare, but also suggests that chemoembolization rarely causes granulomatous reaction in hepatic tissue.

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INTRODUCTION

Hepatic angiomyolipoma (AML) is a rare benign neoplasm composed of variable admixtures of adipose tissue, smooth muscle and thick-walled blood vessels which was first described by Ishak in 1976[1]. Hepatic AML is currently considered to be a tumor of perivascular epithelioid cells (PEComa) having myomatous and lipomatous differentiation and melanogenesis[2,3]. Melanocytic markers, such as HMB-45 and Melan A were considered to be promising markers for AML in immunohistochemistry.

According to the differentiation, predominance of tissue components, and growth patterns, AML were subcategorized into classical/mixed, leiomyomatous, lipomatous, myelolipomatous, angiomatous/angiomyomatous, epithelioid, trabecular, oncocytic, pleomorphic and inflammatory variants[3,4]. Of these, the inflammatory variant is extremely rare, and differential diagnosis with other histologically similar tumors such as inflammatory pseudotumors (IPTs), inflammatory myofibroblastic tumors (IMTs) and follicular dendritic cell (FDC) tumors is necessary[5]. The authors herein present a unique case of spontaneously ruptured hepatic inflammatory AML modified by epithelioid granuloma, the histology of which was previously unreported.

CASE REPORT

A 77-year-old Japanese woman was transported to a nearby hospital due to sudden abdominal pain and transient loss of consciousness. She had no past medical history except for acute appendicitis 20 years earlier. She had no signs of tuberous sclerosis. Abdominal computed tomography (CT) suggested hemoperitoneum and hepatic nodule. The patient was transferred to our hospital for further examination and treatment. Laboratory tests on admission revealed mild anemia (red blood cell count, 3.60 × 10^6/μL; hemoglobin concentration, 10.6 g/dL; hematocrit, 25.6%), while the white blood cell and platelet counts were within the normal ranges (6300/μL and 17.7 × 10^4/μL, respectively). Serology and coagulation tests showed no abnormalities other than the following: total protein, 5.8 g/dL (normal, 6.7-8.3 g/dL); albumin, 3.2 g/dL (normal, 3.8-5.0 g/dL) and C-reactive protein, 1.12 mg/dL (normal, < 0.30 mg/dL). All examined tumor markers were within normal ranges, including alpha-fetoprotein (AFP) and vitamin K absence-Ⅱ (PIVKA-Ⅱ). The patient was negative for HBs antigen and HCV antibody but positive for HBc antibody and positive for antimitochondrial antibody (AMA)-M2. The titer of antinuclear antibody (ANA) was 1:160. The serum IgG level was within the normal range. Additional serum examination revealed no elevation of angiotensin-converting enzyme (ACE) or of the serum IgG4 level. Contrast-enhanced abdominal CT on admission showed subcapsular hematoma at the left lobe of the liver and hemorrhagic ascites around the spleen. A mass-like lesion measuring approximately 1.5 cm in diameter was also noted at segment 2 of the liver adjacent to the simple hepatic cyst (Figure 1A). These findings were confirmed by magnetic resonance imaging (MRI). Because of the stability of the patient's vital signs and the improvement of her anemia, the patient was treated conservatively and discharged on her tenth day in the hospital.

The patient was followed-up with periodic imaging studies. Contrast-enhanced abdominal CT performed two months after the onset revealed an increased mass size (approximately 2.3 cm in diameter) at segment 2 of the liver, and the enhancement pattern (high-low) of the nodule suggested the possibility of hepatocellular carcinoma (HCC). Under a clinical diagnosis of previously ruptured HCC, trancecatheter arterial chemoembolization (TACE) with miriplatin hydrate and lipiodol was performed at three months after the onset. Subsequent CT and MRI images revealed that most of the lipiodol used for the

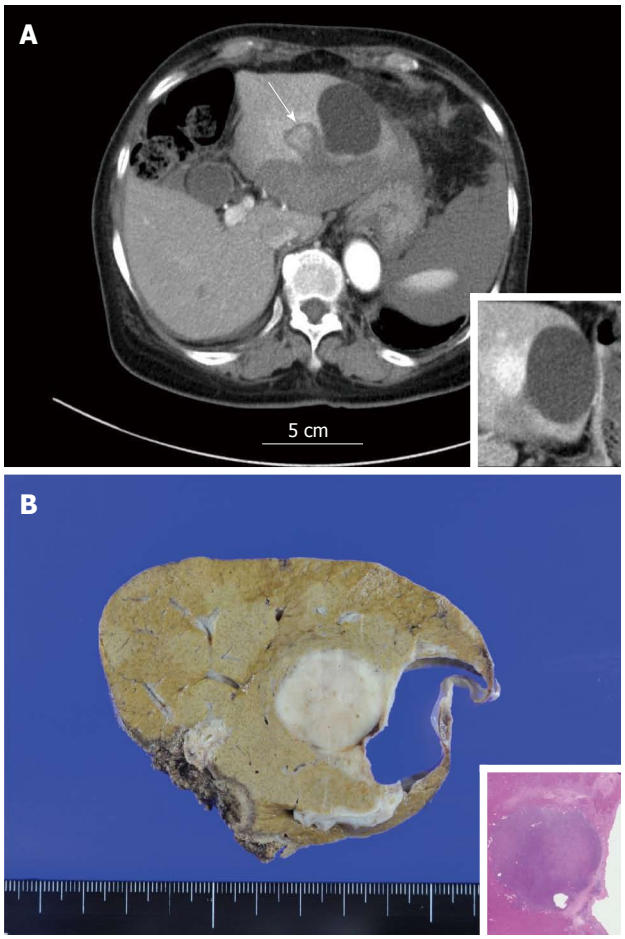

Figure 1 Computed tomography. A: Contrast-enhanced abdominal CT on admission. Subcapsular hematoma at the left lobe of the liver and hemorrhagic ascites around the spleen were observed. The mass-like lesion measuring approximately 1.5 cm is also visible at segment 2 of the liver adjacent to the simple hepatic cyst (arrow). Inset: CT taken two months after onset. The nodule was highly enhanced by contrast medium in the early phase; B: The cut section of the nodule. A well-demarcated brown to whitish solid mass lesion measuring 2.5 cm in diameter was observed. A simple hepatic cyst was adjacent to the nodule. Inset: loupe image of the nodule (HE). CT: Computed tomography.

embolization was washed out. Therefore, surgical resection (laparoscopic left lateral segmentectomy) was performed four months after the onset.

The cut section of the tumor (Figure 1B) revealed a well-demarcated brown to whitish solid mass lesion measuring 2.5 cm in diameter. A simple hepatic cyst was adjacent to the nodule. The nodule contained numerous inflammatory cells including small lymphocytes, plasma cells and macrophages. Thick-walled abnormal arteries were observed peripheral to and inside of the nodule (Figure 2A). Notably, epithelioid granulomatous features, namely epithelioid macrophages and multinucleated giant cells, were frequently observed (Figure 2B). Some of the multinucleated giant cells contained amorphous material which was positively stained by Oil Red O staining (Figure 2C and D). Among infiltrating inflammatory cells, spindle-shaped, histiocytoid or myoid tumor cells with eosinophilic cytoplasm and oval-shaped atypical nuclei with distinct nucleoli were found (Figure 2E). Lipomatous tissue was not apparent in the nodule. Mitotic figures and necrosis were not observed. In the background liver, bridging fibrosis, which would suggest the presence of chronic hepatitis, was not observed (Figure 3A). It should be noted that epithelioid granulomas were observed not only in the nodule, but also in many portal tracts in the background liver surrounding the nodule (Figure 3B). Granulomas were observed even in the intima of some hepatic arteries (Figure 3C). Inflammatory granulation tissue containing hemosiderin-laden macrophages and old blood coagula, which were consistent with a previous rupture and hemorrhage, were observed adjacent to the nodule (Figure 3D). The loss of small bile ducts and chronic non-suppurative destructive cholangitis (CNDSC) suggesting primary biliary cirrhosis (PBC) were not observed.

In the immunohistochemical analysis, the tumor cells were positive for vimentin but negative for pan cytokeratin (AE1/AE3), hepatocyte paraffin 1, c-kit and ALK-1. The tumor cells diffusely expressed melan A with various levels of intensity (Figure 4A) and focally expressed HMB45 (Figure 4B). Few tumor cells weakly expressed alpha-SMA (Figure 4C), but desmin was completely negative. Many IgG4-positive plasma cells were observed in the nodule (Figure 4D). The numbers of IgG- and IgG4-positive plasma cells were counted in three hot spots. The average numbers of IgG- and IgG4-positive plasma cells were 219.0 cells/HPF (range: 177-285) and 72.6 cells/HPF (range: 61-80), respectively. The calculated IgG4: IgG ratio was 33.2%. Based on the above histological and immunohistochemical findings, the nodule was finally diagnosed as inflammatory AML modified by granulomatous reaction with multinucleated giant cells.

The patient's clinical course after surgery was uneventful, and she was discharged from the hospital on the seventh postoperative day. No recurrent tumor has been observed as of the time of this writing (three and a half years after the operation).

DISCUSSION

An inflammatory variant of hepatic AML was first reported by Kojima *et al*[5] in 2004. Since then, only eight cases have appeared in the literature in English (Table 1)[5-8]. To make the diagnosis in these cases, it was essential to recognize histiocytoid or myoid tumor cells with eosinophilic granular cytoplasm among the prominent inflammatory cells and to confirm the expression of melanocytic markers by immunohistochemistry. Recently, a case of hepatic inflammatory AML which suggested an association with IgG4-related pseudotumor was reported[6]. Interestingly, our case also showed an elevated IgG4: IgG ratio; therefore, IgG4-related IPT was initially considered as a differential diagnosis. However, the

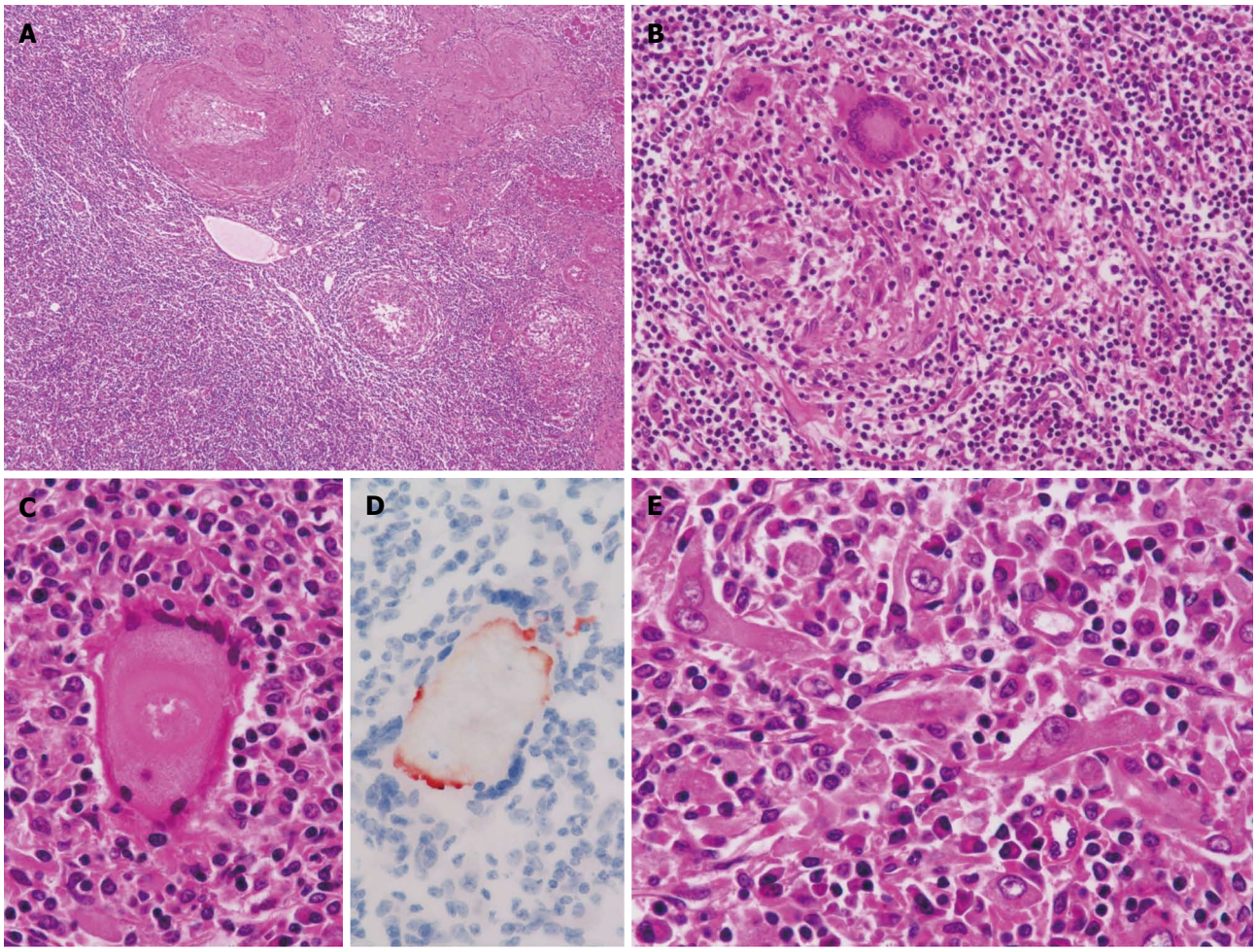

Figure 2 Histopathological features of the nodule. A: The nodule contained numerous inflammatory cells. Thick-walled abnormal arteries were observed peripheral to and inside of the nodule [hematoxylin and eosin (HE), × 40]; B: Epithelioid granulomatous features were frequently observed in the nodule (HE, × 200); C: Some of the multinucleated giant cells contained amorphous material (HE, × 400); D: The amorphous material was positively stained by Oil Red O staining (Oil Red O staining, × 400); E: Among the infiltrating inflammatory cells, spindled, histiocytoid or myoid tumor cells with eosinophilic cytoplasm and oval-shaped atypical nuclei with distinct nucleoli were found (HE, × 400).

lack of prominent reticular or storiform fibrosis, a normal serum IgG4 level and the absence of IgG4-positive plasma cells in the background portal tracts were negative findings for IgG4-related IPT[9]. Although in most of the reported hepatic AML cases IgG4-positive plasma cells were not investigated, the relationship between IgG4-positive plasma cells and inflammatory AML is an interesting subject. Other differential diagnoses that were considered in our case were spontaneous necrosis of HCC and tuberculosis. The former was ruled out due to the negativity of hepatocyte paraffin 1 and the absence of a remaining or wrecked tumor component. The latter was ruled out due to the absence of caseous necrosis and the negative polymerase chain reaction (PCR) findings for formalin-fixed tissue from the background liver and tumor.

Notable features of the present case included the granulomatous reaction and the multinucleated giant cells in both the nodule and the surrounding portal tracts of the background liver. It is known that IPTs sometimes contain multinucleated giant cells[9]. However, hepatic inflammatory AML containing multinucleated giant cells has never been reported[5-8]. The differential diagnoses for multiple granulomas included tuberculosis, systemic sarcoidosis, fungal infection and PBC. Tuberculosis was excluded due to the absence of caseous necrosis and the negative Ziehl-Neelsen stain and negative PCR results obtained using formalin-fixed tissue. Systemic sarcoidosis was ruled out because no lesion was found in radiological examination of the whole body and no elevation of the serum ACE level was observed. Although positive serum AMA-M2 suggested the possibility of PBC, it was ruled out due to the normal levels of serum biliary enzymes (such as γ-glutamyltransferase and alkaline phosphatase) and the negative pathological findings for a lack of small bile ducts and CNDSC. From the above diagnostic ruleouts and the finding that lipid material was confirmed in some multinucleated giant cells, we concluded that the granulomatous reaction of both the tumor and background liver were possibly

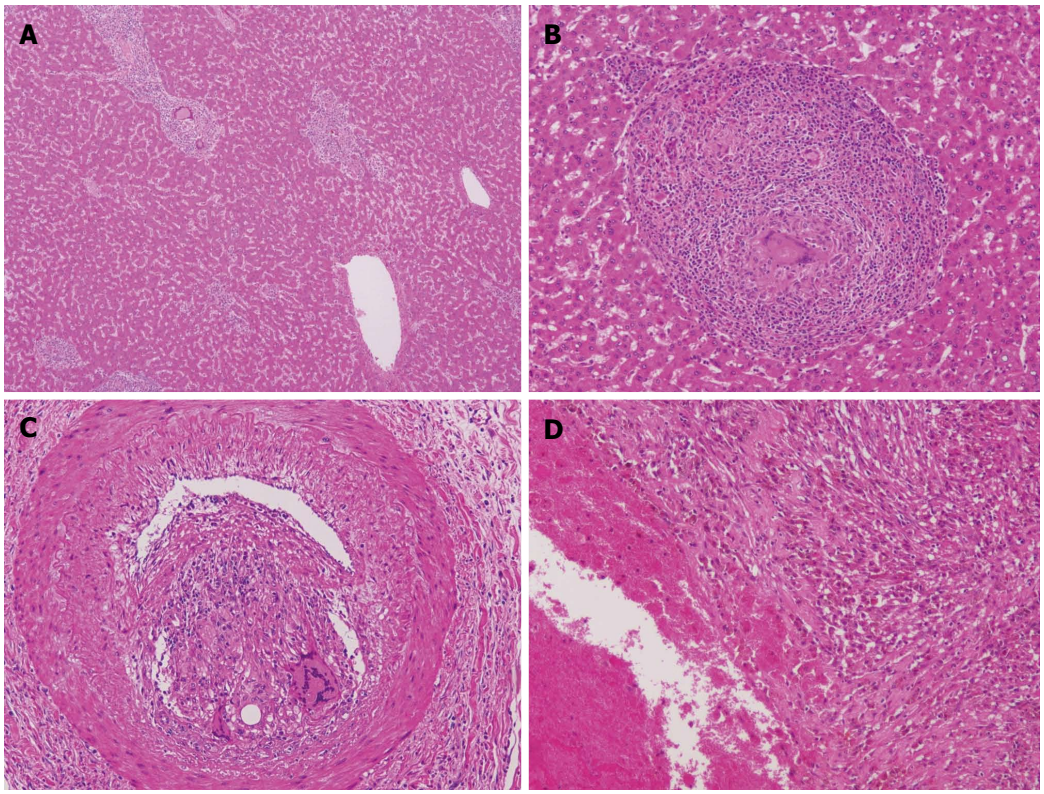

Figure 3 Histopathological features of the background liver. A: Bridging fibrosis was not observed (HE, × 40); B: Many epithelioid granulomas were observed in portal tracts in the surrounding background liver (HE, × 100); C: Granulomas were also observed in the intima of some hepatic arteries (HE, × 100); D: Inflammatory granulation tissue containing hemosiderin-laden macrophages and old blood coagula which were consistent with a previous rupture and hemorrhage were observed adjacent to the nodule (HE, × 100).

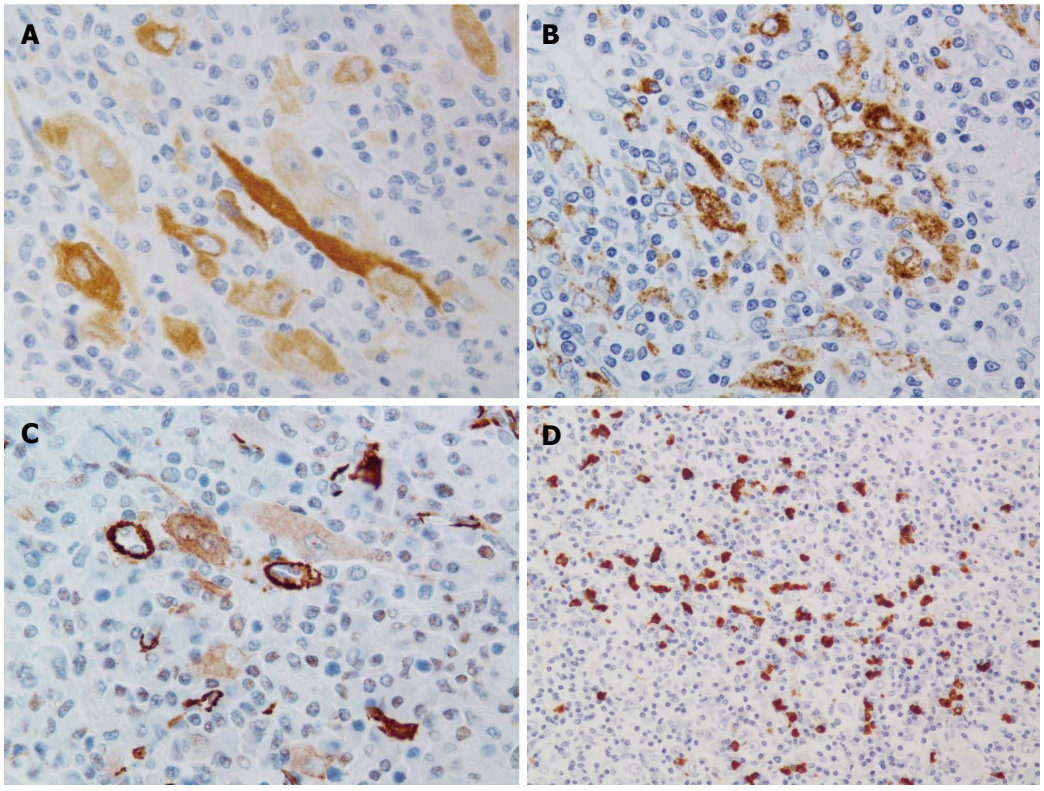

Figure 4 Findings of immunohistochemistry. A: Immunohistochemistry for melan-A (× 400). The tumor cells diffusely expressed melan A; B: Immunohistochemistry for HMB45 (× 400). The tumor cells focally expressed HMB45; C: Few tumor cells weakly expressed alpha-SMA (× 400); D: Many IgG4-positive plasma cells were observed in the nodule (× 200).

Table 1 Summary of previously reported hepatic inflammatory angiomyolipoma and ruptured hepatic angiomyolipoma in the literature in English

	Age	Sex	Size/location	Signs of TSC	Clinical diagnosis	Melanocytic markers	Symptom/onset	Treatment	Follow up after surgery
Previous cases of hepatic inflammatory AML (n = 8)[5-8]	41.3 (mean) 21-63 (range)	Male: 1 Female: 7	6.5 cm (mean) 3-10 cm (range) Lt. lobe: 5 Rt. lobe: 3	None (all cases)	IPT (by biopsy): 1 Mass lesion (without biopsy): 7	HMB 45 (+/-): 8/0 Melan A (+/-): 5/2	Pyrexia: 2 Hepatic mass: 4 Abdominal discomfort: 2	Elective resection (all cases)	All cases alive with no recurrence (follow up: 2-7 yr)
Previous cases of ruptured hepatic AML (n = 7)[3,15-20]	45.1 (mean) 22-74 (range) NA: 1	Male: 3 Female: 3 NA: 1	7.9 cm (mean) 5-10.5 cm (range) Lt. lobe: 2 Rt. lobe: 5	Present: 1 None: 6	Hepatic tumor: 4 Lipomatous tumor: 1 Hemangioma: 1 Hepatic AML: 1	HMB 45 (+): 2 Melan A (+): 1 NA (HMB 45): 5 NA (Melan A): 6	Hemorrhagic shock: 2 Upper abdominal pain: 3 Unkown:2	Emergent resection: 3 Emergent laparotomy for hemostasis: 2 Elective resection after emergent laparotomy for hemostasis: 1 Elective resection after TAE: 1	Alive with no recurrence:1 (follow up: 2 yr) NA: 6
Present case	77	Female	2.5 cm Lt. lobe	None	HCC (without biopsy)	HMB 45: + Melan A: +	Hemorrhagic shock	Elective resection after TAE	Alive with no recurrence (3 yr)

TSC: Tuberous sclerosis complex; IPT: Inflammatory pseudotumor; HCC: Hepatocellular carcinoma; NA: Not available.

induced by the intra-arterial injection of a lipiodolized agent. Although embolization is sometimes performed in renal AML, detailed histology has not been investigated after the embolization[10,11]. A few case reports of granulomatous reactions against lipiodol have been published, including one report of granulomas in HCC induced by lipiodolized styrene maleic acid conjugated neocarzinostatin (SMANCS)[12-14]. Consequently, the question arises as to whether inflammatory cells can secondarily infiltrate an ordinary AML as a result of TACE. However, we believe that the present case originally developed as an inflammatory AML because of its similarity to previous hepatic inflammatory AML series in terms of the morphology of the tumor cells and the composition of the inflammatory cells, with the exception of the granulomatous reaction.

Our case involved an outbreak of intraperitoneal bleeding. Hepatic tumors that cause spontaneous rupture and hemoperitoneum are usually HCCs. However, it is known that hepatic AML has also ruptured spontaneously and caused hemoperitoneum in rare cases. We could find only seven previously reported cases of spontaneously ruptured hepatic AML (Table 1)[3,15-20]. The previously reported cases involved large (5-10.5 cm) subcapsular tumors. Our case was also subcapsular but was the smallest (2.5 cm) ruptured hepatic AML among those reported. No ruptured case was found in the previously reported inflammatory AML series.

In summary, we have presented an extremely rare case of a spontaneously ruptured hepatic inflammatory AML modified by a granulomatous reaction after chemoembolization. Present case would be useful reference for the pathologists or clinicians who encounter similar case in the future.

ACKNOWLEDGMENTS

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COMMENTS

Case characteristics

A 77-year-old woman was transported to a hospital due to sudden abdominal pain and transient loss of consciousness.

Clinical diagnosis
Abdominal computed tomography (CT) revealed hemoperitoneum and hepatic nodule.

Differential diagnosis
Rupture of hepatocellular carcinoma, mass-forming intrahepatic cholangiocarcinoma, metastatic liver tumor and other hepatic tumors.

Laboratory diagnosis
Mild anemia was observed. All examined tumor markers were within normal ranges, including alpha-fetoprotein and vitamin K absence-Ⅱ.

Imaging diagnosis
Clinical diagnosis of ruptured hepatocellular carcinoma was made by the findings of contrast-enhanced abdominal CT.

Pathological diagnosis
Among the infiltrating inflammatory cells with epithelioid granulomatous features, tumor cells which were positive for Melan A and HMB45 were found. The nodule was finally diagnosed as previously ruptured inflammatory angiomyolipoma modified by granulomatous reaction after chemoembolization.

Treatment
Transcatheter arterial chemoembolization was initially performed and then surgical resection (laparoscopic left lateral segmentectomy) was performed.

Related reports
Only eight cases of hepatic inflammatory angiomyolipoma, seven cases of spontaneously ruptured hepatic angiomyolipoma and a few cases of granulomatous reactions against lipiodol have appeared in the literature in English.

Term explanation
Hepatic angiomyolipoma is a rare benign neoplasm having myomatous and lipomatous differentiation and melanogenesis. Its inflammatory variant is extremely rare.

Experiences and lessons
This report provides valuable experience of hepatic inflammatory angiomyolipoma involving spontaneous rupture and granulomatous reaction due to chemoembolization. We believe present case would be useful reference for the pathologists or clinicians who encounter similar case.

Peer-review
This is a well written case series and literature review. Although the pathological features were well described and literatures were reviewed completely, details of CT manifestations were not well revealed.

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