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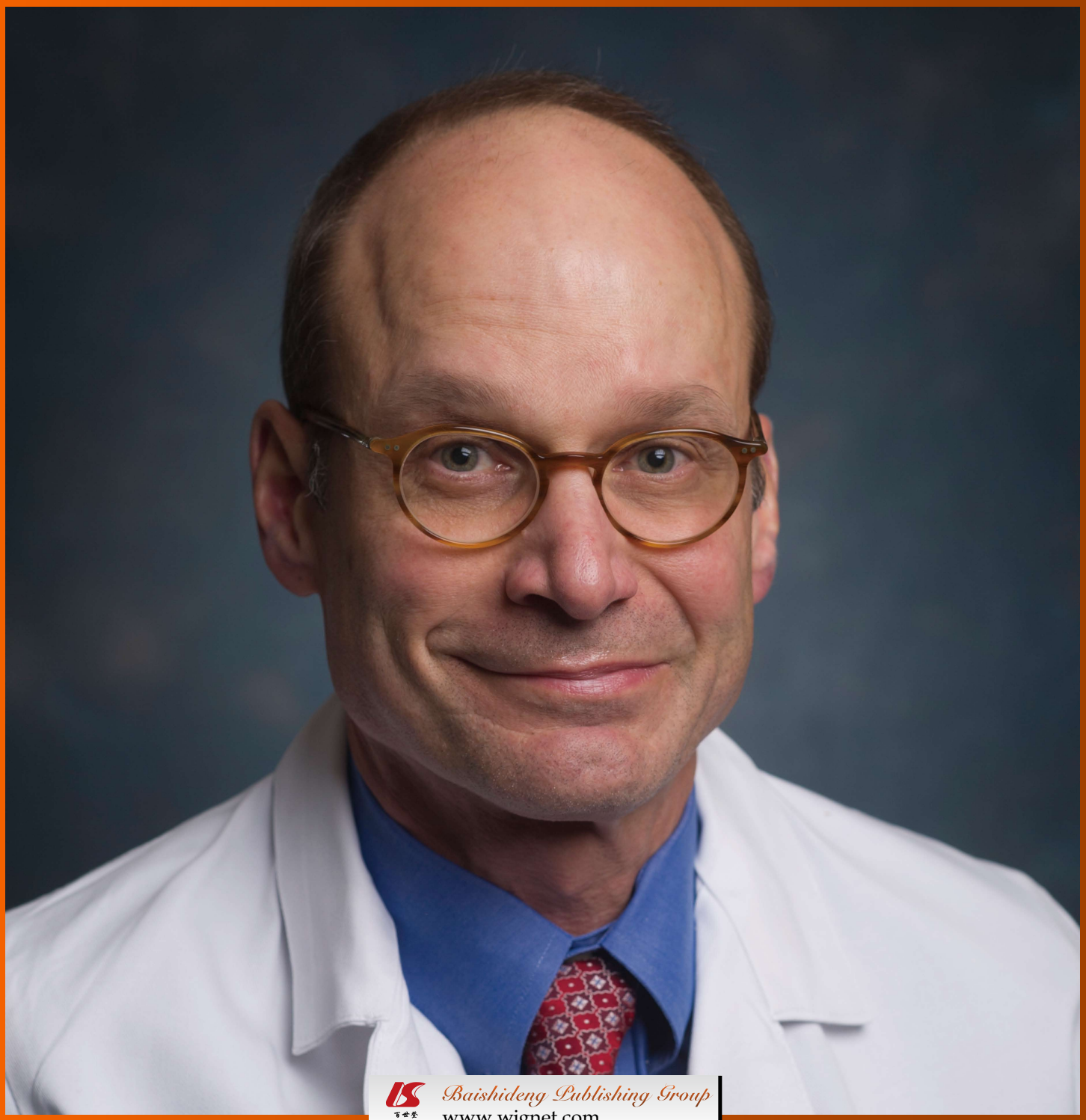

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TOPIC HIGHLIGHT

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Colorectal cancer surveillance: What's new and what's next?

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Abstract

The accumulated evidence from two decades of randomized controlled trials has not yet resolved the question of how best to monitor colorectal cancer (CRC) survivors for early detection of recurrent and metachronous disease or even whether doing so has its intended effect. A new wave of trial data in the coming years and an evolving knowledge of relevant biomarkers may bring us closer to understanding what surveillance strategies are most effective for a given subset of patients. To best apply these insights, a number of important research questions need to be addressed, and new decision making tools must be developed. In this review, we summarize available randomized controlled trial evidence comparing alternative surveillance testing strategies, describe ongoing trials in the area, and compare professional society recommendations for surveillance. In addition, we discuss innovations relevant to CRC surveillance and outline a research agenda which will inform a more risk-stratified and personalized approach to follow-up.

Key words: Colorectal cancer; Surveillance; Follow-up; Recurrence; Relapse; Survivorship

Core tip: We summarize the current state of knowledge and recommended practice around post-treatment surveillance of colorectal cancer survivors. In addition, we describe relevant ongoing trials and the questions which they will and will not answer regarding best surveillance practices. With that background as context, we discuss related practice innovations and propose a number of research questions whose answers could inform more effective, personalized approaches to surveillance.

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INTRODUCTION

Globally, over 1 million individuals develop colorectal

cancer (CRC) each year[1]. Approximately two-thirds will be treated surgically with curative intent[2]. Among those treated curatively, around one-third will experience recurrence of the original cancer or a second primary (*i.e.*, metachronous) colorectal cancer[3]. At least 80% of these recurrences occur within the first three years following initial treatment, while nearly all will have manifested by year five[4,5]. Most patients who recur will survive less than two years[6]. Ultimately, nearly 50000 patients in the United States alone die each year from colorectal cancer[2], with mortality attributable to both advanced stage at initial diagnosis and recurrent disease.

The majority of CRC survivors undergo some form of surveillance to detect recurrence of original disease or development of metachronous CRC. The primary rationale for such surveillance is to improve outcomes by detecting recurrent or metachronous disease before onset of symptoms, at a point where curative reoperation is more likely[7]. Other reasons for conducting surveillance of survivors include psychological benefits to the patient, monitoring patients for side effects of treatment, collecting data on patient outcomes, and detecting other comorbidities.

Despite the theoretical benefits of CRC surveillance, substantial uncertainty still exists around the topic. Though surveillance has been associated with a modest overall survival benefit, improvements in cancer-specific survival have not been shown. Furthermore, the body of research in this area has not consistently pointed to a set of specific best practices for follow-up. Most recurrences detected by surveillance are not curable[8], leading to an increasing sentiment that a more customized, risk-adapted approach to follow-up is needed[9-11]. In this review, we will summarize the evidence which has been gleaned from randomized controlled trials (RCTs) of alternative surveillance testing strategies, provide updates on ongoing trials which promise additional insight, and compare professional society recommendations for surveillance. In addition, we will highlight potential innovations in surveillance-many of which will likely form the basis for a more personalized approach to surveillance in the future-and highlight areas where research is needed to address key unanswered questions. The purpose of this work is not to provide a systematic review or meta-analysis of CRC surveillance studies (others have done so superbly in recent years[10,12-14]). The purpose, rather, is to broadly describe the current state of knowledge and practice around CRC surveillance, and to higihlight the recent developments and key research questions that will shape future practice.

SEARCH STRATEGY

We identified relevant resources based on (1) PubMed searches of randomized controlled trial comparing CRC surveillance strategies; (2) ClinicalTrials.gov searches of ongoing CRC surveillance trials; (3) the authors' personal databases of related publications; (4) related scientific meeting presentations; and (5) the bibliographies of reviewed publications.

WHAT THE TRIALS TELL US

Published data from seven completed randomized controlled trials comparing alternative surveillance regimens describe the experience of some 1938 survivors of Stage Ⅰ-Ⅲ (Dukes A-C) CRC. These subjects, enrolled between 1983 and 2004, experienced 698 recurrences or instances of metachronous CRC[15-21]. Table 1 summarizes the enrollment periods, settings, stage-based inclusion criteria, and follow-up protocols examined in each of these trials. Table 2 summarizes the subject make-up and results of each trial.

Meta analyses by Tjandra *et al*[10] and Jeffery *et al*[12] have incorporated results from these trials. The primary outcome examined by both meta-analyses was overall survival (OS). Tjandra *et al*[10] included all seven available RCTs in their analysis of OS, plus preliminary results from an ongoing trial[22]. Both analyses detected statistically significant improvements in all-cause mortality with respective odds ratios (OR) of 0.74 (95%CI: 0.59-0.93)[10], and 0.73 (95%CI: 0.59-0.91)[12] for the effect of intensive follow-up relative to less intensive follow-up. However, neither meta-analysis found that cancer-specific survival was improved by intensive surveillance (although only two of the constituent RCTs reviewed[15,17] included this key endpoint as an outcome).

The two meta analyses revealed that both intensive and less intensive surveillance led to detection of a similar number of recurrences but that detection occurred between 5.91 mo (95%CI: 3.09-8.74)[12] and 6.75 mo (95%CI: 2.44-11.06)[10] earlier with intensive surveillance. Both analyses also found that curative reoperation ("salvage surgery") was significantly more likely in those subjects who were followed up intensively (OR = 2.41, 95%CI: 1.63-3.54)[10] and (OR = 2.81 95%CI: 1.65-4.79[12]). An earlier meta-analysis by Renehan *et al*[23] included six of the trials described in Tables 1 and 2, and estimated that only about one-fifth of the survival benefit of intensive surveillance was likely due to curative treatment of recurrence. The authors postulated that the remainder of the survival benefit was most likely due to some combination of increased psychological support and well-being, improved health behavior, and improved detection and management of comorbidities[13]. Thus, the increased overall survival, earlier detection of recurrence, and higher reoperation rates seen in trials provide only circumstantial evidence that intensive surveillance extends life by making cure of recurrent disease more likely.

NEXT GENERATION OF CRC SURVEILLANCE TRIALS

The body of RCT-based evidence in the area of CRC surveillance to date has a number of limitations. First, it consists of a series of small studies spanning a period of more than two decades, with no two trials having examined the same surveillance regimen in the same setting (Table 1). Beyond this heterogeneity in interventions, a series of treatment innovations over the years

Table 1 Five-year surveillance regimens tested in reviewed randomized controlled trials

	Enrollment period	Setting	Stages included	Type of regimen	Surveillance regimen
Ohlsson *et al*[15]	1983-1986	2 Swedish centers	Dukes A, B, C	Intensive	History and physical exam, rigid proctosigmoidoscopy, CEA, Alk Phos, liver function tests, fecal hemoglobin, and chest X-ray at 3, 6, 9, 12, 15, 18, 21, 24, 30, 36, 42, 48 and 60 mo; endoscopic visualization of the anastomosis at 9, 21, and 42 mo; complete colonoscopy at 3, 15, 30 and 60 mo; pelvic CT (rectal cancer only) at 3, 6, 12, 18 and 24 mo
				Minimal	No structured follow-up. Advised to obtain fecal hemoglobin tests every 3 mo for 2 years, then annually. Instructed to seek care if a series of warning signs/symptoms were experienced
Mäkelä *et al*[16]	1988-1990	1 Finnish center	Dukes A, B, C	Intensive	History and physical exam CEA, CBC fecal hemoglobin at 3, 6, 9, 12, 15, 18, 21, 24, 30, 36, 48, 54 and 60 mo; Flexible sigmoidoscopy (if rectal/sigmoid tumors) every 3 mo; Liver ultrasound every 6 mo; Colonoscopy and liver CT annually
				Minimal	History and physical exam CEA, CBC fecal hemoglobin, CXR (and rigid sigmoidoscopy if rectal cancer) at 3, 6, 9, 12, 15, 18, 21, 24, 30, 36, 42, 48, 54, 60 mo; Barium enema at 12, 24, 36 48 and 60 mo
Kjeldsen *et al*[17]	1983-1994	A single Danish county	Dukes A, B, C	Intensive	History and physical exam including digital rectal exam and gynecologic exam, hemoglobin, erythrocyte sedimentation rate, liver enzymes, fecal hemoglobin, colonoscopy, and chest X-ray at 6, 12, 18, 24, 30, 36, 42, 48, 54, 60, 120, 150 and 180 mo
				Minimal	The same investigations as above, but only at 60, 120, and 180 mo
Pietra *et al*[18]	1987-1990	1 Italian center	Dukes B, C	Intensive	History and physical exam, liver ultrasound, and CEA at 3, 6, 9, 12, 15, 18, 21, 24, 30, 36, 42, 48, 54, 60 mo; CT, Chest X-ray and colonoscopy annually
				Minimal	History and physical exam, liver ultrasound, and CEA at 6, 12, 24, 36, 48, and 60 mo; Chest X-ray and colonoscopy annually
Schoemaker *et al*[19]	1984-1990	Multiple Australian centers	Dukes A, B, C	Intensive	History and physical exam, CEA, CBC, liver function tests, and fecal hemoglobin at 3, 6, 9, 12, 15, 18, 21, 24, 30, 36, 42, 48, 54, 60 mo; Chest X-ray, liver CT, and colonoscopy annually
				Minimal	History and physical exam, CEA, CBC, liver function tests, and fecal hemoglobin at 3, 6, 9, 12, 15, 18, 21, 24, 30, 36, 42, 48, 54, 60 mo; Chest X-ray, liver CT, and colonoscopy at 60 mo
Secco *et al*[20]	1988-1996	1 Italian center	Low-risk	Intensive risk-adapted	History and physical, CEA, abdominal/pelvic ultrasound at 6, 12, 18, 24, 36, 48, and 60 mo; Chest X-ray annually; Rectal cancer only: Rigid proctosigmoidoscpy at 12, 24 and 48 mo
				Minimal	Telephone follow-up every 6 mo; History and physical exam annually
			High-risk	Intensive risk-adapted	History and physical and CEA at 3, 6, 9, 12, 15, 18, 21, 24, 28, 32, 36, 42, 48, 54, and 60 mo; Abdominal/pelvic ultrasound at 6, 12, 18, 24, 30, 36, 48 and 60 mo; Rigid proctosigmoidoscopy (rectal cancer only) and chest X-ray annually
				Minimal	Telephone follow-up every 6 mo; History and physical exam annually
Rodríguez-Moranta *et al*[21]	1997-2001	3 Spanish centers	TNM Ⅱ and Ⅲ	Intensive	History and physical, CEA, CBC, and liver function tests at 3, 6, 9, 12, 15, 18, 21, 24, 30, 36, 42, 48, 54 and 60 mo; Abdominal/pelvic CT (rectal cancer only) or Abdominal ultrasound (colon cancer only) at 6, 12, 18, 24, 36, 48, 60 mo; Chest X-ray and colonoscopy annually
				Minimal	History and physical, CEA, CBC, and liver function tests at 3, 6, 9, 12, 15, 18, 21, 24, 30, 36, 42, 48, 54 and 60 mo; Colonoscopy at 12 and 36 mo

CEA: Carcinoembryonic antigen assay; CBC: Complete blood count; Alk phos: Alkalilne phosphatase; CT: Computed tomography.

has changed the context of the problem by making recurrence-free survival increasingly more likely (since recruitment of the trials reviewed began in 1983, CRC survival has improved by 5%-10% overall[24]). These innovations include emergence of total mesorectal excision as a standard of care for rectal cancer in many settings, widespread use of adjuvant chemotherapy in Stage Ⅲ and many Stage Ⅱ patients, and the growing practice of attempting to curatively treat oligometastatic hepatic recurrences[25,26]. Whether or not some of these innovations have changed the behavior of recurrent disease itself is difficult to know, but the possibility cannot be excluded. Importantly, improvements in imaging technology have also enabled earlier and more accurate detection of recurrent disease, while increasing the potential for false positives[27,28].

This evolution of technology and practice throws into question the relevance of much of the evidence behind current recommendations for surveillance. Fortunately, there are three large, ongoing RCTs (described below), with targeted sample sizes totaling over 8000 subjects, which will eventually shed additional light on the benefits of CRC surveillance and the comparative effectiveness of a handful of unique follow-up protocols.

FACS (Follow-up after Colorectal Surgery) Trial

The FACS trial (ClinicalTrials.gov identifier NCT00560365) opened in 2004 with a target recruitment of 4890 patients. The primary objective of this factorial trial is to examine the effect of augmenting symptomatic surveillance in primary care with two intensive methods of surveillance [frequent monitoring of carcinoembryonic antigen (CEA) in a primary care setting and intensive computed tomography (CT) imaging in a hospital setting]

Table 2 Results of reviewed randomized controlled trials of colorectal cancer surveillance strategies *n* (%)

	Type of regimen	*n*	Stages[1]	Rectal cancer	Follow-up time	Recurrences[2]	Symptoms were first sign of recurrence	Time to recurrence (mo): mean ± SD /*median*	Reoperated (% of recurrences)	Overall survival at 5 yr	Cancer-related survival at 5 yr	Survival of recurring patients 5 yr after first treated
Ohlsson *et al*[15]	Intensive	53	A/B/C: 19%/40%/41%	36%	6.8 yr median (overall)	17 (32)	8 (47)	*20*	5 (29)	75%	78%	29%
	Minimal	54	A/B/C: 17%/48%/35%	31%		18 (33)	15 (83)	*24*	3 (17)	67%	71%	22%
Mäkelä *et al*[16]	Intensive	52	A/B/C: 24%/46%/29%	31%	NR	22 (42)	3 (14)	10 ± 5	5 (22)	59%	NR	NR
	Minimal	54	A/B/C: 28%/44%/28%	28%	NR	21 (39)	4 (19)	15 ± 10	3 (14)	54%	NR	NR
Kjeldsen *et al*[17]	Intensive	290	A/B/C: 23%/51%/26%	46%	55% still followed at 5 yr (overall)	81[3] (28)	38 (47)	18	17 (21)	70%	78%[4]	NR
	Minimal	307	A/B/C: 23%/47%/30%	49%		83[3] (27)	59 (71)	27	5 (6)	68%	78%[4]	NR
Pietra *et al*[18]	Intensive	104	A/B/C: 0%/60%/40%	30%	100% still followed at 5 yr (overall)	41 (39)	10[5] (42% of local recurrences)	10.3 ± 2.7[5]	21 (51)	73%	NR	38%
	Minimal	103	A/B/C: 0%/58%/42%	36%		42[6] (41)	10[5] (83% of local recurrence)	20.2 ± 6.1[5]	6 (14)	58%	NR	0%
Schoemaker *et al*[19]	Intensive	167	A/B/C: 25%/47%/28%	28%	NR	56 (34)	NR	NR	6 (11)	77%[4]	NR	NR
	Minimal	158	A/B/C: 19%/48%/33%	26%	NR	64 (41)	NR	NR	5 (8)	70%[4]	NR	NR
Secco *et al*[20]	Low-risk–risk-adapted	84	A/B: 100%	NR	Median 42 mo	27 (32)	32%[7]	*16*	6 (22)	80%	NR	NR
	Low risk–minimal	61	A/B: 100%	NR	NR	25 (40)	75%[7]	*14*	6 (24)	60%	NR	NR
	High-risk–risk-adapted	108	A/B: 36% C: 64%	NR	Median 61.5 mo	74 (68)	32%[7]	*13.5*	25 (34)	50%	NR	NR
	High risk–minimal	84	A/B: 20% C: 80%	NR	NR	58 (69)	75%[7]	*8*	7 (12)	32%	NR	NR
Rodríguez-Moranta *et al*[21]	Intensive	127	Ⅱ: 60% Ⅲ:40%	23%	Median 49 mo	35 (27)	NR	39 ± 21	18 (51)	75%[4]	NR	NR
	Minimal	132	Ⅱ: 61% Ⅲ:39%	28%	Median 45 mo	34 (26)	NR	38 ± 19	10 (29)	73%[4]	NR	NR

[1]A, B and C refer to Dukes staging, while Ⅰ, Ⅱ and Ⅲ refer to TNM staging; [2]Includes metachronous colorectal cancers (CRCs); [3]Includes 7 cases of metachronous CRC in the intensive group and 3 in the less intensive group; [4]Estimated visually from survival curve; [5]Reported for local recurrences only; [6]Includes 1 case of metachronous CRC; [7]Reflects combined high-risk and low-risk groups. NR: Not reported; "Overall" describes all trial arms combined.

on survival of patients with stage Ⅰ, Ⅱ or Ⅲ colorectal cancer who have undergone curative resection[29]. In 2013, the FACS investigators presented interim results summarizing a mean 3.7 years of follow-up for 1,202 participants. Only 6.0% of participants had recurrence with subsequent attempted curative resection. Those followed by frequent CEA monitoring had an adjusted OR for attempted cure of recurrence of 2.7 (P = 0.035) relative to the minimal follow-up group which received only a single CT at 12-18 mo. Those followed by serial CT's had an adjusted OR of 3.4 (P = 0.007) relative to the minimum follow-up group. No additional benefit was seen in the group which received both frequent CEA and frequent CT's. In interim analyses, there were no differences seen in overall or cancer-specific mortality between any of the intensive arms and the minimum follow-up arm[30], though the final results are not yet available.

COLOFOL (Assessment of Frequency of Surveillance after Curative Resection in Patients with Stage Ⅱ and Ⅲ Colorectal Cancer)

This multicenter RCT (ClinicalTrials.gov identifier NCT00225641) is comparing two surveillance regimens involving CT-scan or MR scan of the liver, CEA, and CT scan or X-ray of the lungs at intervals of either 12 and 36 mo, or 6, 12, 18, 24 and 36 mo. The study aims to include 2500 subjects[31]. Primary outcomes will be total mortality and cancer specific mortality at five years, while secondary outcomes will include recurrence-free survival, quality of life, and cost effectiveness. Centers from Denmark (n = 15), Sweden (n = 20), Poland (n = 6), Hungary (n = 2) and The Netherlands are participating. Publication is planned for late 2014[32].

Table 3 Summary of United States and European colorectal cancer surveillance guidelines

	ASCO[33] 2005	ASCRS[34,35] 2005	NCCN[36,37] 2014	Denmark[38] 2009	Norway[39] 2012	United Kingdom[40] 2010
Stage	Ⅱ-Ⅲ	Ⅰ-Ⅲ	Ⅰ-Ⅲ	Ⅱ-Ⅲ	Ⅱ-Ⅲ	Ⅰ-Ⅲ
History and physical	q3-6 mo × 3 yr; q 6 mo in year 4-5	At least q4 mo × 2 yr	q3-6 mo × 2 yr; q6 mo in year 3-5	At 1 mo	q6 mo × 3 yr, q12 mo in year 4-5	None
CEA	q3 mo × at least 3 yr	At least q4 mo × 2 yr	q3-6 mo × 2 yr; q6 mo in year 3-5	At 1, 12 and 36 mo	q6 mo × 3 yr, q12 mo in year 4-5	None
CT chest	Annually × 3 yr if high risk	None	Annually up to 5 yr if high risk	At 12 and 36 mo	Annually × 5 yr	None
CT abdomen/ pelvis	Annually × 3 yr if high risk	None	Annually up to 5 yr if high risk	At 12 and 36 mo	At 6 mo and 5 yr	Once within first 2 yr
CEUS liver	None	None	None	None	At 12, 18, 24, 30, 36 and 48 mo	None
Colonoscopy	At 3 yr and q5 thereafter	q3 yr	At 1 and 4 yr, then q5 yr	None	At 5 yr;or CT colonography at 5 yr	q5 yr

NCCN: National Comprehensive Cancer Network; ASCO: American Society of Clinical Oncology; ASCRS: American Society of Colon and Rectal Cancer Surgeons; UK: United Kingdom 2010 guidelines; Nor: Norwegian 2012 guidelines; CEUS: Contrast-enhanced ultrasound; CEA: Carcinoembryonic antigen; CT: Computed tomography.

GILDA (Gruppo Italiano di Lavaro per la Diagnosi Anticipata)

Based in Italy, The GILDA group of investigators is conducting a randomized trial of intensive versus less intensive follow up in patients with Dukes B2-C CRC. Varying between study groups are the frequencies of office visits, CEA and other blood chemistries, colonoscopies, liver ultrasound, chest X-ray, and-in the case of rectal cancer survivors-proctoscopy and abdominal-pelvic CT. Outcomes of interest include overall survival, CRC mortality, quality of life and time to detection of recurrence. The GILDA investigators aim to enroll a minimum of 1500 patients across 45 centers. An interim analysis of 985 patients, published in 2004, did not demonstrate any improvement in overall survival between the two surveillance arms, though mean follow-up at the time was only 14 mo[22].

SURVEILLANCE GUIDELINES

Based on the accumulated trial evidence, a number of organizations have published surveillance recommendations[33-40]. These suggested regimens employ various combinations of carcinoembryonic antigen assays, chest CT, CT abdomen-pelvis, and contrast enhanced ultrasound of the liver. Chest X-ray and plain ultrasound of the liver are not used as a recommended test modality in any of the reviewed guidelines due to their low sensitivity and specificity. Some authors have argued for regular use of Positron Emission Tomography scanning and increased use of tumor markers, but this is not commonly accepted as a standard of practice[17,18]. Table 3 provides a summary of surveillance recommendations from the United States and Europe. There is a moderate amount of variation between the United States recommendations published by the American Society of Clinical Oncology[33], the American Society of Colon and Rectal Surgeons[34,35], and the National Comprehensive Cancer Network[36,37]. Internationally, though, the range in aggressiveness of recommended follow-up is striking, with European societies tending to prescribe much less intensive surveillance-particularly in the case of the United Kingdom's National Health Service[40]-in comparison to United States societies.

It is noteworthy that CEA assay represents the only testing modality whose increased use is associated with higher probability of detection of asymptomatic recurrence, higher curative reoperation rate, and greater mortality reduction in meta-analysis[10]. Ironically, studies of guideline adherence suggest that, across testing modalities used in surveillance, adherence to scheduled CEA testing is among the lowest[41,42]. Future research might focus on better outlining correlates and causes of this non-adherence[41].

INNOVATIONS IN SURVEILLANCE

In the last decade, a handful of investigators have reported on provider care models aimed at delivering more patient-centered, cost-effective survivorship care. These studies have explored alternatives to the conventional model of surgeon-led follow-up in a hospital-based clinic. For instance, Australian investigators randomized 203 recently-treated CRC survivors to identical follow-up regimens led by either surgeons or general practitioners. Rates of recurrence, time to detection, mortality, and quality of life were similar between the groups, but surgeons tended to initiate significantly more colonoscopies and ultrasounds, whereas general practitioners ordered more fecal hemoglobin tests[43]. Similarly, a recent Norwegian trial randomized 110 CRC survivors to either traditional hospital-based surveillance coordinated by surgeons, or community-based surveillance coordinated by general practitioners (GP's). No differences were observed in patient quality-of-life or time to detection of recurrence. Costs, however, were 16.7% lower ($P < 0.001$) in the GP-organized group[44].

Between 2002 and 2005, a Swedish trial randomized CRC survivors to post-treatment follow-up by either a surgeon or a specially-trained nurse. Surgeons and nurses

found similar numbers of recurrences with nearly identical levels of patient satisfaction. Nurses, however, spent an average of eight minutes longer with patients than did surgeons, requiring assistance from surgeons only 7% of the time[45].

Despite these results, whether or not surgeons or patients will allow generalists to direct CRC survivorship care on a large scale remains to be seen. The relationships developed during active treatment can make such handoffs difficult for providers and patients alike[46]. For those adhering to the surgeon-led follow-up model, a promising innovation might be found in the work reported by a British surgeon in the late 1990s[47]. This surgeon developed and measured the impact of a dedicated "one-stop shop" model for a CRC surveillance clinic. This model, which facilitated completion of all scheduled imaging, blood tests, and procedures in a single visit, yielded a substantial improvement in timely receipt of recommended tests compared to the period before establishment of the clinic.

Moving toward risk adapted follow-up

A series of authors over the last two decades have argued for an approach to surveillance that involves tailoring surveillance plans based on recurrence risk[9-11,20,48,49]. Though the idea is intuitively appealing as a way to spare certain patients some of the morbidity associated with surveillance and to reduce costs, little data exists on the topic. Secco and colleagues divided patients who had recently undergone curative treatment for CRC into high-risk and low-risk groups based on a number of prognostic factors. Within each of these risk groups, patients were randomized to either very minimal follow-up or a risk-adapted follow-up protocol (Table 1). Within each risk group, the risk-adapted follow-up patients showed significantly better five-year overall survival[20]. Unfortunately, there was no comparison of an overall strategy of tailoring follow-up to the risk of recurrence versus a one-size-fits-all approach of following all patients using a uniform protocol.

Any version of risk-adapted follow-up in the future will likely employ the use of molecular markers to target patients who might benefit the most form a more intensive level of surveillance. Most work on biomarkers to date has focused on prognostic markers of overall outcome or predictive markers of response to adjuvant chemotherapy. These types of markers hold great promise in informing decision making around adjuvant chemotherapy. Certain prognostic markers which may predict recurrence have the potential to inform surveillance planning after treatment. Vascular Endothelial Growth factor overexpression[50,51] and interleukin-8 overexpression[52] in tumor cells eventually may serve as such markers. Limited evidence suggests that each may signal a heightened risk of recurrence[50-52].

A "Recurrence Score" calculated based on a commercially available tumor gene expression panel (OncotypeDX - Genomic Health, Redwood City, CA, United States) has been validated as a predictor of recurrence in Stage Ⅱ CRC and is advocated as a tool for deciding whether or not to commit these patients to adjuvant chemotherapy[53-55]. Another application of this tool, and an idea which deserves further study, is the use of the recurrence score in individualized surveillance planning. Patients and their providers might opt for more aggressive surveillance if the likelihood of recurrence was high, whereas a low recurrence score might offer reassurance that minimal surveillance was a reasonable course.

The ideal set of recurrence markers would include one or more factors having low correlation with prognosis. In this way, patients could be categorized into four categories based on the two dimensions of recurrence risk and prognosis-given-recurrence. Patient with high recurrence risk but good prognosis-given-recurrence might be followed aggressively since probabilities of both detecting and successfully treating a recurrence would be high. Conversely, patients with low recurrence risk but poor prognosis-given-recurrence might opt for little or no follow-up.

OTHER AREAS FOR FUTURE RESEARCH

After decades of research on the topic, tremendous uncertainty remains concerning how to best monitor CRC survivors for recurrence or metachronous disease. The results of seven randomized controlled trials comparing alternative surveillance strategies have led to a general consensus that more intensive follow-up leads to increased curative treatment of recurrence via earlier detection and to improved overall survival. Whether or not the latter is a result of the former, or whether improved survival instead follows primarily from the benefits of increased contact with healthcare providers in general, remains unclear. In the coming years, we hope to see publication of more trial data on the topic than has been available to date thanks to three ongoing large trials. We will hopefully have a clearer picture of the cancer-specific survival benefit of intensive surveillance as well as the cost-effectiveness and quality-of-life implications of different approaches to surveillance.

Beyond the research questions highlighted above, additional areas for further study are listed below.

Need for model-based research

Even with the new trial evidence, actionable knowledge relevant for clinical practice will remain quite limited. We will still have experimental data on only a tiny fraction of the combinations and schedules of surveillance tests that are possible. Nor will we have a strong translational evidence base to guide risk-adapted follow-up. A promising possibility for leveraging the accumulated trial data may lie in computer simulation modeling. Sophisticated models could help researchers and clinicians examine the impact of virtually any surveillance regimen on patients with differing risk profiles. An example of using such modeling to synthesize what is known about testing and disease progression in such a way that allows virtual experimentation can be found in the numerous models of CRC screening strategies. These models simulate the

adenoma-carcinoma sequence by which benign polyps transform to adenocarcinomas and adenocarcinomas grow and invade healthy tissue, allowing experimentation with a practically infinite number of candidate screening strategies[56-60]. Some of these screening models have informed development of United States Preventive Services Task Force guidelines on colorectal cancer screening[56], have been applied by the Centers for Medicare and Medicaid Services to compare the effectiveness of various CRC screening strategies[57,58], and have spawned vital new research questions[59,60].

Simulating progression of recurrent CRC in such a way that allows the testing of different surveillance regimens is perhaps a more difficult problem, owing to the lack of direct observational data on unchecked recurrence progression (contrasted with the abundant data available on polyp progression and transformation). A few authors have developed recurrence models[61-65], but this line of research has not yet advanced to the point of being able to provide prescriptive recommendations for optimized surveillance regimens as has been the case for CRC screening in a healthy population. The loftiest ambition for applying simulation modeling to the problem of CRC surveillance would be to develop models which incorporate what can be inferred from RCTs about natural history of recurrence, information on test sensitivity and specificity, our best estimates of major complication risks (primarily from colonoscopy and ionizing radiation exposure), and what is known about individual risk factors for recurrence into an individualized decision aid. Such a tool could help providers and their patients reach decisions which incorporate their preferences in light of the estimated benefits and risks of specific surveillance strategies.

What role should colonoscopy play

The possible benefits of surveillance must be considered in light of the potential harms. Colonic perforation and post-procedure bleeding associated with colonoscopy represent the most concrete and serious harms arising from CRC follow-up. Endoscopic surveillance has been endorsed by all reviewed national guidelines, primarily for early detection of metachronous CRC's (which develop in 1.5%-7.7% of CRC survivors[10,66,67]) or adenomas with advanced features. The procedure has a sensitivity of 95% and a specificity of 100% for detecting high-risk polyps or tumors[68]. To date, however, no study has reported increased survival associated with routine colonoscopy after resection. Furthermore, the procedure is relatively invasive and has a major complication rate of 0.2%-1.2%[19,69,70]. This uncertain benefit and potential for harm, as well as the considerable resource demands, have led some to argue against routine endoscopic surveillance after curative CRC resection[71,72]. An area for future research might be to evaluate strategies which select for frequent colonoscopic examination only those patients at high risk for second primary cancers or advanced adenomas. Also worthy of further study is whether CT Colonography, or "virtual colonoscopy", may have the potential to provide a better balance of risks and benefits[71].

What are the quality of life implications of CRC surveillance

A longstanding, and still unresolved, question is to what extent CRC surveillance in general exacts a psychological toll on patients. Such a toll might arise from increased anxiety associated with testing or with the possibility of detection of an unresectable recurrence. Despite these theoretical harms, no negative quality of life impact has yet been demonstrated in studies comparing differing levels of follow-up. The small amount of data available on quality of life impacts of surveillance suggests a neutral or even slightly positive effect[73,74]. A 1997 Dutch study found no diminution in quality of life associated with follow-up of 130 CRC survivors at four hospitals. In fact, the average patient preferences tended to favor follow-up as opposed to no follow-up[73]. Kjeldsen and colleagues reported a slight trend toward increased quality of life among Danish CRC survivors who were followed more intensively compared to counterparts undergoing minimal follow-up[74].

The large surveillance trials underway[22,29,31] should shed further light on the quality of life impacts of CRC surveillance. An area in particular need of further study is the quality of life impact associated with false positive test results. In addition to specific focus on the effect of false positive results, an important, and as-yet-unaddressed question is the loss of quality time brought about by the pre-symptomatic diagnosis of unresectable recurrence. This represents an important concern since a substantial majority of patients whose recurrences are detected by surveillance before symptom onset will have progressed beyond the point of curative treatment[15,16,75].

For how long should crc survivors be followed

The treatment guidelines outlined in Table 3 focus primarily on the period spanning the point of initial treatment through five years post-treatment. There is no clear evidence that this timeframe is the most appropriate, however. Two opposing effects make the choice of an optimal surveillance period difficult. First-arguing for a shorter window-the majority of recurrences occur early; at least 80% of recurrences are detected by three years[4,5]. This fact would suggest that follow-up becomes much lower-yield and that false positive test results would increase drastically after three years of follow-up. On the other hand, time to recurrence appears to be an important prognostic factor for the outcome of recurrent disease[6]. Survival after curative treatment of recurrent disease may increase with later recurrences[76-79]. As such, some have suggested that longer follow-up-while detecting fewer recurrences-would detect a higher rate of curable recurrences[9,11]. The determinant of whether or not such benefit might be realized with longer follow-up is the extent to which late recurrences are treatable when they are detected based on symptoms. The proportion of

symptomatic recurrences which are considered curable is generally quite low (Table 2), but it is possible that the subset of patients whose recurrences manifest later may be an exception. Hopefully the volume of new trial data set to emerge in the coming years will permit this important subanalysis.

CONCLUSION

Optimizing colorectal cancer surveillance represents an incredibly complex medical decision making problem. A heterogeneous and far-from-completely-understood disease occurring in a population with typically advanced age and accompanying morbidity intersect with a surveillance testing framework involving numerous possible combinations of imperfect follow-up modalities. It is not surprising that the accumulation of trial data over the past decades has failed to provide a consistent answer to what strategy of surveillance-if any-most prolongs life by increasing the likelihood that recurrences will be caught early and successfully treated. Nor is it surprising that surveillance recommendations differ considerably across organizations and countries. A series of large, ongoing CRC surveillance trials will begin to produce much-anticipated results in the coming years. Not only will these results shed light on effective follow-up for CRC survivors diagnosed during a modern era of surgical and systemic treatment, but they also promise vital quality-of-life and economic findings. These trials will still have only looked at a small number of possible surveillance regimens. Additional tools, including computer simulation modeling, are needed to synthesize and leverage this new information in conjunction with knowledge of the effects of known and emerging risk factors. By so doing, we can move toward more effective, efficient, and patient-centered follow-up.

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TOPIC HIGHLIGHT

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

Intestinal stem cells and the colorectal cancer microenvironment

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Abstract

Colorectal cancer (CRC) remains a highly fatal condition in part due to its resilience to treatment and its propensity to spread beyond the site of primary occurrence. One possible avenue for cancer to escape eradication is via stem-like cancer cells that, through phenotypic heterogeneity, are more resilient than other tumor constituents and are key contributors to cancer growth and metastasis. These proliferative tumor cells are theorized to possess many properties akin to normal intestinal stem cells. Not only do these CRC "stem" cells demonstrate similar restorative ability, they also share many cell pathways and surface markers in common, as well as respond to the same key niche stimuli. With the improvement of techniques for epithelial stem cell identification, our understanding of CRC behavior is also evolving. Emerging evidence about cellular plasticity and epithelial mesenchymal transition are shedding light onto metastatic CRC processes and are also challenging fundamental concepts about unidirectional epithelial proliferation. This review aims to reappraise evidence supporting the existence and behavior of CRC stem cells, their relationship to normal stem cells, and their possible dependence on the stem cell niche.

Key words: Colon cancer stem cells; DCLK1 protein; Stem cell niche; Cell dedifferentiation

Core tip: Colorectal (CRC) cancer stem cells are a theorized but poorly characterized cell population believed to be crucial for tumor growth, spread, and tenacity. CRC stem cells share many similar characteristics of normal intestinal stem cells and are hypothesized to originate directly from them. It appears, however, that both the regulation of normal intestinal stem cells and the development of CRC are far more complex than previously imagined. Likely pivotal to the success of both are plasticity pathways able to reverse cellular fate, and stem cell niche signals, ultimately leading to self-replenishment and sometimes also unwanted dissemination.

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INTRODUCTION

Colorectal cancer (CRC) remains a highly morbid and fatal disease among both developed nations and globally[1-3]. Based on 2008 world data, CRC is the fourth leading cause of cancer-related mortality behind lung, stomach, and liver cancer, respectively[4,5]. Since Fearon *et al*[6] introduced a model for colorectal tumorigenesis in 1990, the study of the molecular basis of CRC has

been rapidly evolving. While a handful of tumor suppressors and oncogenes (*e.g.*, APC, KRAS, and P53) are commonly found among CRCs, a vast number of low-frequency somatic mutations have since been discovered that are believed to contribute to CRC heterogeneity[7,8]. Given the expanded number of potentially functional mutations, that no CRC therapy is completely curative should come as no surprise[9].

More importantly, individual colorectal cancers can themselves demonstrate phenotypic variability via sub-delegation of constituent cells. Core to this notion are cancer "stem" cells which act as ringleaders that drive CRC proliferation and metastasis[10]. Like normal stem cells, they self-perpetuate and expand in accordance with stem cell hierarchy[10]. Much remains unknown about the origins and regulation of CRC stem cells, though implicated in CRC inception are the signals expressed within the normal intestinal stem cell niche. New light has also been shed onto plasticity pathways that may perhaps be pivotal to CRC metastasis and treatment. The aim of this review is to reappraise current evidence supporting the existence and behavior of CRC stem cells, their relationship to normal stem cells, and their possible dependence on the stem cell microenvironment.

FEARON AND VOGELSTEIN'S MODEL FOR COLORECTAL CARCINOGENESIS

Fearon and Vogelstein's model for colorectal carcinogenesis illustrates how genetic alterations may allow colorectal cells to escape defined behaviors of the normal intestinal epithelium. By the early 1990s, Fearon *et al*[11] established three key features about colorectal cancer. First, cells within a colorectal cancer are monoclonal in nature, suggesting that CRC arises from clonal expansion of a small number of cells. Second, Fearon *et al*[6] surmised that key genetic alterations found commonly among CRC (*e.g.*, RAS, P53, APC) confer functional traits advantageous to the development and expansion of sporadic cancer and are acquired in a sequentially preferred order. For instance, APC mutations often occurred early prior to adenoma formation, whereas P53 mutations frequented tumor phases during the transition of adenomas to overt carcinomas[6]. Finally, based on their own observations and those of others, Fearon *et al*[6] concluded that the number of accumulated mutations in a tumor was the most consistent feature associated with the clinical and histopathological manifestation of CRC[12].

Fearon and Vogelstein's original CRC model has since been greatly expounded upon. Numerous low-frequency candidate mutations have been identified among candidate CRC genes, likely contributing to CRC phenotypic heterogeneity[7,8]. Also, carcinogenesis might not rely strictly on Fearon and Vogelstein's hypothesized mutational gateways. For example, one study found no genetic change between genome-sequenced primary colorectal cancers and their respective metastases, suggesting that insufficient time passed to allow either primary or metastatic lesions to acquire distinguishing mutations[7].

NORMAL INTESTINAL STEM CELLS

Two functionally distinct populations of putative normal epithelial stem cells have been identified in intestinal crypts of humans and mice: Lgr5$^+$ crypt base columnar stem cells and quiescent label-retaining cells[13-17]. These two cell types replenish and maintain the intestinal epithelium[13].

Lgr5$^+$ crypt base columnar cells

Lgr5$^+$ crypt base columnar cells (CBCs) are multipotent stem cells located in crypts of the small intestine and colon[14]. Lgr5 is an orphan G protein-coupled receptor expressed during embryogenesis and among epithelial stem cell populations in the adult intestine, hair follicles, stomach, mammary glands, and taste buds[18]. CBCs were first characterized in 1974 when an electron microscopy study identified a population of crypt cells that shared common secretory components with all differentiated epithelial cell lineages in the mouse intestine[19]. More recently, Barker *et al*[14] demonstrated that Lgr5-mediated activation of a permanent cell-labeling gene identified a line of cells originating from the intestinal crypt that yielded three differentiated cell types. The authors surmised that enteroendocrine cells were too rare to be detected among labeled cells[14]. A subsequent *in vitro* study demonstrated that organoids derived from single Lgr5$^+$ cells form crypt domains containing all lineages of the adult intestinal epithelium including enteroendocrine and crypt paneth cells[20]. Taken together, these findings strongly suggest that multipotent Lgr5$^+$ CBCs are true intestinal epithelial stem cells.

Quite contrary to expected stem cell behavior, evidence suggests that the expansion of Lgr5$^+$ CBCs follows stochastic principles in which cells are equipotent and segregate chromosomes randomly[18,21,22]. Lgr5$^+$ cells are also mitotically-active and demonstrate little asymmetric division[13,21]. Proliferation of these stem cells can at times approximate a square root growth curve, suggesting that they contain potential for rapid, yet very random clonal expansion[13,21,23]. As a likely consequence of their stochastic properties, Lgr5$^+$ stem cells are subject to neutral drift, often resulting in monoclonal or oligoclonal populations in the intestinal crypt[21].

It seems dangerous for a stem cell to propagate in a manner dictated largely by chance. Random chromosomal segregation risks the introduction of genomic errors that can subsequently be passed to both daughters and self-perpetuating clones. Lgr5$^+$ cells also seem to have little control over cell fate, suggesting that they are likely critically regulated by the surrounding milieu.

Quiescent label-retaining cells

Quiescent DNA label-retaining intestinal stem cells (LRCs) have remained controversial since the 1970s when these mitotically-inactive cells were found at and around the

+4 crypt position[24-26]. Although intestinal LRCs express a number of stem cell markers including Hopx, Tert, Lrig1, and Dclk1, they are widely identified by their expression of Bmi1, a member of chromatin-silencing polycomb-repressing complex 1[13,15,27]. Like Lgr5$^+$ CBCs, Bmi1$^+$ LRCs can form spheroids *in vitro* containing all differentiated epithelial cell types[13,20]. The multipotency of Bmi1$^+$ LRCs has also been confirmed *in vivo* through lineage experiments[15]. In contrast to early reports of the radiation sensitivity of +4 position crypt cells, recent evidence suggests that quiescent stem cells are both resistant to and activated by moderate levels of radiation damage, thus suggesting a crucial role in recovery following intestinal injury[13,28]. Notably, Bmi1$^+$ LRCs can single-handedly restore radiation-ablated mouse intestinal epithelium in the total absence of Lgr5$^+$ stem cells[13].

Whether +4 quiescent LRCs are actually stem cells remains a matter of debate. Quiescent stem cells have only been found in the proximal small intestine and to date no presence has yet been found of a corresponding population in the colon[15,29]. Moreover, one study has identified quiescent LRCs not as stem cells, but rather as partially-differentiated secretory precursors[30]. Quiescent stem cell markers (including Bmi1, Tert, Hopx, and Lrig1) have also been found among Lgr5$^+$ stem cells thereby questioning the validity of using such markers to identify a uniquely separate stem cell population[31].

An evolving model of normal intestinal stem cell behavior

In contrast to current single-lineage stem cell theories, the coexistence of two putative intestinal stem cell types may suggest a more complex pathway for the development of the intestinal epithelium (Figure 1)[10,32]. On one hand, evidence exists supporting the subordinancy of LRCs to LGR5$^+$ cells: LRCs have been characterized as secretory precursors and may not share markers unique from Lgr5$^+$ cells[30,31,33]. On the other hand, evidence also exists conversely that Lgr5$^+$ cells may be subordinate to LRCs: Bmi1$^+$ LRCs restore radiation-ablated Lgr5$^+$ cell populations[13,29]. These findings when taken together suggest that LRCs likely interconvert with Lgr5$^+$ CBCs, regardless of whether LRCs are actually stem cells. Such findings suggest that intestinal epithelial development is neither as hierarchical nor as unidirectional as once thought, though the extent of which is not yet known.

Based on the discussion thus far, perhaps the actions of the stem cell pool as we currently understand it are comprised of the combined properties of Lgr5$^+$ and quiescent stem cells in the crypt (Figure 1). Under normal conditions, Lgr5$^+$ stem cells could function to self-sufficiently maintain epithelial homeostasis through high-output cell production in response to trophic niche signals (*e.g.*, Wnt)[34,35]. However, Lgr5$^+$ CBCs are likely as sensitive to genetic damage as they are to injury. In these situations, the quiescent LRC population may assist with recovery from intestinal injury, either directly or by restoring Lgr5$^+$ stem cells.

INTESTINAL STEM CELL NICHE

Like other tissues among higher organisms, all intestinal cells reside within a carefully defined construct of chemical signals that directs genetically identical cell populations towards divergent behaviors[36]. Contained in and around the intestinal crypt are a multitude of molecular and cellular effectors that define a unique microenvironment - a "niche"- that directs the optimal function of stem cells[10]. Components of the niche include the subepithelial stroma, adjacent epithelial cells, natural enteric flora, and soluble epithelium-derived factors. Alteration of niche effectors can also lead to aberrant and dysregulated crypt behavior, which in turn may foster neoplasia.

Wnt signaling pathway

A multitude of signals in the intestinal crypt affect the function and growth of intestinal stem cells (Figure 2A and B)[37]. Of these, Wnt proteins are one of the most crucial for maintaining stem cell homeostasis[34,35,37,38]. Wnt promotes both cellular dedifferentiation and proliferation during embryogenesis and in many adult animal tissues[39-42]. Inhibition of the Wnt pathway results in crypt loss and a marked reduction in epithelial proliferation[43]. Among mice with inducible APC-knockouts, Wnt results in intestinal mucosa populated by undifferentiated cells[44]. Wnt activity is also among the essential signals for the formation of crypt structures from single stem cell cultures as well as for the reprogramming of somatic cells into induced pluripotent stem cells (iPSCs)[20,34,39,41]. Cell-proliferative genes are activated by Wnt *via* nuclear β-catenin intermediaries and include cell migration controllers (EPH), proliferative signals (c-myc, cyclin D1), and stem and cancer cell markers (Lgr5, Bmi1)[10,14,35,45-47].

The Wnt pathway is also a highly influential mediator of cancer (Figure 2C). APC mutations facilitate Wnt activity by dysregulating β-catenin-mediated gene expression[45,48]. APC mutations are common, occurring in over 80% of sporadic colorectal cancer[48]. Vermeulen *et al*[49] showed that primary spheroidal cultures derived from human CRCs are regulated by Wnt signals in the surrounding microenvironment, such as those secreted by intestinal myofibroblasts. They also demonstrated that extrinsic Wnt pathway activation was an important determinant in the cellular acquisition of cancer stem cell features (*e.g.*, formation of tumors when injected into immune-deficient mice and *in vitro* recapitulation of xenograft isolate behavior to that of the original tumor)[49].

Intestinal subepithelial myofibroblasts

Intestinal subepithelial myofibroblasts (ISEMFs), located underneath the basement membrane in the crypt, are stromal cells widely known to promote stem cell self-renewal and differentiation (Figure 2A and B)[20,34,35]. ISEMFs originate from regional intestinal fibroblasts and possibly trans-differentiated bone marrow cells[50]. Intestinal myofibroblasts function as anchors for cell adhesion and provide trophic signals to stem cells *via* cell-

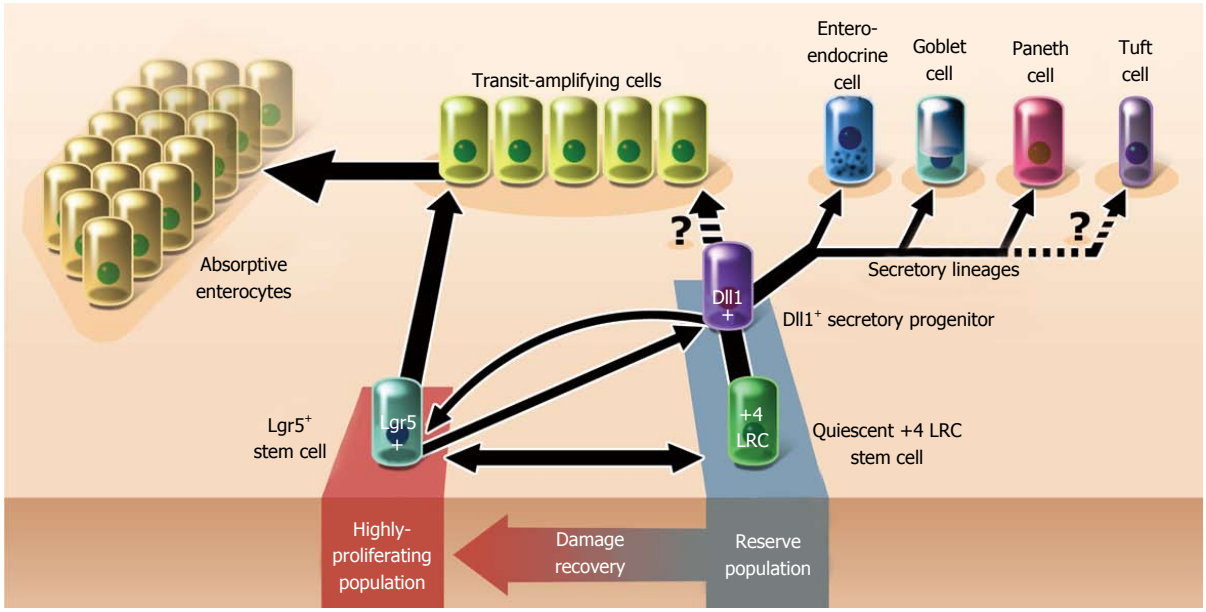

Figure 1 Origin and development of normal intestinal stem cells. Lgr5$^+$ CBCs and +4 LRCs coexist in the crypt. Each stem cell is fully multipotent. Lgr5$^+$ cells likely maintain intestinal homeostasis under normal conditions. Following intestinal injury, the reserve population comprised of +4 LRCs and Dll1$^+$ secretory progenitors restore both the epithelium and Lgr5$^+$ CBCs. Tuft cells are Bmi1$^+$ cells that may be synonymous with or descendants of +4 LRCs. CRC: Colorectal cancer; CBCs: Crypt base columnar cells; LRCs: Label-retaining intestinal stem cells.

cell interactions and secreted mediators[51]. ISEMFs also contribute to wound healing, mucosal protection, fluid and electrolyte transport, and growth of the basement membrane[50,52]. Secreted myofibroblast mediators are numerous: Wnt proteins, hepatocyte growth factor, fibroblast growth factor, TGF-β, keratinocyte growth factor, matrix metalloproteinases, stem cell factor, VEGF, and numerous interleukins, to name a few[52,53].

ISEMFs have long been implicated in promoting colorectal cancer growth and invasion (Figure 2C)[51]. Little clarity exists regarding whether peri-CRC myofibroblasts are derived from normal ISEMFs. Based on knowledge gleaned from other cancer systems, functional differences between normal and CRC fibroblasts do likely exist[54]. Still, even normal myofibroblasts are capable of facilitating CRC growth. Vermeulen *et al*[49] found that normal colonic myofibroblasts prevented both the morphological and molecular differentiation of co-cultured colorectal cancer cells. Furthermore, these myofibroblasts were shown to re-induce tumorigenic potential in subpopulations of CRC cells with low degree of proliferative activity[49].

Paneth cells

Paneth cells are terminally-differentiated secretory cells intermingled between Lgr5$^+$ CBCs at the base of crypts in the small intestinal mucosa[55]. Though unclear why no Paneth cells have been found elsewhere in the intestine, a population of c-kit$^+$/CD117$^+$ goblet cells in the colon may perhaps function analogously[33,56]. Co-culture of c-kit$^+$ cells with Lgr5$^+$ stem cells promotes the growth of organoids in similar fashion to those produced from Paneth/Lgr5$^+$ cell co-cultures[55,56].

Paneth cells contribute to the preservation of the stem cell compartment through the expression of Wnt proteins and other secreted signals such as epidermal growth factor and Notch ligands, all important in the maintenance of the Lgr5$^+$ CBC population[55]. Paneth cells also secrete antimicrobial peptides[57]. Furthermore, they facilitate epithelial repair by deactivating paneth-specific genes and converting to a phase that promotes Bmi1$^+$ cell proliferation[58].

Paneth cells seemingly serve a redundant role in the intestinal crypt. Wnt proteins released from Paneth cells are also derived from other sources in and around the intestinal crypt[59]. Notably, the complete removal of paneth cells in mouse model systems has not been shown to affect the proliferation of Lgr5$^+$ CBCs[60].

INTESTINAL TUMOR/CANCER STEM CELLS

Cells of origin

Is there a population of cells in the intestinal epithelium that reliably serves as the source for most, if not all of colorectal cancers? Intestinal stem cells are prime suspects due to their pre-existing proliferative and self-restorative behavior, making them perhaps more sensitive to overt carcinogenesis[10,35]. In support of this notion, Barker *et al*[61] demonstrated that APC deletions only among Lgr5$^+$ stem cells (6.5% of tumor mass) promoted the formation of adenomas, even in the setting of uniform tumor Wnt target gene activation. Barker and colleagues concluded that Lgr5$^+$ stem cell transformation-especially *via* loss of APC function-is a highly efficient pathway to neoplasia[61]. Multi-color reporter lineage

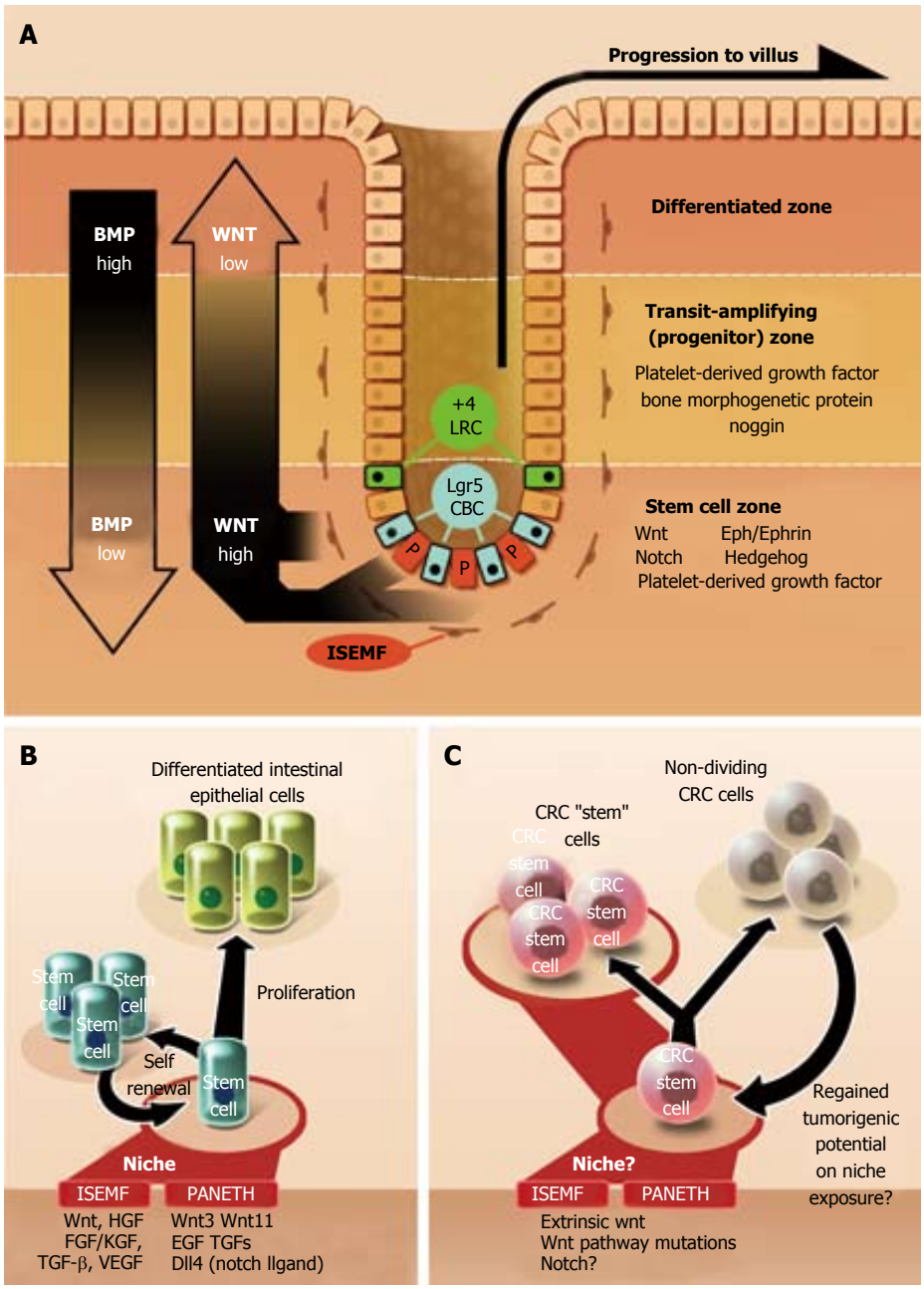

Figure 2 Niche regulation of the normal intestinal epithelium and colorectal cancer. A: Intestinal subepithelial myofibroblasts (ISEMFs) surround the crypt. Along with paneth cells (P), they supply the stem cell niche with trophic signals. Developing intestinal cells migrate upwards towards the villus apex, during which time they are subject to niches among the various strata in the crypt. B: Redundant mediators expressed by ISEMFs and Paneth cells contribute to the preservation of the stem cell compartment and normal intestinal proliferation. C: The local niche immediately around CRC likely fosters tumor growth by activating stem cell pathways. CRC cells lacking proliferative ability may re-awaken upon re-entry into the niche. CRC: Colorectal cancer; BMP: Basic metabolic panel.

retracing experiments by Schepers *et al*[62] have also confirmed that early adenomas are mostly of monoclonal origin, though occasionally oligoclonal. Schepers *et al*[62] also identified stem-like Lgr5$^+$ tumor origin cells at the base of adenomas that shared organizational resemblances to normal stem cells and were 20-fold more efficient at forming cell colonies *in vitro* than Lgr5-poor cells derived from the same population.

Still, evidence suggests that colorectal cancer may also arise from non-stem cells, supporting the idea that ultimately any cell harbors the potential to foster neoplasia. Early observations by Cole *et al*[63] reveal that early adenomatous polyps are positioned at the top of colonic crypts without contact with the stem cell compartment. Schwitalla *et al*[64] have also demonstrated that Wnt-constitutive intestinal cells can re-acquire stem cell properties in an NF-κB dependent manner and lead to tumor formation. These findings are congruent with iPSC research through which differentiated somatic cells have been reprogrammed back to proliferative stem-like states on account of key genetic alterations[41]. As with other non-intestinal cancers, no clear distinction yet

Table 1 Putative colorectal cancer stem cell markers

Marker	Function
ALDH1A1	Enzyme
ALDH1B1	Enzyme
β-catenin	Protein (nuclear)
Bmi-1	Protein (nuclear)
CD24	Cell surface glycoprotein
CD26	Cell surface glycoprotein
CD29	Cell surface glycoprotein
CD44	Cell surface glycoprotein
CD133	Cell surface glycoprotein
CD166 (ALCAM)	Cell surface glycoprotein
CDX-2	Transcription factor
c-myc	Transcription factor
Dclk-1	Serine-threonine kinase (?)
EpCAM	Cell surface glycoprotein
Klf-4	Transcription factor
Lgr-5	Cell surface receptor
Lin-28	Transcription factor
Msi-1	Protein (nuclear)
Nanog	Transcription factor
4-Oct	Transcription factor
Sox-2	Transcription factor

ALDH: Aldehyde dehydrogenase; Bmi-1: B lymphoma Mo-MLV insertion region 1 homolog; CD: Cluster of differentiation; CDX-2: Caudal type homeobox 2; Dclk-1: Doublecortin-like kinase-1; EpCAM: Epithelial cell adhesion molecule; Lgr-5: Leucine-rich repeat-containing G protein coupled receptor 5; Msi-1: Musashi-1.

exists identifying which CRCs, if any, are derived from non-stem cells[65].

What are the triggers that stimulate a cell to progress to cancer? Based on the discussion thus far, the neoplastic potential of a cell might be directly correlated with the combined disruptive impact of affected genes. However, One might imagine a situation in which a cell lacking sufficient functional derangement can be driven to cancer in response to external stimuli. Signals may come from cell placement in a Wnt-rich intestinal crypt, or in response to inflammation in light of concurrent genetic Wnt derangements as Schwitalla *et al*[64] have explored.

Tumor stem cell markers

Not surprisingly, many normal stem markers such as Lgr5, DCLK1, CD133, CD44, CD24, and ALDH1 have also been found among highly proliferating fractions of colorectal cancers[10,52,66,67]. Given the apparent genetic heterogeneity among CRC[7,8], very few, if any, markers are both specific to CRC stem cells and ubiquitous among all CRCs[9]. Table 1 lists putative CRC stem cell markers as previously covered by other authors[10,68-71]. What remains unclear is whether such markers reflect carry-over from intestinal stem cell precursors as with other cancers (*e.g.*, leukemia)[35] or else a re-activation of stem cell pathways. Regardless of the underlying reason, that CRC and normal intestinal epithelial stem cells express many of the same cell surface markers poses a challenge to the isolation of tumor stem cells.

One putative stem cell marker, Doublecortin-like kinase 1 (Dclk1), may be a useful marker for both normal and neoplastic intestinal stem cells. Dclk1 is a complex multi-splicoform transmembrane serine-threonine kinase involved in embryonic neuronal migration through intracellular signaling pathways[72,73]. In the digestive tract, Dclk1$^+$ cells have been found in the stomach and at the +4 position of the intestinal crypt[74,75]. Intestinal Dclk1$^+$ cells are functionally akin to quiescent stem cells via their label retention and radiation-induced activity[74,76]. Some studies contend that Dclk1$^+$ cells are not intestinal stem cells at all. Dclk1 expression may be shared not only by stem cells but also among the enteroendocrine lineage[77]. Alternatively, Gerbe *et al*[78] propose that Dclk1$^+$ cells are actually novel differentiated tuft cells with unidentified function.

Interestingly, cells aberrantly expressing Dclk1 have been found among both mouse intestinal adenomas and human colorectal cancers, suggesting a potential role for Dclk1 to identify neoplastic stem-like intestinal cells[74,79]. Nakanishi *et al*[80] recently demonstrated that Dclk1 specifically identifies abnormal intestinal mucosa found among tumors in the small intestine of APC$^{min/+}$ mice. Not only did Dclk1$^+$ tumor cells co-express Lgr5, they also demonstrated higher expression of other cancer stem cell markers *versus* non-tumor cells[80]. Furthermore, ablation of Dclk1$^+$ cells led to regression of the containing polyps without apparent effect to normal intestine[80]. These results concur with findings from our group showing that siRNA-based Dclk1 interference leads to growth arrest of xenoplanted CRC[81,82]. Also notable is a recent study by Li *et al*[67] demonstrating increased Dclk1$^+$ expression among cell fractions with a higher percentage stem-like HCT116 human CRC cells. Taken together, these findings support the notion that Dclk1$^+$ cells can identify colorectal cancer stem cells and that Dclk1 is critical for tumor growth.

Identifying CRC tumor stem cells

Despite the strong evidence suggesting that only a small fraction of colorectal tumor cells is responsible for maintaining tumor growth, the isolation of "pure" colorectal cancer stem cells has remained an ongoing challenge due to numerous theoretical and practical reasons. In fact, the term "cancer stem cell" may be somewhat of a misnomer. There is no expectation that a dysregulated colorectal cancer cell follows the exact biochemical principles of a normal intestinal epithelial stem cell, even if they share common signaling pathways. So long as the phrase "cancer stem cell" is used loosely to refer to cells in control of the proliferative hierarchy demonstrated by CRC, there is no perceived problem.

The first studies documenting a tumor-initiating CRC subfraction came in 2007 with the identification of CD133$^+$ cells comprising 2.5% of tumor mass[83,84]. However, the significance of CD133 as a specific CRC marker has subsequently been debated[52]. Other markers have further assisted in the enrichment of CRC stem cell fractions (Table 1). Kemper *et al*[66] found that Lgr5$^+$ cells comprised only 1.9%-11.1% of putative stem cells already marked by

Epcam, although admittedly the Lgr5$^+$ fraction was more highly clonogenic. Isolation of DCLK1 among tumor stem cells has been previously discussed, but even Nakanishi *et al*[80] did not find DCLK1 universally among all tumors in their mouse experiments.

The current methods employed to identify CRC stem cells are derived from non-exclusive properties shared by all intestinal stem cells. These methods include: DNA label retention, *in vitro* and *in vivo* proliferation assessments, and detection of cell surface markers[10]. Consequently, the isolation of CRC stem cells is fraught with as much, controversy as normal intestinal stem cells. Not the least of which, subtle differences between humans and animal models may consequently make experimental findings difficult to generalize. The apparent genetic heterogeneity of CRC lends further worry that finding a universal identification standard for CRC stem cells may long remain a daunting task[7,8].

Plasticity

It is becoming increasingly apparent that both the normal intestine and colorectal cancer are subject to "plasticity" processes that convert cells back to less-differentiated forms. Conventional stem cell theory holds that cellular development follows a unidirectional and irreversible hierarchy through semi-differentiated intermediates and concludes with terminal differentiation[85]. The implied goal of such a model is to produce cells capable of specialized organ functions[86]. In the intestine, recent evidence has revealed that short-lived Dll1$^+$ secretory progenitors can readily revert to Lgr5$^+$ stem cells following radiation injury (Figure 1)[87,88]. The apparent conversion of quiescent Bmi1$^+$ LRCs to Lgr5$^+$ stem cells is another clear demonstration of cellular plasticity[30,31]. That differentiated somatic cells, too, can fate-reprogram into iPSCs carries profound implications regarding the exclusivity of stem cell traits and the potential for any cell in an organism to participate in tissue regeneration[41].

Cellular plasticity processes may also depend largely on the cellular microenvironment. For example, extrinsically-derived Wnt signals can sufficiently replace Myc gene mutations during iPSC creation[39]. Also, non-proliferating CRC cells possessing low Wnt activity have been shown to regain proliferative tumorigenic potential when co-cultured with colonic myofibroblasts or the conditioned medium derived from myofibroblast cultures[49]. These results indicate that extrinsic signals -notably activators of the Wnt pathway- are perhaps sufficient to induce behavioral reprogramming, especially in CRC[49].

That fate-reversal occurs in CRC suggests that CRC expansion adheres to a proliferative pattern somewhere in between the classical hierarchical and stochastic growth models[49,85]. Admittedly, however, it is not known to what degree cellular plasticity plays a role in the proliferation of colorectal cancer. Perhaps even among different CRCs there is variation in functional dependence on extrinsic signals, ultimately affecting the growth patterns and behavior of the neoplastic phenotype. In this way, perhaps an extreme disturbance of either genetic derangement or environmental signals alone would also be a sufficient trigger for carcinogenesis[36].

EPITHELIAL MESENCHYMAL TRANSITION: PREVAILING METASTATIC PROGRAM?

The presence of cancer cells in the lymphatic and systemic circulation have long been known to correlate with poor prognosis, even despite the resection of primary lesions and/or chemotherapy[89-95]. With the apparent monoclonality of colorectal cancer[11], one might infer that circulating cancer stem cells originate from a primary colorectal tumor. Because cell migration brings with it certain constraints on adhesion and cellular interactions, circulating cancer stem cells may be functionally divergent from primary tumor cells.

Epithelial-mesenchymal transition (EMT) is a critical extension of cellular plasticity that is believed to govern not only the development of normal tissues but also the growth and spread of colorectal cancer. EMT is defined as the process by which epithelial cells convert to a mesenchymal-like phenotype. *Via* EMT, a cell relinquishes its native cell-cell interactions, loses tissue-specific polarity, and acquires migratory mesenchymal traits[96]. Important aspects of the EMT process such as the loss of E-cadherin (a hallmark of EMT) is mediated by the Wnt pathway[97]. This process is reversible and plays a key role in normal embryonic development as well as normal wound healing and fibrosis in the adult animal. The opposing process of mesenchymal-epithelial transition (MET) likely occurs through inverse regulation of EMT and is critical for final organ formation once embryonic cells have sufficiently migrated via mesenchymal intermediates[96]. Boundaries demarcating the degree of lineage reprogramming during the EMT process remain vastly gray territory. In fact, cells undergoing EMT may not necessarily have re-written fates, for such changes might only involve alterations to cell mobility.

EMT is likely a dominant mechanism driving colorectal cancer metastasis (Figure 3). In fact, CRC cells that display EMT characteristics have been shown to also possess traits of stem cells[98,99]. Critical to both CRC stem cell formation and EMT induction are Wnt mediators (*e.g.*, nuclear β-catenin), most markedly active at the invasive front of colorectal tumors[97]. Microarray analysis has demonstrated up-regulation of EMT-mediating genes among human CRC (*e.g.*, VIM, TWIST 1 + 2, SNAIL, and FOXC 1 + 2)[100]. EMT is also controlled *via* the microRNA miR-200 family[100,101]. MicroRNAs are small, non-coding RNAs that regulate post-transcriptional gene expression and serve to activate oncogenes and silence tumor suppressors. The presence of miR-200 family members (notably miR-200c and miR-141) is associated with a gain of epithelial cell characteristics[101]. In contrast, down-regulation of miR-200 family members

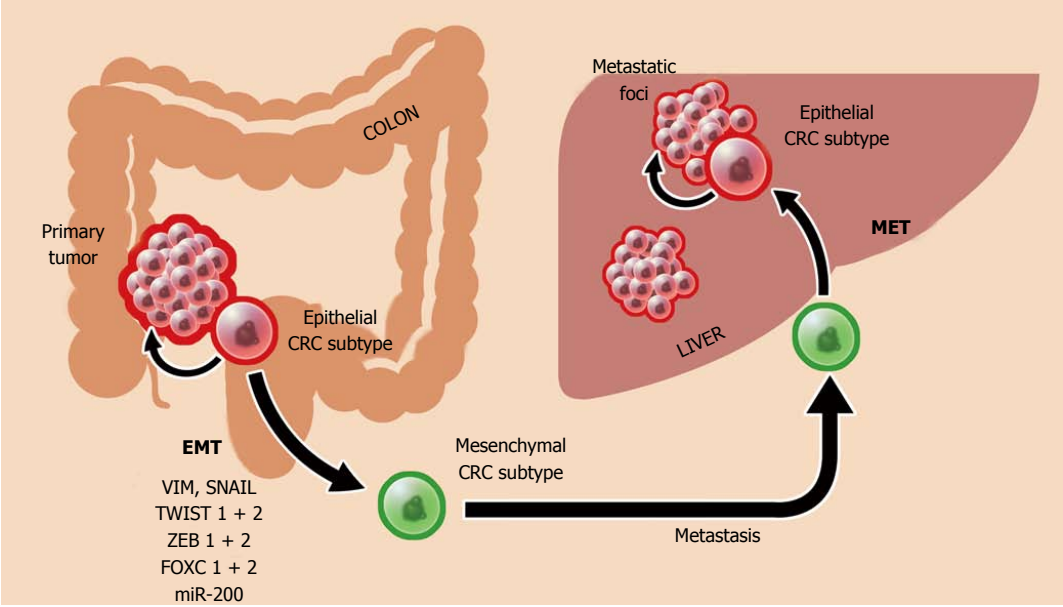

Figure 3 Epithelial-mesenchymal transition and mesenchymal-epithelial transition in colorectal cancer. In a primary tumor, CRC stem cells exist in a stationary phase that promotes growth. EMT transition to a migratory mesenchymal phase deactivates proliferative genes and cell adhesion molecules, generally allowing for metastatic dissemination to occur. Once at distant targets, mesenchymal cells transition back to the stationary phase *via* MET thereby resuming tumor expansion. EMT: Epithelial-mesenchymal transition; MET: Mesenchymal-epithelial transition; CRC: Colorectal cancer.

promotes an invasive mesenchymal phenotype, possibly through the activation of EMT mediators like ZEB1 and ZEB2[96,102,103]. In turn, epigenetic methylation pathways are in control of these miR-200 "switches" that altogether govern the shifting of CRC cells towards either mobile or stationary phases[96,101].

The combined effect of EMT/MET activity is metastatic advancement of a colorectal cancer: EMT enables primary tumor escape and spread by way of mesenchymal intermediates, and MET returns CRC to a highly-proliferative epithelial stem cell phenotype (Figure 3)[101]. In fact, these transitional phases may be the ultimate defining characteristic of CRC and may help direct future CRC therapy. Loboda *et al*[100] demonstrated that colorectal cancer, despite its vast mutational heterogeneity, can be organized principally as either epithelial or mesenchymal subtypes. Admittedly, the extent that EMT contributes to tumor spread remains unknown.

Interfering with EMT at critical phases of cancer growth is thus seemingly an attractive goal. For instance, anti-EMT therapy could be utilized to prevent primary tumor metastasis in early-stage CRC by forcing cells out of a mesenchymal phenotype or else preventing the entry into EMT (as is apparently the case with cetuximab administration)[96,104,105]. However, one concern regarding EMT/MET exploitation is that the two opposing processes may coexist inseparably. As such, unilaterally-directed therapy might lead to undesirable activity of cells in the opposite transitional phase. For instance, EMT processes are in part responsible for chronic resistance to oxaliplatin[106]. Difficulties in controlling mesenchymal processes may be further complicated by plasticity-mediated recruitment of additional CRC stem cells into the mesenchymal pool. Suffice it to say, our understanding of EMT is still in its infancy.

CONCLUSION

Much has been learned about the behavior of colorectal cancer stem cells owing to knowledge gained about normal intestinal stem cell behavior. The limitations inherent in our current isolation methods of pure stem cell fractions will likely bear heavily on how we observe and understand CRC as well. Newer developments in the field of stem cell research have provided insight into the vast potential for stem cells to not only be controlled by environmental factors but also be restored by its descendants. Also critical are core pathways such as Wnt that play an integral role in stem cell function, mesenchymal transition, and metastasis. Given the complexity of CRC "homeostasis", optimal CRC therapy will likely still remain a multi-pronged attack: first by control and/or alteration of trophic niche stimuli, second by the prevention of mesenchymal cell intermediates, and lastly by the elimination of stem cell ringleaders.

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TOPIC HIGHLIGHT

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

Management of locally advanced and metastatic colon cancer in elderly patients

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Abstract

Colon cancer is the second leading cause of cancer mortality in the United States with a median age at diagnosis of 69 years. Sixty percent are diagnosed over the age of 65 years and 36% are 75 years or older. At diagnosis, approximately 58% of patients will have locally advanced and metastatic disease, for which systemic chemotherapy has been shown to improve survival. Treatment of cancer in elderly patients is more challenging due to multiple factors, including disabling co-morbidities as well as a decline in organ function. Cancer treatment of elderly patients is often associated with more toxicities that may lead to frequent hospitalizations. In locally advanced disease, fewer older patients receive adjuvant chemotherapy despite survival benefit and similar toxicity when compared to their younger counterparts. A survival benefit is also observed in the palliative chemotherapy setting for elderly patients with metastatic disease. When treating elderly patients with colon cancer, one has to consider drug pharmacokinetics and pharmacodynamics. Since chronological age is a poor marker of a patient's functional status, several methods of functional assessment including performance status and activities of daily living (ADL) or instrumental ADL, or even a comprehensive geriatric assessment, may be used. There is no ideal chemotherapy regimen that fits all elderly patients and so a regimen needs to be tailored for each individual. Important considerations when treating elderly patients include convenience and tolerability. This review will discuss approaches to the management of elderly patients with locally advanced and metastatic colon cancer.

Key words: Colon cancer; Elderly; Chemotherapy; Management; Toxicity

Core tip: Despite survival benefit, fewer older patients with colon cancer receive chemotherapy, likely due to concerns regarding safety and efficacy of chemotherapy. The decision to treat elderly patients with advanced and metastatic colon cancer requires the incorporation of a thorough evaluation. Fit elderly patients are especially appropriate for treatment and should be offered the same regimens as their younger counterparts. Treatment related toxicities and quality of life should be monitored very closely in elderly patients receiving chemotherapy and more frequent follow-up should be arranged. In frail elderly patients, sequential single agent chemotherapy may be more tolerable than combination therapy.

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INTRODUCTION

An estimated 142820 new cases of colorectal cancer, including 102480 new cases of colon cancer will be diagnosed in 2013 with 50830 deaths expected in the United States[1] Approximately 39% of these patients will have locally advanced disease and 19% will be diagnosed with metastatic disease. In both settings, systemic therapy has been shown to improve survival[2].

Most cancer occurs in the elderly population[1]. Developed countries have accepted the chronological age of 65 and older as a definition of an elderly population[3]. Currently, more than 50 percent of all cancer diagnoses and over 70% of cancer deaths occur in those over age 65[4]. Colon cancer has a median age of 69 years at diagnosis, in which 60% are over the age of 65 and 36% are 75 years or older[1,5].

Adjuvant chemotherapy has been the standard of care for stage III colon cancer following complete surgical resection. Palliative chemotherapy also improves progression free survival (PFS) and overall survival (OS) in patients with metastatic colon cancer. However, since fewer elderly patients are included in clinical trials, establishing a standard adjuvant or palliative treatment regimen may be challenging.

Treatment of cancer in elderly patients often requires greater attention due to multiple factors, including disabling co-morbidities as well as a decline in organs function. Cancer treatment of elderly patients is often associated with more severe toxicities and hospitalizations during treatment[6]. Elderly patients also have a shorter life expectancy. These factors often influence physicians decision to withold chemotherapy. A SEER database analysis showed that the older the patient, the less likely they received chemotherapy[7,8].

BENEFITS OF CHEMOTHERAPY IN LOCALLY ADVANCED AND METASTATIC COLON CANCER

Adjuvant setting in locally advanced disease

In the 1980s, the use of fluorouracil (5-FU) and leucovorin (LV) extended survival for stage III colon cancer, even in elderly patients[9,10]. The use of 5-FU/LV in stage Ⅲ patients age 65 and older provided a survival advantage[8]. Another SEER-Medicare database analysis also found survival benefit for adjuvant therapy in patients age 75 and older[7]. The toxicities of 5FU/LV were similar in older and younger patients.

However, fewer elderly patients received adjuvant chemotherapy[7]. Since older patients are underrepresented in clinical trials, concerns regarding safety and efficacy of chemotherapy have always been raised.

Palliative chemotherapy in metastatic colon cancer

In metastatic disease, treatment options include metastatectomy (particularly in patients with isolated liver metastases) and systemic chemotherapy for palliation. For many years, 5-FU/LV was the only active regimen used in this setting.

Chemotherapy for metastatic colon cancer markedly improves outcomes over best supportive care alone[11]. The availability of newer agents, such as irinotecan, oxaliplatin, and targeted therapies, has markedly improved response rates (RR), time to progression (TTP), and overall survival (OS)[12]. Between 1995 and 2005, an analysis of patients age 65 and older who received chemotherapy for metastatic colon cancer demonstrated a 6-mo improvement in OS[13].

ACTIVE AGENTS FOR LOCALLY ADVANCED AND METASTATIC COLON CANCER

The following represent a list of active agents for colorectal cancer and their most common side effects. In general, strategies to prevent toxicities are to identify the side effects early and provide immediate symptom management as well as dose adjusment as necessary.

5FU/Leucovorin

Flurouracil (5-FU) in combination with leucovorin (LV) has been used alone for decades before the introduction of other agents in the late 1990s and early 2000. To date, 5-FU is still the backbone drug used in combination with other newer agents. Flurouracil is a pyrimidine nucleoside analog that impairs DNA synthesis *via* inhibition of thymidylate synthase and also inhibits RNA synthesis[14]. LV enhances 5-FU cytotoxicity by prolonging the 5-FU enzymatic inhibition of thymidylate synthase[15,16].

The side effects of 5-FU may vary based on the method of administration: Ⅳ bolus *vs* continuous Ⅳ infusion. Bolus 5-FU is more likely to be associated with diarrhea and myelosuppresion, which may be more pronounced in patients with dihydropyrimidine dehydrogenase (DPD) deficiency[17]. Continuous infusion 5-FU is more likely to cause hand-foot syndrome and mucositis, especially in older patients (> 70-year-old)[18,19].

Capecitabine

Capecitabine (fluoropyrimidine carbamate), an orally administered chemotherapeutic agent, is a pro-drug that is converted enzymatically to 5-FU following absorption[20]. Capecitabine is approved in the United States for first-line treatment of metastatic colon cancer as a single agent or in combination with other agents.

As monotherapy capecitabine has similar efficacy when compared to 5-FU/LV for treatment of metastatic colon cancer[21,22]. However, in patients who failed 5-FU-based regimens, replacing 5-FU with capecitabine as a second line monotherapy is an inappropriate treatment strategy due to a low objective response rate[23,24].

The most common side effect of capecitabine is grade 3 or 4 palmar-plantar-erythrodysthesia (PPED) also known as hand-foot skin reaction. Capecitabine may also cause diarrhea and mucositis. However, there is a lower incidence of grade 3 or 4 myelotoxicity when

compared with infusional 5-FU. Therefore, it is generally well tolerated. Dose tolerance is also different among patients treated in the United States *vs* Europe (a lower dose is often given in the United States)[25].

Irinotecan

Irinotecan, a topoisomerase I inhibitor, is used alone or in combination with 5-FU, as well as with targeted agents. In metastatic disease, several phase Ⅲ trials demonstrated a survival benefit for combined irinotecan plus 5-FU/LV compared to 5-FU/LV alone[26-28].

Diarrhea and myelosuppression are the dose-limiting side effects of irinotecan, which may be severe. Premedication with atropine sulfate (0.25-0.5 mg subcutaneous) often prevents the development of irinotecan-induced diarrhea. Early use of a antimotility agent such as loperamide has been shown to decrease the severity of diarrhea and is essential to prevent treatment-related mortality[29]. Blood counts should be monitored and dose modification may be required. Other toxicities include nausea, vomiting, alopecia, and asthenia. Medications for symptom management should be made available if needed[30].

Oxaliplatin

Oxaliplatin is a platinum analog approved for colon cancer in combination with 5-FU or capecitabine, with or without a targeted agent. Three clinical trials have shown a significantly greater RR and PFS but similar overall survival for oxaliplatin plus short-term infusional 5-FU and LV (FOLFOX regimen) compared to 5-FU plus LV alone in the first-line treatment of metastatic colon cancer (mCRC)[31,32].

The dose limiting toxicity of oxaliplatin is peripheral neuropathy. Patients should be closely monitored for the development of neuropathy and educated to avoid cold exposure to prevent worsening of this symptom. Although proposed as a strategy to delay peripheral neuropathy, there is no firm evidence for the use of calcium and magnesium infusions[33,34]. Dose modifications or interruption is often required when symptoms start. Oxaliplatin can also cause pancytopenia, nausea, vomiting, and fatigue. Therefore, complete blood counts should be followed and dose modification may be required[35].

ANTI-ANGIOGENESIS (ANTI-VASCULAR ENDOTHELIAL GROWTH FACTOR)

Bevacizumab

Bevacizumab is a humanized monoclonal antibody (MoAb) targeting vascular endothelial growth factor (VEGF). The addition of bevacizumab to first-line regimens used for metastatic colon cancer improves outcomes modestly. It is usually given with fluoropyrimidines alone or fluoropyrimidines in combination with oxaliplatin (FOLFOX/XELOX) or irinotecan (FOLFIRI)[36-42].

Serious adverse events of this agent include hemorrhage, gastrointestinal perforation, and impaired wound healing. Other significant side effects include hypertension and thromboembolic events (especially in patient age 65 and older). Therefore, the use of this agent should be avoided in high-risk patients (*i.e.* history of bowel perforation, non-healing wounds, history of recent cerebrovascular accident, or uncontrolled hypertension). Blood pressure needs to be monitored and anti-hypertensive agents are often required. Bevacizumab can also lead to proteinuria and regular monitoring of urine protein secretion with urine dipstick or 24-h urine protein to creatinine ratio may be required. Holding the agent at least six to eight weeks prior to elective surgery is recommended[43].

Aflibercept

Intravenous aflibercept is a recombinant fusion protein consisting of VEGF binding portions from key domains of human VEGF receptors 1 and 2 fused to the Fc portion of human immunoglobulin G1. It is approved in the United States for use in combination with FOLFIRI for the treatment of patients with metastatic colon cancer resistant to or who have progressed following an oxaliplatin-containing regimen[44,45].

Due to a similar mechanism of action as bevacizumab (anti-VEGF), aflibercept shares a similar side effect profile including hemorrhage, hypertension, thromboembolism, bowel perforation, and impaired wound healing. Identification of and early symptom management, as well as dose modification as necessary are important in managing toxicities. If patients develop recurrent or severe hypertention, treatment needs to be withheld until blood pressure is controlled and then resumed with a permanent dose reduction. Treatment should be discontinued if patients develop a hypertensive crisis, fistula formation, GI perforation, or severe hemorrhage (see manufacturer package insert).

ANTI-EGFR MONOCLONAL ANTIBODIES

Cetuximab, panitumumab

Activation of epidermal growth factor (EGF) pathway is dependent on ligand binding to its receptor (EGFR), with subsequent homo- and heterodimerization leading to activation of signaling pathways. Cetuximab and panitumumab are monoclonal antibodies directed against EGFR. However, they exert their action on both malignant and normal cells. Cetuximab and panitumumab are only effective in patients who have K-ras wild type tumor[46-48]. While cetuximab is more commonly used in combination with irinotecan based regimens, panitumumab is approved only as a single agent after failure of other regimens[48,49]. Whether panitumumab is of benefit in patients who are refractory to cetuximab is unknown[50].

Since anti-EGFR monoclonal antibodies also bind to EGFR receptors in normal tissue, these agents affect organs with abundant receptors and may cause skin and gastrointestinal toxicities (rash, dryness, pruritus, and diarrhea). Of particular interest, early identification and

proper grading of skin toxicity, as well as symptom management are important. Patients should be educated to recognize the signs and symptoms of toxicity, as well as general prevention strategies such as applying sunscreen and alcohol-free moisturizing creams[51,52]. Hypomagnesaemia is another significant toxicity of this class of drug. Frequent laboratory monitoring and repletion are often required[53,54].

RECEPTOR TYROSINE-KINASE INHIBITOR

Regorafenib

Regorafenib is a new oral multikinase inhibitor that blocks the activity of several protein kinases, including the VEGF and EGFR pathways. It is approved as a single agent for the treatment of patients with refractory mCRC[55].

The most common side effects of Regorafenib are grade 3 or 4 PPED also known as hand-foot skin reaction, fatigue, hypertension, diarrhea, and skin rash. These toxicities tend to occur during the first treatment cycle and then diminish over time[55]. Early identification, intervention, and dose reduction, are key to managing these side effects.

ACTIVE REGIMEN FOR LOCALLY ADVANCED AND METASTATIC COLON CANCER

The following regimens are summarized in Table 1.

FOLFOX

A SEER-Medicare database analysis found that the addition of oxaliplatin to 5-FU/LV adjuvant therapy in elderly patients with stage Ⅲ disease resulted only in a small but non-significant OS benefit[7].

A subset analysis of major adjuvant therapy trials also showed a lack of benefit with the addition of oxaliplatin in older patients. The NSABP C-07 trial found that the addition of oxaliplatin to 5-FU/LV did not prolong survival in patients age 70 and older with stage Ⅱ or Ⅲ colon cancer. There was actually a trend toward decreased survival[56]. A subset analysis of the MOSAIC trial did not show survival benefit with the addition of oxaliplatin for patients of age 70-75 with stage Ⅱ or Ⅲ colon cancer[57]. However, the median age of patients enrolled in the MOSAIC study was 59 with only one-third of these patients were over the age of 65. Due to the small number of elderly patients included in this retrospective analysis, the use of oxaliplatin as adjuvant treatment in elderly patients remains inconclusive.

In the metastatic setting, however, the addition of oxaliplatin to fluoropyrimidine-based regimens significantly improved outcomes without worsening toxicity in elderly and frail patients[58].

If indicated, oxaliplatin 85 mg/m^2 Ⅳ is usually given in combination with LV 400 mg/m^2 Ⅳ over 2 h and 5-FU (400 mg/m^2 Ⅳ bolus on day 1 followed by 2400 mg/m^2 continuous Ⅳ infusion over 46 h)[59]. The cycle is repeated every two weeks for a total of 12 cycles in adjuvant setting.

CAPOX/XELOX

In a randomized trial comparing capecitabine plus oxaliplatin (XELOX) *vs* FOLFOX regimen, XELOX was found to be non-inferior as a first line treatment regimen for mCRC[60]. In the adjuvant setting, the combination of oxaliplatin and capecitabine has been shown to improve disease free and overall survival with less toxicity when compared to standard bolus 5-FU/LV[61-63]. The standard regimen is capecitabine 850-1000 mg/m^2 orally twice daily, from day 1 to 14, with oxaliplatin 130 mg/m^2 Ⅳ on day 1 of every three week cycle.

Single agent capecitabine

The approved dose of oral capecitabine is 1250 mg/m^2 twice daily for 2 wk, every 21 d, either as monotherapy or in combination with other agents[21,22]. The dose is often reduced to 1000 mg/m^2 twice daily (in combination with oxaliplatin) on days 1-14 of a three week cycle[60,62,64]. No clinical trial has yet been done to compare these different dosing regimens. In one study of 51 elderly patients (mean age 76) with advanced CRC, treatment with capecitabine was well tolerated[65].

FOLFOX + bevacizumab

The benefit of adding bevacizumab to an oxaliplatin-containgin regimen has been addressed in several clinical trials and showed an improvement in RR, PFS, and OS[38-40]. However, the use of bevacizumab also increased the risk of bowel perforation, impaired wound healing, grade 3 or 4 hypertension, and bleeding events[38].

In the TREE-2 trial, bevacizumab was added to oxaliplatin and fluoropyrimidine regimens. These regimens were well tolerated as first-line treatment of mCRC with similar overall toxicity. The first-line oxaliplatin and fluoropyrimidine-based regimen with bevacizumab resulted in a median OS of approximately 2 years[38].

The dosing regimen is oxaliplatin 85 mg/m^2 Ⅳ, bevacizumab 5 mg/kg Ⅳ, LV 400 mg/m^2 Ⅳ, and 5-FU 400 mg/m^2 Ⅳ bolus on day 1, followed by 2400 mg/m^2 continuous Ⅳ infusion over 46 h; every 2 wk.

FOLFIRI + bevacizumab

A phase Ⅲ randomized clinical trial comparing the addition of bevacizumab to 5-FU-based combination chemotherapies (irinotecan, bolus fluorouracil, and leucovorin [IFL]) showed improved objective RR, PFS, and OS[42]. Another randomized trial comparing 5-FU given as continuous infusion *vs* bolus (FOLFIRI *vs* IFL), both with bevacizumab, showed a superior result with the former[66]. In the bevacizumab expanded access trial (BEAT), bevacizumab added to first-line chemotherapy showed a comparable efficacy and safety profile compared to chemotherapy alone[39].

Table 1 Chemotherapy regimen in locally advanced and metastatic colon cancer

No.	Regimen	Dosing	Frequency	Adjuvant	Palliative
1	5-FU/LV	Leucovorin 400 mg/m^2 IV over 2 h before 5-FU on day 1 5-FU 400 mg/m^2 IV bolus on day 1, followed by 2400 mg/m^2 IV over 46 h	Every 2 wk	Y	Y
2	Capecitabine	Capecitabine 1000-1250 mg/m^2 by mouth twice daily for 2 wk, then 1 wk off	Every 3 wk	Y	Y
3	FOLFOX	Leucovorin 400 mg/m^2 IV over 2 h before 5-FU on day 1 5-FU 400 mg/m^2 IV bolus on day 1, followed by 2400 mg/m^2 IV over 46 h Oxaliplatin 85 mg/m^2 IV on day 1	Every 2 wk	Y	Y
4	CAPOX	Capecitabine 850-1000 mg/m^2 by mouth twice daily for 2 wk, then 1 wk off Oxaliplatin 130 mg/m^2 IV on day 1	Every 3 wk	Y	Y
5	FOLFIRI	Leucovorin 400 mg/m^2 IV over 2 h before 5-FU on day 1 5-FU 400 mg/m^2 IV bolus on day 1, followed by 2400 mg/m^2 IV over 46 h Irinotecan 180 mg/m^2 IV over 90 min on day 1	Every 2 wk	N	Y
6	FOLFOX + Bevacizumab	Leucovorin 400 mg/m^2 IV over 2 h before 5-FU on day 1 5-FU 400 mg/m^2 IV bolus on day 1, followed by 2400 mg/m^2 IV over 46 h Oxaliplatin 85 mg/m^2 IV on day 1 Bevacizumab 5 mg/kg IV on day 1	Every 2 wk	N	Y
7	FOLFIRI + Bevacizumab	Leucovorin 400 mg/m^2 IV over 2 h before 5-FU on day 1 5-FU 400 mg/m^2 IV bolus on day 1, followed by 2400 mg/m^2 IV over 46 h Irinotecan 180 mg/m^2 IV over 90 min on day 1 Bevacizumab 5 mg/kg IV on day 1	Every 2 wk	N	Y
8	CAPOX + Bevacizumab	Capecitabine 850-1000 mg/m^2 by mouth twice daily for 2 wk, then 1 wk off Oxaliplatin 130 mg/m^2 IV on day 1 Bevacizumab 7.5 mg/kg IV on day 1	Every 3 wk	N	Y
9	Capecitabine + Bevacizumab	Capecitabine 850-1000 mg/m^2 by mouth twice daily for 2 wk, then 1 wk off Bevacizumab 7.5 mg/kg IV on day 1	Every 3 wk	N	Y
10	5-FU/LV + Bevacizumab	Leucovorin 400 mg/m^2 IV over 2 h before 5-FU on day 1 5-FU 400 mg/m^2 IV bolus on day 1, followed by 2400 mg/m^2 IV over 46 h Bevacizumab 5 mg/kg IV on day 1	Every 2 wk	N	Y
11	FOLFIRI + Cetuximab	Leucovorin 400 mg/m^2 IV over 2 h before 5-FU on day 1 5-FU 400 mg/m^2 IV bolus on day 1, followed by 2400 mg/m^2 IV over 46 h Irinotecan 180 mg/m^2 IV over 90 min on day 1 Cetuximab 400 mg/m^2 IV loading on treatment day 1, then 250 mg/m^2 IV every week	Every 2 wk	N	Y
12	FOLFIRI + Ablifercept	Leucovorin 400 mg/m^2 IV over 2 h before 5-FU on day 1 5-FU 400 mg/m^2 IV bolus on day 1, followed by 2400 mg/m^2 IV over 46 h Irinotecan 180 mg/m^2 IV over 90 min on day 1 Aflibercept 4 mg/kg, over 1 h on day 1	Every 2 wk	N	Y
13	Panitumumab	Panitumumab 6 mg/kg IV	Every 2 wk	N	Y
14	Regorafenib	Regorafenib 160 mg by mouth once daily for 3 wk, then 1 wk off	Every 4 wk	N	Y

5-FU: Fluorouracil.

The dosing regimen is irinotecan 180 mg/m^2 IV, bevacizumab 5 mg/kg IV, LV 400 mg/m^2 IV, and 5-FU 400 mg/m^2 IV bolus on day 1, followed by 2400 mg/m^2 continuous IV infusion over 46 h; every two weeks[67].

CAPOX + bevacizumab

The addition of bevacizumab to either XELOX or FOLFOX4 showed improved median PFS when compared to either regimen without bevacizumab[40].

The dosing regimen is oxaliplatin 130 mg/m^2 *iv*, bevacizumab 7.5 mg/kg *iv* on day 1; capecitabine 850-1000 mg/m^2 by mouth twice daily on day 1 to 14, every three weeks.

Fluoropyrimidines + bevacizumab

Bevacizumab adds benefit to first-line 5-FU/LV or capecitabine with improvement in RR, PFT, and OR[36,37]. The addition of bevacizumab to capecitabine also improves PFS compared to capecitabine alone in elderly patients age 70 and older. However, more treatment-related adverse events, inlcuding hand-foot syndrome, diarrhea, venous thrombotic events, and hemorrage were observed with the addition of bevacizumab[68,69].

The dosing regimen is bevacizumab 7.5 mg/kg IV on day 1 with capecitabine 850-1000 mg/m^2 by mouth twice daily on day 1 to 14, every three weeks. When combine with 5-FU/LV containing regimen, the dosing is bevacizumab 5 mg/kg IV, LV 400 mg/m^2 IV, and 5-FU 400 mg/m^2 IV bolus on day 1, followed by 2400 mg/m^2 continuous IV infusion over 46 h, every two weeks.

FOLFOX + cetuximab

Several studies have shown higher RR and prolongation in PFS with the addition of cetuximab, but without significant effect on OS[70]. However, other trials showed no clear benefit in adding cetuximab to a first-line oxaliplatin-containing regimen in patients with K-ras wild-type tumors with only a modest improvement in RR[71,72]. For this reason, the benefit of adding cetuximab to a first-line oxaliplatin-containing regimen remains unclear.

FOLFIRI + cetuximab

Cetuximab can be used in combination with irinotecan for patients with wild-type K-ras tumors. Multiple phase

Ⅲ randomized controlled trials have shown improvement in RR and PFS, but failed to show significant OS benefit[73-75]. Cetuximab is given as a weekly infusion, although some data support the safety and efficacy of every other week dosing, which is often done for patients convenience.

The dosing regimen is cetuximab 400 mg/m^2 Ⅳ loading on first treatment day 1, and then 250 mg/m^2 Ⅳ weekly, with irinotecan 180 mg/m^2 Ⅳ, bevacizumab 5 mg/kg Ⅳ, LV 400 mg/m^2 Ⅳ, and 5-FU 400 mg/m^2 Ⅳ bolus on day 1, followed by 2400 mg/m^2 continuous Ⅳ infusion over 46 h; every 2 wk.

FOLFIRI + aflibercept

Aflibercept in combination with FOLFIRI is approved for treatment of patients with mCRC that is resistant to or has progressed following an oxaliplatin-containing regimen. A placebo controlled trial compared FOLFIRI with or without aflibercept given in patients who failed a oxaliplatin containing regimen. An improved median PFS and OS were observed in patients receiving aflibercept[44].

The dosing regimen is aflibercept 4 mg/kg, followed immediately by the FOLFIRI regimen (irinotecan 180 mg/m^2 Ⅳ, bevacizumab 5 mg/kg Ⅳ, LV 400 mg/m^2 Ⅳ, and 5-FU 400 mg/m^2 Ⅳ bolus on day 1, followed by 2400 mg/m^2 continuous Ⅳ infusion over 46 h) every 2 wk.

Single agent panitumumab

Panitumumab as a single agent is approved for treatment of K-ras wild-type mCRC. Studies evaluating the addition of panitumumab to either FOLFOX or FOLFIRI have shown improvement in PFS, but no survival benefit. However, lower survival and increased toxicity were observed when panitumumab was combined with other agents, including oxaliplatin and bevacizumab[48,76-78]. For this reason, panitumumab is not indicated for use in combination with chemotherapy. The dosing regimen is 6 mg/kg Ⅳ every 2 wk.

Single agent regorafenib

Oral regorafenib is approved for patients with metastatic colon cancer that has progressed after all standard therapies. In a randomized trial comparing regorafenib to best supportive care, regorafenib showed a modest though statistically significant improvement in PFS and median OS[55].

The dosing regimen is 160 mg once daily for 21 d of a 28-d cycle.

METASTASECTOMY

In a large international multicenter cohort study evaluating the outcome of liver surgery for metastatic colon cancer in patients age 70 and older, a 3-year survival rate of 57% and a 60-d perioperative mortality rate of 4% were observed[79]. These results were comparable to previous studies with younger age groups.

Therefore, the management of potentially resectable liver metastases in elderly patients with good performance status should be the same as in younger patients. Older patients may also benefit from neoadjuvant chemotherapy to convert borderline resectable lesions to resectable disease. Several studies showed a similar response rate and five-year OS among younger and older individuals who received neoadjuvant chemotherapy followed by liver resection[80,81].

Although there is no firm data on solitary pulmonary metastases, metastasectomy may be considered for fit older patients with isolated pulmonary metastases[82]. Older age (> 60-year-old), male, and increased lung metastases are negative predictors for survival after pulmonary metastatectomy[83].

TOLERABILITY OF CHEMOTHERAPY IN ELDERLY PATIENTS

When treating elderly patients with cancer one has to consider drug pharmacokinetics and pharmacodynamics. Elderly patients have age related changes in organ function as well as comorbidities. Drug toxicities may be due to a reduction in renal or hepatic function. Also, impaired drug efficacy may be due to age-related decreased intestinal absorption (for oral medications).

ASSESSING FUNCTIONAL STATUS OF ELDERLY PATIENTS

Since chronological age is a poor marker of a patient's functional status, several methods of functional assessment may be used.

Eastern Cooperative Oncology Group performance status

Eastern Cooperative Oncology Group (ECOG) performance status (PS) (Table 2) is useful to assess a patient's ability to tolerate chemotherapy and their short-term prognosis. Patients with a poor performance status (PS) (*e.g.*, ECOG PS > 2) usually tolerate chemotherapy poorly and have shorter median OS. Older patients with poor PS also often have more functional impairment[84].

ADL and IADL scales

Activities of Daily Living (ADL) and Instrumental Activities of Daily Living (IADL) scales are more representative of a patient's functional status. ADL refers to the skills that are necessary for basic living such as self-care and include feeding, grooming, transferring, and toileting. IADL refers to the skills required to live independently in the community including shopping, managing finances, housekeeping, preparing meals, and the ability to take medications.

Comprehensive geriatric assessment

Assessment of functional status with the ADL and IADL

Table 2 Eastern Cooperative Oncology Group performance status

Grade	Description
0	Fully active, able to carry on all pre-disease performance without restriction
1	Restricted in physically strenuous activity but ambulatory and able to carry out work of a light or sedentary nature, *e.g.*, light housework, office work
2	Ambulatory and capable of all self-care but unable to carry out any work activities. Up and about more than 50% of waking hours
3	Capable of only limited self-care, confined to bed or chair more than 50% of waking hours
4	Completely disabled. Cannot carry on any self-care. Totally confined to bed or chair
5	Dead

scales is a component of the comprehensive geriatric assessment (CGA) scale that is used by geriatricians to identify frail older patients at high risk of adverse outcomes such as falls, hospitalization, and death. The task force of International Society of Geriatric Oncology recommends the use of CGA in the care of older cancer patients[85].

CHEMOTHERAPY SELECTION IN ELDERLY PATIENTS

There is no ideal chemotherapy regimen that fits all patients and so a regimen needs to be tailored to each individual. Important considerations when treating elderly patients include convenience and tolerability. While using a 5-FU based regimen, patients will require a portable outpatient infusion pump and an indwelling venous catheter. Otherwise, patients will have to be admitted to the hospital for at least 48 h in order to complete a 5-FU continuous infusion. In our institution, bolus 5-FU is often omitted if there is a concern for increased toxicity in the metastatic setting.

Capecitabine, on the other hand, is given orally. Often times, this drug may be a better alternative for selected patients. However, since capecitabine has to be taken twice daily for 14 d, compliance may be an issue. We recommend that patients who are treated with oral capecitabine use a pill container with scheduled compartments to help with compliance. Nursing staff can also monitor the frequency of refills. In our center, patients are given an individualized chemotherapy calendar.

In the adjuvant setting, we recommend 5-FU continuous IV infusions or oral capecitabine alone for six months for patients age 60 and older.

In the metastatic setting, FOLFOX has a comparable activity to FOLFIRI[86,87]. The choice of which to use should be based upon the expected toxicities of each regimen and the patients comorbidities. If there are no contraindications, bevacizumab may be added to either regimen. A fluoropyrimidine can be given to a patient either *via* an intravenous infusion (5-FU) or by an oral route (capecitabine). If patients are not considered candidates for more intensive therapy due to a poor functional status, then oxaliplatin or irinotecan should not be given. In that case, either an intravenous 5-FU infusion or oral capecitabine with or without bevacizumab is an appropriate option.

Short term 5-FU/LV continuous infusion is preferable to a 5-FU bolus due to a favorable toxicity profile[88].

When patients progress, FOLFOX can be changed to FOLFIRI, or vice-versa, while maintaining treatment with bevacizumab[89]. If the patient is initially treated with a fluoropyrimidine alone, then the addition of either oxaliplatin or irinotecan could be considered. This is especially relevant if the patient has an improvement in functional status. If the patient has a K-ras wild type tumor, cetuximab can be added to FOLFIRI, especially if a FOLFIRI-based regimen was not used first-line. Another alternative is to give FOLFIRI plus ziv-aflibercept when a FOLFOX regimen has already been given as first-line therapy and the patient has progressed.

If the patients functional status declines or does not improve, therapy with single agent panitumumab, cetuximab, regorafenib, or even best supportive care (BSC), are options.

BEST SUPPORTIVE CARE

Many clinical trials were designed to compare drug therapy versus BSC, especially for patients resistant to multiple lines of chemotherapy[90]. BSC is palliative treatment without using chemotherapy with the intent to maximize quality of life (QOL). Appropriate BSC includes antibiotics, analgesics, antiemetics, thoracentesis, pleurodesis, blood transfusions, nutritional support, and also focal external-beam radiation for symptomatic control[91].

Symptom assessment and management is paramount to provide BSC. Once assessed, symptoms should be managed in accordance with one of the many existing evidence-based guidelines[92].

WHOM TO TREAT

There is a general agreement that frail older patients, those with significant functional impairment or an ECOG PS of 3 to 4, should be offered palliative measures aimed at maintaining QOL. Most of the time, they have poor tolerance to aggressive treatment for their cancer. However, active and fit older patients with minimal comorbidities should be treated in the same fashion as younger patients with metastatic colon cancer[93]. Patients with metastatic colon cancer who have a PS of 2 or less should be considered for chemotherapy, particularly if their PS decline is believed to be cancer related.

Table 3 Most common side effects of active agents in colon cancer and their management

Agent	Major side effects	Management
Fluoropyrimidine	Stomatitis, diarrhea, hand-foot syndrome	Identification and early symptom management
5-FU	Vomiting	Dose interuption or reduction if progression (grade 2 or worse)
Capecitabine	Pancytopenia	Adjustment of route of administration: bolus vs continuous infusion
		Predetermined treatment parameter
Oxaliplatin	Peripheral neuropathy (dose limiting)	Education about exposure to cold, dose modification, "stop and go" strategy, and use of neuromodulatory agents
	Pancytopenia	Predetermined treatment parameter
	Nausea, vomiting, diarrhea, fatigue	Identification and early symptom management.
		Dose interuption or reduction if progression (grade 2 or worse)
Irinotecan	Diarrhea	Premedication with atropine sulfate
	Pancytopenia	Proper instruction for the use of anti-motility agent to control diarrhea
		Predetermined treatment parameter
Anti EGFR	Skin toxicity (rash, dryness, pruritus)	Identification and early symptom management
Cetuximab	Mucositis	Proper instruction for the use of anti-motility agent to control diarrhea
Panitimumab	Diarrhea	Dose interuption or reduction if progression (grade 2 or worse).
Anti VEGF	Wound healing impairment	Blood pressure monitoring and adding anti-hypertensive agent if needed
Bevacizumab	Thromboembolism	Avoid in high risk patients.
Ziv-aflibercept	Bowel perforation	Close monitoring if used in patients at risk
	Proteinuria	Regular monitoring of urine protein secretion with urine dipstick or 24HR
	Hypertension	urine protein to creatinine ratio
		Holding medication prior to elective surgical procedure (6-8 wk)
		Appropriate healing time before re-starting medication post-op
Receptor TKI inhibitors	Hand-foot skin syndrome, rash	Identification and early symptom management
Regorafenib	Diarrhea, hypertension	Dose modification

5-FU: Fluorouracil; EGFR: Epidermal growth factor receptor; VEGF: Vascular endothelial growth factor; TKI: Tyrosine-kinase inhibitor.

Although the incidence of postoperative morbidity and mortality increases with advancing age, elderly patients still benefit from surgery and therefore should be evaluated for resectability[94].

STRATEGIES IN TREATING ELDERLY PATIENTS

After carefully selecting an appropriate chemotherapy regimen for elderly patients, the following are additional strategies to improve tolerability and successful completion of a planned treatment.

Prepare the patient for what to expect

Discussing chemotherapy and their side effects during an office visit will encourage patients to read the drug fact information sheets provided. When patients understand what to expect during treatment and what actions to take when they experience side effects they will be reassured and less anxious. In our center, patients are encouraged to participate in the chemotherapy teaching class led by oncology certified nurses.

Early side effect management

Elderly patients are more susceptible to toxicities when receiving chemotherapy. For example, patients age 70 and older with metastatic colon cancer on 5-FU-based chemotherapy are more prone to diarrhea, vomiting, stomatitis, and neutropenia[95,96]. Therefore, a follow up appointment should be scheduled early, especially during the initiation of a new regimen. Patients should have access to immediate medical attention when the expected side effects occur. We summarize the most common side effect profiles of active agents in colon cancer and their managements in Table 3.

CONCLUSION

Treating elderly patients with advanced and metastatic colon cancer is often challenging due to a lack of strong evidence from which to choose the most appropriate regimen. Elderly patients with locally advanced and metastatic colon cancer will benefit from chemotherapy and biologic agents. Fit elderly patients are especially appropriate for treatment and should be offered the same regimens as their younger counterparts. Treatment related toxicities and QOL should be monitored very closely in elderly patients. For this reason, more frequent follow-up of elderly patients receiving chemotherapy should be arranged. In frail elderly patients, sequential single agent chemotherapy may be more tolerable than combination therapy.

The decision to treat elderly patients with advanced and metastatic colon cancer requires the incorporation of a thorough evaluation of the patients functional status, including ECOG PS and also ADL/IADL capacity as well as estimated life expectancy. Chronological age does not always correlate with a patient's functional status. If a patients decline in functional status is due to cancer, chemotherapy should be considered since a treatment response may lead to clinical improvement.

Elderly patients with locally advanced and metastatic colon cancer attain significant benefit from chemotherapy and biologic agents. Chronological age does not always correlate with a patient's functional status. Fit

elderly patients should be offered the same regimens as their younger counterparts. A chemotherapy regimen should be carefully selected based on patients characteristic and underlying medical problems. Frequent follow-up for elderly patients receiving chemotherapy is often required. If a patients decline in functional status is due to cancer, chemotherapy should be considered since a treatment response may lead to clinical improvement.

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TOPIC HIGHLIGHT

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

Targeting cell death signaling in colorectal cancer: Current strategies and future perspectives

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Abstract

The evasion from controlled cell death induction has been considered as one of the hallmarks of cancer cells. Defects in cell death signaling are a fundamental phenomenon in colorectal cancer. Nearly any non-invasive cancer treatment finally aims to induce cell death. However, apoptosis resistance is the major cause for insufficient therapeutic success and disease relapse in gastrointestinal oncology. Various compounds have been developed and evaluated with the aim to meet with this obstacle by triggering cell death in cancer cells. The aim of this review is to illustrate current approaches and future directions in targeting cell death signaling in colorectal cancer. The complex signaling network of apoptosis will be demonstrated and the "druggability" of targets will be identified. In detail, proteins regulating mitochondrial cell death in colorectal cancer, such as Bcl-2 and survivin, will be discussed with respect to potential therapeutic exploitation. Death receptor signaling and targeting in colorectal cancer will be outlined. Encouraging clinical trials including cell death based targeted therapies for colorectal cancer are under way and will be demonstrated. Our conceptual understanding of cell death in cancer is rapidly emerging and new types of controlled cellular death have been identified. To meet this progress in cell death research, the implication of autophagy and necroptosis for colorectal carcinogenesis and therapeutic approaches will also be depicted. The main focus of this topic highlight will be on the revelation of the complex cell death concepts in colorectal cancer and the bridging from basic research to clinical use.

Key words: Colorectal cancer; Apoptosis; Necroptosis; Autophagy; Clinical trial; Bcl-2 proteins; BH-3 mimetics; Inflammatory bowel disease

Core tip: This review highlights current strategies targeting cell death signaling in colorectal cancer. The role of apoptosis, autophagy and necroptosis in the normal colon mucosa as well as in colorectal cancer onset and therapy is defined. Relevant small molecule compounds as well as antisense based approaches for the treatment of colorectal cancer are illustrated. Furthermore, clinical trials investigating new cell death based compounds are discussed. Finally, future directions in translational cell death research are discussed.

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CELL DEATH IN THE NORMAL COLORECTUM

The crypts of the colorectal mucosa are organized in a

polarized fashion. Very few stem cells at the base of a crypt comprise the pool of the regenerative epithelium in which cells travel from bottom to top of the crypt. On the apical edge of the mucosa, about 10^{10} cells per day die by apoptosis and are subsequently shed in the lumen[1]. This fact illustrates the essential need of a proper regulated cell death for the homeostasis of a normal colorectal mucosa. However, defective signaling or dysbalanced regulation of apoptosis is a very likely cause for the initiation and progression of an adenoma to carcinoma sequence ending up in colorectal cancer (CRC). Of note, proteins relevant for apoptosis (*e.g.*, Bak or Bcl-2) are not equally expressed in all parts of the colorectal mucosa pointing on distinct regulation of death in the intestine[2,3].

In addition to apoptosis as the classical form of programmed cell death, autophagy, a controlled process of cellular self digestion of great importance in situations of cellular stress or upon energy deprivation, has been shown to be active and relevant in colorectal glands. In contrast to apoptosis, the autophagic flux intensity decreases in the crypt from bottom to top[4]. This has been indicated by high expression levels of proautophagic protein Beclin-1 and the conversion of LC3- I to LC3- II in lower crypt cells. On their way to the apex of a crypt the epithelial cells lose Beclin-1 expression and accumulate high levels of SQSTM1/p62, which is an ubiquitin-associated adaptor protein maintaining autophagic flux[4].

In summary, the integrity of the complex interplay of cell death signaling is fundamental for mucosal development and homeostasis in the colorectum. Defective or dysbalanced cell death signaling is involved in the pathogenesis of a variety of colorectal diseases from chronic bowel diseases (Crohn's disease as well as ulcerative colitis) to colorectal carcinoma.

CELL DEATH IN INTESTINAL DISEASE AND CARCINOGENESIS

Colorectal carcinoma can occur sporadically, the most common situation, on the base of defined mutations and also as a final consequence of chronic inflammatory diseases of the intestine[5,6]. The intriguing field of cancer related to chronic inflammation will not be in the focus of this review and the reader might refer to comprehensive literature by others addressing this issue[7-11].

During the development of CRCs from benign polyps through adenomas and finally adenocarcinomas, cell death plays a fundamental role. Key regulating proteins of an appropriate mucosal cell death undergo changes in expression during the transition of an adenoma-carcinoma-sequence[12-14]. For instance, antiapoptogenic Bcl-2 gets lost during the development from adenoma to carcinoma[14]. However, especially the value of cell death related proteins as biomarkers for prognosis and prediction of CRC is of great interest, but the available literature is inconsistent and controversial[15-17]. In summary, apoptosis signaling proteins are in the context of biomarkers either ill defined or need further validation[18]. The reason for these contradictory reports might be due to the extraordinary heterogeneity of CRCs and the broad variety of the carcinogenesis driving mutations[5,19,20]. The aim of this review is to identify possible targets in the cell death signaling network and discuss the compounds available to foster killing of colorectal cancer cells.

TARGETING CELL DEATH IN COLORECTAL CANCER

Apoptosis: Implications for therapy

Defects in apoptosis signaling are common in colorectal cancers. An acquired resistance towards cell death may be a key feature of both, carcinogenesis and therapy resistance[21]. However, proteins within the apoptosis signaling pathways have been evaluated for their value as predictive and or prognostic markers as well as targets for therapeutic approaches[18]. Figure 1 shows a synopsis of apoptosis signaling and indicates relevant targets and compounds.

INTRINSIC PATHWAY

Mitochondria are in the very centre of the intrinsic pathway of apoptosis. The mitochondrial membrane integrity is regulated by the Bcl-2 family of proteins. A tight balance of pro- and antiapoptotic Bcl-2 proteins governs cell's fate at the mitochondrial surface. In response to several unfavorable conditions (*e.g.*, growth factor withdrawal, DNA damage), this balance shifts towards death. In this case, the proapoptotic proteins (*e.g.*, BAX and BAK) are released by their antiapoptotic relatives (Bcl-2, Bcl-xL, Mcl-1, Bcl-w and A1)[22]. The proapoptotic proteins finally lead to mitochondrial outer membrane permeabilisation and the immediate release of cytochrome C (cytC) into the cytosol. Together with APAF-1 and Caspase 9, cytC forms a death inducing protein platform called apoptosome which in turn leads to activation of caspase 3 as the central downstream event of cell death execution[23].

BH3-mimetics

Within the intrinsic pathway of apoptosis, the antiapoptotic Bcl-2 proteins have been extensively studied as "druggable" targets. Various small molecules targeting the antiapoptotic proteins by binding to their BH3 cleft. This mechanism of action causes a release of multidomain proapoptotic Bcl-2 proteins (*e.g.*, Bim, Bak or/and Bax) which in turn promote cell death. ABT-737 and its orally available derivate ABT-263 (navitoclax) are potent inhibitors of Bcl-2, Bcl-w and Bcl-xL. ABT-263 has recently been shown to induce cell death in colorectal cancer cells *in vitro* synergistically with the inhibition of the prosurvival kinase MAP kinase/ERK kinase 1/2[24]. This mechanism of death induction by ABT-263 was completely dependent on Bax and Bim. Several phase I trials in solid cancers have proven the safety of ABT263 in combination with established therapy regimes (www.clinicaltrials.gov). ABT-737 has been shown to act synergistically with

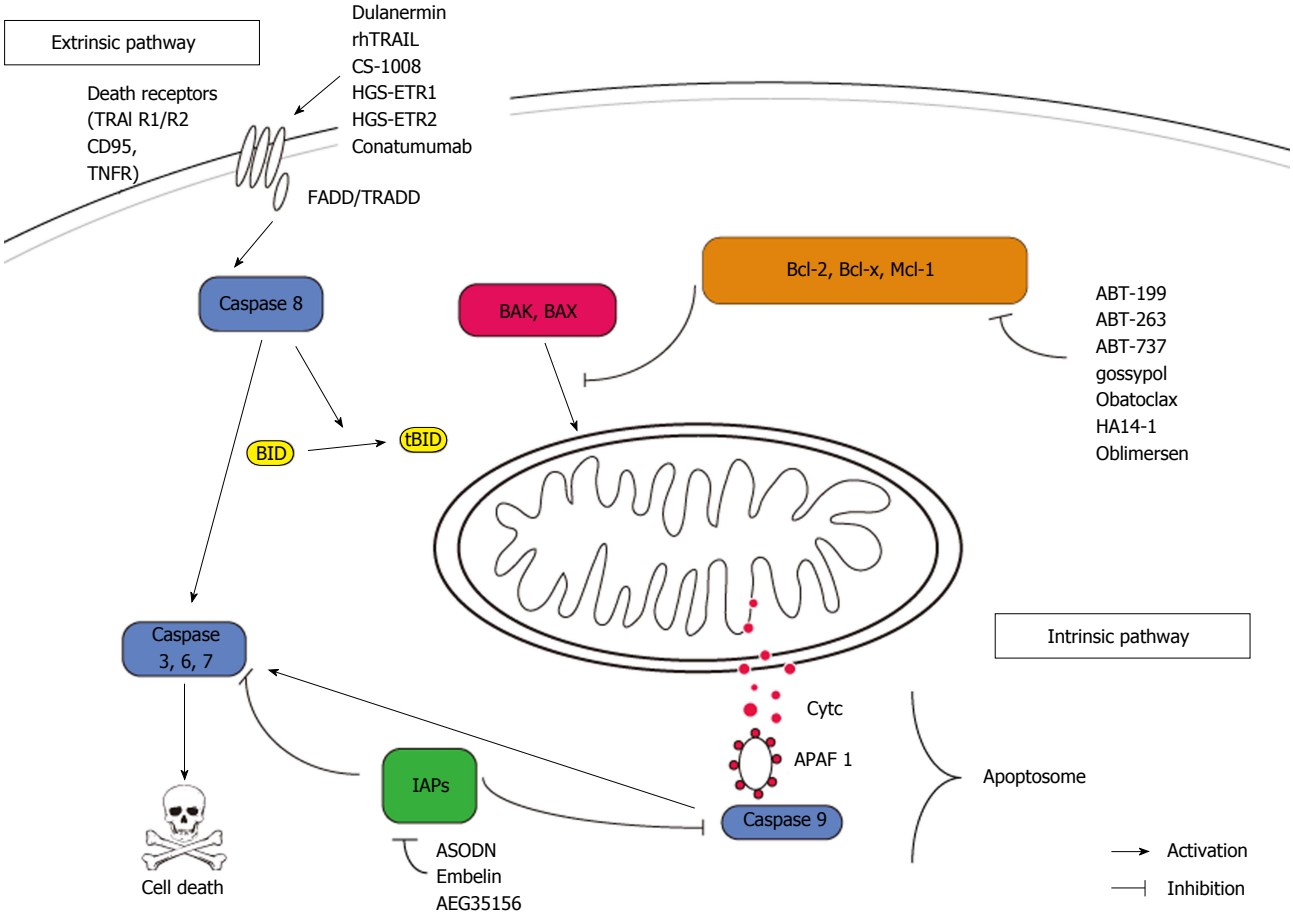

Figure 1 Apoptosis signaling and cell death relevant drugs. Cell death based cancer therapy can be approached by targeting proteins in the extrinsic or intrinsic pathway. Relevant agents, currently under clinical investigation, are listed and placed to their targets. APAF1: Apoptotic protease activating factor-1; CytC: Cytochrome C; IAP: Inhibitors of apoptosis; tBID: Truncated BID; FADD: Fas-associated protein with death domain; TNF: Tumor necrosis factor; TRAIL: Tumor necrosis factor related apoptosis-inducing ligand.

oxaliplatin on CRC cells *in vitro*[25]. An *ex vivo* evaluation of ABT-737 in samples of ovarian tumors is under way (www.clinicaltrials.gov). In addition, ABT-737 enhanced apoptosis in CRC cells induced by cyclo-oxygenase-2 inhibitor celecoxib[26]. Importantly, the sensitivity of cancer cells towards ABT-737 is dictated by the expression of NOXA and its control by Mcl-1, which is not targeted by ABT-737[27,28]. Interestingly, Mcl-1 sparing BH-3 mimetics such as ABT-737, ABT-199 and ABT-263, have been shown to effectively induce apoptosis in hypoxic regions of human colorectal tumor spheres. Hypoxia led to a profound downregulation of Mcl-1 which is responsible for ABT-737 resistance in many settings[29]. This work is of great interest since few normal tissues are exposed to hypoxia, but it is a common challenge for growing tumors[30]. HA14-1 is a highly selective small molecule targeting Bcl-2 only. HA 14-1 has been shown to overcome TRAIL resistance in CRC cells by counteracting Bcl-2 overexpression[31,32].

Obatoclax is a first-in-class BH-3 mimetic with an inhibitory profile including Bcl-2, Bcl-xL, Bcl-w, Mcl-1 and A1 (pan-Bcl-2-inhibitor)[33]. Given the crucial role of Mcl-1 for resistance towards BH-3 mimetics, obatoclax is a promising new agent targeting the complete antiapoptoic Bcl-2 protein family members at once. Few studies investigated the potency of obatoclax for colorectal cancer treatment. It has been recently shown that cell death induction through inhibition of the proproliferative protein Notch by gamma secretase inhibitors is fostered by obatoclax[34].

Oblimersen is an antisense oligonucleotide targeting the first six codons of Bcl-2. Antisense technology represents a highly specific approach for downregulation of antiapoptotic proteins without off-target effects[35]. A phase I trial has shown the safety of oblimersen in combination with irinotecan when intravenously administered in patients with metastatic CRC[36].

In summary, Bcl-2 proteins are context-sensitive targets in colorectal cancer treatment alongside established chemotherapy or radiation. Future studies are urgently warranted to reveal the potential of BH-3 mimetics in colorectal cancer in the clinical setting.

IAP inhibitors

The inhibitor of apoptosis (IAP) family acts by blocking caspase activity (primarily caspase 3). IAPs are found to be overexpressed in several cancer entities including CRC and are able to protect cancer cells from various death

stimuli[37,38]. Several compounds inhibit IAPs (primarily XIAP and Survivin). AEG35156 is a second generation antisense oligonucleotide targeting XIAP. Preclinical and early clinical data revealed a promising death-inducing potential of AEG35156 in several solid tumor entities including CRC[39-42]. Survivin is a second promising target among the IAP family overexpressed in CRC. Survivin antisense oligonucleotides strikingly cleared the way for death induction in CRC cells *in vitro*[43]. Embelin, a naturally occurring benoquinone, has been proven effective in various tumor entities by targeting survivin and other antiapoptotic proteins (Bcl-2 and Bcl-xL)[44]. In the colon, Embelin was able to sufficiently attenuate colitis and carcinoma development in rodents[45,46]. Finally, a double edged approach targeting survivin and XIAP might be a very promising approach for CRC treatment[47].

SMAC mimetics

Second mitochondria activator of caspases (SMAC)/Diablo is a mitochondria derived, proapoptotic protein acting by blocking IAPs thereby promoting caspase dependent cell death[48]. SMAC mimetics have been shown to strongly sensitize CRC cells towards NSAID induced apoptosis through a feedback amplification resulting in the activation of caspase 3[49]. In TRAIL-induced apoptosis in CRC cells, SMAC/Diablo release from the mitochondria plays a pivotal and role and is Bax dependent[50,51]. Further studies are warranted to clarify the exact role of SMAC for colon carcinogenesis and CRC therapy.

EXTRINSIC PATHWAY

The extrinsic pathway of apoptosis becomes activated in case of binding of a specific ligand to its surface death receptor. Most engaged receptors belong to the tumor necrosis factor receptor family (TNFR, CD95/FAS, TRAIL) and share broad similarity in structure and action[52,53].

In response to ligand binding, the receptor homotrimerises and an adaptor molecule (FADD, TRADD) containing a death domain (DD) is recruited to the cytosolic DD of the receptor. Procaspase 8 is hereafter recruited and catalytically activated in its active form. Finally, caspase 8 leads to an activation of caspase 3 where extrinsic and intrinsic pathways of apoptosis converge[54]. In addition to this direct road to death *via* caspase 8 and caspase 3, there is a possible detour integrating mitochondria to enhance the death signal. The BH3 only protein Bid is a direct target of Caspase 8 and after cleavage of Bid truncated Bid (tBid) is able to activate mitochondria herewith involving intrinsic apoptosis[55,56].

The receptors involved in extrinsic cell death signaling have been shown to be promising targets. Various compounds and approaches aim to induce apoptosis *via* direct receptor activation.

Tumor necrosis factor-α/tumor necrosis factor receptor

Recombinant tumor necrosis factor-α (TNF-α) has been approved for regional treatment of melanoma and soft tissue sarcoma in Europe. The use of TNF-α as a systemic approach is hampered by severe toxicity and adverse side effects such as hypotension, organ failure and cachexia[57]. The efficacy of TNF-α for CRC treatment remains to be clarified, but might be restricted due to TNF-α's nature as a proinflammatory cytokine. TNFerade® is an adenoviral delivered, intratumoral therapy with a proven safety in rectal cancer patients[58,59]. In advanced pancreatic cancer, TNFerade® was safe but did not prolong survival of patients[60]. The final investigation of TNFerade® for CRC treatment remains elusive. Furthermore, human monoclonal antibody-cytokine fusion protein L19-TNF has been shown to be safe in solid tumors and effective in sarcomas[61,62]. Again, more studies addressing the efficacy for CRC treatment are needed.

CD95 (Apo1/Fas)

CD95 and its ligand have a highly complex role in the colorectal mucosa as well as in onset and progression of CRC. In CRC tissue, CD95 has been shown to be expressed at higher levels compared to adjacent healthy mucosa[63]. Tumor stromal cells and infiltrating immune cells should be considered as bystander targets of CD95 triggering[64,65]. There is some evidence for a metastasis promoting function of CD95 signaling in colorectal cancer *via* induction of epithelial to mesenchymal transition[66]. As response to hypoxia and radiation, CD95 becomes activated on CRC cells and induces local invasion and promotes liver metastasis in mice[67,68]. In addition, invasive properties of CRC cells have been linked to CD95 signaling[69,70]. At least *in vitro*, CD95 participates in the activity of PEG-liposomal oxaliplatin induced death in CRC[71]. The anti-Fas monoclonal antibody CH-11 showed antitumor activity in CRC cells with high expression levels of CD95. This death inducing effect was effectively prevented by overexpression of Bcl-2 pointing on a pivotal role of mitochondria for CD95 signaling in CRC[72]. Moreover, there is evidence for a regulatory effect of other antitumor drugs [5-fluorouracil (5-FU), mitomycin (MM), cisplatin (CP) and all-trans retinoic acid] on CD95 expression of CRC cells. Here, MM and CP were able to increase CD95-induced apoptosis. By contrast, 5-FU led to a receptor downregulation causing immune escape of CRC cells[73]. In summary, CD95's value as a therapeutic target in CRC is complex and might be limited due to the multifaceted role of CD95 in immune-mediated tumor surveillance[74]. As for TRAIL detailed below, several ways of resistance to CD95-induced death further complicate CD95-based therapeutic approaches[75-77].

Tumor necrosis factor inducing ligand-system

Tumor Necrosis factor inducing ligand (TRAIL) receptors have been considered as extraordinary promising antitumor targets, since activation preferably kills tumor cells while sparing healthy cells[54]. However, normal colon mucosa epithelium is resistant to TRAIL-induced death[78]. TRAIL directly targets death receptor 4 (DR4)

Table 1 Targeting apoptosis in colorectal cancer: An overview of current clinical trials

Drug	Target	Clinical[1]	Ref.
Smac mimetics	IAPs	Phase I (NCT01573780)	[49,139]
Survivin peptide vaccine	survivin	Phase I - II (NCT00108875)	[140,141]
Oblimersen	Bcl-2	Phase I (NCT00004870)	[142,143]
Dulanermin	DR4/5 dual	Phase I b (NCT00671372)	[86]
Tigatuzumab	DR5	Phase I	[144]
CS-1008	DR5	Phase I (NCT01220999)	[145]
HGS-ETR1	DR4	Preclinical *in vivo*	[79]
HGS-ETR2	DR5	Phase I (NCT00428272)	[79,146]
rhApo2L/TRAIL	DR4/DR 5	Phase I - II (NCT00819169)	[147]
Conatumumab	DR5	Phase II (NCT01327612)	[148]
ABT-263	Bcl-2/Bcl-xl	Phase I (NCT00891605, NCT01009073)	[24]
ABT-737	Bcl-2/Bcl-xl	Preclinical *in vivo*	[25,26,30]
Gossypol	Pan-Bcl2	Preclinical *in vivo*	[149]

[1]Further detailed information on clinical trials: www.clinicaltrials.gov. The compounds included in the table directly target apoptotic proteins and show antitumor effects *in vivo*. The phase of the clinical trials is stated and trial identifier indicated in brackets where applicable. IAP: Inhibitors of apoptosis; DR: Death receptor; TRAIL: Tumor necrosis factor related apoptosis-inducing ligand.

and death receptor 5 (DR5). The recombinant, soluble ligand rhApo2L/TRAIL as well as several antibodies targeting DR4 and/or DR5 have been developed and tested for clinical use.

The agonistic DR4 antibody HGSETR1 (Mapatumumab) and the agonistic DR5 antibody HGSETR2 (Lexatumumab) induced apoptosis *in vitro* as well as in xenograft bearing nude mice when combined with radiation[79]. In addition, both agonistic antibodies have strong synergistic effects with the mitosis disrupting agent paclitaxel in CRC cells *in vitro* and *in vivo*. This sensitizing effect is due to an upregulation of the cognate receptors[80]. Several other antibodies targeting DR4 or DR5 have been shown to have strong antitumor potential on CRC cells[81-85]. Dulanermin (rhApo2L/TRAIL), an optimized and soluble form of TRAIL, has been successfully evaluated in early clinical trials[86]. A clinical trial with Dulanermin in combination with a chemotherapy backbone (FOLFIRI) for patients with metastatic CRC has been completed recently and data from this trial should be available soon (www.clinicaltrials.gov).

It is important to have in mind that several CRC cells show intrinsic or acquired resistance towards TRAIL-induced apoptosis. Several proteins have been shown to counteract TRAIL-induced apoptosis. For instance, two decoy receptors within the TRAIL system can counteract DR4 and DR5 activation[87]. Moreover, the interference of antiapoptotic Bcl-2 proteins with TRAIL-receptor-mediated apoptosis has been reported[54,88]. Again at the mitochondrial level, Bax is apparently mandatory for TRAIL's efficiency to kill CRC cells, since Bax deficiency completely abrogates TRAIL-induced death[89]. Furthermore, high levels of XIAP block TRAIL-induced mitochondrial activation[90]. At the receptor level, mutations of caspase 8 have been reported to cause TRAIL resistance[91]. Moreover, high expression levels of FLIP counteract the interaction between the adaptor FADD and Caspase 8 in CRC cells[92,93]. Pennarun and coworkers presented proof of concept of a combined approach: Downregulation of Mcl-1 and FLIP by multikinase inhibitor sorafenib and NSAID aspirin resensitized cells towards TRAIL[94]. These data are indicative for the feasibility of a combination approach of TRAIL receptor targeting and mitochondrial activation, *e.g.*, by BH3-mimetics.

Taken together, a final and clinical proof of concept for individualized TRAIL tailored therapy for CRC is still elusive and large cohort prospective trials addressing this issue are needed. Table 1 provides an overview of strategies and trials targeting TRAIL receptors in CRC. The awaited results from the Dulanermin trial in metastatic CRC might gain important information for further study designs using TRAIL based therapy.

ALTERNATIVE CONTROLLED CELL DEATH IN COLORECTAL CARCINOMA

The conceptual understanding of cell death is under constant expansion and various subtypes of cellular death have been defined[1,95,96]. Among the emerging cell death concepts, this work will deeper discuss necroptosis and autophagy in order to dissect the current knowledge concerning colorectal carcinogenesis and CRC treatment.

Necroptosis

Necrosis has long been considered as a passive, mainly accidental and uncontrolled form of cellular death. To date there is a growing body of literature implicating a tight regulation of necrotic processes similar to apoptosis[97]. Therefore, a programmed form of necrosis, termed necroptosis, has been defined. The signaling events responsible for initiation and execution of necroptosis have been studied best in the context of TNFR signaling. Necroptosis is crucially mediated by receptor-interacting protein 1 (RIP 1) along with its cognate kinase RIP3. Upon TNF induction, a multimeric complex containing FADD, caspase 3, RIP 1 and RIP 3 assembles[98]. This complex is termed complex IIb or necrosome. The determination of cells' fate is complicated by the observation that the ubiquitination status of the engaged proteins (*e.g.*, RIP) appears to be the master switch between apoptosis and necroptosis[99]. Necroptosis has also been demonstrated after activation of TRAIL receptors on hepatocytes and colorectal cancer cells[100]. Mechanistically, there are various central proteins involved in both, apoptosis and necroptosis. Which form of cell death prevails, is cell type and stimulus dependent[101-103]. Necroptosis and its role in various diseases, including CRC and inflammatory bowel disease, are currently under investigation[104-107]. There is evidence for a central role of caspase 8 as a key switch from apoptosis to necroptosis in carcinoma re-

Table 2 Targeting autophagy in colorectal cancer: An overview of current clinical trials

Drug	Target	Clinical[1]	Ref.
Hydroxychloroquine	Autophagosome	Phase I (NCT01206530) Phase II (NCT01006369)	[122,150]
Everolimus/rapamycin	mTOR	Phase II (NCT00419159, NCT01387880)	[126,127,151]

[1]Further detailed information on clinical trials: www.clinicaltrials.gov. The compounds shown target relevant processes or proteins involved in autophagy signaling. The phase of clinical trials is stated and trial identifier indicated in brackets where applicable. mTOR: Mammalian target of rapamycin.

lated inflammatory bowel disease[104].

The relevance of necroptotic cell death for colorectal cancer cells has been evaluated preclinically in the context of azathioprine plus buthionine sulfoximine treatment in CRC and HCC[108]. This work shows a necroptosis phenotype with mitochondrial dependency illustrating the interplay between necroptosis and apoptosis. Another study investigated the role of hypoxia for necroptotic death in colorectal cancer cells. In this study, RIP-dependent necroptosis can be conferred by pyruvate scavenging of mitochondria derived radicals[109]. Finally, targeted approaches to induce necroptotic cell death in cancer cells are still missing due to the absence of appropriate compounds for clinical usage so far. It has been shown that TRAIL receptor ligation causes necroptosis in an acidic extracellular milieu. Necrostatin-1, a chemical inhibitor of RIPK1, sufficiently blocked TRAIL-induced necroptosis in this experimental setting[100]. An indirect or secondary activation of necroptosis has been reported after treatment of CRC cells with TRAIL or inhibition of the multifaceted kinase GSK3-β[100,110].

Autophagy

Autophagy is an evolutionary conserved process by which cells collect proteins and organelles, deliver them to the lysosomal compartment where the cargo is finally degraded for recycling[111]. The implications of autophagy for cell physiology as well as for onset and progression of various diseases including cancer are rapidly emerging[112,113]. A disruption of autophagic flux leads to an intracellular accumulation of organelles, protein aggregates and lipid droplets. These accumulations may lead to the production of reactive oxygen species and cause metabolic insufficiency. Especially in stressful situation and in conditions of energy deprivation, a disruption of autophagic flux can promote carcinogenesis. For instance, the allelic loss of the essential autophagy protein Beclin 1 (also known as Atg6) causes HCC in mice[114,115].

By contrast, autophagy is essential for the survival of cancer cells and cancer cells show an extraordinary high level of autophagy. However, autophagy induction promotes survival under conditions of hypoxia and growth factor withdrawal[116]. Autophagosome formation is most prominent in tumors growing in a hypoxic environment. With regard to these findings, drugs inhibiting autophagy are promising anticancer agents. The anti-malaria drug Chloroquine is a known inhibitor of autophagy and is currently being under investigation in several clinical trials (www.clinicaltrials.gov, Table 2)[117]. Various other compounds or drugs are known regulators of autophagy and have been evaluated preclinically as treatment options for CRC[118-121]. *In vitro*, Chloroquine has been effective in overcoming 5-FU resistance in CRC cells[122,123]. Intriguingly, the approved chimeric anti-EGFR antibody cetuximab exerts its antitumor effect at least partly *via* autophagy-induced cell death[123].

Counterintuitive, drugs directly inducing autophagy are under clinical investigation as therapeutic approaches in CRC, too. Mammalian target of rapamycin is a prominent target to induce lethal autophagy in colorectal cancer cells[124]. The Rapamycin derivate Everolimus has recently been established for the treatment of colorectal neuroendocrine tumors[125]. A Phase II study with Everolimus showed appropriate tolerability, but failed to show meaningful efficacy in heavily pretreated patients with metastatic CRC[126]. Another trial using a combination of vascular endothelial growth factor receptor tyrosine kinase inhibitor tivozanib with everolimus resulted in stable disease of 50 % of all patients with metastatic cancer enrolled[127,128]. These partly contradictory findings highlight the important implication of autophagy in colorectal carcinogenesis.

Importantly, there is a broad overlap of the apoptosis and autophagy signaling network. Most prominently, Bcl-2 proteins function as both, inhibitors of apoptosis and autophagy by binding proautophagic Beclin1. Therefore, it has been shown that BH3-mimetics induce apoptosis and autophagy. For instance, ABT-737 can synergistically induce cell death with the COX2 inhibitor celecoxib in CRC cells by facilitating autophagy and apoptosis[26,129].

CROSSTALK BETWEEN APOPTOSIS, NECROSIS AND AUTOPHAGY: MULTI-DEATH TARGETING STRATEGIES

The past decade of cell death research has shown that necrosis, apoptosis and autophagy are regulated by similar pathways engaging the same proteins. It might be worthwhile targeting the apoptotic and autophagic machinery in a combined approach, since a massive induction of autophagy is able to drive cancer cells in apoptotic death. Recently, various efforts in this direction have been made in order to overcome cell death resistance in colorectal cancer. For instance, silibin, a plant derived

natural compound, is able to induce both, apoptosis and autophagy[130]. In line with these observations, compound C, a small molecule inhibitor of AMP-activated protein kinase, is able to sufficiently suppress colorectal cancer cell growth by inducing apoptosis and autophagy[131]. The capability of such a double-edged approach has been successfully proven *in vivo* in a model of hepatic metastasis in mice[132]. Future studies are needed to further exploit combinatorial approaches for cell death induction in colorectal cancer.

CONCLUSION

From an oncological point of view, it is of outstanding importance to further increase research efforts aiming at more effective and individualized therapies. The effectiveness of monotherapeutic systemic approaches in colorectal cancer treatment is limited. However, combined therapy regimes are now state of the art. Manipulation of cell death represents a promising tool to further amplify response to chemotherapy. In addition to direct cell death induction in cancer cells, triggering cell death *via* cancer-directed immunotherapy or immunomodulation with the aim to overcome major mechanisms of immune resistance, is a newly arising field[133]. For example, recent reports on long-term results from first-in-human clinical trials using anti-PD1 antibody-based immunotherapy are encouraging[134]. Future trials are warranted to identify the best combinatorial approach yielding at cell death induction in cancer cells.

On the way to personalized oncology, it will be mandatory to broaden our knowledge concerning the selection of patients for a specific therapeutic setting. Having in mind that cell death relevant proteins vary in their expression in different subsets and stages of CRC, a stratification of patients to identify those who benefit most of a manipulation of apoptosis requires further research.

Finally, the question whether and how cell death could be measured to monitor therapy in patients needs further attention. There are some elegant and encouraging studies evaluating liquid biopsy markers for cell death in cancer[135,136]. In addition, imaging of cell death on routine basis for non-invasive monitoring of tumor biology and therapeutic response might open new windows for therapy surveillance and outcome prediction in colorectal cancer[137,138].

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TOPIC HIGHLIGHT

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

Intra-operative peritoneal lavage for colorectal cancer

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Abstract

Free cancer cells can be detected in peritoneal fluid at the time of colorectal surgery. Peritoneal lavage in colorectal surgery for cancer is not used in routine, and the prognostic significance of intraperitoneal free cancer cells (IPCC) remains unclear. Data concerning the technique of peritoneal lavage to detect IPCC and its timing regarding colorectal resection are scarce. However, positive IPCC might be the first step of peritoneal spread in colorectal cancers, which could lead to early specific treatments. Because of the important heterogeneity of IPCC determination in reported studies, no treatment have been proposed to patients why positive IPCC. Herein, we provide an overview of IPCC detection and its impact on recurrence and survival, and we suggest further multi-institutional studies to evaluate new treatment strategies.

Key words: Peritoneal carcinomatosis; Colorectal cancer; Free intraperitoneal cells; Immunocytochemistry

Core tip: We provide an overview of intraperitoneal free cancer cells (IPCC) detection and its impact on recurrence and survival, and we suggest further multi-institutional studies to evaluate new treatment strategies. Moreover, while current literature is sufficient to consider positive IPCC as a pejorative prognostic factor, further studies are also needed to propose adjuvant treatment for patients with positive IPCC.

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INTRODUCTION

Intra-operative peritoneal lavage can be used to detect intraperitoneal free cancer cells (IPCC) in order to determine the presence of peritoneal spread in intra abdominal malignancies. IPCC are considered as an important prognostic tool in ovarian[1-3] and gastric cancers[4-7]. Colorectal cancer is one of the most frequent cancers worldwide[8], with development of peritoneal carcinomatosis in 10%-30% of patients[9,10]. The development of curative treatments for peritoneal carcinomatosis, such as cytoreductive surgery and intraperitoneal chemotherapy showed effective outcomes, especially in malignancies of colorectal origin[11,12], and thus raised the interest for free malignant cells detection. In colorectal cancer, different therapeutic strategies could be proposed if IPCC were confirmed to be an important prognostic factor. Several techniques, such as pathological examination, immunocytochemistry (ICC) and polymerase chain reaction (PCR) have been described to determine the presence of IPCC and were used at various times before or after resection. The heterogeneity of peritoneal lavage techniques, timing and samples analysis were the main issues to clarify the impact of IPCC on prognosis and risk to develop recurrence. The aim of this review was to report and discuss

the significance of IPCC detection in patients treated for a colorectal cancer in a curative intent.

PERITONEAL CYTOLOGY TECHNIQUE

Techniques used

Peritoneal cytology can be performed without lavage when free peritoneal fluid is present. In the absence of peritoneal fluid, a lavage with saline serum (NaCl 0.9%) is needed. The volume of fluid used was extremely variable, ranging from 50 to 1000 mL[13-25], but most authors proposed a small amount of liquid (100-200 mL) delivered around the tumor, where most cells are supposed to be.

IPCC were usually sought in peritoneal fluid by conventional cytology. After peritoneal lavage, the collected fluid was centrifuged and the sediment was smeared on slides and stained by the Giemsa or/and Papanicolaou methods. If at least one cancer cell was identified, cytology was considered positive. A clear-cut identification between benign and malignant cases could be achieved in most cases, but in 2% of cases, the analysis was still inconclusive[26]. Yield rate of positive IPCC detection by conventional cytology varied from 4% to 35.5%[14,15,20,26-30]. To increase the sensitivity of conventional cytology, ICC has been proposed with various monoclonal antibodies evaluated Ks20.8, Lu5 and Ber-Ep4[18], C1P83, Ra96, CA19-9[31], CK20[32] and 17-lA14 and Kl-1[22], along with PCR or reverse transcriptase PCR to detect cytokeratin 20, carcinoembryonic antigen, laminin g2, ephrin B4, matrilysin mRNA[17,33], Kras mutation on exon 1 or 2, Braf mutation[34] or human mammaglobin (hMAM) and hMAM-B expression[35], or even fluorescence in situ hybridization[35]. Yield rate of positive IPCC detection varied from 20%-30% and 8%-40% for ICC and PCR, respectively. Bosch *et al*[18] reported one case of positive ICC within a control group of benign lesion, resulting in a specificity of 97% for ICC. PCR techniques present a similar issue by detecting DNA from benign cells[36]. Other techniques such as immunofluorescence for epithelial markers[37] or serosal stamp[38,39] have been proposed and evaluated by a few teams. Even if serosal stamp cytology appeared to be more sensitive than conventional cytology to detect IPCC, its clinical impact was insufficiently evaluated, and its impact on recurrence or survival remains uncertain[39].

To the best of our knowledge, no prior study has compared the different techniques of IPCC detection. Due to the important heterogeneity of these techniques, conventional cytology may be proposed as the standard IPCC detection technique in further clinical trials, given that it is reproducible and widely used. Its specificity is high (100%), while its sensitivity is variable. To improve diagnostic accuracy and sensitivity of conventional cytology, inconclusive cases could be reviewed by an expert panel as suggested by Piaton *et al*[26], or ICC could be associated as suggested by Yang *et al*[32] with the added risk of decreasing specificity[35]. In a study detailing improved effusion analysis, Fiegl *et al*[35] suggested that for gastrointestinal carcinomas, the addition of real time-PCR for hMAM-B to conventional cytology enhanced diagnostic sensitivity from 25.8% to 51.7% and could be considered as the most effective association.

Timing of peritoneal lavage

Peritoneal lavage was mainly performed after the abdomen was opened and before any manipulation of the tumor, but a few series also reported analysis after tumor resection. Two studies reported both pre and postresection IPCC detection by PCR[17,33]. The detection rate before resection was similar in both studies (12%-14%), but the post resection detection rate were contradictory, as it was lower than the pre resection rate in one study (3%)[33], and higher in the other (20%)[17]. Data are missing to recommend a precise timing of sampling. However, the evolution of IPCC detection rate between before and after resection could be a prognostic factor suggesting that peritoneal lavage analysis should be performed before and after resection.

PROGNOSTIC IMPACT

For colorectal cancer, as well as in gastric and ovarian cancer, the objective of IPCC detection was to evaluate the impact on survival and local recurrence, in order to discuss intraperitoneal treatment or adjuvant systemic chemotherapy. Few studies[14,22,31,34,38,40], with less than 200 patients included in each, reported a trend between cancer stage and positivity of peritoneal lavage. The study by Noura *et al*[13] on 697 patients reported a significant correlation between cancer stage and positivity of peritoneal lavage.

Rekhraj *et al*[41] reported a meta-analysis in 2007 in order to determine the impact of IPCC on local and general recurrence of patients treated with curative intent. They analyzed 9 studies for a total of 1182 patients. Three studies included patients with stage Ⅳ colorectal cancer. They reported a significantly higher risk to develop overall recurrence for patients with positive IPCC. The risk rose from 25% for negative pre-resection IPCC to 46% for pre-resection positive IPCC and from 17% for negative post-resection IPCC to 52% for post-resection IPCC. Pre-resection positive IPCC was a significant risk factor for local recurrence (21% *vs* 12% for negative post-resection IPCC), while the risk for post-resection positive IPCC was not significant (18% for positive IPCC *vs* 8% for negative IPCC). Two studies[28,42] demonstrated a higher rate of peritoneal recurrence for positive IPCC compared to negative IPCC.

Alex *et al*[43] reported a more recent meta-analysis that a mean weighted yield of 8.4%, 28.3% and 14.5% for conventional cytology, ICC and PCR, respectively, which aimed to determine the outcome of patients with positive peritoneal lavage treated for colorectal cancer with curative intent. The authors excluded studies that included patients presenting with synchronous peritoneal carcinomatosis. Twelve studies including 6 published after 2007 were analyzed, with 1880, 1711 and 1096 patients for mortality analysis, peritoneal recurrence analysis and

Table 1 Demographic and outcome data from studies involved more than 100 patients

Ref.	Patients (*n*)	Method of IPCC detection	Lavage	Timing of sampling	Yield rate of positive IPCC	Significant impact	
						Overall survival	Global recurrence
Noura *et al*[13]	697	Cyto	100 mL NaCL	Before	2.20%	Yes (5 yr 87% *vs* 50%)	ND
Nishikawa *et al*[21]	410	Cyto	200 mL NaCl	Before	7.60%	Yes (5 yr 68% *vs* 20.6%)	Yes (30% *vs* 60%)
Fujii *et al*[15]	293	Cyto	200 mL NaCl	Before	6.00%	NS	NS
Kristensen *et al*[34]	237	PCR	200-600 mL NaCl	After	8.00%	Yes (median 47 mo *vs* 22 mo)	ND
Lee *et al*[16]	234	Cyto	1000 mL NaCl	Before	8.00%	Yes (mean 32 mo *vs* 25 mo)	ND
Katoh *et al*[14]	226	Cyto	100 mL NaCl	Before	14.60%	Yes (5 yr 79% *vs* 14%)	Yes
Yamamoto *et al*[42]	189	Cyto	50 mL NaCl	Before	5.80%	Yes (5 yr 76% *vs* 46%)	ND (26% *vs* 55%)
Temesi *et al*[23]	145	Cyto		Before	17.00%	ND	ND (23% *vs* 56%)
Vogel *et al*[31]	135	ICC	100 mL NaCl	Before	23.00%	Yes (5 yr 85% *vs* 23%)	ND
Lloyd *et al*[17]	125	PCR	100 mL NaCl	Before After	13.00% 20.80%	NS pre Yes post (mean 88 mo *vs* 44 mo)	ND (4% *vs* 22%)
Schott *et al*[22]	109	ICC	1000 mL NaCl	Before	31.00%	Yes (4 yr 60 mo *vs* 28 mo)	Yes (47% *vs* 85%)

Global recurrence range at end of study follow up. IPCC: Intraperitoneal free cancer cells; ND: Not determinable; NS: Not Significant; Cyto: Conventional cytology; PCR: Polymerase chain reaction; ICC: Immunocytochemistry.

overall recurrence analysis, respectively. Positive peritoneal lavage was associated with an increase in all 3 parameters. Mohan *et al*[24] reported the same findings in a recent review. Other studies reported opposite results[15,19,33,44], but only one[15] of these included more than 200 patients. All other studies including more than 200 patients[13,14,16,21] found a significant impact of positive peritoneal lavage on survival and recurrence. A large multi institutional study is needed to confirm the impact of positive peritoneal lavage on survival and recurrence.

Table 1 reports lavage techniques, yield rate of positive IPCC detection and impact on survival and global recurrences in the main studies.

HOW CAN PERITONEAL CYTOLOGY BE INTEGRATED IN THE OVERALL MANAGEMENT OF COLORECTAL CANCER

Positive peritoneal lavage for stage Ⅰ, Ⅱ and Ⅲ of colorectal cancer appears to be a prognostic factor of local recurrence, overall recurrence and poor survival, but the studies discussed here present an important heterogeneity in lavage techniques and analysis. Standardization is needed in order to integrate peritoneal lavage into routine clinical practice. Peritoneal lavage might be realized twice, after the abdomen has been opened and before closure with 100-200 mL of saline (NaCl 0.9%). Conventional cytology remains the standard to determine positive IPCC, and a panel analysis or ICC or PCR could increase the sensitivity for inconclusive cases.

Positive IPCC appeared to be a pejorative prognostic factor of overall recurrence and survival. These findings might be explained by cell exfoliation into the peritoneal cavity along with systemic diffusion. According to this hypothesis, the presence of IPCC during a curative surgery for stage Ⅰ, Ⅱ or Ⅲ colorectal cancer could be considered as a pejorative prognostic factor. Even if the rate of patients with positive IPCC was variable among the reported studies, adjuvant chemotherapy should be evaluated for these patients in a large multi-institutional study.

The other treatment that could be proposed for patients with positive IPCC could be prophylactic intraperitoneal chemotherapy. Local recurrences were not well described and included lymphatic, anastomotic or peritoneal recurrences. However, the low sensitivity of morphological examinations for peritoneal carcinomatosis diagnosis[45] could under-estimate the rate of peritoneal recurrence in patients with positive IPCC. In a systematic review, Honoré *et al*[46] assumed that patients with positive IPCC have an unknown risk of developing peritoneal carcinomatosis. One issue was the average risk to develop peritoneal carcinomatosis for patient with positive IPCC, with an important variability among reported studies. But this risk remains probably under estimated because of the low sensitivity of morphological examinations to diagnose peritoneal carcinomatosis. Another issue was the large heterogeneity in positive IPCC incidence in reported studies with a mean yield rate of 8%-15%[41,43], raising the question of the efficacy of conventional cytology in routine. Intraperitoneal chemotherapy combined with surgery is an aggressive treatment[47] associated with

an increased morbidity, and therefore requires expertise. Data available about peritoneal recurrence and the impact of intra-peritoneal chemotherapy are insufficient to propose intraperitoneal chemotherapy routinely. The risk to develop peritoneal carcinomatosis for this patient population could be evaluated by a second look surgery, as proposed by Sugarbaker[48]. In the author's series, patients treated for stage Ⅰ, Ⅱ or Ⅲ colorectal cancer with limited surgical history underwent a laparoscopic second look in order to limit morbidity. The exploration enabled the detection of limited carcinomatosis and could lead to a curative treatment combining systemic chemotherapy, cytoreductive surgery +/- intraperitoneal chemotherapy. This study showed that patients with positive IPCC had a higher risk of developing peritoneal carcinomatosis, and could therefore benefit from a prophylactic treatment with intra-peritoneal chemotherapy.

CONCLUSION

Positive intraperitoneal free cancer cells are a prognostic factor of recurrence and survival for patients treated for stage Ⅰ, Ⅱ and Ⅲ colorectal cancer. These findings should be supported by a large multi-institutional study to determine the real prevalence of positive IPCC. Moreover, while current literature is sufficient to consider positive IPCC as a pejorative prognostic factor, further studies are also needed to propose adjuvant treatment for patients with positive IPCC.

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TOPIC HIGHLIGHT

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

Telomeres, telomerase and colorectal cancer

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Abstract

Colorectal cancer (CRC) is the third most common cancer worldwide and, despite improved treatments, is still an important cause of cancer-related deaths. CRC encompasses a complex of diseases arising from a multistep process of genetic and epigenetic events. Besides heterogeneity in the molecular and biological features of CRC, chromosomal instability is a hallmark of cancer and cancer cells may also circumvent replicative senescence and acquire the ability to sustain unlimited proliferation. Telomere/telomerase interplay is an important mechanism involved in both genomic stability and cellular replicative potential, and its dysfunction plays a key role in the oncogenetic process. The erosion of telomeres, mainly because of cell proliferation, may be accelerated by specific alterations in the genes involved in CRC, such as *APC* and *MSH2*. Although there is general agreement that the shortening of telomeres plays a role in the early steps of CRC carcinogenesis by promoting chromosomal instability, the prognostic role of telomere length in CRC is still under debate. The activation of telomerase reverse transcriptase (TERT), the catalytic component of the telomerase complex, allows cancer cells to grow indefinitely by maintaining the length of the telomeres, thus favouring tumour formation/progression. Several studies indicate that TERT increases with disease progression, and most studies suggest that telomerase is a useful prognostic factor. Plasma TERT mRNA may also be a promising marker for the minimally invasive monitoring of disease progression and response to therapy.

Key words: Telomere; Telomerase; Telomerase reverse transcriptase; Colorectal cancer; Prognostic marker

Core tip: Telomere/telomerase interplay is an important mechanism involved in both genomic stability and cellular replicative potential. Telomere shortening is an early event that contributes to genetic instability, which plays a key role in the early steps of carcinogenesis. The activation of telomerase, which preserves replicative potential by maintaining the length of telomeres, occurs during the adenoma-carcinoma sequence and increases during tumour progression. While the prognostic value of telomere length is controversial, most studies agree that the level of telomerase in tumours represents a useful prognostic marker. Circulating telomerase reverse transcriptase is a promising marker for the minimally invasive monitoring of disease and response to therapy.

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INTRODUCTION

Colorectal cancer (CRC) is the third most common cancer worldwide; over 1.2 million new cancer cases and nearly 600000 deaths are estimated to have occurred in 2008[1]. Despite improved treatments, increased awareness and early detection, which have all contributed to prolonged survival, CRC is still an important cause of cancer-related deaths[1]. CRCs encompass a complex of diseases with different molecular pathways and biological characteristics arising from a multi-step process that involves several genetic and epigenetic events[2,3]. The stepwise change in morphology from normal epithelium to carcinoma occurs through a multi-step genetic model with the loss of the functions of tumour suppressor genes, such as adenomatous polyposis coli (*APC*) and *TP53*, and the gain of the function of oncogenes, such as *KRAS*. Recent genome-wide sequencing analyses have estimated as many as 80 mutated genes in CRC. Although a smaller number of mutations are considered drivers of tumourigenesis, multiple genetic hits are required for tumour onset and progression[4]. Many efforts have been made to identify molecular markers that predict the outcome of CRC patients, and several genetic and epigenetic alterations that are involved in the development of CRC have been proposed as prognostic markers of disease progression; however, no agreement has been reached[5,6]. Besides great heterogeneity of the molecular and biological features, chromosomal instability may play a key role in the early steps of carcinogenesis[7]. Cancer cells may also circumvent replicative senescence and acquire the ability to sustain unlimited proliferation[8]. Telomere/telomerase interplay is an important mechanism involved in the genomic stability and cellular replicative potential, and telomere/telomerase dysfunction has emerged as playing a key role in carcinogenesis. Here, we review the role of telomeres and telomerase in the genesis and progression of CRC.

TELOMERES AND TELOMERASE

Telomeres are specialised DNA structures located at the end of chromosomes; they are essential for stabilising chromosomes by protecting them from end-to-end fusion and DNA degradation[9]. In human cells, telomeres are composed of (TTAGGG)n tandem repeats that are associated with the capping proteins Telomeric Repeat binding Factor (TRF)1, TRF2, Repressor/Activator Protein1 (RAP1), TRF1-interacting Nuclear protein 2 (TIN2), TTP1 (also known as TINT1, PTOP, PIP1), and Protection Of Telomers 1 (POT1), which constitute the shelterin complex[10]. Telomeres are progressively shortened during each cell division by replication-dependent loss of sequences at the DNA termini, caused by the failure of DNA polymerase to completely replicate the 3' end of chromosomes[11]. When telomeres become critically short (*i.e.*, the Hayflick limit), they are no longer protected by the shelterin complex; at that point they are recognised as DNA double-strand breaks that trigger a DNA damage response (DDR), and the cells undergo replicative senescence and apoptosis[10]. If protective mechanisms, such as that of the TP53 protein, are inactive, cells continue to proliferate; the further erosion of telomeres impairs their role in protecting chromosome ends and ultimately causes chromosomal instability[12]. Thus, telomere erosion may play two conflicting roles: tumour suppression by inducing cell death, and tumour promotion by causing genetic instability, a key event in the initiation of carcinogenesis. It has been recently advanced that short telomeres may also affect genome-wide DNA methylation, which may modulate oncogene and oncosuppressor gene expression[13]. However, cell division-associated telomere shortening prevents unlimited cell proliferation and thus tumour development/progression. To escape this proliferation barrier, cells must stabilise their telomeres. Most tumours maintain their ability to grow indefinitely through the inappropriate expression of telomerase, a ribonucleoprotein complex containing an internal RNA component [telomerase RNA (TR), or telomerase RNA component] and a catalytic protein with telomere-specific reverse transcriptase activity [telomerase reverse trancriptase (TERT)][14]. TERT which synthesises *de novo* telomere sequences by using TR as a template, is the rate-limiting component of the telomerase complex, and its expression is correlated with telomerase activity[15]. While TR has broad tissue distribution and is constitutively present in normal and tumour cells, expression of TERT, which is usually repressed in normal somatic cells, occurs in germ-line cells and most cancer cells. TERT is essential for unlimited cell growth and thus plays a critical role in tumour formation and progression[16].

Regulation of telomerase operates at several biological levels: transcription, mRNA splicing, subcellular localisation of each component and the assembly of TR and TERT in an active ribonucleoprotein. Transcription of the *TERT* gene is most likely the key determinant in the regulation of telomerase activity; notably, TERT transcriptional activity is specifically up-regulated in cancer cells, but is silent in most normal cells. The *TERT* gene consists of approximately 35 kb DNA and comprises 16 exons and 15 introns. At the transcriptional level, more than 20 transcription factor-binding sites that act as activators or repressors have been identified within the TERT promoter. The cooperation of MYC and SP1 is required for the full activation of the *TERT* promoter, while TP53, through its interaction with SP1, down-regulates TERT. *TERT* is also directly activated by nuclear factor-κB, hypoxia-inducible factor (HIF)-1, and the ETS/MYC complex. The histone methyltransferase SMYD3 also directly contributes to inducible and constitutive TERT expression in normal and malignant human cells. TERT expression is suppressed by the oncosuppressor genes *WT127* and *MEN1,* and through the MAD/MYC and TGF-β/SMAD pathways. The cell cycle inhibitors p16INK4a and p27KIP1 have also been shown to down-regulate TERT expression in cancer cells[17]. Regulation of *TERT* transcription may also involve DNA methylation, because the *TERT* promoter contains

Table 1 Telomeres and telomerase: outstanding questions regarding their role in the genesis and progression of colorectal cancer

Is the shortening of telomeres an early or late event in colorectal carcinogenesis?
Does telomere shortening play a role in genomic instability?
Do telomere lengths correlate with telomerase expression/activity?
Do telomere lengths correlate with disease progression?
Do levels of telomerase expression/activity increase with disease progression?
Do telomere and/or telomerase act as prognostic markers for disease outcome?

a cluster of CpG sites. At the post-transcriptional level, modulation of telomerase may occur by alternative splicings that may be tissue-specific; at least 10 different variants of TERT mRNA have been described, and some of these splicing products may exert a dominant negative function by competitive interaction with components of the telomerase complex[18,19]. Telomerase activity is also controlled through post-translational modifications of the TERT protein. Phosphorylation of the protein at critical sites by the PI3K/AKT kinase pathway seems to be crucial for telomerase activity[20]. Telomere-associated shelterin plays a role in the activity of telomerase; TPP1 is heterodimerised with POT1 and the POT1-TPP1 complex can recruit and stimulate telomerase activity, thereby regulating telomere length through the TPP1-telomerase interaction[21]. Notably, recent studies have suggested that, in addition to maintaining telomere length, TERT is involved in several other cell functions. The expression of TERT increases replicative kinetics[22,23], promotes cell growth under adverse conditions and may also act as an anti-apoptotic agent[24-26]. High levels of telomerase confer resistance to several antineoplastic drugs[27,28].

We direct our attention here to the questions listed in Table 1. The answers to these questions are important in defining the role of telomere/telomerase interplay in the CRC carcinogenesis.

TELOMERES AND GENETIC INSTABILITY IN THE GENESIS OF COLORECTAL CANCERS

There are at least two major pathways by which molecular events can lead to CRC; most CRCs (approximately 85% of cases) are characterised by chromosomal instability (CIN), while the other CRCs have a microsatellite instability (MSI) phenotype. CIN is a dynamic process of allelic imbalance at several chromosomal loci, with chromosome amplification and translocation, and it is an efficient mechanism for causing the loss of oncosuppressor genes, such as *APC*, *TP53*, and *SMAD* family member 2 and 4 involved in the TGF-β signaling pathway, and the activation of oncogenes, such as *KRAS* and *BRAF*, which activate the mitogen-activated protein kinase signalling pathway[29]. The MSI phenotype is generated by a deficient DNA mismatch repair (MMR) system. Alterations to one of the seven known *MMR* genes (*MSH2*, *MLH1*, *MSH6*, *PMS1*, *PMS2*, *MSH3*, and *MLH3*) cause unrepaired errors in the nucleotide repeat sequences, known as microsatellites. Methylation of promoters of *MMR* genes, particularly *MLH1*, is the most frequent mechanism for silencing *MMR* genes in sporadic CRCs, which in fact is frequently associated with the GpG island methylator phenotype[4,30]. While the significance of telomere alterations in MSI is unclear, telomere dysfunction may be considered a major driving force in the generation of CIN.

Several studies have demonstrated that telomeres are shorter in CRCs than in the adjacent mucosa (Table 2). While telomere length in somatic cells primarily reflects cellular proliferation, in tumour cells it reflects the balance between cellular proliferation with telomere loss and telomerase activity with *de novo* synthesis of telomeric sequences. Evidence that telomeres are shorter in CRCs than in adjacent mucosa, even in well-differentiated tumours, strongly supports the concept that telomere erosion is a critical initial event in colorectal carcinogenesis. TRF1 is a main negative regulator of telomere length; over-expression of TRF1 in colorectal cells is correlated with shorter telomeres[38]. Telomere shortening in colorectal polyps was recently correlated with large-scale genomic rearrangements[43]. Notably, telomere shortening in adenomas is not correlated with polyp size. In addition, the great differences in telomere length (differences of up to 4.6 kb between normal mucosa and polyps) are too large to be explained by replicative telomere erosion alone. Thus, the telomere length in CRC may reflect the short telomere length in the cells that originated the tumours, and telomere erosion may even precede the colorectal adenomagenesis[43]. Because this pattern has been observed in colorectal adenomas from patients with familial adenomatous polyposis, it remains to be established whether it also occurs in sporadic CRCs.

Approximately 15% of CRCs present MSI, whereas the *TP53* gene is the known major genetic alteration in CRCs with chromosomal instability and stable microsatellites (MSS)[5,44]. A study performed on a large number of CRCs demonstrated that both MSI and MSS tumours have shorter telomeres compared with adjacent mucosa, but MSI cancers have shorter telomeres than MSS cancers[41]. This result matches another study[45]. The MSI pathway involves the failure of the MMR system[46], which maintains genetic stability by repairing DNA replication errors and preventing chromosomal recombinations; a deficiency in MMR helps cells overcome cellular crises caused by the critical shortening of telomeres[47]. Thus, cells from MSI cancers may undergo more replicative cycles and more pronounced shortening of telomeres before stabilising compared with cells from MSS cancers. The difference is particularly great and significant when MSI tumours are compared with MSS tumours carrying the wild-type *TP53* gene. Notably, MSS tumours with a mutated *TP53* gene have slightly shorter telomeres than MSS tumours with the wild-type *TP53* gene do. In cells

Table 2 Telomere lengths and colorectal cancer

Ref.	Cases	Main findings
Hastie *et al*[31], 1990	23 (20 CRCs, 3 adenomas) and patient-matched non-cancerous mucosa (frozen samples)	TL Decrease with age in non-cancerous cells (33 bp per year) Shorter in CRCs and adenomas than in normal mucosa
Engelhardt *et al*[32], 1997	80 (50 CRCs, 20 polyps, 10 colitis) and CRC patient-matched non-cancerous mucosa (frozen samples)	TL Shorter in CRCs than in normal mucosa Shorter in CRCs than in polyps and colitis Longer in late-stage cancer with higher telomerase activity Do not differ between colon and rectum cancer
Takagi *et al*[33], 1999	61 CRC (including 12 non-ulcerating and 39 ulcerating tumours, according to Borrmann's classification) and patient-matched non-cancerous mucosa (frozen samples)	TL Shorter in non-ulcerating CRCs than in normal mucosa Shorter in non-ulcerating than in ulcerating tumours Not correlated with tumour stage or grade Not correlated with telomerase activity
Katayama *et al*[34], 1999	35 (26 CRCs, 9 polyps) (frozen samples)	TL Do not differ between CRCs and polyps
Nakamura *et al*[35], 2000	124 CRC and patient-matched non-cancerous mucosa (frozen samples)	TL Shorter in CRCs than in normal mucosa Decrease with age in both cancer and non-cancerous cells (44 and 50 bp/yr)
Plentz *et al*[36], 2003	10 (adenoma-carcinoma transition) (paraffin-embedded samples)	TL Shorter in high-grade dysplastic areas than in the surrounding adenoma
Gertler *et al*[37], 2004	57 CRC and patient-matched non-cancerous mucosa (frozen samples)	TL Shorter in CRCs than in adjacent mucosa Decrease with age only in non-cancer cells (19 bp per year) Correlate with tumour stage, being longer in advanced tumours Correlate with TERT mRNA levels Lead to a poor prognosis if TL cancer/TL non-cancer > 0.9 Do not differ between colon and rectum cancer
Garcia-Aranda *et al*[38], 2006	91 CRC (23 right-colon, 13 left-colon, 55 rectum) and patient-matched non-cancerous mucosa (frozen samples)	TL Shorter in CRC than in adjacent mucosa Shorter in right-colon cancers than in tumours located in other sites Shorter in poorly differentiated tumours Tend to be longer in telomerase-positive CRCs Have prognostic value (longer telomeres: poor clinical outcome) Correlated with the expression of TRF1 protein
O'Sullivan *et al*[39], 2006	38 (26 adenomas, 12 CRCs) (paraffin-embedded samples)	TL Shorter in adenomas than in adjacent and distant mucosa Similar in CRCs and adjacent and distant mucosa
Raynaud *et al*[40], 2008	15, each case with normal mucosa, low-grade dysplasia, high-grade dysplasia and carcinoma (paraffin-embedded samples)	TL Shorter in low-grade and high-grade dysplasia than in carcinoma Inversely correlated with activation of the DDR pathway
Rampazzo *et al*[41], 2010	118 CRC (53 right-colon, 30 left-colon, 35 rectum) and patient-matched non-cancerous mucosa (frozen samples)	TL Shorter in CRCs than in adjacent mucosa Shorter in right-colon cancers than in tumours located in other sites Shorter in MSI than in MSS tumours Decrease with age only in non-cancer cells Not correlated with tumour stage or grade Not correlated with TERT mRNA levels
Valls *et al*[42], 2011	147 CRC and patient-matched non-cancerous mucosa (frozen samples)	TL Shorter in CRCs than in adjacent mucosa In cancer correlate with TL in normal mucosa Do not differ between colon and rectum cancer Not correlated with tumor stage Have prognostic value (TL cancer/TL non-cancer ⩽ 1: higher OS)
Roger *et al*[43], 2013	135 (85 polyps from 10 patients with FAP, 50 CRCs) (frozen samples)	TL Shorter in polyps than in normal mucosa Correlated with genomic rearrangement in polyps Independent of adenoma size In polyps may reflect the TL of the originating cells

TL: Telomere lengths; CRC: Colorectal cancer; DDR: DNA damage response; OS: Overall survival; FAP: Familial adenomatous polyposis.

with mutated *TP53*, telomeres may protract their shortening with cell proliferation. However, TP53 is a well-known negative regulator of the *TERT* promoter, and mutated TP53 protein may also result in TERT activation, so telomere stabilisation may occur earlier than it does in MSI tumours[41].

The down-regulation of MSH2 is associated with greater telomere shortening than in control cells; thus

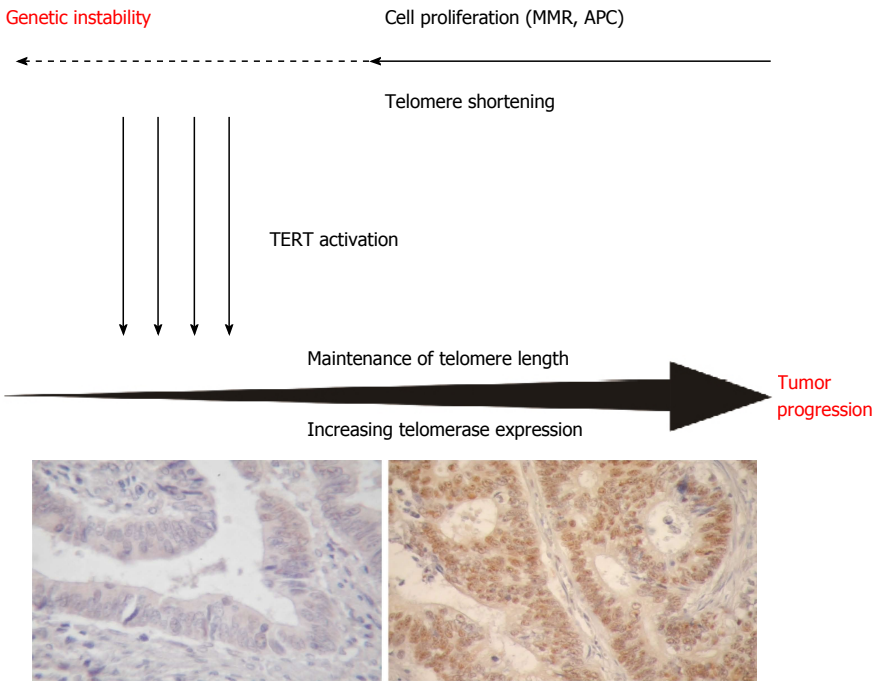

Figure 1 Model of telomere/telomerase interplay in the carcinogenesis of colorectal cancer. Telomere shortening is mainly caused by cell proliferation in preneoplastic lesions. Erosion of telomeres may be accelerated by mutations in specific genes, such as the adenomatous polyposis coli (*APC*) gene or DNA mismatch repair (*MMR*) system genes. The activation of telomerase reverse transcriptase (TERT), the catalytic unit of the telomerase, occurs during the adenoma-carcinoma sequence; TERT and telomerase activity levels increase with tumour progression. Inserts: Immunohistochemical analysis of TERT expression in stage Ⅰ (left) and stage Ⅳ (right) tumours. Mayer's haematoxylin counterstaining; original magnification × 20.

MSH2 deficiency may accelerate telomere shortening[48]. It is worth noting that the leukocyte telomeres of patients with Lynch syndrome, a hereditary CRC syndrome caused by germline mutations in *MMR* genes are shorter than those of age-matched controls[49]. Whether a shorter telomere length in leukocytes is a risk factor for CRC or a consequence of either disease treatment or disease burden is a controversial question[50-52], but there is general agreement that telomere shortening is an early event in colorectal carcinogenesis, even in sporadic CRC (Figure 1). Activation of the DDR is almost universal during the earliest stages of carcinogenesis[53,54]. A recent study suggested that telomere length is inversely correlated with activation of the DDR pathway, and telomere fusion may lead to general genomic instability[40].

While there is general agreement that telomere shortening, which is mainly caused by high proliferation of preneoplastic lesions and most likely accelerated by alterations in genes such as *APC* and *MSH2*, is an early event in the CRC carcinogenesis, there is no agreement concerning the role of telomere length as a marker of disease progression. Only a few studies report that telomeres are longer in late stage cancer than in preneoplastic lesions and/or early neoplastic stages; the activation of telomerase and/or high levels of telomerase expression may explain the increase in telomere length with disease progression[37,38]. However, other studies have not indicated any correlation between telomere length and tumour stage or grade (Table 2). Telomere lengths may stabilise with tumour progression because of increased telomerase activity that compensates for replicative telomere loss[41,55].

TELOMERASE AS A MARKER OF DISEASE PROGRESSION IN COLORECTAL CANCER

Two main strategies are used to estimate telomerase levels: quantification of TERT mRNA and quantification of telomerase activity. The telomerase level, even in telomerase-positive tumour cells, is estimated to be relatively low (approximately 100 molecules per cell), so its detection, either as mRNA or activity, requires methods based on polymerase chain reaction (PCR) amplification. In general, all quantitative data acquired with real-time PCR must be normalised by a housekeeping gene. The ideal housekeeping gene should not vary with disease progression. The glyceraldehyde 3-phosphate dehydrogenase gene, which is often employed as housekeeping gene, is activated by HIF and is thus expressed at higher levels in advanced disease than in tumours at early stages. Other genes, such as the hypoxanthine-guanine phosphoribosyltransferase 1 (*HPRT1*) gene, which does not vary with tumour stage[56], allow a more reliable estimation of TERT levels. In CRC, a study by real-time PCR with *HPRT1* as a housekeeping gene demonstrated that there is a good relationship between the levels of all TERT transcripts and the full-length TERT transcript; in addition, levels of TERT mRNA correlated with telomerase activity, as estimated with a telomere repeat amplification

protocol (TRAP) assay[54]. Although there are no clinically approved telomerase assays, several promising approaches have recently been published[57].

There is general agreement that TERT levels and telomerase activity increase with the adenoma-carcinoma sequence[60,64,70], and are higher in CRCs than in adjacent non-cancerous mucosa (Table 3). Normal adjacent mucosa may have some detectable TERT mRNA and telomerase activity, mainly because of intestinal crypt basal cells[55,58]. These findings strongly support the hypothesis that telomerase activation is subsequent to telomere erosion (Figure 1).

Most studies have demonstrated that TERT expression and/or telomerase activity increase with tumour progression (Figure 2A and Table 3). Well-differentiated and moderately differentiated tumours have significantly lower TERT levels than poorly differentiated tumours do, and late-stage tumours (Dukes C and D) show higher telomerase activity than early-stage tumours[63,67]. Only a few studies have found no correlation between levels of telomerase activity, as assessed by the semi-quantitative TRAP assay, and tumor progression[38,58,61]. Unlike telomere length, levels of telomerase expression/activity do not correlate with MSI status and increase with disease progression in both MSI and MSS tumours[68,73]. The finding that TERT mRNA is higher in tumours bearing *TP53* mutations[66] may support the hypothesis that high TERT expression is a marker of poor outcome and poor response to therapy[27,73].

TELOMERASE, BUT NOT TELOMERES, MAY ACT AS A PROGNOSTIC FACTOR IN COLORECTAL CANCERS

Pathologic tumour staging remains a key determinant of CRC prognosis and treatment. Invasive cancers are confined within the wall of the colon (stages I and Ⅱ), but if untreated they spread to regional lymph nodes (stage Ⅲ) and then metastasise to distant sites (stage Ⅳ). Although radical resection and adjuvant therapy are effective curative treatments, the risk of disease recurrence cannot be foreseen, even among patients at the same tumour stage. Although 5-fluorouracil-based adjuvant chemotherapy is the standard care for stage Ⅲ patients, the role of adjuvant therapy for stage Ⅱ is still debated. The controversial results obtained in various studies[74-78] may reflect the molecular and biological heterogeneity of CRC and highlight the need for definitive prognostic markers able to stratify patients.

While most studies do not confirm the prognostic role of telomere length (Table 2), there is general agreement that high levels of TERT and/or telomerase activity are associated with poor prognosis (Table 3) Only two studies do not confirm the prognostic value of TERT[72] or telomerase activity[62]. High levels of TERT mRNA and/or telomerase activity have been associated with worse overall survival (OS) and this negative prognostic effect is independent of pathologic stage. In particular, over a median follow-up of 70 mo, patients with high levels of TERT mRNA (above the median) had approximately double the risk of death compared with patients with low levels of TERT (below the median) did[73]. Only two studies analysed stage Ⅱ patients in detail. In one study, in which telomerase activity was determined with TRAP assay, patients with telomerase-positive CRCs had longer disease-free survival (DFS) than did patients with telomerase-negative tumours[62]. In the second study, TERT levels estimated using real-time PCR significantly stratified stage Ⅱ patients; stage Ⅱ patients with high TERT levels showed significantly worse median OS and DFS than patients with low TERT levels did[73].

In recent years, great efforts have been made to identify markers for minimally invasive early diagnosis and/or monitoring of disease. The expression of epithelial cell adhesion molecules has been used primarily to detect CRC cells in the hematopoietic milieu, and the detection of circulating cancer cells is a promising approach, although its diagnostic/prognostic role needs to be established[79]. The detection of cancer-related RNA molecules in plasma has recently been proposed as a marker of cancer onset and outcome, and ongoing studies indicate that circulating microRNAs may be biomarkers for the early detection of CRC[80,81]. Within this framework, recent studies suggest that cell-free circulating TERT mRNA is also a potential marker of disease.

Transcripts of TERT have been detected in the plasma of patients with different tumours, including CRC[82,83]. In a series of CRCs (stage I to stage Ⅳ), the TERT mRNA levels in plasma were related to those in tumours[55] (Figure 2B). In addition, while 95% of patients with tumours had detectable cell-free circulating TERT, aged-matched controls were negative in almost all cases[55]. This finding suggests that TERT levels in plasma reflect those in tumours. Very promising findings have been reported in patients with rectal cancer who underwent chemoradiotherapy (CRT) prior to surgery; plasma TERT was significantly decreased in patients who underwent a complete pathologic response, but remained unchanged or increased in patients who did not respond to CRT[84] (Figure 2C). These findings also suggest that circulating TERT is a useful marker for monitoring the response to therapy. However, further studies with a prospective design and with a large sample sizes are required to clearly define the prognostic role of telomerase in CRC patients and to ascertain the cut-off values and reliability of circulating TERT as a marker for monitoring disease outcome and response to therapy.

CONCLUSION

Besides extensive heterogeneity in the molecular and biological features of CRC, chromosomal instability plays a key role in the early steps of carcinogenesis. The majority of studies agree that telomere shortening is an early event in the oncogenetic process and that telomere erosion

Table 3 Telomerase as a marker of disease in colorectal cancer

Ref.	Cases	Main findings
Engelhardt *et al*[32], 1997	80 (50 CRCs, 20 polyps, 10 colitis) cancerous and 50 CRC patient-matched non-cancerous mucosa specimens	Telomerase activity Absent in normal tissues Higher in CRCs than in nonneoplastic lesions Higher in late-stage than in early-stage tumours
Tatsumoto *et al*[58], 2000	100 CRC and patient-matched non-cancerous mucosa specimens	Telomerase activity Higher in CRC than in adjacent non-cancerous mucosa Detectable in adjacent non-cancerous mucosa derived from intestinal crypt basal cells Not correlated with CRC stage or grade Has prognostic value for OS and DFS (high telomerase activity: poor prognosis)
Niiyama *et al*[59], 2001	140 CRC and patient-matched non-cancerous mucosa specimens; 20 adenomas	TERT mRNA and telomerase activity Higher in CRCs than in adenomas Higher in adenomas than in normal mucosa
Naito *et al*[60], 2001	66 (50 adenomas, 6 mucosal carcinomas, 10 invasive carcinomas) specimens	Positive correlation between TERT mRNA and telomerase activity TERT levels increase with adenoma-carcinoma sequence
Gertler *et al*[61], 2002	57 CRC and patient-matched non-cancerous mucosa specimens	Both CRC and adjacent non-cancerous mucosa are positive for TERT TERT levels lower in tumours than in non-cancerous mucosa in most cases TERT levels not correlated with tumour stage TERT has prognostic value for OD and DFS (high telomerase activity: poor prognosis)
Kawanishi-Tabata *et al*[62], 2002	122 CRCs, stage Ⅱ (52 colon, 70 rectum)	80% of CRC are telomerase-positive Higher percentage of telomerase-positive tumours in the colon than in the rectum High telomerase activity: Good prognosis
Ghori *et al*[63], 2002	30 CRCs and 20 patient-matched non-cancerous mucosa specimens	Telomerase activity Higher in CRCs than in adjacent non-cancerous mucosa Correlated with Duke's stage
Boldrini *et al*[64], 2002	36 CRC and patient-matched non-cancerous mucosa specimens, 8 adenomatous polyps, 9 dysplastic polyps	Telomerase activity Absent in normal mucosa and adenomas Higher in CRCs than in dysplastic polyps Higher in late-stage than in early-stage tumours
Maláska *et al*[65], 2004	41 CRC and patient-matched non-cancerous mucosa specimens	Telomerase activity Present in 83% of CRCs Absent or at very low level in normal mucosa Higher in metastatic tumours
Boldrini *et al*[66], 2004	43 CRCs	TERT levels and telomerase activity higher in tumours with mutated *TP53*
Sanz-Casla *et al*[67], 2005	103 CRCs	Telomerase activity increases with tumour progression (Duke's stage) Higher percentage of telomerase-positive tumours in the colon than in the rectum Telomerase activity has prognostic value for DFS (high telomerase activity: poor prognosis)
Garcia-Aranda *et al*[38], 2006	91 CRC and patient-matched non-cancerous mucosa specimens	Telomerase activity Present in 81% of CRCs Present at very low levels in 15% of normal samples Not correlated with tumour progression No prognostic value
Vidaurreta *et al*[68], 2007	97 CRCs	Telomerase activity Present both in MSI and MSS tumours Has prognostic value for OS (high telomere activity: poor prognosis)
Bautista *et al*[69], 2007	108 rectal cancer and patient-matched non-cancerous mucosa specimens	Telomerase activity Higher in rectal cancer than in normal mucosa Not correlated with tumour stage and grade Has prognostic value for DFS and OS
Terrin *et al*[55], 2008	85 CRC and 42 patient-matched non-cancerous mucosa specimens, 49 plasma samples	TERT levels Higher in CRCs than in adjacent non-cancerous mucosa Increase with tumour stage and grade Not correlated with MSI status Not correlated with tumour location Plasma TERT levels correlated with tumour TERT levels
Valls Bautista *et al*[70], 2009	6 cases, each with cancer, polyps and normal mucosa; 8 polyps and normal mucosa	Telomerase activity Increases with adenoma-carcinoma sequence
Kojima *et al*[71], 2011	106 CRC and paired adjacent non-cancerous mucosa specimens	Elongation of the 3′OH of telomere by telomerase may increase Malignant potential of cancer cells Telomerase activity has prognostic values for OS (telomeraseactivated without 3'OH shortened telomeres: poor prognosis)
Safont *et al*[72], 2011	48 CRC and adjacent non-cancerous mucosa specimens and 48 plasma samples	Plasma TERT levels correlated with tumour TERT levels Higher circulating TERT levels in stage Ⅳ tumours No correlation between telomerase expression and prognosis

Bertorelle *et al*[73], 2013	137 CRCs	TERT levels: Increase with tumour stage and grade Not correlated with MSI status Not correlated with tumour location Have prognostic value for OS and for both OS and DFS for stage Ⅱ patients (high TERT levels: poor prognosis)

CRC: Colorectal cancer; DFS: Disease free survival; OS: Overall survival; TERT: Telomerase reverse transcriptase; MSI: Microsatellite instability.

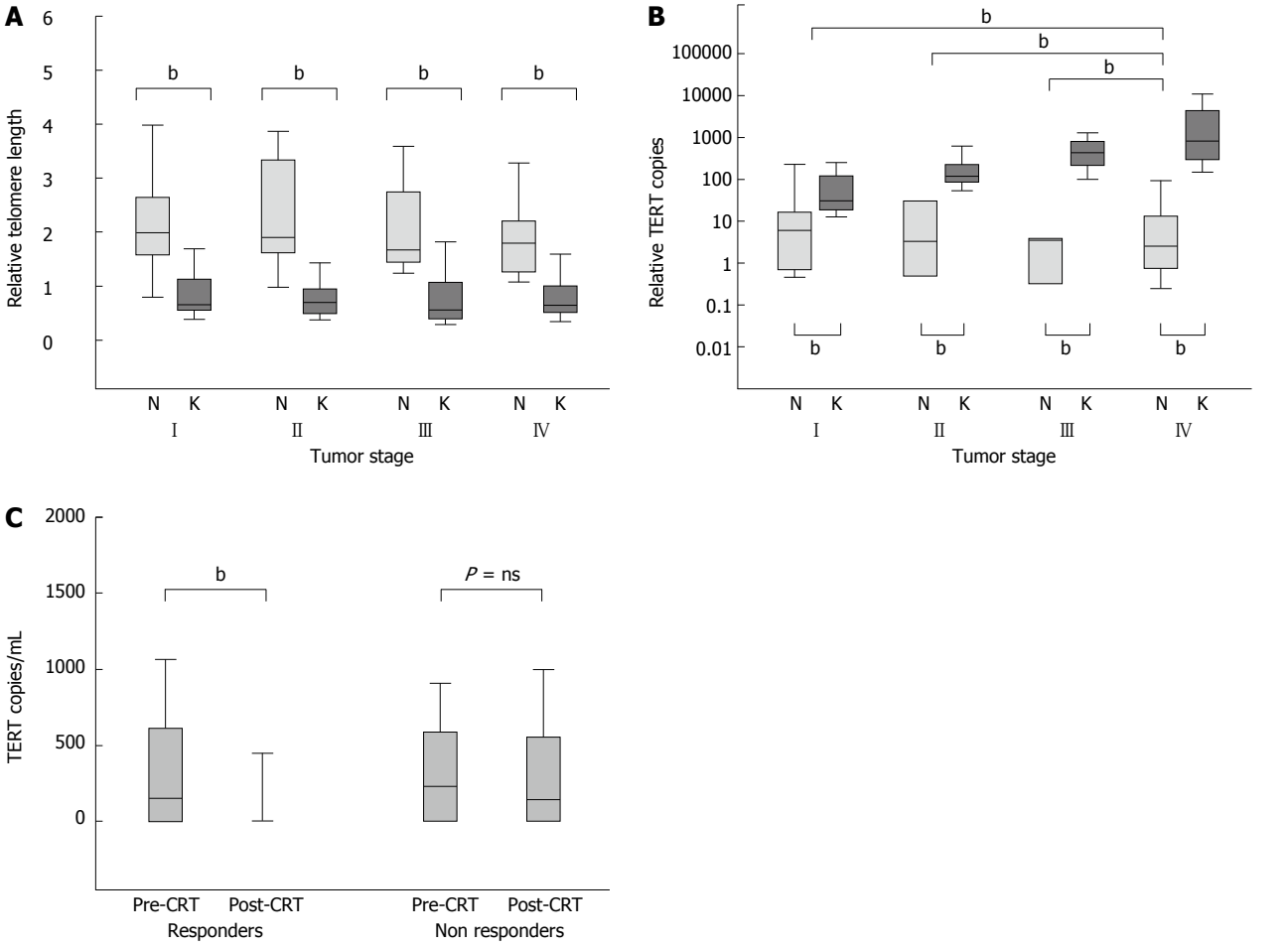

Figure 2 Representative panels of telomere length and telomerase reverse transcriptase levels. A: Relative telomere length in tumours (K) and adjacent mucosa (N) according to tumour stages Ⅰ (30 samples), Ⅱ (45 samples), Ⅲ (29 samples), and Ⅳ (29 samples). The cases included those reported in Rampazzo *et al*[41]. Telomere length was significant shorter in tumours than in adjacent mucosa ($^bP < 0.0001$) at all tumour stages, but telomere lengths did not significantly differ with tumour stage. Relative telomere length was estimated using real-time polymerase chain reaction (real-time PCR)[41]; B: Telomerase reverse transcriptase (TERT) levels in tumours (K) and adjacent mucosa (N) according to tumour stages Ⅰ (K: 25 samples, N: 17 samples), Ⅱ (K: 35 samples; N: 10 samples), Ⅲ (K: 15 samples; N: 5 samples), and Ⅳ (K: 30 samples; N: 22 samples). The cases included those reported in Terrin *et al*[55]. TERT levels were significantly higher in tumours than in adjacent mucosa and significantly increased ($^bP < 0.01$) with tumour stage. TERT levels were estimated using real-time PCR[41,55]; C: Plasma TERT levels before and after the chemoradiotherapy prior to surgery in responders (35 samples) and non-responders (42 samples) with rectal cancer. The cases included those reported in Pucciarelli *et al*[84]. TERT levels in plasma were estimated using real-time PCR[84]. Boxes and whiskers: 25th-75th and 10th-90th percentiles, respectively; the median is the central line in each box.

leads to genetic instability. Telomerase, which maintains telomere length and preserves the cell's replicative potential, is activated during the adenoma-carcinoma sequence and its activity increases during tumour progression.

While most studies do not confirm the prognostic role of telomere length, there is general agreement that high levels of TERT and/or telomerase activity are associated with poor prognosis. Emerging data also suggest that circulating TERT levels reflects tumour TERT levels. Overall, there is sufficient evidence to indicate that telomerase is a useful marker for monitoring and predicting disease outcome. A caveat to the use of telomerase as a marker is the availability of simple and reliable assays to quantify telomerase expression and/or activity. The use of reliable assays will allow researchers to compare data and to define useful cut-off values to discriminate between patients at low and high risk of disease progression. Further studies with a prospective design and large

sample sizes are required to clearly define the prognostic role of telomerase and to acertain its reliability as a circulating biomarker for the minimally invasive monitoring of disease and the response to therapy.

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TOPIC HIGHLIGHT

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

Colorectal cancer and lymph nodes: The obsession with the number 12

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Abstract

Lymphadenectomy of colorectal cancer is a decisive factor for the prognostic and therapeutic staging of the patient. For over 15 years, we have asked ourselves if the minimum number of 12 examined lymph nodes (LNs) was sufficient for the prevention of understaging. The debate is certainly still open if we consider that a limit of 12 LNs is still not the gold standard mainly because the research methodology of the first studies has been criticized. Moreover many authors report that to date both in the United States and Europe the number "12" target is uncommon, not adequate, or accessible only in highly specialised centres. It should however be noted that both the pressing nature of the debate and the dissemination of guidelines have been responsible for a trend that has allowed for a general increase in the number of LNs examined. There are different variables that can affect the retrieval of LNs. Some, like the surgeon, the surgery, and the pathology exam, are without question modifiable; however, other both patient and disease-related variables are non-modifiable and pose the question of whether the minimum number of examined LNs must be individually assigned. The lymph nodal ratio, the sentinel LNs and the study of the biological aspects of the tumor could find valid application in this field in the near future.

Key words: Colorectal cancer; Lymphadenectomy; Lymph node count; Lymph node ratio; Staging

Core tip: Lymphadenectomy of colorectal cancer is a decisive factor for the prognostic staging of the patient. A limit of 12 lymph nodes (LNs) is still not the gold standard and accessible only in highly specialized centers. There are different variables that can affect the retrieval of LNs; some are non-modifiable and pose the question of whether the minimum number of examined LNs must be individually assigned.

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INTRODUCTION

In 1998, Curti *et al*[1] stressed that continually talking about the lymphadenectomy of colorectal cancer makes for incredibly monotonous reading. In fact, even though it has been proven that the excision of lymph nodes (LNs) in colorectal cancer is a crucial measure, in the last decade the problem has mainly shifted its focus to the physical dimensions of the lymph nodal excision and, more specifically, to the number of LNs to be removed. Even if in this area there are precise indications, in reality, they

are not always respected due to, above all else, the large number of variables that can interfere with the sampling of the LNs.

MEANING OF LYMPHADENECTOMY

All histological staging, even the recent seventh edition of the AJCC[2], has considered the metastatic involvement of the LNs a determining factor for the staging of the colorectal tumor[3-28] as long as examined in sufficient numbers to ensure the "certainty" of a patient's prognostic classification[13]. Actually, this is not the case, since as we shall see, the lack of reliable data makes the current staging systems inadequate. This lack creates episodes of "stage migration," which are likely responsible for the 20%-25% of the cases in which a node-negative patient relapses[29-34], as well as for better prognoses for patients staged IIIa than for those staged Ⅱb[31-34].

The correct staging of a patient treated for colorectal cancer is also critical in the planning of adjuvant therapies that certainly, especially for stage Ⅲ, ensure improved outcomes and may not be prescribed to a patient who has a falsely-judged more "favorable" staging[3,8,10,11,15,17,19,22-24,27,31,33,35-37]. In this context, some authors as well as some organizations such as the American Society of Clinical Oncology (ASCO) and the National Comprehensive Cancer Network (NCCN) recommend adjuvant chemotherapy to patients for whom the LN study proved insufficient[7,21,25,35,38,39].

In addition to its accuracy in staging, the LNs excision also seems to be an independent prognostic factor. Many case-study reviews[3-5,10,11,14,19,20,24-28,35,40-49], particularly in patients with stage Ⅱ, report a directly proportional relationship between the number of LNs removed and survival. In this regard, it seems appropriate here to refer to the systematic review of Chang *et al*[24] who report that in 16 of 17 studies the increased survival of patients with stage Ⅱ colon cancer was associated with increased numbers of LNs evaluated. The most likely explanation is that the higher the number of LNs examined the better select the group of node-negative patients with a better prognosis for which surgery alone should be curative. Other authors[1,21,40,50], however, believe that in patients with more advanced stages, the lymphadenectomy can be therapeutic both by improving tumor clearance by the surgeon and by reducing the metastatic spread through lymphatic drainage. Not all authors agree with this latter view[5,11].

Last but not least, it must be noted that organizations such as the American College of Surgeons, the ASCO, and the National Quality Forum consider the entity of the lymphadenectomy as a way to gauge the quality of a center dealing with this type of pathology[51-55]. Concerning this issue, not all are in agreement mainly because the number of LNs removed may not reflect the quality of the surgeon or the pathologist but, as we shall see later, may be tied to unchangeable factors inherent in the patient or the tumor[6,50].

NUMBER OF LNs NECESSARY FOR A CORRECT STAGING

Many authors[17,27,28,56] claim that in clinical practice there should be no set limit to the number of LNs examined since in addition to survival, as has already been mentioned, there is a direct correlation between the number of examined LNs and the number of LNs with metastasis[4,14,24,28, 57-59].

However, in light of this observation, we have to ask ourselves what the minimum number of LNs is, beyond which there is no change in the staging if not within acceptable limits. Therefore, along with McDonald *et al*[6] we believe that a "ceiling effect may be reached", above all for the purposes of allowing pathologists to realize the point at which they can feel satisfied with their search no matter how many LNs may be left in the piece removed. In fact, there is no doubt that in the "real world", pathologists, with the methods that are presently available, do not or simply cannot sample all removed LNs especially when they are small[15].

In light of this, the number of LNs to be sampled still varies widely even though it has been discussed for over 20 years. In fact, since 1990, at the World Congress of Gastroenterology in Sydney, 12 was established as the minimum standard of LNs to be examined since this number would allow for a correct diagnosis of N0 in 90% of cases[6,21,51,60].

This number, referred to as "magic"[50], was later included in many guidelines and has been endorsed by a large number of United States and European organizations[7,9,17-19,36,40, 50,51,61,62].

In this regard, what Stocchi *et al*[41] have recently reported seems paradigmatic. He claims that, considering only patients treated for stage II colon cancer, the examination of at least 12 LNs is associated with an improvement in results; this improvement reduces if a smaller sample of LNs get examined, but it does not increase with a larger sample of LNs.

Other data reported by Nelson *et al*[51], Norwood *et al*[19], and Lee *et al*[58] show data compatible with Stocchi's theory. Nelson *et al*[51] report that by examining 12 LNs, the lymph nodal positivity is correctly identified in 90% of patients; Norwood *et al*[19] claim that only when the number of LNs is < 12 there is a reduction in the survival rate; finally, Lee *et al*[58] reports that the examination of a number of LNs ⩾ 12 increases the chance of diagnosing positive LNs by 30%.

In light of this data, the number 12 indeed seems correct, but this is not the case of course if in 2012 Fingerhut[4] still asked himself, "Why all the fuss?"

The debate is certainly still open if we consider that a limit of 12 LNs is, as of today, still not the gold standard mainly because the research methodology of the first studies[40,63,64], which do not go beyond a level of Ⅲ or Ⅳ and a grade C recommendation, has been criticized[6,51].

In fact, in the literature there is no uniformity in determining what the minimum lymph nodal sampling is to allow for a greater diagnosis of positive LNs, a different

Table 1 Minimum lymph node sampling recommended for a correct staging

Under 12 LNs	LNs *n*	At least 12 LNs	LNs *n*	Over 12 LNs	LNs *n*
Caplin *et al*[65]	7	Nir *et al*[18]	12	Swanson *et al*[57]	13
Maurel *et al*[66]	8	Norwood *et al*[19]	12	Wong *et al*[71]	14
Mekenkamp *et al*[67]	8	Stocchi *et al*[41]	12	Tepper *et al*[72]	14
Yoshimatsu *et al*[26]	9	Wong *et al*[46]	12	Wong *et al*[73]	14
Sarli *et al*[35]	9	Kukreja *et al*[50]	12	Chen *et al*[48]	15
Cianchi *et al*[68]	9	Nelson *et al*[51]	12	Mukai *et al*[74]	15
		Lee *et al*[58]	12	Goldstein *et al*[75]	17
		Bilimoria *et al*[69]	12	Tsai *et al*[25]	18
		Storli *et al*[70]	12	Le Voyer *et al*[27]	20
				Joseph *et al*[76] 03 (T3)	30
				Joseph *et al*[76] 03 (T4)	40

LN: Lymph node.

staging that justifies adjuvant chemotherapies, or, ultimately, a better survival rate (Table 1).

This confusion is also documented by McDonald *et al*[6] who, citing 10 observational studies that analyzed more than 43000 patients, points out that not only is there no agreement on what the LN cut-off point should be, but that in a wide range of LNs examined (between 6 and 21) the actual cut-off point fluctuated. This range is similar to the one reported by Valsecchi *et al*[21] (between 6 and 17) and lower than the one reported by Noura *et al*[42] (between 6 and 40).

This variety of data leads us to ask what our main objective is when we examine LNs? If the goal is to "certify" a node-negative patient, then evaluating a number of LNs equal to 12 or perhaps even higher is likely to be necessary; if instead metastatic LNs are identified, it is then possible to "settle" for a smaller number of LNs which, according to some authors[46], are easier to identify as they are more visible and palpable. However, even in this respect "everything and the opposite of everything" can be said if considering what is reported by some authors[35,60,77] who claim that about 50% of enlarged LNs are negative or only an expression of a vigorous immune response, while 45%-78% of metastatic LNs have a less than 5 mm diameter.

Gelos *et al*[78], however, focuse on yet another aspect, arguing that in patients with a malignancy at an earlier stage which can lead to a lower immune response, it is likely that we can settle for a sample of less than 12 LNs.

The fact that even today the number "12" target is "uncommon, "not adequate," or accessible only in highly specialized centers[21,41,50] is demonstrated by the fact that in the United States in 2001[4], the number of 12 sampled LNs was reached for only 44% of patients and that the target of patients increased to 75% in only 38% of hospitals in 2004-05[69], 15 years after Sydney. Moreover, once again in the United States, reports published between 2005 and 2010 revealed that, despite the "dense forest of articles"[79] lymphadenectomy was still considered inadequate in 48% to 63% of cases[79,80].

Similar experiences are also reported in Europe; in fact, in Germany in 2009, the Dutch Surgical Colorectal Audit[77] reported that in 73% of colon cancers and 58% of rectum cancers, the number of LNs examined was ⩽ 10; in England, Johnson *et al*[81] and Mitchell *et al*[82] have recently pointed out that the limit of 12 LNs was not reached between 33% and 50% of colorectal cancer cases.

It should however be noted that both the pressing nature of the debate and the dissemination of guidelines have been responsible for a trend that, over the years, has allowed for a general increase in the number of LNs examined, thus enabling the U.S. to increase the number of hospitals that reach the target of 12 LNs from 15% in 1995-96 to 38% in 2004-05[69] and in 2005-2008[52] reach the figure of 92% albeit only in centers that are members of the NCCN and are thus made up of top institutions[69]. This, however, is probably not the case in smaller hospitals[9,21].

Table 2 What "could interfere" with the lymph node count

Modifiable factors	Surgeons	Pathologist
	Specialization	Lack of training
	Case volume	Lack of time
	Surgical technique	Techniques
	Emergency	
	Extension	
	Laparoscopy	
Unmodifiable factors	Patient related	Disease related
	Advanced age	Tumor site
	Female	Tumor staging
	Obesity	Pre-operative radiotherapy

WHAT CAN INTERFERE WITH LYMPH NODAL COUNT

Ideally, the surgeon should remove all the LNs pertaining to the tumor and the pathologist should sample and examine them thoroughly. However, even if this were carried out, all authors agree that there would be "unmodifiable" factors patient-related and disease-related that could make a node-count problematic (Table 2).

In our opinion, all the variables, modifiable and unmodifiable, that can affect lymph nodal sampling should be examined so as to make the work of both the surgeon and of the pathologist more efficient.

Modifiable factors

Surgeons and surgery: The extent to which a surgeon's experience, specialization and case volume impact the quality of a performed surgery has often been considered a possible factor which can affect the number of removed LNs[3,11,21,23,41,83] (Table 3).

Even if this seems logical[35], if we consider Table 3 we can see that although the "surgeon variable" is considered an "independent factor", there is no clear differ-

Table 3 Surgeon's experience *vs* lymph nodes harvested

	Surgeon's experience	Statistical analysis	LN harvested (expert *vs* no expert)
Leung *et al*[11]	> 15 yr	MA: se independent factor ($P < 0.05$)	13 *vs* 11 LN
Valsecchi *et al*[21]	5 colon/yr	MA: se significant predictor ($P = 0.001$)	Not specified
Shaw *et al*[23]	Colorectal surgeon	UA: se $P = 0.002$	11 *vs* 9 LN
Stocchi *et al*[41]	Largest case volume	MA: se independent variable ($P = 0.018$)	86% *vs* 83.6% pts with ⩾ 12 LN

MA: Multivariate analysis; UA: Univariate analysis; LNs: Lymp nodes; se: Surgeon's experience.

Table 4 Lymph node sampling in laparoscopic *vs* open approaches

	Laparoscopy		Open	
	Patients	LNs	Patients	LNs
	n	*n*	*n*	*n*
COST[91]	435	12	428	12
Veldkamp *et al*[92]	627	10	621	10
Guillou *et al*[93]	526	12	268	13.5
Kang *et al*[94]	170	17	170	18
Braga *et al*[95]	134	14.5	134	15.3
Hewett *et al*[96]	294	13	298	13
Liang *et al*[97]	135	15.6	134	16
Leung *et al*[98]	203	11.1	200	12.1
Benhaim *et al*[99]	235	26.8	296	25.9

Only prospective or prospective randomized trials in over 250 patients. COST: Clinical Outcomes of Surgical Therapy Study Group; LNs: Lymp nodes.

ence between surgeon with greater and lesser experience compared to harvested LNs.

Indeed other authors consider this correlation inconsistent[8,19,22,53,78,84-86] as it does not record statistical differences related to surgeon expertise or between colorectal surgeons and general surgeons, thus giving other authors[9,87,88] the opportunity to dwell, instead, on the importance of an educational strategy that allows for a more accurate surgical technique. From this perspective, we must keep in mind that even though American studies[36,46,69] have reported that a hospital's volume of surgery could affect lymph nodal sampling, Porter *et al*[37] and Dejardin *et al*[89] have recently reported that the simple implementation of guidelines within a center in addition to recommendations or the application of audit strategies may eliminate any differences between hospitals. This leads us to believe that a correct approach to the problem can bridge the "gap" between "current and best available evidence." The above authors' implication seems to reveal that a diligent surgical technique, which above all else ensures an adequate and "potentially measurable" sample of mesocolon[21,41,87,88], can simply guarantee a sufficient number of LNs to be sampled. Whether the greater length of the intestine removed can determine more lymph nodal sampling is, in fact, a matter of controversy. While some authors lean toward this hypothesis[19,21,78], others refute it completely[90].

Although, as of present, literature has not offered conclusive data as to whether emergency surgeries are responsible for limited resections and hence smaller numbers of collected LNs[19,40,56,86], more reliable data is available with regards to the influence of laparoscopic surgery on lymph nodal sampling, whose efficacy has been questioned. Actually, in addition to the COST[91], COLOR[92] and CLASICC[93], other prospective randomized trials have opted for an overlap of the two techniques (Table 4). More significantly, a recent meta-analysis[100] of 24 randomized trials has shown no significant differences between the two approaches concerning the number of LNs examined. On the contrary, Lujan *et al*[101] has reported advantages in favor of laparoscopic surgery with regards to the number of LNs sampled in patients suffering from rectal cancer (13.63 *vs* 11.57, $P = 0.026$); similarly El-Gazzaz *et al*[102] have reported, a greater number of metastatic LNs removed (2.2 ± 3.8 *vs* 1.6 ± 4, $P = 0.03$), despite not being able to pin-point the exact reasons.

Pathologists: A review of the literature, even if there are still conflicting view points[22,41,84], soon reveals that the diligence of a "pathology staff" (pathologists, pathology assistants, pathology residents, pathology technicians) could affect the number of LNs sampled[10,11,21,35,50,53,60,61,79,86] and that the simple lack of time, more than the lack of educational training, especially seems to interfere with this data[6,8,27,60,77]. This is indeed confirmed by the fact that, paradoxically, first-year "pathology residents"[8] or "pathologists' assistants" who have "more time with fewer distractions"[77] carry out better lymph nodal samplings than "pathologists", especially for cancer of the rectum.

Moreover, from the multivariate analysis of Leung *et al*[98] we can deduce that pathologists and surgeons independently affect the lymph nodal sampling ($P < 0.05$ and $P = 0.01$ respectively) and that pairing surgeon/pathologist does not serve to compensate for the differences. Valsecchi *et al*[21] also, in his analysis, support Leung's data[98], but report for "surgeon's experience" a major risk factor ("OR = 2.33; 95%CI: 1.4-3.9, $P = 0.001$" *vs* "OR = 1.9; 95%CI: 1.1-3.2, $P = 0.01$" respectively) in contrast, Evans *et al*[86] find no significant differences among surgeons but does so only among pathologists.

The possibility of having more time may also be useful for the implementation of procedures which have been widely recommended[7,27,30,40]. Such procedures include the fat clearance technique or the intra-arterial injection of blue methylene, among others, which seem to improve performance. However, in addition to being costly[11,23,27,60], these procedures are difficult to carry out

in centers with a high case volume[3,23]. Therefore, while the NCCN[39] recommends that if a pathologist samples < 12 LNs, a greater amount of tissue must be examined, the American College of Pathologists[103] adds that in these cases the use of additional techniques is necessary even though there is still no consensus on the precise technique to be recommended.

In light of these considerations, especially for pathologists, it seems that, particularly in today's society, the "ceiling effect" must be reached (which is 12 or greater) in order to optimize time, costs and human resources.

Unmodiafiable factors

Patient-related: The patient-related variables are among those for which there is less debate and difference of opinion. In the literature, in fact, it is agreed that advanced age could negatively affect lymph nodal sampling[10,40,41,44,48, 50,53,60,80,104-108], decreasing by 9% for every 10 years of age[107]. Among the hypotheses put forth, we must remember that surgery performed on a patient of advanced age cannot be extensive because of the presence of comorbidities[3,10,40], in addition to the physiological involution of LNs[10,40,56] and the weaker response of the immune system[41].

Similarly, most authors[19,22,36,85,90,107], with regards to gender, do not report a different LN retrieval while only some[60,79,109] mention greater sampling in females.

Not all authors, on the other hand, are in agreement on the role that obesity may have during lymphadenectomy; some authors[5,110], in fact, have shown either a higher LN retrieval in non-obese patients or a lower one in patients with high body mass index (BMI), probably due to the more difficult surgical dissection[9,40,51,84]. Kuo *et al*[5], which refer in his experience as the BMI is associated with LNs harvest, highlights that the larger LN retrieval in non-obese patients is due to a bigger number of right colon cancers. However, the relationship between BMI and LN sampling still remains an open question. In fact many authors do not report such a correlation[9,22,61,84].

Disease-related: Also with regards to the unchangeable disease-related variables, the literature is mostly consistent. All authors, in fact, agree that it is more difficult to achieve the target of 12 LNs when the tumor is located in the rectum, possibly due to the smaller size of the LNs, in spite of the higher percentage of malignant nodes retrieved[3,53]. With regards to the colon, the number of LNs sampled is definitely higher in the right colon[3,5,6,10,18,21,22,25,36,41,44,52,60,61,78,80,84,107,108] either because of the greater length of the mesentery root[5,90] or due to a different embryological development that would ensure a greater number of LNs[78].

Tumor characteristics have often been thought to have an effect on lymph nodal sampling; the greater the size and the more advanced the tumor staging (T and grading), the greater the number of LNs retrieved[9,10,21,22,25,31,78,86,107], this probably due either to a greater immune response[78] or to more aggressive surgery[9,10].

When we consider, instead, the non-advanced tumors interesting is that recently report by Benhaim *et al*[99], the first in the literature, that determine the total number of LNs examined after colectomy for an endoscopically removed malignant polyp. In these patients the mean number of LNs examined was significantly lower compared to both patients operated for colon cancer at any stage (11.63 *vs* 26.23, $P = 0.0006$) and patients operated for colon cancer at pT_1 stage (11.85 *vs* 19.21, $P = 0.018$). Considering the fact that none of the patients who underwent a colectomy after endoscopic polypectomy showed a relapse, the authors suggest that the rule of 12 LNs can not be applied to malignant polyps as more than 12 LNs were examined in only 41% of patients who underwent a colectomy for such lesions.

It is also generally agreed[3,56,80,86,107,111] that pre-operative radiotherapy is responsible for either a minor, absent, or at best widely variable lymph nodal sampling, irrespective of the characteristics of the patients or treatment[17]. Evans *et al*[86], Deodhar *et al*[3], Tekkis *et al*[56] therefore refer to an average lymph nodal sampling of 7, 9.54 e 9.8 LNs respectively, while Doll *et al*[111], Govindarajan *et al*[112] and Rullier *et al*[113] report a statistically significant difference between patients treated with neoadjuvant radiochemotherapy or surgery alone (respectively 12.9 *vs* 21.4, $P > 0.0001$, 10.8 *vs* 15.5, $P > 0.001$, 13 *vs* 17, $P > 0.001$).

This appears to be due to inflammatory post-radiotherapy processes which cause stromal fibrosis of the LNs and of their subsequent reduction in size[6,17,67]. Rullier *et al*[113] report that for every Gy of radiation, the sampled LNs number will be less than 0.21% and Norwood *et al*[19] show that this reduction is evident especially when pre-operative radiotherapy is used in combination with chemotherapy.

It is perhaps interesting to note that, in this case, the reduction in the number of sampled LNs, although oncologically favorable does not affect the survival rate but rather must be viewed as a positive response to neoadjuvant treatment[6,111-113]. This has led some authors[17] to conclude that the limit of 12 LNs is unrealistic for the stage of rectal cancer of patients who are treated with neoadjuvant therapy.

CAN THE "LYMPH NODAL RATIO" BE USEFUL IN THE EVENT OF INADEQUATE SAMPLING?

The seventh edition of the AJCC classification[2], as mentioned previously, subdivides patients treated for colorectal cancer into prognostic categories according to the number of metastatic LNs. The accuracy of the staging is, however, influenced by the number of retrieved LNs that must be ⩾ 12. The lymph nodal ratio (LNR)[49], which is the relationship between positive nodes divided by the total number of retrieved nodes, is in our opinion, justified mainly because it means not having to reach the so called "magic number." The LNR prognostic validity could in

fact be effective also in cases of reduced lymph nodal sampling[12,16,45,114]. However, not all authors who have written on the subject agree[6,43,49]. The LNR, independent of the number of LNs sampled, is also justified since, taken with the AJCC classification, it would allow us to subdivide, according to the risk involved, stage Ⅲ patients reducing the excessive prognostic heterogeneity[12,16,45].

In light of this, reviewing and taking into consideration the work of Bamboat *et al*[8], Qiu *et al*[12], Song *et al*[13], and Greenberg *et al*[114] in 2011, the LNR seems to be an independent prognostic factor in colorectal cancer, superior to the classification based only on N stage (number of positive modes). In fact, based on the LNR analysis, Greenberg *et al*[114] himself state that the survival rate of stage Ⅲ patients with favorable LNR is similar to that of stage Ⅱ patients.

Conversely, Noura *et al*[42] only one year before published an interesting and somewhat more cautious editorial. In fact, the author reported that even though the LNR seemed to be a more reliable prognostic factor, its validity, in actuality, could not be completely agreed upon. In fact, clinical records were very different, randomized and multi-centric studies were lacking, and, most importantly, a uniformly valid cut-off was missing.

One thing is certain, given the importance of both the lymph nodal sampling and of the evidence of the lymph nodal metastasis, it is unthinkable that a pathologist could stop after "having found" the first neoplastic LN[78]. However, it is not exactly clear what the "ceiling effect" is even in this case.

CONCLUSION

Despite the fact that numerous authors have expressed their opinions on the number of LNs sampled, it can be gathered that the number is between 6 and 40[42]. Therefore, only in light of this wide range, should we all refer to the minimum number of LNs (now the obsessive "12") which can allow us to avoid the so-called "Will Roger's phenomenon"[115] responsible for understaging.

Even if the surgeon and pathologist, as variables in the equation, could improve simply by standardizing surgical technique and by increasing the amount of time dedicated to this procedure, the other, more important variables[22,31,114], namely patient and cancer-related, are not as easily modifiable. It is with these latter two variables in mind that we still pose the question whether it is possible, as we hope, to establish a universally valid cut-off node for all patients or whether it should instead be varied according to individual cases[6,78].

Today, a valid perspective is still necessary for the identification of the sentinel LNs (at least 3)[116]. This approach is based on the idea that the lymphatic flow originating from the tumor occurs "step by step"[35,117] and the purpose of this technique in colorectal cancer would be not so much to modify the size of the resection, as has happened with other diseases, but for its "potential" effects on improving the staging[29,31,40,41] since it would allow for more involved and expensive techniques on only a few LNs[29,30,32-34] which would reveal micrometastases or isolated tumor cells.

The identification of the sentinel LNs, actually still remains a controversy among those authors who consider the mesenteric lymph drainage, especially in the rectum, too complicated[32,40], and the majority of authors who, on the other hand, maintain that an aberrant lymphatic drainage occurs only in a small percentage of cases[27,29,33,34].

As certainly interesting, the biological aspects of the tumor still remain the subject of speculation. Some authors suggest that reduced survival is not necessarily due to an inappropriate dissection performed by the surgeon and the pathologist, but may be linked to a cancer that is quite virulent and is hence responsible for a low immune response from the patient[35,40,57,117]. Still, some other authors[11,40,41,46,47,118,119] maintain that an elevated sampling could be determined by a vigorous immune response; this, in turn, is determined by the molecular instability of the tumor, which manifests itself as a high rate of "neoantigens" and therefore causes a more limited neoplastic progression. Not coincidentally, these malignancies are located in the right colon[41,44,118], where, as mentioned, more LNs are found.

As has already been pointed out, the obsession with the number 12 has its origins in studies which lack clear statistical evidence. Just as Curti *et al*[1] asserted that, as of 1998, not even a single prospective study had been published, authors still today are calling for prospective controlled studies that are, without question, difficult to predict both for a number of ethical reasons and for the sheer volume of clinical records. Hence, obtaining reliable data that would allow us to go beyond this obsession with the number 12 will not be easy.

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TOPIC HIGHLIGHT

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

New genes emerging for colorectal cancer predisposition

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Abstract

Colorectal cancer (CRC) is one of the most frequent neoplasms and an important cause of mortality in the developed world. This cancer is caused by both genetic and environmental factors although 35% of the variation in CRC susceptibility involves inherited genetic differences. Mendelian syndromes account for about 5% of the total burden of CRC, with Lynch syndrome and familial adenomatous polyposis the most common forms. Excluding hereditary forms, there is an important fraction of CRC cases that present familial aggregation for the disease with an unknown germline genetic cause. CRC can be also considered as a complex disease taking into account the common disease-commom variant hypothesis with a polygenic model of inheritance where the genetic components of common complex diseases correspond mostly to variants of low/moderate effect. So far, 30 common, low-penetrance susceptibility variants have been identified for CRC. Recently, new sequencing technologies including exome- and whole-genome sequencing have permitted to add

a new approach to facilitate the identification of new genes responsible for human disease predisposition. By using whole-genome sequencing, germline mutations in the *POLE* and *POLD1* genes have been found to be responsible for a new form of CRC genetic predisposition called polymerase proofreading-associated polyposis.

Key words: Colorectal neoplasm, genetic predisposition to disease; Next generation sequencing; Genotype-phenotype correlation; Genetic variant; Single nucleotide polymorphism

Core tip: Colorectal cancer (CRC) is caused by both genetic and environmental factors although 35% of the variation in CRC susceptibility involves inherited genetic differences. Mendelian syndromes account for about 5% of the total burden of CRC. Excluding hereditary forms, there is an important fraction of CRC cases that present familial aggregation for the disease with an unknown germline genetic cause. Recently, new sequencing technologies have permitted to add a new approach to identify new genes responsible for human disease predisposition. By doing so, germline mutations in the *POLE* and *POLD1* genes have been found to be responsible for a new form of CRC genetic predisposition.

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INTRODUCTION

Colorectal cancer (CRC) is one of the most frequent neoplasms and an important cause of mortality in the developed world. Approximately 5% of the population develops CRC and this figure is expected to rise as life expectancy increases[1]. For 2015, approximately 473200 new cases are predicted and 233900 individuals will die from this disease in Europe[2]. When taking into account both genders together, it corresponds to the most frequent neoplasm in Spain. Although there has been recent progress in CRC clinical management and treatment that has permitted to reduce the number of cases in the developed countries, it is foreseen that its incidence will increase worldwide with developing nations bearing the brunt of the rise. The incidence of CRC varies widely between countries, depending on their degree of development and also on the quality of their cancer registries[3]. Around 60% of cases are diagnosed in the developed world[3]. The highest incidence rates are found in Australia and New Zealand, North America and Europe, whereas the lowest rates are registered in Africa and South-Central Asia[2] (Figure 1).

CRC survival depends on the stage of disease at diagnosis and typically ranges from a 90% 5-year survival rate for cancers detected at the localized stage to 10% for people diagnosed of a distant metastatic cancer[4]. The lifetime risk of CRC in the general population is about 5% in Western countries, but the likelihood of CRC diagnosis increases progressively with age, being more than 90% in individuals over age 50, and 70% of these over 65[4].

CRC is believed to develop from polyps, which have been traditionally classified as either hyperplastic or adenomatous. Until recently, according to the adenoma-carcinoma sequence proposed by Vogelstein *et al*[5] the adenoma was considered the exclusive precursor lesion while hypeplastic polyps were deemed to have no malignant potential. However, it is now recognized that lesions, formerly classified as hyperplastic, represent a heterogeneous group of polyps with a characteristic serrated morphology, some of which have a significant risk of malignant transformation through the serrated neoplasia pathway[4].

GENETIC AND ENVIRONMENTAL RISK FACTORS

As other complex diseases, CRC is caused by both genetic and environmental factors. The role of environmental factors on colorectal carcinogenesis is indicated by the increase in CRC incidence in parallel with economic development and adoption of Western diets and lifestyles, responsible for the high incidence of CRC in industrialized countries[7]. Although the majority of CRC occur mostly in industrialized countries, their incidence rates are rapidly rising in economically transitioning countries in the world[8]. These observations highlight the importance of environmental influences on CRC development and suggest that Western lifestyle risk factors play an important role in the etiology of the disease. However, although environmental causes such as smoking and diet are undoubtedly risk factors for CRC, twin studies have shown that 35% of the variation in CRC susceptibility involves inherited genetic differences[9,10]. In that sense, a minority of CRC cases (about 5%) show strong familial aggregation and belong to the well-known hereditary CRC forms mainly caused by germline mutations in *APC*, *MUTYH* and the DNA mismatch repair genes[11]. Approximately 30% of CRC cases show some family history of the disease but do not fit in the previous category and are regarded as familial CRC, whereas a majority of cases do not show any familial aggregation and correspond to sporadic CRC. For instance, familial CRC accounted for about 30% of all CRC cases in an

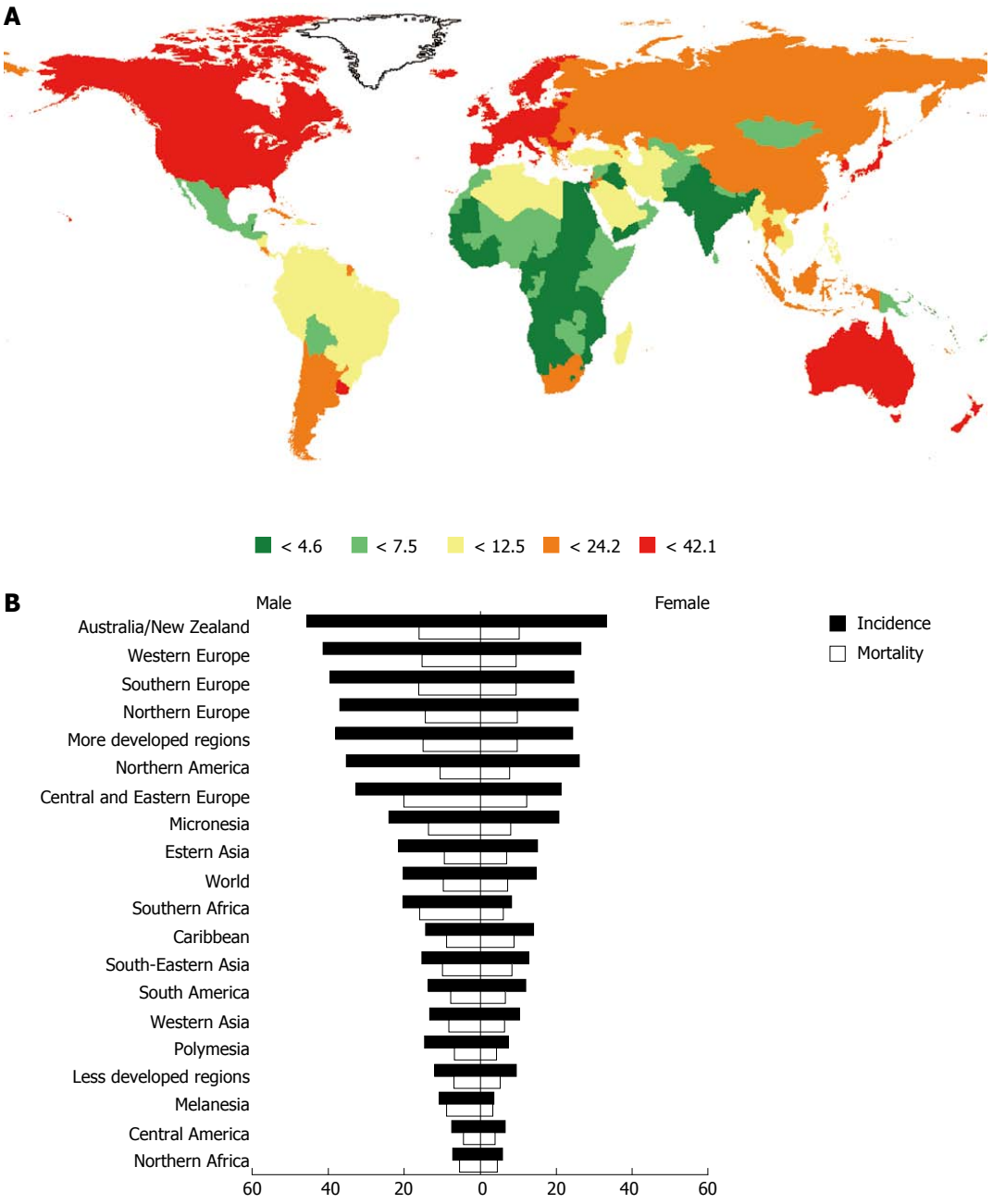

Figure 1 Colorectal cancer in the world. A: Estimated age-standarized incidence rate per 100000 individuals (both genders and all ages); B: Estimated age-standarized incidence and mortality rate per 100000 individuals by genders (data adapted from Ferlay *et al*[2]).

epidemiological study in the Spanish population[12].

HEREDITARY CRC

Mendelian cancer syndromes account for about 5% of the total burden of CRC[11]. The genetic components involved in these less frequent hereditary forms were successfully identified using linkage analysis in the past two decades and they correspond to rare highly penetrant alleles that predispose to CRC. Two major subgroups can be clinically divided on the presence or absence of colorectal polyposis. An overview of all CRC syndromes is provided in Table 1. The most frequent forms are hereditary nonpolyposis colorectal cancer and familial polyposis syndrome, which are further described below.

Hereditary Nonpolyposis Colorectal Cancer (HNPCC; MIM No.120435), also known as Lynch syndrome, is the most common form of hereditary CRC accounting for at least 3% of all CRC. HPNCC is an autosomal dominant syndrome defined clinically by the Amsterdam criteria (Table 2), which are used in clinical practice to identify individuals at risk for this disease who require further evaluation and are based on strong familial aggregation and early onset. It is characterized by early-onset CRC (mean age at diagnosis, approximately 45 years), excess synchronous and metachronous colorectal neoplasms and right-sided predominance compared to sporadic neoplasms. In addition, there is an increased

Table 1 Hereditary colorectal cancer genes

	Gene	Chromosome	Mendelian pattern	Function
Familial adenomatous polyposis	*APC*	5q	AD	Regulation of canonical Wnt signaling pathway
	MUTYH	1p	AR	Base-excision repair
Hereditary non-polyposis CRC (Lynch syndrome)	*MLH1*	3p	AD	Mismatch repair
	MSH2	2p	AD	Mismatch repair
	MSH6	2p	AD	Mismatch repair
	PMS2	7p	AD	Mismatch repair
Peutz-Jeghers	*LKB1*	19p	AD	Regulation of Wnt signaling pathway
Juvenile polyposis	*SMAD4*	18q	AD	TGFBR signaling pathway
	BMPR1A	10q	AD	TGFBR signaling pathway
Cowden's disease	*PTEN*	10q	AD	Negative regulation of PI3K signaling
	KLLN	10q	AD	Apoptotic process

CRC: Colorectal cancer; AD: Autosomal dominant; AR: Autosomal recessive; TGFBR: Transforming growth factor beta receptor; PI3K: Phosphatidylinositol 3-kinase.

Table 2 Amsterdam criteria in Lynch syndrome

Amsterdam criteria I	Amsterdam criteria II
At least three relatives with CRC; all of the following must be met:	At least three relatives with colorectal, endometrial, small bowel, ureter, or renal pelvis cancer; all of the following must be met:
One affected individual is a first degree relative of the other two	One affected individual is a first degree relative of the other two
At least two successive generations affected	At least two successive generations affected
At least one CRC diagnosed before the age of 50 years	At least one tumor diagnosed before the age of 50 years
Familial adenomatous polyposis has been excluded	Familial adenomatous polyposis has been excluded

CRC: Colorectal cancer.

incidence of extracolonic neoplasms (endometrial, small bowel, gastric, upper urinary tract, ovarian, brain and pancreatic tumors) being endometrial cancer the most common malignancy associated with Lynch syndrome. Indeed, Lynch syndrome is responsible for approximately 2% of all endometrial cancers[13]. The lifetime risk for developing CRC in individuals affected with Lynch syndrome have been estimated in approximately 66% for men and about 43% for women. The cumulative risk of endometrial cancer is approximately 40% and the lifetime risk of endometrial cancer or CRC in women is approximately 73%[14]. Lynch syndrome tumors develop as a consequence of defective DNA mismatch repair (MMR) associated with germline mutations in the MMR genes, including *MSH2* on chromosome 2p16, *MLH1* on chromosome 3p21, *MSH6* on chromosme 2p16, and *PMS2* on chromosome 7q11. In addition, germline epigenetic inactivation of *MLH1*, by hypermethylation of its promoter, can also lead to Lynch syndrome[15]. Recently, germline deletions of the 3' region of *EPCAM* gene were found in a subset of families with Lynch syndrome. This deletion leads to promoter hypermetilation of *MSH2*, located upstream of the deleted gene[16]. The MMR system is necessary to maintaining genomic fidelity by correcting single-base mismatches and insertion-deletion loops during DNA replication. As a consequence, Lynch syndrome tumors accumulate errors in short repetitive sequences, a phenomenon called microsatellite instability (MSI), which is considered a landmark for this disease. It is noteworthy to mention that in sporadic MSI CRC cancers, loss of expression of MLH1 due to hypermethilation of its promoter is a frequent event, and it is linked with the somatic mutation V600E in the *BRAF* gene[17].

Familial Adenomatous Polyposis (FAP; MIM No.175100) is the most common polyposis syndrome, classically characterized by the development of hundreds to thousands of adenomatous polyps in the rectum and colon. FAP is an autosomal dominant disease and accounts for approximately 1% of all CRC cases. In the majority of patients polyps begin to develop during the second decade of life and nearly 100% of untreated patients will have malignancy by ages 40-50 years. Individuals with FAP can also develop a variety of extracolonic manifestations, including cutaneous lesions such as fibromas, lipomas, sebaceous and epidermoid cysts, facial osteomas, congenital hypertrophy of the retinal pigment epithelium, desmoid tumours and extracolonic cancers (tyroid, liver, biliary tract and central nervous system)[18]. Duodenal cancer is the second most common malignancy in FAP, with a lifetime risk of approximately 4%-12%. *Adenomatous polyps* are also found in the stomach and duodenum, especially the *periampullary area* and can develop into adenocarcinomas. After colectomy, periampullary carcinoma is the most common malignancy, occurring in approximately 5%-6% of the patients[19]. Some lesions such as skull and mandible osteomas, dental abnormalities and fibromas are indicative of the Gardner syndrome, a clinical variant of FAP where the extracolonic features are prominent. FAP is caused by germline mutations in the *APC* gene on

chromosome 5q22, which encodes a tumor suppressor protein that plays an important role in the *Wnt* signaling pathway. Most patients have a family history of colorectal polyps and cancer, but de novo *APC* mutations are responsible for approximately 25% of cases[11].

APPROACHES TO IDENTIFY GENETIC VARIANTS FOR CRC RISK

Among CRC cases of unknown inherited cause, there are large families with a clear positive family history of CRC, which are likely caused by highly penetrant risk loci. In the last few years, it has been described that approximately 40%-50% of CRC families that fulfill the Amsterdam Criteria for Lynch syndrome do not show evidence of MMR deficiency. Studying relatives in such families showed that CRC risk is lower than in those families with Lynch syndrome, that CRC diagnosis is in average 10 years later and that there is no increased incidence of extracolonic malignancies[20,21]. The designation of Familial CRC type X was proposed to describe this type of CRC clustering[20]. Meanwhile, genes responsible for this new entity are unknown, and most patients are included in the heterogeneous group of non-syndromic familial CRC.

Recently, there have been several efforts to identify additional genetic factors that predispose to CRC with uneven success. Linkage analysis in affected families were able to pinpoint chromosomal regions of interest such as 9q22 and 3q22 but no clear CRC predisposition genes were identified after screening for interesting candidates within these areas[22,23].

Since the known high-risk syndromes only account for a small minority of CRC cases, there has been an intensified search for low-penetrance genetic variants that probably underlie part of the hereditary predisposition and together with environmental interactions are responsible for CRC as a complex disease. Therefore, the common disease-common variant hypothesis has been also considered, being a polygenic model of inheritance where the genetic components of common complex diseases correspond mostly to variants of low/moderate effect (typically < 1.5-fold increased risk) that appeared at an elevated frequency in the population (> 5%), each exerting a small influence on disease risk. In this regard, case-control genome-wide association studies (GWAS) have been more successful by discovering up to now 31 common, low-penetrance genetic variants involved in CRC susceptibility[24-32] (Table 3).

OVERVIEW OF NEW SEQUENCING TECHNOLOGIES

Until recently, the Sanger method was the dominant approach and gold standard for DNA sequencing[33]. Next generation sequencing (NGS), also called massive parallel sequencing, is based in sequencing millions of DNA fragments at the same time[34]. It consists in a mix of techniques of DNA shearing, PCR amplification and sequencing through modified nucleotides attached to a reversible terminator and a fluorophore, which permits fluorescent detection with an imaging system. Once the fragments are sequenced, they are assembled de novo or aligned with a reference genome by bioinformatics tools and positions that differ are designated as variants. Variants are annotated assigning their position in a gene, retrieving frequency information from genetic variation databases and categorizing them by their functional class (nonsense, missense, synonymous, frameshift, splicing, intronic, untranslated regions, regulatory).

The advantage of NGS comparing with conventional Sanger sequencing is that millions of DNA fragments are sequenced at the same time which permits to have an entire human genome sequenced in few days, and the cost is greatly reduced. However, data analysis that includes filtering of the false positives and prioritization of the candidate variants for the studied phenotypic condition is the main bottleneck of NGS, being time consuming and requiring different strategies that will be discussed later. Another disadvantage of NGS is that PCR amplification and sequencing reaction steps systematically introduce mistakes, producing base-calling errors and shorter sequenced fragments that difficult the mapability to the reference sequence. Due to recent technology and variant calling algorithm improvements, NGS is probably nowadays more accurate than conventional Sanger sequencing[35]. However, although there is a very small error rate associated with NGS, a huge amount of false positives are still detected since millions of variants are sequenced per genome[36]. Thus, after data analysis and selection of the candidate variants it is necessary to validate them using a technology with a different systematic error associated, such as conventional Sanger sequencing, which increases the costs and time of the analysis.

In order to detect genomic sequence variation by NGS, it is possible to sequence the entire genome (whole-genome sequencing, WGS) or capture and sequence only specific regions of interest (targeted enrichment). The most commonly used application for NGS target enrichment in the human genome is whole-exome sequencing (WES) that captures and amplifies the entire protein coding sequence (1% genome), flanking intronic regions and some noncoding RNAs[37]. It is a cost effective approach for detecting rare high penetrance variants based on the fact that for Mendelian disorders over the 85% of causative mutations are in coding regions. One advantage of WES is that is about much cheaper than WGS, which allows sequencing a larger number of samples with better accuracy or coverage. The term coverage corresponds to the read depth or depth and it is the average number of times that a nucleotide has been sequenced in a different sequencing read. Also, the data analysis pipelines are simpler in WES than for

Table 3 Genetic variants associated with colorectal cancer susceptibility identified by genome-wide association studies (as for September 2013)

SNP	Region	Gene	Sample size (cases/controls)	Effect size OR (95%CI)
rs693267	8q24.21	*MYC*	8264/6206	1.21 (1.15-1.27)
rs4939827	18q21.1	*SMAD7*	8413/6949	1.18 (1.12-1.23)
rs16892766	8q23.3	*EIF3H*	18831/18540	1.25 (1.19-1.32)
rs3802842	11q23.1	*?*	14500/13294	1.12 (1.07-1.17)
rs4779584	15q13.3	*GREM1*	7922/6741	1.26 (1.19-1.34)
rs10795668	10p14	*?*	18831/18540	1.12 (1.10-1.16)
rs4444235	14q22.2	*BMP4*	20288/20971	1.11 (1.08-1.15)
rs9929218	16q22.1	*CDH1*	20288/20971	1.10 (1.06-1.12)
rs10411210	19q13	*RHNP2*	20288/20971	1.15 (1.10-1.20)
rs961253	20p12.3	*BMP2*	20288/20971	1.12 (1.08-1.16)
rs6691170	1q41	*DUSP10*	18185/20197	1.06 (1.03-1.09)
rs10936599	3q26.2	*TERC*	18185/20197	0.93 (0.91-0.96)
rs11169552	12q13.3	*?*	18185/20197	0.92 (0.90-0.95)
rs4925386	20q13.33	*LAMA5*	18,185/20,197	0.93 (0.91-0.95)
rs1957636	14q22.2	*BMP4*	24910/26275	1.08 (1.05-1.11)
rs4813802	20p12.3	*BMP2*	24910/26275	1.09 (1.06-1.12)
rs2736100	5p15.33	*TERT*	16039/16430	1.07 (1.04-1.10)
rs1321311	6p21	*CDKN1A*	21096/19555	1.10 (1.07-1.13)
rs3824999	11q13.4	*POLD3*	21096/19555	1.10 (1.07-1.13)
rs5934683	Xp22.2	*SHROOM2*	21096/19555	1.07 (1.04-1.10)
rs12080929	1p33	*SLC5A9*	2317/2447	0.86 (0.78-0.95)
rs11987193	8p12	*DUSP4*	2317/2447	0.78 (0.70-0.87)
rs10774214	12p13.32	*CCND2*	11870/14190	1.04 (1.00-1.09)
rs647161	5q31.1	*PITX1*	11870/14190	1.07 (1.02-1.11)
rs2423279	20p12.3	*HAQ1*	11870/14190	1.07 (1.03-1.12)
rs11903757	2q32.3	*NABP1*	15752/21771	1.16 (1.10-1.22)
rs10911251	1q25.3	*LAMC1*	15752/21771	1.09 (1.06-1.13)
rs3217810	12p13.32	*CCND2*	13654/16022	1.20 (1.12-1.28)
rs3217901	12p13.32	*CCND2*	15752/21771	1.10 (1.06-1.14)
rs59336	12q24.21	*TBX3*	15752/21771	1.09 (1.06-1.13)

WGS. However, WES need for larger amounts of DNA sample and only covering coding variants are among the shortcomings for this technique. It is noteworthy mentioning that NGS target enrichment can also be used to sequence a panel of known genes for clinical diagnosis[33] or regions of linkage disequilibrium for a disease.

The election of individuals to sequence is a critical process to take into account for further analysis and will depend of the disease phenotype and pattern of genetic inheritance. Also, it should be noted that is possible to obtain good results with NGS when using carefully selected patients in contrast to GWAS, where number of cases and controls that are compared needs to be much higher in order to obtain statistically significant findings. For diseases with genetic heterogeneity as human cancers, different strategies can be used including the selection of families with strong disease aggregation or sequencing sporadic cases with early onset for the disease. Both situations are suggestive of the involvement of a germline predisposition. When focusing in families with several affected members, sequencing can be performed in several cases in each family and only those shared variants will be taken into account. On the other hand, if sporadic early-onset cases are chosen, genes with variants in different individuals can be selected. Sequencing non affected individuals of the same family can be useful to discard the variants shared with patients, as long as the disease has complete penetrance or it is quite likely that the non affected individuals will not express the disease in their lifetime.

Data filtering and prioritization in NGS

Based on several recently sequenced individual genomes a pattern has been recognized that, in general, approximately 3-4 million variants are expected to be found in a human genome by WGS[38] and 20000 single nucleotide variants are to be found in a human exome by WES[39], so it is necessary to do a filtering strategy in order to eliminate as many false positives as possible. The first filter to apply is for those variants that do not pass a coverage threshold (typically 5-10x).

The second filtering process is based on the kind of inheritance, penetrance and frequency of the disease. Regarding the inheritance, for monogenic diseases where unrelated affected individuals have been sequenced, it is necessary to select only the genes that have variants in all of them. If a disease with genetic heterogeneity is studied, variants shared between the affected members of the same family and not shared by the unaffected ones will be chosen. Also, if dominant inheritance is present heterozygous mutations will be expected, whereas homozygous or compound heterozygous mutations will be selected in the case of recessive inheritance. However, variants in the non pseudoautosomal regions of X chromosome for dominant inheritance have to take into account also. In men, they will be annotated as

homozygous and it is necessary to select these variants too and not filter them out. Regarding variant effect on protein, it is assumed that high penetrance mutations are causative of Mendelian disorders with a large effect on protein function. Therefore, a positive selection for variants with a strong effect on the protein is advised including those affecting canonical splice sites, as well as frameshift, nonsense and missense mutations.

Proportionally, more deleterious than polymorphic variants are expected to be rare so a causative mutation is not expected to be present at a high frequency in the general population[40]. Thus, variants present at high frequency at reference genetic variation databases can be removed as potential candidates to be causative mutations.

However, many variants can still remain for each individual as putative causative mutations for the disease after filtering. A logical approach to reduce the number of candidate variants is to prioritize the mutations in genes previously implicated with the studied disease. Also, since the protein products of genes responsible for the same disorder tend to physically interact with each other so as to carry out certain biological functions, another approach for the prioritization strategy will be to include genes interacting with those previously implicated with the studied disease[41]. Finally, knowledge of the pathways implicated in a disease can be helpful also to prioritize those genes related with those pathways. After filtering and prioritization, a list of candidate variants will be available.

Sequencing validation by Sanger sequencing or any other PCR technology designed to detect a specific nucleotide change is necessary after NGS to confirm the prioritized variants and exclude sequencing artifacts. Also, segregation analysis in families permits to check if a candidate variant segregates correctly with the disease. Therefore, affected members need to be carriers and non-affected individuals old enough to be expressing the disease should be non-carriers in order to find correct segregation of the candidate variant with the studied disease. Additionally in the case of hereditary cancer, when heterozygous candidate variants with correct segregation are identified, it is necessary to confirm if there is loss of the second allele in the tumor DNA in order to establish the candidate gene as a tumor suppressor gene. Case-control screening studies can also be performed in order to identify additional carriers of the candidate variants in ample disease cohorts and further demonstrate its absence in controls. Finally, functional assessment of the candidate variant and affected gene will be also necessary to further confirm the negative effect of the variant in the protein and prove its involvement in disease development by *in vitro* studies and animal models.

NEW GENES IDENTIFIED FOR CRC GENETIC PREDISPOSITION

New sequencing technologies made available recently including exome- and whole-genome sequencing have permitted to add a new approach to facilitate the identification of new genes responsible for human disease predisposition. Indeed, some seminal efforts have been already completed very recently for CRC. However, before these high-throughput technologies have yielded results in CRC families, some previous low-throughput sequencing studies reported directed screening of some plausible gene candidates for various reasons. Most studies have not been replicated in additional cohorts and, therefore, there is a strong need to further validate them before considering these genes as hereditary CRC genes perse.

A truncating mutation was found in the *CDH1* gene in a family with predisposition to CRC and gastric cancer, suggesting that germline mutations in this gene could contribute to early onset CRC and gastric cancer[42]. Later on, the *AXIN2* gene, a component of the Wnt signaling, was found to be mutated in a Finnish family with severe permanent tooth agenesis and CRC[43]. In a subsequent study in patients with unexplained hamartomatous or hyperplastic/mixed polyposis, two early-onset disease patients were found to have germline mutations in *ENG*, encoding endoglin, previously associated only with hereditary hemorrhagic telangiectasia[44]. This study suggested *ENG* as a new predisposition gene for juvenile polyposis, however this gene was found to be mutated in an additional study only in patients with $\geqslant$ 5 cumulative lifetime gastrointestinal polyps but not in juvenile polyposis[45]. *EPHB2* was also evaluated as a candidate tumor suppressor gene for CRC and found mutated in 3 out 116 population-based familial CRC cases, suggesting this gene may contribute to a small fraction of hereditary CRC[46]. In 2009, the *GALNT12* gene was also found mutated in the germline of 6 CRC patients[47]. This gene encodes one of the proteins involved in mucin type O-linked glycosylation and it is located in chromosomal region 9q22, previously involved in familial CRC. A more recent study detected additional deleterious variants in this gene reinforcing its role as a new candidate gene for hereditary CRC[48]. Also, an inherited duplication affecting the protein tyrosine phosphatase *PTPRJ* and causing epigenetic silencing of this gene was detected in a CRC family without polyposis and MMR alteration, being indicative of its contribution to a fraction of hereditary CRC with unknown basis[49]. Afterwards, *BMP4*, a gene close to 2 of the CRC genetic susceptibility variants identified by GWAS, was also screened in 504 genetically enriched CRC and 3 pathogenic mutations were identified[50]. Then, it could be plausible that some genes identified by CRC GWAS could be also involved in hereditary CRC. In 2011, the *BMPR1A* gene, previously involved in juveline polyposis and mixed polyposis germline predisposition, was also found mutated in familial CRC type X cases, expanding its phenotype also to this CRC hereditary form[51]. Finally, Cowden syndrome individuals without germline *PTEN* mutations were found to carry germline mutations in *PIK3CA* and *AKT1*, expanding the genetic spec-

trum of this hereditary CRC condition[52].

Regarding NGS studies to identify new CRC predisposition genes, Palles *et al*[53] reported very recently the identification of germline mutations in the *POLE* (polymerase (DNA directed), epsilon, catalytic subunit) and *POLD1* (polymerase (DNA directed), delta 1, catalytic subunit) genes in individuals with multiple colorectal adenomas, carcinoma or both, using whole-genome sequencing[54]. *POLE* and *POLD1* encode the catalytic and proofreading activities of the leading-strand DNA polymerase ε and the lagging-strand polymerase δ. The proofreading capacity of the exonuclease domain is essential for the maintenance of replication fidelity and may act not only on newly misincorporated bases but also on mismatches produced by non-proofreading polymerases. They identified a heterozygous p.Leu424Val missense variant in *POLE* DNA polymerase in a family affected with adenomas and CRC and a p.Ser478Asn missense variant in *POLD1* in a second family with CRC. The same *POLD1* p.Ser478Asn variant was also identified in the affected members of an independent family. These findings were further validated in a screen of 3,085 individuals with CRC, enriched for a family history of colorectal tumors, in which they detected 12 individuals with the p.Leu424Val variant in *POLE* and one additional individual with the pSer478Asn in *POLD1*. Functional assessment supported the importance of these mutations in *POLE* and *POLD1*. Mutagenesis studies of Polδ and Pol3 in yeast showed that the mutation of the equivalent residue produces a mutator phenotype and loss of the proofreading activity of the protein[53,55,56]. Also, mice expressing proofreading-impaired Pole and Pold1 in a homozygous state developed spontaneous intestinal adenocarcinomas or a spectrum of cancers[57]. Thus, germline variants in *POLE* and *POLD1* predispose to individuals to either a multiple colorectal adenoma phenotype similar to that observed in MUTYH-associated polyposis or a HNPCC phenotype, in which carriers develop early-onset CRC. Although additional studies will be needed to evaluate these rare germline variants in *POLD1* and *POLE* and their associated phenotypes, the authors suggest that screening for these variants should be considered in patients with an unexplained personal or family history of multiple adenomas, early onset CRC or both. On the other hand, carriers are potential candidates for regular and frequent colonoscopic surveillance starting at an early age.

Two additional reports using exome sequencing have also been published very recently but their results are not as solid as those for the polymerase genes previously mentioned. A cohort of 50 sporadic CRC patients was sequenced including 18 early-onset cases with a relatively low coverage in the first study[58]. Variants were biased selected when found in a list of 1,138 genes likely to play a role in CRC. Further selection to include only those genes undergoing bialleic inactivation yielded *FANCM*, *LAMB4*, *PTCHD3*, *LAMC3* and *TREX2* as potential tumor suppressor candidates. In the second study, exome sequencing was completed for 40 familial cases from 16 families by selecting distant relatives to decrease the number of shared, non-predisposition variants[59]. Data was analyzed firstly by an agnostic search for CRC predisposition genes not taking into account a biased list of candidates, and secondly by selecting genes previously involved in CRC predisposition or within CRC linkage regions. Two missense variants in the *CENPE* and *KIF23* genes that complied with family segregation and belong to regions on chromosomes 1 and 15 formerly linked to CRC were considered the more plausible candidates for CRC predisposition but additional studies are needed to further elucidate their role.

CONCLUSION

CRC is one of the most frequent neoplasms and an important cause of mortality in the developed world. CRC is caused by both genetic and environmental factors although 35% of the variation in CRC susceptibility involves inherited genetic differences. Mendelian cancer syndromes account for about 5% of the total burden of CRC, being Lynch syndrome and familial adenomatous polyposis the most common forms. Familial CRC type X is an example of CRC with unknown inherited cause. A clear positive family history of CRC is present (Amsterdam criteria for Lynch syndrome are fulfilled) although MMR is proficient. When considering CRC as a complex disease, low-penetrance genetic variants probably underlie part of the hereditary predisposition together with environmental interactions. So far, 30 susceptibility variants have been identified for CRC. New sequencing technologies made available recently including exome- and whole-genome sequencing have permitted to add a new approach to facilitate the identification of new genes responsible for human disease predisposition. Germline mutations in the *POLE* and *POLD1* genes are responsible for a new form of CRC genetic predisposition called polymerase proofreading-associated polyposis.

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TOPIC HIGHLIGHT

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

Perioperative anemia management in colorectal cancer patients: A pragmatic approach

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Abstract

Anemia, usually due to iron deficiency, is highly prevalent among patients with colorectal cancer. Inflammatory cytokines lead to iron restricted erythropoiesis further decreasing iron availability and impairing iron utilization. Preoperative anemia predicts for decreased survival. Allogeneic blood transfusion is widely used to correct anemia and is associated with poorer surgical outcomes, increased post-operative nosocomial infections, longer hospital stays, increased rates of cancer recurrence and perioperative venous thromboembolism. Infections are more likely to occur in those with low preoperative serum ferritin level compared to those with normal levels. A multidisciplinary, multimodal, individualized strategy, collectively termed Patient Blood Management, minimizes or eliminates allogeneic blood transfusion. This includes restrictive transfusion policy, thromboprophylaxis and anemia management to improve outcomes. Normalization of preoperative hemoglobin levels is a World Health Organization recommendation. Iron repletion should be routinely ordered when indicated. Oral iron is poorly tolerated with low adherence based on published evidence. Intravenous iron is safe and effective but is frequently avoided due to misinformation and misinterpretation concerning the incidence and clinical nature of minor infusion reactions. Serious adverse events with intravenous iron are extremely rare. Newer formulations allow complete replacement dosing in 15-60 min markedly facilitating care. Erythropoiesis stimulating agents may improve response rates. A multidisciplinary, multimodal, individualized strategy, collectively termed Patient Blood Management used to minimize or eliminate allogeneic blood transfusion is indicated to improve outcomes.

Key words: Colorectal cancer; Anemia; Allogeneic blood transfusion; Intravenous iron; Erythropoiesis stimulating agents; Patient Blood Management

Core tip: Anemia, usually due to iron deficiency, is highly prevalent among patients with colorectal cancer. Both anemia and allogeneic blood transfusion are associated with poorer outcomes. Anemia management, within a multidisciplinary, multimodal, individualized strategy to minimize or eliminate allogeneic blood transfusion, is indicated to improve outcomes. Intravenous iron is safe and effective but underused, despite the extremely low risk of causing serious adverse events. Newer intravenous iron formulations allow complete replacement dosing in 15-60 min markedly facilitating care. Erythropoiesis stimulating agents may improve response rates.

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PREVALENCE OF ANEMIA AND IRON DEFICIENCY

Anemia is one of the most frequent extraintestinal manifestations of colorectal cancer (CRC), and may be present in 30%-75% of patients, predicated on the level of hemoglobin (Hb) used to define anemia and tumor localization and stage[1-7]. A study on 358 patients with CRC reported a 25% prevalence of moderate to severe anemia (Hb < 10 g/dL). The multivariate analysis revealed that age, tumor site (right colon), and tumor size (large size), but not clinical stage or histological type, were significant contributing factors[2]. These results were corroborated by a study of 1189 Norwegian patients[7]. Lastly, a recent study showed that iron deficiency (ID) in CRC was associated with poor performance and more advanced disease[8].

CONSEQUENCES OF ANEMIA

Preoperative anemia is the major predictive factor for allogeneic blood transfusion (ABT) in surgeries with moderate to high perioperative blood loss, which is causative in postoperative anemia and aggravates pre-existing anemia[9]. In CRC resection, a hematocrit of less than 30% has been shown to be an independent risk factor for perioperative ABT[6,7,10,11]. Even mild-to-moderate preoperative anemia has been linked to increased postoperative morbidity and length of hospital stay as well as decreased disease-free survival after resection[12-14]. This is supported by a recent publication in which preoperative anemia significantly worsened overall survival ($P = 0.040$) in the univariate analysis[15]. However, in the multivariate analysis the difference did not approach statistical significance.

Both preoperative anemia and ID without anemia increase the rate of postoperative nosocomial infection. Following abdominal surgery, infections were significantly more likely with low preoperative serum ferritins compared with normal levels. The data were especially poignant in that confounders including Hb level were taken into account in the analysis[16]. Zago *et al*[17] evaluated the relationship of vitamin and mineral levels to wound complications in 100 abdominal surgical procedures, and noted low plasma retinol (a marker of low vitamin A intake) and high erythrocyte protoporphyrin (an early marker of ID) to be surrogates of increased complications. Further evaluation of the benefits of these measurements as a standard of care appears warranted.

PATHOPHYSIOLOGY OF ANEMIA

In CRC, preoperative anemia can be attributed to chronic hemorrhage, neoadjuvant chemotherapy or radiotherapy, and nutritional deficiencies. These may be exacerbated by activation of the immune system with release of inflammatory cytokines such as tumor necrosis factor alpha (TNF-α), interferon gamma (IFN-γ), and interleukins (IL)-1, -6, -8 and -10[18-20]. These inflammatory mediators cause anemia *via* a variety of pathophysiological mechanisms (Figure 1): (1) decreased red cell half-life due to dyserythropoiesis with red cell damage and increased erythrophagocytosis (TNF-α); (2) inadequate EPO response for the severity of anemia; (3) impaired responsiveness of erythroid cells to EPO (IFN-γ, IL-1, and TNF-α); (4) inhibited proliferation and differentiation of erythroid cells (IFN-γ, IL-1, TNF-α, and α-1-antitrypsin); and (5) pathologic iron homeostasis due to increased divalent metal transport 1 (IFN-γ) and transferrin receptor expression (IL-10) in macrophages, reduced ferroportin 1 expression (IFN-γ and IL-6-induced high hepcidin levels) in enterocytes and macrophages, and increased ferritin synthesis (TNF-α, IL-1, IL-6, IL-10).

Transferrin-bound iron is the primary iron source for erythropoiesis, entering the erythroblast by a process involving transferrin receptor-mediated endocytosis. This iron may be obtained by absorption of dietary iron and/or mobilization of iron stores at macrophages and liver. Dietary non-hem iron primarily exists in an oxidised (Fe^{3+}) form which is reduced to the Fe^{2+} form by a ferrireductase enzyme, before being transported across the intestinal epithelium by a carrier protein called divalent metal transporter 1. Dietary heme iron enters the enterocyte by heme carrier protein, is metabolised by heme oxygenase to release Fe^{2+}, which enters a common pathway with dietary non-hem iron before being exported by ferroportin 1 across the basolateral membrane of the enterocyte (absorbed iron). Iron export from the stores at macrophages and hepatocytes is also accomplished primarily by ferroportin 1. Iron is then oxidized, released into the circulation, bound to transferrin and transported to sites of use (Figure 2)[19,20].

The amount of iron required for daily renewal of red blood cells (20-30 mg) is provided mostly by senescent erythrocyte iron recycling at macrophages. Therefore, as daily absorption (1-2 mg) just balances daily loss, internal turnover of iron is essential to meet the bone marrow requirements for erythropoiesis.

Hepcidin, a 25-amino acid peptide produced mainly by hepatocytes in response IL-6 levels, plays a major role in dysregulation of iron homeostasis during inflammation. Once synthesised, hepcidin is secreted into the bloodstream and interacts with ferroportin 1 (the only known iron exporting protein) at enterocyte basolateral membrane, hepatocytes and macrophages (Figure 2). The binding of hepcidin to ferroportin 1 causes internalization and lysosomal degradation of the carrier protein. Thus, hepcidin regulates the rate of iron absorption by villous enterocytes and the rate of iron recirculation from macrophages and hepatocytes, resulting in hypoferremia. In addition, inflammatory mediators increased divalent metal

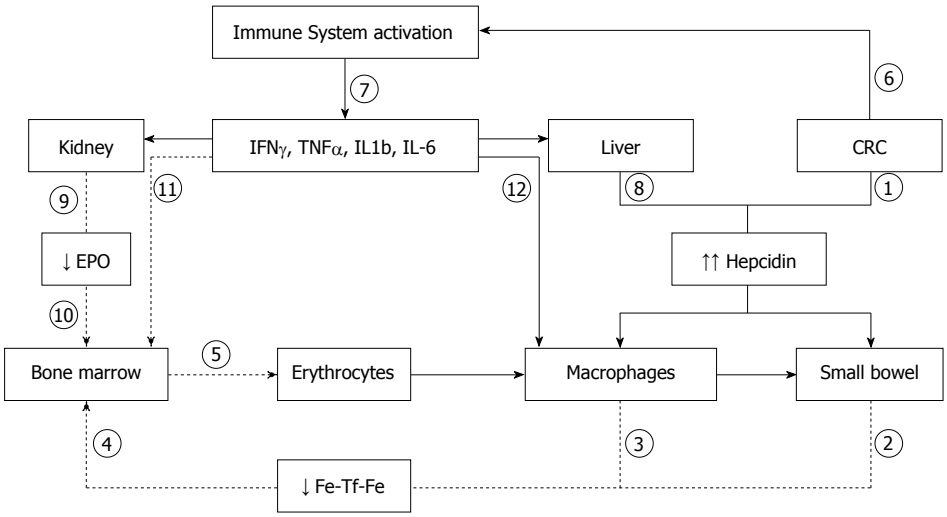

Figure 1 Pathophysiological mechanisms of anemia of inflammation in colorectal cancer. 1: Hepcidin release by colorectal cancer cells (CRC); 2,3: Decreased release of iron via ferroportin: leading to decreased transferrin-bound iron; 4: Decreased iron availability; 5: Reduced erythrocyte production; 6: Activation of immune system by CRC; 7: Release of immune and inflammatory cytokines; 8: Interleukin-6 (IL-6) induced hepcidin release; 9: Decreased erythropoietin (EPO) production; 10: Decreased erythropoietic stimulation; 11: Inhibition of erythroid cell proliferation; 12: Augmented erythrofagocytosis. IFN-γ: Interferon-γ; TNF-α: Tumor necrosis factor-α.

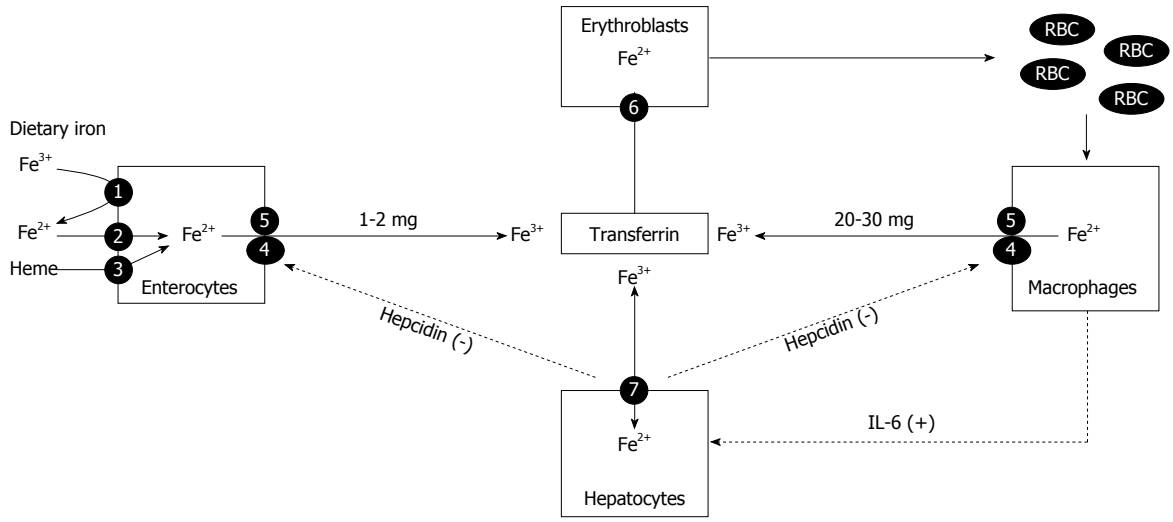

Figure 2 A simplified scheme of main pathways of iron metabolism. 1: Ferrireductase; 2: Divalent metal transporter (DMT1); 3: Heme protein carrier 1; 4: Ferroportin; 5: Hephastin/ceruloplasmin; 6: Transferrin receptor-1 (TfR1); 7: Several mechanisms; IL-6: Interleukin 6; RBC: Red blood cell.

transporter 1 (IFN-γ), transferrin receptor expression (IL-10) and ferritin synthesis (TNF-α, IL-1, IL-6, IL-10) in macrophages leading to increased iron storage[18-20].

Hypoferremia and increased reticuloendothelial iron result in decreased iron availability referred to as iron restricted erythropoiesis or functional iron deficiency (FID); formerly referred to as anemia of chronic disease. This is characterized by low serum iron and decreased transferrin saturation, in the face of adequate body iron stores defined by the presence of stainable iron in the bone marrow and/or a serum ferritin value within or above normal limits. Finally, when persisting decreased iron absorption and/or chronic blood loss are present, FID may evolve to absolute iron deficiency (FID + ID). While hepcidin affects iron trafficking in FID and FID + ID, individuals suffering from FID + ID have significantly lower hepcidin levels than those with FID without ID[21]. Individuals with both, in contrast to FID alone, absorb some dietary iron from the gut and mobilize some iron from macrophages. Thus, hepcidin levels may be useful in differentiating between FID and FID + ID and in selecting appropriate therapy for these patients[21]. This is supported by a recent presentation by Steensma *et al*[22] who noted a 92% response rate to intravenous (*iv*) iron in chemotherapy induced anemia patients with low pretreatment hepcidin levels. Hepcidin levels have been also shown useful in predicting non-responsiveness to oral iron therapy in patients with IDA[23]. Ward *et al*[24], in a cohort of 56 CRC patients, measured hepcidin in urine and determined hepcidin mRNA expression and hepcidin cellular localization

Table 1 Main laboratory tests for the assessment of iron depletion

Laboratory test	Normal values		
	Conventional units	Conversion factor[2]	SI units
Iron status in the body			
Serum iron	50-180 g/dL	× 0.179	9-32 mol/L
Transferrin	200-360 mg/dL	× 0.01	2-3.6 g/L
Transferrin saturation	20%-50%		
Ft	30-300 ng/mL	× 2.247	65-670 pmol/L
sTfR[1]	0.76-1.76 mg/L		6.4-25.7 nmol/L
sTfR/log Ft	< 1		
Iron deficient red cell production			
Hb	12-16 g/dL ♀	× 0.6206[3]	7.5-10 mmol/L
	13-17 g/dL ♂		8-10.5 mmol/L
Mean corpuscular volume	80-100 fL		
Red cell distribution width	11-15		
Mean corpuscular Hb	28-35 pg		
Hypochromic red cells	< 5%		
Reticulocyte Hb content	28-35 pg		

[1]Normal values may differ depending on the assay used; [2]To convert the concentrations values in conventional unit into SI units multiply figures by the conversion factor; [3]In fact, although widely used, this factor allows for the calculation the molar concentration of hemoglobin subunits. Thus, the molar concentration of hemoglobin (Mw 64 kDa) is 4-fold times lower (2-3 mmol/L). Ft: Ferritin; sTfR: Soluble transferrin receptors; Hb: Hemoglobin; sTfR/log Ft: Ratio of sTfR to serum Ft.

in CRC tissue. Hepcidin immunoreactivity was found in 34% specimens from patients with CRC and was correlated with ferroportin inhibition. Urinary hepcidin was positively associated with increasing CRC tumor stage, but not with anemia. This suggests that CRC hepcidin, rather than hepatic hepcidin, is involved in a proportion of cases of CRC-associated anemia more likely to be IDA (or FID + ID) rather than FID and will respond to *iv* iron rather than oral iron[25].

DIAGNOSIS

The prevalence of gastrointestinal (GI) pathology among IDA patients varies from 43% to 86%, the most common being benign erosive lesions in the upper GI tract, accounting for 39%-57% of upper GI with CRC, accounting for 42%-69% of lower GI lesions[26]. IDA is a known harbinger of CRC mandating evaluation. Whenever clinically feasible, at four weeks prior to elective CRC surgery an anemia evaluation including standard iron parameters should be performed. If indicated, appropriate intervention should be implemented, as they decreases perioperative morbidity[27,28]. Laboratory screening for anemia in CRC should include, at least, a CBC, reticulocytes and assessment of iron parameters (Fe, TSAT, ferritin) (Table 1), and C-reactive protein (CRP), which is useful in determining the presence or absence of inflammation[29-33]. The presence of anemia should be considered when the Hb level is < 13 g/dL for men and < 12 g/dL for women. However, a normal Hb level does not exclude ID, as blood loss is nearly always the cause and a significant amount must occur before iron deficient erythropoiesis begins. In non-anemic ID patients, the symptom of chronic fatigue is non-specific and a laboratory finding of a low serum ferritin provides an indirect estimate of body iron stores (1 ng/mL of ferritin = 8 mg of stored iron)[34]. ID is defined as a ferritin level < 30 ng/mL regardless patient's inflammatory status.

In the presence of inflammation, TSAT < 20% and ferritin 30-100 ng/mL suggest absolute ID, whereas FID is generally defined by TSAT < 20% and ferritin ⩾ 100 ng/mL[30,32,33,35] (Figure 3). However, though ferritin values > 100 ng/mL argue against concurrent absolute ID in the setting of inflammation, this test is imperfect due to its acute phase reactivity. Other tests can be utilized to evaluate for a component of ID, which if present suggests a benefit toward iron supplementation. These tests include measurement of the ratio of the sTfR to log ferritin and percent circulating hypochromic erythrocytes (increased in IDA and FID + ID), and the reticulocyte Hb content (low with IDA and FID + ID) (Table 1). Where available, hepcidin will help the diagnostic evaluation in this regard; if suppressed, a component of absolute ID is implied. IDA should be considered if anemia with a TSAT < 20% and/or ferritin 30 ng/mL, with or without inflammation (Figure 3). Low MCV (< 80 fL) is a reliable and routinely part of the automated CBC, but is a late indicator. In addition, IDA without microcytosis may occur early in iron deficiency anemia prior to the development of iron deficient erythropoiesis, when there is coexistinmg vitamin B_{12} or folate deficiency, post-bleeding reticulocytosis, initial response to iron treatment, alcohol intake or mild myelodysplasia.

When anemia in CRC cannot be explained by IDA or FID + ID, it is important to consider other causes that would demand specific treatment. In these cases, further testing should include B_{12}, lactate dehydrogenase, and serum creatinine in order to exclude other nutritional deficiencies, hemolysis or renal disease[29-33]. If malabsorption or severe malnutrition, a red cell folate may also be useful.

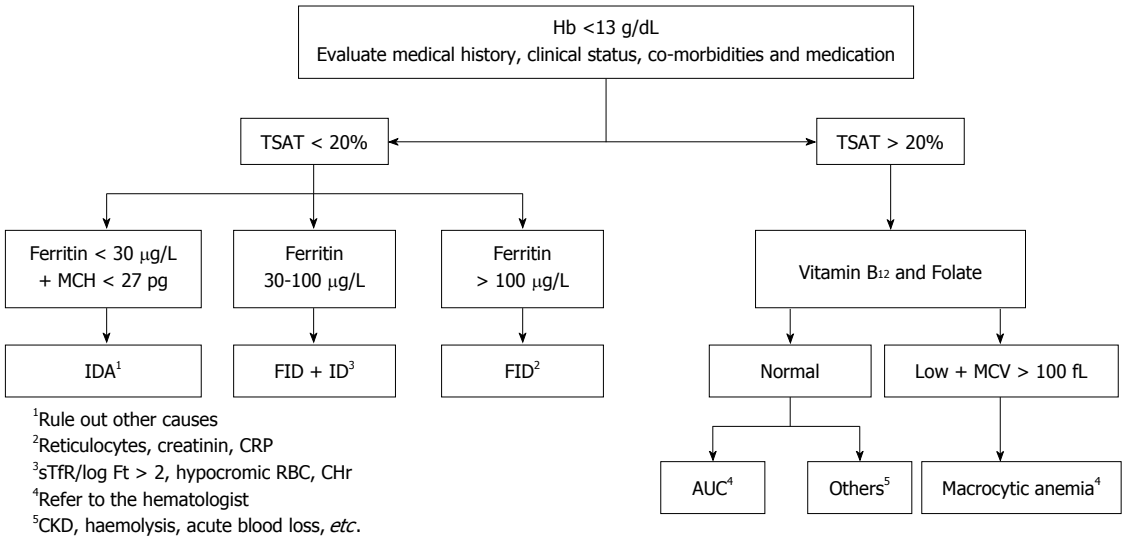

Figure 3 An algorithm for anemia diagnosis. Modified from Muñoz *et al*[20]. ACD: Anemia of chronic disease; AUC: Anemia of unknown cause; CHr: Reticulocyte hemoglobin; CKD: Chronic kidney disease; CRP: C-reactive protein; Ft: Ferritin; Hb: Hemoglobin; ID: iron deficiency; IDA: Iron deficiency anemia; MCH: Mean corpuscular hemoglobin; MCV: Mean corpuscular volume; sTfR: Serum transferrin receptor; TSAT: Transferrin saturation; FID: Functional iron deficiency.

TREATMENT OPTIONS

Allogeneic blood transfusion

After CRC surgery, perioperative blood loss and postoperative blunted erythropoiesis, due to surgery-induced inflammation, may lead to severe postoperative anemia, especially in those presenting with low preoperative Hb. In this context, ABT continues to be the most frequently used treatment for acute intra- and post-operative anemia, although its quick and effective increase in Hb levels is transitory, and is associated with poorer outcomes. Subsequently, ABT should be restricted to those with severe anemia, poor physiological reserve and/or acute symptoms requiring immediate correction.

Perioperative ABT is associated with increased rates of cancer recurrence[36,37]. In a meta-analysis, 23 out of 36 studies on 12127 patients showed a detrimental effect of ABT. After ABT a higher rate of tumor recurrence compared to those not transfused with a clustered OR of 1.42[38] was observed. In a more recent meta-analysis, ABT has been shown to increase all-cause mortality (OR = 1.72), cancer-related mortality (OR = 1.71) and morbidity, such as wound infection (OR = 3.27), after CRC resection[39]. Subsequently, many medical scientific societies recommend a restrictive approach for perioperative ABT, in which the level of Hb below which an ABT unit is transfused (*i.e.*, the "transfusion trigger") should be intimately related to the ability to tolerate normovolemic anemia relative to available cardiopulmonary reserve[40,41]. In non-bleeding, euvolemic anemic patients, ABT is recommended to maintain Hb levels between 7 and 9 g/dL (8-10 g/dL for those with cardiac and/or central nervous system dysfunction)[40,41].

Malignancy and surgery are known prothrombotic stimuli, and perioperative ABT may further increase the risk for venous thromboembolism (VTE). The analysis of two databases of almost 3000 CRC resections demonstrated that intraoperative ABT was a significant risk factor for the development of VTE, increasing with increased number of units transfused[42,43]. In this analysis the risk for VTE in women was statistically greater than in men. Preoperative hematocrit did not enter the multivariable model as an independent predictor of VTE, or did open versus laparoscopic resection or wound class[43]. The diagnosis of almost one third of postoperative VTE occurred after discharge[44]. Subsequently, the Enhanced Recovery after Surgery (ERAS) program recommends extended postoperative prophylaxis with low-molecular weight heparin for 28 d in CRC patients[45].

In summary, available data strongly recommend minimizing ABT in CRC surgery, using restrictive transfusion policies and implementing ABT alternatives, with emphasis on thromboprophylaxis after discharge.

Pharmacologic treatment

Objectives: The goal of preoperative anemia therapy should be normalization of the Hb levels, in accordance with World Health Organization criteria. However, as CRC resections are procedures with a moderate-to-high blood loss, it would be desirable to achieve a Hb of 13 g/dL for both genders to minimize the risk for transfusion. Similarly, postoperative anemia treatment should be aimed to attain Hb levels which avoid or reduce the exposure to ABT, followed by its correction in the shortest possible period, to favourably influence sensitivity to adjuvant treatments, facilitate the functional recovery and improve quality of life.

Iron replacement: In CRC, ID with or without anemia should be corrected pre-operatively by *iv* iron, with or without an erythropoiesis stimulating agent (ESA), preferably two to four weeks prior the scheduled procedure. While at least four studies explored the efficacy of preoperative oral iron[46-49] (Table 2), the results are routinely

Table 2 Characteristics of the clinical studies examining the role of preoperative iron replacement in colorectal cancer included in this review

Study	Study design	Patients	Baseline Hb (g/dL)	Iron compound dose (mg)	Duration (wk)	Hb (g/dL)	ABT (% or U/pt)
Oral iron							
Okuyama *et al*[46]	OBS	Iron: 32	8.1 ± 1.4	Ferrous citrate	⩾ 2	2.0	9.4%
		No iron: 84	8.0 ± 1.6	(200 mg/d)		0.9	27.4%
Lidder *et al*[47]	RCT	Iron: 23	13.4 ± 1.9	FS	2-8	-0.3	26.0%
		No iron: 22	12.4 ± 2.1	(200 mg TDS)		-0.6	59.0%
Quinn *et al*[48]	OBS	Iron: 103	12 (10-14)	FS	1-9		0.69 U/pt
		No iron: 167	NS	(200 mg TDS)			1.69 U/pt
Ferrari *et al*[49]	RCT	FB: 12	11.6 ± 1.6	FB	8	1 (2) mo	NS
				(28-14 mg/d)		0.8 (1.4)	
		FS: 12	11.3 ± 1.2	FS		0.7 (1.4)	
				(105 mg/d)			
Intravenous iron							
Edwards *et al*[57]	RCT	Iron: 34	13.7 ± 0.5	IS	2	-0.2	14.7%
		Placebo: 26	13.4 ± 0.4	(2 × 300 mg)		-0.5	19.2%
Bisbe *et al*[64]	OBS	IS: 30	10.1 ± 1.2	IS	2-6	0.9	7.0%
				(100-200 mg, 6 ± 3 doses)			
		FCM: 15	9.2 ± 1.0	FCM		2.5	40.0%
				(500-1000 mg, 3 ± 1 doses)			
Todman *et al*[68]	Case series	Iron: 22	< 12	Iron isomaltoside-1000	2-6	0.7, 1-2 w	NS
				(20 mg/kg bw)		1.4, 3-4 w	
						3.1, 6-8 w	

RCT: Randomized controlled trial; OBS: Observational cohort study; Hb: Increment from baseline; ABT: Allogeneic blood transfusion; FB: Ferrous bisglicinate; FS: Ferrous sulphate; IS: Iron sucrose; FCM: Ferric carboxymaltose.

inferior to those with the *iv* iron route[50].

Okuyama *et al*[46] compared preoperative Hb levels and transfusion requirements of anemic patients (Hb ⩽ 10 g/dL), 32 who received oral iron supplementation (sodium ferrous citrate, 200 mg/d) for at least 2 wk preoperatively with those of 84 who did not. While iron supplementation resulted in higher Hb levels immediately before surgery (+1.2 g/dL; $P < 0.05$), and fewer required intraoperative ABT (9.4% *vs* 27.4%, $P < 0.05$), there were no significant differences in postoperative Hb levels or ABT volumes between the two groups. Lidder *et al*[47] conducted a randomized controlled trial (RCT) of oral ferrous sulphate (200 mg TDS) for a mean of 14 d pre-operatively (12-56 d) *vs* no iron therapy in patients with IDA or ID scheduled for CRC surgery. Oral iron was found to prevent Hb decrease from recruitment to admission, and to reduce ABT (25% *vs* 59%, for iron and control, respectively; $P = 0.031$), although these differences were not statistically significant for patients with IDA.

In a series of 103 patients receiving oral ferrous sulphate (200 mg TDS) for a median of 39 d pre-operatively (interquartile range = 7-63 d) and no preoperative ABT, Quinn *et al*[48] observed that: (1) fifty-eight (56.3%) patients who were anemic at presentation had a mean increment in Hb of 1.7 g/dL ($P < 0.001$); (2) those with right-sided tumors (lower mean Hb at presentation) responded more often to oral iron than those with left-sided tumors ($P < 0.017$); (3) increase in Hb was unrelated to tumor stage, but was greater when iron was administered for more than 14 days; and (4) ABT rate for all curative resections was 0.69 units/patient (compared to 1.69 units/patient using an historical cohort).

Several *iv* iron formulations are currently available (Table 3). *iv* iron therapy, with or without ESAs, as a safe and efficacious tool for treating anemia and reducing transfusion requirements in surgical and medical patients, has been extensively reviewed[51-54]. Randomized clinical trials have shown superior efficacy of *iv* iron over oral or no iron in reducing ABT, increasing Hb, and improving quality of life in ESA-treated anemic cancer patients[53,55]. In contrast, studies examining *iv* iron as sole anemia treatment in cancer patients are only just starting to emerge, and the role of *iv* iron for correcting perioperative anemia is frequently overlooked in the surgical care of cancer patients[56-60].

Two case series illustrates the potential benefits of pre- or peri-operative iron supplementation in CRC resections. Campos *et al*[61] studied 43 CRC patients who received preoperative oral iron (100 mg/d) if Hb > 14 g/dL and iron deficiency was present; iron sucrose (200 mg/wk) if Hb 10-14 g/dL; or iron sucrose (200 mg twice a week) if Hb < 10 g/dL, during weeks 3-4. Seventeen received postoperative iron sucrose (200 mg on days 0, 2, and 4). A retrospective series not receiving iron was used as a control ($n = 66$). Despite a lower baseline Hb (12.3 g/dL *vs* 11.5 g/dL; $P < 0.05$), iron therapy reduced the transfusion index (4.0 unit/patient *vs* 1.3 unit/patient; $P < 0.05$) and the percentage of patients who received preoperative ABT (33% *vs* 9%; $P < 0.05$), but not the percentage of patients administered perioperative ABT (48% *vs* 35%; $P = 0.161$). However, the treatment was ineffective in patients with a high transfusion index (> 5 units/patient).

Díaz Espallardo *et al*[62] analyzed data from 437 CRC surgeries from 2005-2009. Patients presenting with Hb

Table 3 Some characteristics of the different intravenous iron formulations

	Iron gluconate	Iron sucrose	High molecular weight iron dextran	Low molecular weight iron dextran	Ferric carboxymaltose	Iron isomaltoside 1000	Ferumoxytol
Brand name	Ferrlecit®	Venofer®	Dexferrum®	Cosmofer® INFeD®	Ferinject® Injectafer®	Monofer®	Rienso® FeraHeme®
Carbohydrate shell	Gluconate (monosaccharide)	Sucrose (disaccharide)	Dextran (branched polysaccharide)	Dextran (branched polysaccharide)	Carboxymaltose (branched polysaccharide)	Isomaltoside (linear oligosaccharide)	Polyglucose sorbitol carboxymethylether
Molecular weight (kDa)	289-440	30-60	265	165	150	150	750
Plasma half-life (h)	1	6	60	20	16	20	15
Direct iron donation to transferrin (% injected dose)	5-6	4-5	1-2	1-2	1-2	< 1	< 1
Test dose required[1]	No	Yes/No	Yes	Yes	No	No	No
Iron content (mg/mL)	12.5	20	50	50	50	100	30
Maximal single dose (mg)	125	200-300	20 mg/kg	20 mg/kg[2]	20 mg/kg (max 1000 mg in one infusion)	20 mg/kg	510[3]
Premedication	No	No	TDI only	No	No	No	No
Life-threatening ADE ($\times 10^6$ doses)	0.9	0.6	11.3	3.3	??	??	??

[1]A test dose is no longer recommended by the European Medicines Agency (2013); [2]Low molecular weight iron dextran can be safely administered at doses of 1000 mg over 1 h[101]; [3]Preliminary data indicate that Ferumoxytol may be administered at doses of 1020 mg over 15 min[102].

<13 g/dL and/or abnormal iron parameters, (group A, *n* = 242) received preoperative iron supplementation (178 received a mean of 867 mg *iv* iron sucrose, and 64 oral iron), whereas those presenting with Hb ≥ 13 g/dL and normal iron status, received no treatment (group B, *n* = 195). From diagnosis to surgery, Hb increased by 0.6 g/dL in group A, while it decreased by 0.8 g/dL in Group B ($P < 0.05$). From diagnosis to discharge, Hb decreased by 0.4 g/dL in group A, and by 2.5 g/dL in group B ($P < 0.05$). This tendency to progressive anemia observed in both groups may be secondary to the effects of CRC on erythropoiesis, chemo-radiotherapy treatment, and blood loss due to the tumor and later surgery. However, the differences between groups strongly suggest that iron therapy prevented patients from group A from reaching low Hb levels. The overall ABT rate was 8.6% (32/244, 13.1% *vs* 6/195, 3.1%; P = NS) and no differences in complications were observed.

In contrast, in a retrospective paired case-control study, Titos-Arcos *et al*[63] observed that postoperative administration of *iv* iron sucrose (592 ± 445 mg) did not decrease ABT rates (28.8% *vs* 30.8%, for case and control, respectively). In addition, for patients not receiving ABT, there were no differences in Hb concentration decrease between the first postoperative day and discharge (0.88 g/dL *vs* 0.82 g/dL, for case and control).

Higher *vs* lower dose intravenous iron administration: Bisbe *et al*[64] compared clinical and laboratory data of 15 anemic CRC receiving preoperative ferric carboxymaltose (FCM, 500-1000 mg/session; 3 ± 1 sessions) to those from a previous series of 30 CRC receiving preoperative iron sucrose (100-200 mg/session; 6 ± 1 sessions). Even though those in the FCM group had lower baseline Hb levels (9.2 g/dL *vs* 10.1 g/dL; $P < 0.05$), those from the FCM group showed a higher post-treatment Hb increment (+2.5 g/dL *vs* +0.9 g/dL; $P < 0.05$), and received fewer perioperative ABT (7% *vs* 40%; $P > 0.05$). While the total amount of *iv* iron infused in the FCM group was higher (1550 mg *vs* 1140 mg; $P < 0.05$) most clinical trials suggest 1000 mg is an adequate replacement dose[65-67]. Todman *et al*[68] administered a single dose iron infusion (Iron isomaltoside-1000, 20 mg/kg body weight) to 22 major cancer surgery patients with IDA either 2-6 wk before surgery, or post-operatively. Hemoglobin levels were monitored for up to 8 wk after infusion, or up to next blood transfusion, whichever was earlier. Mean Hb rise at 1-2 wk was 0.7 g/dL, 1.4 g/dL at 3-4 wk and 3.1g/dL at 6-8 wk, and no serious adverse effects were noted (Table 2).

This apparent superiority of "higher dose" over "lower dose" *iv* iron supplementation in improving erythropoiesis has been also reported for inflammatory bowel disease[69], and several factors might have account for the observed differences. Firstly, the "extra" amount of iron administered to the FCM group could have compensated for the ongoing blood loss from recruitment to surgery. Secondly, macrophage iron loading may also inhibit pro-inflammatory immune effector pathways[18,70]. Lastly, in iron balance, high hepcidin also reduces IL-6 production by macrophages, thus limiting the potential damage of an excessive inflammatory response[71]. As ID is highly prevalent among CRC, it is possible that rapid repletion with higher dose of *iv* iron can contribute in restoring an adequate immune response, improving the erythropoietic response.

As summarized in Table 2, initial results with preoperative oral or *iv* iron replacement therapy in CRC have been mixed, highlighting the need for large randomized controlled trials in preoperatively anemic patients.

Safety of intravenous iron: Although no serious ad-

verse drug events (SAEs) have been reported in the studies reviewed, both the numbers and follow-up time were not large enough to draw definitive conclusions regarding the safety of *iv* iron agents in this clinical setting. In all published evidence extant, including millions of dialysis patients, no long term toxicity has been reported over the last two decades[72].

As of June 28th 2013, the European Medicines Agency's Committee for Medicinal Products for Human Use (CHMP) concluded that the benefits of *iv* exceed their risks, provided that adequate measures are taken to minimize the risk of allergic reactions. Data on the risk of hypersensitivity comes mainly from post-marketing spontaneous reports and the total number of life-threatening and fatal events reported is low, and cannot be used to detect any differences in the safety profile of the different iron formulations available in Europe (high molecular weight iron dextran and ferumoxytol were not included). The CHMP has issued recommendations for health care professionals which include: (1) intravenous iron medicines should only be administered when staff trained to evaluate and manage anaphylactic and anaphylactoid reactions are immediately available as well as resuscitation facilities; (2) a test dose is no longer recommended, as there are data indicating that allergic reactions may still occur in patients who have not reacted to a test dose; (3) patients should be closely observed for signs and symptoms of hypersensitivity reactions during and for at least 30 min following each injection of an *iv* iron medicine; and (4) intravenous iron-containing products are contraindicated in patients with hypersensitivity to a specific active substance or excipients, or other parenteral iron products.

Early formulations of high molecular weight iron dextran were associated with rare occurrences of anaphylaxis and even death. The newer formulations, LMW ID, ferric gluconate, iron sucrose, ferumoxytol, iron isomaltoside and ferric carboxymaltose are much safer with SAEs vanishingly rare. None the less minor infusion reactions still occur and are often misinterpreted as SAEs[53]. Premedication with antihistamines has been reported to cause the majority of perceived reactions to *iv* iron in one large cohort[73]. Antihistamines can cause somnolence, diaphoresis, hypotension and tachycardia ostensibly attributed to the administered iron. Tryptase a marker of mast cell degranulation, levels are virtually always normal and subsequently the use of premedication with antihistamines should be proscribed. In contradistinction, all of the formulations can be associated with acute chest and back tightness, without accompanying hypotension, tachypnea, tachycardia, wheezing, stridor or periorbital edema[51,74]. These infrequent reactions abate without therapy and rarely recur with rechallenge. The reactions are more frequent in those with allergic diatheses[65]. It is important not to overreact in the event of these minor AEs. A few patients will experience self-limited arthralgias and myalgias the day after iron infusions. These reactions abate without therapy and never leave residua. Non-steroidal anti-inflammatory drugs may shorten their duration. When these tenets are adhered to the administration of *iv* iron is safe and much safer than most physicians realize.

Erythropoiesis stimulating agents: The role of *iv* iron for CRC-related anemia remains unclear. It is possible that the effectiveness of perioperative iron treatment could be enhanced by concomitant ESA administration, although in Europe this is an off-label use of these growth factors. Pooled data from six trials (621 patients) showed that perioperative treatment with recombinant human erythropoietin did not reduced ABT (33% *vs* 37%; OR = 0.89; $P = 0.206$) in GI cancer surgery[75-80] (Table 4). Norager *et al*[80] explored the effect of perioperative ESA administration in CRC surgery (Table 4). No significant benefits were found for postoperative fatigue, quality of life, muscle strength or ABT use, but improved work capacity and early restoration of postoperative Hb concentrations to preoperative levels were observed. The treatment was uniformly well tolerated. Unfortunately, the impact of ESA therapy on anemic CRC patients was not analyzed separately. However, a reduction of both the percentage of transfused patients and the number of transfused units was only observed for those receiving ESAs plus *iv* iron. Additionally, the use of *iv* iron allowed for a significant reduction in the total dose of ESAs (Table 4).

Safety of erythropoiesis stimulating agents: ESA prevent transfusions among chemotherapy-associated anemia patients. An ESA-associated increase in mortality and/or disease progression has also been reported in eight controlled studies conducted in CIA however in each of these the ESA use was off-label. Another meta-analysis of 60 studies (15323 patients) showed no significant effect of ESAs on survival or disease progression, but increased the risk for venous-thromboembolic events (44 studies: OR = 1.48; 95%CI: 1.28-1.72)[81]. However, venous-thromboembolic events in cancer patients receiving ESAs for chemotherapy induced anemia may be linked to thrombocytosis due to ESA induced iron restricted erythropoiesis, which can be reversed by administration of *iv* iron[82,83]. Nevertheless, product labels advise against administering ESAs with potentially curative chemotherapy (United States) or to conduct risk-benefit assessments (Europe/Canada) and, since 2007, fewer chemotherapy-associated anemia patients in the United States and Europe receive ESAs[84-87].

In CRC surgery, a recent systematic review and meta-analysis of 4 RCTs also found insufficient evidence to support the use of ESAs in the preoperative and postoperative period for improving anemia and decreasing ABT. There were no significant differences in postoperative mortality or thrombotic events between groups, but no included study evaluated recurrences, survival, or quality of life[88].

Vitamin replacement: Deficiencies of vitamin B_{12}, with or without anemia, should be appropriately managed. The intramuscular route is preferred (hydroxylcobalamin, 1 mg/wk, 4-6 wk), except for vegans (oral route) or anticoagulated patients (*iv* route).

Table 4 Effects of perioperative administration of erythropoietin and iron on transfusion requirements in patients undergoing elective colorectal cancer resection

Study	+ ESA		Placebo		Iron	ESA
	n	ABT, *n* (%)	*n*	ABT, *n* (%)	(route, type, dose, d)	(type, total dose, route, day)
Braga *et al*[75]	10	1 (10)[1]	10	5 (50)	*iv* iron gluconate, 125 mg/d, 4 d	Epoetin alfa, 500 IU/kg, SC (from day -12 to day +8)
Kettelhack *et al*[76]	48	16 (33)	54	15 (28)	Oral, NS, 5-10 d preOP *iv*, NS, 40 mg, 1 d postOP	Epoetin beta, 3000-4500 IU/kg, SC (from day -10 to day +4)
Qvist *et al*[77]	38	13 (34)[2]	43	23 (53)	Oral, NS, 200 mg/d, 4 d	Epoetin alfa, 1350 IU/kg, SC (from day -4 to day +7)
Kosmadakis *et al*[78]	31	9 (29)[1]	32	19 (59)	*iv* iron sucrose, 100 mg/d, 14 d	Epoetin alfa, 4200 IU/kg, SC (from day -7 to day +7)
Christodoulakis *et al*[79]	69 (a) 67 (b)	34 (49) 27 (40)[2]	68	35 (51)	Oral, NS, 200 mg/d, 10 d	Epoetin alfa, 1800 IU/kg, SC (a) 3600 IU/kg, SC (b) (from day -10 to day +1)
Norager *et al*[80]	75	10 (13)	76	9 (12)	Oral, NS, 200 mg/d, 7d	Darbepoetin alfa, 750-1500 g, SC (from day -10 to day +25)
Overall	338	110 (33)	283	106 (37)	OR = 0.89 (95%CI: 0.58-1.12; *P* = 0.206)	

[1]Reduction in both percentage of transfused patients and number of transfused units; [2]Reduction in the number of transfused units only. ABT: Allogeneic blood transfusion; ESA: Erythropoiesis stimulating agent; NS: Not stated; preOP: Preoperative; postOP: Postoperative; SC: Subcutaneous.

Adjuvant measures

Nutritional support: Poor pre-operative nutritional status has been linked consistently to an increase in post-operative complications and poorer surgical outcome. Patients should be screened for nutritional status and, if deemed to be at risk of under-nutrition, given active nutritional support[45].

Meta-analyses were undertaken on trials evaluating different preoperative nutritional interventions. Benefits on post-operative complications and length of hospital stay of preoperative immune enhancing nutrition or parenteral nutrition may not be generalized or are not applicable to current clinical practice, whereas trials evaluating enteral or standard oral supplements were inconclusive[89]. Therefore, except for the severely malnourished, whether or not nutritional intervention should be initiated earlier in the preoperative period remains unclear.

In contrast, post-operative management in gastrointestinal surgery is becoming well established with ERAS protocols starting 24 h prior to surgery with carbohydrate loading, minimization of preoperative fasting and early oral or enteral feeding given to patients the first day following surgery (with oral nutritional supplements if necessary). ERAS is aimed to reduce surgical stress, insulin resistance, unnecessary protein losses and postoperative complications. In comparison with traditional care, ERAS programs were associated with significantly decreased length of hospital stay and total and general complications, without affecting readmission rates, surgical complications, and mortality[90].

Preventing perioperative hypothermia: Perioperative maintenance of normothermia with a suitable warming device and warmed *iv* fluids to keep body temperature > 36 ℃ decreased intraoperative blood loss and postoperative shivering, and it has been associated with lower rates of postoperative infection and better pain scores[45,91-94].

Restrictive fluid replacement (fluid balance): Hypovolemia can lead to hypoperfusion of vital organs and the bowel, which can lead to complications, and appropriated fluid reposition with balanced crystalloid solutions should be performed. However, administering too much may result in bowel edema, increased interstitial lung water, and dilution anemia which can also lead to complications[95]. The evidence suggests that patients being in a state of "fluid balance" (goal-directed fluid replacement) fared better than those with "fluid imbalance"[96-99]. Postoperative *iv* fluids should be aimed to maintain normovolemia and avoid fluid excess. The enteral route should be used in preference and the drip taken down at the earliest opportunity (preferably no later than the morning after surgery)[45].

Perioperative supplemental oxygen: Although the role of perioperative supplemental oxygen in anemia tolerance has not been properly investigated, it has been proposed to decrease the incidence of surgical site infection in CRC surgery. This positive effect was not confirmed by a recent meta-analysis of 5 RCTs[100]. However, supplemental oxygen appears to confer a mortality benefit, a previously unreported finding that needs to be confirmed.

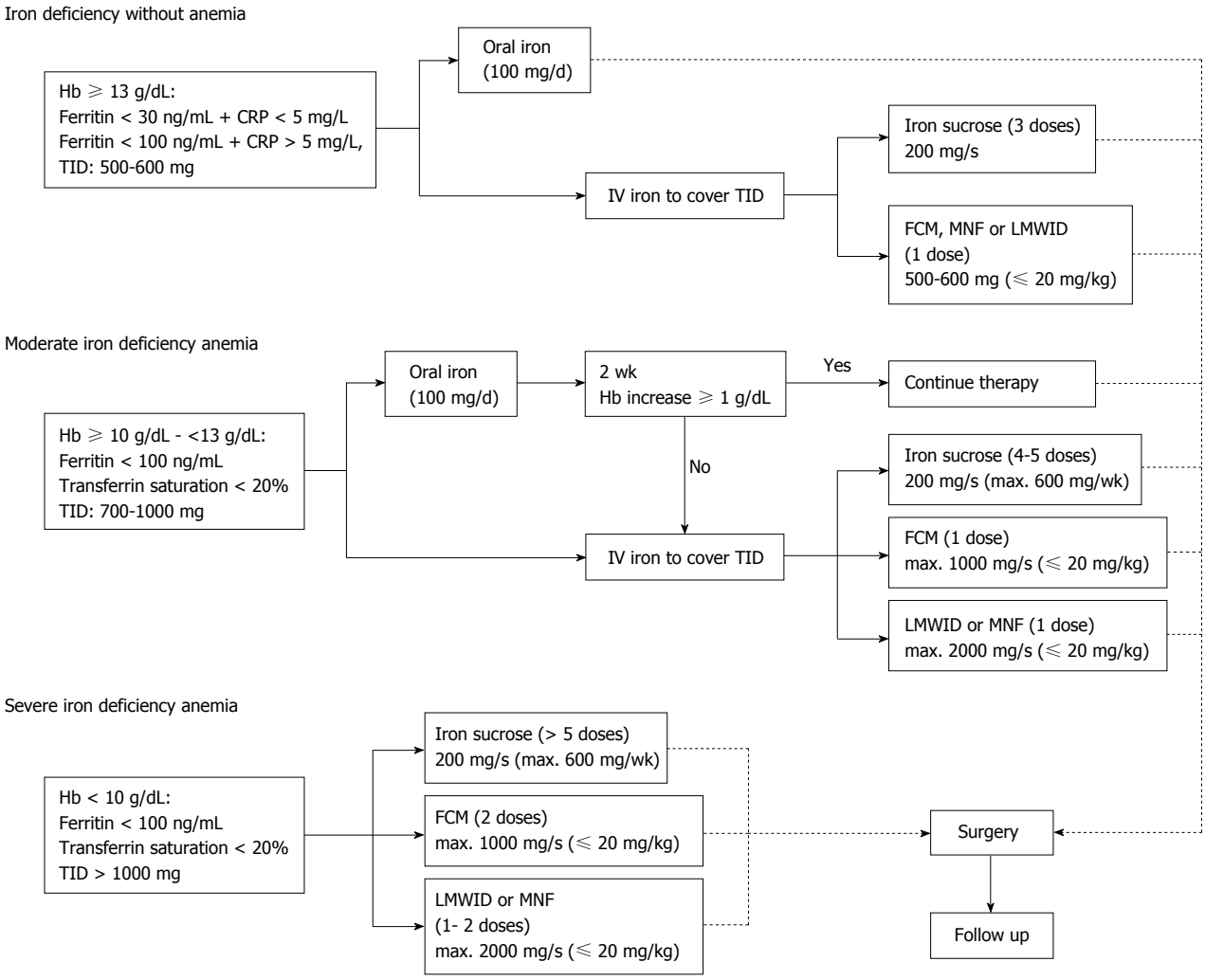

Figure 4 An algorithm for iron replacement. Modified from Muñoz *et al*[31]. FCM: Ferric caboxymaltose; Hb: Hemoglobin; LMWID: Low molecular weight iron dextran; MNF: Iron isomaltoside-1000; s: Session; TID: Total iron deficiency; CRP: C-reactive protein.

FROM LITERATURE TO BED-SIDE: A PRAGMATIC APPROACH TO CRC ASSOCIATED ANEMIA

"Time is gold for anemic patients waiting for CRC resection"

Early and aggressive treatment of anemia in CRC enables optimization of preoperative Hb, thus transforming a high transfusion risk to a low transfusion risk, which improves outcomes. Therefore, we developed a pragmatic, easy-to-follow protocol for diagnosis and treatment of preoperative CRC associated anemia, which is based upon the following considerations.

Diagnosis

Basic laboratory screening for anemia in CRC should comprise Hb, full blood counts (including reticulocytes), and assessments of body iron store (serum ferritin), iron availability (TSAT) and level of inflammation (CRP). Should anemia not be explained by initial work-up, further testing could comprise vitamin B_{12} and folic acid, haptoglobin, lactate dehydrogenase, and serum creatinine if other laboratory tests indicate their usefulness (Figure 3). These are low-cost, widely available tests which allow for correctly classifying most cases of CRC-associated anemia.

Treatment

Iron therapy: As IDA and FID + ID are the most frequent types of anemia in CRC, iron supplementation is of paramount importance and can be accomplished by following the algorithm depicted in Figure 4. The estimated total iron deficiency take into account the amount of iron needed to restore a Hb level of 13 g/dL and to replenish iron stores, as well as estimated iron loss due to ongoing chronic bleeding and perioperative blood loss.

Erythropoiesis stimulating agents: Until more safety data in CRC are available, ESAs should be only used in the approved indications and following the recommendations of international guidelines.

Restrictive transfusion protocol: In most surgical CRC

patients, ABT could be considered for maintaining Hb concentrations between 7 and 9 g/dL; for those with cardiac and/or central nervous system dysfunction, ABT could be considered for patients with symptoms or a Hb level of 8 g/dL or less, and ABT given for maintaining Hb concentrations between 8 and 10 g/dL[40,41] Carson 2011. However, whenever possible, avoidance of ABT is preferable.

Adjuvant therapies: All of above mentioned measures aimed to decrease blood loss, hemodilution and post-operative hyper-catabolism should also be implanted, as they may contribute to reduce the severity of and to hasten the recovery from postoperative anemia.

Follow-up

Patients should be followed-up for documenting the recovery from postoperative anemia, especially if adjuvant chemotherapy and/or radiotherapy were administered.

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TOPIC HIGHLIGHT

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

Clock genes: Their role in colorectal cancer

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Abstract

Clock genes create a complicated molecular time-keeping system consisting of multiple positive and negative feedback loops at transcriptional and translational levels. This circadian system coordinates and regulates multiple cellular procedures implicated in cancer development such as metabolism, cell cycle and DNA damage response. Recent data support that molecules such as CLOCK1, BMAL1 and PER and CRY proteins have various effects on c-Myc/p21 and Wnt/β-catenin pathways and influence multiple steps of DNA damage response playing a critical role in the preservation of genomic integrity in normal and cancer cells. Notably, all these events have already been related to the development and progression of colorectal cancer (CRC). Recent data highlight critical correlations between clock genes' expression and pathogenesis, progression, aggressiveness and prognosis of CRC. Increased expression of positive regulators of this circadian system such as BMAL1 has been related to decrease overall survival while decreased expression of negative regulators such as PER2 and PER3 is connected with poorer differentiation, increased aggressiveness and worse prognosis. The implications of these molecules in DNA repair systems explain their involvement in the development of CRC but at the same time provide us with novel targets for modern therapeutic approaches for patients with advanced CRC.

Key words: Clock genes; Colorectal cancer; Development; Prognosis

Core tip: Clock genes are involved in numerous cellular activities such as cell cycle and DNA repair with various implications in the development of colorectal cancer. Multiple clinical and epidemiological data support these correlations and suggest that altered expression of these genes may be critical for the initiation and progression of this disease while their levels may predict bad response to traditional therapeutic approaches and poor clinical outcome. Finally, the defective circadian system may represent an attractive and currently unknown pathway which can be targeted by novel agents in aggressive colorectal cancers.

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INTRODUCTION

Colorectal cancer (CRC) is the third leading cause of cancer death in the United States and one of the most common types of cancer in the western societies[1]. Surgical intervention remains the mainstay of therapy for patients

without metastatic disease while adjuvant or neo-adjuvant chemotherapy and radiation are considered to improve the survival of patients with stage 2 and 3 disease[2]. Despite the improvements of secondary prevention and surgical intervention, the prognosis for patients with metastatic disease remains poor. The role of adjuvant therapy for patients with stage 2 is still not clear suggesting that better stratification of these patients may be critical to improve the survival rates[2]. Finally, therapeutic approaches for patients with stage 4 are mainly palliative[3] which clearly implies that better understanding of the molecular biology of this disease may reveal new targets for the development of novel agents for CRC patients with poor response to the conventional chemotherapy.

Epidemiologic and genetic studies have shown that there is a clear link between the disruption of circadian rhythms and cancer development and progression in humans including breast, endometrial, prostate and colon cancer[4,5]. Interestingly, alterations of the circadian rhythm have been related to modulations of tumor growth in animal models[6], differences in recurrence rates, stage and prognosis in human cancers[7,8]. The master circadian clock generating and sustaining 24 h periodicity is located in the suprachiasmatic nucleus (SCN) in the anterior hypothalamus orchestrating peripheral clocks located in other organs and tissues[9]. The existence of a circadian clock in the cellular level generating and regulating multiple activities related to metabolism, cell cycle, DNA synthesis and repair has been recently identified. In particular, the molecular mechanisms underlying circadian clock involve transcriptional and translational positive and negative feedback loops[10].

Recent molecular and genetic data strongly suggest that among the most important targets downstream of circadian clock are molecules related to DNA damage response (DDR) such as ATM, CHK2 [11] and BRCA1[12], cell cycle progression such as c-Myc and p21[13,14] and Wnt/β-catenin pathway[15]. Given that all these pathways are involved in the molecular biology of CRC it is not surprising that numerous epidemiological, genetic and molecular studies highlight the implication of clock genes not only in the initiation and progression of CRC, but in the development of resistance to chemotherapeutic agents as well. In particular, Soták *et al*[16] using a model of chemically induced CRC, recently found that the circadian rhythmicity of critical mediators of the circadian system, namely PER1, PER2, REV-ERBA is significantly decreased in CRC tissues while the rhythmicity of BMAL1, another circadian rhythm component is completely abolished not only in the CRC tissues but in the surrounding healthy colon tissue as well in tumor bearing animals. These results clearly support that deregulation of the Circadian system is strongly implicated in the development of CRC.

The aim of this review is to summarize the involvement of clock genes in the molecular pathways related to the development and progression of CRC and the implication of clock genes' genetic alterations in the aggressiveness, therapeutic response and prognosis of the disease.

MOLECULAR DETERMINANTS OF THE CIRCADIAN RHYTHM

Recent data from expression pattern analysis and generation of transgenic mice with hyperactive clock genes such as *PER1*, *PER2* and *BMAL1* have shown that the activity of the SCN is not essential for the peripheral oscillation but is critical for the synchronization of these "peripheral clocks"[17,18]. These results suggest that each individual cell exhibits an independent regulation of its own circadian system.

As mentioned above the circadian system and its downstream effects on various cellular activities are regulated by positive and negative feedback loops which are tightly connected. BMAL1, CLOCK1 and NPAS2 form heterodimers, which bind to the promoters of *PER, CRY, REV-ERBa, RORa, DEC1* and *DEC2* genes activating their transcription[19] (Figure 1). PER and CRY proteins are negatively regulate the BMAL1/CLOCK1 and BMAL1/NPAS2 heterodimers activity suppressing their own expression and inhibiting the circadian system[9] (Figure 1). Moreover, the DEC1 and DEC2 proteins compete with the above mentioned heterodimers for a common DNA binding site and supress the expression of clock genes[20]. It should be noted that PER proteins can also form heterodimers with the TIM protein maintaining their own integrity[21]. Finally, being involved in an additional pathway of the circadian system, REV-ERBα and RORα compete each other for binding to RORE elements inhibiting or activating the expression of the *BMAL1* gene respectively[22].

Interestingly, various posttranscriptional and posttranslational modulations of the above mentioned proteins further confer to the complexity of this system. In particular, protein kinases CK1ε and CK1δ phosphorylate multiple molecules implicated in this signaling altering their nuclear translocation and subsequently their transcriptional activities[23] (Figure 1). Moreover, epigenetic modifications through acetylation, deacetylation and methylation of histones in the promoter of various clock genes are critical for the activation of this cellular clock. In particular, it is known that the BMAL1/CLOCK1 heterodimers promote the acetylation activating the expression of their downstream targets[24] while PER/CRY heterodimers induce deacetylation and methylation of histones downregulating the expression of the clock genes.

CIRCADIAN SYSTEM REGULATES CELL CYCLE THROUGH c-MYC, P21 AND WEE1

Recent studies support that the circadian system in the regulates the cell cycle progression through c-Myc/p21 signaling which has been implicated in the development

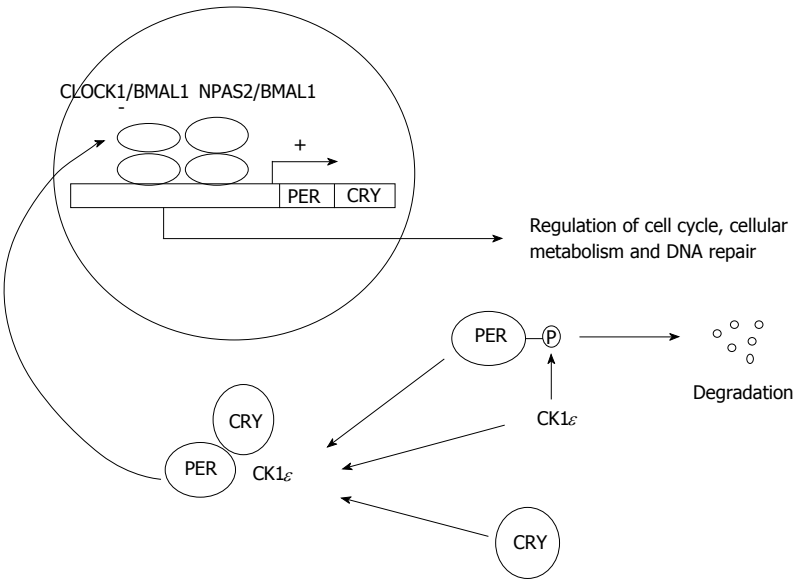

Figure 1 The molecular mechanism of the cellular circadian system. CLOCK1/BMAL1 and NPAS2/BMAL1 complexes promote the expression of PER and CRY. PER is phosphorylated by CK1ε kinase resulting to its degradation while the accumulation of CRY leads to the formation of PER/CRY/CK1ε complex which inserts to the nucleus and down-regulates the CLOCK1 and BMAL1 activity. Among the transcriptional targets of CLOCK1 and BMAL1 are some cell cycle mediators, tumor suppressor genes and oncogenes regulating cell cycle, cellular metabolism and DNA repair.

of CRC since c-Myc is found to be overexpressed in 70% of colon cancers[25]. In particular, microarray data revealed that critical molecules involved in the cell cycle machinery such as p21, cyclin D1, cyclin B1, c-Myc, p53, Wee1 and Mdm2 are regulated by the circadian system[9]. *BMAL1* deletion results in an imbalance in the expression of *REV-ERBa* and *RORa* which are positive and negative regulators of p21 leading to inhibition and promotion of the cell cycle respectively[14]. It has also been shown that *Bmal1*$^{-/-}$ transgenic mice exhibit dramatically increased expression of p21 which is no longer rhythmic in their liver cells suggesting that the peripheral oscillators control critical biological processes such as cell cycle progression[14]. On the other hand, Zeng *et al*[13] showed that downregulation of *BMAL1* in colon cancer cells leads to increased cyclin B1, CDC2, cyclin D1 and E expression accelerating tumor growth. Finally, Alhopuro *et al*[12] using ChIP technology showed that one of the targets of *CLOCK1* gene is p21, while its regulation is believed to be p53 independent.

c-Myc is a critical regulator of the cell cycle through downregulation of p21 and activation of cyclin D1. Its promoter contains multiple E-box sequences, which are controlled by molecular components of the circadian system including PER1 and PER2[26]. Of note, *Per1*$^{-/-}$ and *Per2Brdm1* mutant mice exhibit increased expression of c-Myc leading to elevated cyclin D1 and induced cell cycle[27,28]. Moreover, overexpression of *PER2* in K562 leukemic cells led to downregulation of c-Myc and cyclin B1 suppressing cell's proliferation and inducing their apoptosis[29]. Finally, BMAL1/CLOCK1 and BMAL1/NPAS2 activate *WEE1* expression activating the phosphorylation of CDK1/Cyclin B complex which leads to G_2/M arrest and inhibition of cell proliferation[30]. Consistent with these data *Clock1* deficient mice present significantly decreased levels of WEE1 mRNA[9] while *CRY1* and *CRY2* deletion leads to higher WEE1 levels inhibiting cell proliferation[30,31]. Notably, *WEE1* has been found to be suppressed in colon cancer tissues and cell lines[32]. Collectively these data suggest that potential alterations of critical clock genes such as BMAL1 and CLOCK1 can modulate the G2-to-M transition with subsequent effects on cell cycle progression and cell proliferation through c-Myc, p21 and Wee1.

WNT/β-CATENIN SIGNALING AND CIRCADIAN SYSTEM

Wnt/β-catenin singaling is frequently de-regulated in colorectal cancer and APC, a central component of this pathway is mutated in 50% of sporadic CRCs[33]. Wnt ligand binds the N terminal domain of a Frizzled family receptor, a G-protein coupled receptor. This interaction disrupts the function of the APC/Axin/GSK-3β destruction complex inhibiting the degradation of β-catenin. This results in increased nuclear accumulation of β-catenin inducing the expression of several mediators of cell proliferation such as c-Myc and cyclin D1[34], and activates cadherin cell adhesion complexes promoting migration and metastasis. Patients with Familial Adenomatous Polypodiasis harbor APC mutations resulting in dysfunctional destruction complex and sustained β-catenin signaling. Overexpression of *BMAL1* in NIH-3T3 fibroblasts leads to increased β-catenin expression and Wnt activation contributing to induced cell proliferation[15]. Moreover, it was found that downregulation of *PER2* in HCT116 and SW480 colon cell lines induces β-catenin expression and accelerates cell proliferation mediated by increased cyclin D levels[35]. Consistently, deletion of *PER2* was related to increased colonic and small intestine polyps formation in mice with APC mutation[35]. Interestingly, the same group showed that activation of β-catenin signaling leads to destabilization of PER2 in the intestinal mucosa of mice with APC mutation altering the circadian rhythm and its downstream targets such as *WEE1*[36]. Finally, Sahar *et al*[37] showed that under Wnt signaling activation, the absence of GSK-3β mediated

phosphorylation leads to increased BMAL1 stabilization and activity while active GSK-3β promotes BMAL1 ubiquitylation. These results suggest that Wnt/β-catenin interacts with clock system probably through a positive feedback mechanism maintaining and de-regulating colon cancer cell proliferation. Further studies are needed to validate the effect of circadian system on cell migration and metastasis through de-regulated β-catenin pathway.

DNA DAMAGE RESPONSE AND CLOCK GENES

It is known that 90% of hereditary non-polyposis colon cancers and 10%-15% of sporadic CRCs carry inactivating mutations in genes involved in the mismatch repair (MMR) system such as *MLH1* and *MSH2* leading to microsatellite instability (MSI) related to deficient DNA repair[38]. It should be mentioned that Alhopuro *et al*[12] in a recent study showed that *CLOCK1* gene is somatically mutated in 53% of CRC characterized by MSI. According to this study, CLOCK1 promotes growth arrest, DNA repair and apoptosis upon genotoxic stress caused by UV radiation suggesting that this molecule may represent an important "caretaker" promoting cell cycle arrest upon DNA damage. Further studies are needed to establish the important effects of clock genes and circadian system on the cellular responses following DNA damaging events.

Apart from MMR other components of DDR such as repair of double strand breaks through ATM and CHK2 activation have been implicated in the development of CRC. In particular, reduced expression of BRCA1 and ATM, which are critical nodes in the double strand break repair system, is more frequent in CRC compared to normal colonic mucosa and related to decreased overall survival in patients with CRC[39,40]. Moreover, Takabayashi *et al*[41] showed that DNA damage response is significantly reduced during CRC progression. These results suggest that DNA repair and its defects are correlated to CRC development, progression and potentially clinical outcome since the deficient DNA damage response can be proved to be the Achilles' heel of these cells.

Interestingly, Gery *et al*[11] showed that PER1, a critical component of the circadian system promotes the ATM mediated CHK2 activation upon exposure to radiation leading to increased G_1/S arrest in colon cancer cell lines while PER1 levels are significantly reduced in human colorectal cancer samples. Moreover, it has been shown that TIM protein, another mediator of the circadian regulation at the molecular level, is also required for CHK2 activation promoting arrest of the cancer cell in the G2 phase upon DNA damage[42]. Collectively, these data support that circadian system regulates the ATM/CHK2 signaling which is critical for the repair of DNA.

More reports demonstrated that circadian system modulates other aspects of DNA repair upon genotoxic stimuli. In particular recent studies have also highlighted the role of PER1/TIM complex in the ATR mediated CHK1 activation which also leads to cell cycle arrest upon genotoxic events[43,44]. Hoffman *et al*[45] found that knockdown of the circadian gene *NPAS2*, which as described above creates heterodimers with BMAL1 and CLOCK1, leads to impaired DNA repair and inhibition of cell cycle delay upon mutagen treatment. Finally, loss of circadian *CRY1* and *CRY2* genes increases the sensitivity to DNA damage induced apoptosis in p53 deficient cancer cells through increased expression of the p53 related gene p73. These results suggest that impaired DNA damage response, which is partially related to altered peripheral circadian function, promotes genomic instability contributing to the development of CRC.

CLINICAL CORRELATIONS BETWEEN CLOCK GENES AND COLORECTAL CANCER

Multiple recent studies correlating the expression of clock genes with clinical outcomes highlight the role of Circadian system in the development and progression of CRC. In particular, Mazzoccoli *et al*[46] demonstrated that *PER1, PER2, PER3* and *CRY2* are significantly downregulated in CRC tissues compared to healthy colonic mucosa while lower *PER1* and *PER3* expression was associated with poorer survival rates. These results support the hypothesis that these genes act mainly as tumor suppressors and their downregulation is implicated in CRC development and progression. Consistent with these data, Oshima *et al*[47] showed that *PER1* and *PER3* are downregulated in CRC tissue compared to adjacent normal mucosa while reduced expression of *PER1* is related to increased incidence of liver metastasis highlighting the potential negative impact of clock genes in the aggressiveness of CRC. On the contrary the same group showed that *CLOCK1* and *CK1ε* are upregulated in CRC compared to healthy mucosa, while increased expression of *BMAL1* is related to decreased overall survival. These results were further supported by our group showing in a recent study that *CLOCK1* and *BMAL1* are upregulated while *PER1* and *PER3* are downregulated in CRC tissues compared to healthy mucosa[48]. These conclusions suggest that different components of the circadian system may have different effects on the development of this disease based on their implications in different oncogenic pathways.

It is known that the expression and activity of dihydropyrimidine dehydrogenase (DPD) which determine the efficacy and outcome of 5-fluorouracil (5-FU) treatment in CRC are regulated by a circadian rhythm. This conclusion led to the introduction of the "chronomodulated chemotherapy" with variable rate infusions of 5-FU for treatment of advanced CRC[49,50]. Interestingly, Krugluger *et al*[51] found that reduced *PER1* mRNA levels are correlated with decreased DPD expression in undifferentiated CRC, a result which was more pronounced in female patients. This result suggest that in advanced CRC characterized by lower PER1 mRNA levels the circadian regulation of DPD is probably lost making cancer cells

more susceptible to 5-FU treatment especially in female patients.

The role of PER2 as a negative regulator of circadian system and potential tumor suppressor for CRC has been supported by a study by Zeman *et al*[52] who found that tumor staging is negatively correlated with *PER2* gene expression. Interestingly, a recent study demonstrated that immunohistochemical staining for PER2 is weaker in CRC cancer cells following a heterogenous pattern compared to normal colonic cells[53]. In the same study, the well differentiated cancer cells were found to have comparable PER2 levels with that in non-cancerous cells suggesting that loss of PER2 is related to increased aggressiveness of CRC[53]. Finally, the authors showed that decreased PER2 mRNA and protein levels are correlated with higher histological grade, deeper tumor invasion, lymph node metastasis, advanced TNM stage and higher Ki67 score, which suggests that reduction of PER2 may lead to attenuated cancer cell growth[53]. Collectively these data support that low expression of PER2 and potentially activated circadian system is implicated in the CRC development and progression.

On the contrary, according to a recent report by Yu *et al*[54], CRY was found to be upregulated in CRC cell lines and human CRC samples while higher CRY expression was associated with lymph node metastasis, increased TNM staging and poorer prognosis. At the molecular level the authors showed that upregulation of CRY increased CRC cell proliferation and migration while downregulation of CRY significantly decreased the colony formation and migration in a CRC cell line[54]. Further studies are needed to clarify the role of CRY since as mentioned above it negatively regulates the BMAL1 and CLOCK1 activities, which is more consistent with a role of tumor suppressor in CRC.

In contrast to PER proteins which are important negative regulators of the circadian system, CLOCK1, NPAS2 and BMAL1 molecules are forming heterodimers controlling the transcription of about 10% of genes implicated in cell proliferation, apoptosis and cell cycle such as *c-MYC*, *p21* and *WEE1*[55]. As mentioned above, CLOCK1 regulates a complicated response to DNA damage caused by UV radiation protecting cells from acquiring additional DNA alterations, which can promote the development of a cancerous phenotype. Finally, it has been shown that 2 single nucleotide polymorphisms (rs3749474 and rs1801260) located in the 3'UTR of the *CLOCK1* gene decreasing its mRNA levels are related to decreased overall survival of CRC patients[56]. In a recent study evaluating the association between clock genes polymorphisms and CRC susceptibility we showed that the rs1801260 polymorphism in the 3'UTR of the *CLOCK1* gene significantly increases the risk for CRC development but it does not alter the clinical outcome in CRC patients[57]. It should be noted that BMAL1 is the Clock gene most strongly related to poor prognosis in CRC patients. According to a recent report by Tan *et al* the micro RNA (miRNA) mir-142-3p directly targets the 3'UTR of BMAL1 while its expression is controlled by the CLOCK1/BMAL1 heterodimers[58]. miRNAs are believed to be a novel reasonable therapeutic approach in numerous cancerous diseases[59]. Based on the above mentioned results showing that high BMAL1 is associated with poor prognosis in CRC the introduction of mir-142-3p for high grade metastatic CRC could be considered as a novel therapeutic approach in the future.

CONCLUSION

The aim of this review was to highlight the implications of the circadian system to various intracellular events related to CRC development and progression explaining multiple epidemiological findings correlating clock genes' expression with CRC progression. According to the literature the oscillating circadian clock with its components regulates numerous cellular activities such as cell cycle, cellular metabolism and DNA damage response with known implications in carcinogenesis. In particular, clock genes have been related to p21, c-Myc and Wee1 regulation explaining their effect on cell cycle progression and proliferation while recent evidence suggest that the circadian system influences the Wnt/β-catenin signaling which is a critical pathway for the development and progression of CRC. Moreover, it has been shown that clock genes have important implications in the regulation of DNA damage response. A defective circadian system may be related to impaired DNA repair which related to the initiation and development of CRC but at the same time may make these cells susceptible to various DNA damaging agents. Finally, we can conclude that clock genes are implicated in the pathogenesis of CRC with important correlations with prognosis but may constitute an important pathway for the identification of novel agents for modern therapeutic approaches in this type of cancer.

In general, PER proteins are considered to be inhibitors of cell cycle progression and β-catenin activation and mediators of DDR maintaining genomic integrity explaining the clinical data suggesting that lower levels of PER2 are associated with poorer prognosis and metastatic disease. BMAL1 on the other hand activates cell proliferation, β-catenin pathway and is strongly associated with CRC initiation and poor clinical outcome. Targeting BMAL1 with miRNA may be a reasonable approach for patients with metastatic disease. CLOCK1 is more complicated since molecular data suggest that it inhibits cell cycle progression and promotes DNA repair upon genotoxic stress but clinical correlations show that CLOCK1 expression is higher in CRC tissues. It should be noted though that polymosphisms related to dysfunctional CLOCK1 have been shown to increase the risk for CRC and are associated with poorer prognosis. Further studies are needed to clarify the role of this gene in CRC development but it could be hypothesized that the role of CLOCK1 as an inhibitor of cell proliferation upon genotoxic stimuli can be beneficial for cancer cells' survival after the development of the disease. Finally, the discovery of the biological roles of these genes in disease's initiation and progression may provide valuable

prognostic biomarkers which can be particularly useful for patients with stage 2 disease regarding the addition of chemotherapy for their management.

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Sequencing of treatment in metastatic colorectal cancer: Where to fit the target?

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Abstract

Colorectal cancer is a lethal disease if not discovered early. Even though appropriate screening and preventive strategies are in place in many countries, a significant number of patients are still diagnosed at late stages of the disease. The management of metastatic colorectal cancer remains a significant clinical challenge to oncologists worldwide. While cytotoxic regimens constitute the main treatment of choice in this patient population, addition of the five biologics (bevacizumab, cetuximab, aflibercept, panitumumab and regorafenib) to these regimens has improved clinical outcomes. The most commonly used cytotoxic regimens include doublet combinations (FOLFOX/XELOX or FOLFIRI). Many clinical trials have been published and others are underway to compare the biologic agents with one another in order to prove the superiority of one regimen over another. Metastatic colorectal cancer patients have many treatment options; however, the optimal use and sequence of targeted agents remain to be determined. This review entails concise and updated clinical data on the management of metastatic colorectal cancer. The aim of the review is to determine where to fit the five biologic targets into the treatment algorithm of metastatic colorectal cancer patients and to derive treatment sequences that would achieve best clinical outcome based on the current available data.

Key words: Metastatic colorectal cancer; Chemotherapy; Anti-epithelial growth factor receptor; Anti-vascular endothelial growth factor; Treatment sequence

Core tip: Metastatic colorectal cancer patients have many treatment options; however, the issue of best treatment sequence remains a challenge in this population. This review involves an in depth analysis of previous and most recent clinical advances in this field and aims to come out with treatment sequences that identify patient groups who are most likely to benefit from such sequences based on the current available data.

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INTRODUCTION

Colorectal cancer (CRC) is a lethal disease if not discovered early. Even though appropriate screening and preventive strategies are in place in many countries, a significant number of patients are still diagnosed at late stages of the disease. It is reported that approximately 20%-25% of patients present with distant metastatis at diagnosis[1,2]. Treatment goals for these patients are usually palliative rather than curative with the exception of

a small number of patients with stage Ⅳ disease, liver-confined disease who may be surgically cured.

Recent advances in chemotherapy-based regimens have increased median overall survival (OS) for patients with metastatic CRC (mCRC) from 11-12 mo in the 5-fluorouracil (5-FU) era[3] to more than 24 mo in the era of biologic compounds and doublet/triplet chemotherapy regimens[4-6].

The continuum of care approach to the management of patients with metastatic rectal cancer is the same as that for patients with metastatic colon cancer. The three active conventional chemotherapy agents for mCRC are fluoropyrimidines, irinotecan and oxaliplatin. The most widely used cytotoxic backbone involves double-agent chemotherapy with either FOLFOX/XELOX or FOLFIRI with no significant differences between either regimen[7,8], while triple-agent chemotherapy (FOLFOXIRI), although achieving better progression free survival (PFS), response rate (RR) and OS than FOLFIRI in some trials[9,10], is only reserved to patients who can tolerate such an aggressive regimen. 5-FU/LV or capecitabine, which have been shown to be inferior to FOLFOX[11-13] and FOLFIRI[14,15] in terms of OS (with FOLFIRI regimen), PFS and RR, are still a treatment of choice in patients who cannot tolerate treatment with oxaliplatin and irinotecan. The addition of biological targets to these four cytotoxic regimens has shown better treatment outcomes in the majority of patients; however, debate still exists with regards to the best sequence of treatment, and which agents to be used in first line and then following progression. In the discussion that follows, we review the literature of clinical trials to come out with treatment sequences that achieve the best outcome in mCRC patients.

Data for this review were compiled using MEDLINE/PubMed, American Society of Clinical Oncology and European Society of Medical Oncology abstract databases published before July 2013. The search terms included colorectal cancer, bevacizumab, panitumumab, cetuximab, aflibercept and regorafenib. Information regarding ongoing clinical trials was obtained using the United Stated National Institute of Health's online resource clinicaltrials.gov. Only articles published in English were considered.

FIRST-LINE THERAPY

Single-agent fluoropyrimidine regimens: Can the addition of anti-angiogenic therapy improve outcomes?

Addition of bevacizumab to "weaker" cytotoxic regimens such as 5-FU/LV or to capecitabine yielded better PFS compared to the cytotoxic regimen alone in 3 clinical trials. The first phase Ⅱ trial assessing the efficacy of adding bevacizumab to 5-FU/LV revealed that bevacizumab at 5 mg/kg every 2 wk resulted in increases of 3.8 mo in PFS (from 5.2 to 9.0 mo; $P = 0.005$) compared with 5-FU/LV alone. A statistically significant increase in RR was demonstrated for the bevacizumab arm compared with the control arm (40% *vs* 17%, $P = 0.029$). Median OS was improved in the bevacizumab arm but did not reach statistical significance[16]. In another phase Ⅱ trial by Kabbinavar *et al*, patients were randomly assigned to 5-FU/LV/placebo ($n = 105$) or 5-FU/LV/bevacizumab ($n = 104$). RR and OS were better in the bevacizumab arm but they did not reach statistical significance. PFS was significantly better in the bevacizumab arm with 9.9 mo *vs* 5.5 mo in the placebo arm ($P = 0.0002$)[17]. Patients in this trial were non-eligible to receive irinotecan based-therapy and were ≥ 65 years. In the recent phase Ⅲ trial by Cunningham et al, addition of bevacizumab to capecitabine in elderly patients ≥ 70 years was associated with significantly prolonged PFS, the primary end point, compared with capecitabine alone (9.1 mo *vs* 5.1 mo, $P < 0.001$)[18]. RR was also significantly improved in the bevacizumab plus capecitabine arm (19.3% *vs* 10.0%, $P = 0.042$). OS, a secondary endpoint, was longer in patients in the bevacizumab arm (20.7 mo *vs* 16.8 mo, $P = 0.182$) but did not reach statistical significance and the study was not powered to show a difference in OS between treatment arms. Therefore, patients receiving fluoropyrimidine regimens as part of their first-line treatment have prolonged PFS of about 9 mo from the addition of bevacizumab. The toxicity profile from adding bevacizumab was generally well tolerated in all 3 trials.

First-line irinotecan-based regimens: What is the evidence for the addition of targeted therapy?

Bevacizumab: In a phase 3 trial by Hurwitz *et al*[19], patients were assigned to either receive irinotecan, bolus 5-FU and leucovorin (IFL) plus bevacizumab or the same cytotoxic regimen with placebo. Median OS (20.3 mo *vs* 15.6 mo, $P < 0.001$), PFS (10.6 mo *vs* 6.2 mo, $P < 0.001$) and RR (44.8% *vs* 34.8%, $P = 0.004$) were all superior in the bevacizumab group. Results from a phase Ⅲ study that was initially meant to compare the safety and efficacy of 3 different irinotecan containing regimens in the first-line treatment of mCRC was later amended to compare FOLFIRI plus bevacizumab with mIFL plus bevacizumab. At the time when the results were first published, the median OS was not reached in the FOLFIRI arm[20]. A year later, the authors report a median OS of 28 mo in the FOLFIRI plus bevacizumab arm compared to 19.2 mo in the mIFL plus bevacizumab arm ($P = 0.037$). Differences in PFS and RR were not statistically significant between the 2 arms[21]. Based on the results from this trial, FOLFIRI plus bevacizumab was found to be superior to mIFL plus bevacizumab in the first-line treatment of mCRC. Two other clinical trials, the PACCE and AVIRI trials, of FOLFIRI plus bevacizumab thereafter reported consistent data with PFS reported to be 11.7 and 11.1 mo, OS 20.5 mo and 22.2 mo and RR 40% and 53.1%, respectively[22,23]. The median OS of 28 mo reported by Fuchs *et al*[20] was the highest survival reported when bevacizumab was added to FOLFIRI. The cytotoxic regimen FOLFIRI was shown to be superior to IFL, and addition of bevacizumab to both regimens yielded better results with FOLFIRI as is expected. Nevertheless, bevacizumab

and FOLFIRI in the first-line treatment of mCRC is a superior regimen and is hence recommended in patients who can tolerate such a combination.

Panitumumab: In a single arm phase Ⅱ trial, FOLFIRI plus panitumumab in the first line setting resulted in an overall RR of 49%, PFS of 7.6 mo and an R0 resection rate of hepatic metastasis of 7%. When stratified according to KRAS status, those with wild-type KRAS had better PFS (8.9 mo *vs* 7.2 mo), RR (56% *vs* 38%) and R0 resection rate (8% *vs* 5%) than those with mutated KRAS tumors[24].

Cetuximab: Cetuximab with FOLFIRI in the first line treatment of mCRC demonstrated significant clinical activity. In the CRYSTAL (Cetuximab Combined with Irinotecan in First-Line Therapy for Metastatic Colorectal Cancer) trial, addition of cetuximab to FOLFIRI in patients with KRAS wild-type resulted in significantly better OS (23.5 mo *vs* 20 mo, $P = 0.0093$), PFS (9.9 mo *vs* 8.4 mo, $P = 0.0012$), RR (57.3% *vs* 39.7%, $P < 0.001$) and R0 resection rate (5.1% *vs* 2%, $P = 0.0265$) compared with FOLFIRI alone[25]. However, patients with mutated KRAS status failed to achieve improvement in survival and RRs.

Cetuximab *vs* bevacizumab: The German AIO (Arbeitsgemeinschaft Internistische Onkologie) KRK-0306 (FIRE-3) phase Ⅲ randomized multicenter trial compared the efficacy of FOLFIRI-cetuximab to FOLFIRI-bevacizumab in 592 patients with wild-type KRAS mCRC who were not previously treated for metastatic disease[4]. The primary endpoint was the overall RR. Among the intent to treat (ITT) population, overall RR (62% *vs* 58%, $P = 0.183$) and PFS (10.0 mo *vs* 10.3 mo, $P = 0.547$) were similar between the cetuximab and bevacizumab arms, respectively. In those 526 patients assessable for efficacy, the overall RR was significantly higher in the FOLFIRI-cetuximab arm (72.2% *vs* 63.1%, $P = 0.017$). OS was significantly longer in patients treated with FOLFIRI-cetuximab (28.7 mo) compared with patients who received FOLFIRI-bevacizumab (25 mo, $P = 0.017$). The lack of correlation between PFS and OS in this trial is unclear and may be related to the subsequent therapies used after first-line treatment and also highlights the importance of choice of primary endpoint. In a subgroup analysis of the same trial for patients with mutated KRAS tumors, neither strategy demonstrated a clearly superior outcome[26]. Results from the US intergroup phase Ⅲ C80405 trial which randomized patients to either cetuximab or bevacizumab with FOLFOX or FOLFIRI will help address this issue as well. But for now, and until data from other trials become available, the optimum biologic to be used with FOLFIRI based on the current available data seems to be cetuximab. In patients with mutated KRAS tumors, and even though bevacizumab did not seem to incur additional benefits over cetuximab in the subgroup analysis, it is still not recommended to use cetuximab/panitumumab-based regimens. And hence, FOLFIRI plus bevacizumab is a treatment option in patients with mutated KRAS tumors.

First-line oxaliplatin-based regimens: What is the evidence for the addition of targeted therapy?

Bevacizumab: Addition of bevacizumab to FOLFOX or XELOX in the NO16966 trial reported only an increase in PFS when bevacizumab was added to FOLFOX or XELOX compared to the cytotoxic regimen alone (9.4 mo *vs* 8.0 mo, $P = 0.0023$). Median OS was 21.3 mo in the bevacizumab group and 19.9 mo in the placebo group ($P = 0.07$) and RR was similar between the two arms (47% *vs* 49%, $P = 0.31$)[27]. Other trials suggest that the addition of bevacizumab to an oxaliplatin-based regimen yields a similar magnitude of efficacy to that seen when bevacizumab is added to a FOLFIRI regimen. In four clinical trials, addition of bevacizumab to XELOX or FOLFOX resulted in PFS ranging between 10.3-11.4 mo, OS ranging between 20.3-24.5 mo and a RR ranging between 46%-50%[22,28-30]. However, in all these trials, addition of bevacizumab to oxaliplatin-based regimens was not compared to the cytotoxic regimen alone. The NO16966 trial was the only trial that involved this comparison and has shown that addition of bevacizumab improved PFS as reported in other phase Ⅲ trials, but the observed trend in an improvement in OS did not reach statistical significance, which may be attributed to a shorter treatment duration in the bevacizumab arm (about 6 mo) as compared to other trials and that treatment until disease progression may be necessary to maximize the clinical benefit derived from bevacizumab therapy.

Results of the large observational BEAT trial of bevacizumab concluded that median PFS, TTP (time to treatment progression) and OS were consistent across the doublet regimens (FOLFOX, XELOX and FOLFIRI), suggesting thatthe efficacy of bevacizumab is not related to thechemotherapy regimen used[31]. Results of this have been confirmed in doublet combinations but not in triplet regimens. In a recent phase 2 trial of a head-to-head comparison between XELOX plus bevacizumab and XELIRI plus bevacizumab, the addition of bevacizumab to these two cytotoxic regimens yielded similar PFS (10.4 mo *vs* 12.1 mo, $P = 0.3$) and OS (24.4 mo *vs* 25.5 mo, $P = 0.45$) with no superiority of one regimen over the other[32]. Another clinical trial, MAVERICC, is underway comparing FOLFIRI plus bevacizumab *vs* FOLFOX plus bevacizumab. In this phase 2 prospective study, tumoral excision repair cross-complementation group 1 and plasma vascular endothelial growth factor A are employed as potential biomarkers for oxaliplatin- and bevacizumab-containing regimens, respectively (ClinicalTrials.gov Identifier: NCT01374425). While the magnitude of effect seems to be equivalent between FOLFIRI and FOLFOX, only further clinical trials addressing biomarkers of response to these cytotoxic regimens could stratify patients to either cytotoxic regimen.

Aflibercept: In a phase Ⅱ trial assessing the efficacy of aflibercept when added to FOLFOX in the first-line treatment of mCRC, no significant improvement in RR and PFS was achieved. OS in that trial was not reported[5]. Hence, for now, aflibercept is not recommended in the first line treatment when added to a FOLFOX regimen. Its efficacy in the second-line setting was achieved when added to FOLFIRI which may also be of benefit if used in the first-line. However, no clinical trial has yet addressed this issue and so aflibercept's use is limited to second-line treatment regimens that involve irinotecan naïve patients.

Panitumumab: In the phase Ⅲ Panitumumab Randomized Trial in Combination with Chemotherapy for Metastatic Colorectal Cancer to Determine Efficacy (PRIME) study, addition of panitumumab to FOLFOX in the first-line treatment of patients with KRAS wild-type significantly improved PFS (9.6 mo *vs* 8.0 mo, $P = 0.02$). The overall increase in survival was not significant but was higher in the panitumumab group (23.9 mo *vs* 17.9 mo, $P = 0.072$) as well as the overall RR (55% *vs* 48%; $P = 0.068$) and R0 resection rate (8.3% *vs* 7.0%)[5].

Wild-type RAS (wild-type KRAS exons 2, 3, 4 and wild-type NRAS exons 2, 3, 4) was associated with significantly better OS (26 mo *vs* 20.2 mo, $P = 0.04$) and PFS (10.1 mo *vs* 7.9 mo, $P < 0.01$) in the panitumumab plus FOLFOX arm than the FOLFOX arm alone. In patients with wild-type KRAS exon 2 but mutated other RAS (KRAS exons 3, 4 or NRAS exons 2, 3, 4), the PFS and OS were not different between the two arms. Hence, patients with wild-type RAS have a statistically significant OS benefit when treated with panitumumab plus FOLFOX *vs* FOLFOX alone. Panitumumab is unlikely to benefit patients with any *RAS* mutations and *BRAF* mutation had no predictive value[33].

Cetuximab: Unlike the synergy seen between cetuximab and irinotecan, data on the efficacy of cetuximab with oxaliplatin-based regimens report conflicting results ranging from additive to detrimental effects of these two drugs. The phase 2 oxaliplatin and cetuximab in first-line treatment of metastatic colorectal cancer (OPUS) trial demonstrated that addition of cetuximab to FOLFOX4 regimen resulted in significant improvement in PFS (8.3 mo *vs* 7.2 mo, $P = 0.0064$), RR (57% *vs* 34%, $P = 0.0027$), R0 resection rate (12% *vs* 3%, $P = 0.0242$) but only a trend toward improvement in OS (22.8 mo *vs* 18.5 mo, $P = 0.39$)[34]. However, two recent phase 3 trials, the Medical Research Council Continuous Chemotherapy plus Cetuximab or Intermittent Chemotherapy with Standard Continuous Palliative Combination Chemotherapy with Oxaliplatin and Fluoropyrimidine in First-Line Treatment of Metastatic Cancer (MRC COIN) and Nordic Colorectal Cancer Biomodulation Group Study 7 (NORDIC Ⅶ) trials have raised more questions with regards to the efficacy of cetuximab with oxaliplatin-based regimens. The MRC COIN study involved 357 patients with KRAS wild-type in the cetuximab arm plus FOLFOX or XELOX and 358 patients with KRAS wild-type in the control arm (FOLFOX or XELOX without cetuximab). The investigators reported no differences in OS (17 mo *vs* 17.9 mo, $P = 0.67$) and PFS (8.6 mo *vs* 8.6 mo, $P = 0.6$) between cetuximab arm and control group, respectively. RR, on the other hand, was increased from 57% with chemotherapy alone to 64% with addition of cetuximab ($P = 0.049$)[35]. A post-hoc analysis; however, demonstrated improvement in PFS in the infusional FOLFOX plus cetuximab ($P = 0.037$) but not in the XELOX plus cetuximab group ($P = 0.88$). A PFS benefit was restricted to those patients with wild-type KRAS and those with no or only one metastatic site treated with 5-FU infusion therapy ($P = 0.011$).The number of patients receiving XELOX ($n = 240$) far exceeded those receiving FOLFOX ($n = 117$) which may have contributed to the negative outcomes seen in the cetuximab arms. Moreover, the COIN trial reported significant dose reductions in infusional 5-FU in the FOLFOX plus cetuximab arm compared to the control group ($P = 0.016$) and the XELOX plus cetuximab group received significant dose reductions of both oxaliplatin ($P = 0.0018$) and capecitabine ($P = 0.004$) compared to the control arm which may explain in part the lack of efficacy in the cetuximab arms. The Nordic Ⅶ trial investigated the efficacy of cetuximab when added to bolus 5-FU/LV/oxaliplatin (FLOX)[36]. The trial included 194 patients with wild-type KRAS; 97 patients received FLOX plus cetuximab and 97 received FLOX alone. An additional 130 patients with mutant KRAS tumors were randomized between the two arms. In patients with wild-type KRAS, a trend towards worse outcome was seen in terms of OS (20.1 mo *vs* 22 mo, $P = 0.48$) and PFS (7.9 mo *vs* 8.7 mo, $P = 0.66$) between the cetuximab arm and the control arm, respectively. Additionally, the RR did not differ between the two groups (46% *vs* 47%, $P = 0.89$). On the other hand, patients with mutated KRAS tumors exhibited a trend toward better prognosis when they were treated with cetuximab; PFS (9.2 mo *vs* 7.8 mo, $P = 0.07$), OS (21.1 mo *vs* 20.4 mo, $P = 0.89$) and RR (35% *vs* 23%, $P = 0.31$). Hence, both the COIN and NORDIC Ⅶ trials did not demonstrate an efficacy from the addition of cetuximab to oxaliplatin-based regimens. However, this was not the case in the OPUS trial which demonstrated a significant improvement in PFS when cetuximab was added to FOLFOX regimen. It seems that cetuximab is efficient when added to infusional 5-FU as seen in the OPUS trial, while capecitabine or bolus 5-FU are not associated with significant improvement in PFS. The PRIME trial also demonstrated a significant improvement in PFS when panitumumab was added to the FOLFOX regimen. The AIO KRK-0104 study randomly assigned 198 patients to either cetuximab plus XELIRI ($n = 93$) or cetuximab plus XELOX ($n = 92$)[37]. The trial was not powered to compare the two treatment regimens; however, the RR was similar for the two arms (46.1% in XELIRI *vs* 47.7% in XELOX arm). The PFS reported in this trial is lower than that reported in both the OPUS and CRYSTAL

trials probably further indicating that cetuximab is more efficient with infusional 5-FU regimens than either bolus 5-FU or capecitabine regimens. It is of note that the OS reported in the trial was comparable to that observed in OPUS and Crystal trials. A recent meta-analysis that pooled results ofthe PRIME, OPUS, COIN, and NORDIC Ⅶ revealed that addition of cetuximab and panitumumab to oxaliplatin-based regimens in the first line setting significantly improved PFS (P = 0.03) and RR (P = 0.009) compared to chemotherapy alone but the difference in OS was not significant. OS and PFS were not significant when cetuximab and panitumumab were added to bolus 5-FU or capecitabine-based regimens compared with chemotherapy alone[38].

The recent results of the new EPOC study revealed detrimental results with the addition of cetuximab to chemotherapy (fluoropyrimidine and oxaliplatin) in patients with liver resectable metastases and KRAS wild-type tumors thus questioning the role of cetuximab in upfront therapy with oxaliplatin based regimens in this setting[39]. The study randomized 272 patients to chemotherapy alone or chemotherapy with cetuximab. The trial was stopped when the study met a protocol pre-defined futility analysis. PFS was significantly worse in the cetuximab arm (14.8 mo *vs* 24.2 mo, P < 0.048). The phase 2 OPUS trial was the only trial that supported the addition of cetuximab to FOLFOX and so until a phase 3 trial of cetuximab plus FOLFOX demonstrates superior clinical activity over FOLFOX alone, this cytotoxic regimen is still not recommended in the first-line treatment of mCRC patients and particularly in patients with resectable liver metastases.

In a pooled, retrospective analysis by Roock *et al*[40] of 579 mCRC patients who received cetuximab, patients with mutation in codon 13 (G13D) had significantly longer OS (7.6 *vs* 5.7 mo; P = 0.005) and PFS (4.0 mo *vs* 1.9 mo, P = 0.004) than patients with other KRAS mutations. In addition, OS was similar between patients with the G13D mutation and patients with wild-type KRAS. Moreover, pooled data from 1378 evaluable patients from the CRYSTAL and OPUS studies revealed significant variations in treatment effects for RR (P = 0.005) and PFS (P = 0.046) in patients with G13D-mutant tumors *vs* all other mutations[41]. Cetuximab plus chemotherapy *vs* chemotherapy alone significantly improved PFS (7.4 mo *vs* 6.0 mo, P = 0.039) and RR (40.5% *vs* 22.0%, P = 0.042) but not OS (15.4 mo *vs* 14.7 mo, P = 0.68) in patients with *G13D*-mutant tumors. However, the efficacy of cetuximab in patients with *G13D* mutations was inferior to those with wild-type KRAS. A study by Gajate *et al*[42] reported different results, patients with mutation in *G13D* did not differ significantly in PFS (4.96 mo *vs* 3.1 mo, P = 0.72) and OS (8.2 mo *vs* 14.6 mo, P = 0.084) from other *KRAS* mutations. Also, as seen in pooled data from the CRYSTAL and OPUS studies, patients with KRAS wild-type tumors have a longer PFS (7.3 mo, P = 0.025) and OS (19.0 mo, P = 0.004) than patients with *G13D*-mutated tumors[42]. Moreover, the finding of cetuximab benefit in patients with *G13D* mutations was not reproducible with panitumumab in other pooled retrospective analysis of 3 trials with the use of FOLFOX with or without panitumumab in the first-line setting (PRIME trial), FOLFIRI with and without panitumumab in the second-line setting and best supportive care with and without panitumumab in the salvage setting[43]. No mutant *KRAS* allele was consistently identified as a predictive factor for PFS or OS in either the control arm or the panitumumab arm[43]. Prospective randomized trials in patients with G13D mutations are needed before any conclusions could be made about the potential benefit from cetuximab (or panitumumab). One such trial is currently open to accrual[44].

Panitumumab *vs* bevacizumab: The PEAK study was the first prospective trial to compare bevacizumab to an anti-EGFR monoclonal antibody in combination with an oxaliplatin-based regimen[45]. Median PFS was 10.9 mo with panitumumab and 10.1 mo with bevacizumab (P = 0.35). Median OS has not been reached with panitumumab and was 25.4 mo with bevacizumab (P = 0.14). The overall RRs were 58% and 54% and the resection rates were 13% and 11% for the panitumumab and bevacizumab arms, respectively.

In a prospective-retrospective analysis of the PEAK, patients with wild-type RAS receiving panitumumab had a PFS of 13.1 mo while those receiving bevacizumab had a PFS of 9.5 mo (P = 0.02)[46]. OS in the panitumumab arm was not reached while in the bevacizumab arm OS was 29 mo (P = 0.06). In patients with wild-type KRAS exon 2 but mutated KRAS (exons 3 or 4) or mutated NRAS (exons 2, 3 or 4), both the PFS (7.8 mo *vs* 8.9 mo, P = 0.44) and OS (not reached *vs* 21.6 mo, P = 0.5) were comparable between the panitumumab and bevacizumab arms. In this first-line estimation study in patients with wild-type RAS mCRC, PFS and OS favored panitumumab plus FOLFOX relative to bevacizumab plus FOLFOX.

First-line FOLFOXIRI: Should targeted agents be added to this chemotherapy combination?

Bevacizumab: Bevacizumab with triple cytotoxic regimens seems to be superior to doublet regimens. Recently, Falcone *et al*[6] reported the results of the Tribe trial where they sought to confirm the superiority of FOLFOXIRI over FOLFIRI when bevacizumab is added to both regimens. FOLFOXIRI plus bevacizumab significantly increased PFS (median 9.5 mo *vs* 11.9 mo, P = 0.001) and RR (53% *vs* 64%, P = 0.015) when compared to FOLFIRI plus bevacizumab. Median OS for FOLFOXIRI/bevacizumab was 31.0 mo compared with 25.8 mo in the FOLFIRI/bevacizumab group (P = 0.054). Grade 3-4 neurotoxicity, diarrhea, stomatitis, and neutropenia were significantly higher (P < 0.05) in patients receiving FOLFOXIRI/bevacizumab; while the incidence of febrile neutropenia, serious adverse events, and treatment-related deaths were similar among the two groups. Pre-

liminary results of the OPAL trial assessing the safety of FOLFOXIRI with bevacizumab in the first-line setting in 96 patients revealed that the incidence of adverse events was as previously reported by Falcone *et al*[6] and that the regimen was well tolerated among the patient population included in the study. An interesting activity of FOLFOXIRI/bevacizumab was seen in BRAF mutated cancers; however, the numbers were low to derive any definite conclusions[6]. FOLFOXIRI regimen has been shown to be superior to FOLFIRI alone in the first line treatment of mCRC[9,10] and whether an additional benefit is employed from the addition of bevacizumab is unclear. The superiority of FOLFOXIRI plus bevacizumab over FOLFOX plus bevacizumab has also been reported in the phase 2 OLIVIA trial[48]. The R0 resection rate was significantly higher (48.8% *vs* 23.1%, $P = 0.017$), RR was higher but did not reach statistical significance and PFS data are still immature but favor the FOLFOXIRI arm. The results suggest that FOLFOXIRI-bevacizumab improves resection rates, RR, and long-term outcomes *vs* FOLFOX-bevacizumab in patients with initially unresectable colorectal liver metastases. Grade $\geqslant 3$ adverse events occurred in 84% of patients in the FOLFOX arm compared to 95% in the FOLFOXIRI arm and included neutropenia (35% *vs* 48%), febrile (8% *vs* 13%) and diarrhea (14% *vs* 28%).

A clinical trial comparing FOLFOXIRI plus bevacizumab to FOLFOXIRI alone could define the magnitude of effect from the addition of bevacizumab. Moreover, BRAF-mutated microsatellite stable tumors have a poor prognosis[49] and could hence be good candidates to an aggressive regimen such as FOLFOXIRI plus bevacizumab. Also, receiving FOLFOXIRI-bevacizumab as first-line treatment limits choices in subsequent treatment arms, an issue that questions the importance of second and third line treatments. Among elderly Medicare metastatic CRC patients who survived at least 1 year after diagnosis, first-line therapy improved both short and long-term survival[50]. Second and subsequent chemotherapy lines reduced short-term mortality (2 years); however, they didn't add any additional long term survival benefit (5 years) as compared to first-line therapy. So, should we worry about the sequential treatment strategy or should we provide the best upfront treatment? Only clinical trials addressing the benefit of first and subsequent lines of therapy between several treatment sequences can answer this question.

Cetuximab: Data on cetuximab with FOLFOXIRI is still premature. Two small trials reported high RRs of 79 and 81%, OS of 35 and 24.7 mo, and one trial reported a PFS of 9.5 mo[51,52]. Toxicity will likely be a problem with such a combination. But till now, the only biologic target whose efficacy with FOLFOXIRI has been proven in phase Ⅲ trials is bevacizumab. A trial comparing the FOLFOXIRI regimen alone to FOLFOXIRI plus biologics is needed to assess the efficacy of biologics with this cytotoxic regimen.

SUBSEQUENT TREATMENT OPTIONS

Subsequent treatment options following progression on the 4 aforementioned cytotoxic backbones and their associated targets are summarized in Figure 1.

Progression following treatment with 5-FU or capecitabine plus bevacizumab: What are the options?

Patients progressing on 5-FU or capecitabine with bevacizumab in the first-line are unlikely to receive any regimen containing irinotecan or oxaliplatin in subsequent lines of therapy. Therefore, patients progressing on first line 5-FU or capecitabine-bevacizumab have only the option of EFGR monoclonal antibodies in the second line setting if they have KRAS wild type tumors then regorafenib as their last treatment line[53-56]. Patients with mutated KRAS can only receive regorafenib as their second treatment line since anti-EGFR therapy in this patient population is not recommended.

Progression following FOLFOX plus bevacizumab: What are the options?

Patients receiving the FOLFOX regimen with bevacizumab in the first-line setting receive the alternative cytotoxic regimen FOLFIRI following progression[57-59]. The TML trial enrolled 820 patients with unresectable mCRC who progressed within 3 mo after discontinuing first-line treatment with a bevacizumab-containing chemotherapy regimen. Patients were randomized to receive either oxaliplatin-based or irinotecan-based chemotherapy (depending on what they received first line) plus bevacizumab ($n = 409$) or chemotherapy alone ($n = 411$). Results of the primary analysis showed a significant improvement in OS (11.2 mo *vs* 9.8 mo, $P = 0.006$) and PFS (5.7 mo *vs* 4.1 mo, $P < 0.0001$) in favor of the bevacizumab plus chemotherapy arm[60]. RR were comparable between the two treatment arms (5.4% *vs* 3.9%, $P = 0.3113$). In a post hoc subgroup analysis of the trial, patients progressing on oxaliplatin-based chemotherapy with bevacizumab and crossing over to irinotecan-based chemotherapy with bevacizumab had a prolonged OS (12 mo *vs* 10 mo, $P = 0.052$) and PFS (6.2 mo *vs* 4.2 mo, $P = 0.0005$) compared to the chemotherapy alone arm. The BEBYP trial, conducted by the Gruppo Oncologico Nord Ovest, also supported the results of the TML trial[61]. A significant clinical benefit was associated with continuing bevacizumab after first-line bevacizumab-containing chemotherapy. At a median follow-up of 18 mo, median PFS was 6.77 mo in the bevacizumab arm compared to 4.97 mo in the chemotherapy-alone arm ($P = 0.006$). In the phase 3 VELOUR trial, addition of aflibercept to FOLFIRI in patients who progressed on an oxaliplatin-based regimen resulted in significant improvement in OS (13.5 mo *vs* 12.06 mo, $P = 0.0032$) and PFS (6.90 mo *vs* 4.67 mo, $P < 0.0001$) compared to FOLFIRI plus placebo[62]. The OS and PFS were comparable to those achieved with bevacizumab and FOLFIRI and prove the superiority of aflibercept with FOLFIRI over FOLFIRI alone. Hence,

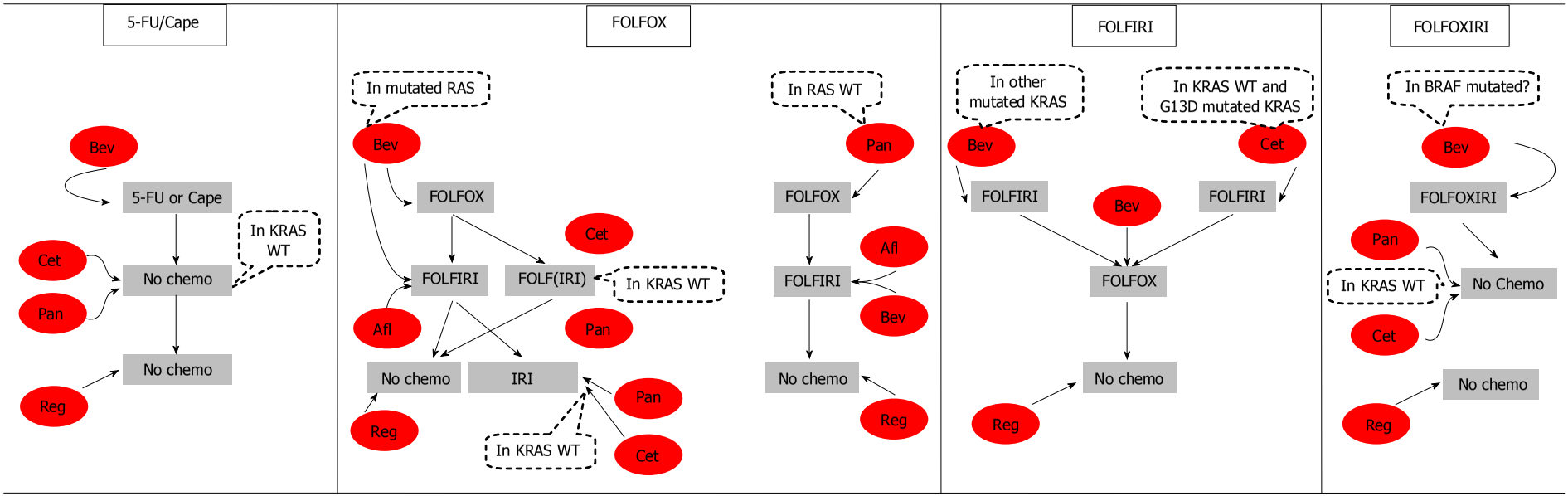

Figure 1 Treatment sequences achieving best clinical outcomes in specified patient population based on current clinical data. Red circles show where biologic targets fit into the cytotoxic treatment sequence (shown in grey boxes) and the specific circumstances that favor its use (shown in dashed comments). Bev: Bevacizumab; Cet: Cetuximab; Pan: Panitumumab; Reg: Regorafenib; Afl: Aflibercept; Cape: Capecitabine; WT: Wild-type; RAS WT: wild-type KRAS and NRAS. 5-FU: 5-fluorouracil.

aflibercept with FOLFIRI constitutes another treatment of choice in patients progressing on first-line FOLFOX plus bevacizumab. Following progression on this regimen and having received all standard therapies, patients with mutated KRAS can be administered regorafenib monotherapy as their final treatment line.

A subset of patients with mutated RAS includes patients with wild-type KRAS. In this subset of patients, panitumumab or cetuximab plus FOLFIRI or irinotecan could be a treatment option. FOLFIRI plus panitumumab resulted in significantly better PFS (5.9 mo *vs* 3.9 mo, $P = 0.004$) and a trend toward improved OS compared to FOLFIRI alone that did not reach statistical significance and which may have been attributed to the large number of patients receiving anti-EGFR therapy following progression[63]. The EPIC trial, which evaluated irinotecan monotherapy with irinotecan plus cetuximab in patients pre-treated with FOLFOX, revealed that cetuximab added to irinotecan significantly improved PFS (4.0 mo *vs* 2.6 mo, $P \leqslant 0.0001$) and RR (16.4% *vs* 4.2%, $P < 0.0001$) but not OS[64]. In these trials, cetuximab and panitumumab resulted in significantly better PFS and RR but not OS while bevacizumab and aflibercept were associated with significantly better OS compared to chemotherapy alone. In a head-to-to head comparison between panitumumab and bevacizumab with FOLFIRI following progression on oxaliplatin-based chemo and bevacizumab, the SPIRITT trial revealed no significant difference in OS and PFS between the two arms. However, RR was higher in the panitumumab arm (28% *vs* 16%)[65]. The worst of grade 3/4 adverse events were recorded for 78% of the panitumumab arm *vs* 65% of the bevacizumab arm but this did not appear to impact discontinuation rates (29% *vs* 25% rates of discontinuation due to adverse events, respectively). Another phase 2 trial is currently recruiting participants to compare the efficacy of cetuximab *vs* bevacizumab with chemotherapy following progression on bevacizumab and chemotherapy in the first-line setting (ClinicalTrials.gov Identifier: NCT01442649). At this point, the choice of whether to use anti-EGFR therapy or bevacizumab with FOLFIRI partly depends on the patient's clinical situation. If the patient is suffering from large tumor burden and is progressing rapidly, then panitumumab may be a better choice since it is associated with a higher response rate. But if skin toxicity is a concern, bevacizumab should be used.

Patients with wild-type KRAS have two options; either bevacizumab/aflibercept with FOLFIRI or panitumumab/cetuximab with FOLFIRI. Patients receiving bevacizumab/afliberceptplus FOLFIRI have the chance to be given irinotecan plus cetuximab as a third treatment line. In this setting, 55 heavily pretreated patients whose disease had

progressed during or within an oxaliplatin-based first-line chemotherapy and an irinotecan-based second-line regimen were given irinotecan and cetuximab. This regimen in the third-line treatment resulted in a median PFS of 4.7 mo and median OS of 9.8 mo[66]. Finally, their last treatment line will involve regorafenib. On the other hand, patients with wild-type KRAS receiving panitumumab or cetuximab in the second line setting with FOLFIRI can only be administered regorafenib following progression.

Progression following FOLFOX plus panitumumab: What are the options?

Patients with wild-type RAS who receive first-line therapy with panitumumab and FOLFOX, are administered either aflibercept or bevacizumab with the FOLFIRI regimen which both have shown a survival benefit over chemotherapy alone[60,62]. Following progression on either of these lines, the last treatment of choice remaining for these patients is regorafenib since they have progressed on all standard therapies.

Progression following FOLFIRI plus cetuximab: What are the options?

Patients with KRAS wild-type tumors progressing on FOLFIRI plus cetuximab should receive the FOLFOX regimen with bevacizumab. Aflibercept with FOLFOX did not show any significant improvement in the first-line setting and so it is not recommended in the second-line setting. Moreover, the GOIM (Gruppo Oncologico Dell' Italia Meridionale) trial is underway to assess the efficacy of FOLFOX with or without cetuximab following progression on cetuximab plus FOLFIRI[67]. Until the results of this trial become available, bevacizumab is used in this setting with the FOLFOX regimen. The ECOG (Eastern Cooperative Oncology Group) Study E3200 assessed the efficacy of bevacizumab plus FOLFOX in patients previously treated with fluoropyrimidine and irinotecan to FOLFOX alone and found that OS (12.9 mo *vs* 10.8 mo, $P = 0.0011$), PFS (7.3 mo *vs* 4.7 mo, $P < 0.0001$) and RR (22.7% *vs* 8.6%, $P \leqslant 0.0001$) were all significantly higher in the bevacizumab group compared to the FOLFOX regimen alone[68]. Patients progressing on bevacizumab and FOLFOX benefit from regorafenib monotherapy in the third-line setting. Regorafenib is approved for the treatment of mCRC patients who progressed on standard therapies and was shown to be superior to supportive care in the CORRECT trial[69].

Progression following FOLRIRI plus bevacizumab: What are the options?

Patients with mutated *KRAS*, who cannot receive anti-EGFR therapy as part of their treatment, receive FOLFIRI plus bevacizumab and then cross over to FOLFOX plus bevacizumab after progression. In the TML trial, the post hoc analysis revealed that patients receiving irinotecan-based regimens with bevacizumab and then receiving bevacizumab with oxaliplatin-based regimens after progression had prolonged PFS (5.4 mo *vs* 3.8 mo, $P < 0.0001$) and OS (10.9 mo *vs* 9.3 mo, $P = 0.0454$) than patients in the chemotherapy alone arm. The last line of therapy available for these patients involves regorafenib which yielded an OS of 6.4 mo compared to best supportive care alone which yielded an OS of 5.0 mo ($P = 0.0052$)[69].

Progression following FOLFOXIRI plus bevacizumab: What are the options?

Patients progressing on the FOLFOXIRI plus bevacizumab regimen and having wild-type KRAS status benefit from irinotecan and cetuximab in the second treatment line. In a phase 2 trial of 40 patients progressing on at least one line of chemotherapy, biweekly cetuximab biweekly and irinotecan resulted in a RR of 22.5%, PFS of 3.4 mo and OS of 8 mo[70]. As their last treatment line, patients could receive regorafenib. On the other hand, if patients had mutated KRAS tumors, then their second treatment option would be regorafenib.

DISCUSSION

First-line treatment involves four cytotoxic backbones to which biologic targeted agents have been added. The effect of these targeted agents ranges from synergistic to detrimental and hence it is crucial to know where to fit these compounds into the management of mCRC patients. 5-FU or capecitabine is a weak regimen limited to elderly patients and those who cannot tolerate aggressive regimens. The addition of bevacizumab to this cytotoxic regimen yielded better PFS of up to 9 mo[16-18].

FOLFOX (or XELOX) is arguably the doublet cytotoxic regimen most commonly used in the first-line treatment of mCRC. The combination of EGFR-targeted therapy with this regimen has shown conflicting results with cetuximab but not with panitumumab. Addition of panitumumab to this regimen yielded an OS and PFS benefit in patients with wild-type RAS compared to bevacizumab[46]. Hence, patients with wild-type RAS are good candidates for FOLFOX plus panitumumab regimens while patients exhibiting any RAS mutation are candidates for FOLFOX plus bevacizumab. The other doublet cytotoxic regimen used in the first-line treatment is FOLFIRI. In a head-to-head comparison between bevacizumab and cetuximab with this regimen, cetuximab seems to be superior to bevacizumab[4]. Hence, cetuximab with FOLFIRI is limited to patients with KRAS-wild type and possible mutated KRAS with G13D mutations while other mutated KRAS tumors are more likely to benefit from FOLFIRI with bevacizumab. The results of the Intergroup C80405 study are eagerly awaited and it is hoped that results of this study will reveal the optimal first-line regimen for chemotherapy doublet plus targeted therapy. As for the triplet cytotoxic regimen FOLFOXIRI, and even though it was associated with significantly more adverse events when added to bevacizumab than either FOLFIRI or FOLFOX regimen, it resulted in the longest reported PFS and OS[6,48]. Cetuximab with this

regimen yielded very high RRs but the data are still immature in this setting[51,52].

As outlined in Figure 1, second and third-line treatment options will depend on the drugs used in the first line setting. Biomarkers such as *RAS* mutation status remain of key importance. For patients with RAS wild-type tumors who have received anti-angiogenic rather than EGFR-targeted therapy in the first-line setting there is a choice to be made whether to continue anti-angiogenic therapy and switch the chemotherapy backbone, reserving EGFR-targeted therapy to the third line, or switch both chemotherapy and targeted therapy. We have no definitive data to guide this decision however there appears to be an advantage to the use of cetuximab in combination with irinotecan over oxaliplatin. Regorafenib has shown a survival advantage over placebo in heavily pretreated patients and we are awaiting further work to identify biomarker that might help us select which patients are more likely to benefit from this therapy.

CONCLUSION

Current options for the management of metastatic CRC involve the use of four cytotoxic chemotherapy regimens and five targeted therapeutic agents. The optimal use and sequencing of these agents has yet to be determined. A major concern regarding clinical trials designed to compare one regimen with another is the large number of patients crossing over to the alternative regimen which may hinder the exact interpretation of OS. To overcome such a drawback, treatment sequences should be compared from line one up to subsequent treatment lines. In such a way, the efficacy of the whole treatment sequence is compared to another treatment sequence with the OS, PFS, RR and R0 resection rates compared across all treatment lines. Such trials are beginning to emerge and are currently underway (ClinicalTrials.gov Identifier: NCT01910610 and NCT01878422). As we learn more about the biology of this disease and biomarkers for treatment selection, we hope to improve outcomes for all patients.

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TOPIC HIGHLIGHT

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

Stereotactic body radiotherapy for oligo-recurrence within the nodal area from colorectal cancer

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Abstract

Recurrence of colorectal cancer (CRC) often presents as solitary metastases, oligometastases or oligo-recurrence. Surgical resection became the preferred treatment for patients with CRC lung and hepatic metastases. However, surgical treatment for oligo-recurrence within nodal area is not a widely accepted treatment due to due to their relative rarity and high postoperative morbidity. Stereotactic body radiotherapy (SBRT) is one of the emerging radiation treatment techniques in which a high radiation dose can be delivered to the tumor. High-dose SBRT can ablate the tumor with an efficacy similar to that achieved with surgery, especially for small tumors. However, there have been very few studies on SBRT for oligo-recurrence within nodal area, although several studies have evaluated the role of SBRT in the treatment of liver and lung metastases from CRC. This article reviews the current clinical status of and treatment methods for oligo-recurrence within nodal area from CRC, with particular emphasis on SBRT.

Key words: Colorectal cancer; Oligo-recurrence; Oligometastases; Stereotactic radiotherapy; Lymph node

Core tip: Surgical treatment for oligo-recurrence of colorectal cancer (CRC) within nodal area is not a widely accepted treatment due to due to their relative rarity and high postoperative morbidity. High-dose stereotactic body radiotherapy (SBRT) can ablate the tumor with an efficacy similar to that achieved with surgery, especially for small tumors. Recently, several investigators successfully treated oligo-recurrence of CRC within nodal area with SBRT. This article reviews the current clinical status of and treatment methods for oligo-recurrence within nodal area from CRC, with particular emphasis on SBRT.

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INTRODUCTION

Colorectal cancer (CRC) remains a major health problem worldwide and is the third most common cause of cancer-related death globally[1]. It is more common in developed than in developing countries. However, in Asia, the incidence of CRC is rising rapidly, and it is now the third most common malignant disease in both men and women[2-4]. Although surgery, chemotherapy, and radiotherapy (RT) for CRC have all developed rapidly in recent decades, approximately 20%-50% of CRC patients still

develop recurrence after definitive treatment[5-7]. This recurrence often presents as solitary metastases or oligometastases, and indeed a study by Tepper *et al*[6] found that approximately 70% of CRC recurrences were solitary.

The term oligometastases, introduced in 1995[8] and expanded upon more recently[9], describes an intermediate state of cancer spread between localized disease and widespread metastases. The implication of this intermediate state is that metastatic disease might be cured using metastasis-directed therapy. As a further conceptual refinement, Niibe *et al*[10] suggested the concept of oligo-recurrence as a disease stage in which there are a limited number of metastases and in which the primary tumor has been controlled. Patients with oligo-recurrence have an improved prognosis compared to those with limited metastasis but uncontrolled primary tumors.

Evidence from a number of clinical studies has suggested that surgical resection of lung and hepatic metastases from CRC prolongs survival[11-15]. As a result, surgical resection, became the preferred treatment for patients with CRC lung and hepatic metastases. However, surgical treatment for oligo-recurrence within the nodal area is not a widely accepted treatment, even when lesions are localized, due to their relative rarity, high postoperative morbidity, and unsatisfied surgical margin etc. If patients with oligo-recurrence do not receive treatment, their median survival is typically only 6-15 mo and the disease is frequently accompanied by refractory pain[16-19].

Stereotactic body radiotherapy (SBRT) is one of the emerging radiation treatment techniques in which a high radiation dose can be delivered to the tumor. It allows for high precision with tight planning margins and a sophisticated treatment plan allowing rapid dose fall-off away from the treatment area. Therefore, this technique provides higher tumor dose description with smaller irradiated volumes of normal tissue. And high-dose SBRT in a single or small number of fractions can ablate the tumor with an efficacy similar to that achieved with surgery, especially for small tumors[16,20-24]. However, SBRT can correspondingly cause more damage to normal tissue if it is included in the radiation field because repair mechanism is not expected in high ablative radiation dose. Therefore, it is important to select the optimal indication for SBRT, and one of these may be nodal metastases as they usually have clearly demarcated margins and allow very little movement. However, there have been very few studies on SBRT for oligo-recurrence within the nodal area, although several studies have evaluated the role of SBRT in the treatment of liver and lung metastases from CRC. This article reviews the current clinical status of and treatment methods for oligo-recurrence within the nodal area from CRC, with particular emphasis on SBRT.

SURGERY FOR OLIGO-RECURRENCE WITHIN THE NODAL AREA FROM CRC

Approximately 50% of local recurrences are restricted to the pelvis or associated with operable oligo-recurrence and are thus potentially amenable to curative re-operation[25-27]. Nevertheless, radical surgery is challenging, not commonly performed, and historically associated with high morbidity and mortality. The most important prognostic factor is whether R0 resection can be achieved. Previous studies have reported 5-year overall survival rate for R0 surgical resection ranging from 19% to 53%, whilst the rate is only between 0% and 32% when complete resection cannot be achieved[28-37]. However, in most cases, recurrence is detected as a fixed mass that invades the pelvic wall or sacrum. Pelvic sidewall recurrence in particular is associated with the worst prognosis and the least likelihood of achieving an R0 resection[38]. The disease often involves key structures such as the ureters, iliac vessels, the sciatic nerve, or the bony pelvis itself, and extensive involvement of the sidewall is a relative contraindication for the surgical treatment of recurrent rectal cancer.

Isolated paraaortic lymph node (PALN) recurrences are rarely encountered from CRC, and consequently its treatment is not well established. Recently, Min *et al*[39] categorized PALN recurrence as a retroperitoneal malignancy, which in turn is a type of locoregional recurrence. Furthermore, several studies[16,40,41] have investigated the therapeutic efficacies of surgery for retroperitoneal, intraabdominal, and PALN recurrences, and several reported outstanding survival rates, which appear to have resulted from the selection of patients with a resectable mass at time of recurrence. In these studies, the reported 5-year survival rates approached a maximum of 56% after complete resection, whereas they ranged from 0% to 7% after incomplete resection. Because radical surgery is rarely feasible for PALN recurrence, they have usually been treated using chemotherapy.

SBRT FOR OLIGO-RECURRENCE WITHIN THE NODAL AREA FROM CRC

Radiobiological aspects of SBRT

SBRT may differ biologically from conventional RT, which is administered in small doses of 1.8-2 Gy per fraction over 6-8 wk. In addition to the direct cell killing within the high-dose region, vascular and stromal effects also likely contribute to tumor control[42]. Experimental models have demonstrated the importance of sphingomyelinase-mediated endothelial apoptosis of tumor cells when high-dose RT is used[43,44]. Another host factor of potential importance after a high single dose (or a few doses) of RT is the activation of the innate and adaptive immune responses against the tumor[45-47]. Lee *et al*[47] reported that a single ablative dose of radiation (20 Gy) to the tumor dramatically increased T-cell priming in the draining lymphatic tissues. This CD8(+) T-cell response was essential for the antitumor effects of irradiation and resulted in a reduction in primary tumor size and an abscopal effect[48,49] on distant metastases. The clearance of

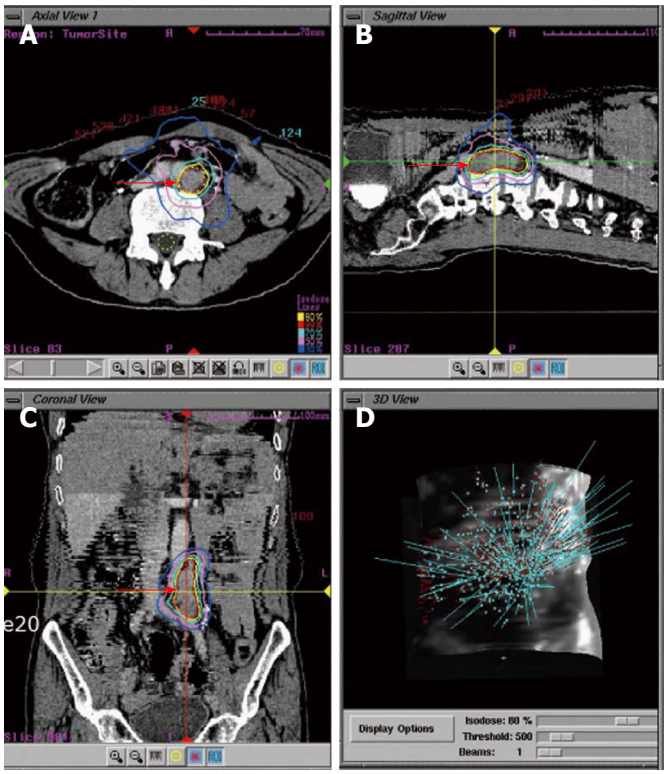

Figure 1 CyberKnife planning of stereotactic body radiotherapy for paraaortic lymph node metastases from colorectal cancer. A: Axial view 1; B: Sagittal view; C: Coronal view; D: 3D view. Gross tumor volume (red arrow) was defined as the visualized lymph node. The radiation dose, 48 Gy in 3 fractions, was prescribed to the 80% isodose line of the maximum dose in order to cover the planning target volume.

nonirradiated tumors after localized radiation therapy is known as the abscopal effect. Activation of an antitumor immune response has been proposed as a mechanism for the abscopal effect. The abscopal effect has been reported in several malignancies[50-52]. Stamell *et al*[52] reported a patient with metastatic melanoma who received palliative radiation to his primary tumor with subsequent clearance of all his nonirradiated in-transit metastases. Anti-MAGEA3 antibodies were found upon serological testing, demonstrating an association between the abscopal effect and a systemic antitumor immune response. While these antitumor effects hardly observed with conventional fractionated RT or with chemotherapy[53,54]. On the basis of these findings, the authors suggested that a new therapeutic strategy may be developed that combines RT with immunotherapy for oligometastasis.

Technical aspects of SBRT

Previously, the delivery of truly ablative doses of radiation has been limited by the risk to normal tissue, and the need for extended fractionation. However, SBRT utilizes stereotactic principles for dose localization and delivers multiple beams to well defined targets in a few fractions. As a result, this technique can deliver higher doses to tumors due to reduced mechanical error margins, and thus cause less normal tissue damage[55] (Figure 1). Regardless of the SBRT treatment delivery unit used, image-guided therapy enables verification of the location of the tumor or target volume before treatment delivery[56]. This image-guided therapy can be performed using three-dimensional volume imaging, using for example cone beam computed tomography (CT). If two-dimensional imaging is used, invasive fiduciary markers positioned in or close to the tumor are required. These image-guidance procedures substantially reduce treatment setup errors, using the tumor itself as a fiducial (frameless SBRT), and will in turn enable the planning target volume to be reduced.

Patient selection

The use of appropriate selection criteria for SBRT in the radical treatment of oligo-recurrence within nodal area remains crucial. In general, indications for SBRT are the same as those for metastasectomy, but without the limits imposed by the need for patients to be fit for surgery. In several reports, the eligibility criteria for SBRT for oligometastatic cancer were described as a limited number of metastases (between 1 and 5), a tumor diameter less than 4 cm, a locally controlled primary tumor, and no additional metastatic sites[57]. Other more specific and recently proposed criteria for the use of SBRT to treat patients with various oligometastatic tumors include a controlled primary tumor, a favorable histology, limited metastatic disease, a metachronous appearance of metastases, young age, and a good performance status[58-60].

As isolated or oligo-recurrence within nodal area is a very rare in CRC cases, clinical trials of SBRT for these recurrences are correspondingly also rare. Kim *et al*[55] published the results of a study in which SBRT was used to treat isolated PALN recurrence from CRC. The patients criteria for this study included a single conglomerate recurrent node or 2-3 recurrent nodes within 1 cm of each other; and excluded a tumor attached to the stomach or intestine (as determined by CT), or more than 3 separate affected LNs affected. This criteria is focusing to preserve normal tissue surrounding lymph node metastasis.

Table 1 Streotactic body radiotherapy for oligo-recurrence within nodal area in colorectal cancer

Ref.	Study year	No. of patients	Proportion of oligo-nodal metastases	SBRTdose (Gy)[1]; range (median)	Outcomes		
					LC	OS	Severe GI toxicity
Bae *et al*[69]	2012	41	44%	45-60 (48)	57% (5 yr)	38% (5 yr)	7%
Kang *et al*[68]	2010	59	53%	36-51 (42)	24% (5 yr)	29% (5 yr)	3%
Kim *et al*[55]	2009	7	100%	36-51 (48)	(-)	71% (3 yr)	14%
Kim *et al*[75]	2008	23	100%	30-51 (39)	74% (4 yr)	25% (4 yr)	4%
Hoyer *et al*[66]	2006	64	5%	45 (45)	63% (2 yr)	38% (2 yr)	5%

[1]Three fractions of streotactic body radiotherapy were used in all studies. LC: Local control; OS: Overall survival; GI: Gastrointestinal.

A further important consideration is the identification of patients with truly oligo-recurrence. Most published surgical oligo-recurrence series describe patients managed in an era before modern imaging techniques such as Positron emission tomography (PET)/computerized tomography (CT) became widely available. Thus, many patients were probably understaged, potentially leading to an underestimation of the effect of aggressive management on truly oligo-recurrence, since some of those patients treated aggressively would have had more extensive disease than was visible on CT or magnetic resonance imaging. Improved imaging will enable better selection of patients. Indeed, these advanced imaging methods (PET/CT scan) and molecular diagnostic techniques were used in some of the most recent studies[14] and are likely to have contributed to better patient selection and improved 5-year survival in this study compared with previous trials[15,61,62].

Clinical outcome

There is only a little published data on the treatment outcome of using SBRT for CRC oligo-recurrence within nodal area. An overview of published case series and phase 2 trials are presented in Table 1. However, several studies included cohorts that were too heterogeneous to evaluate the effect of SBRTs on these lesions. Greco *et al*[63] and Milano *et al*[64,65] studied heterogeneous in terms of the treated site or primary tumor histology. Hoyer *et al*[66] and Kim *et al*[55] studied including only a very small number of cases of nodal metastases although all enrolled patients had oligometastases from CRC.

In review of SBRT for oligometastases in all primary and all treated sites, Tree *et al*[67] indicated that generally around 20% of patients remain disease-free 2-4 years after treatment. Kang *et al*[68] reported the results of a study including 59 CRC patients with LN (31), lung (13), liver (10), and other (5) metastases, which were confined to 1 organ and treated by SBRT (median 42 Gy in 3 fractions). The 3-year overall survival, disease progression free survival, and local control rates were 49%, 25% and 66%, respectively, and the 5-year overall survival, disease progression free survival, and local control rates were to 29% and 19% and 24%, respectively. Focusing to the 31 patients with oligo-recurrence within nodal area, progression-free survival was 25% at 3 years and 19% at 5 years. In further study using high dose SBRT (median 48 Gy in 3 fractions) in same institute, Bae *et al*[69] reported better survival in the cohort of 41 CRC patients with LN (18), lung (12), and liver (11) metastases confined to a single organ. The 5-year overall survival, disease progression free survival, and local control rates were to 38%, 40% and 57%, respectively. The difference of outcomes between these studies may come from different dose of SBRT. These will be discussed further in the section of "SBRT dose".

Despite the heterogeneous nature of these studies with respect to the methods used to categorize oligometastatic disease from CRC, the findings indicate that a substantial proportion of patients, generally over 20%, remain disease-free 4-5 years after SBRT (Figure 2). These findings support the idea of an oligometastatic state in which aggressive local therapy could improve cause-specific survival.

SBRT dose

The efficacy of SBRT had primarily been investigated in the context of the treatment of early stage non-small cell lung cancer (NSCLC), in which disease a dose-control relationship has been established. Onishi *et al*[70] reported that the local control and survival rates for patients with stage I NSCLC were significantly better using a biologically effective dose larger than 100 Gy (α/β = 10 Gy). On the basis of this result, dose escalation was performed in a number of primary and metastatic cancer patients, and there were also efforts to escalate the SBRT dose to abdominal LN metastases from CRC. In the study conducted by Kim *et al*[55], the SBRT dose was escalated in a stepwise manner by 3 Gy from 36 Gy in 3 fractions. During escalation of dose, however, the 2 severe complication resulted in when 48 or 51 Gy was delivered in 3 fractions. They therefore did not escalate the radiation dose over 51 Gy during the treatment of paraaortic LN or pelvic LN. They also found that the radiation dose to tumor was a significant prognostic factor of overall survival. The median survival time was 32 and 72 mo with a SBRT dose of $\leqslant$ 42 Gy and > 42 Gy in 3 fractions, respectively. Bae *et al*[69] also found that SBRT dose was a significant prognostic factor for local control in multivariate analysis and that a dose of $\geqslant$ 48 Gy in 3 fractions resulted in a 5-year local control rate of 69%.

In several studies to evaluate SBRT result for oligometastases from heterogeneous primary cancers[71-75], all reports did not suggest that the SBRT dose was a prognostic factor of survival or local control. The SBRT

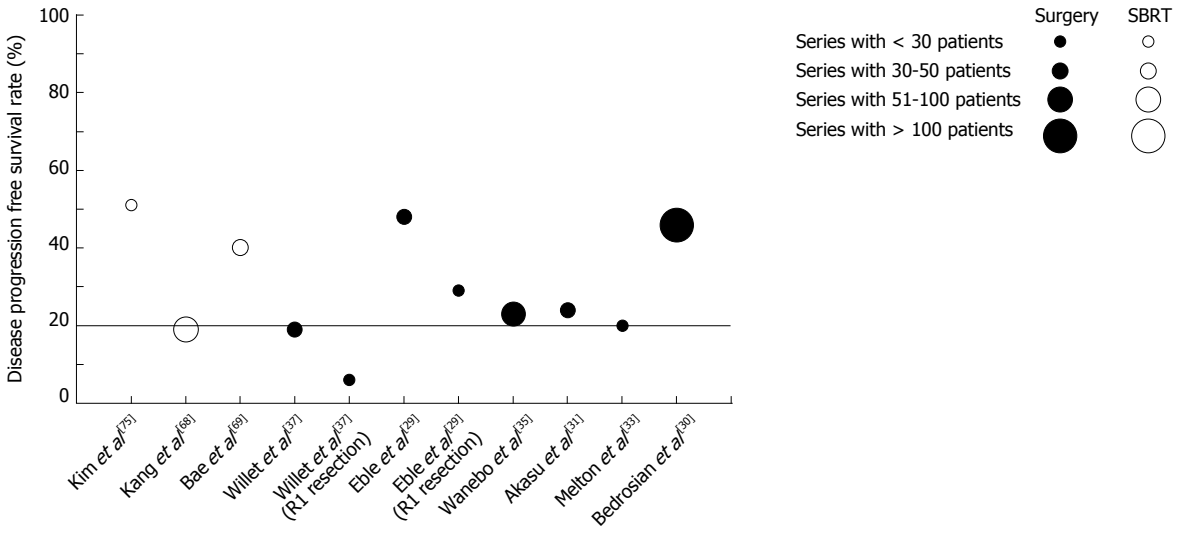

Figure 2 Disease progression free survival in patients with oligo-recurrence within nodal area from colorectal cancer treated with streotactic body radiotherapy or surgical resection. The cohort of Kang and Bae's studies are mostly composed of nodal metastases but additionally include lung and liver metastases. The cohort of surgical series include not only oligo-recurrence within nodal area but also central recurrence at anastomosis site. Dot size was weighted for number of patients in each cohort. SBRT: Streotactic body radiotherapy.

dose ranged from 30 to 51 Gy delivered in 3-6 fractions, and the highest dose was 51 Gy in 3 fractions[76]. Lower doses, such as those used successfully in the study by Bignardi *et al*[72], might be sufficient to eradicate viable tumor cells. Interestingly, Herfarth *et al*[77] performed a separate analysis of patients with metastatic disease and found that CRC metastasis had worse local control than metastases from other histological tumor types (45% *vs* 95%, respectively). In particular, in patients who had previously undergone systemic chemotherapy, tumors may have been radioresistant. Our data[55] support the radiocurative dose for metastases from CRC may be higher than those from other primary tumors as a result of induced cross-resistance from prolonged chemotherapy (discussed above)[77-79]. One hypothesis to explain these phenomenon may be Epidermal growth factor receptor (EGFR), which is reported to be overexpressed in approximately 70%-75% of CRCs[80]. A recent study using CRC-derived cell lines showed that cells with high constitutive EGFR-positive cells within a colorectal adenocarcinoma may have an intrinsic susceptibility to chemotherapy like oxaliplatin and 5-fluorouracil[81] as well as anti-EGFR agents. While, Khalifa *et al*[78] reported that recurrences following postoperative chemotherapy were approximately 5 times more likely to have lower levels of EGFR expression. In similar pattern, several studies have shown that an absence of EGFR expression is associated with radioresistance[82,83]. Furthermore, in a study of CRC treated with preoperative RT, Zlobec *et al*[79] reported that a complete pathological response was nearly 6 times more likely in EGFR-positive tumors than in EGFR-negative cases. In this point, the lower EGFR status of recurrent CRC after intensive chemotherapy may induce radio-resistance, requiring higher SBRT dose to achieve local control.

Results from a study of patients with oligo-recurrence within abdominopelvic nodal area suggested that a SBRT dose of more than 42 Gy in 3 fractions is a favorable prognostic factor for overall survival and local control, and dose escalation was recommended. However, there is as yet no consensus on the optimal dose and number of fractions, and further study with larger patient numbers is therefore required[55,69].

Toxicity

When oligo-recurrence within nodal area in the abdominopelvic area is treated with SBRT, the gastrointestinal tract is one of the most important dose-limiting organs. Since Timmerman *et al*[84] complied unvalidated normal tissue dose constraints for SBRT, most published studies have considered this recommendation or individual empiric data to be the permitted dose constraints for gastrointestinal toxicity. Surely, dosimetric parameters such as maximal point dose (D_{max}) and absolute volume of gastrointestine to receive some radiation dose, or fraction number affect complication. Unfortunately, because prospective study to control these variable factors were not available till now, there was no definite conclusion for gastrointestinal tolerance dose. Based on extensive experience to give SBRT to tumor located in abdominopelvic site, using 3 fraction, we suggested the dose constraint for gastroduodenum and intestine[85,86]. For severe gastroduodenal toxicity, D_{max} was found to be the best dosimetric predictor. A D_{max} of 35 Gy and 38 Gy were respectively associated with a 5% and 10% probability of the development of severe gastroduodenal toxicity. For intestinal toxicity, absolute volume to receive 20 Gy, 25 Gy, 30 Gy, or 35 Gy and D_{max} of the intestine were all the valuable predictor of severe toxicity. At D_{max} below 37 Gy, no severe intestinal toxicity was not detected. These tolerance dose are higher than expected for SBRT to some extent. Based on limited individualized clinical data, Kavanagh *et al*[87] and Rusthoven *et al*[88] suggested D_{max}

below 30 Gy in 3 fractions for stomach and intestine as the constraint. Timmerman *et al*[84] suggested D_{max} < 27 Gy in 3 fractions for the intestine and < 30 Gy for the colon, which based on the data of the biological effective dose using universal model, not validated by clinical data. One reason to cause discrepancy from these data based on dosimetric uncertainty. Intrafractional and interfractional gastrointestinal movement make it difficult to define accurate radiation dose of gastrointestine. In addition, as the volume of gastrointestine may vary according to the food consumed and respiration, the dose-volume histogram endpoint for pretreatment planning might not accurately reflect the actual dose distribution. In spite of these uncertainties, about D_{max} of 30 Gy in 3 fractions in gastroduodenum is supposed to be safe dose constraint

CONCLUSION

Oligo-recurrence within nodal area from CRC are rarely lethal in themselves. However, aggressive local treatment such as SBRT could prevent further extensive widespread metastatic disease. Several investigators have suggested that higher SBRT doses are associated with a better prognosis with respect to local control and survival. However, there is still no consensus on the optimal dose, number of fractions, or planning constraints. Given the radioresistant nature of CRC oligo-recurrence, increasing the SBRT dose may be a necessity, although because LNs are usually surrounded by radiosensitive normal tissue, the possibility of complications, especially gastrointestinal toxicity, should be carefully considered in treatment planning with SBRT for oligo-recurrence within nodal area in the abdominopelvic area. The constraints for the gastrointestinal tract and colon, a D_{max} of 30 Gy could prevent severe gastrointestinal toxicity during SBRT for tumors located in this area.

The outcomes of SBRT for oligo-recurrence within nodal area from CRC appear to be similar to those obtained after surgery despite the fact most studies have only included a small number of patients with a heterogeneous clinical profile. A substantial proportion of patients, generally over 20%, remain disease free 4-5 years after SBRT. This finding supports the idea of an oligo-recurrence state in which aggressive local therapy could improve the cure rate in appropriately selected patients. However, the general aim of oncological interventions for metastatic disease is not cure, but improvement in the quality of life and prolongation of overall survival. To this end, the use of SBRT, which is less invasive, better tolerated, and of a shorter duration than conventional radiation therapy, could have a number of advantages. These include the preservation of the quality of life through delaying further systemic treatment or preventing pain and prolonging survival through reducing subsequent metastatic spread to important organs.

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TOPIC HIGHLIGHT

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

CT colonography in the diagnosis and management of colorectal cancer: Emphasis on pre- and post-surgical evaluation

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Abstract

This article addresses the use of computed tomographic colonography (CTC) for the diagnosis and management of colorectal cancer, focusing on presurgical evaluation of the colon proximal to an occlusive cancer and surveillance after cancer resection surgery. The key evidences accumulated in the literature and future work needed are summarized. CTC is a technically robust and the most practical method to evaluate the colon proximal to an occlusive cancer, which prevents colonoscopic examination past the occlusion, either before or after metallic stent placement. The high sensitivity of CTC for detecting cancers and advanced adenomas in the proximal colon can help prevent additional surgical procedures in patients showing negative results. However, the accuracy of CTC for distinguishing intramural cancers from adenomas is low, and the technique is limited in guiding management when a medium-sized lesion that do not show invasive features such as pericolic extension or nodal metastasis is found in the proximal colon. A maximal diameter ⩾ 15 mm has been proposed as a criterion for surgical removal of proximal lesions. However, this needs to be verified in a larger cohort. In addition, the influence of presurgical CTC results on the current post-cancer resection colonic surveillance timeline remains to be determined. CTC can be readily added to the routine abdominopelvic CT in the form of contrast-enhanced CTC, which can serve as an effective stand-alone tool for post-cancer resection surveillance of both the colorectum and extracolonic organs. Although the accuracy of CTC has been demonstrated, its role in the current colonoscopy-based postoperative colonic surveillance protocols remains to be determined. Readers of CTC also need to be knowledgeable on the colonic lesions that are unique to the postoperative colon.

Key words: Computed tomographic colonography; Colonic cancer; Rectal cancer; Surgery; Colonoscopy

Core tip: Computed tomographic colonography (CTC) is technically robust and the most practical method to evaluate the colon proximal to an occlusive cancer either before or after metallic stent placement. Contrast-enhanced CTC may serve as an effective stand-alone tool for post-cancer resection surveillance of both the colorectum and extracolonic organs. However, several issues discussed in this article should be addressed further and clarified.

Hong N, Park SH. CT colonography in the diagnosis and management of colorectal cancer: Emphasis on pre- and post-surgical evaluation. *World J Gastroenterol* 2014; 20(8): 2014-2022 Available from: URL: http://www.wjgnet.com/1007-9327/full/v20/

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INTRODUCTION

Computed tomographic (CT) colonography (CTC) (also known as virtual colonoscopy) is a recently developed radiological imaging technology for the evaluation of the colorectum, enabled by advances in CT scan and three-dimensional image processing technologies[1,2]. CTC is less invasive and generally safer than optical colonoscopy[1,3]. CTC can visualize the lumen of the colorectum in various three-dimensional views in addition to the conventional colonoscopy-like endoluminal navigation as well as in two-dimensional multiplanar cross-sectional views[2,4,5]. This variety in visualization modes allows for accurate and efficient evaluation of the colorectum. Unlike optical colonoscopy, which is limited to the endoluminal examination of the colorectum, CTC enables the evaluation of extracolonic organs, particularly when performed with intravenous contrast enhancement. The clinical usefulness of CTC has been studied extensively, largely focusing on screening/surveillance of the general population for colorectal cancer, and CTC has repeatedly shown acceptably high accuracy comparable to colonoscopy for detecting clinically-relevant colorectal neoplasms[6-12]. Accordingly, CTC has now been included in the guidelines for colorectal cancer screening in several countries, for instance, the Joint Guideline from the American Cancer Society, the US Multi-Society Task Force on Colorectal Cancer, and the American College of Radiology[13] and Korean guidelines[14]. On the other hand, CTC has yet to be completely accepted as a tool for population screening in terms of reimbursement as CTC is only incompletely reimbursed in some countries[15,16]: the decision by the Centers for Medicare and Medicaid Services in the United States to deny coverage for CTC in the recent past was such an example[17,18]. Nevertheless, new clinical evidences and data have been accumulated and are likely to resolve the prior concerns regarding widespread adoption of CTC in population screening for colorectal cancer[19,20]. Likewise, CTC is steadily gaining clinical acceptance and increasingly utilized as a screening examination [13-15].

In addition to the role in general screening/surveillance for colorectal cancer, the dual function of CTC in colorectal and extracolonic evaluation suggests that this technique could be applicable to other clinical scenarios. One particular area of interest is the role of CTC in the management of patients who have already been diagnosed with colorectal cancer[21], and multiple studies have addressed this use of CTC, albeit not as extensively as the research on the general screening/surveillance role of CTC. The present review summarizes and discusses the results of such studies, placing emphasis on (1) the use of CTC for presurgical evaluation of the colonic segments proximal to an occlusive cancer preventing colonoscopic examination beyond the level of occlusion, and (2) the use of CTC for post-cancer resection surveillance. The review highlights key evidence accumulated in the literature and further work that needs to be done. This article does not address the general technical issues or principles of CTC, as these are already well explained in the literature elsewhere[1,2,21]. A few technical issues unique to the practice of CTC for such non-screening indications will be briefly addressed.

EVALUATION OF THE COLON PROXIMAL TO AN OCCLUSIVE CANCER

Patients with colorectal cancer may present with an occlusive mass that prevents colonoscopic examination beyond the level of the occlusion. Complete presurgical evaluation of the entire colon is important in patients diagnosed with colorectal cancer because identification of synchronous cancers, which are present in 1%-7% of these patients[22,23], may determine the extent of surgical resection. The presurgical diagnosis of these synchronous cancers is important to prevent a second surgery or even failure of curative treatment. Various options are available for proximal colonic evaluation, including double-contrast barium enema, CTC, intraoperative colonoscopy, and surgical palpation. Of these, CTC is currently regarded as the standard procedure (Figure 1). Double-contrast barium enema, despite its historical use for proximal colonic evaluation in occlusive colorectal cancers[24], has low sensitivity even in the absence of an occlusive cancer[25], in which case bowel preparation is relatively easier compared with in patients with occlusive cancer. In addition, barium is associated with a risk of barium desiccation in the colon proximal to an obstructing cancer. Intraoperative colonoscopy is possible but is not a practical option[26]. By contrast, CTC is a technically robust method that can be performed successfully if the insufflated gas can be delivered across the tumor-induced occlusion to adequately distend the colonic segments proximal to the occlusion. This is in contrast to colonoscopy, which requires the passage of the scope across the narrowing. Therefore, almost all cases of failed colonoscopy due to occlusive cancer can be examined successfully with CTC using the low-pressure carbon dioxide colon insufflation system widely adopted for screening CTC[27-29]. CTC is known to be a safe procedure, particularly when it is performed using the low-pressure carbon dioxide insufflation, as the reported rates of overall procedure-related colonic perforations ranged from 0.009% to 0.06% and nearly all the perforated cases were associated with manual insufflation[3,30-32]. However, the data were largely from screening CTC practices or from patients who did not have colonic obstruction; and, in fact, there is no large data regarding the risk of colonic perforation of CTC performed for patients with an occlusive cancer. The majority of the reported cases of colonic perforations associated with CTC had underlying colonic lesions including inflammatory and/or obstructive lesions[31,33,34]. Also, according to a recent systematic review, large bowel

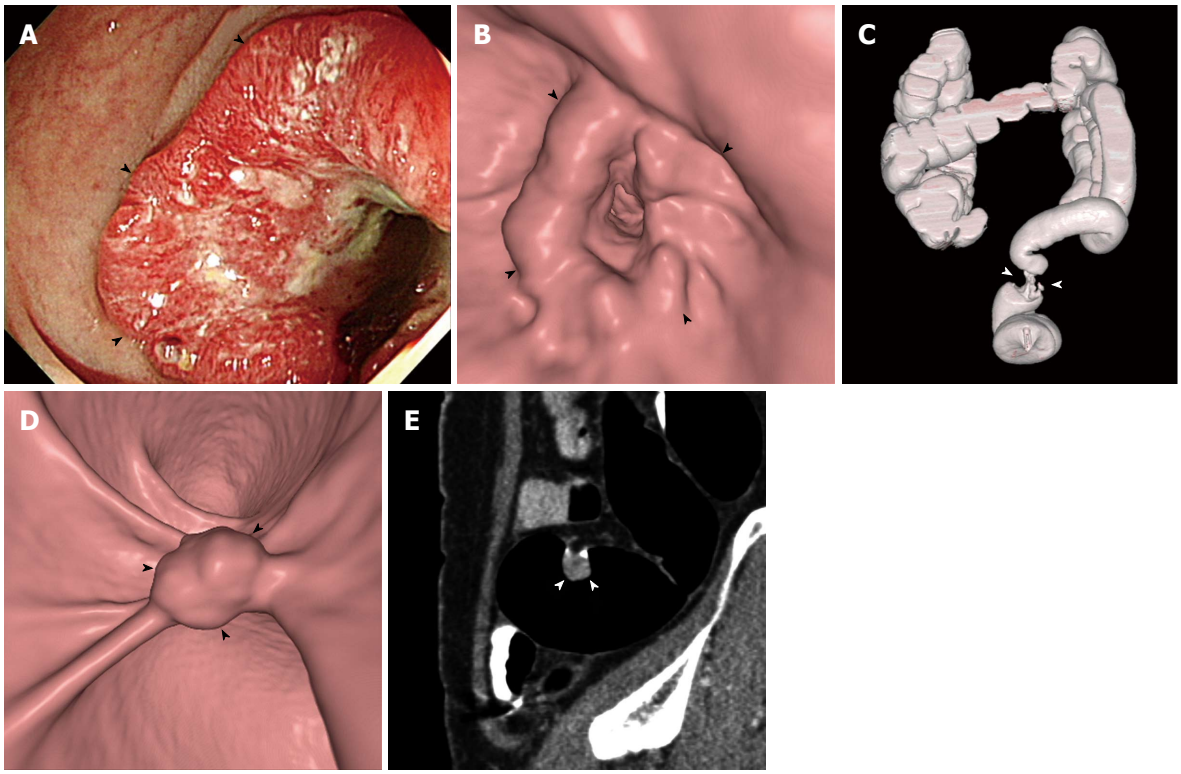

Figure 1 A 55-year-old woman with occlusive cancer in the upper rectum and a 17-mm synchronous cancer in the sigmoid colon. A-C: Colonoscopic (A), three-dimensional endoluminal computed tomographic (CT) colonographic (*i.e.*, virtual colonoscopic) (B), and three-dimensional volume-rendered (C) images of the colon show a luminal-encircling occlusive mass (arrowheads) in the upper rectum that impeded the passage of a colonoscope; D, E: Three-dimensional endoluminal (D) and two-dimensional sagittal (E) CT colonographic images show a 17-mm polyp (arrowheads) unassociated with invasive features in the sigmoid colon. The lesion was removed by surgery and pathologically confirmed as a cancer confined to the mucosa.

Table 1 Computed tomographic colonography accuracy for diagnosing synchronous cancers in the colon proximal to an occlusive cancer

	Patients with occlusive cancer	Sensitivity			Specificity
		Target lesions	Per-patient	Per-lesion	
Park *et al*[28]	284	Adenocarcinoma	100% (6/6)	100% (8/8)	87.9 (181/206)
		Advanced neoplasia[1]	88.6% (39/44)	80% (52/65)	
Fenlon *et al*[35]	29	Adenocarcinoma	100% (2/2)	100% (2/2)	NA
Neri *et al*[36]	17	Adenocarcinoma	100% (3/3)	100% (3/3)	NA
Coccetta *et al*[37]	43	Adenocarcinoma	100% (1/1)	100% (1/1)	NA
Galia *et al*[38]	19	Adenocarcinoma	100% (2/2)	100% (2/2)	NA
Kim *et al*[39]	67	Adenocarcinoma	100% (3/3)	100% (3/3)	95 (NA)

Data are percentages with the actual numbers of patients and lesions are presented in parentheses. [1]Advanced neoplasia includes both advanced adenomas (≥ 10 mm in size or with a substantial villous component or high-grade dysplasia) and adenocarcinomas. NA: Not available.

obstruction is among the risk factors for colonic perforation following CTC[33]. Therefore, more careful attention while performing the procedure would be prudent.

Several studies have investigated the accuracy of CTC for detecting synchronous colonic lesions proximal to an occlusive cancer, and have demonstrated a high sensitivity of CTC for the detection of proximal synchronous cancers[28,35-39](Table 1). Most of these studies were preliminary studies that included a small number of patients[35-39]; however, one recent large study (the largest report thus far)[28] included 427 consecutive patients with stenosing colorectal cancer, of which 284 were ultimately analyzed to determine the accuracy of CTC. The results showed 100% and 88.6% sensitivities of CTC for detecting patients harboring synchronous colorectal cancer and advanced neoplasia (*i.e.*, advanced adenoma[40] or cancer), respectively, in the proximal colon. As a result, the corresponding negative predictive values of CTC (*i.e.*, the probability of the proximal colon being devoid of the lesions when CTC is negative) were 100% for proximal synchronous cancer and 97.4% for advanced neoplasia. Therefore, negative CTC findings in the proximal colon exclude the need for additional surgical procedures in the proximal colon with high confidence. These results are highly promising. Nevertheless, given the low prevalence of proximal synchronous cancers[22,23], future multi-

institutional efforts aimed at accumulating additional data and evidence would be indicated.

Another advantage of CTC to this particular group of patients is that it can serve as a one-stop examination for the proximal colonic evaluation as well as for overall pretreatment cancer staging of the abdomen and pelvis when performed with intravenous contrast enhancement. Contrast-enhanced CTC is essentially the same imaging method as the routine contrast-enhanced abdominopelvic CT used for abdominopelvic staging of colorectal cancer[41,42], except for the use of gaseous colonic distention in the former. Therefore, the two methods are expected to be similarly effective and accurate for tumor staging, although published data on the accuracy of contrast-enhanced CTC for general TNM staging of colorectal cancers are limited. According to several published studies, the accuracy of contrast-enhanced CTC for tumor staging is 83%-95% for T-staging, 80%-85% for N-staging, and 100% for M-staging[43-46].

Despite the high accuracy of CTC for detecting synchronous lesions in the colon proximal to an occlusive cancer, the clinical impact of CTC in the management of occlusive cancer patients remains a bit unclear. First, even if CTC accurately detects proximal colonic lesions, unless it can clearly tell which of the detected lesions should be removed by surgery rather than endoscopy after resection of the occlusive cancer, the patient management remains ambiguous. The distinction would be straightforward for small polyps (*i.e.*, endoscopic removal) or large invasive advanced cancers (*i.e.*, surgical excision). However, it is difficult for CTC to distinguish adenomas from relatively small medium-sized cancers confined within the colonic wall without pericolic extension or nodal metastasis[28]. Therefore, a certain degree of over-interpretation (*i.e.*, overcalling noncancerous polyps as cancers) or under-interpretation (*i.e.*, undercalling small cancers as noncancerous polyps) at CTC, which may result in unnecessarily extensive surgery or repeat colonic surgery, respectively, seems inevitable. Robust criteria for the selection of surgical removal versus postsurgical endoscopic resection for a proximal colonic lesion detected by CTC remain to be developed. One study[28] suggested a maximum lesion diameter of 15 mm or greater as the criterion for surgical removal, which yielded 87.5% sensitivity and 70% positive predictive value for proximal synchronous cancers. The need for specific characterization of the colonic lesions detected by CTC is a unique aspect of CTC performed in occlusive cancer patients. By contrast, the general screening/surveillance CTC is only concerned with detecting colonic lesions, as its key clinical role is to determine who should be sent for colonoscopy. Secondly, it is unclear if and how the adoption of CTC in the presurgical evaluation of occlusive colorectal cancer patients should affect the current postsurgical colonoscopic surveillance timeline. The current guidelines for the management of colorectal cancer (as proposed by The National Comprehensive Cancer Network, the American Cancer Society, and the US Multi-Society Task Force on Colorectal Cancer) stipulate that early postoperative follow-up colonoscopy to evaluate the proximal colon should be performed 3-6 mo after surgical removal of an occlusive cancer in addition to the routine colonoscopic surveillance approximately 1 year after surgery or perioperative clearance of the colon[41,42,47]. These "current" guidelines are largely based on the data and experience from the pre-CTC era. Given the higher accuracy of CTC compared with other methods, particularly the high sensitivity of CTC for detecting cancer that is approaching 100%[48], negative preoperative CTC findings in the proximal colon could potentially provide a confident clearance for the proximal colon and could potentially eliminate the need for early postoperative colonoscopy. If this notion is proven, it would help reduce redundancy and the costs of postsurgical colonic surveillance, and would also mean a substantial convenience factor for patients who are recuperating from major surgery. Further investigations in this area would therefore be worthwhile.

PROXIMAL COLONIC EVALUATION AFTER METALLIC STENT PLACEMENT

Patients with advanced colorectal cancer causing acute severe colonic obstruction require urgent decompression to avoid colonic perforation. Self-expandable metallic colonic stents are currently widely used in patients with acute severe colonic obstruction caused by colorectal cancer, as a bridging treatment to one-stage elective surgery[49,50]. In these cases, proximal colonic evaluation requiring passage through the metallic stent to find synchronous colonic lesions becomes an issue[51-53]. Colonoscopy involving passage through the stent can be performed safely without any major complications and a success rate of 88.9%-93.4% has been reported[51,52]. However, the extent of clinical application of this procedure is unknown. Among the concerns raised, long-term instrumental damage to a colonoscope caused by passing it through a metallic stent appears to be one important reason for the reluctance in performing colonoscopy under these conditions[54]. CTC could provide an alternative tool for this diagnostic task. According to one study[53], which included 50 consecutive patients who underwent CTC after metallic stent placement for acute severe cancer obstruction, CTC was performed adequately in 94% of the patients using the standard techniques used for screening or other indications and no procedure-related adverse events were reported. Although the diagnostic performance of CTC in this setting was not evaluated thoroughly because of the small number of patients analyzed, the preliminary results were promising. The per-patient and per-lesion sensitivities for lesions 6 mm or larger in diameter in the colon proximal to the stent were 90% and 85.7%, respectively, and CTC correctly identified two proximal synchronous cancers present in the study cohort[53]. One potential diagnostic pitfall noted in the study was some degree of lesion obscuration by colonic obstruction-related mural edema[53], which may need further clarifica-

Table 2 Computed tomographic colonography accuracy for colonic surveillance after colorectal cancer resection

	Patients		Sensitivity			Specificity
	n	Characteristics	Target lesions[1]	Per-patient	Per-lesion	
Amitai *et al*[64]	29	Routine surveillance	(Peri) anastomotic recurrence	100% (2/2)	100% (2/2)	NA
			Metachronous polyps	100% (NA)	93% (28/30)	71% (NA)
Fletcher *et al*[65]	50	Routine surveillance	(Peri) anastomotic recurrence	100% (2/2)	NA	94% (45/48)
			Metachronous polyps ≥ 5 mm	60% (3/5)	NA	84% (38/45)
You *et al*[67]	80	Suspicion of recurrence	(Peri) anastomotic recurrence	100% (51/51)	100% (51/51)	83% (24/29)
Kim *et al*[68]	548	Routine surveillance	Metachronous cancer and (peri) anastomotic recurrence	100% (6/6)	100% (7/7)	93.1% (421/452)
			Advanced neoplasia[2]	81.8% (18/22)	80.8% (21/26)	
			All adenomatous lesions[3] ≥ 6 mm	80% (52/65)	78.5% (62/79)	

Data are percentages with the actual numbers of patients and lesions are presented in parentheses. [1]Histology and size are not specified unless provided in the original studies; [2]Advanced neoplasia includes both advanced adenomas (≥ 10 mm in size or with a substantial villous component or high-grade dysplasia) and adenocarcinomas; [3]Both adenomas and adenocarcinomas are included. NA: Not available.

tion. Furthermore, a technical consideration is that an additional scout CT scan of the abdomen and pelvis using low-dose radiation prior to gaseous colonic distention is recommended in this group of patients to detect any clinically silent colonic perforation, given the relatively high risk of colonic perforation associated with the metallic stent placement procedure (3.8% according to one systematic review[50]). The scout scan would be a prudent step to avoid the risk of exacerbating a clinically silent perforation by inadvertently performing CTC.

POST-CANCER RESECTION SURVEILLANCE

Colorectal cancer is unique in that, unlike other gastrointestinal malignancies, timely second curative-intent treatment of the recurred/metastatic cancer that developed after the initial curative-intent treatment can improve the ultimate patient survival[55-57]. Therefore, preemptive (*i.e.*, performed for all postsurgical patients regardless of their symptoms) surveillance for recurrent disease after curative-intent treatment of colorectal cancer is crucial in the management of this disease. Both colonic and extracolonic surveillance are important, as the recurrent disease may occur in any location. Most recurrences occur as distant extracolonic metastatic disease and, in the case of local or (peri-)anastomotic recurrence, more often than not without an intraluminal colonic component[58-60]. As a result, current postsurgical surveillance guidelines generally include a combination of clinical assessment, serum carcinoembryonic antigen measurement, colonoscopy, and contrast-enhanced CT[41,42,61]. Considering that contrast-enhanced abdominopelvic CT is already a standard postoperative surveillance examination[41,42], and that CTC can be readily added to the routine abdominopelvic CT in the form of contrast-enhanced CTC, which would effectively cover both the colorectum and extracolonic organs simultaneously, contrast-enhanced CTC may potentially represent an attractive stand-alone examination for combined colonic and extracolonic postoperative surveillance of colorectal cancer patients[62,63]. Adding the essential colonographic techniques (*i.e.*, bowel preparation and colonic distention) to contrast-enhanced abdominopelvic CT would not incur much extra cost, another hospital visit, or other complexity in patient management.

At present, a relatively small amount of data regarding the use of CTC as a tool for post-cancer resection surveillance exists (Table 2), and most such research reports were feasibility studies in nature that only included a small number of patients[64-67]. On the other hand, one recent study[68] analyzed a large retrospective cohort of 742 consecutive patients who had no apparent clinical or laboratory evidence of recurrent disease after curative-intent colorectal cancer surgery and underwent contrast-enhanced CTC for postsurgical surveillance[68]. In the study, the per-patient sensitivity of CTC was 100% for metachronous or anastomotic recurrent cancers and 81.8% for advanced neoplasia. The corresponding negative predictive value of CTC was 100% for metachronous or anastomotic recurrent cancers and 99.1% for advanced neoplasia. The maximum referral rate for colonoscopy after CTC in this asymptomatic postsurgical population was 19%. These results imply that performing CTC as an adjunct to the routine postsurgical contrast-enhanced abdominopelvic CT could theoretically prevent surveillance colonoscopy in as much as approximately 80% of the patients (on an assumption that colonoscopy is to be performed at a similar time to CT) by confidently excluding those patients who would not need colonoscopy because they do not harbor advanced neoplasia or cancer. As the frequency and timing of surveillance colonoscopy and surveillance abdominopelvic CT do not always coincide in the real-world clinical setting, the actual benefit of contrast-enhanced CTC would be smaller. However, the study at least demonstrated that CTC could be a viable alternative to colonoscopy for postsurgical surveillance and may therefore help decrease the burden or redundancy of the colonoscopic surveillance.

Although CTC may have diagnostically acceptable accuracy for postsurgical colonic surveillance, how it may fit into the current colonoscopy-based colonic surveillance practice remains to be determined. The current guidelines for colonic surveillance recommend

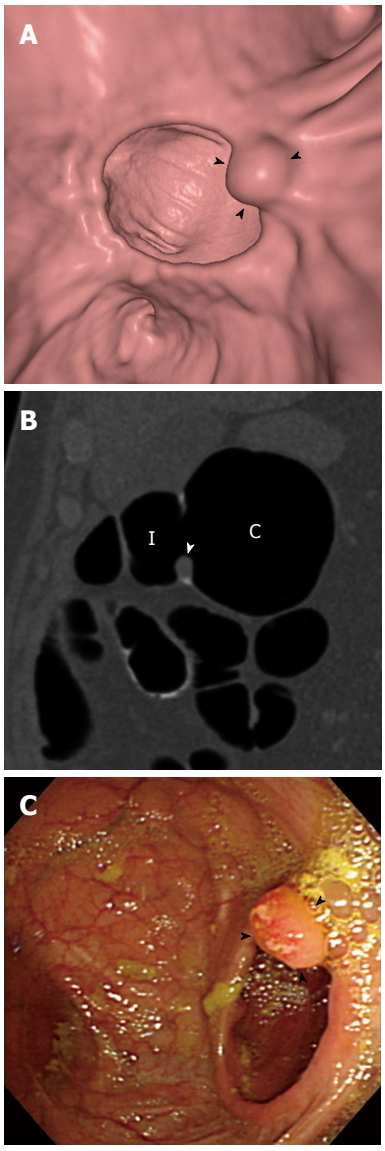

Figure 2 A 79-year-old man with a 9-mm inflammatory polyp at ileocolic anastomosis. Three-dimensional endoluminal (A) and two-dimensional coronal (B; I = Ileum and C = Colon) computed tomographic colonographic images and a colonoscopy image (C) obtained 3 years after colorectal cancer resection show a well-defined sessile polypoid lesion (arrowheads) at the anastomotic line. Colonoscopic biopsy revealed nonspecific chronic inflammation with edema and no evidence of tumor recurrence.

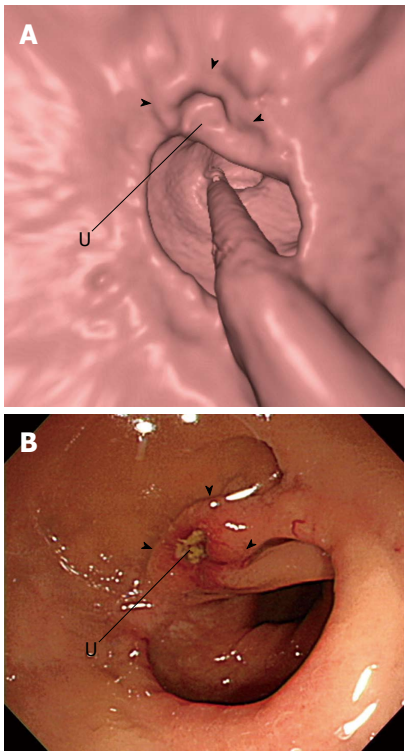

Figure 3 A 66-year-old man with a 16-mm ulcerating anastomotic recurrence. Three-dimensional endoluminal computed tomographic colonographic (A; U = Ulcer) and colonoscopic (B; U = Ulcer) images obtained 10 mo after cancer resection surgery show an ill-defined elevated lesion with central ulceration (arrowheads) at the anastomosis site. Subsequent surgical resection and pathologic analysis confirmed recurrent adenocarcinoma (reprint with permission[68]).

colonoscopy at 1 year after the curative-intent surgery or after perioperative colonoscopic clearance of synchronous lesions, then in 3 years if negative at 1 year, and every 5 years if negative at the prior colonoscopy[41,42,47]. However, as revealed in a recent study[69], postsurgical colonoscopies are being performed more frequently than recommended by the guidelines at many institutions. Considering the relatively higher rates of metachronous cancers in the early postsurgical period[47], the use of colonoscopy for surveillance during the early postsurgical period, such as at 1 year, and CTC at later times may be appropriate. In addition, because CTC is less sensitive for small and subtle lesions than colonoscopy, while colonoscopy has a greater amount of blind areas compared with CTC[70], the alternating use of colonoscopy and CTC for postsurgical surveillance may be worth investigating, as it could capitalize on their complementary strengths and may contribute to improved patient survival. Another issue that may need to be addressed for the successful implementation of CTC in post-cancer resection surveillance is the reader familiarity with colonic lesions that are unique to the postoperative colon and are unencountered in general screening practice, including anastomotic inflammatory polyps (Figure 2) and anastomotic recurrences (Figure 3). Inflammatory polyps are by far the most common type of polypoid lesion occurring in the anastomosis that do not require treatment and typically manifest as well-circumscribed discrete 5- to 15-mm polyps located in the anastomotic line[62,71]. Anastomotic recurrent tumors may present as friable mucosa, irregular mucosa with shallow ulceration, sessile-to-flat infiltrative lesions, or luminal stenosis instead of showing mass-like or polypoid appearance, as they do not develop through the polypoid growth of the adenoma-carcinoma sequence[62,72].

CONCLUSION

In summary, CT colonography has important current and potential roles in the management of patients who have been diagnosed with colorectal cancer. It is technically robust and the most practical method for the evaluation of the colon proximal to an occlusive cancer, either before

or after metallic stent placement. CT colonography may also serve as an effective stand-alone tool for post-cancer resection surveillance of both the colorectum and extra-colonic organs.

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TOPIC HIGHLIGHT

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

Current issues in locally advanced colorectal cancer treated by preoperative chemoradiotherapy

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Abstract

In patients with locally advanced rectal cancer, preoperative chemoradiotherapy has proven to significantly improve local control and cause lower treatment-related toxicity compared with postoperative adjuvant treatment. Preoperative chemoradiotherapy followed by total mesorectal excision or tumor specific mesorectal excision has evolved as the standard treatment for locally advanced rectal cancer. The paradigm shift from postoperative to preoperative therapy has raised a series of concerns however that have practical clinical implications. These include the method used to predict patients who will show good response, sphincter preservation, the application of conservative management such as local excision or "wait-and-watch" in patients obtaining a good response following preoperative chemoradiotherapy, and the role of adjuvant chemotherapy. This review addresses these current issues in patients with locally advanced rectal cancer treated by preoperative chemoradiotherapy.

Key words: Colorectal cancer; Rectal cancer; Preoperative chemoradiotherapy; Conservative; Response

Core tip: In the era of preoperative chemoradiotherapy for rectal cancer, issues such as treatment plan according to response which included application of organ preserving strategies, prediction of response, and role of adjuvant treatment were need to be discussed under circumstances that preoperative chemoradiotherpay spread widely as a standard treatment of rectal cancer.

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INTRODUCTION

Preoperative chemoradiotherapy (PCRT) has been used increasingly to treat locally advanced rectal cancer since it was proven to be beneficial in reducing the rate of local recurrence. A German trial[1] has reported that patients treated with PCRT had significantly lower local failure rates and toxicity rates than those receiving postoperative chemoradiotherapy (CRT), and PCRT was also found to produce a better outcome in terms of sphincter preservation. These findings led to a paradigm shift from postoperative to preoperative CRT so that PCRT has now become the standard treatment for cT3-4 and/or node-positive rectal cancer. This shift has however raised a series of concerns that have practical clinical implications such as a prediction of the responsiveness to PCRT, the application of conservative management such as local excision in patients obtaining a good response to this intervention, sphincter preservation, and the role of adjuvant chemotherapy. In this review, we discuss these issues.

Table 1 Local excision after preoperative chemoradiotherapy for rectal cancer *n* (%)

Ref	Year	*n*	inclusion	Complete remission	Local recurrence	Follow-up duration, mo	Overall survival
Kim *et al*[10]	2001	26	cT2-3 CR after PCRT	17 (65.4)	1 (3.8)	19	100%
Bonnen *et al*[11]	2004	26	cT3N0 or N1 CR after PCRT	14 (53.8)	2 (7.7)	46	5 yr OS; 85%
Huh *et al*[58]	2008	9	cT2-3N0 or N1	4 (44.4)	1 (11.1)	91	10 yr OS; 88.9%
Nair *et al*[59]	2008	44	cT2-3N0 or N1	19 (43.2)	4 (9.1)	64	5 yr OS; 84
Guerrieri *et al*[9]	2008	145	CR after PCRT cT2-3N0	17	8 (4)	81	100% (pT0-1) 90% (pT2) 77% (pT3)
Kundel *et al*[60]	2010	14	CR after PCRT	All	0	47	100%
Yu *et al*[17]	2013	40	cT2-3N0	19 (47.5)	4 (7.5)	38	3 yr DFS: 85.9%
Perez *et al*[61]	2013	27	cT2-3N0-2	3 (11.1)	4 (14.8)	15	1 yr DFS: 68%

CR: Complete remission; PCRT: Preoperative chemoradiotherapy (CRT); OS: Overall survival; DFS: Disese-free survival.

ORGAN PRESERVING STRATEGIES

Local excision

Although the standard management of locally advanced rectal cancer treated by PCRT is radical surgical resection, conservative management (local excision or close observation) has been used in some cases. The local excision of rectal cancer has been employed as surgical procedure for patients with early rectal tumors limited to the mucosa and submucosa. In early T1 tumors without high risk features, full thickness local excision alone has been shown to produce comparable long-term outcomes to radical surgery[2]. Complete regression of the tumor was reported to occur in up to 20% of patients with rectal cancer after PCRT[3-6]. Some investigators have performed local excisions to avoid possible morbidities such as permanent stoma formation and functional impairments in patients who showed a good response to PCRT, with many studies reporting that such cases subsequently had acceptably low rates of local recurrence and long-term survival outcomes comparable to radical surgery[7-11]. The promising results from these studies have encouraged interest in the possibility of avoiding radical surgery in some patients after PCRT and thus preserving sexual and urinary function, sparing rectal function, and, in cases of low rectal cancer, avoiding permanent stoma (Table 1).

However, the interpretation of the above data is confounded by the predominantly retrospective nature of the studies on rectal cancer to date. Moreover, these earlier studies cannot be directly compared due to the significant heterogeneity with respect to patient and tumor characteristics resulting from a lack of consistent staging and selection criteria. In addition, no mesorectal lymphadenectomies were undertaken for these previous study cohorts and the lymph node stages were undefined. More importantly, the extent and quality of the local surgery is likely to have significantly varied between studies, depending on the individual techniques used and the skills of the surgeons involved.

One of the great uncertainties when conducting local surgery is the status of the mesorectal lymph nodes. Some studies have confirmed that there can be differential responses between the primary tumor and the mesorectal lymph nodes[12,13]. The proportion of lymph node metastases reported in pathological complete response (pCR) cases is low, with a median rate of 7% ranging from 2% to 11%[12-14]. The potential caveat of using mural response as the only criterion for selecting patients for local excision was highlighted in a retrospective study of 242 patients following PCRT[15]. The incidence of lymph node involvement was 3.2% in patients developing mural pCR (ypT0) compared to 11% for ypT1 tumors and increased further as the ypT stage increased (ypT2 = 29.2%; ypT3 = 37.3%). When nodal involvement is understaged and patients undergo local excision, the prognosis is poorer. Recently, the American College of Surgeons Oncology Group has completed the Z6041 phase Ⅱ trial of patients with clinical T2N0 rectal cancer who received PCRT (total dose, 54 Gy) with capecitabine and oxaliplatin followed by transanal local excision 6 weeks after the completion of PCRT[16]. Of the 77 patients in that report who underwent local excision, 34 achieved a pCR (44%), 49 (64%) had ypT0-1, and 4 (5%) had ypT3 tumors. All but one patient had negative margins. Acute toxicity of at least grade 3 during PCRT occurred in 39% of these patients, and rectal pain was the most common postoperative complication. Colorectal Cancer Study Group in Korea also reported results of multicenter study for local resection after PCRT[17]. They reviewed 40 patients with cT2-3N0M0 treated with PCRT followed by local excision retrospectively. Among them, Four patients (7.5%) had recurrence [local recurrence (1 patient) and systemic metastasis (3 patients)]. The 3-year disease-free survival rate was 85.9%. Only pCR was a recurrence-related prognostic factor (P = 0.040). Based on these findings, a longer follow-up is clearly needed to assess the oncologic outcome. Moreover, local excisions need to be performed with great care for sub-group of patients and credible methods to measure the treatment response or remaining disease after PCRT are required.

"Wait and watch"

Possibly the other challenge for improving conservative

treatment regimens for rectal cancers is to try to preserve not only the anal sphincter but also the whole organ. Habr-Gama is proposing a strategy comprising PCRT and "watch and wait" in cases of a clinical complete response (cCR) with no radical surgery[18]. Data from a Brazilian series have demonstrated excellent long-term local control and OS rates in patients developing cCR after PCRT[18]. The long-term outcome of the observation group (5-year OS 100%, DFS 92%) was similar to that of the resection group (5-year OS 88%, DFS 83%) with a histologic complete response.

The ability to identify patients with a cCR who are also likely to have a pCR would have major clinical implications. If such information were available and accurate, it could obviate the need for radical surgery and possibly prevent a permanent stoma in selected patients. The limitations of clinical assessments after PCRT were demonstrated in a prospective series of 94 patients who underwent an assessment with digital rectal examination (DRE) and sigmoidoscopy both prior to and after the completion of PCRT[19]. These clinical assessments underestimated the pathologic response in 73 patients and DRE was able to identify only three of 14 cases (21%) with a pCR. The overall concordance between clinical evaluation and actual pathologic response was only 22%[19]. In another retrospective review of 488 patients with rectal cancer following PCRT, the cCR rate for the entire cohort was 19%, but only 10% had a true pCR[20]. Glynne-Jones *et al*[21] reviewed 218 phase Ⅰ / Ⅱ and 28 phase Ⅲ trials of preoperative radiotherapy or PCRT. They concluded that a clinical and/or radiological response does not sufficiently correlate with the pathologic response to recommend a 'wait and see' approach to surgery following preoperative therapy.

It is not surprising therefore that the Brazilian experience has generated intense debate with some investigators expressing concerns about employing a policy of watchful expectancy based entirely on the presence of cCR after PCRT[22,23].

It is notable that other investigators have been unable to reproduce these aforementioned results. Hughes *et al*[22] reported a 60% intrapelvic recurrence rate in 10 cases with a cCR and concluded that a 'wait and see' policy could not be justified in T3 or 4 rectal cancers after PCRT. Nakagawa *et al*[24] also reported a high (80%) local recurrence rates and suggested that an exclusive PCRT approach is not safe for treating patients with low locally advanced rectal cancer. Such a strategy, however, could be of specific interest in elderly and vulnerable patients who are not fit for conventional surgery. It is possible that (full thickness) trans-anal local excision could be more relevant than observation alone after PCRT in such cases. Some phase Ⅱ and Ⅲ trials (ACOSOG Z 6041; GRECCAR 2; CONTEM 2) are currently ongoing to test this strategy.

PREDICTION OF TREATMENT RESPONSE

The response to PCRT differs among individual tumors and there currently is no effective method of predicting which patients will respond favorably to this treatment. Although positive responders to PCRT will experience the benefits of this intervention approach, patients who do not respond to PCRT will be exposed to unnecessary toxicities and surgery delay. It is therefore of the utmost importance to predict the treatment response and outcomes before initiating PCRT. Although a number of postsurgical prognostic factors have been proposed, patients with pCR after PCRT cannot at present be predicted by clinical examination or radiologic imaging procedures. The identification of basal resistance biomarkers could offer great help in this regard. Directed strategies that explore individual markers have not so far yielded clinically validated assays[25-27]. Past efforts to develop a predictive assay of tumor radio-sensitivity have been recently reviewed[28] and can be grouped into three categories: assays to determine intrinsic radiosensitivity (*ex vivo* determination of tumor survival fraction at 2 Gy)[29-32]; assays to determine tumor oxygen levels (electrodes to measure tumor pO2)[33,34]; and determination of tumor proliferative potential[35,36]. Unfortunately, although initial clinical data supported each of these approaches, none has become routine. A central reason for this has been that all of these approaches are highly impractical as a routine clinical application. The generation of high-throughput data sets has provided an opportunity to address the identification of biomarkers from a different perspective.

ADJUVANT CHEMOTHERAPY IN ADDITION TO PCRT AND SURGERY

There is no uniform agreement regarding the role of chemotherapy in addition to PCRT although current guidelines recommend additional adjuvant chemotherapy after PCRT regardless of the tumor response. Since most locally advanced rectal cancer patients have pathologically negative nodes following PCRT, some clinicians have argued that systemic therapy is not indicated. This argument is in part due to the lack of a proven survival benefit of chemotherapy in node negative colon cancer cases. The controversy is further illustrated by the fact that the European Organization for the Research and Treatment of Cancer (EORTC) is conducting a phase III trial in which patients are randomized to receive either 5-fluorouracil (5-FU) based chemotherapy or no further therapy following PCRT and radical resection.

The authors of the EORTC 22921 study reported that subgroups of patients achieving a pCR or who were downstaged to a ypT1-2 tumor category after preoperative radiation, benefited from adjuvant chemotherapy, whereas those with residual ypT3-4 disease did not[37]. These authors suggested the beneficial effects of adjuvant chemotherapy based on pathologic results, but they analyzed ypT and ypN categories separately. They also reported that adjuvant chemotherapy provided a benefit in patients who received a ypT downstage, but not in ypN0 or ypN-positive patients. Some data did not

confirm results of EORTC 22921 especially in terms of the effect of adjuvant chemotherapy on patients achieving pCR[38,39]. Chemotherapy is rarely indicated when the 5-year free-from recurrence rate exceeds 95%, which occurs in a complete pathological response. Considering the favorable outcome of patients with a complete response, survival outcomes with adjuvant chemotherapy is difficult to be improved than those of patients without adjuvant chemotherapy.

When evaluating subgroups of patients who may or may not benefit from adjuvant therapy after PCRT followed by resection, the benefit of adjuvant therapy for node-negative patients on final pathologic staging (ypN0) would be expected to be especially questionable. There is a paucity of information in the literature on whether adjuvant therapy improves survival for locally advanced rectal cancer patients with a stage ypN0 tumor. These findings are consistent with the suggestion by Fietkau *et al*[39] that postoperative chemotherapy may be unnecessary in ypN0 cases. Das *et al*[40] have insisted that postoperative chemotherapy may be of greater benefit for high-risk patients. However, their results are contrary to those of Janjan *et al*[41], who found a significant improvement in cancer-specific survival in response to PCRT and the addition of postoperative chemotherapy. In that study, it was suggested that patients who responded to 5-FU during PCRT would probably also respond to 5-FU-based postoperative chemotherapy.

Adjuvant chemotherapy for patients who do not show a good response to PCRT needs to be different from that administered to patients showing a good response to this treatment. Das *et al*[40] have recommended adjuvant FOLFOX for high-risk patients, and adjuvant FL or capecitabine for low-risk patients. This seems to be a reasonable approach to the postoperative adjuvant treatment of rectal cancer patients treated with PCRT. Until now, however, oxaliplatin has been the drug of focus in terms of outcome benefits as part of a preoperative multimodality treatment regimen[42-44]. The role of postoperative adjuvant chemotherapy following PCRT and radical resection for patients with locally advanced rectal cancer thus remains unclear.

SPHINCTER PRESERVATION

Avoiding permanent stoma is an important quality of life issue for rectal cancer patients[45]. An advantage of tumor shrinkage after PCRT is supposedly an increased chance of sphincter preservation[46,47]. However, this is a very complex issue involving the stage and location of the tumor, the patient habitus and desire, and the surgeon`s experience. Although an increase in the rate of sphincter preservation was reported in early PCRT trials, no such trials since 1980 have been able to demonstrate this. This may be due to the immediateness of the surgery after the end of a short-course of PCRT[48-51] which gives little opportunity for tumor shrinkage. However, despite an increased rate of pCR of up to 16%-19% in the latest PCRT trials[42,52], no benefit has been evident in terms of the sphincter preservation rate.

Two randomized trials[1,53] of preoperative and postoperative CRT for clinically resectable locally advanced rectal cancer have reported opposing results. In a German trial[1], of the 194 patients assessed by the surgeon before treatment as requiring APR, there was a significant improvement in sphincter preservation with preoperative therapy. However, in the National Surgical Adjuvant Breast and Bowel Project (NSABP) R-03 trial[53], based on a prospective assessment by the operating surgeon, there was no reported improvement in sphincter preservation (PCRT: 47.8%; postoperative CRT = 39.2%; $P = 0.227$). The results of the NSABP R-03 trial, however, were obtained from only 267 of the 900 intended patients. The positive findings from the German trial were based on results from a sufficient number of patients, and the possibility of improved sphincter preservation by preoperative CRT remains one of the important potential benefits of this approach. In the recent Australian[54] trial where the two treatment arms were quite different (short course with immediate surgery *vs* chemoradiotherapy and delayed surgery) there was a reported increase in sphincter preservation of 8% in the delayed surgery arm. However, this was not significant because the number of patients assessed was too small. Weiser *et al*[55] reported a benefit of PCRT in terms of sphincter preservation from a retrospective analysis of 148 rectal cancer patients (within 6 cm of the anal verge).

The pooled data from 19 trials[56] favors PCRT, although not in a statistically significant way (0.94, 95%CI: 0.88-1.04) (Comparison 01:09). These data were borderline however in terms of homogeneity ($P = 0.05$), indicative of variations in the magnitude of effect across reports. In a recent review that analyzed the findings of 17 randomized trials the authors concluded that none of the neoadjuvant treatments tested could demonstrate an increase in the rate of sphincter-preserving surgery[57]. However, the effects of conservative treatments such as local excision or "wait-and-watch" on sphincter preservation were not considered in these analysis.

Until now, the evidence has been that an improved sphincter preservation benefit of PCRT was unclear. As described earlier, however, the link between PCRT and sphincter preservation needs to be evaluated with great care with consideration of tumor, patients and surgeon factors together. In addition, the effect of conservative management after PCRT need to be considered under condition the oncologic safety of this strategy is confirmed. The influence of PCRT on sphincter preservation needs to be re-evaluated under recent circumstances.

CONCLUSION

PCRT for locally advanced rectal cancer has been established as a standard treatment, but some issues regarding its practical application still need to be evaluated. In ad-

dition, an accurate prediction of the response to PCRT before administering this intervention, as well as an evaluation of nodal involvement after PCRT, remain important issues. An acceptable prediction of the response to PCRT should be integral to the decision making regarding an extension or selection of this treatment option.

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TOPIC HIGHLIGHT

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

Magnetic resonance imaging in rectal cancer: A surgeon's perspective

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Abstract

Magnetic resonance imaging (MRI) in rectal cancer was first investigated in 1999 and has become almost mandatory in planning rectal cancer treatment. MRI has a high accuracy in predicting circumferential resection margin involvement and is used to plan neoadjuvant therapy. The accuracy of MRI in assessing mesorectal lymph nodes remains moderate, as there are no reliable criteria to assess nodal involvement. MRI seems to be good in assessing peritoneal involvement in upper rectal cancer; this however has been assessed in only a few studies and needs further research. For low rectal cancers, mesorectum is thin at the level of levator ani especially in relation to prostate; so predicting circumferential resection margin involvement is not easy. However high spatial resolution coronal imaging shows levator muscles, sphincter complex and intersphincteric plane accurately. This is used to stage low rectal tumors and plan plane of surgery (standard surgery, intersphincteric resection, Extralevator abdominoperineal resection). While most centres perform MRI post chemoradiotherapy, its role in accurate staging post neoadjuvant therapy remains debatable. THe role of Diffusion weighted MRI post neoadjuvant therapy is being evaluated in research settings.

Key words: Magnetic resonance imaging; Rectal cancer; Surgeon

Core tip: Magnetic resonance imaging in rectal cancer is mandatory for a surgeon to plan neoadjuvant therapy. It also helps in planning surgical approach especially in low rectal cancer.

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INTRODUCTION

Over the last two decades outcomes of rectal cancer surgery has improved. The principle of sharp dissection in the total mesorectal excision (TME) plane as advocated by Bill Heald and implementation of national training programmes have improved outcomes of rectal cancer surgery[1,2]. The German Rectal Cancer Study Group trial showed that preoperative long course chemoradiotherapy (LCRT) improves 5-year locoregional recurrence rates compared with postoperative LCRT in stage T3, T4, or node-positive patients and with less toxicity[3].

The success of pre-operative therapy over post-operative treatments meant that a technique identifying prognostic factors pre-operatively is of potential benefit in modifying the intensity of pre-operative therapy ac-

cording to risk of local or distant failure. Careful staging of rectal tumors results in selective pre-operative treatment strategies aimed at reducing local failure and distant failure in high risk patients[4].

In rectal cancer staging, magnetic resonance imaging (MRI) has played a crucial role. In this review, we discuss in brief the history and relevance of rectal MRI through a surgeon's perspective.

RESEARCH

A systematic search of PubMed, MEDLINE and the Cochrane Library databases was performed from January 1995 to March 2013 using the terms: "MRI and rectal cancer" to identify studies investigating role of MRI in rectal cancer surgery. Using the criteria listed above 1231 articles were identified. After records were screened by abstract, 137 articles were eligible for full text evaluation and 72 were included in the reference list. This review included brief history of MRI in rectal cancer with its role in staging, selecting patients for neoadjuvant therapy, classification rectal cancers and other relevant topics.

History

The first MRI of human body was performed in 1977. In 1980 GE built the first high-field whole body MRI scanner. Blomqvist *et al*[5] in 1999, performed MRI on rectal cancer specimens concluded that presence of tumor free lateral resection margin could be predicted by MRI of resected specimen when this exceeds 1 mm[5]. However it is Brown *et al*[6] who used thin section MRI imaging to identify mesorectal fascia in all patients and accurately stage tumors especially T3 tumors. The same group performed MRI in cadaveric sections and in patients before they underwent total mesorectal excision surgery to establish criteria for visualization of the structures relevant to anterior resection of the rectum[7]. The MERCURY (Magnetic Resonance Imaging and Rectal Cancer European Equivalence) Study Group is a multicenter multidisciplinary collaboration formed in 2001. This group evaluated association between MRI and histopathology in measuring depth of tumour invasion beyond the bowel and involvement of the circumferential resection margin (CRM) in rectal cancer specimens[8,9]. Low Rectal Cancer study group (LOREC) is undertaking a study with the primary aim to reduce rate of incomplete excision in these patients from 30% to less than 10%[10].

Principles of MRI scan

Clinical MRI uses the magnetic properties of hydrogen and its interaction with both a large magnetic field and radio waves to produce highly detailed image of human body. By changing parameters on scanner a contrast between tissues can be obtained. T1 images-water and fluid containing tissues dark and fat brighter, basic scan; T2 images-water and fluid containing tissues bright, fat dark suited to show edema; FLAIR sequence-water dark but edema bright.

Special MRI: MR imaging of the rectum may be performed with either an endorectal coil or a phased-array surface coil. While endorectal coil gets better resolution of lesion, it is uncomfortable and cannot be used for stenosing lesion and rectosigmoid tumors. Hence standard MRI includes images with phased-array body coil only.

Diffusion MRI: Diffusion-weighted MRI (DW-MRI) is a functional imaging technique that displays information about the extent and direction of random water motion in tissues. Preclinical and clinical data indicate a number of potential roles of DW-MRI in the characterization of malignancy, including determination of lesion aggressiveness and monitoring response to therapy[11-13].

MRI with super paramagnetic iron oxide: In structures such as lymph nodes, insufficient contrast between normal and diseased tissues requires development of contrast agents. Super paramagnetic iron oxide (SPIO) structure is composed of ferric iron and ferrous iron (coated with a layer of dextran or other polysaccharide). SPIO particles are taken up by phagocytic cells such as monocytes, macrophages, and oligodendroglial cells but not by tumor cells. This SPIO enhanced MRI to enhance nodal resolution is under investigation and has shown promising results in rectal cancer[14,15]. Use of this agent in patients who have complete response after LCRT can be potentially used to identify patients (no lymph node involvement) who may be candidates for local excision yPT0/T2[16]. This agent is not FDA approved for rectal MRI.

Guidelines to perform MRI rectum

The technique for MRI in rectal cancer has been described by Taylor *et al*[17] (MERCURY study). The clinician provides location of tumor on rigid sigmoidoscopy. There is need for bowel preparation or intravenous contrast. We use 1.5-T system with phased array coil with the coil positioned from sacral promontory to 10 cm below pubic symphysis. Rectal distention with water may improve the depiction of a primary rectal tumor and the assessment accuracy of a perirectal tumor extension, but it does not improve the accuracy for determining the presence of regional lymph node involvement[18]. This however is not used routinely except selected centres: (1) the first series-sagittal, T2-weighted, fast spin echo from one pelvic sidewall to other, which locates tumor and relation to peritoneal reflection; (2) the second series- large field of view axial sections whole pelvis; (3) the third series-T2 weighted thin slice (3 mm) axial images through rectal cancer perpendicular to long axis rectum; and (4) for low rectal cancers, high spatial resolution coronal imaging to show levator muscles, sphincter complex and intersphincteric plane.

There is no need for post contrast MRI. Only T2 weighted non-fat suppressed sequences in all three orthogonal planes to tumor axial, coronal and sagittal should be used (Figure 1). The findings on scan are re-

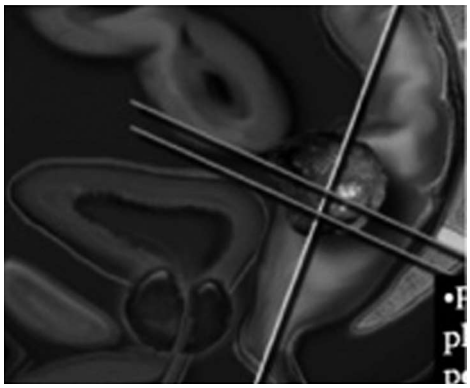

Figure 1 Magnetic resonance imaging technique. T2 weighted non-fat suppressed sequence in all three orthogonal planes to tumor axial, coronal, sagittal should be used.

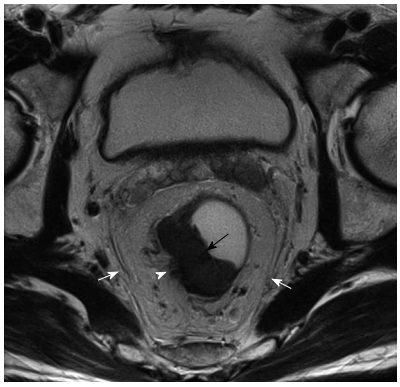

Figure 2 T3 tumor, circumferential resection margin not threatened. T2W axial magnetic resonance imaging image shows a mildly hyperintense proliferative tumor along the right lateral and posterior wall (black arrow). Arrow head shows the tumor reaching upto the muscularis with spiculation in the adjacent perirectal fat. The white arrows show the mesorectal fascia which is not involved/threatened.

corded on set proforma, which shows MRI based classification of rectal tumors, classification for low rectal tumors, tumor regression grades after LCRT on MRI[19].

Normal MRI findings: Anatomy, T2-weighted MR imaging sequences are the most suitable for depicting the rectal wall anatomy. The rectal wall consists of three different layers that can be recognized at MR imaging. Inner hyper-intense layer, which represents the mucosa and submucosa (no differentiation is possible between these two components); an intermediate hypointense layer, which represents the muscularis propria; and an outer hyperintense layer, which represents the perirectal fat tissue. The mesorectal fascia appears as a thin, hypointense line surrounding the hyperintense perirectal fat (Figure 2). At the level of levator ani/prostate mesorectum is thin anteriorly and mesorectal fascia is close to muscularis propria, so accuracy is low[20]. At the level of anal canal, even if the spatial resolution is low compared with endoanal coil imaging, all of the major anatomic structures (levator ani muscle, puborectal muscle, internal and external anal sphincters, anal canal) can easily be evaluated. CRM

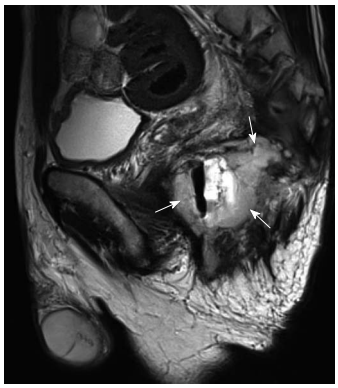

Figure 3 Mucinous adenocarcinoma of rectum. Sagittal T2 weighted MRI image showing a circumferential rectal tumour with high signal intensity (arrows) characteristic of a mucinous tumor. MRI: Magnetic resonance imaging.

is considered as closest distance from tumor to MRF (mesorectal fascia and around the levator, tumor invading the intersphincteric plane or extends to within 1 mm of the levator muscle is considered to potentially involve the CRM.

Besides normal anatomy, MRI pelvimetry can be used to anticipate problems during TME dissection. Kim *et al*[21] analysed factors related to difficult Laparoscopic TME (pelvic dissection time). In a prospective study enrolling 74 patients, tumor and patient characteristics (including pelvic dimensions) were analysed with respect to pelvic dissection time. Multivariate analysis showed that patients with longer sacral length, narrow intertuberous diameter and shallow sacral angle on MRI had longer pelvic dissection time but were not associated with increased postoperative complications[21]. Also variations in pelvic dimensions did not predict the risk of CRM involvement in rectal cancer[22].

MRI rectal cancer

It is mandatory to have location of tumor on rigid sigmoidoscopy prior to performing a MRI scan. Location of tumor on MRI and rigid sigmoidoscopy have a 10% discrepancy in location, the difference being 3 cm for anterior tumors while it is 1.2 cm for posterior tumors[23]. For upper rectal cancers, relation to peritoneal reflection is looked out for. Mucinous and non--mucinous rectal tumors can be differentiated with MR Imaging. Mucinous tumors are hetrogenous with intermediate and high SI (signal intensity) on T2-weighted FSE (fast spin echo) images reflecting the mucin content[24,25] Figure 3.

Staging: Tumor staging and EMD (extramural depth of tumor) assessment, There is seldom any dispute about Endo anal ultrasonography being more accurate when compared to MRI for T1/T2 lesions[26]. A recent meta-analysis of MRI staging of rectal cancer (T1/T2 *vs* T3/T4) revealed a sensitivity and specificity of T staging to be 87% and 75%[27], Table 1. Previous studies have described staging failures due to overstaging of T2 lesions with difficulty in the distinction of spiculation in the perirectal fat caused by fibrosis alone compared with that caused by

Table 1 Metanalysis of magnetic resonance imaging staging of rectal cancer-Al-Sukhni *et al*[27]

	Sensitivity% (95%CI)	Specificity% (95%CI)	DOR (95%CI)
T stage	87 (81-92)	75 (68-80)	20.4 (11-37)
N stage	77 (69-84)	71 (59-81)	8.3 (4.6-14.7)
CRM	77 (57-90)	94 (88-97)	56.1 (15-205)

DOR: Diagnostic odds ratio; CI: Confidence interval; CRM: Circumferential resection margin.

fibrosis that contains tumor cells[28]. Peritumoral fibrosis is represented by spiculation while broad based nodular growth is tumor spread.

Although tumor staging with use of the T component of the TNM classification is the traditional method of prognostically stratifying patients, this approach has limitations[26]. The main limitation of T staging is that T3 tumors comprise the majority of rectal cancers seen at presentation, and the outcome of patients with these tumors depends on the depth of extramural spread.

The maximal extramural depth (EMD) of tumor spread, defined on histopathologic analysis as the distance from the outer edge of the longitudinal muscularis-propria to the outer edge of the tumor is more related to tumor prognosis and preoperative therapy than T stage alone. In one of the largest series published by a University of Erlangen group, T3 tumors with extramural spread of more than 5 mm were associated with a 5-year cancer-specific patient survival rate of only 54%, but T3 tumors with 5 mm or less of extramural spread regardless of whether lymph node involvement was present were associated with a 5-year cancer-specific survival rate of greater than 85%. T3 tumors with 5 mm or less of extramural spread and pT2 patients showed very similar 5-year survival rates (both lymph node positive and negative patient)[29].

In a prospective study of 679 patients with rectal cancer, MERCURY group demonstrated EMD invasion to be equivalent on MRI and histopathology to a mean difference of less than 0.5 mm[9]. Pederson *et al*[30] evaluated 168 patients with rectal cancer MRI and histopathological examination and felt measurements of extramural tumor spread are more reproducible among different observers than are 5 mm distance measurements to the anticipated CRM. This EM spread is the basis of classification of T3 tumors on MRI (T3a EMD < 1 mm, T3b 1-5 mm, T3c 5-15 mm, T4 > 15 mm).

NCCN guidelines recommend neoadjuvant LCRT for all T3/T4 tumors irrespective of CRM involvement. However in the United Kingdom, LCRT would be reserved for only T3 tumors with threatened CRM. However it can be suggested, that for T3 tumors with EMD invasion > 5 mm (bad T3) but with clear CRM can undergo preoperative short course radiotherapy rather than surgery alone.

Nodes: Size is not a criteria for lymph node involvement[27]. In fact, one study found that 15% of lymph nodes less than or equal to 5 mm on MRI were involved with metastatic disease[31,32]. A suspicious nodebased on an irregular border or mixed signal intensity had a superior accuracy with a sensitivity of 85% and a specificity of 97%[31] (Figure 4). However, these can be subjective with inter observer variability. Distance of involved node to CRM is important. If suspicious nodes are present, one to three nodes is stage N1 and four or more is stage N2.

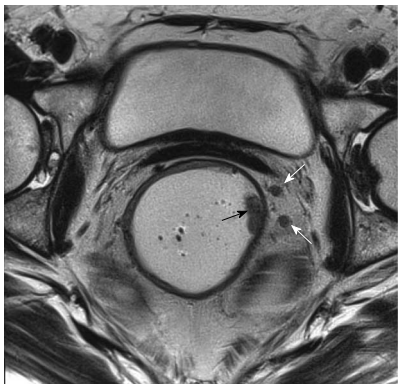

Figure 4 T1 N+ tumor. T2W axial magnetic resonance image shows two small nodes in the mesorectal fat on the left (white arrows) with irregular borders and signal intensity similar to primary tumor along left lateral wall (black arrow).

A metanalysis of MRI staging of rectal cancer revealed a sensitivity and specificity of N staging to be 77% and 71%[27]. This indicates limitation of MRI in assessing mesorectal lymph nodes, which is exacerbated by the lack of agreement on optimal criteria to assess lymph nodes.

However, high-resolution pelvic MRI was more accurate than PET/CT for the prediction of regional nodal status. Magnetic resonance imaging had a high sensitivity and PET/CT had a high specificity for N staging in rectal cancer[33].

Mesorectal fascia and CRM: Although the tumour stage on MRI is an important prognostic factor, it alone may not alter preoperative or operative management. Prediction of the CRM, by contrast, could be clinically useful to select patients for preoperative radiotherapy. MRI prediction of CRM mesorectal fascia (MRF) with final histology was performed by Beets-Tan *et al*[28]. They concluded that tumour-free margin of at least 1.0 mm could be predicted when the measured distance on MRI was at least 5.0 mm, and a margin of at least 2.0 mm when the MRI distance was at least 6.0 mm. Inter observer agreement was better for CRM than for T stage. However nodes threatening CRM can were difficult to evaluate.

CRM margin 5 mm or 1 mm: While the original study by Beets-tan concluded that MRI prediction of CRM involvement is reliable but suggested the use of a wider threshold on MRI compared to pathology[28]. The MERCURY group based their predicted CRM involvement on MRI to be less than 1 mm. A prospective study by Taylor *et al*[34] also showed that a cutoff of 1 mm on MRI could be used to predict clear margins with a low positive histologic CRM rate (3.3%)[34].

Table 2 Classification of low rectal cancers Shihab *et al*[41]

Level	Tumor height	Tumor depth	Operative plane
1	Tumor height between levator origin and puborectalis sling	Confined to muscle	LAR/intersphincteric APE
		Beyond muscle	LAR/intersphincteric APE
		Tumor < 1 mm MRF/levator	Extralevator APE
		Tumor extending beyond levator	Extralevator APE
2	Tumor at or below puborectalis sling	Submucosal/partial thickness muscle	LAR/intersphincteric APE
		Full thickness muscle	Extralevator APE
		In to intersphincteric plane	Extralevator APE
		In to external sphincter	Extralevator APE
		Beyond external sphincter into ischiorectal tissue	Pelvic exenteration

APE: Abdominoperineal excision; LAR: Low anterior; MRF: Mesorectal fascia.

In the MERCURY group study, the accuracy for predicting the status of CRM by initial imaging or imaging after treatment but before surgery in 408 patients was 88%. Of the 408 patients, 311 underwent primary surgery. The accuracy for prediction of a clear margin was 91% with a negative predictive value of 93%. This compared with an accuracy of 77% and negative predictive value of 98% in patients who had received preoperative chemoradiotherapy or long course radiotherapy[8].

A recent meta analysis to assess accuracy of MRI staging rectal cancer based on 21 studies concluded that MRI specificity was significantly higher for CRM involvement (94%) than for T category (75%) and lymph nodes (71%)[27] Table 2. However MRI can overestimate the CRM involvement in low and anterior tumor with the risk of over treating the patients[35,36].

Extramural vascular invasion

Extramural vascular invasion is an important and independent prognostic feature that can be readily identified on MRI. The morphologic features of extramural venous invasion on baseline T2 weighed MRI range from discrete serpiginous or tubular projections of intermediate signal intensity into perirectal fat following the course of a visible vessel to, in more advanced cases, the vessel being expanded by intermediate-signal-intensity tumor and having an irregular contour, Figure 5. The degree of extramural venous invasion system predicts relapse-free survival, with a 3-year relapse-free survival rate of 35% for patients with advanced extramural venous invasion, compared with 74% for those with no or early extramural venous invasion[37].

Pelvic lymph nodes: While lateral pelvic lymph node dissection (LPLND) is not performed routinely in United Kingdom, this is a good prognostic indicator as evaluated as part of MERCURY study. Patients with rectal cancer and suspicious pelvic side-wall lymph nodes (PSW) on MRI had significantly worse Disease free survival (DFS) that improved with the use of preoperative therapy. Five-year DFS was 42% and 70.7% respectively for patients with, and without suspicious PSW nodes ($P < 0.001$), but the presence of suspicious nodes had no impact on survival among patients who received preoperative therapy[38]. Based on this, most Mutidisciplinary teams would advocate preoperative LCRT for patients with rectal cancer and suspicious PSW nodes. While most nodes shrink with LCRT, management of persistent PSW nodes after LCRT is not standardized. In South Korea, selective unilateral/bilateral LPLND is performed for persistent PSW after LCRT[39]. From a surgeon's perspective, localization of these lymph nodes by preoperative MRI is important. At Yonsei university, between 2007 and 2012, of 1686 patients who underwent TME for rectal cancer, 92 (5.4%) patients underwent TME and LPND (unpublished). This however is not evidence based and requires further research.

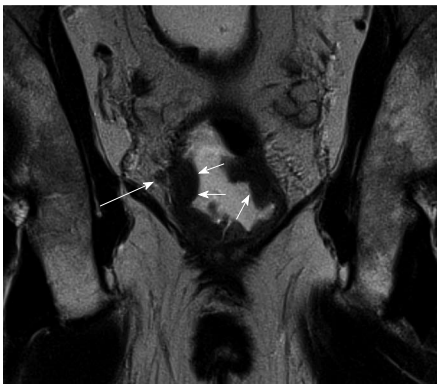

Figure 5 T2 tumor coronol views with extramural venous invasion. Coronal T2W magnetic resonance imaging shows a cicumferential tumor in the rectum (small arrows) reaching the muscularis with extramural venous invasion (long arrow).

Peritoneal involvement: Burton *et al*[40] in a small study showed that tumors of the distal sigmoid, rectosigmoid, and upper rectum can be staged accurately using high spatial resolution MRI and that those with poor prognostic disease including upper rectal cancer (anterior) may benefit from preoperative therapy. However further trials regarding this may be worthwhile.

MRI BASED CLASSIFICATION AND PLANNING THE SURGICAL PROCEDURES FOR LOW RECTAL CANCER

Currently, rectal cancer is classified based on the distance from the anal verge (upper, middle and lower). However, a selection of the optimal surgical procedures by tumor height is not enough. Low rectal tumors especially those treated by abdominoperineal excision (APE), have a high rate of margin involvement when compared with tumors elsewhere in the rectum. Correct surgical management to minimise this rate of margin involvement is reliant on highly accurate imaging, which can be used to plan the

Table 3 Classification low rectal cancer (Taylor *et al*[34] 2008)

Stage	
1	Tumor confined to bowel wall, outer muscle intact
2	Tumor occupies muscle coat but does not enter intersphincteric plane
3	Tumor enters intersphincteric space or lies within within 1 mm of levator muscle
4	Invades external anal sphincter or is 1 mm or beyond levator with/without adjacent organ involvement

planes of excision. Two staging systems for low rectal cancers have been proposed[17,41] (Tables 2 and 3). Shihab *et al*[42] reported a retrospective analysis of MRI and histopathology data of 33 patients with low rectal cancer. They felt defining plane of surgery preoperatively would be the best way to avoid a positive margin[42].

Similarly, based on Taylor classification, positive resection margins of patients undergoing APER/Low anterior resections were analysed. Of 101 patients with low rectal cancers (70 APER, 30 ant resection), positive resection margin odds were higher for magnetic resonance Stages 3 to 4 than Stages 1 to 2 by a factor of 17.7 ($P < 0.001$)[43]. Based on this classification we can plan a tailored procedure as shown below: Stage 1-TME; Stage 2-TME + intersphincteric resection; Stage 3-APER; Stage 4-APER or pelvic exenteration.

MRI for radiotherapy planning

CT scan remains a gold tool for planning radiotherapy for rectal cancer (conformal radiotherapy using 3 dimensional views). Most Radiation oncologists would also have access to MRI views to enable planning *i.e.*, delineation of target volume. Tumour volumes defined on MRI are smaller, shorter and more distal from the anal sphincter than CT-based volumes. For radiotherapy planning, this may result in smaller treatment volumes, which could lead to a reduction in dose to organs at risk and facilitate dose escalation[44,45]. Co-registration of the images where MR images are used for optimal outlining while retaining the CT data for dose calculations is now considered the gold standard in prostate cancer radiotherapy[46]. However for rectal cancer, further research is required regarding this.

MRI as prognostic factors

Following can be used as a prognostic factors on MRI: (1) tumor EMD/T4/CRM; (2) extramural vascular invasion; (3) inflammatory reaction; (4) mucinous tumours; (5) pelvic lymphnodes; and (6) MRI assessment of TRG (tumor regression grade) and CRM are imaging markers that predict survival outcomes for good and poor responders[47]. Practically it translates in to following.

Good tumor no adjuvant therapy: The preoperative identification of good prognosis tumors using MRI allows stratification of patients and targeting of preoperative therapy. MRI can also identify T3 rectal cancer patients who are likely to have a good outcome with primary surgery alone[34].

Poor prognostic tumors may benefit from: (1) preoperative extrastaging: Adverse features found on rectal MRI identify patients at increased risk of synchronous metastatic disease. Hunter *et al*[48] evaluated the incidence of synchronous metastatic disease on FDG-PET/CT and contrast-enhanced multiple-row detector computed tomography in MRI-stratified high- and low-risk rectal cancers. Incidence of confirmed distant metastases was significantly greater in the MRI high-risk group, with 20.7% *vs* the low-risk group, with 4.2% (odds ratio 6.0). This group may benefit from additional preoperative investigation for synchronous metastases such as FDG-PET/CT or liver MRI; and (2) trials involving Novel therapies such as Neoadjuvant capecitabine and oxaliplatin followed by synchronous chemoradiation and total mesorectal excision in magnetic resonance imaging-defined poor-risk rectal cancer[49].

MRI variation in countries

While there is good evidence supporting the role of MRI in rectal cancer, resource limitations and lack of National guidelines mean many patients with rectal cancer are still operated upon without preoperative MRI leading to suboptimal results. In United Kingdom in 2005, less than 50% of the units studied were able to offer preoperative MRI to all of their rectal cancer cases[50]. This however has changed to near 84% in 2012[51]. In an international questionnaire regarding use of MRI in rectal cancer, only 35% respondents used MRI routinely[52]. In South Korea, rectal MRI has been used for local staging since 2005, now it is very popular (90%). Furthermore cost of MRI is covered with national insurance system. In our institution, usually MRI and trans rectal USG are performed together for all mid and low rectal cancer patients.

POST LCRT MRI

MRI in rectal cancer is sometimes performed after radiotherapy (MRI 2) to evaluate tumor response and to choose alternative forms of surgery (Figure 6). With regards to MRI 2, there are two schools of thought.

MRI2 mandatory

MERCURY group believe MRI 2 should be mandatory for post treatment staging. This special radiology group felt with appropriate training, radiologists were able to differentiate tumor and fibrosis and even acellular mucin from cellular mucin on MRI scans. On post CRT T2 weighted images areas of fibrosis have very low signal intensity (similar to muscularis propria), whereas areas of residual tumor have intermediate signal intensity (similar to baseline tumor). Careful examination of high-resolution images enables delineation of small foci of intermediate signal intensity tumor in areas of low intensity fibrosis. Low intensity spicules in perirectal fat radiating from residual tumor represent desmoplastic reaction whereas advancing tumor has more nodular intermediate signal intensity. The guidelines for reporting MRI2 have been published by Patel *et al*[53], Table 4. MRI for restaging

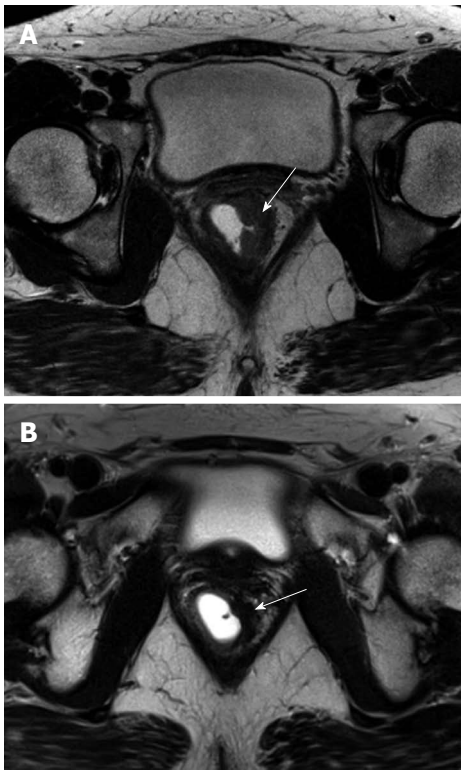

Figure 6 (A) Pre-chemoradiotherapy and (B) postchemoradiotherapy status. Arrows point to tumor along left lateral wall tumor which is mildly hyperintense in A that has become darkly hypointense in B indicating response (fibrosis).

has established accuracy of involvement of intersphincteric plane[42,43,47]. Another argument on which MRI was based was that endoluminal USG cannot differentiate tumor and fibrosis[54,55]. In a study by this group, the negative predictive value of CRM for MRI 2 was 98%. In this study both posttreatment MRI T staging and posttreatment MRI assessment of tumor regression grade showed statistical correlation with pathologic T stage, which in turn was strongly associated with overall and disease-free survival as well as local recurrence[56].

As a consequence, reassessment of MRI scans after preoperative therapy has implications for surgical planning, the timing of surgery, sphincter preservation[53,56]. Patients with CRM positivity on MRI 2 may require excision of adjacent organs (exenteration). While it has been suggested development of further preoperative treatments for radiologically identified poor responders and deferral of surgery for good responders, this can happen only in context of a trial. Phase Ⅱ trials are currently evaluating the safety of deferring surgical resection in patients with a good response as shown on MRI[57]. However there are drawbacks, while MRI 2 has an negative predictive value of 98% for CRM, its specificity for CRM was 73% (compared to 92% for MRI 1)[8]. This would mean, excision performed based on MRI2, chances of involved margin would be less, but at the same time, you would be likely to overtreat and excise outside TME plane/exenteration. Sphincter preservation (ISR) would also be less likely.

Table 4 Post long course chemoradiotherapy magnetic resonance imaging 2-Parameters are assessed on post treatment magnetic resonance images

	Pre treatment	Post treatment
Height of tumor from anal verge		
Length of tumor		
Tumor stage		
(sub stage based on extramural spread)		
Nodal spread		
Nodes pelvic sidewall		
Involvement peritoneal reflection		
Distance to potential CRM		
Depth of maximum extramural spread		
(distance from outer edge of muscularis propria)		
tumor and fibrosis separately		
Extramural venous invasion		

MRI tumor regression grade, Grade Ⅴ: No response (same as original tumor); Grade Ⅳ: Slight response (litte area of fibrosis/mucin, mostly tumor); Grade Ⅲ: Moderate response (more than 50% fibrosis, mucin but mostly tumor); Grade Ⅱ: Good response (dense fibrosis, no obvious residual tumor); Grade Ⅰ: Radiological complete response (no evidence of ever treated tumor); CRM: Circumferential resection margin.

MRI2 optional/unnecessary

MRI accuracy is poor after LCRT, T stage (43%), N stage (71%), On MRI it is difficult to differentiate tumor cells in scar tissue[58]. In a recent multicentric evaluation of MRI post neoadjuvant chemoradiotherapy (MERRION) found limited use of MRI post therapy[59]. In a small series of 16 patients with locally advanced cancer MRI before and after LCRT (MRI 1 and MRI 2) were compared to final histology. The accuracies of both MRI before and after radiotherapy were moderate, with no additional value of MRI after radiotherapy. They concluded that morphological assessment of pelvic MRI after preoperative radiotherapy does not provide any significant new information about tumor extent in patients with locally advanced rectal cancer[60]. Similarly, in another study, authors felt that accuracy of MRI 2 in distinguishing tumor delineation might be difficult due to fibrosis[61]. Post treatment MRI is a poor predictor of final histology and should not be relied upon to guide the extent of surgical resection. Larsen *et al*[62] felt that to achieve R0 resection, optimal surgery should be based on pre-treatment MRI. The study has initiated a new approach to histopathological classification of the removed specimen where they introduce a MRI assisted technique for investigating the areas at risk outside the mesorectal fascia in the specimen[62]. Kang *et al*[63] concluded that the tumor volume reduction ratio was not significantly associated with T and N downstaging. MRI is unable to detect the majority of patients who have a complete histopathological response as MRI appearances of ypT0 tumours are heterogeneous[64].

There is little consensus on the use of MRI after LCRT. Martellucci *et al*[65] suggested against restaging with MRI and recommended TRUS. They found that regarding the depth of invasion after treatment, TRUS agreed

with histopathology in 25/37 patients (67.5%), CT agreed in 22/37 cases (59.5%), and MRI in 12/20 cases (60%). They therefore suggested limiting the use of MRI for restaging to selected cases. There however is uniform consensus on current guidelines, which advocate removing areas of fibrosis on MRI 2, as they could harbor tumor cells.

Even in United Kingdom, ACPGBI guidelines do not enforce MRI 2[8] prior to surgery. Ultimately it would be a joint decision by individual MDT as there seems to be no uniform consensus across the board. In low resource economies MRI 2 may be performed only in selected cases for locally advanced unresectable cases to plan operability, extent of resection or consideration of more non-cross resistant chemotherapy prior to surgery.

Timing of MRI 2: There remains some controversy regarding the optimum time for imaging prior to surgery after the chemoradiotherapy treatment has been completed. As surgery is planned for approximately 6-8 wk after the final chemoradiotherapy treatment, the MRI needs to be performed during this time, too early and the oncologists will argue that the chemoradiotherapy will still be having effects and potentially decrease the tumour size further; therefore the closer to surgery the better. Many centres aim for approximately 1 mo after LCRT to enable the surgery to be planned/organised.

MRI for surveillance

Surgical treatment offers the best prospect of survival for patients with recurrent colorectal cancer. Unfortunately, most local recurrences are diagnosed at an advanced (unresectable) stage, when traditional follow-up methods are used. The impact of MRI surveillance on the early diagnosis of local recurrences has been evaluated by Titu *et al*[66] In this unique study, 226 patients who underwent curative surgery for rectal and left-sided colon tumors were included in a program of surveillance using pelvic MRI in addition to standard follow-up protocol. Twenty-six (13%) local recurrences were identified. These were then analyzed based on mode of diagnosis, resectability, and overall survival. Recurrent pelvic cancer was diagnosed by MRI with a 87% sensitivity and 86% specificity. In 19 (63%) cases, CEA was abnormally elevated, and 9 patients (30%) were symptomatic. Surgical resection was possible in only 6 patients (20%). There was no difference between MRI and conventional follow-up tests in their ability to detect cases suitable for surgery. Hence they concluded, pelvic surveillance by MRI is not justified as part of the routine follow-up after a curative resection for colorectal cancer and should be reserved for selectively imaging patients with clinical, colonoscopic, and/or biochemical suspicion of recurrent disease[66].

Functional outcome: MRI pelvis may predict functional outcome in patients undergoing anterior resection. In a small series of patients undergoing anterior resection, How *et al*[67] evaluated functional outcome and co-related it with preoperative MRI sphincter morphology and anal manometry. They found only puborectalis thickness showed a significant ($P = 0.01$) relationship with the number of adverse symptoms suffered postoperatively[67].

MRI in recurrent cancers

MRI scans of pelvis are mandatory for selection for exenterative surgeryfor recurrent cancers, as CRM co-relates with survival. In primary cancers, assessing the preservation of fat plane can be used to predict invasion. However this is difficult in post-operative/post-radiotherapy pelvis, where fat planes are often grossly distorted or absent. Pelvic oncology unit in Leeds consider definite invasion in three specific circumstances on MRI: (1) when tumour tissue is clearly seen to invade or destroy adjacent anatomy; (2) when signal change in adjacent tissue is comparable with the signal intensity of the recurrent tumour; or (3) where muscle enlargement is evident. In their experience when patients are assessed between 21 and 48 mo post-primary surgery, muscle enlargement seen on MRI is related to recurrence rather than haematoma/inflammatory changes[68].

MRI directed MDT: MRI directed MDT improves outcome and is mandatory for recognition as a cancer centre. In 2006, Burton *et al*[69] compared CRM involvement of rectal cancer patients who were operated with/without MDT discussion and found low CRM involvement in patients discussed at MDT. This opinion however is not uniform. Review By Danish MDT team found increased detection of metachronous cancers through MDT but no difference in overall survival. Similar results were shown by Department of Health care policy, Harvard Medical school[70,71].

CLINICAL APPLICATION OF MRI FOR THE SURGEON

T1/T2 cancer. MRI can't differentiate, hence need endorectal usg to identify T1 cancers. Theses may be candidates for local excision (transanal or TEO) (Figure 4).

T3 minimal (no CRM involvement) can go for surgery either per primum or after shortcourse radiotherapy (Figure 2).

T3/N0, + (Figures 3, 7 and 8) with circumferential margin involvement and T4 tumors (Figure 9) are treated with LCRT and reassesses with MRI 2.

All rectal cancer with pelvic side wall nodes needs to undergo LCRT as this leads to improved survival and avoids need for lateral pelvic node dissection (Figure 8).

All low rectal cancers (5 cm from anal verge) are assesses with coronal images of MRI T2 sequences, to see involvement of intersphincteric plane and extension to levator ani: (1) those tumors free from intersphincteric plane and more than 1 mm from levator plate can be subjected to intersphincteric dissection and sphincter saving procedures (Figure 10A); (2) tumors involving intersphincteric space and external sphincters undergo

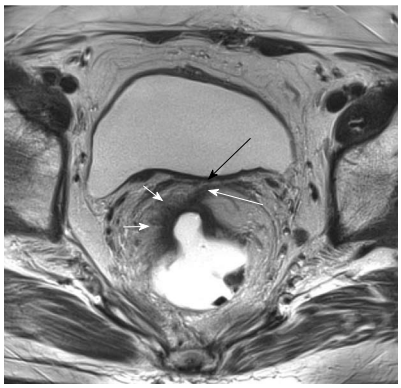

Figure 7 T3 tumor with spiculation reaching mesorectal fascia. Peritumoral fibrosis. T2W axial magnetic resonance imaging shows rectal tumor along anterior wall with spiculations (small white arrows) into the perirectal fat. The spiculation reaches up to mesorectal fascia (black arrow) at 12' o'clock position (long white arrow) causing focal fascia retraction.

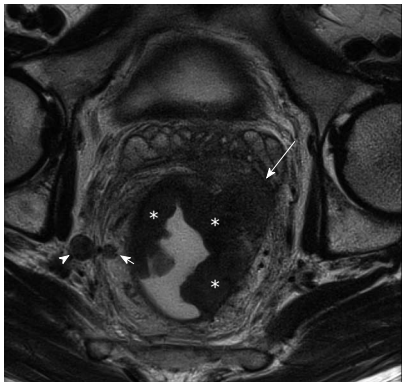

Figure 8 T3 tumor with lateral pelvic nodes. Axial T2W magnetic resonance imaging shows a large right lateral pelvic wall node (arrowhead). Short arrow shows perirectal node. The primary rectal tumor (asterisk) is seen extending into left mesorectal fat upto the mesorectal fascia (long arrow).

standard APER (Figure 10B); and (3) tumors involving levators need extralevator APER (Figure 11).

DISCUSSION

One of the most important factors that governs the success of TME surgery is the relationship of tumour to the CRM. Tumour involvement of the CRM in patients undergoing TME surgery is related to poor survival and local recurrence. Tumor relation to CRM on MRI (predicted CRM) helps in planning preoperative LCRT in selected cases. MRI may help plan plane of resection for low rectal tumors and may decreases CRM involvement and ultimate outcome (LOREC project).

While not in line with NCCN (National comprehensive cancer Network) guidelines, it helps avoid radiotherapy for T3 patients where predicted CRM is not threatened. This will mitigate radiotherapy related complications and improve bowel function. In addition, MRI will identify tumours exhibiting other poor prognostic features, namely, extramural spread > 5 mm, extramural venous invasion by tumour, nodal involvement, and

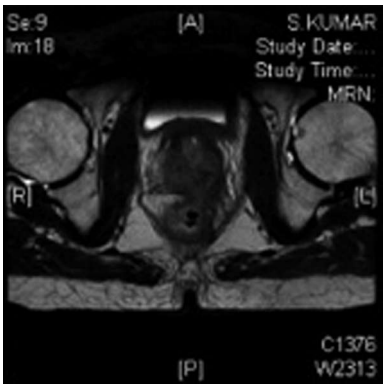

Figure 9 T4 lesion rectum involving prostate.

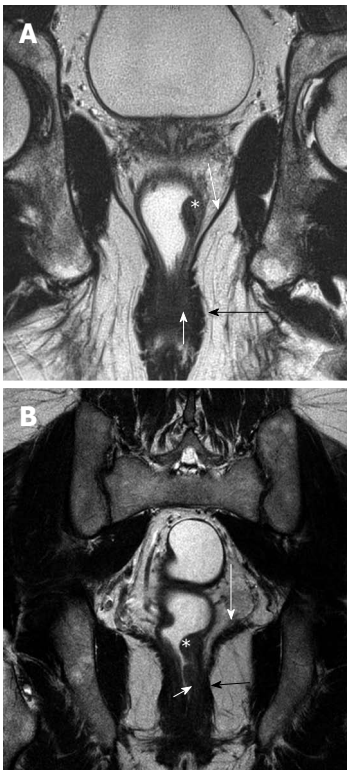

Figure 10 Coronal section T2 weighted magnetic resonance imaging to see level of tumor for planning surgery. A and B show tumor (asterisk) along the left lateral wall, that reaches up to the internal sphincter (short white arrows) in B, but spares it in A. The uninvolved external sphincter (darkly hypointense) is shown by black arrows. The long white arrows in A and B shows the spared levator ani.

peritoneal infiltration who may be candidates for trials regarding intensive chemotherapy/biological therapy along with radiotherapy to improve DFS/OS/LR.

For advanced tumors (Non-resectable) on MRI, targeted preoperative therapy may not only reduce the size of the primary tumour and render potentially unresectable tumour resectable. This would also enable patients at high risk of systemic failure to benefit from intensive combined modality therapy aimed at eliminating micrometastatic disease[72]. The Role of MRI post LCRT is uncertain but is often used in selected centres planning to offer sphincter preservation based on tumor response.

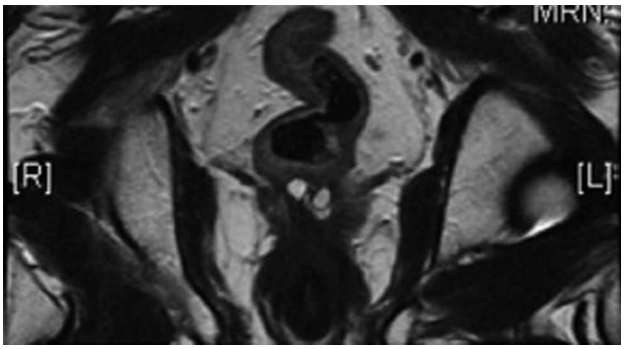

Figure 11 Low rectal cancer involving left levator.

CONCLUSION

MRI should be mandatory in planning radical surgery for rectal cancer. This improve R0 resection rates, decreases local recurrences with improved oncological outcomes. There is uncertainty over the role of MRI post LCRT. While MRI directed MDT has shown improved outcome in most studies, this however is debatable. The role of MRI in early rectal cancer seems to be limited, and needs complimentary endorectal ultrasound.

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Targeted therapy in gastric cancer: Personalizing cancer treatment based on patient genome

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Abstract

Gastric cancer is the second leading cause of cancer-related deaths worldwide. Conventional cytotoxic chemotherapy has limited efficacy for metastatic gastric cancer, with an overall survival of approximately ten months. Recent advances in high-throughput technologies have enabled the implementation of personalized cancer therapy for high-risk patients. The use of such high-throughput technologies, including microarray and next generation sequencing, have promoted the discovery of novel targets that offer new treatment strategies for patients lacking other therapeutic options. Many molecular pathways are currently under investigation as therapeutic targets in gastric cancer, including those related to the epidermal growth factor receptor family, the mesenchymal-epithelial transition factor axis, and the phosphatidylinositol 3-kinase-AKT-mammalian target of rapamycin factors. Advances in molecular diagnostic tools further support the discovery of new molecular targets. Limitations exist, however; not all patients can be tested for biomarkers, and numerous challenges hamper implementation of targeted therapy in clinical settings. Indeed, the scale of tumor genomic profiling is rapidly outpacing our ability to appropriately synthesize all the information in order to optimally refine patient care. Therefore, clinicians must continue to educate themselves regarding new tools and frameworks, and to utilize multidisciplinary team science, comprised of oncologists, geneticists, pathologists, biologists and bioinformaticians, to successfully implement this genomic approach therapeutically.

Key words: Gastric cancer; Targeted therapy; Biomarker; Microarray; Sequencing

Core tip: Understanding the molecular mechanisms governing carcinogenesis, progression and prognosis of gastric cancer is a prerequisite for development of effective management strategies. Analysis of genomic and proteomic expression profiles of oncogenic signaling pathways have revealed different molecular subtypes of gastric cancer. Development of personalized cancer therapy regimens will specifically target aberrations that drive tumor growth and survival. Therefore, identifying and administering the appropriate drug based on genetic profiling will improve clinical outcomes and decrease toxicity. We anticipate that identification of novel cancer targets will further aid in understanding of cancer heterogeneity and in refinement of personalized therapeutic strategies.

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INTRODUCTION

Advances in high-throughput technologies, such as microarray and next generation sequencing (NGS), have led to the discovery of novel therapeutic targets and revealed the power of predictive and prognostic markers in patient care. In addition, such comprehensive genomic approaches have increased our understanding of critical cellular and molecular mechanisms of cancer[1,2]. To date, hundreds of cancer-causing mutations have been discovered by genome-wide sequencing of the entire exome of more than 3000 tumors[3-5]. Most of the mutated cancer-causing genes may already have been identified, as the more recent NGS studies have reported previously identified mutants from different tumor types[5]. Because of this depth of knowledge regarding cancer genomes, we can now target specific aberrations that drive tumor growth and survival and customize drug combinations for individual patients. Despite the feasibility and clinical advantages of personalized cancer therapy, only a small minority of patients are being tested for biomarkers and treated accordingly. Moreover, most emerging drug candidates with no predictive biomarkers fail in clinical trials. Therefore, it is essential to determine which specific targeted therapies are most efficacious for particular sets of patients, and optimization of these treatment regimens should be a priority of future research.

Gastric cancer is one of the most common malignancies worldwide and is the most frequent cancer diagnosed in East Asian countries[6]. Since gastric cancer is a heterogeneous disease, both histologically and genetically, it is difficult to predict patient outcomes using classical histologic and molecular classifications[7]. Surgery is the only curative treatment strategy; yet, even when the primary tumor is resected, some early gastric cancer patients will ultimately succumb to the disease as a result of recurrence of local or distant tumors. Although not necessary in all patients, adjuvant chemotherapy has been shown to benefit some patients with early gastric cancer, whereas conventional chemotherapy has limited efficacy for advanced gastric cancer, with an overall survival of approximately ten mo. Therefore, to improve prognosis of these high-risk patients, it is important to identify predictive biomarkers and to develop refined treatment strategies.

MOLECULAR HETEROGENEITY OF GASTRIC CANCER

Human epidermal growth factor receptor 2 (HER2; also known as ERBB2) is a receptor associated with cell survival, proliferation, migration, adhesion, and differentiation. In the trastuzumab for gastric cancer trial, 594 patients with gastric cancer with overexpression of the HER2 protein were randomly assigned to two treatment groups, chemotherapy (capecitabine/fluorouracil plus cisplatin) or chemotherapy in combination with trastuzumab (a monoclonal antibody that inhibits HER2)[8]. Trastuzumab extended the median overall survival from 11.1 to 13.8 mo (HR = 0.74; P = 0.0046). This finding satisfied the primary objective of the trial and was later referenced in the National Comprehensive Cancer Network guideline.

In addition to the HER2 pathway, there are many other pathways abnormally regulated in gastric cancer. These include the fibroblast growth factor receptor (FGFR) family, the hepatocyte growth factor (HGF)-mesenchymal epithelial transition factor axis, the phosphatidylinositol 3-kinase (PI3K)-AKT-mammalian target of rapamycin (mTOR) factors, and the RAS/RAF/MEK/mitogen-activated protein kinase factors.

Genetic amplification, translocation or mutation of FGFR accelerates growth in a variety of cancers. More specifically, FGFR2 has been reported as amplified in 9% of gastric cancer specimens[9]. For the HGF/Met pathway, dysregulation can occur by aberrant paracrine and autocrine activation via inappropriate ligand production, activating mutations, genomic amplification, increased transcription, or Met receptor overexpression. Overexpression of Met has been detected in human gastric cancers and is associated with a more aggressive phenotype[10-12]. Notably, rilotumumab (a monoclonal antibody targeting the Met-HGF axis) yielded superior overall survival rates in a subgroup analysis of a phase Ⅱ randomized study. Patients with high levels of Met expression were treated with either epirubicin, cisplatin, and capecitabine (ECX) in combination with rilotumumab or ECX alone in the first-line setting (11.1 mo *vs* 5.7 mo, HR = 0.29; 95%CI: 0.11-0.76, P = 0.012)[13]. Activation of the PI3K/Akt/mTOR signaling pathway is correlated with poor prognosis and has also been studied as a therapeutic target[14,15]. A phase Ⅲ trial that evaluated supportive care in combination with either everolimus (an inhibitor of mTOR) or placebo for patients with advanced stage gastric cancer yielded negative results, where there was no significant difference in overall survival between the treatment arms (5.4 mo *vs* 4.3 mo for the everolimus and placebo groups, respectively)[16]. Histone deacetylase and poly (ADP-ribose) polymerase (PARP; a family of proteins involved in a number of cellular processes involving DNA repair and programmed cell death) have been investigated as treatment targets for gastric cancer[17,18]. Angiogenesis is essential in cancer development, growth, and proliferation, and the vascular endothelial growth factor (VEGF) and receptor (VEGFR) have been spotlighted as therapeutic targets. Recently, the REGARD Trial reported that ramucirumab (a VEGFR-2 monoclonal antibody) improved both progression-free survival and overall survival (*vs* placebo)[19]. Many clinical trials have evaluated molecular targeting agents that correspond to the various aforementioned signaling pathways (Figure 1), and many of them are ongoing (Table 1)[8,18-23].

Due to large-scale molecular techniques, our understanding of the molecular complexity underlying gastric cancer has increased, and the development of prognostic

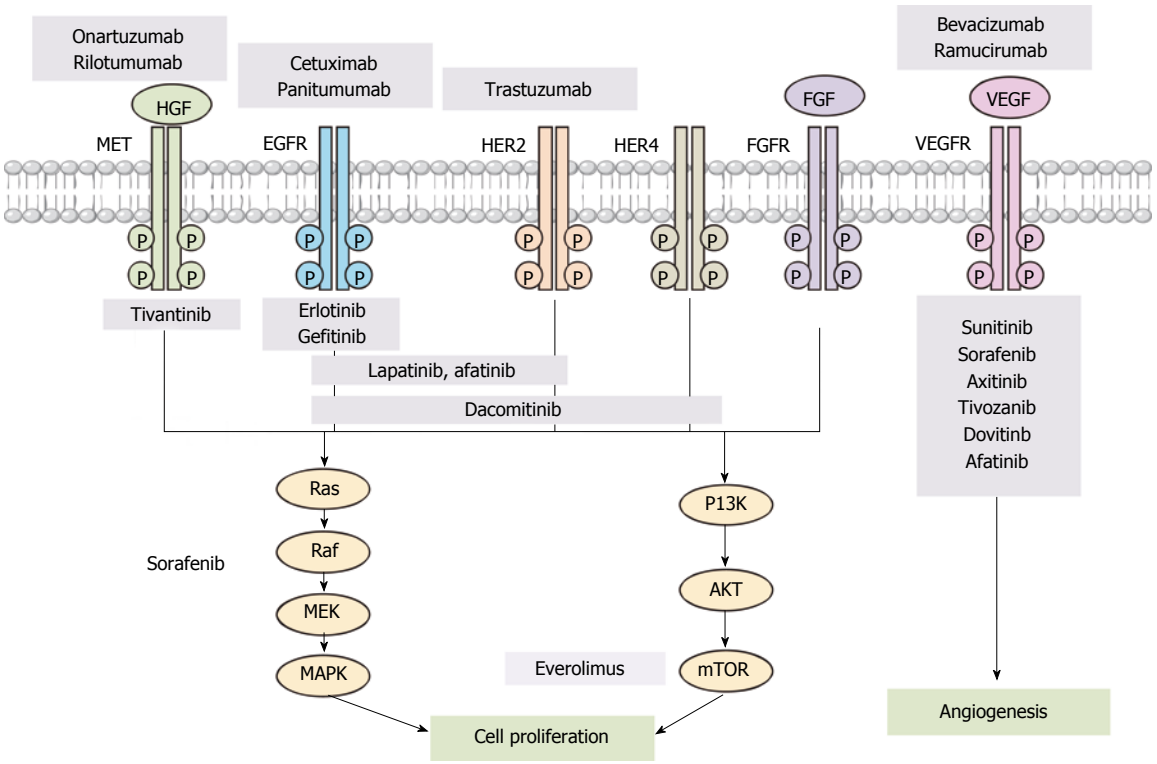

Figure 1 Molecular targeting agents for different signaling pathways in gastric cancer. PI3K: Phosphatidylinositol 3-kinase; HER: Human epidermal growth factor receptor; FGFR: Fibroblast growth factor (FGF) receptor; mTOR: Mammalian target of rapamycin; HGF: Hepatocyte growth factor; VEGFR: Vascular endothelial growth factor (VEGF) receptor; EGF: Epidermal growth factor; MAPK: Mitogen-activated protein kinase.

Table 1 Recent phase Ⅲ clinical trials investigating molecular targeting agents in gastric cancer

Clinical trial	Biomarker	*n*	Results	Achievement of primary objective	Ref.
HER2 inhibitor					
Capecitabine/cisplatin ± trastuzumab (ToGA)	HER2	584	PFS 6.7 mo *vs* 5.5 mo, $P = 0.0002$ OS 13.8 mo *vs* 11.1 mo, $P = 0.0046$	Positive	[8]
Capecitabine/oxaliplatin ± lapatinib (LOGiC)	HER3	545	Enrollment done	NP (NCT00680901)	
Paclitaxel ± lapatinib (TYTAN)	HER4	261	OS 11.3 mo *vs* 8.8 mo, $P = 0.2088$	Negative	[20]
EGFR inhibitor					
Capecitabine/cisplatin ± cetuximab (EXPAND)	NA	904	PFS 5.6 mo *vs* 4.4 mo, $P = 0.3158$ OS 10.7 mo *vs* 9.4 mo, $P = 0.9547$ Increased toxicity	Negative	[21]
Epirubicin/oxaliplatin/capecitabine ± panitumumab (REAL-3)	NA	553	PFS 6.0 mo *vs* 7.4 mo, $P = 0.068$ OS 8.8 mo *vs* 11.3 mo, $P = 0.013$ Increased toxicity	Negative	[22]
Angiogenesis inhibitor					
Capecitabine/cisplatin ± bevacizumab (AVAGAST)	NA	774	PFS 6.7 mo *vs* 5.3 mo, $P = 0.0037$ OS 12.1 mo *vs* 10.1 mo, $P = 0.1002$	Negative	[23]
Ramucirumab *vs* placebo (REGARD)	NA	355	PFS 2.1 mo *vs* 1.3 mo, $P < 0.0001$ OS 5.2 mo *vs* 3.8 mo, $P = 0.0473$	Positive	[19]
Paclitaxel ± ramucirumab (RAINBOW)	NA	665	Enrollment done	NP (NCT01170663)	
Afatinib *vs* placebo	NA	270	Enrolling	NP (NCT01512745)	
C-MET/HGF pathway inhibitor					
Epirubicin/cisplatin/capecitabine ± rilotumumab (RILOMET-1)	MET	450	Enrolling	NP (NCT01697072)	
Fluorouracil/folinic acid/oxaliplatin ± onartuzumab (MetGastric)	MET HER2	800	Enrolling	NP (NCT01662869)	
PI3K/Akt/mTOR pathway inhibitor					
Everolimus *vs* placebo	NA	648	PFS 1.68 mo *vs* 1.41 mo, $P < 0.00001$ OS 5.39 mo *vs* 4.34 mo, $P = 0.1244$	Negative	[16]
Paclitaxel ± everolimus (AIO-STO-0111)	NA	480	Enrolling	NP (NCT01248403)	

NA: Not applicable; NCT: ClinicalTrials.gov identifier; NP: Not published; OS: Overall survival; PFS: Progression-free survival; PI3K: Phosphatidylinositol 3-kinase; HER: Human epidermal growth factor receptor; mTOR: Mammalian target of rapamycin.

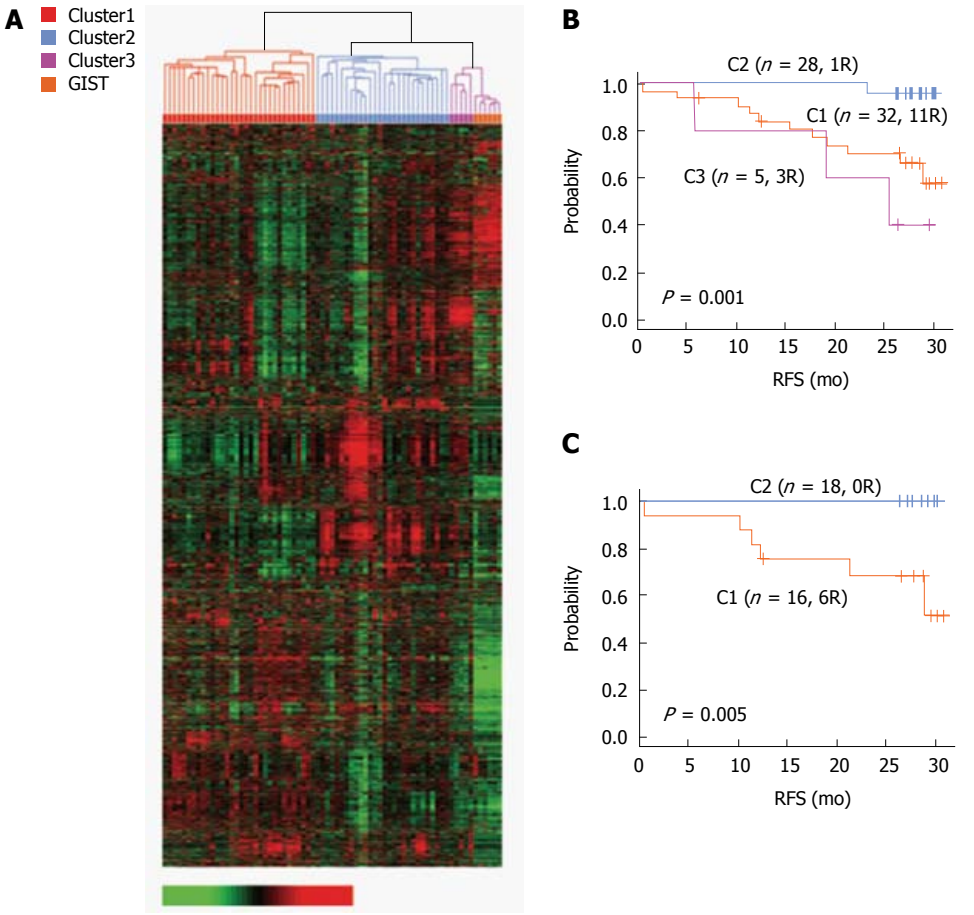

Figure 2 Prognostic expression signatures of genes associated with survival. A: Hierarchical clustering of gene expression data from 65 gastric cancer and 6 gastrointestinal stromal tumors patients in the Yonsei gastric cancer (YGC) cohort; B: Kaplan-Meier plots of 3 gastric cancer clusters in the YGC cohort; C: Kaplan-Meier plots of stage III patients in 2 clusters (C1 and C2) in the YGC cohort. RFS: Recurrence-free survival.

classifications based on gene expression profiles is rapidly evolving[24-28]. For example, two distinct gastric cancer subclasses that were strongly associated by prognosis were linked by analyzing gene expression profiles[29]. Interestingly, whole exome sequencing of a gastric adenocarcinoma revealed recurrent somatic mutations in both cell adhesion and chromatin remodeling genes[30]. The characterization of more molecular processes and interactions will yield effective tailored therapies to improve patient outcome and reduce drug toxicity.

GENE EXPRESSION MICROARRAY

A recent study by Ahn *et al*[29] identified the prognostic gene expression signatures of six genes significantly associated with survival and relapse and developed a scoring system based on these genes. Specifically, reverse transcriptase polymerase chain reaction was performed on paraffin-embedded tissues, and microarray was used to generate and analyze gene expression profiling data from 65 gastric cancer patients. Two distinct subgroups were strongly associated with prognosis (Figure 2). The C1 subgroup was linked with a poor prognosis, and six genes were identified whose expressions were unique to this subgroup (*CTNNB1*, *EXOCS3*, *TOP2A*, *LBA1*, *CCL5*, and *LXTR1*). Next, a scoring system based on the six genes was developed that independently predicted the likelihood of relapse after curative resection. This suggested that the distinct gene expression signature accurately reflected the clinical differences between patient subgroups and that such screens can provide information on disease trajectory. In the future, the efficacy of the risk score will be evaluated as a predictive marker for clinical response to adjuvant chemotherapy.

In another study using a gene expression microarray, the M2 isoform of pyruvate kinase (PKM2) was found to be overexpressed in gastric cancers at both the mRNA and protein levels relative to normal gastric tissues. Its expression was negatively correlated with survival in signet-ring cell gastric cancer patients. PKM2 expression may be an adverse prognostic factor for signet-ring cell carcinomas, and the biological role of PKM2 in gastric cancer development and its prognostic value need to be further elucidated[31].

MICRORNA MICROARRAY

MicroRNA (miRNA) play a role in the pathogenesis of various human cancers[32]. Although some miRNAs have been shown to function as oncogenes and oth-

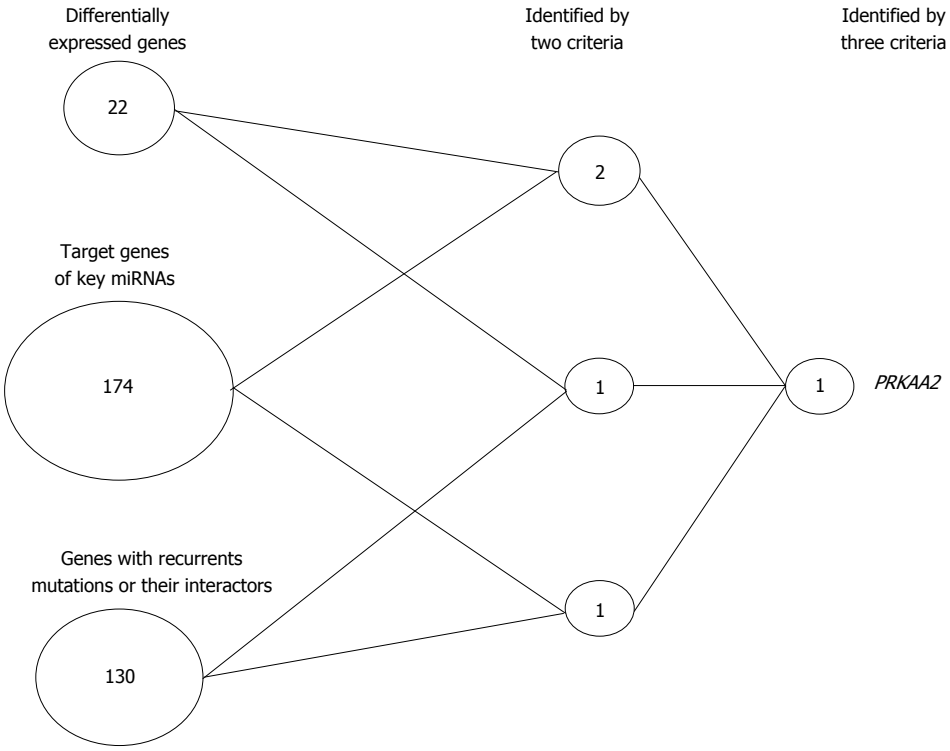

Figure 3 Simple scoring analysis of Asian gastric cancer. Three criteria were used to select key genes: (1) Genes that were identified in both 5-group and 4-stage differential expression analysis; (2) Target genes of six key differentially expressed miRNAs, where the target genes were predicted by TargetScan (Rs < -0.4, $P < 0.05$); and (3) Genes with recurrent somatic mutations or their interactors (Ingenuity Pathway Analysis program annotation). Only two genes met two criteria, and PRKAA2 was the only gene that met all three criteria. miRNA: MicroRNA.

ers as tumor suppressors, their mechanisms remain to be elucidated[33,34]. The relationship between miRNA expression profile and gastric cancer prognosis[35,36] and pathogenesis[37,38] has been actively explored. For example, miR-196b may be a useful marker, as overexpression of miR-196b has been linked to leukemia and several solid cancers, including gastric cancer[39,40]. Whether miR-196b is an oncogene or tumor suppressor and whether it has a role in gastric carcinogenesis and progression have not yet been confirmed[41]. Recently, miR-21, miR-106b, miR-17, miR-18a and miR-20a were identified as the five most consistently identified miRNAs in screens of gastric cancer. The association between expression level of these miRNAs and clinicopathological features of gastric cancer was significant, and these miRNAs are potential diagnostic and/or prognostic markers that warrant further investigation[42].

RNA-sequencing

RNA-sequencing (RNA-seq) technology enables investigators to simultaneously quantify gene expression levels, assess alternative splicing and gene fusion events, and detect nucleotide variations in transcribed regions. In particular, whole-transcriptome RNA-seq provides a detailed view of the spectrum of expressed transcripts of both coding and noncoding mRNA[43]. Recently, a central metabolic regulator AMP-activated protein kinase (AMPK) was identified as a potential functional target in Asian gastric cancer. As seen in Figure 3, a simple scoring analysis found that only *PRKAA2* (AMPKα2) was identified as a potential key modulator of gastric cancer progression. Importantly, the translational relevance of this gene as a target for early-stage gastric cancer was suggested by functional studies in gastric cancer cell lines[44]. Relative to previous RNA-seq studies, this whole-transcriptome RNA-seq approach has several advantages. First, two protocols were used that complementarily covered RNA fragments of different sizes. In this manner, quantification of mRNA, long noncoding RNA and miRNA expression could be conducted simultaneously. Second, ribosome-depleted RNA samples, rather than poly A-enriched RNA samples, were sequenced, thereby generating a less biased population of transcribed molecules. Third, strand-specific short reads were used, and this allowed for more accurate quantification of gene expression. In the future, further functional studies will be necessary to elucidate whether AMPKα2 is an effective therapeutic target.

CONNECTIVITY MAP

The Connectivity Map is a web-based interface (http://www.broadinstitue.org/cmap) that contains more than 7000 expression profiles representing effects of 1309 compounds on several cultured human cells[45]. Analyses using the Connectivity Map reveal functional connections between drugs, genes, and disease and provide a novel approach for cancer treatment. Candidate agents

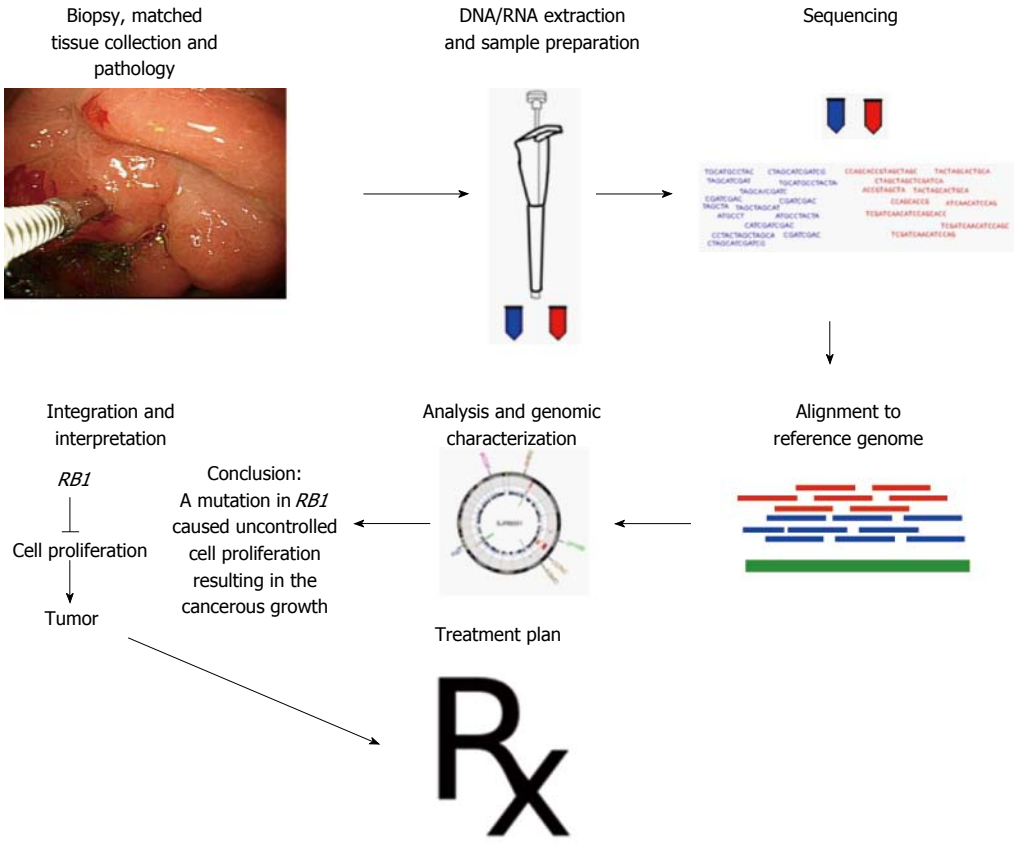

Figure 4 Schematic for genomics-driven cancer medicine where the cancer treatment regimen is adjusted according to each patient's genome.

against a specific disease can be recognized by applying disease-specific gene expression profiles to Connectivity Map analysis[46]. Recently, a gastric cancer gene signature was applied to the Connectivity Map, and analysis revealed that histone deacetylase inhibitors, including vorinostat and trichostatin A, were potential drugs for the treatment of gastric cancer. These findings were validated *in vitro* using gastric cancer cell lines, wherein vorinostat significantly inhibited cell viability in a dose-dependent manner[17]. Therefore, application of unique gene expression profiles to the Connectivity Map may be a viable strategy for the discovery of novel therapeutic agents for gastric cancer.

CONCLUSION

Advances in molecular diagnostics rely more on classifying tumors based on the pathways that drive the oncogenic process, rather than by the tissue of origin. Such molecular tumor classification schemes are very useful in selecting the appropriate pathway inhibitor to apply to an individual patient. Genomic technologies enable robust tumor genomic profiling in the clinical arena (Figure 4) and make it possible to match plausible genetic alterations with rational therapeutic regimens. This means that data from cancer genomes may dictate rational treatment decisions that are tailored for a specific tumor. Importantly, the patients benefit from use of these biomarkers, as the most effective therapy would be selected upfront, sparing the patient from the considerable toxicity associated with conventional "trial and error" therapy. However, the genome era also poses clinical challenges. This unprecedented flow of tumor and germline genomic information needs to be supported by clinical-grade data interpretation. Oncologists will be required to conduct a new generation of evidence-based clinical trials to accommodate smaller numbers of patients with discrete genetic alterations. To aid in this discovery, platforms for repeat biopsy and tissue banking should be established. Moreover, the roles of personalized surgery and radiotherapy should not be underestimated. Defining a subgroup of patients who benefit from radiotherapy and the potential interactions between patient characteristics and the efficacy of radiotherapy should be further explored in the future. We believe this confluence of science, technology, drug discovery, and clinical trial will lead to successful implementation of informed personalized cancer medicine.

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ORIGINAL ARTICLE

Longitudinal analysis of inflammation and microbiota dynamics in a model of mild chronic dextran sulfate sodium-induced colitis in mice

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Abstract

AIM: To characterize longitudinally the inflammation and the gut microbiota dynamics in a mouse model of dextran sulfate sodium (DSS)-induced colitis.

METHODS: In animal models, the most common method used to trigger colitis is based on the oral administration of the sulfated polysaccharides DSS. The murine DSS colitis model has been widely adopted to induce severe acute, chronic or semi-chronic colitis, and has been validated as an important model for the translation of mice data to human inflammatory bowel disease (IBD). However, it is now clear that models characterized by mild intestinal damage are more accurate for studying the effects of therapeutic agents. For this reason, we have developed a murine model of mild colitis to study longitudinally the inflammation and microbiota dynamics during the intestinal repair processes, and to obtain data suitable to support the recovery of gut microbiota-host homeostasis.

RESULTS: All plasma cytokines evaluated, except IL-17, began to increase ($P < 0.05$), after 7 d of DSS administration. IL-17 only began to increase 4 d after DSS withdrawal. IL-1β and IL-17 continue to increase during the recovery phase, even when clinical signs of colitis had disappeared. IL-6, IL-10 and IFN-γ reached their maxima 4 d after DSS withdrawal and decreased during the late recovery phase. TNFα reached a peak (a three- fold increase, $P < 0.05$), after which it slightly decreased, only to increase again close to the end of the recovery phase. DSS administration induced profound and rapid changes in the mice gut microbiota. After 3 d of DSS administration, we observed a major reduction in Bacteroidetes/Prevotella and a corresponding increase in Bacillaceae, with respect to control mice. In particular, Bacteroidetes/Prevotella decreased from a relative abundance of 59.42%-33.05%, while Bacillaceae showed a concomitant increase from 2.77% to 10.52%. Gut microbiota rapidly shifted toward a healthy profile during the recovery phase and returned normal 4 d after DSS withdrawal. Cyclooxygenase 2 expression started to increase 4 d after DSS withdrawal ($P < 0.05$), when dysbiosis had recovered, and continued to increase during the recovery phase. Taken together,

these data indicated that a chronic phase of intestinal inflammation, characterized by the absence of dysbiosis, could be obtained in mice using a single DSS cycle.

CONCLUSION: Dysbiosis contributes to the local and systemic inflammation that occurs in the DSS model of colitis; however, chronic bowel inflammation is maintained even after recovery from dysbiosis.

Key words: Colitis, Dysbiosis; Dextran sulfate sodium; Inflammation; Cyclooxygenase 2

Core tip: Experimental animal models of colitis are important for investigating the physiopathological mechanisms underlying inflammatory bowel disease (IBD) in humans. Murine dextran sulfate sodium colitis models have been widely adopted and validated as relevant models for the translation of mice data to human IBD. Nevertheless, it is clear that models characterized by mild intestinal damages are more accurate for studying the effects of therapeutic agents. In this study, we developed a reproducible mild chronic colitis model, which allows the evaluation of the intestinal repair processes, the modulation of systemic inflammation and the recovery of the gut microbiotic homeostasis.

De Fazio L, Cavazza E, Spisni E, Strillacci A, Centanni M, Candela M, Praticò C, Campieri M, Ricci C, Valerii MC. Longitudinal analysis of inflammation and microbiota dynamics in a model of mild chronic dextran sulfate sodium-induced colitis in mice. *World J Gastroenterol* 2014; 20(8): 2051-2061 Available from: URL: http://www.wjgnet.com/1007-9327/full/v20/i8/2051.htm DOI: http://dx.doi.org/10.3748/wjg.v20.i8.2051

INTRODUCTION

Inflammatory bowel disease (IBD), comprising Crohn's disease (CD) and ulcerative colitis (UC), is more common in the populations of developed countries. Assessment of the efficacy of novel and adjunct IBD therapies requires experimental animal models resembling human IBD. There is no ideal animal model for IBD, and myriad methods have been designed to induce colitis in mice, rats and other animals. Dextran sulfate sodium (DSS)-induced colitis is one of the most commonly used models. DSS-colitis reflects many of the clinical features of UC[1-3]. For example, changing the DSS concentration or administration cycles can easily induce acute, chronic or relapsing colitis. Moreover, the dysplasia that frequently occurs after the chronic phase of DSS colitis resembles the clinical course of human UC[4]. Recent reports have focused on the multifunctional role of DSS for *in vivo* colitis modeling[5]. In the most widely used DSS murine model, animals are treated with 3% DSS in their drinking water for seven days. This provides a model of acute intestinal injuries that permits clinical monitoring of colitis using parameters such as weight loss, stool consistency and blood in the stool. Together these parameters yield an average clinical score that is a powerful comparable number for identifying potential differences among groups over the total duration of the experiment. However, the devastating intestinal injuries caused by 3% DSS remain for up to ten days after DSS administration. Therefore, they do not provide a sensitive system to evaluate the role of therapeutic agents with different efficacies[5], or the role of the various parameters involved in intestinal repair. By contrast, the milder seven-day 1% DSS treatment model seems to be a powerful means of evaluating the effect of most therapeutic agents and the repair phase of colitis. However, the 1% DSS model would not be characterized as a disease according to traditional disease activity indices and hence prevents clinical monitoring[5]. Histopathology, with quantification of morphological and immunological changes in the colon during and after 1% DSS treatment, is necessary to identify differences among groups.

The molecular events taking place after DSS ingestion and those leading to established colitis are not completely understood, but are of primary importance to understand the strengths and weaknesses of this model. DSS is a sulfated polysaccharide with a variable molecular weight (MW) ranging from 5 to 1400 kDa. DSS is rapidly depolymerized in the stomach, reaching the cecum with a MW between 750 and 5000 Da, and it is reasonable to assume that these smaller sulfated polysaccharides are responsible for the observed colon damage[6]. Hence, the MW of DSS is considered a major factor in the induction of colitis. The most severe colitis was obtained in BALB/c mice using DSS with a MW of 40 kDa; higher or lower MWs resulted in milder forms of colitis[7]. For this reason, some companies have developed DSS specifically designed for the induction of colitis, and these specific products are strongly recommended to obtain much more repeatable results. DSS metabolism in the gut also involves the formation of complexes between DSS fragments and medium-chain fatty acids (MCFA), which are enriched in the large bowel[8]. The high toxicity of DSS-MCFA complexes explains why only the large bowel, and especially the terminal colon, is inflamed by the DSS moieties. Once it enters into colonocytes, DSS impairs major cellular functions by inhibiting the activity of cellular enzymes, such as ribonuclease[8] and iNOS[9], and ultimately causes cell cycle arrest and apoptosis in colonocytes[6], and probably in other colonic wall cells. By interfering with the intestinal barrier function, DSS is also able to stimulate local and systemic inflammation by locally increasing the expression of cyclooxygenase-2 (COX-2) and by inducing the secretion of a variety of cytokines and other inflammatory mediators that spread from the colon to the blood[10].

The importance of the microbiota and microbe-mucosa crosstalk in the pathogenesis of IBD is supported by several animal model studies. Colitis severity is dependent on the commensal bacterial strains maintained in gnotobiotic animals[11], and DSS treatment has been asso-

ciated with a major shift in the composition of the intestinal microbiota, whose dynamics rapidly shift toward an unhealthy state[12-14]. Moreover, antibiotic administration has been shown to improve both IBD and DSS-induced colitis[15], indicating that the microbiota play a critical role in this disease, as well as in the DSS model system. This view is supported by evidence showing that the simple ingestion of a lysate of microbial cells belonging to the Firmicutes, considered a healthy-type phylum, reduced DSS-induced experimental colitis in mice[14].

The present study aimed to characterize longitudinally the inflammation and the gut microbiota dynamics in a highly sensitive DSS-induced murine model of colitis.

MATERIALS AND METHODS

Animal treatment

Twenty-four male 8-week-old C57BL/6 mice were purchased from Charles River Laboratories (Lecco, Italy). The animals were housed in a controlled environment in collective cages at 22 ± 2 ℃ and 50% humidity, under a 12-h light/dark cycle. Mice were allowed to acclimate to these conditions for at least 14 d before inclusion in the experiments and had free access to food and water throughout the study. Colitis was induced in 12 mice by oral administration of dextran sulfate sodium (DSS for colitis, TdB Consultancy, Sweden, MW 40000). DSS was added at a concentration of 1.5% in tap water. DSS-tap water was freshly prepared every day and administered to the mice for 9 d (day 0-9), followed by 21 d of tap water (day 10-29). The average amount of DSS taken was recorded daily. The control group (n = 12) received only tap water. A schema of the experimental design is shown in Figure 1. The experiment, which was approved by the institutional review board of the University of Bologna and performed according to Italian and European guidelines, was repeated three times.

Disease activity index

The disease activity index (DAI) was calculated by the combined score of weight loss, stool consistency and bleeding, as detailed in Table 1. All parameters were scored from day 1 to day 29.

Histological evaluation of colitis

Mice (n = 2) were anesthetized and sacrificed by cervical dislocation on day 3 (after 3 d of DSS intake), 7 (after 7 d of DSS intake), 13 and 19. The colon was excised, rinsed with saline solution, fixed in 4% formalin and embedded in paraffin. Of 4 μm sections were obtained, stained with hematoxylin-eosin and observed for a histological assessment of epithelial damage by a pathologist who was blinded to the samples' origins.

Determination of plasma cytokine levels

Blood samples (200 μL) were taken from the tail vein on days 3, 7, 13, 19, and 29. Blood, collected in Eppendorf tubes containing sodium citrate, was centrifuged at 1000 RPM for 10 minutes, and the plasma was collected and stored at -80 ℃ until BioPlex analysis. Cytokine levels were determined using a multiplexed mouse bead immunoassay kit (Bio-Rad, CA, United States). The six-plexed assays (IL-1α, IL-6, IL-10, IL-17A, IFN-γ and TNFα) were performed in 96-well filter plates, as previously described[16], following the manufacturer's instructions. Microsphere magnetic beads coated with monoclonal antibodies against the different target analytes were added to the wells. After incubation for 30 min, the wells were washed and biotinylated secondary antibodies were added. After incubation for 30 min, the beads were washed and incubated for 10 min with streptavidin-PE conjugated to the fluorescent protein, phycoerythrin (streptavidin/phycoerythrin). After washing, the beads (a minimum of 100/analyte) were analyzed in the BioPlex 200 instrument (BioRad). The concentrations of the samples were estimated from a standard curve using a fifth-order polynomial equation and expressed as pg/ml after adjusting for the dilution factor (Bio-Plex Manager software 5.0). Samples below the detection limit of the assay were recorded as zero. The intra-assay coefficient of variance averaged 15%.

Table 1 Disease activity index score parameters

Stool consistency	Bleeding	Weight loss
0 = Formed	0 = Normal color stool	0 = No weight loss
1 = Mild-soft	1 = Brown color	1 = 5%-10% weight loss
2 = Very soft	2 = Reddish color	2 = 11%-15% weight loss
3 = Watery stool	3 = Bloody stool	3 = 16%-20% weight loss
		4 ⩾ 20% weight loss

RNA extraction and real-time polymerase chain reaction

Total RNA from colon samples was extracted using the Trizol® reagent (Life Technologies, CA, United States), according to the manufacturer's instructions. Extracted RNA samples were treated with DNase I to remove any genomic DNA contamination using DNA-free kit (Ambion, United States) and reverse-transcribed using RevertAid™ First Strand cDNA Synthesis Kit (Fermentas, Canada). COX-2 and β-actin mRNAs were reverse-transcribed using random hexamer primers (Fermentas, Canada). Real-time polymerase chain reaction (PCR) was used to analyze COX-2 and β-actin mRNA levels using the SYBR® Select Master Mix (Life Technologies, CA, United States) and StepOnePlusTM system (Applied Biosystems, CA, United States), according to the manufacturer's instructions. The melting curve data were collected to check PCR specificity. Each cDNA sample was analyzed as triplicate. COX-2 mRNA levels were normalized against β-actin mRNA and relative expressions were calculated using the $2^{-\Delta\Delta Ct}$ formula. The COX-2 primer pair was: 5'- TTC TCT ACA ACA ACT CCA TCC TC -3' and 5'- GCA GCC ATT TCC TTC TCT CC -3' (247 bp product); the β-actin primer pair was: 5'- ACC AAC TGG GAC GAC ATG GAG -3' and 5'- GTG GTG GTG AAG CTG TAG CC -3' (380 bp product).

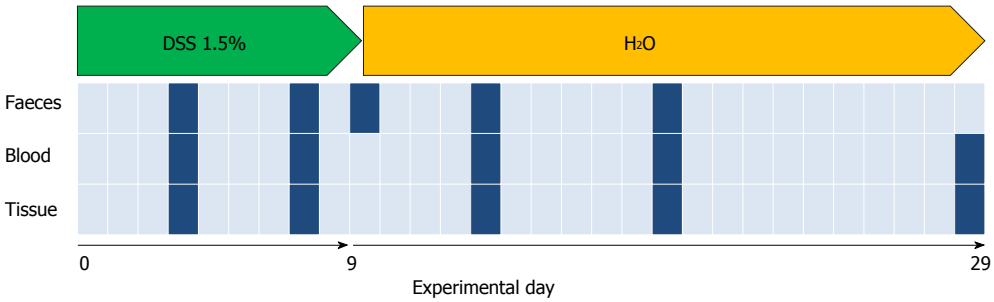

Figure 1 Experimental design of the study. Feces, blood and tissue collection are indicated (dark blue) in the grid. DSS: Dextran sulfate sodium.

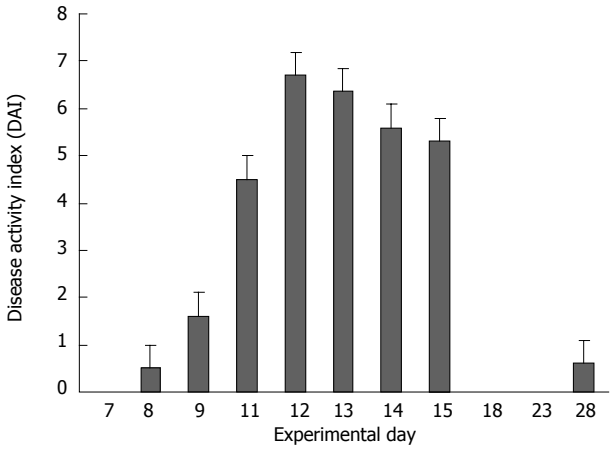

Figure 2 Disease activity index score of colitis in 1.5% dextran sulfate sodium-treated mice. At day 28, an average disease activity index (DAI) score of 0.5 is still present.

Immunohistochemistry

Tissue sections (4 μm) were mounted on slides, sections were deparaffinized with xylene and rehydrated through a series of graded alcohols, and then incubated overnight at 4 ℃ with anti-COX-2 antibody (Cayman Chemicals, Ann Arbor, MC, United States) at a 1:200 dilution in PBS/BSA-1.5%. The primary antibody was omitted from control slides. Sections were then incubated with secondary anti-rabbit antibody for 15 min at room temperature and then reacted with 3,3-diaminobenzidine tetrahydrochloride for 1 min. Sections were then counterstained with hematoxylin.

Characterization of the intestinal microbiota by HTF-Microbi.Array

The intestinal mice microbiota were characterized using the fully validated diphylogenetic DNA microarray platform, HTF-Microbi.Array[17]. Targeting 33 phylogenetically related groups, this ligation detection reaction (LDR)-based Universal Array covers up to 95% of the mammalian gut microbiota[18]. Gut microbiota analysis was performed at day 3, 7, 9, 13 and 19. The QIAamp DNA Stool Mini Kit (Qiagen) was used to extract total DNA from fecal material, according to the modified protocol reported previously[17]. The final DNA concentration was determined using a NanoDrop ND-1000 instrument (NanoDrop Technologies). A nearly full-length portion of the 16S rDNA gene was amplified using universal forward primer 27F and reverse primer 1492R, according to a protocol described previously[17]. PCR amplifications were performed in a Biometra Thermal Cycler T Gradient (Biometra, Göttingen, Germany). The PCR products were purified using the High Pure PCR CleanupMicrokit (Roche, Mannheim, Germany), eluted in 30 μL of sterile water and quantified using a NanoDrop ND-1000. Slide chemical treatment, array production, the LDR protocol and hybridization conditions were performed as reported previously[19,20]. Briefly, LDR reactions were carried out in a final volume of 20 μL containing 500 fmol of each LDR-UA HTF-Microbi.Array probe[18], 50 fmol of PCR product and 25 fmol of the synthetic template (5'-AGCCGCGAACACCACGATCGACCGGCGCGCGGCAGCTGCAGCTTGCTCATG-3'). The LDR products were hybridized on Universal Arrays, setting the probe annealing temperature at 60 ℃. All arrays were scanned and processed according to the protocol and parameters described previously[17]. Fluorescence intensities were normalized on the basis of the synthetic ligation control signal[18]. The relative abundance of each bacterial group was obtained by calculating the relative fluorescence contribution of the corresponding HTF-Microbi. Array probe as a percentage of the total fluorescence.

Statistical analysis

Al data were expressed as the mean ± SEM of at least three independent determinations. Statistical differences between groups were determined by one-way ANOVA by using GraphPad Prism 6 (GraphPad Software Inc., San Diego, CA, United States). Differences were considered statistically significant at $P < 0.05$.

RESULTS

Colitis activity indexes

Mice started to show mild clinical signs of disease after the end of the 1.5% DSS treatment (day 9), as indicated by the simultaneous increase in stool consistency and bleeding index (maximum DAI score = 2). The most evident clinical signs were recorded between days 11 and 15 (Figure 2), with a significant weight loss that peaked

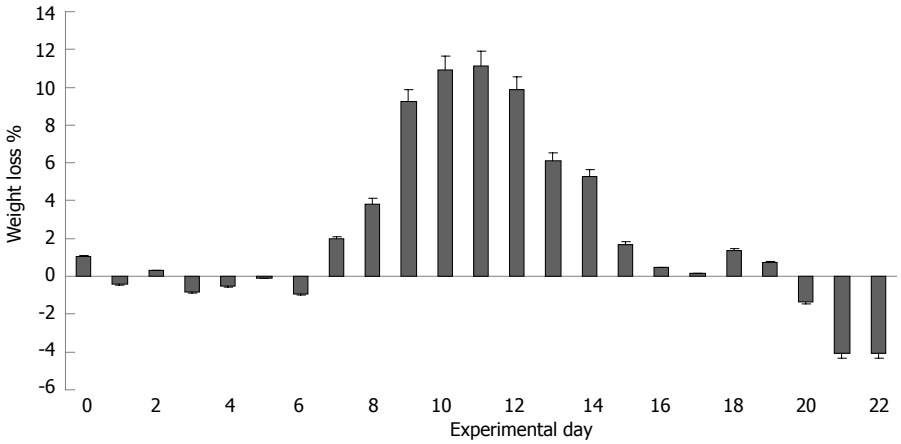

Figure 3 Weight loss in dextran sulphate sodium-treated mice. The maximum weight loss (11%) was recorded between days 9 and 12. Weight recovery ended at days 19-20.

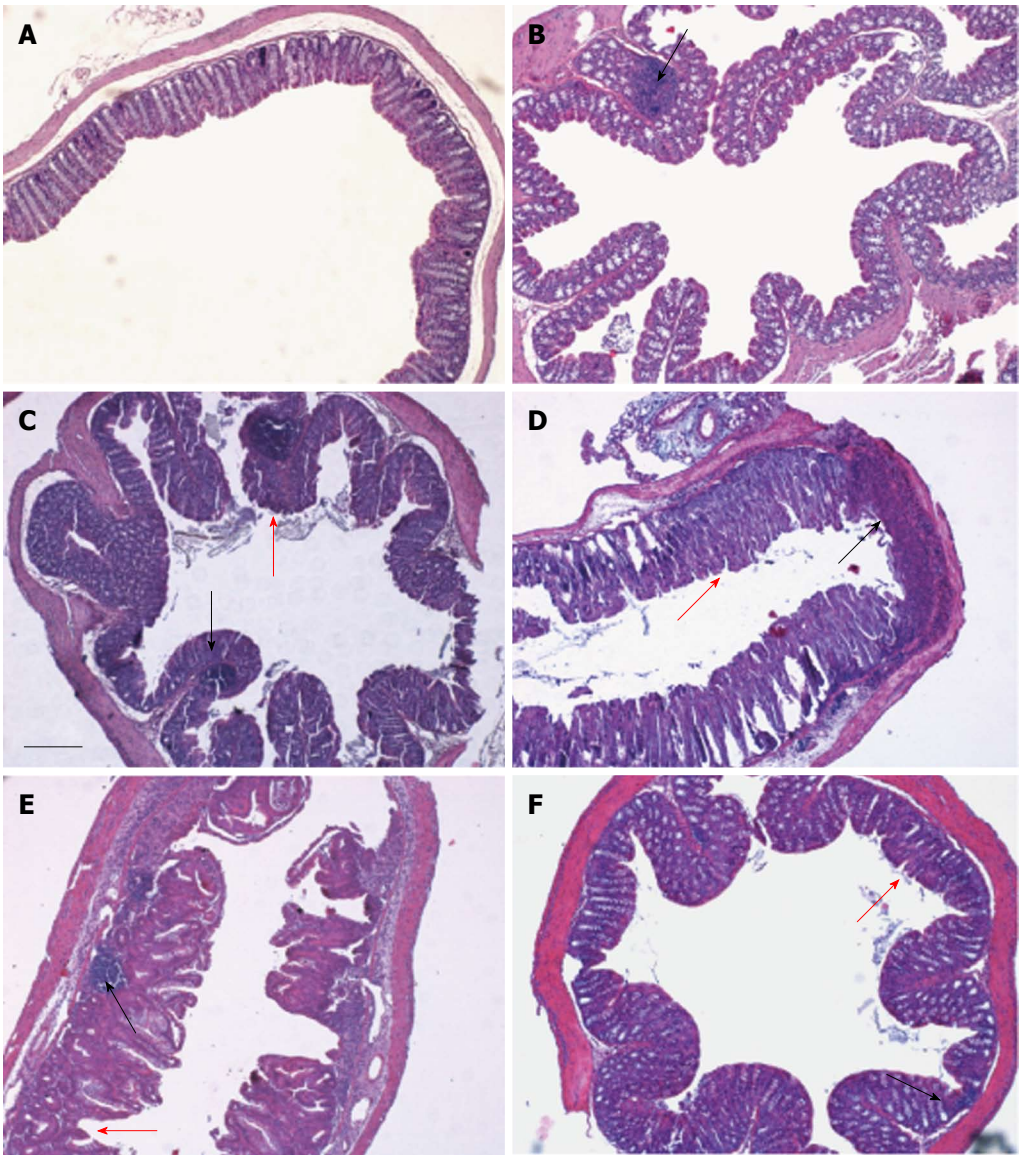

Figure 4 Differences in histological parameters during experimental colitis. Colons were collected from DSS-treated mice on days 3 (B), 7 (C) 13 (D), 19 (E) and 29 (F). In comparison to control mice (A), histopathological changes in individual crypts are shown in representative hematoxylin and eosin-stained sections. Loss of crypt architecture associated with epithelial damage and flattened villi (red arrows) and leukocyte infiltration (black arrows) are evident following DSS treatment (bar = 200 μm).

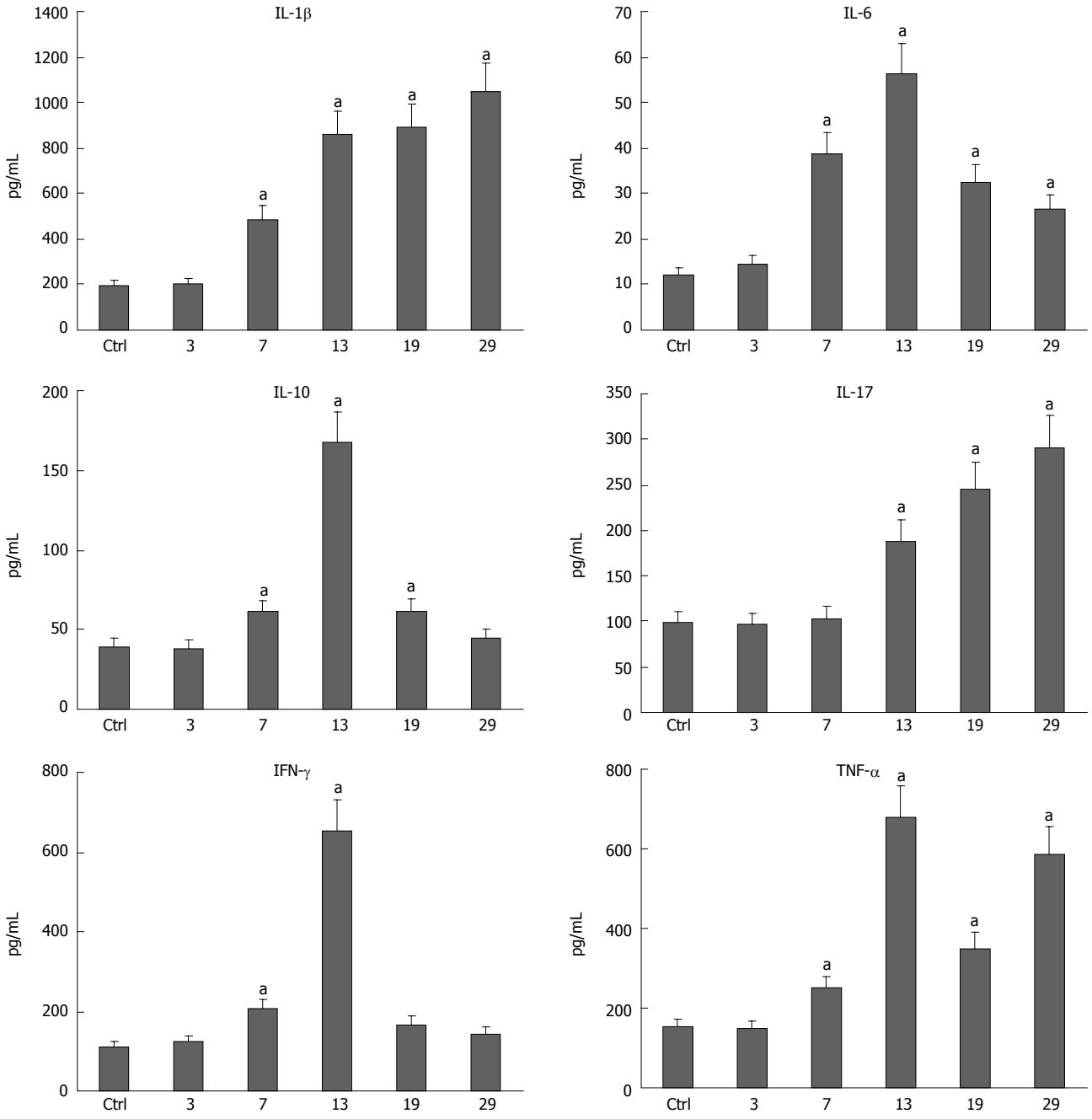

Figure 5 Plasma cytokine variations during experimental colitis. Data are expressed as mean ± SEM of at least three animals. $^{a}P \leq 0.05$ with respect to controls.

between days 9 and 12 (Figure 3) with a maximum DAI score = 7.

Histological evaluation of colitis

Histological evaluation of the colon was made from the colocecal junction to the anus. Overall, the tissue damage tended to be limited to the terminal colon and rectum regions, and could be classified as mild to moderate colitis (Figure 4). After 3 d of DDS treatment (Figure 4B), the colonic mucosa appeared normal, except for focal inflamed areas in which we observed leukocyte infiltration, with a prevalence of granulocytes, indicating active inflammatory colitis, and solid lymphatic follicles. After 7 d of DDS treatment (Figure 4C), the terminal colonic mucosa showed the same features of spotted focal leukocyte infiltrations, with a prevalence of lymphocytes, showing the colitis had shifted toward a chronic status, with mucus discharge, architectural abnormalities and depletion of goblet cells. At day 13 (Figure 4D), the lymphocyte infiltration was widespread, and epithelial damage was evident, with complete crypt disappearance, mucosal erosion in some areas and a mild thickening of the muscularis mucosa. During weight recovery, at day 19 (Figure 4E), leukocyte infiltration tended to return to being focalized. Mucosal erosions were still evident, with flattened villi. At day 29 (Figure 4F), significant focal infiltration, also involving the glands, was still present, with villi that still appeared flattened.

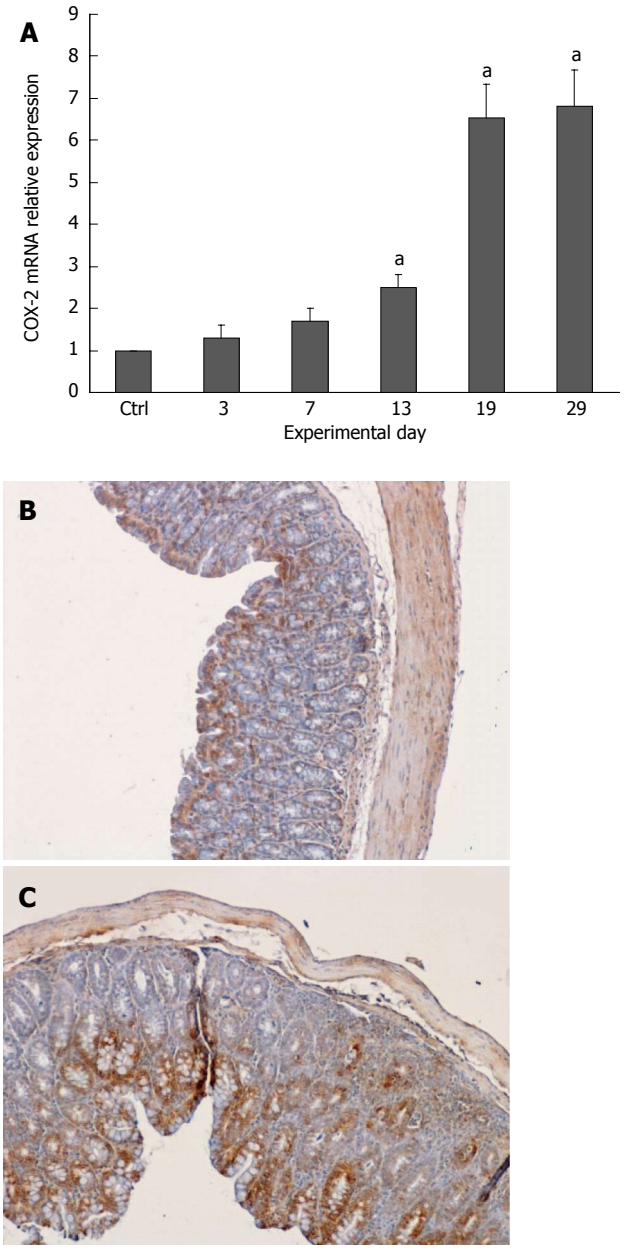

Figure 6 Evaluation of cyclooxygenase-2 mRNA during dextran sulfate sodium-induced colitis. On days 3, 7, 13, 19 and 29 colon tissue was collected and processed for real-time PCR. The COX-2 mRNA significantly increased during the recovery phase (A). $^{a}P \leq 0.05$ *vs* control. Immunohistochemical analysis confirmed that COX-2 expression is limited to the apical mucosa in healthy mice (B), while it is increased and spreads over the entire thickness of the mucosa at day 29 (C).

Inflammatory cytokine profile of colitis

All cytokines evaluated, except IL-17, started to increase after 7 d of DSS administration (Figure 5). The IL-17 plasma level started to increase at day 13. IL-1β (Figure 5A) had a sudden four-fold increase at day 13, when colitis reached its maximum DAI score, and slightly increased until day 29, when clinical signs of colitis had disappeared, reachingβ its peak plasma level. IL-6 (Figure 5B) started to increase at day 7 and reached a peak at day 13 (a 4.5 fold increase), after which it decreased to reach a value twice the normal level by the end of the experiment (day 29). IL-10 (Figure 5C) reached a peak at day 13 (a 4.2 fold increase), after which it decreased to resume a physiological level at the end of the experiment (day 29). IL-17 (Figure 5D) started to increase at day 13 and continuously increased to a peak at day 29 (a three-fold increase), which was the end of the experiment. IFN-γ (Figure 5E) increased at day 7, reaching a peak at day 13 (a six-fold increase) and then rapidly decreased to resume a physiological level at the end of the experiment (day 29). Finally, TNFα (Figure 5F) increased at day 7, reaching a peak at day 13 (a three-fold increase), after which it slightly decreased at day 19 to increase again to the peak level at the end of the experiment (day 29). At the end of the recovery (day 29), IL-1β, IL-17 and TNFα were at their maximum levels, indicating that colitis had become chronic.

Colitis induces COX-2 overexpression in colonocytes and in the colon wall

COX-2 plays a crucial role in the production of many lipid mediators involved in intestinal inflammation and is one of the targets of IBD pharmacological therapy; therefore, we analyzed COX-2 mRNA expression in colon tissue during DSS-induced colitis. Interestingly, COX-2 mRNA started to increase (by two-fold) only 5 d after DSS removal and continue to increase (up to seven-fold) until the end of the recovery phase, a trend that mirrored the chronicization of the inflammatory process (Figure 6).

Intestinal microbiota modifications induced by colitis

To characterize the reaction of the gut microbiota in our DSS murine model of mild colitis, the temporal dynamics of the fecal microbiota of DSS-treated mice was compared with that of healthy controls. In particular, for DSS-treated mice, the gut microbiota was characterized in control mice and at day 3, 7, 9, 13, 19 (Figure 7). The fecal microbiota of healthy control mice was sampled at the same time points. According to our data, DSS administration prompted profound and rapid changes in the mice microbiota (Figure 7). After 3 d of DSS treatment, we observed a major reduction of Bacteroidetes/Prevotella and a corresponding increase in Bacillaceae, with respect to control mice. In particular, Bacteroidetes/Prevotella decreased from a relative abundance (rel.ab.) of 59.42% to 33.05%, while Bacillaceae showed a concomitant increase from 2.77% to 10.52%. During the course of colitis, from day 7 to day 9, there was a progressive increase in the rel.ab. of Bacillaceae (from 10.52% to 17.90%), Lactobacillaceae (from 2.07% to 6.55%), Verrucomicrobiae (from 0.82% to 1.07%), Enterococcales (from 1.52% to 2.07%) and Enterobacteriaceae (from 0.66% to 1.18%), and a parallel progressive reduction of members of the Clostridium cluster XIVa (from 23.79% to 7.19%). On the other hand, the progression of colitis did not affect Bacteroidetes/Prevotella, which remained constant at the low rel.ab value detected 3 d after DSS administration. Unlike DSS-treated mice, healthy control mice showed a constant gut microbiota profile throughout the study. Interestingly, during recovery from DSS-induced colitis,

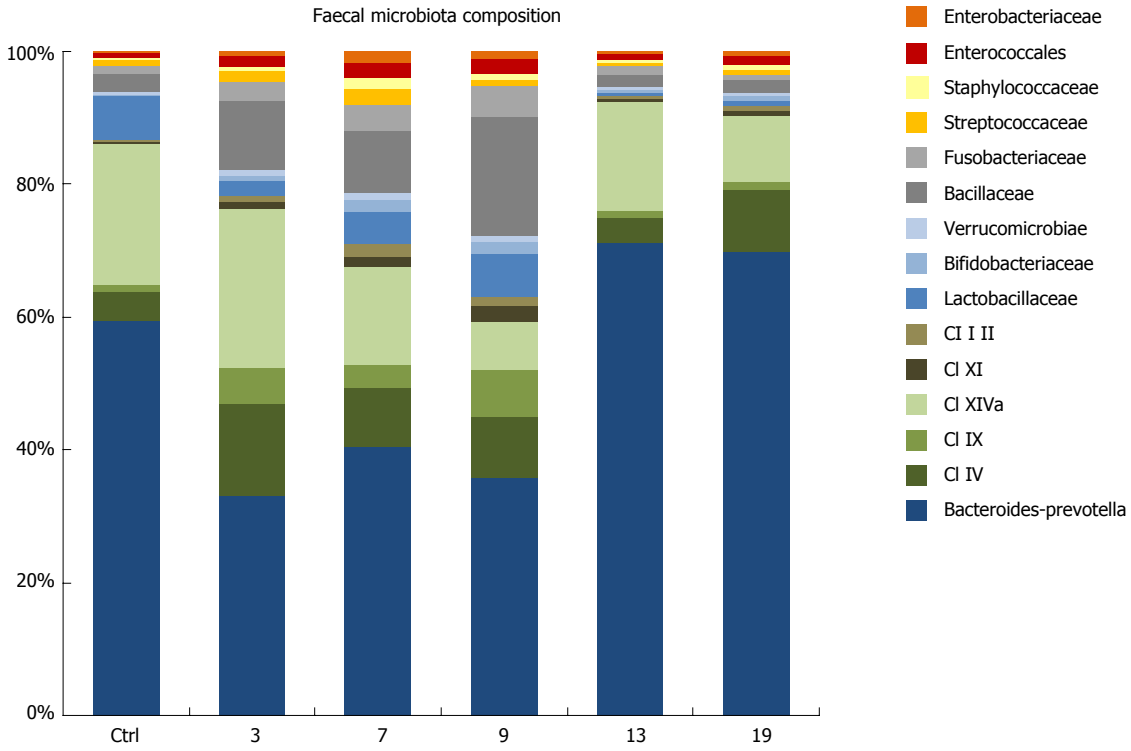

Figure 7 Temporal dynamics of the fecal microbial community of dextran sulfate sodium-treated mice and healthy controls. Cl: *Clostridium* cluster.

at day 13 and 19, we observed a rapid shift of the gut microbiota toward a healthy profile, comparable to that shown by healthy control mice. In particular, 5 d after the interruption of DSS administration, the mice microbiota recovered a rel.ab. value of Bacteroidetes/Prevotella and Bacillaceae similar to that observed in healthy controls (71.08% and 1.79%, respectively).

DISCUSSION

The DSS murine model described here induces a milder colitis than the classical 3% DSS model, with a mortality rate very close to zero. Moreover, the signs and symptoms of colitis induced by this model are much more homogeneous in all the treated mice. We are aware that the responses to DSS observed in laboratory animals not only depend on DSS type and treatment protocol[21]; however, in our hands, this model has proven to be highly reproducible.

Histological examination of the colon of 1.5% DSS-treated mice showed that the mucosal damage starts to be evident 6 d before the DAI started to increase. This early damage was limited to the terminal colon mucosa and ascended toward the proximal colon when colitis severity increased. Colitis showed peak histological damage at day 13, in association with the maximum DAI. Histological damage remained evident even when the DAI score had returned to zero and the weight loss had been completely recovered. The persistence of histological damage, lymphocyte infiltration and COX-2 overexpression after complete clinical recovery (day 29) mimics the features of chronic UC in humans. The histological changes in this model are much easier to follow compared with those observed in the 3% DSS model, in which epithelial architecture is constantly lost for many days[5].

Circulating cytokine levels are indicative of the whole inflammatory profile. While studies in IBD patients have focused on serum cytokines, most investigations in mice models tended to evaluate tissue-derived cytokines, losing information on systemic inflammation. Circulating IL-1β, IL-6, IL-17 and TNFα play a key role in the pathogenesis of IBD[22]. IL-1β and IL-6 levels correlate with IBD activity. IL-17 is a delayed-type immune reaction cytokine produced by Th17 and by CD8+ T cells during chronic inflammation. Even if its role in IBD remains controversial[23], it seems to have a prominent pro-inflammatory role in the DSS model[24,25]. IL-10 is the most important antinflammatory cytokine in humans. Its role has been extensively studied in IL-10 knockout mice, and IL-10 mRNA expression in the inflamed mucosa is increased in UC patients, but decreased in CD patients[26,27]. IFNγ secretion has been linked to IL-17 secretion[28] in experimental colitis and its relative mRNA expression transiently increases during DSS-induced acute colitis, with a peak close to the maximum DAI score[5]. TNFα is a master cytokine in IBD pathogenesis, and its orchestrating role in colonic inflammation was verified by the efficacy of anti-TNFα therapy in IBD[29]. The serum TNFα level correlates with clinical activity both in UC and CD[29]. Alex and collaborators[30] reported that acute DSS colitis in mice significantly increases circulating IL-1β, TNFα, IL-6 and IL-17, while chronic colitis increases IL-4, IL-10, IL-6 and IFNγ; however, they only analyzed a single time point for each condition. In our model, while IL-6, IFNγ

and IL-10 peaked at day 13 (maximum DAI score) and decreased during the recovery of colitis, IL-1β, IL-17 and TNFα levels remained high, even when the symptoms and signs of colitis had disappeared. Thus, while circulating IL-6, IFNγ and IL-10 levels seem to correlate with the major clinical signs, IL-1β, IL-17 and TNFα mainly correlate with histological damage that persists and becomes chronic.

DSS (2%-5%) administration for 5-7 d has been used to develop an acute form of colitis, while an inflammatory condition reminiscent of human chronic IBD can be induced by repeated DSS cycles[31]. Our model permits the development of chronic colitis using a single DSS cycle.

To the best of our knowledge, this is the first study to characterize the gut microbiota trajectory in a mouse model of DSS-induced colitis. In particular, the longitudinal approach allowed the assessment of microbiotic changes immediately after the induction of colitis, during the course of disease progression and during the recovery phase. According to our data, the induction of colitis rapidly compromises the homeostasis of the gut microbial ecosystem, leading to a dramatic reduction of Bacteroidetes/Prevotella, a major mutualistic group of the mice gut microbiota, and a corresponding increase in Bacillaceae. Confirming these findings, a rapid decrease in Bacteroidetes was previously observed in mice models of DSS-induced colitis[32]. Moreover, disease progression in our model was associated with a gradual, but weak, increase in the pro-inflammatory gut microbiotic components Enterococcales and Enterobacteriaceae, the minor symbiotic member Lactobacillaceae, and the mucus-degrading Verrucomicrobiae *Akkermansia muciniphila*. On the other hand, during the progression of colitis, we also noted a gradual decrease in members of the Clostridium cluster XIVa. As a major component of a healthy gut microbiota, this cluster is involved in the production of short-chain fatty acids, which are microbial metabolites essential for several aspects of the host physiology: nutrition, immune modulation and protection from pathogen colonization[33]. Taken together, these data demonstrate a progressive impairment of the gut microbiota with advancing colitis, resulting in a dysbiotic profile that can violate mutualism and support the disease. Interestingly, at the end of DSS administration, during weight recovery, we observed a rapid shift of the gut microbial community toward a healthy profile. Within two days of the end of DSS administration, the mice gut microbiota showed rel.ab. values of Bacteroidetes/Prevotella, Bacillaceae, Enterococcales, Enterobacteriaceae, Lactobacillaceae, Verrucomicrobiae and Clostridium cluster XIVa similar to those observed in healthy controls. These data demonstrated the high degree of resilience of the gut microbiota, which showed a potential for rapid recovery of its healthy mutualistic profile after DSS-induced dysbiosis.

One of the unanswered questions regarding IBD and DSS-induced colitis is to establish to what extent the dysbiosis is a contributory cause of the local and systemic inflammation, especially during the recovery phase. Dysbiosis can cause increased mucus secretion[7] and exacerbate intestinal inflammation, which further contribute to the microbiota shift. In DSS-colitis, microbiota homeostasis is rapidly compromised. After 3 d of DSS treatment, the microbiota is profoundly changed, and this alteration is maintained during DSS administration. When the maximum DAI was reached, the microbiota were observed to be returning to a healthy composition. Thus, dysbiosis precedes the systemic inflammation that starts to increase after 7 d of DSS treatment, reaching its maximum when the maximum DAI is reached and remaining high until the late recovery phase.

COX-2 is a very good marker of colonocytes and colon mucosa inflammation. Its expression in the colon of DSS-treated mice starts to increase only when the maximum DAI is reached and remains very high until the late recovery phase. The 10 d delay between the dysbiosis and the increased COX-2 expression in colonocytes suggests that dysbiosis alone is not able to trigger COX-2 expression. On the other hand, recovery from dysbiosis is not sufficient to ameliorate the inflammatory profile of DSS colitic mice, nor the inflammation of their colonocytes.

These results emphasize that the microbiota certainly contribute to intestinal inflammation, but also that the pro-inflammatory response elicited by DSS in the colon wall continues even when the animals recover from dysbiosis. It is therefore reasonable to assume that the observed deviations in the gut microbiota structure can foster changes in cytokine expression[34]. However, the interactions between the microbiota and the immune system are very complicated, and remain to be elucidated in detail[35]. More studies are required to be able to draw conclusions regarding this point.

The overexpression of COX-2 in colonocytes associated with leukocyte mucosal infiltration creates a pro-inflammatory loop from which it is difficult to escape. Moreover, circulating IL-10, one of the major anti-inflammatory cytokines, decreases during the recovery phase until it returns to basal levels at the end of the recovery, when both COX-2 expression and circulating IL-1β, IL-17 and TNFα are at their maximum levels. It is very likely that this kind of pro-inflammatory loop, which is responsible for the chronicity of DSS-induced colitis, is also activated in UC patients.

Concluding remarks

Decreasing the DSS concentration to 1.5% and increasing the treatment's duration to 9 d induces chronic colitis with a short milder acute phase, followed by a mild chronic active disease. This mild disease is a much more accurate condition for studying the dynamics of colitis during clinical remission. This model also represents a step forward in reducing the suffering of animals and, given the very low mortality rate, it allows a reduction in the number of animals required to obtain statistically significant results.

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COMMENTS

Background

Progress in understanding the molecular basis of inflammatory bowel disease (IBD) in humans has accelerated, thanks to the generation of animal models of colitis. Experimental colitis permits the study of complex physiopathological mechanisms, which cannot be simulated *in vitro* or *in silico*. The most commonly used method to trigger colitis in animal models is based on oral administration of a sulfated polysaccharide called dextran sulfate sodium (DSS). This model has been validated as a relevant model for the translation of mice data to human inflammatory bowel diseases.

Research frontiers

The etiology of IBDs remains largely unknown, and their prevalence is increasing in developed countries, with the total number of IBD patients estimated as between 1 and 1.5 million in the United States. A genetic basis for IBD has long been recognized, because of the increased familial risk. However, significant discordance for Crohn's disease (CD) in twins, and a much less robust phenotypic concordance for ulcerative colitis (UC), suggest that environmental factors play a major role in IBDs pathogenesis. Among these, the gut microbiota seems to have a crucial role in CD and UC, because an altered immune response to normal microbiota has been identified as a common feature in IBD patients.

Applications

This study represents a step forward in the use of the DSS model in preclinical studies. It describes new experimental procedures for dissecting the role of microbiome-immune system interactions in the pathogenesis of colitis and the evaluation of new possible IBDs treatments.

Terminology

IBDs, including CD and UC, are chronic inflammatory disorders of the intestine. DSS is a synthetic sulfated polysaccharide composed of dextran with sulfated glucose. It is capable of triggering colitis in mice by binding to medium-chain-length fatty acids present in the colon, thereby inducing inflammation.

Peer review

The study is well designed and very interesting for evaluating treatments for ulcerative colitis with mild to moderate activity. It describes a new murine model of colitis, based on the administration of 1.5% DSS. The results are very interesting and have a strong potential for being used as a benchmark for further studies that evaluate the possible treatments of colitis.

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ORIGINAL ARTICLE

Impaired balance of T helper 17/T regulatory cells in carbon tetrachloride-induced liver fibrosis in mice

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Abstract

AIM: To investigate the effect of T helper (Th) 17/T regulatory (Treg) cells on hepatic fibrosis in mice and its possible mechanism.

METHODS: Hepatic fibrosis was induced by intraperitoneal injection of carbon tetrachloride. Hepatic pathological changes were observed by hematoxylin and eosin staining; the protein levels of interleukin (IL)-6, transforming growth factor (TGF)-β and α-smooth muscle actin (SMA) in liver tissue were determined by Western blotting; and the frequency of Th17 and Treg cells in the liver was estimated by flow cytometry. In addition, hepatic stellate cells were isolated from healthy mouse liver and co-cultured with Th17 or Treg cells. Immunofluorescence staining and Western blotting were performed to determine the change in HSC activation.

RESULTS: In the model group, there were different degrees of fibroplasia, degeneration and necrosis. The protein levels of IL-6, TGF-β and α-SMA in liver tissue were significantly higher than those in the control group at 12 wk ($P < 0.05$). Compared with the control group, the frequency of Th17 cells in the model group was increased but the frequency of Treg cells decreased gradually. Furthermore, at 4, 8 and 12 wk, there were significant differences in the number of Th17 cells (0.52% ± 0.16%, 1.46% ± 0.24%, and 2.60% ± 0.41%, respectively, $P < 0.05$) and Treg cells (2.99% ± 0.40%, 2.16% ± 0.50%, and 1.49% ± 0.34%, respectively, $P < 0.05$). *In vitro*, Th17 cells promoted, whereas Treg cells inhibited the expression of α-SMA, both in a dose-dependent manner, compared with the control group.

CONCLUSION: Th17/Treg imbalance exists in mice with liver fibrosis, which potentially promotes liver fibrosis *via* HSC activation.

Key words: T helper 17 cell; Treg cell; Carbon tetrachloride; Hepatic fibrosis; Hepatic stellate cell

Core tip: It has been reported that T helper (Th) 17/T regulatory (Treg) cell imbalance is closely related to many autoimmune diseases. The role of Th17/Treg imbalance in liver fibrosis has seldom been reported. Our study focused on the change in Th17/Treg balance in a liver fibrosis model in mice, and explored the possible mechanism through which the development of fibrosis is regulated. The frequency of Th17 cells increased, while the frequency of Treg cells decreased in liver fibrosis. These changes promote the occurrence of liver fibrosis *via* hepatic stellate cell activation.

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INTRODUCTION

Liver fibrosis is a chronic progressive disease that is characterized by the formation and accumulation of extracellular matrix that lead to the remodeling of the hepatic architecture. It is the final common pathway in a variety of chronic liver diseases that can be reversed at an early stage, but when it is irreversible, the patients with liver fibrosis are at increased risk of developing cirrhosis. However, the pathogenesis of fibrosis is not entirely clear at present.

Helper $CD4^+$ T cells can orchestrate host immune responses through the release of distinct cytokine profiles. Recent studies have described two additional subsets-interleukin (IL)-17-producing $CD4^+$ T helper (Th) 17 cells and T regulatory (Treg) cells[1]. Th17 cells expressing retinoic-acid-related orphan receptor (ROR)-γt play critical roles in the development of autoimmunity and allergic reactions by producing IL-17[2-4], while Treg cells expressing the forkhead/winged helix transcription factor P3 (FoxP3) have an anti-inflammatory role and maintain tolerance to self-components[5] by contact-dependent suppression or releasing anti-inflammatory cytokines [IL-10 and transforming growth factor (TGF)-β][6,7]. Recently, many studies have found that imbalance of Th17/Treg cells is closely related to a variety of autoimmune diseases[8-11]. However, the role of Th17/Treg imbalance in liver fibrosis has seldom been reported.

The objectives of this study were to evaluate whether Th17/Treg balance is disrupted in mice with liver fibrosis, and to explore the potential mechanism through which Th17/Treg imbalance promotes the development of liver fibrosis. We used carbon tetrachloride (CCl_4) to induce liver fibrosis in a mouse model, and mice were sacrificed at 4, 8 and 12 wk. We first measured the protein levels of IL-6, TGF-β and α-smooth muscle actin (SMA) by Western blotting, and the frequency of Th17 and Treg cells in the liver was evaluated by flow cytometry. Finally, we investigated the effect of Th17 and Treg cells on the activation of hepatic stellate cells (HSCs) *in vitro*.

MATERIALS AND METHODS

Animals

Male C57BL/6 mice (aged 6-8 wk, 18-21 g) were purchased from the Shanghai Slac Experimental Animal Centre (Shanghai, China). Mice were maintained under specific pathogen-free conditions with a 12-h light/dark cycle and unlimited supplies of food and water. The experimental procedures conformed to the guidelines outlined in the Guide for the Care and Use of Laboratory Animals and were approved by the Research Ethics Committee of Renji Hospital [SYXY (hu) 2011-0121]. Sixty mice were randomly divided into a control group (n = 30) and model group (n = 30), and then the mice in each group were randomly divided into 4, 8 and 12-wk groups of 10 mice each.

Liver fibrosis model and sample collection

Mice in the model group were injected intraperitoneally, twice a week, with 10 μL/g of 30% CCl_4 (Shanghai Jiahe Biotechnology, Shanghai, China) dissolved in olive oil. Mice in the control group were given the same volume of olive oil for the indicated time intervals. Mice were sacrificed 72 h after the final CCl_4 injection at 4, 8 and 12 wk, and liver tissues were collected. The liver tissues were divided into two parts. One part was kept for histological examination and Western blotting, and the other was used for the detection of Th17 and Treg cells.

Histological examination

The liver tissues were fixed in 10% neutral buffered formalin and embedded in paraffin. Slices 4-μm thick were prepared and stained with hematoxylin and eosin (HE) according to standard procedures. The degree of fibrosis was assessed based on Scheuer's scoring system[12].

Western blotting

Total protein was extracted according to the manufacturer's instructions (Pierce, United States) and the protein concentration was determined. Proteins were separated by 12% SDS-PAGE and then transferred onto a polyvinylidene difluoride membrane. The membrane was blocked with 5% non-fat milk for 2 h followed by incubation with primary antibody in Tris-buffered saline with Tween overnight at 4 ℃ (anti-IL-6 1:300 dilution; anti-TGF-β 1:300 dilution; and anti-α-SMA 1:500 dilution); all the antibodies were purchased from Abcam (Cambridge, United Kingdom). The membrane was incubated with a horseradish peroxidase-conjugated secondary antibody (1:10000 dilution, LI-COR, Lincoln, NE, United States) The membrane was scanned by Odyssey machine and quantified using Image J version 1.4.3.67 software.

Flow cytometric analysis of T cell subsets

Single-cell suspensions were prepared from liver by dissecting the tissue into small pieces, grinding them, and then filtering them through stainless steel meshes. Lymphocytes were obtained through Percoll density gradient centrifugation (Beijing Dingguo Biotechnology, Beijing, China) from the cell suspensions. For Th17 cell detection, lymphocytes were stimulated for 5 h with 50 ng/mL phorbol myristate acetate (PMA), 1 μmol/L ionomycin (both from Sigma, St Louis, MO, United States) and 10 μg/mL brefeldin A (eBioscience, San Diego, CA, United States) in RPMI-1640 (Hyclone, Logan, UT, United States) supplemented with 10% fetal bovine serum (FBS; Hyclone). Upon harvest, cells were first stained with fluorescein isothiocyanate (FITC)-anti-CD4 at 4 ℃ for 20 min, then fixed in paraformaldehyde, permeabilized in Perm/Fix solution, and finally stained

Table 1 Dynamic changes in hepatic fibrosis score in mice

Time	Group	Number	Liver fibrosis score			
			-	+	++	+++
4 wk	Model	9	2	7		
	Control	10	10			
8 wk	Model	8		2	6	
	Control	10	10			
12 wk	Model	6			1	5
	Control	10	9	1		

intracellularly with PE-anti-IL-17A. For Treg cell detection, lymphocytes were incubated with FITC-anti-CD4 and APC-anti-CD25 at room temperature for 20 min[13]. Cells stained with IgG isotype control were used as controls. All antibodies and fixation/permeabilization agents were purchased from Bioscience. Cells were analyzed by FACSCalibur flow cytometer. The percentage of positive cells was determined using CXP analysis software.

Isolation of HSCs

Mouse HSCs were isolated from the livers of C57BL/6 by *in situ* collagenase perfusion and differential centrifugation on Percoll density gradients (Beijing Dingguo Biotechnology) as described previously[14]. The fresh isolated HSCs were resuspended in RPMI-1640 (Hyclone) containing 10% FBS and penicillin/streptomycin, and then plated onto 24-well plates (plastic plates were used for self-activation of HSCs). The HSCs were cultured for 6 d and harvested for subsequent use. The purity of HSC cultures was > 92% as determined by the fluorescence of vitamin-A-containing lipid droplets[15-17]. All cells were cultured in a humidified incubator with 5% CO_2 at 37 ℃.

Preparation of Th17 and Treg cells

Th17 and Treg cells were isolated from the spleen of C57BL/6 mice using a cell isolation kit (Miltenyi Biotec, Bisley, Surrey, United Kingdom). For Th17 cell isolation, fresh spleen cells were stimulated with ionomycin (1 μg/mL) and PMA (10 ng/mL) for 3 h; labeled with Mouse IL-17 Catch Reagent, IL-17 Detection Antibody (Biotin) and Anti-Biotin-PE; and collected by magnetic separation. For Treg cell isolation, non-$CD4^+$T cells were depleted after magnetic labeling of non-$CD4^+$ cells and fluorescent labeling of $CD25^+$ cells, finally the cells were labeled with Anti-PE MicroBeads, and $CD4^+CD25^+$ regulatory T cells were selected with MS Columns. Cells were cultured and expanded for 5 d using CD3/CD28 MACSiBead Particles and 2000 U/mL IL-2. RPMI-1640 medium supplemented with 10% FBS and penicillin/streptomycin were used for cultures. After culturing, cells were harvested and beads were removed.

Cell co-culture

Forty-eight-well plates (Corning, Corning, NY, United States) were used for cell co-cultures. First, the HSCs were plated at a density of 1×10^4 cells per well and cultured for 12 h. Th17 and Treg cells were added separately to the culture system. The cells were co-cultured for 3 d and expression of α-SMA was determined by Western blotting and immunofluorescene staining.

Immunofluorescence staining

Immunofluorescence staining was performed as described previously[16,17]. The fixed cells were stained with rabbit anti-mouse α-SMA monoclonal antibody (1:100 dilution; Abcam) followed by CY3-conjugated goat anti-rabbit antibody (1:400 dilution) (Jackson ImmunoResearch, West Grove, PA, United States). DAPI was used for nuclear staining. Expression of α-SMA was observed under fluorescent microscopy and fluorescence intensity was determined by Image Pro Plus software.

Statistical analysis

Statistical analysis was performed using SPSS version 17.0 software. The results were presented as mean ± SD. One-way analysis of variance was applied, and $P < 0.05$ was considered statistically significant.

RESULTS

Histopathology of liver tissue

Hepatic fibrosis scores are shown in Table 1. In the model group, hepatic fibrosis score increased gradually after CCl_4 injection; however, the scores in the control group were largely unchanged. These results indicated that olive oil was not harmful to the mouse liver.

Mouse liver surface in the control group was smooth, the structure of the hepatic lobules and portal area was complete, and there was no cellular degeneration, inflammatory reaction and fiber cords as shown by HE staining (Figure 1A). After CCl_4 administration, mice developed different degrees of hepatic inflammation, cellular degeneration, necrosis, and distorted hepatic architectural (Figure 1B-D). At the end of 12 wk, mouse liver showed diffuse hyperplasia of fibrous tissue, the arrangement of the hepatic cells was disordered, and there was a lot of degeneration and necrosis of liver cells, with deficiency of the central vein (Figure 1D). As the time of CCl_4 injection was increased, the liver fibrosis model was gradually established.

Expression of IL-6, TGF-β and α-SMA in liver tissue

After CCl_4 administration, the expression of IL-6, TGF-β and α-SMA was increased as compared with the controls (Figure 2A). Relative protein levels are shown in Figure 2B. Expression of TGF-β at 4 wk was higher than in the control group but the difference was not significant ($P > 0.05$). The level of TGF-β at 8 and 12 wk was significantly increased ($P < 0.05$). Compared with the controls, the expression of IL-6 and α-SMA in the model group was significantly increased ($P < 0.05$), and the differences at 4, 8 and 12 wk were significant ($P < 0.05$).

Increased Th17 cells and decreased Treg cells in CCl_4-induced hepatic fibrosis model

To investigate whether injection of CCl_4 influenced the number of Th17 cells ($CD4^+IL\text{-}17^+$ T cells) and Treg

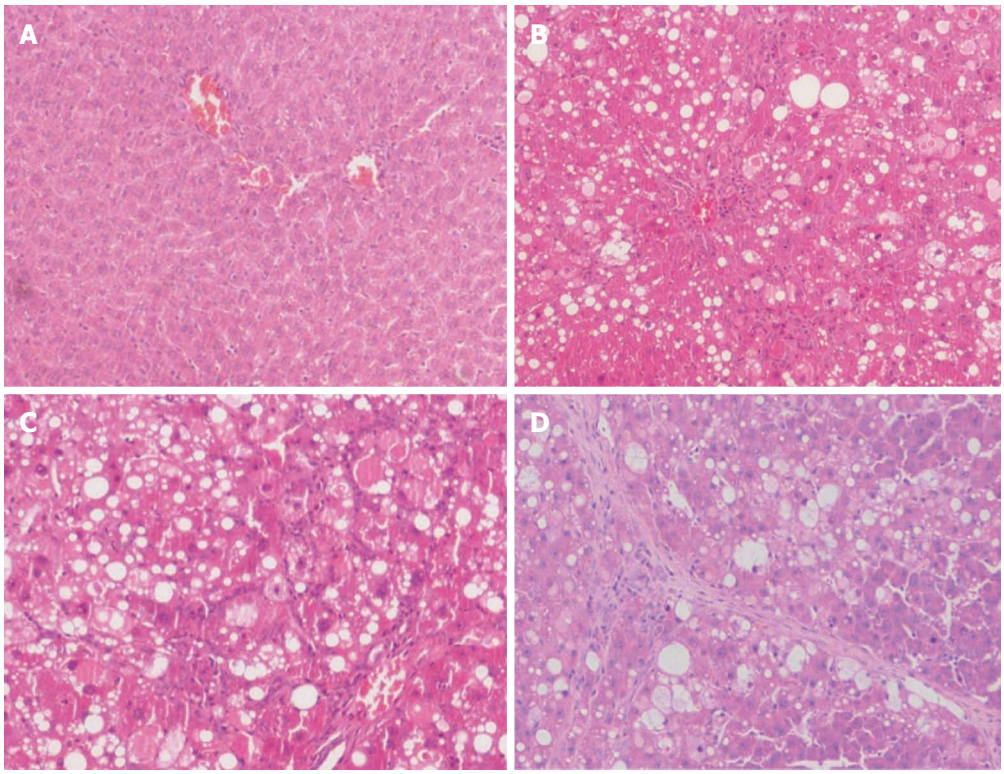


Figure 1 Pathological changes in the liver after carbon tetrachloride administration (hematoxylin and eosin, original magnification, × 100). A: Control group; B: 4-wk group; C: 8-wk group; D: 12-wk group.

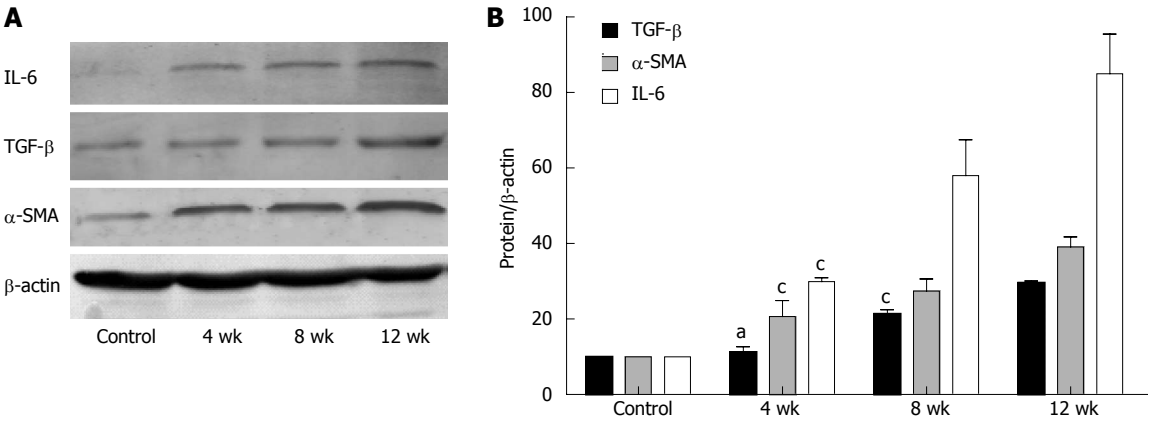


Figure 2 Expression of proteins in the liver at different times. A: Western blotting; B: Quantitive analysis, $^{a}P > 0.05$ *vs* control, $^{c}P < 0.05$ *vs* control. α-SMA: α-Smooth muscle actin; IL: Interleukin; TGF: Transforming growth factor.

cells ($CD4^{+}CD25^{+}$ Treg cells), the percentages of Th17 and Treg cells in mouse liver were measured by flow cytometry (Figure 3).

In the control group, the frequency of Th17 cells at 4, 8 and 12 wk was 0.30% ± 0.15%, 0.34% ± 0.16% and 0.26% ± 0.08%, respectively. The frequency of Treg cells was 3.31% ± 0.32%, 3.42% ± 0.27% and 3.25% ± 0.38%, respectively. The differences between the 4, 8 and 12-wk groups for the number of Th17 and Treg cells were not significant ($P > 0.05$). The results indicated that olive oil was not harmful to mouse liver. The data from the 4-wk group were used as controls for the model group.

In the model group (Figure 3A and C), the frequency of Th17 cells was increased gradually. The difference between the 4-wk group (0.52% ± 0.16%, $n = 9$) and the controls (0.30% ± 0.15%, $n = 10$) was not significant ($P > 0.05$). The frequency of Th17 cells was significantly increased in both the 8-wk (1.46% ± 0.24%, $n = 8$) and 12-wk (2.60% ± 0.41%, $n = 6$) groups compared with the controls ($P < 0.01$). Moreover, there was a significant difference between the 4-, 8- and 12-wk groups ($P < 0.05$).

As shown in Figure 3B and D, the frequency of Treg cells in the 4-wk group (2.99% ± 0.40%, $n = 9$) was not significantly decreased compared to the controls (3.31%

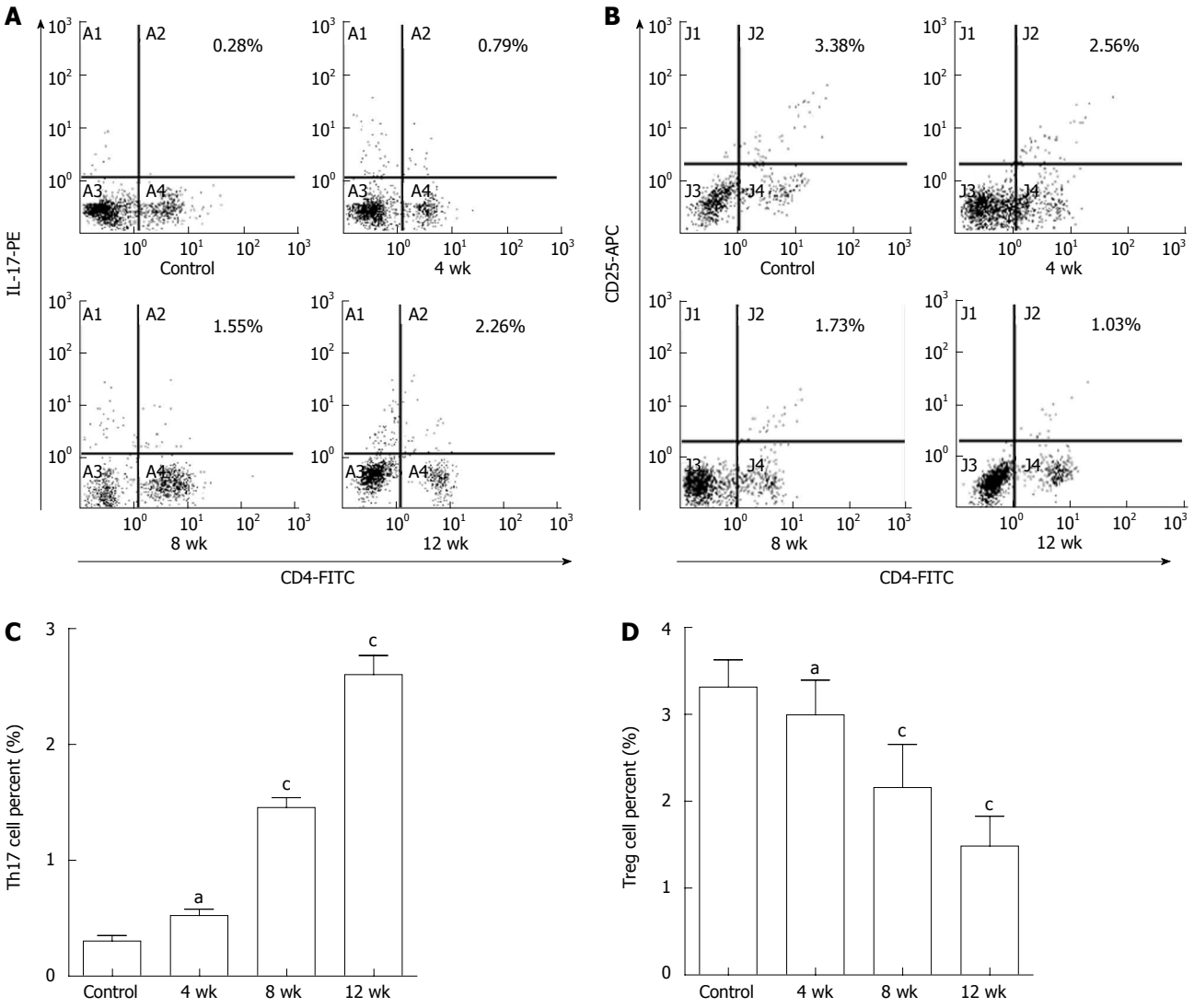


Figure 3 Changes in T helper 17 and T regulatory cells in the liver after carbon tetrachloride administration. A and B: Representative images of Th17 (A) and Treg (B) cells at different times after CCl_4 administration; C and D: Proportions of Th17 (C) and Treg (D) cells. $^aP > 0.05$ *vs* control, $^cP < 0.05$ *vs* control. CCl_4: Carbon tetrachloride; Th17: T helper 17; Treg: T regulatory; IL: Interleukin; FITC: Fluorescein isothiocyanate.

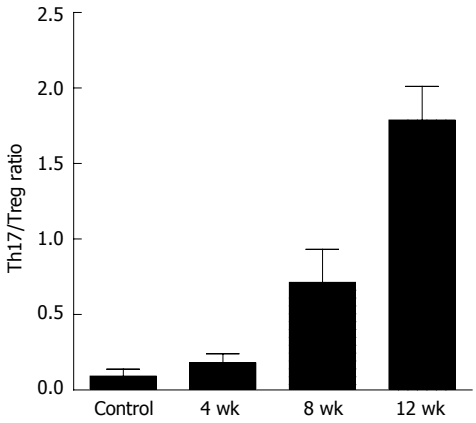


Figure 4 T helper 17/T regulatory ratio in the control and 4-, 8- and 12-wk groups. Th17: T helper 17; Treg: T regulatory.

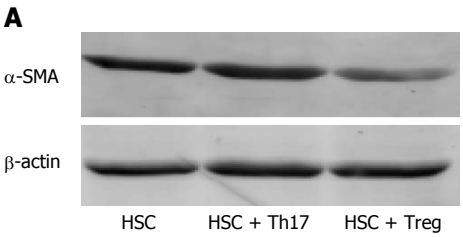
± 0.32%, $n = 10$) ($P > 0.05$). However, the frequency of Treg cells was significantly decreased in both the 8-wk (2.16% ± 0.50%, $n = 8$) and 12-wk (1.49% ± 0.34%, $n = 6$) groups ($P < 0.01$). Also, the difference between the 4-, 8- and 12-wk groups was significant ($P < 0.05$).

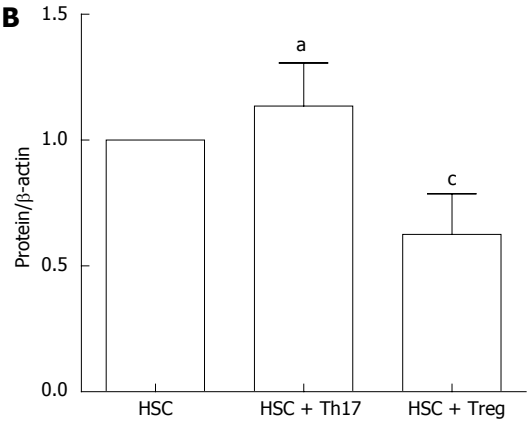
Th17/Treg ratios indicated severity of fibrosis in mice

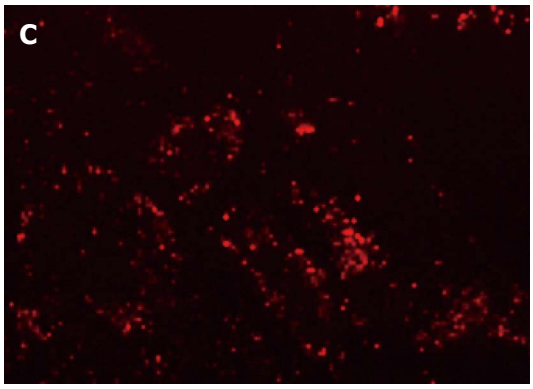
As shown in Figure 3, we detected the frequency of Th17 and Treg cells, and then we calculated the Th17/Treg cell ratios using the frequencies at different times. We found that the ratios in the model group were higher than in the controls ($P < 0.05$) (Figure 4) and the ratios increased gradually in the 4-, 8- and 12-wk groups. This indicated that the Th17/Treg cell balance in the liver was disrupted and more conducive to Th17 cell production and progression of fibrosis.

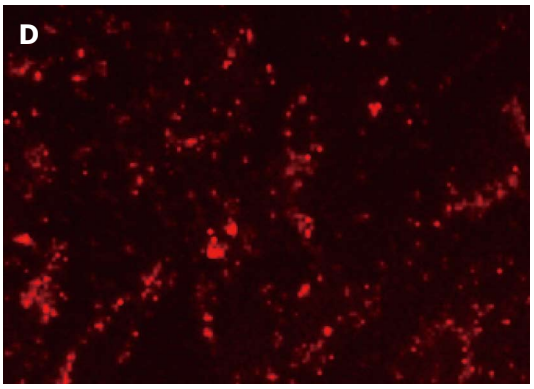
Effect of Th17/Treg cells on activation of HSCs

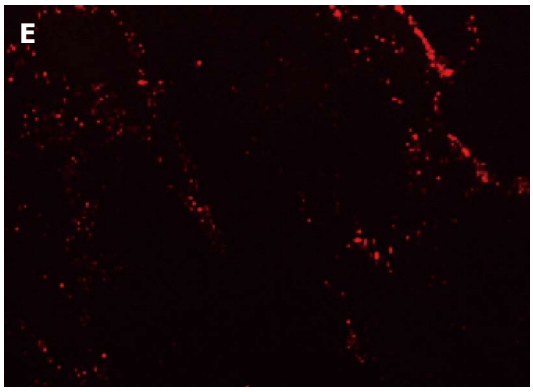
To study whether Th17/Treg cells could modulate the activation of HSCs, we isolated HSCs from the mouse liver, and treated then with Th17 cells (2×10^4/well) or Treg

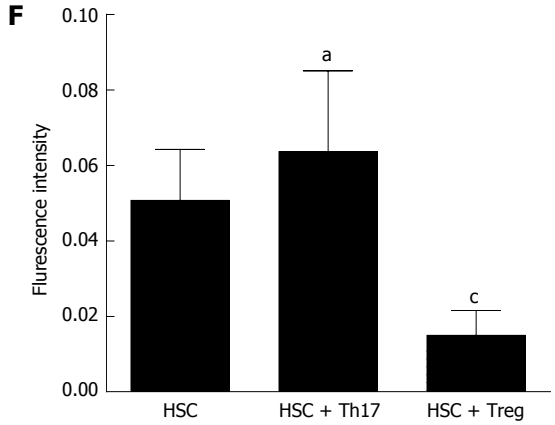

A
α-SMA
β-actin
HSC
HSC + Th17
HSC + Treg

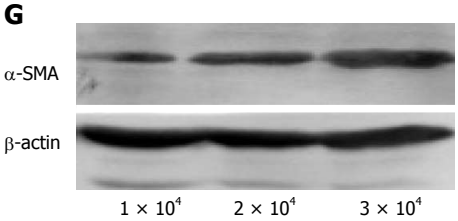

B
1.5
1.0
0.5
0.0
Protein/β-actin
a
c
HSC
HSC + Th17
HSC + Treg

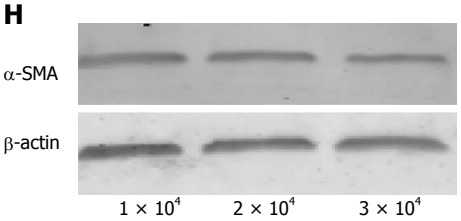

C


D


E


F
0.10
0.08
0.06
0.04
0.02
0.00
Flurescence intensity
a
c
HSC
HSC + Th17
HSC + Treg


G
α-SMA
β-actin
1 × 10⁴
2 × 10⁴
3 × 10⁴


H
α-SMA
β-actin
1 × 10⁴
2 × 10⁴
3 × 10⁴

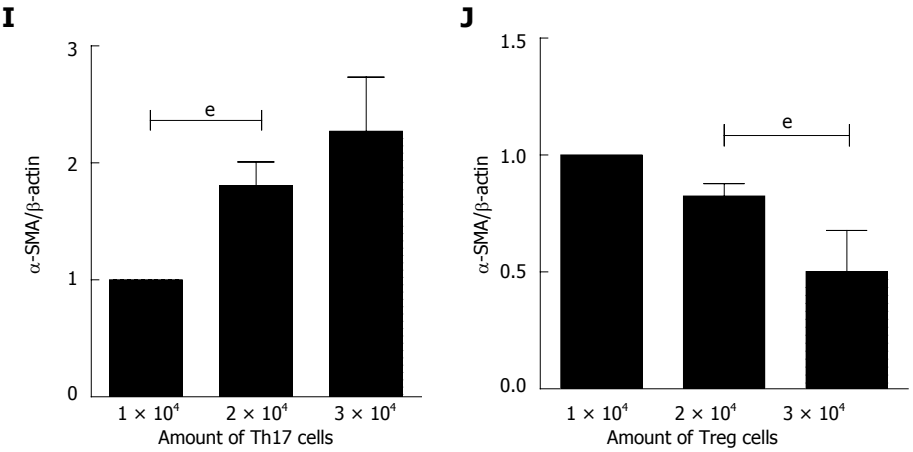


Figure 5 α-Smooth muscle actin expression after different treatment. A and B: Western blotting and quantitative analysis of α-SMA; C-E: Immunofluorescence staining (C: HSCs; D: HSCs + Th17 cells; E: HSCs + Treg cells. Original magnification, × 200); F: Fluorescence intensity of α-SMA; G and I: Western blotting and quantitative analysis after HSCs treated with different numbers of Th17 cells; H and J: Western blotting and quantitative analysis after HSCs treated with different numbers of Treg cells. [a]*P* > 0.05 *vs* control, [c]*P* < 0.05 *vs* control; [e]*P* < 0.05 *vs* 1 × 10^4 cells, *P* > 0.05 *vs* 2 × 10^4 cells. α-SMA: α-Smooth muscle actin; HSCs: Hepatic stellate cells; Th17: T helper 17; Treg: Th17/T regulatory.

cells (2 × 10^4/well). Western blotting and immunofluorescence staining were performed to determine changes in the expression of α-SMA. As illustrated in Figure 5A and B, in comparison with the control group, α-SMA expression was induced following exposure to Th17 cells but the difference was not significant ($P > 0.05$). α-SMA expression was significantly reduced after exposure to Treg cells ($P < 0.05$). Figure 5C-E shows α-SMA expression after different treatments, and Figure 5F shows the fluorescence intensity of each group, which had the same trends as described above.

To detect further the influence of Th17 and Treg cells on HSCs, we cultured HSCs with various amounts of Th17 or Treg cells (1 × 10^4, 2 × 10^4 or 3 × 10^4/well). Results indicated that Th17 cells promoted, whereas Treg cells inhibited the expression of α-SMA in a dose-dependent manner.

DISCUSSION

Recent findings demonstrate that Th17 and Treg cells are T lymphocyte subgroups with unique immunoregulating functions and play opposing roles[18], and both of them participate in the regulation of a variety of diseases.

Wang *et al*[19] have found that the frequency of peripheral Th17 cells, as well as the level of IL-17 mRNA in PBMCs, was significantly increased in patients with acute-on-chronic hepatitis B liver failure compared with patients with chronic hepatitis B and healthy controls. Amoroso *et al*[20] suggested that the presence of Treg cells infiltrating the liver is associated with high levels of activated/effector T cells in the peripheral blood and lower activity of hepatitis. Therefore, Th17 and Treg cells are closely related with liver fibrosis. In addition, Th17 and Treg cells are both differentiated from naive CD4$^+$ T cell precursors. TGF-β is a central cytokine to the differentiation of both cells and can induce the expression of FoxP3 and ROR-γt at the same time[21]. Low concentrations of TGF-β synergize with IL-6 to promote ROR-γt expression, thereby favoring Th17 cell differentiation. However, increased concentrations of TGF-β repress ROR-γt expression and favor the generation of Treg cells[22,23]. Considering all this evidence together, we hypothesize that an imbalance of Th17 cells and Treg cells may exist and play a role in regulating the immune response during liver fibrosis.

In our study, we determined the protein levels of IL-6 and TGF-β during liver fibrosis to understand their impact on Th17/Treg cell balance. Our data showed that after CCl_4 administration, the expression of IL-6 and TGF-β was increased compared with the controls (Figure 2). In addition, the frequency of Th17 cells was increased while the frequency of Treg cells was decreased with the aggravation of liver fibrosis (Figure 3). To a large extent, the results of our study confirmed the above inference; that in the process of liver fibrosis, IL-6, TGF-β and other cytokines are produced in large quantities, and these changes are more conductive to the production of Th17 cells. Increased Th17 cells also induced the production of IL-6[24], so the Th17/Treg balance was disrupted (Figure 4), which led to immune disorder and promotion of liver fibrosis. Whether decreased Treg cells lead to the upregulation of TGF-β is controversial at present, and requires further study for confirmation.

As described previously, the transdifferentiation of HSCs into myofibroblasts is characterized by expression of α-SMA and is a critical event during liver fibrogenesis[25]. Our study also confirmed that the expression of α-SMA was increased gradually during the process of liver fibrosis (Figure 2), which indicates that the activation of HSCs in liver fibrosis is a continuous process. In addition, HSCs are not only the regulatory cells of liver inflammation but also the target cells of immunoregulation[26]. Li *et al*[27] have reported that CD4$^+$CD25$^+$ cells from chronic hepatitis B patients inhibit HSC activation in a dose-dependent manner, whereas recombinant IL-17

promotes the proliferation and activation of HSCs. Sun *et al*[28] have also found that IL-17 together with IL-17-activated monocytes promote the activation of HSCs *in vitro*.

To investigate whether Th17/Treg imbalance increased the activation of HSCs, we co-cultured HSCs with Th17 or Treg cells to explore their effects on HSCs. Our results indicated that Th17 cells promote HSC activation, while Treg cells have the opposite effect on HSC activation; both in a dose-dependent manner (Figure 5). In addition, we demonstrated that Th17 cells were increased in association with the reduction in Treg cells during liver fibrosis, and these changes were more conducive to HSC activation, which in turn, further promotes the development of fibrosis by secreting various inflammatory cytokines. Furthermore, fibrogenic factors (IL-6 and TGF-β) involved in the activation of HSCs induce the production of matrix proteins[29]. Therefore, a closed loop is formed between fibrogenic factors, Th17/Treg cells and HSCs, which influence each other and mediate the development of liver fibrosis jointly. However, the accurate mechanism by which Th17 and Treg cells play their pivotal roles in the activation of HSCs remains to be elucidated.

In summary, our data demonstrated that a Th17/Treg cell imbalance existed in mice with liver fibrosis. Therefore, the dynamic interaction between Th17 and Treg cells may be important in the development of liver fibrosis, suggesting a potential role for Th17/Treg imbalance in the pathogenesis of liver fibrosis.

COMMENTS

Background

Liver fibrosis is a chronic progressive disease that seriously affects human health, so it is important to prevent its occurrence. However, the pathogenesis of fibrosis is not entirely clear to date. T helper (Th) 17 and T regulatory (Treg) cells are T lymphocyte subgroups. Many studies have reported that Th17/Treg imbalance is closely related to many autoimmune diseases. However, its role in liver fibrosis has seldomly been reported.

Research frontiers

The imbalance of Th17/Treg cells is closely related to a variety of autoimmune diseases. In the area of preventing liver fibrosis, the research hotpot is to explore how Th17/Treg balance changes and then prevent the occurrence of liver fibrosis by modulating the imbalance in Th17/Treg cells.

Innovations and breakthroughs

This is believed to be the first study to explore the effect of Th17/Treg cells in carbon tetrachloride (CCl_4)-induced liver fibrosis in mice and its potential mechanisms. The results indicate that Th17/Treg imbalance exists and may play a potential role in the pathogenesis of liver fibrosis.

Applications

Correcting Th17/Treg imbalance may be a potential therapeutic approach in the management of liver fibrosis.

Terminology

Th17 (IL-17-producing $CD4^+$T helper cells) and Treg cells (regulatory T cells) are described as two additional subsets of T lymphocytes that can orchestrate host immune responses through releasing distinct cytokine profiles.

Peer review

This was a good descriptive study in which the authors analyzed the effect of Th17/Treg cells in CCl_4-induced liver fibrosis in mice. The results are interesting and suggest that Th17/Treg imbalance is a potential therapeutic target that could be used for preventing the occurrence of liver fibrosis.

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ORIGINAL ARTICLE

Chloride intracellular channel 1 regulates colon cancer cell migration and invasion through ROS/ERK pathway

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Abstract

AIM: To investigate the mechanisms of chloride intracellular channel 1 (CLIC1) in the metastasis of colon cancer under hypoxia-reoxygenation (H-R) conditions.

METHODS: Fluorescent probes were used to detect reactive oxygen species (ROS) in LOVO cells. Wound healing assay and transwell assay were performed to examine the migration and invasion of LOVO cells. Expression of CLIC1 mRNA and protein, p-ERK, MMP-2 and MMP-9 proteins was analyzed by reverse transcription-polymerase chain reaction and Western blot.

METHODS: H-R treatment increased the intracellular ROS level in LOVO cells. The mRNA and protein expression of CLIC1 was elevated under H-R conditions. Functional inhibition of CLIC1 markedly decreased the H-R-enhanced ROS generation, cell migration, invasion and phosphorylation of ERK in treated LOVO cells. Additionally, the expression of MMP-2 and MMP-9 could be regulated by CLIC1-mediated ROS/ERK pathway.

CONCLUSION: Our results suggest that CLIC1 protein is involved in the metastasis of colon cancer LOVO cells via regulating the ROS/ERK pathway in the H-R process.

Key words: Colon cancer; Intracellular chloride channel 1; Hypoxia-reoxygenation; Reactive oxygen species; Extracellular signal-regulated kinase; Cancer invasion

Core tip: Hypoxia-reoxygenation (H-R) treatment increases the intracellular reactive oxygen species (ROS) level to activate the MAPK/ERK pathway, resulting in the promotion of migration and invasion in colon cancer LOVO cells. Inhibition of chloride intracellular channel 1 (CLIC1) using specific inhibitor IAA94 can markedly decrease the H-R-enhanced ROS generation, migration, invasion and phosphorylation of ERK. The results presented in the current study suggest that CLIC1 is involved in the metastasis of colon cancer LOVO cells via regulating the ROS/ERK pathway in the H-R process.

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INTRODUCTION

Colorectal cancer is one of the most common malignancies that result in the death of people in the world[1,2]. It is important for patients with colorectal cancer to get early detection and treatment. The 5-year survival rate in patients with colorectal cancer at early stages is higher than 90%; however, it drops to less than 10% in patients with distant metastases[3]. It is well known that the tumor microenvironment can influence the progression and metastasis of various cancer models, including colon cancer. Emerging evidence has suggested that local hypoxia is a common feature in the solid tumor microenvironment. Tumor cells exposed to hypoxic conditions may undergo transformation to a more aggressive phenotype to promote the metastasis of cancer[4].

However, little is known about hypoxia and reoxygenation (H-R) microenvironment that occurs in tumors. Recent discoveries have shown that cancer cells are under the H-R microenvironment attributing to irregular microvascular network and blood flow patterns[5,6]. Permanent or transient limitations in blood perfusion may contribute to the migration and invasion of cancer cells under the H-R process[7,8]. It is known that during the H-R process the cells can produce abundant reactive oxygen species (ROS), leading to the injury of membrane proteins and nucleic acids of organisms and the damage to cells and tissues[9]. However, recent reports indicated that ROS production in cancer cells can function as a secondary signaling molecule, which has been shown to play a role in cell proliferation, apoptosis, differentiation[10], migration and invasion[4] in cancer.

Chloride channel 1 (CLIC1, formerly NCC27), a member of the CLIC family, was first cloned and identified by subtractive cloning in 1997[11]. Recent studies have indicated that CLIC1 is significantly up-regulated in tumor tissues, such as gastric carcinoma and lung carcinoma[12,13]. In addition, CLIC1, proposed as a novel potential prognostic factor, was significantly up-regulated in gastric cancer and strongly correlated with lymph node metastasis[12]. It was presumed that elevated expression of CLIC1 can modulate cell division and anti-apoptosis signaling, resulting in cellular transformation[14,15]. Moreover, CLIC1 may act as a "sensor" and an "effector" of the redox state of the cells caused by oxidative stress[16], and it is known that the initiation and progression of cancer are closely correlated with redox state disequilibrium in cells[10]. CLIC1 also contributes to acquisition of the radioresistant phenotype of laryngeal cancer through regulation of ROS production[17], and ROS up-regulation can result in increasing cell motility and invasiveness of cancer cells[4]. In our previous study, we found that CLIC1 participates in colonic carcinoma metastasis under H-R conditions[18]. However, the molecular mechanisms of CLIC1 in colon cancer metastasis remain unclear.

In this study, our data showed that H-R treatment increased the intracellular ROS level to activate the MAPK/ERK pathway, resulting in the promotion of migration and invasion in colon cancer LOVO cells. The mRNA and protein expression of CLIC1 was elevated under H-R conditions. Functional inhibition of CLIC1 using specific inhibitor IAA94 markedly decreased H-R-enhanced ROS generation, cell migration, invasion and phosphorylation of ERK in treated colon cancer LOVO cells. Additionally, the expression of MMP-2 and MMP-9, two important mediators of cancer metastasis, could be regulated by CLIC1-mediated ROS/ERK pathway. The results presented in the current study suggest that CLIC1 protein is involved in the metastasis of colon cancer LOVO cells *via* regulating the ROS/ERK pathway in the H-R process.

MATERIALS AND METHODS

Cell line and cell culture

The human colon cancer cell line LOVO was incubated in Dulbecco's modified Eagle's medium (DMEM) plus 10% (v/v) fetal calf serum (FCS) (Hyclone, United States), at 37 ℃ in a humidified atmosphere of 5% CO_2 in air. The generation of H-R conditions was performed as previously described[7,8]. Briefly, cells were cultured in an air-tight hypoxic (5% CO_2 and 95% N_2) chamber incubator (Thermo Electron, Waltham, MA, United States) for 4 h, rapidly transferred to an incubator with a humidified atmosphere of 5% CO_2, and additionally cultured for 20 h. For normoxia (N) control treatment, cells were maintained in a humidified incubator with a 95% air/5% CO_2 atmosphere for the same period of time as the H-R groups.

Reagents and antibodies

IAA94 was purchased from Sigma and prepared in dimethylsulphoxide. Specific inhibitor of NADPH [diphenyleneiodonium (DPI)] was from Sigma Chemical Co. (St. Louis, MO, United States). Fluorescent probe DCFH-DA, inhibitors of ROS [N-acetylcysteine (NAC)] and MAPK/ERK (PD98059) were purchased from Beyotime Institute of Biotechnology (Nantong, Jiangsu, China). Antibodies against CLIC1, MMP-2, MMP-9, total-ERK and phospho-ERK were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, United States).

Measurements of ROS production

LOVO cells were trypsinized and cultured in 96 well plates (1×10^4 cell/well). To determine the effect of specific inhibitors on ROS production, cells were pretreated with DPI (15 μmol/L), NAC (30 mmol/L) or IAA94 (1, 20 and 40 μmol/L) for 1 h before H-R treatment. For DCF-DA ROS measurements, culture medium was replaced with regular culture medium without FCS containing 10 μmol/L of DCF-DA for 30 min. Cells were rinsed with DMEM without FCS, and fluorescence was

then measured at 488 nm for excitation and 525 nm for emission with the Fluoroscan Ascent FL fluorimeter (Labsystems, France). All measurements were performed at 37 ℃.

Wound healing assay

Cells were cultured to a confluent monolayer in 6-well plates. A sterile 200 μL pipette tip was used to scratch the cell monolayer to form a wound. For the wound healing assays under H-R conditions, cells were pretreated with DPI (15 μmol/L), NAC (30 mmol/L), PD98059 (50 μmol/L) or IAA94 (1, 20 and 40 μmol/L) for 1 h. Pictures of the wound area were taken at 0 and 24 h at × 100 magnification.

Cell invasion assay

The *in vitro* invasive ability of LOVO cells was tested by the Boyden chamber invasion assay. Matrigel (BD Biosciences) was diluted with cold filtered distilled water, and added to 8-μm pore size poly-carbonate membrane filters. The cells were trypsinized and seeded to the upper part of Boyden chamber at a density of 3×10^5 cells/mL in 300 μL of serum-free medium. The bottom chamber contained medium with 10% FCS as a chemoattractant. Cells were preloaded with DPI (15 μmol/L), NAC (30 mmol/L), PD98059 (50 μmol/L) or IAA94 (1, 20 and 40 μmol/L) for 1 h before H-R. After the incubation time was complete (6 h hypoxia followed by 18 h reoxygenation or 24 h normoxia), the cells that had invaded to the lower surface of the membrane were fixed with paraformaldehyde, and stained with crystal violet. The cells were counted in five randomly selected fields under a microscope at × 400 magnification.

Real-time reverse transcription-polymerase chain reaction (RT-PCR)

Total RNA was extracted from cells using the Simply P RNA Extraction kit (Bioer Biotech Co., Latd) according to the manufacturer's instructions. Total RNA (1 μg) was reverse-transcribed into cDNA using the Reverse Transcript Kit (Cwbio Biotech Co, China), and amplified by polymerase chain reaction (PCR). For PCR, 1/25 of the reverse transcription reaction mixture was amplified using 35 cycles for CLIC1 and 35 cycles for GAPDH. Amplified products were separated by electrophoresis on a 2% agarose gel and photographed. The sequences of the primers (Sagon biotech Co., China) used in the real-time RT-PCR were as follows: CLIC1: 5'-GTATTGACCAGTCTCCCTTCCAGC-3' (forward) and 5'-GGTCTTTATGAGGAGGTCGTGGG-3' (reverse); GAPDH: 5'-TCATGAAGTGTGACGTTGACATCCGT-3' (forward) and 5'-CCTAGAAGCATTTGCGGTGCACGATG-3' (reverse).

Western blot

Briefly, proteins were separated on a 10% denaturing polyacrylamide gel and electro-transferred to Immuno-Blot nitrocellulose membranes. The membranes were blocked in TBS-T containing 5% fat-free dry milk and then incubated with a primary antibody overnight at 4 ℃, followed by incubation with a horseradish peroxidase-conjugated secondary antibody for 1 h. Primary antibodies against ERK (1:400), p-ERK (1:400), MMP-2 (1:200), MMP-9 (1:200) and GAPDH (1:1000) were used. Proteins were detected using ECL reagents (GE Healthcare, NJ, United States).

Statistical analysis

All data are expressed as mean ± SD. The data for each condition were subjected to analysis of variance followed by the Student-Newman-Keuls test for comparisons between the means. Differences were considered significant when $P < 0.05$.

RESULTS

Involvement of CLIC1 in ROS production induced by H-R treatment in LOVO cells

Cells were cultured with DPI (15 μmol/L), NAC (30 mmol/L) or IAA 94 (1, 20 and 40 μmol/L) for 1 h before H-R, and control cells were only incubated in a humidified atmosphere of 95% air/5% CO_2. Intracellular ROS were measured by preloading the cells with DCF-DA followed by H-R or normoxia treatment. As shown in Figure 1, the level of intracellular ROS was significantly increased in cells after exposure to H-R compared with normoxia ($P < 0.01$). Further investigation indicated that the ROS level was significantly abated in cells pretreated with DPI (15 μmol/L) or NAC (30 mmol/L) compared with H-R cells ($P < 0.01$) (Figure 1A). These data provide evidence that ROS production is involved in the H-R process in colon cancer LOVO cells, which is consistent with the finding of a previous study[8].

To determine whether inhibition of CLIC1 could reduce ROS generation during the H-R treatments, cells were cultured in the presence of CLIC1 blocker IAA94. As shown in Figure 1B, the intracellular ROS level was significantly increased under H-R conditions. However, preloading with 20 or 40 μmol/L of IAA94 significantly decreased the ROS levels in LOVO cells ($P < 0.01$). These results suggest that CLIC1 can regulate the intracellular ROS production in colon cancer cells in the H-R process.

H-R up-regulates the expression of CLIC1 in human colonic cancer LOVO cells

It has been previously documented that CLIC1 may act as a "sensor" and an "effector" of the oxidative stress of the cells[16], and H-R circumstances are closely correlated with metastasis of colon cancer[7]. Therefore, in our present study, the mRNA and protein expression of CLIC1 was determined by RT-PCR and Western blot under H-R conditions, respectively. As shown in Figure 2, CLIC1 mRNA and protein expression was significantly elevated in the H-R group compared with the normoxia control group ($P < 0.01$). However, we found no signifi-

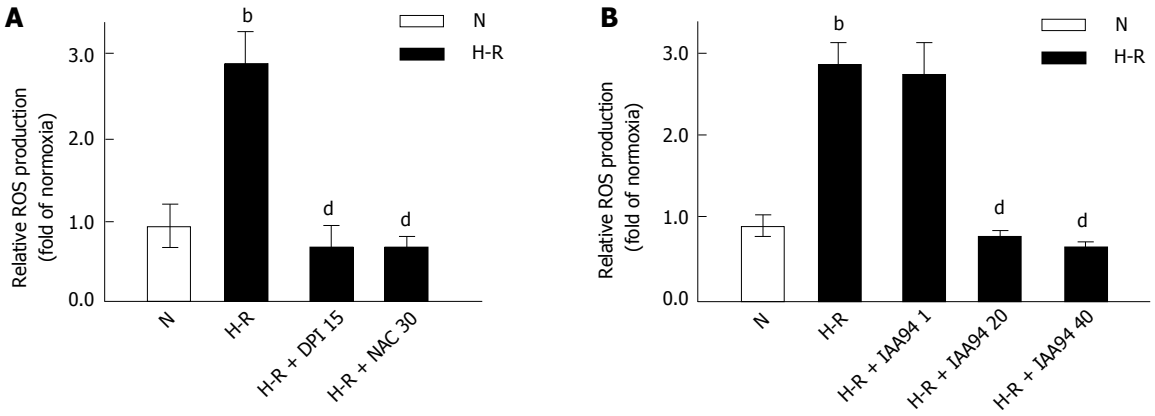

Figure 1 Increased reactive oxygen species production in LOVO cells under hypoxia-reoxygenation conditions. LOVO cells were cultured in normoxia (N) for 24 h or under hypoxia for 4 h followed by reoxygenation for 20 h (hypoxia-reoxygenation, H-R). DPI (15 μmol/L), NAC (30 mmol/L) (A) and IAA94 at various concentration (1, 20, and 40 μmol/L) (B) decreased the reactive oxygen species production under H-R conditions. Results are expressed as fold of normoxia. Values represent the mean ± SD from three independent experiments. $^{b}P < 0.01$ *vs* N group, $^{d}P < 0.01$ *vs* H-R group. ROS: Reactive oxygen species; DPI: Diphenyleneiodonium; NAC: N-acetylcysteine.

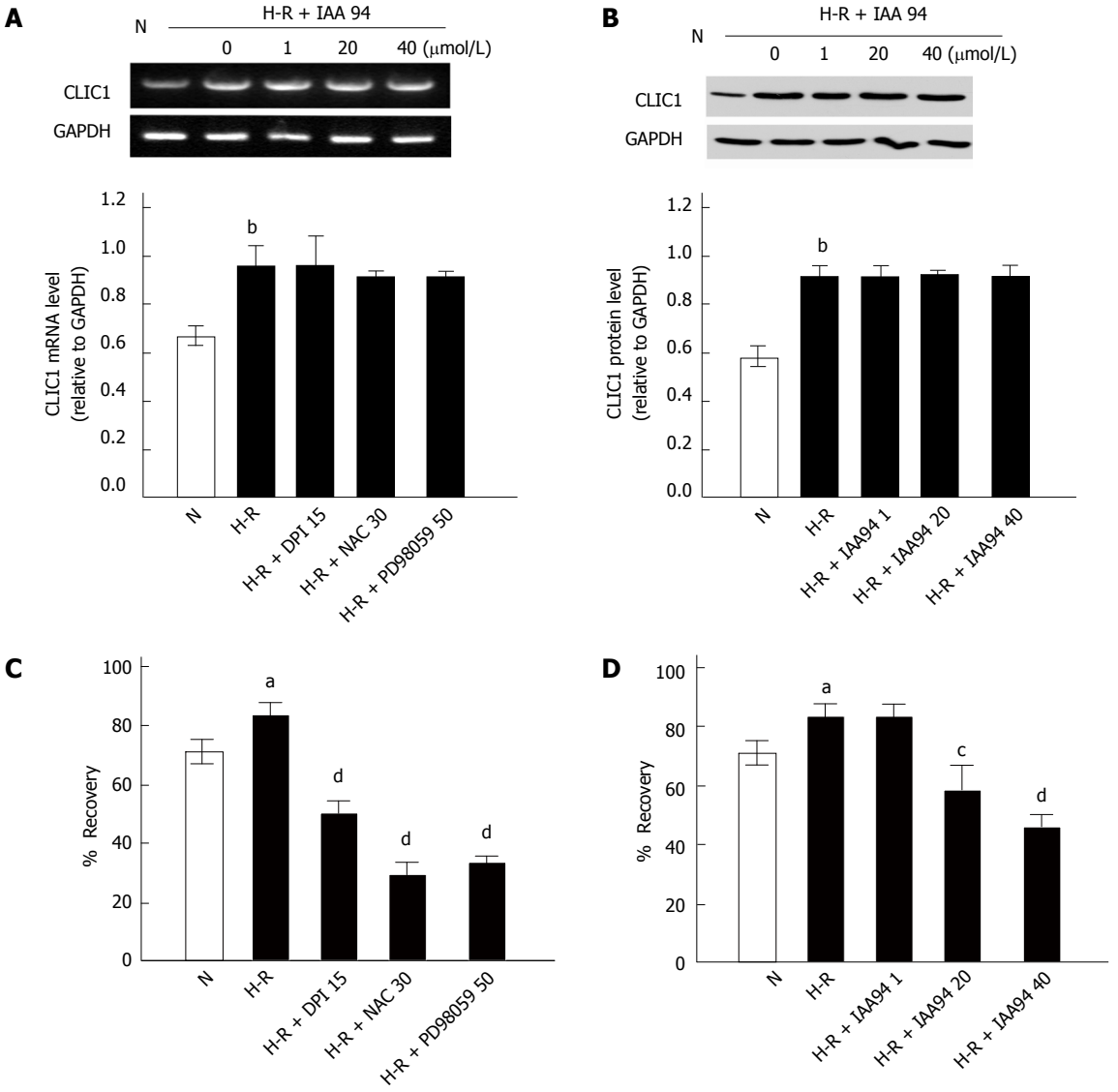

Figure 2 Effect of hypoxia-reoxygenation on the mRNA and protein expression of CLIC1 and wound healing assays in LOVO cells. A, B: mRNA (A) and protein (B) expression of CLIC1 was significantly increased under hypoxia-reoxygenation (H-R) conditions as revealed by RT-PCR or Western blot analysis, respectively. Results were normalized to GAPDH; C, D: Wound recovery (%) of LOVO cells treated with DPI (15 μmol/L), NAC (30 mmol/L), PD98059 (50 μmol/L) or with IAA94 at various concentrations (1, 20 and 40 μmol/L) for 24 h under H-R conditions, respectively. Values represent the mean ± SD from three independent experiments. $^{a}P < 0.05$, $^{b}P < 0.01$ *vs* N group, $^{c}P < 0.05$, $^{d}P < 0.01$ *vs* H-R group. DPI: Diphenyleneiodonium; RT-PCR: Reverse transcription-polymerase chain reaction.

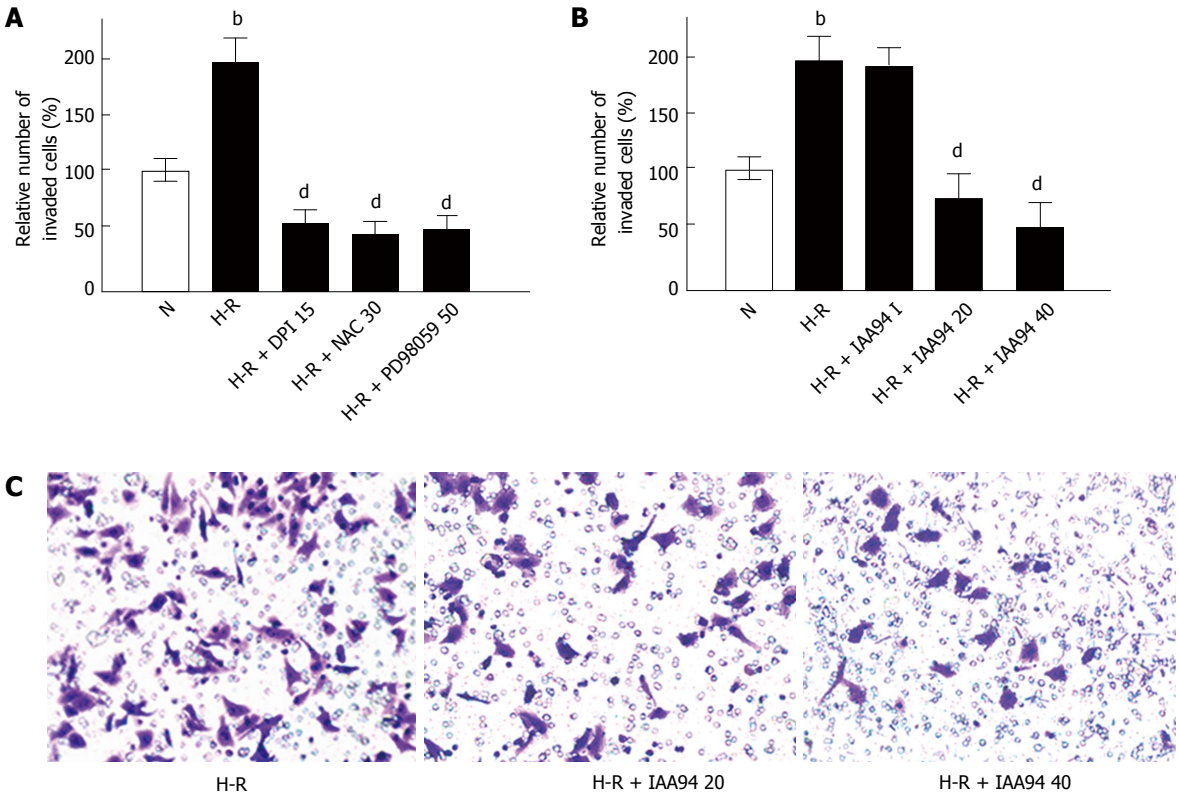

Figure 3 Effect of various treatments for 24 h on LOVO cell invasiveness under hypoxia-reoxygenation conditions. A, B: Pretreatment with DPI (15 μmol/L), NAC (30 mmol/L), PD98059 (50 μmol/L) or with IAA94 at 20 and 40 μmol/L decreased the invasiveness of LOVO cells compared with H-R group. Results are expressed as fold of normoxia (N); C: LOVO cells incubated with IAA94 for 24 h under hypoxia-reoxygenation (H-R) conditions. The invaded cells were fixed and stained with crystal violet. Values represent the mean ± SD from three independent experiments. $^{b}P < 0.01$ *vs* N group, $^{d}P < 0.01$ *vs* H-R group. DPI: Diphenyleneiodonium.

cant changes in CLIC1 mRNA or protein expression in LOVO cells treated with IAA94 at different concentrations (1, 20 or 40 μmol/L) (Figure 2). Our results suggest that functional inhibition of CLIC1 can play a role in down-regulating ROS generation in colon cancer.

Involvement of CLIC1 in the migration of colon cancer cells under H-R conditions

During cancer metastasis process, tumor cells must firstly undergo several morphological changes so that they could pass through narrow extracellular spaces to metastasize[18]. Recent studies showed that ROS up-regulation could lead to morphological transformation and increase cell motility[19]. The effect of the ROS/ERK pathway on cell motility during the H-R treatments was then assessed using wound-healing assays. As shown in Figure 2C and D, the mobility of LOVO cells was increased after exposure to H-R conditions ($P < 0.05$). This effect was significantly suppressed by NAC, DPI or PD 98059, respectively (Figure 2C). These results implied that the ROS/ERK pathway could regulate the migration of colon cancer cells under H-R conditions. Furthermore, as shown in Figure 2D, preloading LOVO cells with IAA 94 (20 or 40 μmol/L) could also decrease the cell motility potential for H-R, and the effect was dose-dependent. All these data provide evidence that CLIC1 is involved in the migration of colon cancer cells under H-R conditions.

Inhibiting CLIC1 inhibits invasion of colon cancer cells

The effect of CLIC1 on colon cancer cell invasiveness was examined by Matrigel invasion assays *in vitro*. It was found that H-R caused a remarkable increase in the invasiveness of LOVO cells (Figure 3A). However, treatment with DPI (15 μmol/L), NAC (30 mmol/L) or PD 98059 (15 μmol/L) could significantly prevent colonic cancer cells from invading after H-R exposure. As expected, a similar effect of inhibiting CLIC1 on LOVO cells invasion was observed. After pretreatment with 20 or 40 μmol/L of IAA94 for 1 h, the invasiveness of LOVO cells was significantly decreased under H-R conditions (Figure 3B and C). These results suggest that H-R can promote the invasiveness of colon cancer cells, and the effect can be inhibited by CLIC1 under H-R conditions.

CLIC1 regulates MAPK/ERK pathway, MMP-2 and MMP-9 in LOVO cells following exposure to H-R conditions

It has been reported that MAPK/ERK and ROS/ERK pathways are involved in the metastasis of cancer[4,20]. However, it is still unknown whether those pathways are correlated with metastasis of colon cancer under H-R conditions. In our present study, the expression of phosphorylated ERK (p-ERK), MMP-2 and MMP-9 was monitored by Western blot analysis in the H-R process. Our results suggested that p-ERK, MMP-2 and MMP-9 proteins were significantly elevated in H-R conditions

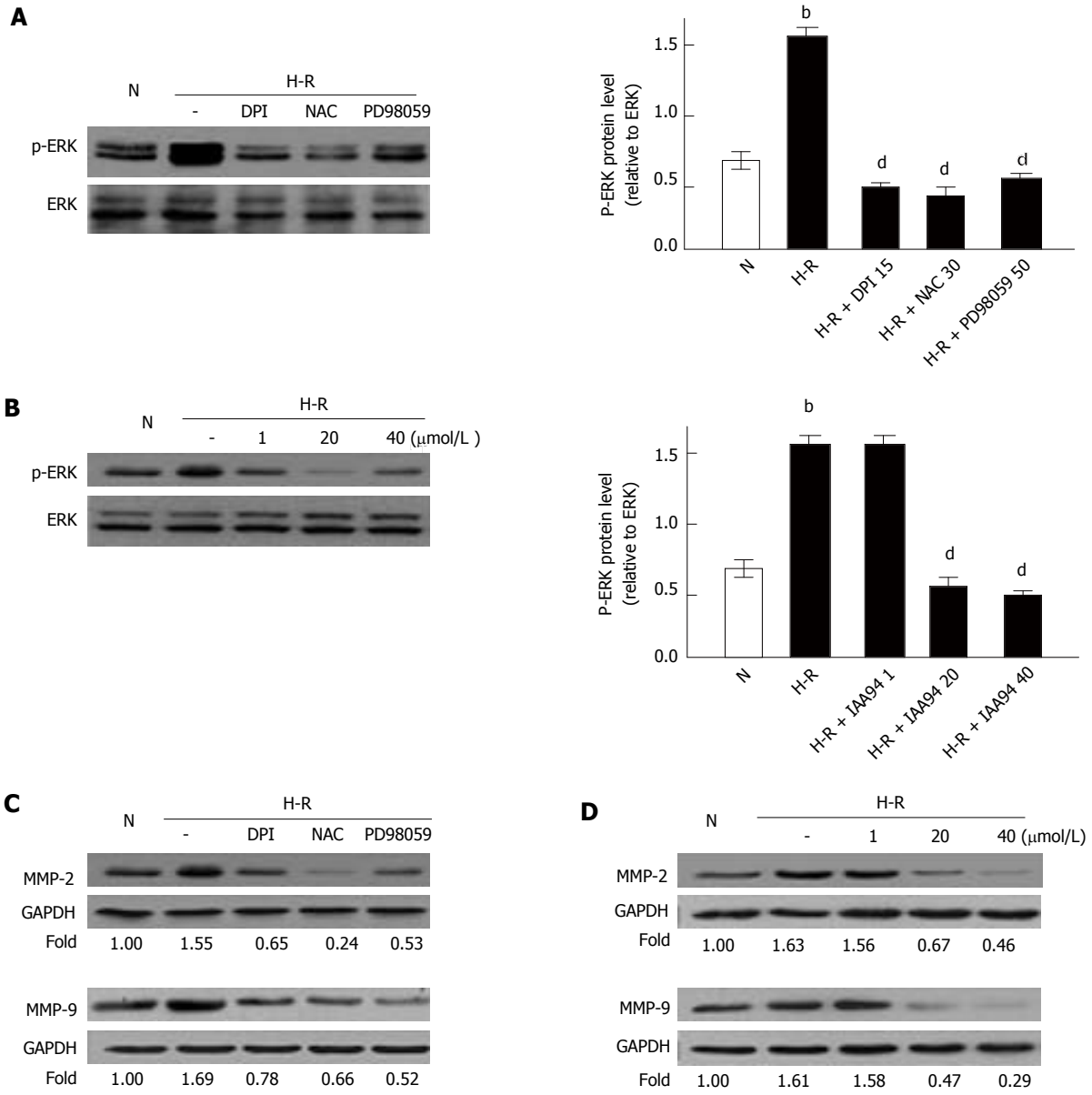

Figure 4 MEK/ERK/MMPs pathway involved in the metastasis of LOVO cells under hypoxia-reoxygenation conditions. A, C: Treatment with DPI (15 μmol/L), NAC (30 mmol/L) or PD98059 (50 μmol/L) decreased the protein expression of p-ERK or MMP-2 and MMP-9 in LOVO cells compared with H-R group, respectively; B, D: Treatment with 20 and 40 μmol/L IAA94 also decreased the expression of p-ERK or the protein expression of MMP-2 and MMP-9 in LOVO cells, respectively. Results were normalized to GAPDH. Values represent the mean ± SD from three independent experiments. $^{b}P < 0.01$ *vs* N group, $^{d}P < 0.01$ *vs* hypoxia-reoxygenation (H-R) group.

when compared with the normoxia control (Figure 4). However, p-ERK, MMP-2 and MMP-9 protein levels in H-R conditions were strongly down-regulated with addition of DPI or NAC (Figure 4A and C). These findings implied that ROS could activate the ERK/MMPs pathway in H-R conditions. We also examined the effect of CLIC1 on the ERK/MMPs pathway by Western blot analysis. As shown in Figure 4, pretreatment with IAA 94 (20 or 40 μmol/L) significantly decreased p-ERK protein, MMP-2 and MMP-9 protein levels under H-R conditions in a dose dependent manner. ERK inhibitor PD 98059 could also decrease MMP-2 and MMP-2 protein levels under H-R conditions. Taken together, our findings demonstrated that the ROS/ERK pathway is involved in the metastasis of colonic cancer, and ERK/MMPs pathway is regulated by CLIC1 *via* regulating ROS production under H-R conditions.

DISCUSSION

Under physiological circumstances, ROS can be continually eliminated by endosomatic antioxidase, which is dependent on the stable system of cellular redox state. It has been demonstrated that cancer cells are characterized by the defect of redox system development, and persistently elevated intracellular ROS can function as second messengers and are involved in proliferation, differentiation, migration, and invasion of cancer cells[4,10,20]. Many

solid tumors possess irregular microvascular network and blood flow patterns, which is the main cause that cancer cells are usually present under H-R conditions[5,6]. In the present study, we confirmed that the ROS level was significantly elevated under the H-R conditions in LOVO cells when compared with the normoxia control. Moreover, ROS production was significantly decreased by pretreatment with DPI or NAC under the H-R conditions. Further investigations by wound healing and invasion assays showed that the metastasis potential of colon cancer cells was significantly increased in response to H-R treatments, and this effect was abrogated by preloading with DPI or NAC. Taken together, our results confirmed that ROS play an important role in regulating the migration and invasion of colon cancer cells under H-R conditions. Our findings also suggested that colon cancer cells under H-R microenvironment can undergo transformation to a more aggressive phenotype to promote cancer metastasis.

Previous studies of CLIC1 are mostly focused on its physiological function, its association with non-tumorous disease, and its ability to act as an ion channel[21]. However, some recent studies suggest that the expression of CLIC1 is up-regulated in tumor tissues. In addition, CLIC1 was significantly up-regulated and correlated with metastasis of tumor cells in gastric cancer[14]. Additionally, a recent study has indicated that CLIC1 is highly expressed in colorectal cancer tissues[17]. However, it is still unknown whether CLIC1 plays a role in the metastasis of colon cancer under H-R conditions. In this study, it was found that CLIC1 mRNA and protein expression was significantly elevated under H-R conditions. Preloading with CLIC1 blocker IAA 94 could not affect the expression of CLIC1, but the ROS level was significantly decreased by IAA 94 treatment, suggesting that functional inhibition of CLIC1 channel activity could reduce the intracellular ROS production in the H-R process. This is supported by previous findings that CLIC1 channel activity is increased in the oxidative environment and inhibition of CLIC1 reduces the ROS production *via* blocking NADPH oxidization[16], and testified by structural analysis of CLIC1 showing that it can dimerize in the presence of strong oxidizing stress[22,23]. In addition, the migration and invasion of colon cancer cells were obviously decreased in the presence of IAA94 under H-R conditions. Taken together, our results indicated that CLIC1 was involved the metastasis of colon cancer through regulating intracellular ROS levels.

We further explored the molecular signaling pathways that may be involved in H-R conditions. It is known that MAPK/ERK and ROS/ERK pathways can promote the metastasis of cancer. The ROS/ERK pathway, involved in cell migration and activation of matrix metalloproteinases, is activated by cell oxidation[4,20]. In the present study, Western blot analysis showed that the MAPK/ERK pathway was activated in H-R conditions. The effect could be suppressed by DPI, NAC or PD98059 treatment. As expected, the MAPK/ERK pathway was significantly blocked by treatment with IAA94. Taken together, our findings implied that CLIC1 promoted the mobility and invasive capacity of cancer by regulating NADPH-derived ROS *via* the MAPK/ERK pathway. MMPs can degrade the basement membrane and play main roles in promotion of cancer invasion and metastasis[24]. MMP-2 and MMP-9, members of the MMPs family, are thought as the two important mediators of cancer metastasis in patients with colorectal carcinoma[25]. In the present study, we found that both MMP-2 and MMP-9 proteins were up-regulated in LOVO cells under H-R conditions, and the effect was abated by suppressing the ROS production, MAPK/EER pathway or CLIC1. Our results demonstrated that MMP-2 and MMP-9 played an important role in the invasion of colon cancer in the H-R process.

In summary, our findings provide the evidence that H-R conditions act as a relevant key factor for the promotion of colonic cancer metastasis. CLIC1 is an important candidate protein that may serve as an effective metastasis-associated regulator for colon cancer. However, the accumulating evidence suggests that ROS derived from NADPH oxidase can mediate tumor growth and angiogenesis, which is essential for tumor metastasis[26]. Our findings warrant further investigation to explore some other possible molecular mechanisms of CLIC1 in colon cancer metastasis.

COMMENTS

Background

Colorectal cancer is one of the most common malignancies that result in the death of people in the world. The tumor microenvironment hypoxia-reoxygenation (H-R) can influence the progression and metastasis of colon cancer but the molecular basis for such a link has not been well understood.

Research frontiers

Chloride intracellular channel 1 (CLIC1) is highly expressed in colorectal cancer, and our previous study has reported that CLIC1 participates in the metastasis of colorectal cancer. However, the precise mechanisms of CLIC1 in the metastasis of colonic cancer under H-R conditions is still unknown. In this study, the authors demonstrated that CLIC1 protein was involved in the metastasis of colon cancer LOVO cells *via* regulating reactive oxygen species (ROS)/ERK pathway in the H-R process.

Innovations and breakthroughs

Although previous studies have demonstrated that CLIC1 is correlated with tumor metastasis, the precise mechanisms are not well understood. In this study, it was found that tumor H-R microenvironment increased the intracellular ROS levels to activate the MAPK/ERK pathway, resulting in the promotion of migration and invasion in colon cancer LOVO cells. The findings, for the first time, provide the evidence that CLIC1 is involved in the metastasis of colon cancer LOVO cells *via* regulating ROS/ERK pathway under H-R conditions.

Applications

The tumor H-R microenvironment can promote the progression and metastasis of colon cancer. By understanding how CLIC1 is involved in the metastasis of colon cancer in the H-R process, this study may represent a future strategy for the treatment of patients with colon cancer.

Terminology

CLIC1 can act as a "sensor" and an "effector" during changes in the redox state of the cells caused by oxidative stress *via* regulating the oxidation of GAPDH because of its similar structure to GAPDH. Non-surprisingly, CLIC1 may have some effects on the production of ROS in the H-R process.

Peer review

In this study, the authors have assessed the relationship between protein expression of CLIC1, which has previously shown to be increased in tumor metastasis, and intracellular ROS level, which in turn activates the MAPK/ERK pathway. The activation of this pathway resulted in the promotion of migration

and invasion of colon cancer LOVO cells. This is a new topic which merits further investigation; on this subject, the study is well written and the results are interesting.

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BRIEF ARTICLE

Platelet count/spleen diameter ratio to predict esophageal varices in Mexican patients with hepatic cirrhosis

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Abstract

AIM: To validate whether the platelet count/spleen size ratio can be used to predict the presence of esophageal varices in Mexican patients with hepatic cirrhosis.

METHODS: This was an analytical cross-sectional study to validate the diagnostic test for hepatic cirrhosis and was performed between February 2010 and December 2011. Patients with a diagnosis of hepatic cirrhosis were included and stratified using their Child-Pugh score. Biochemical parameters were evaluated, and ultrasound was used to measure the longest diameter of the spleen. The platelet count/spleen diameter ratio was calculated and analyzed to determine whether it can predict the presence of esophageal varices. Upper gastrointestinal endoscopy was used as the gold standard. Sensitivity and specificity, positive and negative predictive values, and positive and negative likelihood ratios were determined, with the cutoff points determined by receiver-operating characteristic curves.

RESULTS: A total of 91 patients were included. The mean age was 53.75 ± 12 years; 50 (54.9%) were men, and 41 (45.0%) women. The etiology of cirrhosis included alcohol in 48 (52.7%), virally induced in 24 (26.3%), alcoholism plus hepatitis C virus in three (3.2%), cryptogenic in nine (9.8%), and primary biliary cirrhosis in seven (7.6%). Esophageal varices were present in 73 (80.2%) patients. Child-Pugh classification, 17 (18.6%) patients were classified as class A, 37 (40.6%) as class B, and 37 (40.6%) as class C. The platelet count/spleen diameter ratio to detect esophageal varices independent of the grade showed using a cutoff value of $\leqslant$ 884.3, had 84% sensitivity, 70% specificity, and positive and negative predictive values of 94% and 40%, respectively.

CONCLUSION: Our results suggest that the platelet count/spleen diameter ratio may be a useful tool for detecting esophageal varices in patients with hepatic cirrhosis.

Key words: Platelet count/spleen diameter ratio; Esophageal varices; Hepatic cirrhosis; Hepatitis C virus; Mexican patients

Core tip: Using noninvasive parameters for high-risk esophageal varices may reduce the need for endoscopies. The ratio of platelet count/diameter of the spleen (PC/SD ratio) is the principal noninvasive predictor of esophageal varices for stratifying patients with cirrhosis. These parameters are easy to obtain, reproducible and noninvasive. In our study, the cutoff point for PC/SD ratio was lower than that reported in previous literature (< 884.3 and < 909, respectively), with a sensitivity of 84% and specificity of 70%. The differences are probably influenced by racial characteristics. The PC/SD ratio should be considered to identifying patients with esophageal varices.

González-Ojeda A, Cervantes-Guevara G, Chávez-Sánchez M, Dávalos-Cobián C, Ornelas-Cázares S, Macías-Amezcua MD, Chávez-Tostado M, Ramírez-Campos KM, Ramírez-Arce AR, Fuentes-Orozco C. Platelet count/spleen diameter ratio to predict esophageal varices in Mexican patients with hepatic cirrhosis. *World J Gastroenterol* 2014; 20(8): 2079-2084 Available from: URL: http://www.wjgnet.com/1007-9327/full/v20/i8/2079.htm DOI: http://dx.doi.org/10.3748/wjg.v20.i8.2079

INTRODUCTION

Portal hypertension is the principal complication of hepatic cirrhosis[1]. More than 80% of patients with cirrhosis will develop esophageal varices at some point, and 30% of these patients will have at least one bleeding episode because of rupture of a varix[2]. Most first bleeding episodes happen during the first year after the detection of the varices, with a 5%-10% mortality attributed to the initial hemorrhage[3]. For this reason, identifying the presence of esophageal varices is a fundamental part of the diagnostic work-up in patients with cirrhosis, and it is also a prognostic marker of the illness. The first crucial preventive step is the identification of those patients with bleeding risk and selection for prophylactic treatment.

Today's guidelines are clear that there are no substitute markers to determine the presence and size of esophageal varices, and endoscopy is still the only valid method to investigate varices[4]. However, access to endoscopy and other resources is limited in some countries. At any given time, a variable proportion of patients will not have varices, and the reported prevalence of esophageal varices is 24%-80%[5]. The use of noninvasive methods to predict the presence of esophageal varices would help restrict endoscopic studies to those with a high probability of having varices. Until a few years ago, little information was available on this subject; however, a recent consensus on the definitions, methodology, and therapeutic strategies in portal hypertension[6] recommended that all patients with cirrhosis should be assessed with endoscopy to verify the presence of varices.

Recent studies[7-11] have emphasized the use of noninvasive methods to identify patients with the intention of avoiding endoscopy in low-risk cases. The fact that distinct predictors of the presence of varices have been identified in different studies probably reflects differences between the study populations and spectrum of the disease and this makes it difficult to develop a widely applicable predictive model.

Giannini *et al*[12] proposed the use of the platelet count (PC)/spleen diameter (SD) ratio as a noninvasive tool for predicting the presence of varices. The use of the PC/SD ratio for the noninvasive assessment of varices seems to meet strict methodological criteria and is based on pathophysiological criteria. The diagnostic precision of this parameter was validated using endoscopic diagnosis in a follow-up of patients free of esophageal varices[13].

The preliminary results obtained by other authors have demonstrated that the diagnostic accuracy of the PC/SD ratio is maintained in patient subgroups with different hepatic disease etiologies and when applying different methodologies[14], suggesting the universality of the diagnostic method. However, no studies have confirmed the results of these earlier studies in the Mexican population. Previous studies were performed in Caucasian populations, and the ratio may differ between populations. Thus, different predictive values may be needed to indicate the presence of esophageal varices. The objective of our study was to validate the PC/SD ratio as a predictor of the presence and absence of esophageal varices in Mexican patients with chronic hepatopathy.

MATERIALS AND METHODS

This study was an analytical cross-sectional validation study of a diagnostic test. Patients were included from the Department of Enteral and Parenteral Nutrition, Gastroenterology Service, Hospital Civil de Guadalajara "Fray Antonio Alcalde." The inclusion criteria were a diagnosis of hepatic cirrhosis by histology or physical, biochemical, and imaging examinations compatible with the disease and treatment from February 1, 2010 to December 31, 2011.

Both men and women with a diagnosis of hepatic cirrhosis of any etiology were included. The exclusion criteria were hepatocellular carcinoma, use of medications for the primary prophylaxis of variceal bleeding, history of esophageal variceal bleeding, alcohol consumption within the admission and a history of ligation, sclerotherapy, and/or portal hypertension surgery.

Once the patients were included in the study, a complete medical history was taken, and biochemical parameters were measured. All patients were classified according to their Child-Pugh grading.

To calculate the PC/SD ratio, a blood count and PC was obtained using a CELL-DYN 3700 automated he-

Table 1 Clinical and laboratory characteristics of the study sample *n* (%)

Characteristics	Value
Patients (*n*)	91 (100)
Age (yr)	53.75 ± 12
Cirrhosis etiology	
Alcoholism	48 (52.7)
Viral hepatitis	24 (26.3)
Viral hepatitis + alcoholism	3 (3.2)
Other	16 (17.5)
Grading of varices	
I	21 (23)
II	30 (32.9)
III	22 (24.1)
Child-Pugh classification	
A	17 (18.6)
B	37 (40.6)
C	37 (40.6)

matology analyzer (Abbott Laboratories, Abbott Park, IL, United States) Afterwards, the patient underwent an upper abdominal echographic examination using a GE Logiq P5 ultrasound system (General Electric Company, Fairfield CT, United States); the spleen's longest diameter was measured in millimeters.

The PC/SD ratio was calculated by dividing the number of platelets/μL by the maximum bipolar diameter of the spleen in millimeters, estimated with abdominal ultrasound. Normal values in healthy mexican adults are: Maximum bipolar diameter of 115-130 mm and platelet count of 167000-431000/mm^3[15].

Finally, the patient received a upper gastrointestinal endoscopy to determine the presence and grade of esophageal varices according to Westaby's grading system. All endoscopies were performed using one endoscopy unit and an Olympus GIF-Q150 gastrointestinal videoscope (Olympus Corporation, Tokyo, Japan). The endoscopy and echography operators were blinded to the biochemical parameters. The interobserver variation coefficients for spleen measurements and presence of varices were evaluated in all the patients (1.4% and 1.2%, respectively).

Ethical considerations

The study was conducted according to the principles of the Declaration of Helsinki (1989) and the Mexican Health Guidelines. Full written informed consent was obtained from all patients before their inclusion in the study. The protocol was approved by the Local Research Committee of the Hospital Civil de Guadalajara "Fray Antonio Alcalde" (registry number, 138/09).

Statistical analysis

The results are expressed as frequencies, average percentages, and standard deviations. Student's *t* test was used to compare the quantitative variables. The test validity was determined with sensitivity, specificity, positive predictive value (PPV), negative predictive value (NPV), and positive and negative likelihood ratios (LR+ and LR-) of the PC/SD ratio. The statistical analysis was performed using SPSS for Windows version 17.0 (SPSS Inc., Chicago, IL, United States).

Table 2 Patient distribution according to the presence of esophageal varices

	EV present	EV absent	*P* value[1]
Platelets/μL	117517 ± 50275	175788 ± 88448	0.014
SD (average mm)	148.16 ± 21.88	130.61 ± 14.41	0.002
PC/SD ratio	824.56 ± 412.27	1390 ± 905.49	0.018

[1]Using Student's *t* test. EV: Esophageal varices. PC/SD: Platelet count/diameter of the spleen.

RESULTS

A total of 91 patients with hepatic cirrhosis were included. Forty-one (45%) were women, and 50 (54.9%) were men. The average age was 53.75 ± 12 years. The most common hepatic cirrhosis etiology was alcoholism in 48 (52.7%) patients, followed by hepatitis C virus (HCV) in 24 (26.3%), no hepatitis B virus cirrhosis were included, alcoholism plus HCV in three (3.2%), cryptogenic in nine (9.8%), and primary biliary cirrhosis in seven (7.6%).

In the Child-Pugh classification, 17 (18.6%) patients were classified as class A, 37 (40.6%) as class B, and 37 (40.6%) as class C. During the endoscopic procedure, esophageal varices were detected in 73 patients (Table 1).

The average PC/SD ratios were 824.56 ± 412.27 and 1390 ± 905.49 ($P = 0.018$) for patients who showed evidence of esophageal varices during the endoscopy and in those who did not, respectively. The respective PCs were 117517 ± 50275/μL and 175788 ± 88448/μL ($P = 0.014$). The respective SD values were 148.16 ± 21.88 mm and 130.61 ± 14.41 mm ($P = 0.002$) (Table 2).

The PC/SD ratio to detect esophageal varices independent of the grade showed that a value of 884 for the cutoff point had an 84% sensitivity, 70% specificity, 94% PPV, 40% NPV, 4.13 LR+, and 0.37 LR-. The exactitude was 0.72 (Table 3, Figure 1).

According to Westby's classification, 21 (23%) patients had grade I varices, 30 (32.9%) had grade II varices, and 22 (24.1%) had grade III varices. The intra- and interobserver variation coefficients for the spleen bipolar measurement were evaluated in all 91 patients using the kappa index (1.2 and 0.98, respectively).

DISCUSSION

There is an ever-growing demand for endoscopic studies, and the capacity to satisfy this demand is limited, especially in developing countries. It is difficult to provide endoscopy for the detection of esophageal varices every year or two in high-risk patients. Finding noninvasive parameters associated with high-risk esophageal varices may help reduce the need for endoscopy, and, above all, lower the cost. These parameters could be used to distinguish between high- and low-risk patients; the high-risk patients

Table 3 Results of the platelet count/diameter of the spleen ratio diagnostic test using the cutoff value of ≤ 884 to predict esophageal varices

	EV present	EV absent
Cutoff ratio of ≤ 884 *vs* > 884	73	18
Sensitivity	84%	84%
Specificity	84%	84%
Positive predictive value	94%	94%
Negative predictive value	40%	40%
Positive likelihood ratio	4.1	4.1
Negative likelihood ratio	0.37	0.37

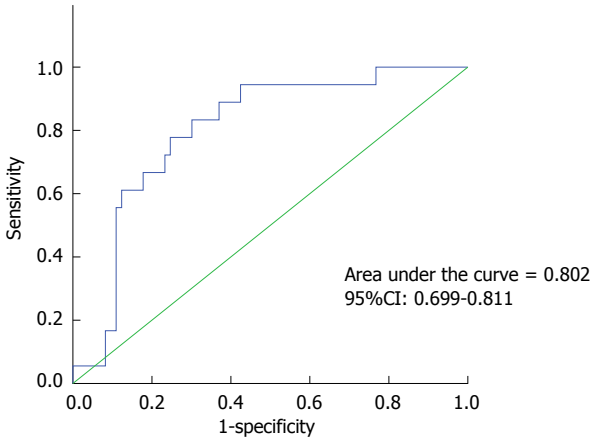

Figure 1 Receiver operating characteristic curve for the platelet count/diameter of the spleen ratio independent of esophageal varix size.

could then be followed up with endoscopic examinations.

The principal noninvasive predictor of esophageal varices may be the PC/SD ratio because it has high sensitivity and specificity in patients with hepatic cirrhosis. This ratio could represent an acceptable parameter of clinical relevance in patients with portal hypertension[12,13,16,17].

We tried to validate whether a simple score could predict the presence of esophageal varices in mexican patients with hepatic cirrhosis of any etiology. Large-scale assessment that takes into account the etiology of cirrhosis is needed to define the role of the PC/SD ratio and to compare the diagnostic exactitude of this ratio with that of other noninvasive parameters. At present, the available data do not support the substitution of another method for upper gastrointestinal endoscopy when identifying esophageal varices, but the PC/SD ratio may be helpful for stratifying patients with cirrhosis into different risk categories. This may be especially relevant to those whose general health conditions do not permit the use of an invasive study, but whose history suggests the possibility of esophageal varices, thus reducing the number of endoscopies.

A large number of patients undergo an endoscopic study to diagnose chronic hepatic illness, and there is a particular interest in finding noninvasive predictors of esophageal varices that could replace the need for scrutinizing endoscopies and thus lower the economic, medical, and social costs[1-12,14,18].

Particular groups of patients have been studied, for example, patients waiting for a liver transplant[18]. In some cases, there is no uniformity in the classification of the varices, or the statistical analysis has been inadequate, and most studies have a retrospective design. In the literature, a low PC and splenomegaly are the most studied noninvasive predictors of esophageal varices, and these parameters produce better results than do parameters such as serum bilirubin and albumin levels, Child-Pugh functional classification, and portal vein diameter[9-12,18-20].

In our study, the analysis of the noninvasive predictors was based on the maximum diameter of the spleen, measured in millimeters using abdominal ultrasound, and the PC. These two parameters were used to calculate the PC/SD ratio. A cutoff point of ≤ 884 produced a sensitivity and specificity of 84% and 70%, respectively.

These results are similar to those of a recent meta-analysis[21], that included 1275 patients and reported a sensitivity of 89% (95%CI: 87-92) and specificity of 74% (95%CI: 70-78). These parameters are easy to obtain, reproducible, and above all, noninvasive. Another advantage is that no additional expense is involved because these studies are performed routinely in patients with hepatic cirrhosis.

The cutoff point for the PC/SD ratio in our study was lower than that reported by Giannini *et al*[16] (< 884.3 and < 909, respectively). This difference is probably influenced by racial characteristics. The higher values in the study by Giannini *et al*[16] were obtained from studies of a principally Caucasian population; the patients were taller than in our population and therefore had larger internal organs. The patient population included in our study is representative of the population with hepatic cirrhosis who have signs of portal hypertension seen in clinical practice. The difference in PC/SD ratio between our study and previous studies indicate that it is important to determine a cutoff point for the PC/SD ratio in any specific population.

Thrombocytopenia can be caused by splenic sequestration or by a decrease in hepatic production of thrombopoietin caused by a failing liver and antibody platelet destruction[1-4,9-11,18]. Some authors who have evaluated noninvasive parameters in the diagnosis of esophageal varices have found that splenomegaly can have a high sensitivity but a low specificity, whereas thrombocytopenia shows the opposite, that is, a low sensitivity and intermediate specificity. In the study by Chalasani *et al*[22], the PC and splenomegaly independently predicted the presence of esophageal varices. In the study by Madhotra *et al*[8], 32% of the patients had a PC < 68000/μL without splenomegaly. These differences may reflect differences in the etiology of cirrhosis, action of immunological mediators, or reduction in thrombopoietin and not just splenic sequestration[4,11].

The positive predictive value (or the proportion of patients with a positive test result who have the disease) was 94%, but the negative predictor value (or the proportion of people with a negative test results who do not

have the disease) was 40% as a reflection of our specificity (70%), the prevalence of esophageal varices in patients in cirrhosis and the different etiologies of the chronic liver diseases.

The use of the PC/SD ratio will help create a lower-cost and more effective method to identify esophageal varices in patients with portal hypertension. The ideal tool should have high sensitivity and specificity as close as possible to 100% to obtain an accurate profile a high-security profile and to avoid the need for endoscopy in patients without esophageal varices. The PC/SD ratio should be considered when identifying patients with a high risk of developing esophageal varices.

In conclusion, the PC/SD ratio cannot substitute for upper gastrointestinal endoscopy in the scrutiny of esophageal varices. However, this ratio can be a useful noninvasive method for identifying patients with esophageal varices and thereby may help reduce the number of unnecessary endoscopies.

COMMENTS

Background

Portal hypertension is the principal complication of hepatic cirrhosis. More than 80% of patients with cirrhosis will develop esophageal varices at some point, and 30% of these patients will have at least one bleeding episode because of rupture of a varix. Most first bleeding episodes happen during the first year after the detection of the varices, with a 5%-10% mortality attributed to the initial hemorrhage. For this reason, identifying the presence of esophageal varices is a fundamental part of the diagnostic work-up in patients with cirrhosis, and it is also a prognostic marker of the illness.

Research frontiers

Recent studies have emphasized the use of noninvasive methods to identify patients with the intention of avoiding endoscopy in low-risk cases. The fact that distinct predictors of the presence of varices have been identified in different studies probably reflects differences between the study populations and spectrum of the disease and this makes it difficult to develop a widely applicable predictive model.

Innovations and breakthroughs

The cutoff point for platelet count/spleen diameter ratio (PC/SD) ratio was lower than that reported in previous literature (< 884.3 and < 909, respectively), with a sensitivity of 84% and specificity of 70%. The differences are probably influenced by racial characteristics. The PC/SD ratio should be considered to identifying patients with esophageal varices.

Peer review

This is an interesting study in which whether ratio of platelet count to spleen diameter can be a predictive factor for existence of esophageal varices was examined in Mexican patients with hepatic cirrhosis. This paper validated the useful of platelet count/spleen diameter ratio and determined a cutoff value for the Mexican, which is a little smaller than the value found in the Caucasian population; suggest that this ratio may be racial specific.

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BRIEF ARTICLE

Protective effects of D-002 on experimentally induced gastroesophageal reflux in rats

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Abstract

AIM: To investigate the effects of beeswax alcohols (D-002) on the esophageal damage induced by gastroesophageal reflux (GER) in rats.

METHODS: Sixty male rats were randomized into six groups (10 rats/group): a negative control and five groups with experimentally induced GER: a positive vehicle control, three treated with D-002 (25, 100 and 200 mg/kg, respectively), and one with omeprazole 10 mg/kg. All treatments were given by gastric gavage. One hour after dosing, GER was produced by simultaneous ligation of the pyloric end and the forestomach. Esophageal lesions index (ELI), gastric secretion volume and acidity, and esophageal malondialdehyde (MDA) and sulfhydryl (SH) group concentrations were measured. Statistical significance was considered at $P < 0.05$.

RESULTS: As compared to the negative control, the positive control group exhibited increased ELI (5.2 ± 0.33 *vs* 0 ± 0, $P = 0.0003$), gastric secretion volume (2.69 ± 0.09 *vs* 0.1 ± 0.0, $P = 0.0003$) and acidity (238 ± 19.37 *vs* 120.0 ± 5.77, $P = 0.001$), and esophageal concentrations of MDA (2.56 ± 0.1 *vs* 1.76 ± 0.28, $P = 0.001$) and SH groups (1.02 ± 0.05 *vs* 0.56 ± 0.08, $P = 0.0003$). D-002 (25, 100 and 200 mg/kg) reduced ELI (3.36 ± 0.31, 2.90 ± 0.46 and 2.8 ± 0.23, respectively) *vs* the positive control (5.2 ± 0.33) ($P = 0.004$; $P = 0.002$; $P = 0.001$, respectively). There were no significant changes in acidity with D-002 treatment, and only the highest dose reduced the volume of the gastric secretion (1.92 ± 0.25) *vs* the positive control (2.69 ± 0.09, $P = 0.013$). D-002 (25, 100 and 200 mg/kg) lowered the esophageal MDA (2.05 ± 0.16, 1.98 ± 0.22 and 1.93 ± 0.22, respectively) ($P = 0.01$; $P = 0.03$; $P = 0.03$, respectively) and SH group concentration (0.87 ± 0.06, 0.79 ± 0.08 and 0.77 ± 0.06, respectively) ($P = 0.04$; $P = 0.04$; $P = 0.02$) *vs* the positive control (2.56 ± 0.10 and 1.02 ± 0.05, respectively). Omeprazole decreased ELI (2.54 ± 0.47), gastric secretion volume (1.97 ± 0.14) and acidity (158.5 ± 22.79), esophageal MDA (1.87 ± 0.13) and SH group (0.72 ± 0.05) concentrations *vs* the positive control ($P = 0.002$; $P = 0.001$; $P = 0.02$; $P = 0.003$; $P = 0.002$, respectively).

CONCLUSION: Acute oral administration of D-002 decreased macroscopic esophageal lesions and oxidative stress in rats with experimentally induced GER, without modifying gastric secretion acidity.

Key words: D-002; Beeswax alcohols; Esophagitis; Gastroesophageal reflux; Oxidative stress

Core tip: Beeswax alcohols (D-002) has gastroprotective effects in experimental and clinical studies. How-

ever, the effects of D-002 on gastroesophageal reflux (GER) have not been investigated. We demonstrated that acute oral administration of 25, 100 and 200 mg/kg D-002 decreased the esophageal lesion index, and esophageal malondialdehyde and sulfhydryl group concentrations. Only the highest dose of D-002 reduced the gastric secretion volume, but none modified the acidity. D-002 decreased esophageal lesions and esophageal concentrations of lipid peroxidation and protein oxidation markers in rats with experimental GER, without modifying gastric secretion acidity.

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INTRODUCTION

Gastroesophageal reflux disease (GERD), a chronic and relapsing disease that affects 40% of the adult population worldwide, emerges when the gastric acid flows back into the esophagus[1-4]. GERD progression leads to the erosion of the esophageal mucosal epithelium, which is implicated in the development of Barrett's esophagus and the subsequent increased risk of developing esophageal cancer[5,6].

Although the etiology of the abnormal reflux of the gastric contents from the stomach to the esophagus is complex and involves multiple causes, the disease mainly results from weak anti-reflux barriers at the gastroesophageal junction that become incompetent to protect against increased reflux, thus leading to esophageal erosion and inflammation. The imbalance between aggressive (refluxed gastric acid secretion and duodenal juice) and defensive factors (esophageal acid clearance and tissue resistance) contributes to the esophageal damage[7-9]. The acid secretion into the esophagus trigger this process, but when it acts together with small amounts of pepsin it increases the risk of esophageal mucosal damage[10]. Gastroesophageal reflux (GER)-induced increase in inflammatory mediators and reactive oxygen species have been shown to contribute to the mucosal damage[11,12].

Current management of GERD includes the use of antisecretory treatments aimed primarily at reducing gastric acidity, such as the proton pump inhibitors (PPIs) or H_2 receptor antagonists (H_2RAs)[13,14]. In particular, acid suppression achieved with PPIs is the mainstay of therapy for reflux disease, but despite this, symptoms and damage persist and recur in many patients[15].

In terms of safety, PPIs and H_2RAs both have a good safety profile[13,14], but recent data suggest a link between their use and some long-term effects of clinical relevance, of which the increased risk of fractures, mainly in the elderly, seems to most supported by the evidence available[16-18]. The benefits of current therapy to manage GERD, however, outweigh the risks, but the search for new effective and safer treatments is ongoing.

Beeswax alcohols (D-002), a mixture of six higher aliphatic primary alcohols purified from the beeswax, in which traicontanol is the major component[19], has been shown to produce gastroprotective effects through multiple mechanisms that mainly involve increased gastric mucus secretion and improved mucus composition (increased content of mucus proteins, glycoproteins and sulfated macromolecules)[20-22]. In addition, D-002 exhibits antioxidant and anti-inflammatory effects on the gastric mucosa[23,24], which could contribute additionally to the gastroesophageal protection.

Oral administration of D-002 reduces the generation of hydroxyl radicals *in vivo*[24], the concentration of malondialdehyde (MDA) (a lipid peroxidation marker)[23,24] and carbonyl groups (a protein oxidation marker), and myeloperoxidase activity (a marker of inflammation), but increases the activity of glutathione peroxidase, superoxide dismutase and catalase in the gastric mucosa of rats with indomethacin-induced ulcers[24].

In light of these findings, we supposed that D-002 could be beneficial for ameliorating GER, which has not yet been investigated. The present study was undertaken to investigate the effect of D-002 on experimentally induced GER in rats.

MATERIALS AND METHODS

Animals

Male Sprague-Dawley rats (200-250 g) purchased from the National Centre for Laboratory Animals Production (CENPALAB, Havana, Cuba) were adapted for 7 d to the experimental conditions: temperature 25 ℃ ± 2 ℃, humidity 60% ± 5% and light/dark cycles of 12 h. Standard chow pellets from CENPALAB and water were given *ad libitum*. Rats were deprived of food for the 24 h prior to GER induction, but with free access to water.

Animal experiments were conducted in accordance with the Cuban Guidelines of Animals Handling and the Cuban Code of Good Laboratory Practices, which follow international guidelines for the use and care of laboratory animals. The study protocol and animals use were approved prior to the study by the Institutional Animal Ethics Committee.

Chemicals and test substance

Omeprazole was purchased from DOMER (Mexico). The batch of D-002, supplied by the Plants of Natural Products (Havana, Cuba), had the following composition: tetracosanol (7.0%), hexacosanol (11.5%), octacosanol (12.1%), triacontanol (34.8%), dotriacontanol (22.5%) and tetratiacontanol (2.6%). Purity (total content of these alcohols) was 90.0%.

Dosage and administration

D-002 and omeprazole were suspended in 1% acacia

gum/water. Rats were randomized into six groups of 10 rats each: a negative vehicle control and five exposed to GER induction: a positive vehicle control, three treated with D-002 (25, 100 and 200 mg/kg, respectively), and one with 10 mg/kg omeprazole. All treatments (D-002, omeprazole, or vehicle) were given orally by gastric gavage (1 mL/200 g body weight), 1 h prior to GER induction.

The chosen doses of D-002 were those effective in the model of pylorus-ligation-induced gastric ulceration[20], and the omeprazole dose was that reported as effective in a model of GER in rats[25].

Induction of GER

Under pentobarbital anaesthesia (40 mg/kg intraperitoneally), a midline incision was performed on the abdomen and the pylorus and transitional junction between the forestomach and corpus were simultaneously ligated to induce reflux of gastric juice into the esophagus[26]. The abdominal cavity was then closed and 5 h later, rats were sacrificed under anaesthesia. The gastric content was collected and centrifuged at 3000 r/min for 10 min. The volume of the supernatant was measured and its acidity estimated by titration with 0.1 mol/L NaOH to pH = 7.0[20]. The esophagus was removed, incised lengthwise, and then the macroscopic esophageal lesions were observed under a microscope and measured. The esophageal tissue was stored at -20 ℃ until performing the biochemical analyses.

Esophageal lesion index

The esophageal lesion index (ELI) score was calculated (macroscopic degree of injury 0-6) after gross inspection of the esophagus under a magnifying glass (× 3) by two independent blinded observers. The lesions were scored as follows: 0: no visible lesion; 1: some erosion and bleeding; 2: total area of lesions < 15 mm^2; 3: total area of lesions < 30 mm^2; 4: total area of lesions < 40 mm^2; 5: total area of lesions < 45 mm^2; and 6: perforation[27].

Oxidative variables

For the estimation of oxidative variables, the excised esophageal tissue was transferred to ice-cooled test tubes and homogenized in 150 mmol/L Tris-HCl buffer (pH = 7.4) containing 0.25 mol/L sucrose-EDTA (1 g tissue/9 mL buffer) by Ultra-Turrax homogenizer T25 (Germany). The homogenates were centrifuged at 5000 *g* for 10 min at 4 ℃, and the supernatants stored at -80 ℃ until analysis. All the assays were performed in triplicate in an Ultrospec Plus LKB spectrophotometer (Pharmacia LkB Biotechnology; Uppsala, Sweden). Protein concentrations were measured by a modification of the Lowry method[28].

Lipid peroxidation assessment: MDA level, a marker of lipid peroxidation in esophageal homogenates, was measured as thiobarbituric acid reactive species (TBARS)[29] Homogenate aliquots (1 mL) were added to a mixture containing 0.2 mL 8.1% SDS plus 1.5 mL 20% acetic acid solution adjusted to pH = 3.5, 1.5 mL of thiobarbituric acid solution, and 1 mmol/L butylated hydroxytoluene, heated at 95 ℃ for 45 min and cooled. One milliliter of distilled water plus 5 mL *n*-butanol: pyridine (15:1 v/v) mixture was added to the mixture, shaken and centrifuged. The organic layer was used for TBARS determination at 535 nm using freshly diluted MDA bis (dimethyl acetal) as a standard. TBARS concentrations were expressed as nmoL MDA/mg protein.

Protein oxidation assessment: SH groups were measured using the 5'5-dithio-bis (2-nitrobenzoic acid) (DTNB) assay[30]. Homogenate aliquots (200 μL) were treated with 600 μL 20 mmol/L Tris-EDTA buffer (pH = 8.2), 40 μL 10 mmol/L DTNB and 3.16 mL absolute ethanol. This mixture was then incubated to ambient temperature for 20 min and centrifuged at 3000 *g* for 10 min. The optical density of the supernatant was measured at 412 nm, using a 13.6/cm/mol coefficient of absorptivity and SH concentrations were reported in mmol/L.

Statistical analysis

Data were expressed as the means ± SE. Paired comparisons between control and treated groups were done with the nonparametric Mann-Whitney *U* test. The level of statistical significance was set at $\alpha = 0.05$. The analyses were carried out using the Statistic software for Windows (Release 4.2, Stat Soft, United States). Dose-effect relationships were assessed by using dose regression linear analysis on the Primer of Biostatistics program (Stanton A, Glantz; McGraw-Hill, Inc Version 3.01).

RESULTS

Effects on esophageal lesions

Five hours after ligation, the positive group displayed macroscopic lesions quantitatively assessed in term of ELI values that were significantly increased as compared to the negative control group, which did not have visible lesions. By contrast, treatment with D-002 (25-200 mg/kg) and omeprazole (10 mg/kg) ameliorated GER-induced esopagheal injury (Table 1).

Acute oral administration of D-002 (25, 100 and 200 mg/kg) reduced the severity of GER-induced oesophagitis (ELI) by 35.4%, 44.2% and 46.1%, respectively, as compared to the positive control group. Despite the fact that the effects increased slightly with dose, the statistical analysis did not show dose dependence. Omeprazole (10 mg/kg), the reference drug, reduced the ELI significantly by 51.1% as compared to the positive control group. The positive control group also exhibited an increase in the volume and acidity of gastric secretion vs the negative control group. Oral administration of the highest dose of D-002 (200 mg/kg) reduced significantly the volume of gastric secretion, but all doses failed to affect the acidity of the gastric secretion. Omeprazole (10 mg/kg) decreased significantly the volume and acidity of the gastric secretion as compared to the positive control

Table 1 Effects on esophageal lesions, gastric secretion volume and acidity, oxidative variable in rats with gastroesophageal reflux

Groups	Doses (mg/kg)	ELI (mean ± SE)	I	Volume (mL)	I	Acidity (meq/L/100 g)	MDA (nmol/mg protein)	I	SH (mmol)	I
Negative control	-	0 ± 0[c]		0.1 ± 0[c]	--	120 ± 5.77[b]	1.76 ± 0.28[b]		0.56 ± 0.08[b]	--
Positive control	-	5.2 ± 0.33		2.69 ± 0.09	--	238 ± 19.37	2.56 ± 0.10		1.02 ± 0.05	--
D-002	25	3.36 ± 0.31[b]	35.4%	2.63 ± 0.36	2.3%	220 ± 22.57	2.05 ± 0.16[a]	64.0%	0.87 ± 0.06[a]	32.6%
D-002	100	2.90 ± 0.46[b]	44.2%	2.28 ± 0.45	19.0%	210 ± 19.79	1.98 ± 0.22[a]	72.5%	0.79 ± 0.08[b]	50.0%
D-002	200	2.8 ± 0.23[b]	46.1%	1.92 ± 0.25[a]	29.7%	233.7 ± 44.7	1.93 ± 0.22[a]	79.0%	0.77 ± 0.06[a]	54.3%
Omeprazole	10	2.54 ± 0.4[b]	51.1%	1.97 ± 0.14[b]	27.8%	158.5 ± 22.79[a]	1.87 ± 0.13[b]	86.3%	0.72 ± 0.05[b]	65.2%

These data were obtained from groups of 10 rats. Values are represented as mean ± SE. [a]$P < 0.05$, [b]$P < 0.01$ *vs* the positive control (Mann–Whitney *U* test). I: Inhibition; ELI: Esophageal lesions index; MDA: Malondialdehyde; SH: Sulfhydryl.

group (Table 1).

Effects on oxidative markers

Experimentally induced GER increased significantly the MDA and SH concentrations in the esophageal homogenates of the positive control as compared to negative control group; a change also ameliorated by D-002 (25, 100 and 200 mg/kg) and omeprazole (10 mg/kg). Oral treatment with D-002 (25, 100 and 200 mg/kg) decreased significantly the esophageal levels of MDA (64%, 72.5% and 79%, respectively) and SH (32.6%, 50% and 54%, respectively). No dose-effect relationship, however, was seen. Oral omeprazole (10 mg/kg) reduced significantly MDA (86.3% decrease) and SH (65.2% decrease) levels in the esophageal tissues of rats with experimentally induced GER (Table 1).

DISCUSSION

The results of this study demonstrated, for the first time, that oral supplementation of D-002 significantly ameliorated GER-induced esophageal damage in rats. Our data confirm that GER induction causes esophageal lesions and increases the volume of gastric secretion, gastric acidity, and the extent of lipid peroxidation and protein oxidation in esophageal homogenates, as demonstrated by the increased levels of MDA and SH groups. In addition, omeprazole significantly reduced GER-induced changes, as expected[25], all of which confirms the validity of this model in our experimental conditions.

Oral treatment with D-002 (25-200 mg/kg) significantly (approximately 45%) reduced ELI. A dose of 100 mg/kg achieved the ceiling effect (44.2% decrease) and 200 mg/kg produced roughly the same effect (46.1% decrease), which in turn was similar to the reduction (51.1%) induced by 10 mg/kg omeprazole.

All doses of D-002 failed to modify the acidity of the gastric secretion, in agreement with previous results, in the model of pylorus-ligation-induced ulcers in rats, confirming the cytoprotective effect of D-002[20,21]. The highest dose (200 mg/kg) reduced significantly (29.7%) the gastric secretion volume, as did omeprazole (27.8%), but by different mechanisms as proven by the marked reduction of the acidity produced by this drug. The mechanism by which D-002 produces this effect has not been explained to date.

GER-induced ELI has been shown to correlate with the increased production of free radicals that triggers membrane lipid peroxidation, impairs cell functions, and reinforces the oxidative stress through the production of lipid-derived radicals[31]. Accordingly, natural approaches with antioxidant effects have been investigated for the management of GERD[32], and the efficacy of PPIs on mucosal protection is currently explained by the inhibition of acid secretion, as well as its antioxidant and anti-apoptotic effects[33].

Our results support that D-002 was able to attenuate the GER-induced increase in MDA and SH concentrations to 72.5% and 50%, respectively. A dose of 100 mg/kg was the maximal effective dose for both effects, and its effects on oxidative markers were more pronounced than its ability to lower ELI. D-002 was effective at reducing GER-induced esophagitis and the increased oxidative stress in this model, without modifying gastric secretion acidity.

Although the safety of current first-line therapies for GERD is good, the lack of antisecretory activity of D-002 could be beneficial for avoiding the adverse effects associated with long-term acid suppression[15-18,35]. Experimental toxicology has demonstrated the safety of acute, subchronic and chronic oral administration of D-002. A dose of 1000 mg/kg was found to have no observable adverse effects in a long-term (1 year) study in rats[36], and the same was true for a dose of 250 mg/kg in beagle dogs[37]. Clinical studies have shown that D-002 is safe and well tolerated when administered in the short and long term to humans[38-42].

Nevertheless, the present results are a preliminary demonstration of the efficacy of oral D-002 treatment in an experimental model of GER. The efficacy and safety of D-002 for GERD management, therefore, merits extensive further experimental and clinical research.

In conclusion this study demonstrates that acute oral administration of D-002 (25-200 mg/kg) was effective to reduce esophageal lesions and oxidative stress markers in rats with experimentally induced GER, without modifying gastric secretion acidity.

COMMENTS

Background

Beeswax alcohols (D-002), a food supplement, has been shown to produce gastroprotective effects in experimental and clinical studies, but its efficacy against gastroesophageal reflux (GER) has not been investigated before. The authors investigated the effects of a single oral administration of D-002 in a model of experimentally induced GER in rats.

Research frontiers

Several experiments have reported that D-002 protects against gastric mucosa ulceration induced by different agents through multiple mechanisms, including an increase in gastric mucus secretion, improvement of mucus quality, and antioxidant effects on the gastric mucosa. It is unresolved whether D-002 can protect against GER.

Innovations and breakthroughs

This is believed to be the first study to show that acute oral administration of D-002 reduced esophageal lesions as well as esophageal concentrations of malondialdehyde and sulfhydryl groups in rats with experimentally induced GER, without modifying gastric secretion acidity. These findings suggest that supplementation with D-002 protects against esophageal injury induced by GER in rats.

Applications

D-002 is a dietary supplement that has gastroprotective and antioxidant effects. However, its potential effect on GER, a common condition in routine practice, has not been investigated before. This study is a preliminary step in demonstrating whether D-002 could be an alternative to help manage GER. However, extensive experimental and clinical research is still required to demonstrate its application in this field.

Terminology

The esophageal lesion index is a validated score for quantifying the severity of the macroscopic lesions present in the esophageal tissue.

Peer review

This study evaluated the effect of D-002, a defined mixture of higher primary alcohols purified from bees wax, on experimentally induced GER in rats. The possible influence and protective effect of D-002 on experimental gastric ulcer and colitis in rats and on nonalcoholic fatty liver disease in humans has been evaluated by the same group of researchers in recent years. The anti-inflammatory activity of D-002, by reducing the generation of harmful hydroxyl radicals, has been well studied, but it is difficult to acknowledge this substance as a further option in the management of GERD.

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BRIEF ARTICLE

ZD 7288, an HCN channel blocker, attenuates chronic visceral pain in irritable bowel syndrome-like rats

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Abstract

AIM: To investigate the effects of ZD 7288, a hyperpolarization-activated cyclic nucleotide-gated (HCN) channel blocker, on rats with chronic visceral pain.

METHODS: Rats with visceral hypersensitivity were generated using neonatal colon irritation during postnatal days 8-15 as described previously. Visceral hypersensitivity was evaluated using electromyographic (EMG) responses of abdominal external oblique muscles to 20-80 mmHg colorectal distentions (CRD). Abdominal withdrawal reflex (AWR) scores and pain thresholds were also detected in adult rats. Different doses of ZD 7288 (25, 50, and 100 nmol/L) were intrathecally administered in rats to study the role of spinal HCN channel in chronic visceral hypersensitivity.

RESULTS: EMG responses to 20-80 mmHg CRD and AWR scores under 20-60 mmHg CRD significantly increased in rats with visceral hypersensitivity compared to control rats ($P < 0.05$). The pain threshold in rats with visceral hypersensitivity significantly decreased compared to control rats ($P < 0.05$). Treatment with 50-100 nmol/L ZD 7288 significantly inhibited EMG responses (16%-62%, 80-20 mmHg CRD, $P < 0.05$) and AWR scores (24%-37%, 40-20 mmHg CRD, $P < 0.05$; 12%-61%, 80-20 mmHg CRD, $P < 0.05$, respectively), and significantly increased pain thresholds (32%-77%, $P < 0.05$).

CONCLUSION: Spinal HCN channels may play an important role in chronic visceral hypersensitivity.

Key words: Abdominal withdrawal reflex; Electromyography; Hyperpolarization-activated cyclic nucleotide-gated channel; Visceral hypersensitivity; ZD 7288; Irritable bowel syndrome

Core tip: Intrathecal administration of ZD 7288, a hyperpolarization-activated cyclic nucleotide-gated channel blocker, significantly inhibited electromyographic responses and abdominal withdrawal reflex scores and significantly increased pain thresholds in rats with chronic visceral hypersensitivity. These results are important for clinicians and the fundamental scientific community and provide scientific evidence for ZD 7288 as a novel treatment for visceral pain in irritable bowel syndrome.

Chen Y, Lin C, Tang Y, Chen AQ, Liu CY, Lu DL. ZD 7288, an HCN channel blocker, attenuates chronic visceral pain in irritable bowel syndrome-like rats. *World J Gastroenterol* 2014; 20(8): 2091-2097 Available from: URL: http://www.wjgnet.com/1007-9327/full/v20/i8/2091.htm DOI: http://dx.doi.org/10.3748/wjg.v20.i8.2091

INTRODUCTION

Irritable bowel syndrome (IBS) is a common disorder characterized by abdominal pain, bloating and altered bowel habit[1]. No effective drug treatment is currently available for IBS[1-4]. The persistent colon hypersensitivity in IBS is associated with neuronal sensitization, which manifests as an increase in neuronal excitability[5]. Some ion channels may be the primary determinants of neuronal excitability[6,7]. Emery *et al*[8] stated "The rate of action potential firing in nociceptors is a major determinant of the intensity of pain. Possible modulators of action potential firing include the hyperpolarization-activated cyclic nucleotide-gated (HCN) ion channels". The HCN channel was first identified in the sinoatrial node (SAN) in rats[9]. These channels are permeable to both Na^+ and K^+. The HCN current, hyperpolarization-activated cation current (Ih), was found in neurons under hyperpolarization of the cell membrane[10]. Ih typically contributes to the pacemaker activity of cardiac SAN cells and a variety of spontaneously firing neurons. Ih is crucial in determining the frequency of firing of action potentials[11]. Much attention has focused on HCN channels and somatic pain in the past decade[8,12], and Ih is an important contributor of neuropathic pain. The HCN channel might be a valuable target for the treatment of neuropathic pain[10].

ZD 7288 [4-(N-Ethyl-N-phenylamino)-1,2-dimethyl-6-(methylamino) pyrimidinium chloride] completely blocked Ih in a previous study, and eliminated the depolarizing sag of transmembrane potential[13]. ZD 7288 may reduce neuropathic pain and provide significant analgesic effects[14-16]. Ectopic discharges may be involved in the development of neuropathic pain[10]. A low concentration of ZD 7288 significantly suppressed ectopic discharges generated from DRG neurons[10]. HCN ion channels are called pacemakers of pain[11], but all conclusions about the relationship between HCN channels and pain have come from somatic pain research. No studies on the role of HCN channels in visceral pain have been conducted. The contribution of HCN channels to the genesis of visceral hypersensitivity must be explored.

Most studies on chronic visceral pain mechanisms focused on inflammatory mediators, neuron-active compounds, neurotransmitters and receptors in peripheral and central sensitization[17]. However, ion channels may be the primary determinants of neuronal excitability[18]. The HCN channel current creates a more positive resting membrane potential and up-regulates neuronal excitability[10]. ZD 7288 provides significant analgesic effects against neuropathic pain[10], and it may attenuate visceral pain through a similar mechanism. However, the role of ZD 7288 in chronic visceral pain has not been investigated.

An IBS-like rat model was established in the present study using neonatal colon irritation. Abdominal withdrawal reflex (AWR) scores and electromyographic (EMG) amplitudes were recorded. The effects of ZD 7288 on visceral sensitivity in IBS-like rats were evaluated.

MATERIALS AND METHODS

Animal preparation

Male Sprague-Dawley rats were obtained as preweanling neonates (5 d old) from the Shanghai SLAC Laboratory Animal Co., Ltd. (Animal approval number: SCXK 2007-0005). Twelve neonates were housed in a cage with 1 adult female rat until they were 28 d old. The adult female rat had access to food and water *ad libitum*. After separation, 4 young rats were housed in a plastic cage with sawdust bedding, given access to food and water *ad libitum* and maintained on a 12-h light/dark cycle. The irritation procedure and experimental testing were conducted during the light cycle. Experiments were performed when the rats were 8 wk old.

Animal model of visceral hypersensitivity

Neonatal colon irritation was applied once daily using colorectal distention (CRD) during postnatal days 8-15. The CRD procedure was modified from Lin and Al-Chaer[19]. The distention was applied using angioplasty balloons (length, 20.0 mm; diameter, 2.5 mm) inserted rectally into the descending colon. The balloon was distended with air exerting a pressure of 60 mmHg for 1 min, deflated and withdrawn. The control rats were handled similarly to the model group except that no CRD was applied. The experiments were approved by the Animal Care and Use Committee of Fujian Medical University.

EMG recordings and AWR scores

EMG recordings were performed as described previously[20]. Rats were anesthetized with ether, and distention balloons (5 cm in length; made of the finger of a latex glove attached to polyethylene tubing) were inserted through the anus into the rectum and descending colon of adult rats. The tubing was taped to the tail to hold the balloon in place. Two silver bipolar electrodes were inserted into the external oblique muscle of the abdomen (EOMA). The rats were maintained in a supine position in a self-made restrainer and allowed at least 30 min to recover from inhalational anesthesia. The tubing was attached through a T-connector to a sphygmomanometer pump and a pressure gauge. Distention was produced by rapid inflation of the balloon to the desired pressure (20, 40, 60 or 80 mmHg) for 10 s followed by a 4-min rest. The magnitude of EMG was measured using an RM6240BD multi-channel physiological signal acquisition and processing system (Chengdu, China; high-frequency filter: 3 KHz; time constant: 0.001 s; sampling frequency: 40 KHz; sensitivity: 500 μV; scanning speed: 200 ms/div). Twenty amplitudes were measured during each 10 s distention period, and the mean amplitude represented the magnitude of EMG. EMG data were derived by subtracting the mean baseline amplitude (10-s pre-distention period) from the mean EMG amplitude of each pressure.

Behavioral responses to CRD were assessed via AWR measurement using a semi-quantitative score[21]. AWR is

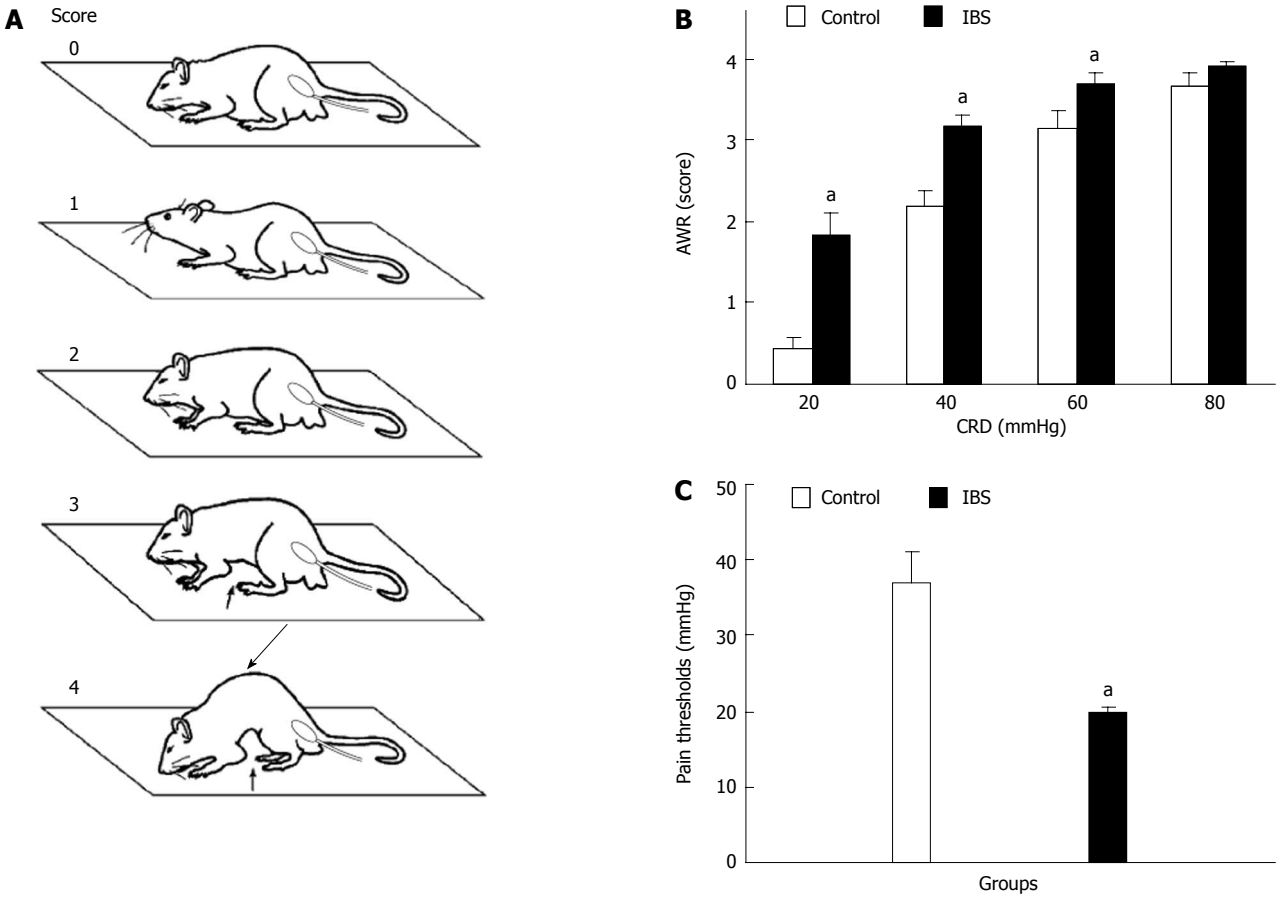

Figure 1 Behavioral evaluation of visceral pain. A: Schematic drawings illustrating the visually based behavioral scale of the abdominal withdrawal reflex (AWR) in response to graded colorectal distension (CRD). AWR scores are 0, 1, 2, 3, and 4; B: AWR scores of control and irritable bowel syndrome (IBS)-like rats; C: Pain thresholds measured by AWR in rats. The AWR threshold indicates CRD intensity when the AWR score is 3. $^{a}P < 0.05$ compared with control rats.

Table 1 Abdominal withdrawal reflex scoring criteria

Score	
0	No behavioral response to colorectal distention
1	Immobile during colorectal distention and occasional head clinching at stimulus onset
2	Mild contraction of the abdominal muscles but absence of abdomen lifting from the platform
3	Observed strong contraction of the abdominal muscles and lifting of the abdomen off the platform
4	Arching of the body and lifting of the pelvic structures and scrotum

an involuntary motor reflex that is similar to the viscero-motor reflex[22]. AWR measurement consisted of visual observation of animal responses to graded CRD (20, 40, 60, and 80 mmHg) by blinded observers who assigned AWR scores (Figure 1A, Table 1).

Determination of pain thresholds

Visceral pain thresholds were evaluated via measurement of the pressure thresholds (increments of 5 mmHg, 3-min duration, 1-min rest) required to evoke abdominal contractions that caused the rat to lift its abdomen off the platform. The recordings were repeated three times for each animal, and the arithmetic mean value was calculated.

Intrathecal cannulation and drug administration

Rats were anesthetized with barbanylum (8%, 0.1 mL/100 g). A sterile polyethylene catheter (PE10 tubing, Becton Dickinson) was introduced at the L6/S1 interspace and threaded to the lumbar enlargement. Rats recovered for 1 wk after intrathecal cannulation. Various doses (25, 50, and 100 nmol/L) of ZD 7288 (molecular weight 292.81, Tocris Cookson, Ellisville, MI) diluted in sterile 0.9% saline were administered at a volume of 10 μL followed by a 10-μL saline flush. Visceral pain measurements were performed 30 min after intrathecal administration.

Statistical analysis

Data are expressed as means ± SEM (standard error). A two-way repeated measures analysis of variance (ANOVA) with Bonferroni post hoc analysis was used to assess changes in AWR or EMG in different groups across pressures. Comparisons of pain thresholds between IBS-like rats and control rats were analyzed using Student's *t* test. One-way ANOVA followed by the Student-Newman-Keuls post-hoc test was used to compare differences in pain thresholds of IBS-like rats before and after different ZD 7288 doses. Data analysis was performed using SPSS 13.0. A P value < 0.05 was considered statistically significant.

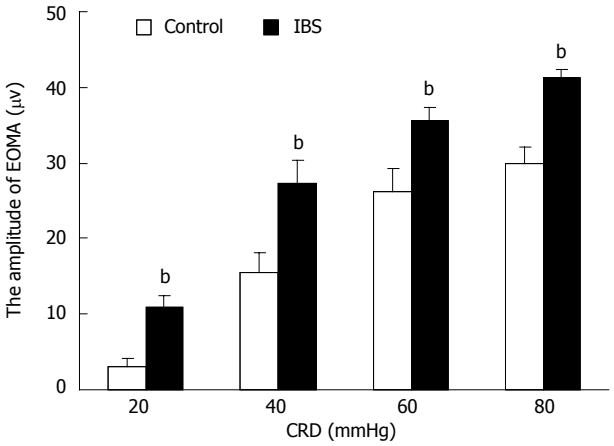

Figure 2 Electromyographic responses to colorectal distension in rats. The average responses to graded colorectal distension (CRD) were significantly increased in irritable bowel syndrome (IBS)-like rats compared with control rats. $^{b}P < 0.01$, compared with control rats at the same CRD intensity. EOMA: External oblique muscle of the abdomen.

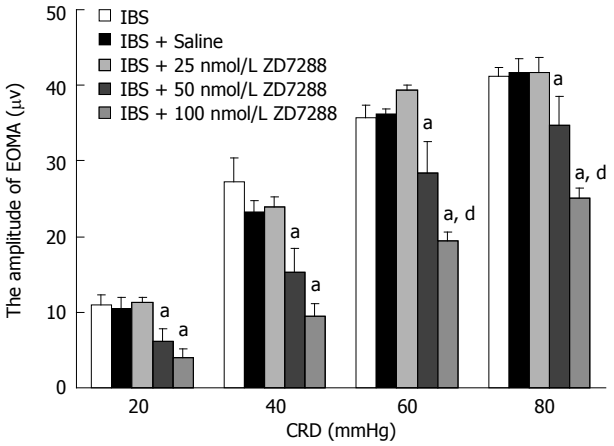

Figure 3 Inhibitory effects of ZD 7288 on amplitudes of external oblique muscle of the abdomen in irritable bowel syndrome-like rats. $^{a}P < 0.05$, compared with saline-treated irritable bowel syndrome (IBS)-like rats; $^{d}P < 0.01$, compared with IBS-like rats receiving 50 nmol/L ZD 7288. EOMA: External oblique muscle of the abdomen; CRD: colorectal distension.

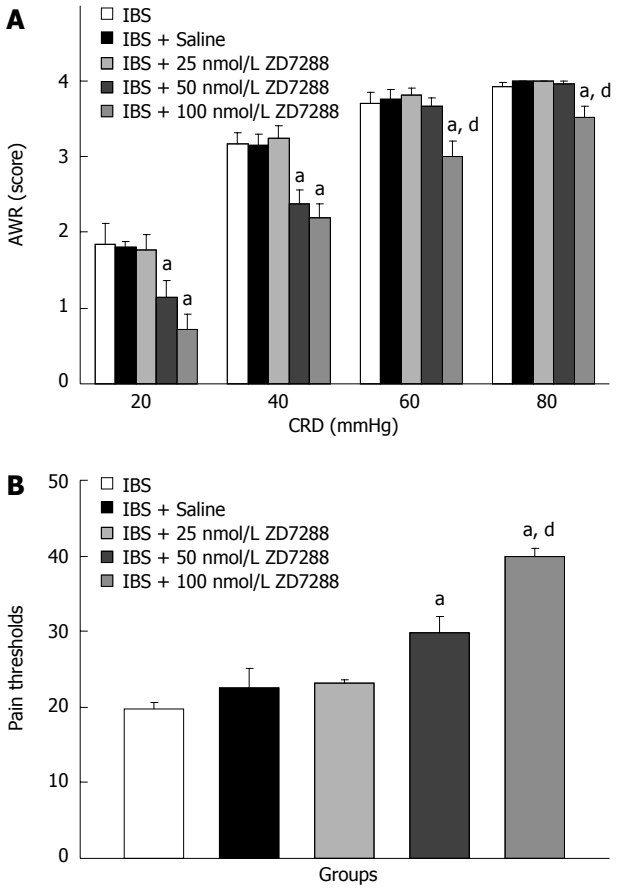

Figure 4 Inhibitory effects of ZD 7288 on abdominal withdrawal reflex scores (A) and pain thresholds (B) of irritable bowel syndrome-like rats. $^{a}P < 0.05$, compared with saline-treated IBS-like rats; $^{d}P < 0.01$, compared with IBS-like rats receiving 50 nmol/L ZD 7288. CRD: Colorectal distension; AWR: Abdominal withdrawal reflex.

RESULTS

Visceral sensitivity increased in IBS-like rats

The AWR scores at 20-60 mmHg CRD were significantly higher in IBS-like rats than those in control rats ($P < 0.05$, Figure 1B). The pain threshold of IBS-like rats significantly decreased compared to control rats ($P < 0.05$, Figure 1C). EMG amplitudes in IBS-like rats significantly increased at 20-80 mmHg CRD compared to control rats ($P < 0.05$, Figure 2).

Intrathecal ZD 7288 administration inhibited visceral hypersensitivity in IBS-like rats

No differences in EMG amplitudes were observed between the IBS-like, IBS-like + saline and IBS-like + 25 nmol/L ZD 7288 groups. IBS-like rats given 50 and 100 nmol/L ZD 7288 demonstrated a significant CRD-dependent (20-80 mmHg) decrease in amplitudes compared to the IBS-like + saline group (50 nmol/L: $P < 0.05$; 100 nmol/L: $P < 0.01$, Figure 3). IBS-like rats given 50 nmol/L ZD 7288 showed a significant reduction in EMG amplitudes compared to IBS-like rats given normal saline (41%, 35%, 22%, and 16% at 20, 40, 60, and 80 mmHg CRD, respectively). Treatment with 100 nmol/L ZD 7288 significantly inhibited EMG amplitudes (62%, 59%, 46%, and 40% at 20, 40, 60, and 80 mmHg CRD, respectively) compared to IBS-like rats given normal saline.

Furthermore, no differences in AWR scores were observed between the IBS-like, IBS-like + saline and IBS-like + 25 nmol/L ZD 7288 groups. IBS-like rats given 50 nmol/L (20-40 mmHg) and 100 nmol/L (20-80 mmHg) ZD 7288 showed a significant CRD-dependent decrease in AWR scores compared to the IBS-like + saline group. IBS-like rats given 50 nmol/L ZD 7288 exhibited significantly reduced AWR scores compared to IBS-like rats given normal saline (37% at 20 mmHg CRD and 24% at 40 mmHg CRD). ZD 7288 at 100 nmol/L significantly inhibited AWR scores (61%, 30%, 20%, and 12% at 20, 40, 60, and 80 mmHg CRD, respectively) (Figure 4A).

The pain thresholds of rats treated with 50 nmol/L ZD 7288 increased by 32% compared to rats given normal saline, and the pain thresholds of rats treated with 100 nmol/L ZD 7288 increased by 77% (Figure 4B).

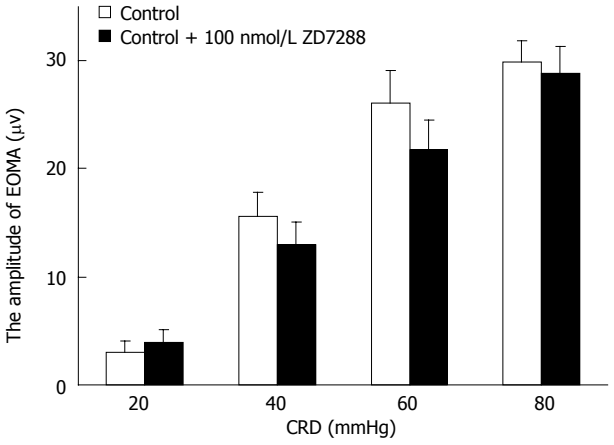

Figure 5 Effects of ZD 7288 on amplitudes of external oblique muscle of the abdomen in control rats. EOMA: External oblique muscle of the abdomen; CRD: Colorectal distension.

No significant differences in EMG amplitudes were observed between the control and control + 100 nmol/L ZD 7288 groups (Figure 5).

DISCUSSION

This study tested the effect of intrathecal ZD 7288, an HCN channel blocker, on the visceral hypersensitivity of IBS-like rats. The results of EMG, AWR and pain thresholds clearly demonstrate that 50-100 nmol/L ZD 7288 significantly attenuated chronic visceral hypersensitivity in IBS-like rats. These data indicate that spinal HCN channels contribute to visceral hypersensitivity in IBS-like rats.

Effect of ZD 7288 on spinal central sensitization in chronic visceral pain rats

Emery *et al*[8] reported that HCN channels play a central role in neuropathic pain. ZD 7288 effectively attenuates neuropathic pain[16]. Most of the drugs that are specifically approved for the treatment of visceral pain syndromes are effective treatments for chronic neuropathic pain states[23], which suggests that chronic visceral pain and chronic neuropathic pain share a common mechanism. Treatment with 50 nmol/L and 100 nmol/L ZD 7288 significantly inhibited EMG amplitudes in IBS-like rats in our study (16%-41% and 40%-62%, 80-20 mmHg CRD, respectively). Intrathecal administration of 100 nmol/L ZD 7288 significantly relieved mechanical allodynia in neuropathic pain rats with spinal nerve ligation, but 50 nmol/L ZD 7288 had no effect[16]. These results indicated that HCN channels may be the common mechanism in visceral and neuropathic pain, and these channels may play a more important role in visceral hypersensitivity.

Neuropathic pain is characterized by ectopic discharges, which are similar to the discharges observed in IBS-like rats[19]. The spontaneous activity of lumbosacral afferents and the number of dorsal roots activated by CRD are significantly enhanced in IBS-like rats compared to controls[19,21]. Spinal HCN channels contribute to the maintenance of neuropathic pain, most likely at the primary afferent terminals[6]. Ih amplitude is augmented in the ventral-lateral periaqueductal gray neurons in neuropathic pain models, and an increase in the frequency of ZD 7288-attenuated action potential firing is observed[24]. Therefore, we inferred that the hyperexcitability of spinal ascending neurons due to an up-regulation of HCN channels might underlie spinal sensitization in chronic visceral pain. However, more electrophysiological studies, such as whole-cell patch clamp recordings, are required.

Potential of ZD 7288 as a treatment for chronic visceral pain

Several treatments, such as anti-spasm medications, antidepressants, probiotics and acupuncture, are efficacious for IBS, but patients and clinicians question their efficacy due to the recurrence of abdominal pain, diarrhea and other symptoms[1-4]. For example, acupuncture is clinically effective for visceral pain due to bowel obstruction, inflammation or ulcer, but controversies exist due to the high recurrence rate[25,26]. Scientific evidence of acupuncture treatment efficacy is lacking, and its mechanisms require to be investigated[25]. Antagonists to NMDA receptors, such as MK-801[27] and AP-7[5], inhibit visceral hypersensitivity. However, the use of these agents in the treatment of chronic pain is restricted due to their serious side effects, including hallucinations, learning and memory impairments, and sensorimotor disturbances[28,29].

In this study, 100 nmol/L ZD 7288 exhibited stronger analgesic effects without apparent side effects, which is consistent with the results of Wan's study on neuropathic pain[16]. Intrathecal administration of ZD 7288 increased pain thresholds in IBS-like rats in a dose-dependent manner. Visceral hypersensitivity includes allodynia and hyperalgesia. Allodynia indicates that an originally non-noxious stimulation induces pain, and hyperalgesia indicates that an originally noxious stimulation induces a supernormal reaction[21,30,31]. Neonatal CRD in the present study may result in allodynia and hyperalgesia, which is consistent with previous studies[20]. ZD 7288, an HCN channel blocker, attenuated visceral pain at 20-40 (non-noxious stimulation) and 60-80 mmHg CRD (noxious stimulation). Therefore, ZD 7288 attenuated allodynia and hyperalgesia in rats with chronic visceral pain. Our results suggest that ZD 7288, an HCN channel blocker, is a useful drug for the treatment of chronic visceral pain in the future.

Comparison of AWR and EMG findings

Neonatal CRD produced allodynia and hyperalgesia in the present study, which supports the hypothesis that early life stress may trigger visceral hyperalgesia and colonic dysfunction[32]. Our AWR scores were *ca.* 1.8, 3.1, 3.8, and 3.9 at 20-80 mmHg CRD in IBS-like rats, which is partially different from the results obtained by Li *et al*[33], who reported AWR scores of *ca.* 0.9, 2.5, 3.1 and 3.5 in visceral hypersensitivity rats. These differences may be attributed to the distinct models. Li *et al*[33] used neonatal

maternal deprivation to produce visceral hypersensitivity, and our study used neonatal CRD to induce IBS-like symptoms. These results demonstrate that neonatal CRD may induce allodynia and hyperalgesia, but neonatal maternal deprivation may preferentially induce hyperalgesia. Zhou *et al*[4] proposed that a 9-d heterotypic intermittent stress generates visceral hypersensitivity. The results of these three studies suggest that stimulus intensity is more important than stimulus type for the induction of visceral hypersensitivity.

EMG and AWR scores are two different methods to assess visceral hypersensitivity in rats[4,20,33]. AWR scores are semi-measurement data, and it is sometimes difficult for an observer to distinguish the difference between scores of 3 and 4. EMG recording results are pure measurement data that are not influenced by objective factors. EMG recording is a relatively sensitive method to evaluate visceral pain. However, other signals easily interfere with EMG recordings during the experimental process. Most AWR scores in our study were consistent with the EMG findings. Therefore, the combined application of EMG and AWR scores improves the credibility of results.

In conclusion, intrathecal administration of ZD 7288, an HCN channel blocker, attenuated visceral hypersensitivity in IBS-like rats without motor disorders. Therefore, ZD 7288 might be a potential treatment for IBS.

COMMENTS

Background

Irritable bowel syndrome (IBS) is a common gastrointestinal disorder that is characterized by chronic visceral pain, bloating and altered bowel habit. Several treatments, such as anti-spasm medications, antidepressants, and probiotics, are efficacious for IBS, but patients and clinicians question their efficacy due to the recurrence of abdominal pain. Hyperpolarization-activated cyclic nucleotide-gated (HCN) ion channels are called pacemakers of pain. ZD 7288, an HCN channel blocker, may reduce neuropathic pain and provide significant analgesic effects. However, the role of ZD 7288 in chronic visceral pain remains unknown.

Research frontiers

The present study used ZD 7288 to treat visceral hypersensitivity in rats with IBS-like symptoms induced using neonatal colorectal distention. Treatment with 50-100 nmol/L ZD 7288 attenuated chronic visceral pain and increased pain thresholds in IBS-like rats.

Innovations and breakthroughs

This is the first study to demonstrate that ZD 7288 treatment produces an analgesic effect on visceral hyperalgesia in rats with IBS-like symptoms induced using neonatal colorectal distention.

Applications

This study showed that ZD 7288 administration produced an analgesic effect on visceral hyperalgesia in IBS-like rats and provide scientific evidence for ZD 7288 as a novel treatment for visceral pain in IBS.

Terminology

IBS is a common gastrointestinal disorder that is characterized by chronic visceral pain, bloating and altered bowel habit. The HCN channel is a hyperpolarization-activated cyclic nucleotide-gated channel. The HCN current is a crucial determinant of the firing frequency of action potentials. ZD 7288 is an HCN channel blocker.

Peer review

This study describes an analgesic effect of ZD 7288 in IBS-like rats and elucidates that ion channels may be the primary determinants of neuronal excitability. The HCN channel current creates a more positive resting membrane potential and up-regulates neuronal excitability. The presented results are important for clinicians and the fundamental scientific community.

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BRIEF ARTICLE

Cancer stem cell markers correlate with early recurrence and survival in hepatocellular carcinoma

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Abstract

AIM: To investigate whether expression of cancer stem cell (CSC) markers is associated with recurrence and survival in hepatocellular carcinoma (HCC) patients.

METHODS: A consecutive series of 90 HCC patients who underwent curative hepatectomy between April 2007 and April 2009 were analyzed. Of the 90 patients, 38 (42%) experienced recurrence within two years of surgery. To adjust for baseline differences between this early recurrence group and the other patients, propensity-score matching was used to generate 25 pairs of patients. Immunohistochemistry was used to compare expression of CD133, CD90, and epithelial cell adhesion molecule (EpCAM) in liver tissues from propensity score-matched patients and from 10 healthy adults. Associations of the three markers with HCC, clinicopathological characteristics, early recurrence, and survival time were explored.

RESULTS: The expression of all three CSC markers was significantly higher in HCC tissue than in healthy liver tissue ($P < 0.001$ for all). Among the HCC clinicopathology characteristics examined, the absence of tumor capsule was associated with CD133 expression ($P = 0.005$); higher histopathology grade and larger tumor size were associated with CD90 expression ($P = 0.010$ and 0.034, respectively); and elevated serum alpha-fetoprotein levels were associated with EpCAM expression ($P = 0.021$). Expression of CD90 and EpCAM was significantly higher in the early recurrence group than in other patients ($P = 0.001$ and 0.045, respectively), whereas CD133 expression was not significantly different between the two groups ($P = 0.440$). Multivariate analysis identified only CD90 expression as significantly associated with early recurrence. Log-rank analysis identified expression of both CD90 and EpCAM as significantly associated with survival time of HCC patients. Cox regression identified EpCAM expression as an independent predictor of survival time.

CONCLUSION: Expression of CD133, CD90, and EpCAM CSC markers may be linked to HCC tumor onset and/or progression. In addition, EpCAM expression is associated with shorter survival time, while CD90 expression is associated with early HCC recurrence.

Key words: Hepatocellular carcinoma; Cancer stem cells; CD133; CD90; Epithelial cell adhesion molecule

Core tip: Cancer stem cells have been proposed as the cells responsible for initiating tumor formation, recurrence and metastasis, and liver cancer stem cells have been found to carry the surface markers CD133, CD90, and epithelial cell adhesion molecule (EpCAM). This paper addresses the clinical impact of CD133, CD90, and EpCAM in propensity score-matched patients with hepatocellular carcinoma. Our findings revealed that expression of CD133, CD90, and EpCAM may be linked to hepatocellular carcinoma (HCC) tumor onset and/or progression. In addition, EpCAM expression is associated with shorter survival time, while CD90 expression is associated with early HCC recurrence.

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INTRODUCTION

Hepatocellular carcinoma (HCC) is one of the most common cancers worldwide, with nearly one million new cases diagnosed every year around the world[1]. Hepatic resection remains the most effective and practical treatment for HCC patients[2,3]; single-site studies indicate that this method can achieve five-year survival rates as high as 50%[4,5]. However, postoperative recurrence, which can be as high as 45% within two years of surgery[6], and the associated poor prognosis remain challenges for HCC management[7,8].

The cancer stem cell (CSC) hypothesis stipulates that primary tumors are initiated and maintained by a small population of cancer cells with "stem cell-like" characteristics[9]. In support of this hypothesis, CSCs have been identified in many tumor types, including HCC[10]. Numerous cell surface antigens, such as CD133, CD90, and epithelial cell adhesion molecule (EpCAM) have been identified as CSC markers, and all three are expressed by liver CSCs (LCSCs) in HCC. Such markers may prove useful for predicting the prognosis of HCC patients, since the CSC hypothesis predicts that CSCs drive tumor recurrence and metastasis after hepatic resection[9]. Therefore we aimed to analyze the expression of CD133, CD90, and EpCAM in patients with HCC and to search for associations with early recurrence and survival time.

MATERIALS AND METHODS

This study was approved by the ethics committee of the Tumor Hospital, Guangxi Medical University, and written informed consent was obtained from participants prior to enrollment.

Patients and healthy controls

A consecutive sample of 90 HCC patients treated by curative hepatectomy at our hospital between April 2007 and April 2009 was enrolled in our study. To be enrolled, patients had to have pathology-confirmed HCC that had not been treated with any other anticancer modality, and their hepatectomy had to be confirmed as curative based on the following criteria: (1) the surgery was limited to a solitary nodular tumor; (2) the resection margin was greater than 10 mm; (3) post-surgical imaging did not show residual tumor[11], extrahepatic metastases or portal tumor thromboses[12]; and (4) levels of alpha-fetoprotein (AFP) in patients with elevated serum AFP levels before surgery decreased to normal within two months after the procedure. Patients with multiple tumors were excluded, such as those with macroscopic intrahepatic metastases adjacent to the primary tumor, or those with extrahepatic metastases. Liver samples from 10 adult patients treated surgically for hepatic injury or hemangioma were collected as controls.

Propensity-score matching based on early recurrence

Since clinicopathological characteristics related to tumor recurrence have been shown to cause significant baseline differences in cancer patients, which can bias subsequent analyses[13], we used propensity-score matching[14] to generate pairs in which one patient experienced recurrence within two years of hepatectomy and the other did not. These pairs were generated using one-to-one matching without replacement and a 0.2 caliper width[15].

Follow-up

All patients were followed one month after hepatectomy, then every three months during the first year after surgery, and every six months thereafter. During follow-up visits, patients were subjected to a physical examination, liver function tests, assay of serum AFP, abdominal ultrasonography, and computed tomography (CT) or magnetic resonance imaging (MRI) of the liver. Patients were diagnosed with recurrence when ultrasonography, dynamic CT or MRI detected a new hepatic lesion. The end-point for follow-up was defined as three years, and the survival time was defined as 36 mo for those who survived more than three years.

Immunohistochemistry of CSC markers

Surgical tissues were fixed in 10% formalin, embedded in paraffin, cut into 4-ìm sections, deparaffinized in xylene and rehydrated through graded alcohol solutions. Antigen retrieval was performed for 5 min at 100 ℃ in citrate buffer (10 mmol/L, pH 6.0) in a microwave oven. Endogenous peroxidases were blocked by immersing the sections in 3% hydrogen peroxide for 15 min. Sections were then incubated at 37 ℃ for one hour with a rabbit monoclonal antibody against human CD133 (1:100; Miltenyi, CA, United States), a mouse monoclonal antibody against human CD90 (1:100; Eptomics, CA,

Table 1 Baseline characteristics

Characteristic	ER group $n = 38$	NER group $n = 52$	*P* value
Age (yr)	48.4 ± 10.6	47.2 ± 12.9	0.637
BMI (kg/m^2)			
< 23	28	34	0.401
≥ 23	10	18	
Total bilirubin (mg/dL)	14.0 ± 6.3	13.3 ± 5.7	0.585
Albumin (g/L)	40.0 ± 3.9	41.0 ± 4.9	0.300
ALT (U/L)	38.1 ± 31.5	40.2 ± 39.7	0.767
AST (U/L)	51.9 ± 40.0	47.3 ± 46.1	0.850
Prothrombin time (s)	13.0 ± 2.1	12.6 ± 1.3	0.346
Platelet count (10^9/L)	150.2 ± 49.0	196.7 ± 83.5	0.003
AFP (ng/mL)			
< 400	24	37	0.423
≥ 400	14	15	
Tumor size (cm)	7.0 ± 3.0	6.0 ± 2.9	0.093
Liver cirrhosis			
Present	26	22	0.014
Absent	12	30	
Tumor capsule			
Present	23	32	0.038
Absent	15	20	
Edmondson grade			
Ⅰ or Ⅱ	17	28	0.393
Ⅲ or Ⅳ	21	24	

Baseline characteristics of hepatocellular carcinoma (HCC) patients who experienced recurrence (ER) within two years of hepatectomy (ER group) and HCC patients who did not (NER group). AFP: Alpha-fetoprotein; ALT: Alanine aminotransferase; AST: Aspartate aminotransferase; BMI: Body mass index.

United States), or a mouse monoclonal antibody against human EpCAM (1: 100; Eptomics). Sections were rinsed with phosphate-buffered saline (PBS), incubated with biotinylated anti-rabbit or anti-mouse immunoglobulin diluted in PBS for 20 min at room temperature, and rinsed again with PBS. Sections were incubated with anti-horseradish peroxidase conjugate for 10 min, rinsed in PBS, and incubated with diaminobenzidine for 10 min. Finally, sections were counterstained with hematoxylin. Negative controls were prepared in the same way except that they were incubated with PBS instead of primary antibodies.

Stained sections were examined by two experienced hepatopathologists (Ou C, Zeng LX) who were blinded to the clinicopathological data of the tissue samples. To assess CD133, CD90, and EpCAM expression, the numbers of cells positive for these markers were counted in five non-overlapping, randomly selected × 400 fields containing a total of at least 1000 cells. Expression levels in each patient or control were quantified as the percentage of the total number of cells in the fields that were positive for CD133, CD90, or EpCAM. For statistical analysis, patients were categorized as negative for these markers if the percentage of CD133, CD90, or EpCAM-positive cells was below 5%, or positive if the percentage was 5% or greater.

Statistical analysis

All statistical analyses were performed using SPSS 19.0 (IBM, United States). Results were reported as averages or relative risk (RR) ratios with 95% CIs. The chi-squared test was used to compare categorical data, while the *t* test was used to compare continuous data. Survival curves were constructed using the Kaplan-Meier method, and differences between curves were analyzed using the log-rank test. Multivariate Cox proportional hazard regression was used to assess the ability of variables to predict overall survival. All statistical tests were two-sided, with the threshold for significance defined as $P < 0.05$.

RESULTS

During the study period, 729 HCC patients were treated at our hospital and 215 (29.5%) were excluded because they had been treated initially for HCC at other centers. Among the remaining 514 patients, 157 (30.5%) had solitary nodular tumors without extrahepatic metastases or portal tumor thromboses. We excluded 55 of these (35.0%) because they had received only local ablation therapy, ethanol injection or transarterial chemoembolization, and we excluded another 12 (7.6%) because they did not participate in follow-up. The remaining 90 (57.3%) patients satisfied our inclusion criteria and were included in our study. Of these, 38 (42%) experienced intrahepatic recurrence within two years after curative hepatectomy; these patients were assigned to the early recurrence (ER) group. The remaining 52 patients did not experience recurrence within two years and were therefore assigned to the non-ER (NER) group. Table 1 summarizes the demographic and clinicopathological characteristics of both groups. To provide a comparison with HCC patients, we also collected liver tissue samples from 10 healthy adults who had undergone surgery for non-HCC problems.

The NER group showed a significantly higher frequency of tumor capsule and significantly elevated platelet count than did the ER group. However, the ER group showed a significantly higher frequency of liver cirrhosis. The two groups did not differ significantly in age, body mass index (BMI), serum bilirubin, albumin, alanine aminotransferase (ALT), aspartate aminotransferase (AST), prothrombin time, AFP, tumor size or Edmondson grade.

To reduce confounding due to covariates related to cancer recurrence, previously observed in studies of HCC[13,16], we used propensity-score matching to generate 25 pairs of ER and NER patients. No significant baseline differences were observed between these pairs (Table 2), which were then used in subsequent analysis.

Association of CD133, CD90, and EpCAM expression with HCC

Of the 50 tumor samples examined, 44 (88%) showed CD133 expression by immunohistochemistry, with each sample showing an average of 37.7% ± 26.0% of CD133-positive cells. A total of 42 samples (84%) were

Table 2 Baseline characteristics of experienced recurrence and non-experienced recurrence patients with hepatocellular carcinoma, after propensity-score matching

Characteristic	ER group *n* = 25	NER group *n* = 25	*P* value
Age (yr)	46.6 ± 8.9	45.7 ± 13.7	0.784
BMI (kg/m^2)			
< 23	18	16	0.544
≥ 23	7	9	
Total bilirubin (mg/dL)	13.2 ± 2.5	15.4 ± 6.4	0.989
Albumin (g/L)	39.3 ± 3.5	40.3 ± 4.7	0.371
ALT (U/L)	39.6 ± 16.9	34.6 ± 12.4	0.232
AST (U/L)	50.4 ± 35.0	47.8 ± 24.9	0.767
Prothrombin time (s)	13.2 ± 2.5	12.7 ± 1.6	0.466
Platelet count (10^9/L)	141.5 ± 42.0	157.4 ± 64.6	0.308
AFP (ng/mL)			
< 400	17	20	0.333
≥ 400	8	5	
Tumor size (cm)	6.9 ± 3.0	6.0 ± 2.9	0.164
Liver cirrhosis			
Present	18	18	1.000
Absent	7	7	
Tumor capsule			
Present	7	7	1.000
Absent	18	18	
Edmondson grade			
Ⅰ or Ⅱ	8	12	0.248
Ⅲ or Ⅳ	17	13	

AFP: Alpha-fetoprotein; ALT: Alanine aminotransferase; AST: Aspartate aminotransferase; BMI: Body mass index; ER: Early recurrence; NER: Non-ER.

categorized as CD133-positive because the percentage of CD133-positive cells was at least 5%. The protein was present mainly in the cytoplasm (Figure 1A). CD90 expression was observed in 47 samples (94%), with each sample showing an average of 5.5% ± 2.9% of CD90-positive cells. However, only 32 samples (64%) were categorized as CD90-positive. The protein was observed exclusively in the cytoplasm (Figure 1B). EpCAM expression was detected in 39 samples (78%), with each sample containing an average of 4.6% ± 3.8% of EpCAM-positive cells. Only 21 samples (42%) were categorized as EpCAM-positive. The protein was observed mostly on the membrane of tumor cells (Figure 1C).

In contrast to these results with CSC markers in tumor tissues, none of the 10 normal adult liver samples showed detectable expression of CD133, CD90, or EpCAM under the same antibody staining conditions used with the tumor samples (data not shown; $P < 0.001$ for all).

To explore whether expression of CD133, CD90, or EpCAM may correlate with HCC oncogenesis, we examined potential associations of CSC marker expression with the following dichotomized HCC clinicopathological variables: age, < 50 or ≥ 50 years; BMI, < 23 or ≥ 23 kg/m^2; serum bilirubin, ≤ 17.1 or > 17.1 g/L; albumin, < 35 or ≥ 35 g/L; ALT, < 2 times the upper normal limit or ≥ 2 times the upper normal limit; AST, < 2 times the upper normal limit or ≥ 2 times the upper normal limit; platelets, < 100 or ≥ 100 × 10^9/L; prothrombin time, < 14 or ≥ 14 s; AFP, < 400 or ≥ 400 ng/mL; tumor size, < 5 or ≥ 5 cm; tumor capsule, present or absent; cirrhosis, present or absent; and Edmondson grade, Ⅰ-Ⅱ or Ⅲ-Ⅳ.

Among all these variables, only the absence of tumor capsule showed a significant association with CD133 expression ($P = 0.005$; Table 3). CD90 expression was significantly more frequent in stage Ⅲ or Ⅳ tumors than in stage Ⅰ or Ⅱ tumors ($P = 0.010$), and it was more frequent in larger tumors ($P = 0.034$). EpCAM expression was significantly more frequent in patients with elevated serum AFP levels ($P = 0.021$).

Association of CD133, CD90, and EpCAM expression with early recurrence

Univariate analysis showed that early HCC recurrence was associated with expression of CD90 ($P = 0.001$) and EpCAM ($P = 0.045$; Table 4). However, multivariate analysis showed that only CD90 expression correlated significantly with early recurrence (RR = 9.333; 95%CI: 2.207-39.463, $P = 0.002$).

Association of CD133, CD90, and EpCAM expression with overall survival

The association of CD133, CD90, and EpCAM expression with overall survival was evaluated by calculating Kaplan-Meier survival curves separately for patients positive or negative for each CSC marker and then comparing the curves using the log-rank test (Table 4). The survival curve analysis was then verified using Cox regression. Survival rates at one, two, and three years were similar between CD133-negative patients (87.5%, 72.9%, 54.7%) and CD133-positive patients (85.7%, 71.2%, 68.6%, $P = 0.732$; Figure 2A). In contrast, the corresponding survival rates were significantly higher for patients negative for CD90 expression (100%, 94.1%, 88.2%) than for CD90-positive patients (78.8%, 60.2%, 56.4%, $P = 0.018$; Figure 2B). Similarly, survival rates were significantly higher for EpCAM-negative patients (86.2%, 86.2%, and 82.3%) than for EpCAM-positive ones (85.7%, 51.3%, 46.2%, $P = 0.010$; Figure 2C). Multivariate Cox regression showed that only EpCAM expression (RR = 4.857; 95%CI: 1.648-14.313, $P = 0.004$) was a significant predictor of survival time in patients with HCC.

DISCUSSION

In this study, we evaluated the relationship between expression of three putative CSC markers and the most clinically relevant features of HCC. Our findings suggest that CD133, CD90, and EpCAM expression correlates with the onset and/or progression of HCC, because they are expressed to a significantly greater extent in HCC tissue than in normal liver tissue. In addition, EpCAM expression is associated with shorter survival, and CD90 expression predicts early recurrence.

The biology of several human cancers, including HCC, is driven by self-renewal, unlimited prolifera-

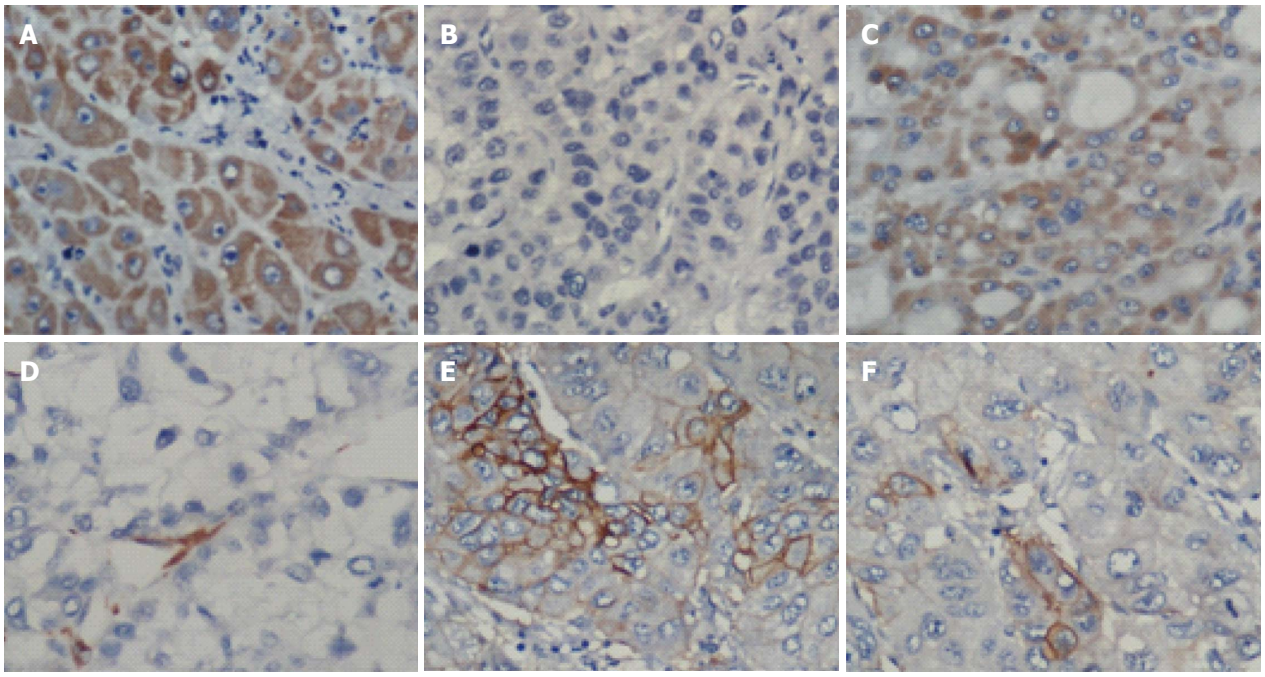

Figure 1 Representative images of hepatocellular carcinoma tissue. Representative images of hepatocellular carcinoma (HCC) tissue showing positive and negative cytoplasmic staining for CD133 (A, B) and CD90 (C, D), or positive and negative membrane staining for epithelial cell adhesion molecule (EpCAM) (E, F). Magnification, × 400.

Table 3 Association of CD133, CD90, and epithelial cell adhesion molecule expression with clinicopathological variables

Variable	*n*	CD133		*P* value	CD90		P value	EpCAM		*P* value
		Positive	Negative		Positive	Negative		Positive	Negative	
Tumor size (cm)										
< 5	22	20	2	0.439	11	11	0.034	11	11	0.310
⩾ 5	28	22	6		22	6		10	18	
Tumor capsule										
Present	14	8	6	0.005	8	6	0.623	8	6	0.176
Absent	36	34	2		25	11		13	23	
Edmondson grade										
Ⅰ or Ⅱ	20	17	3	0.875	9	11	0.010	7	13	0.413
Ⅲ or Ⅳ	30	25	5		24	6		14	16	
AFP (ng/mL)										
< 400	37	32	5	0.721	25	12	0.957	12	25	0.021
⩾ 400	13	10	3		8	5		9	4	

Association of CD133, CD90, and epithelial cell adhesion molecule (EpCAM) expression with dichotomized clinicopathological variables in HCC patients after propensity-score matching; AFP: Alpha-fetoprotein.

tion, and differentiation, all of which are stem cell-like properties[17]. In fact, CSCs have been proposed to initiate tumorigenesis and contribute to cancer resistance, metastasis, and recurrence[18]. Several surface markers, including CD133, CD90, and EpCAM, have been identified as putative markers of LCSCs associated with HCC, though these markers are also present on other types of CSCs.

CD133, known as a 5-transmembrane domain glycoprotein, is expressed in various types of tumors[19]. This marker has been used to identify and isolate CSCs in malignant cancers such as acute myeloid leukemia[20] and brain and colon cancers[21-23]. In addition, increased CD133 expression may be a prognostic marker in many human malignancies[24-26]. Suetsugu *et al*[27] first identified CD133 in HCC cells; those authors demonstrated that CD133-positive Huh-7 cells showed higher tumorigenic potential *in vivo* and greater proliferative ability *in vitro* than did CD133-negative cells. CD133 is now widely recognized as a CSC marker in HCC tissues. Our findings are consistent with this idea: in the 50 HCC samples that we examined, the average percentage of CD133-positive cells was 37.7% ± 26.0%. This percentage should be interpreted with caution, since not all CD133-positive cells correspond to LCSCs. Only a relatively small and well-defined subset of cells with enhanced ability to proliferate and form tumors should be considered CSCs[9]. Identifying CSCs may require analyzing multiple markers. Indeed, a survey of HCC cell lines found that cells co-expressing both CD133 and CD44 were more likely

Table 4 Association of CD133, CD90, and epithelial cell adhesion molecule expression with recurrence and survival

Marker	ER group *n* = 25	NER group *n* = 25	*P* value	Overall survival			*P* value
				1 yr	2 yr	3 yr	
CD133							
Positive	20	22	0.440	85.9%	71.2%	68.6%	0.732
Negative	5	3		87.5%	72.9%	54.7%	
CD90							
Positive	22	11	0.001	78.8%	60.2%	56.4%	0.018
Negative	3	14		100.0%	94.1%	88.2%	
EpCAM							
Positive	14	7	0.045	85.7%	51.3%	46.2%	0.010
Negative	11	18		86.2%	86.2%	82.3%	

Association of CD133, CD90, and epithelial cell adhesion molecule (EpCAM) expression with dichotomized clinicopathological variables in HCC patients after propensity-score matching; ER: Early recurrence; NER: Non-ER.

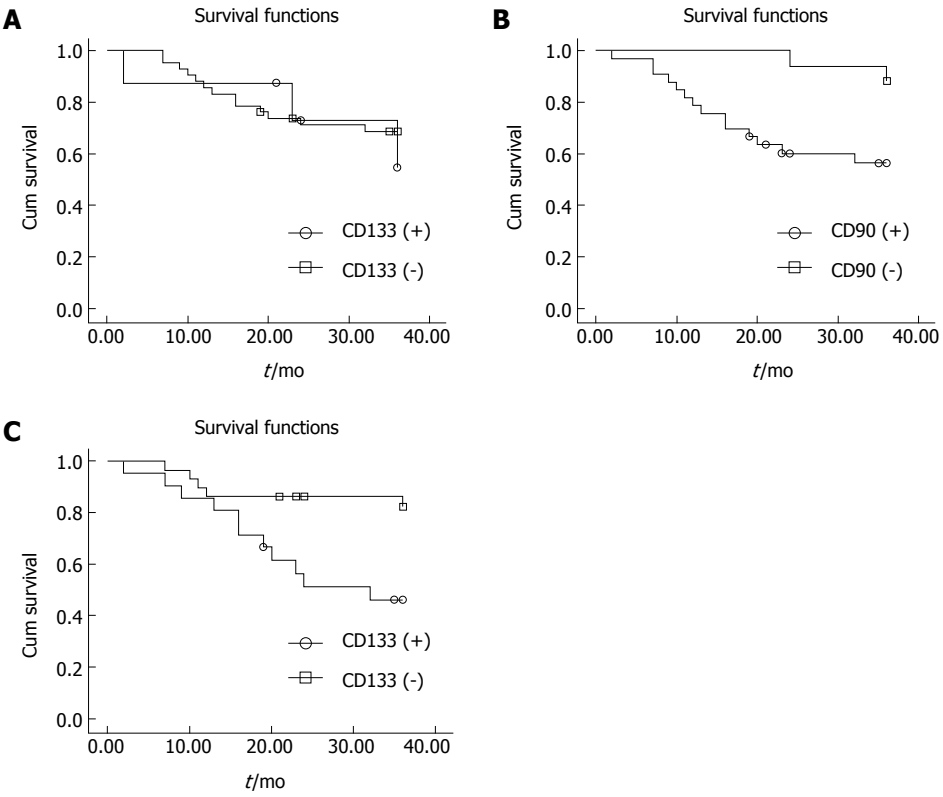

Figure 2 Overall survival curves. A: For patients whose hepatocellular carcinoma (HCC) tissue was positive or negative for CD133 expression. The two curves were not significantly different, based on the log-rank test ($P = 0.732$); B: For patients whose HCC tissue was positive or negative for CD90 expression. Survival times were significantly shorter for CD90-positive patients ($P = 0.018$); C: For patients whose HCC tissue was positive or negative for epithelial cell adhesion molecule (EpCAM) expression. Survival times were significantly shorter for EpCAM-positive patients ($P = 0.010$).

to be LCSCs than cells expressing CD133 alone[28].

In our study, CD133 expression was associated with the absence of tumor capsule. The tumor capsule acts as a barrier to prevent the spread of tumor cells[29], giving it an important role in tumor suppression. Our findings suggest that CD133 tends to be expressed in tumors showing stronger potential for invasion and metastasis.

CD90, a cell surface glycoprotein of 25-37 kDa, plays an important role in cell-cell and cell-matrix interactions[30]. The protein has been used as a surface marker of many types of stem cells[31,32]. CD90-positive cells isolated from HCC cell lines, human HCC specimens, and blood samples are tumorigenic in a mouse xenograft model, suggesting that CD90 is also an LCSC marker[33]. Here we report that CD90 expression is not only significantly higher in HCC tissues than in normal adult liver tissue, but also correlates with higher histopathologic grade and larger tumors. These findings are similar to a former study[34] which found higher expression of CD90 in poorly differentiated HCC than in well-differentiated ones with staining intensity correlating to degree of differentiation. These results suggest that CD90 is involved in the onset and/or progression of HCC.

EpCAM is another cell surface glycoprotein, and it functions as a homophilic, epithelial-specific intercellular adhesion molecule[35,36]. More recent work has shown

that the protein also contributes to cell signaling, proliferation, differentiation, and migration[37,38]. Yamashita *et al*[39] were the first to characterize EpCAM in HCC cell lines and tumor specimens; they demonstrated that EpCAM-positive HCC cells possess LCSC-like abilities to self-renew and differentiate. In the present study, we show that EpCAM expression is significantly higher in HCC tissues than in normal adult liver tissues, and that it correlates with elevated serum AFP levels. Since AFP level correlates with the degree of HCC malignancy[40], these findings indicate that EpCAM tends to be expressed in more malignant HCC tissues. Therefore EpCAM may well play a role in HCC progression.

Postoperative recurrence is the main cause of death for HCC patients in the long term[7,8], and most recurrences occur within two years after resection[16]. Evidence suggests that CSCs may drive postoperative recurrence and metastasis[41]. First, CSCs detach from the primary mass and enter the lymph and peripheral blood. Then they sense a chemoattractant gradient that directs them to a particular point, where they attach to the endothelium and penetrate the microvessel wall. Outside the vasculature, CSCs find an environmental niche that protects them from damage and allows them to establish a recurrent or metastatic tumor.

Based on the literature, we defined two years as a cut-off for early recurrence, and we divided our patients into two groups: those who experienced early recurrence (n = 38) and those who did not (n = 52). Since the two groups showed several significant differences at baseline (Table 1), as has been observed to be related to tumor recurrence in other studies of HCC[13,16], we generated balanced pairs of ER and NER patients using propensity-score matching. Analysis of these 25 pairs showed that CD90 and EpCAM were expressed to a significantly greater degree in ER patients than in NER patients; CD133 expression, however, was similar between the two groups. Multivariate analysis showed that of the three putative LCSC markers, only expression of CD90 was significantly associated with early recurrence. This finding is consistent with previous work linking CD90 up-regulation to HCC tumor invasion and metastasis[34]. In that study, CD90-positive cells were found to be more likely than CD90-negative cells to detach from the primary tumor and establish recurrent tumors in appropriate environmental niches in the residual liver.

In the present study, we also evaluated the ability of CD133, CD90, and EpCAM to predict survival time in HCC patients. Univariate analysis showed expression of both CD90 and EpCAM to be associated with shorter survival time, while multivariate analysis showed only EpCAM expression to predict shorter survival time. Expression of EpCAM has also been associated with shorter survival time in cancers of the breast[42], renal clear cells[43], ovaries[44], and gallbladder[45]. The poor prognosis associated with EpCAM expression may indicate that EpCAM-positive cells possess the CSC properties of self-renewal, unlimited proliferation and differentiation.

The present study has several limitations, including small cohort size from a single site, short follow-up, and an observational design. Future studies should verify the insights from the present work and extend them by defining the optimal mixture of surface markers (including CD133) for identifying and isolating LCSCs. Future studies should also explore how CD133, CD90, and EpCAM - and potentially other CSC markers - influence postoperative recurrence and prognosis in patients with HCC.

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COMMENTS

Background

The cancer stem cell (CSC) hypothesis stipulates that primary tumors are initiated and maintained by a small population of cancer cells with "stem cell-like" characteristics. And it also states that tumor recurrence and metastasis after surgical resection are driven by CSCs. Recently, numerous cell surface markers, such as CD133, CD90, and epithelial cell adhesion molecule (EpCAM), have been identified as CSC markers in hepatocellular carcinoma (HCC). However, the association of CSC markers with early HCC recurrence and survival time is still unclear.

Research frontiers

Liver cancer stem cells (LCSCs) have been identified by numerous surface markers, including CD133, CD90 and EpCAM. However, definite LCSC markers are still controversial, because none of these markers are exclusively expressed by LCSCs in HCC. In addition, the research hotspot is to develop specific therapies targeting LCSCs.

Innovations and breakthroughs

To date, many clinical researches have attempted to investigate whether the existence of LCSCs is associated with clinical outcomes in HCC. However, the clinical relevance of LCSCs remains a major challenge for current anti-cancer therapy. In this study, authors aimed to analyze the expression of CD133, CD90 and EpCAM in patients and to search for association with early recurrence and survival time. To reduce the bias in patient selection, propensity-score matching was used to generate pairs of early recurrence (ER) and non-ER patients. The data indicated that EpCAM and CD90 are associated with shorter survival time and early HCC recurrence, respectively.

Applications

The study results suggested that the expression of the three LCSC markers CD133, CD90 and EpCAM is linked to HCC tumor onset and/or progression. In addition, EpCAM is associated with shorter survival time, while CD90 is associated with early recurrence.

Peer review

This is an interesting study in which the authors investigated the association of three CSC markers CD133, CD90 and EpCAM with the early HCC recurrence and survival time in patients with HCC. The results suggested that the EpCAM expression is associated with shorter survival time, while the expression of CD90 is associated with early recurrence.

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BRIEF ARTICLE

3.0T 31P MR spectroscopy in assessment of response to antiviral therapy for chronic hepatitis C

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Abstract

AIM: To investigate the utility of phosphorus-31 (31P) magnetic resonance spectroscopy (MRS) as a noninvasive test for assessment of response to interferon and ribavirin treatment in patients with different severities of hepatitis C virus infection.

METHODS: Sixty chronic hepatitis C patients undergoing antiviral therapy with interferon and ribavirin underwent 31P MRS at 3.0T before treatment, 6 mo after the start of treatment, and 1 year after the start of treatment.

RESULTS: The phosphomonoester (PME)/phosphodiester (PDE) ratio at 6 mo after the start of antiviral therapy in the Child-Pugh B and C groups were significantly higher than those before therapy, but this was not seen in the Child-Pugh A group. In the antiviral therapy group, the PME/PDE ratios had decreased on follow-up MR spectroscopy. However, in the virological nonresponder group, the PME/PDE ratios on follow-up imaging were similar to the baseline values.

CONCLUSION: 31P MRS can be used to provide biochemical information on hepatic metabolic processes. This study indicates that the PME/PDE ratio can be used as an indicator of response to antiviral treatment in chronic hepatitis C patients.

Key words: 31P; Magnetic resonance spectroscopy; Hepatitis C; Antiviral therapy

Core tip: This study assessed the value of 3.0T 31P magnetic resonance spectroscopy, a noninvasive technique, in testing response to antiviral therapy for chronic hepatitis C. The technique can provide biochemical information on hepatic metabolic processes. The phosphomonoester/phosphodiester ratio can be used as an indicator of response to antiviral treatment in chronic hepatitis C patients.

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INTRODUCTION

Hepatitis C virus (HCV) is one of the leading causes of liver disease worldwide. It is estimated that approximately 3% of the global population is infected with HCV. Many of the cases develop into chronic liver disease, cirrhosis,

or even hepatocellular carcinoma[1]. Liver biopsy remains the gold standard for providing the stage (extent of fibrosis) and grade (degree of NI activity) of HCV-related liver disease, but this invasive procedure is not without risk[1]. There is a low mortality rate but a high error rate, predominantly owing to undersampling, whereby typically, less than 1/50000 of the liver volume is obtained for histological evaluation[2-5]. These factors highlight the need for a noninvasive test to characterise diffuse liver disease.

For ethical reasons and because most patients are unwilling to undergo repeated procedures, treatment algorithms rarely allow serial liver biopsy. Thus, the impetus to find a reliable and repeatable biomarker of disease activity and response to treatment has a renewed focus[6].

Clinical (*in vivo*) phosphorus-31 magnetic resonance spectroscopy (31P MRS) is the only noninvasive technique that can be used to provide direct localised biochemical information on hepatic metabolic processes. A typical 31P MR spectrum of the human liver *in vivo* contains resonances that can be assigned to phosphomonoesters (PMEs), containing information from sugar phosphates in the glycolytic pathway and from cell membrane precursors such as phosphoethanolamine and phosphocholine; and to phosphodiesters[7], containing information from the endoplasmic reticulum and from cell membrane degradation products such as glycerophosphorylcholine and glycerophosphorylethanolamine, in addition to signals from inorganic phosphate and nucleotide triphosphates, including adenosine triphosphate. Many studies have reported a good correlation between elevated PME resonance and decreased phosphodiester (PDE) resonance in cirrhosis[8-10]. The ratio of PME to PDE has traditionally been viewed as an index of cell membrane turnover and thus provides an indirect measure of grading of liver histology[9].

The aim of the current study was to investigate the utility of 31P MRS as a noninvasive test for assessment of response to interferon and ribavirin treatment in patients with different severities of HCV.

MATERIALS AND METHODS

Patients

From January 2010 to June 2010, 120 patients with chronic hepatitis C were enrolled. The diagnosis of decompensated HCV-induced cirrhosis was based on the American Association for the Study of Liver Diseases Clinical Guideline for Hepatitis C (2004).

All enrolled patients were naive to antiviral treatments. Other inclusion criteria were: (1) HCV RNA >500 copies/mL; (2) absence of complications such as gastrointestinal bleeding, hepatic encephalopathy, and primary liver cancer; and (3) liver function defined as Child-Pugh grade B or C based on serum bilirubin, serum albumin, presence of ascites, presence of hepatic encephalopathy, and prothrombin time. Patients with hypersplenism were also enrolled. Exclusion criteria were: (1) infection with hepatitis A, B, D, or F virus, Epstein-Barr virus, cytomegalovirus, or human immunodeficiency virus; and (2) presence of alcoholic or drug-induced liver diseases, or severe heart, brain, or kidney disease.

A total of 120 patients meeting the inclusion criteria were enrolled. Patients were considered as part of the treatment group (n = 90) or control group (n = 30), based on whether they opted to receive antiviral therapy. The study was approved by the Institutional Review Board of the hospital, and informed consent was obtained from all study participants.

Clinical evaluation

Determination of therapeutic efficacy: The primary endpoints were: (1) SVR, defined as HCV RNA undetectable or < 500 copies/mL for at least 24 wk after treatment discontinuation[11]; and (2) relapse, defined as HCV RNA undetectable or < 500 copies/mL during antiviral therapy, but becomes detectable at 24 wk after treatment discontinuation. The secondary endpoints were disease progression (defined as an increase of 2 or more in the Child-Pugh score), presence of primary hepatocellular carcinoma, renal dysfunction, spontaneous bacterial peritonitis, variceal bleeding, or death due to liver disease[12].

Measures: Patients in the treatment group were evaluated for serum HCV antibodies, liver function, HCV RNA, coagulation function, thyroid function, and alpha foetoprotein as well as liver computed tomography. Routine blood and urine tests were performed before the start of the study. Routine blood and liver function tests were performed weekly in the first month, then once every 4 wk during the study period and once every 8 wk for 24 wk after discontinuation of treatment. Quantitative detection of HCV RNA was done immediately prior to treatment (baseline), at 24 and 48 wk after treatment, and 6 mo after discontinuation of treatment. HCV RNA levels were quantitated by real-time polymerase chain reaction using a kit from the Roche company.

Patients in the control group were evaluated for liver function and HCV RNA levels. Routine blood tests and colour ultrasonography of the liver were done every 12 wk. All patients were assessed for disease progression.

Treatment regimen and follow-up: All participants received symptomatic and supportive treatment, including treatment for reducing levels of transaminase and bilirubin and supplemental albumin. For patients in the treatment group, those who had a neutrophil count $\geqslant 1.0 \times 10^9$/L, platelet count $\geqslant 50 \times 10^9$/L, and haemoglobin > 10 g/L were treated additionally with both pegylated interferon α 2a (Peg-IFNα-2a) and ribavirin (RBV). The initial dose of Peg-IFNα-2a was 180 μg/kg subcutaneously. Peg-IFNα-2a dosage was reduced to 90 μg/kg once weekly when neutrophil or platelet counts decreased to $\leqslant 0.75 \times 10^9$/L or $< 50 \times 10^9$/L, respectively. The dose was returned to 180 μg/kg if neutrophil and platelet counts increased to $> 0.75 \times 10^9$/L and $\geqslant 50 \times 10^9$/L,

Table 1 Patient demographics and baseline characteristics *n* (%)

	Treatment (*n* = 90)	Control (*n* = 30)	*P*-value
Age (yr)	52.7 ± 10.1	58.3 ± 12.5	< 0.001[1]
Gender			
Male	36 (40.0)	14 (46.7)	0.573
Female	54 (60.0)	16 (53.3)	
Baseline HCV RNA level (log10 copies/mL)	5.30 ± 1.18	5.23 ± 1.15	0.681
Baseline MELD score	12.6 (9.8, 15.2)	12.5 (9.4, 15.8)	0.654
Baseline Child-Pugh score	9.0 (7.0, 10.0)	8.0 (7.0, 10.0)	0.809
Total bilirubin (mg/dL)			
< 2	9 (10.0)	5 (16.67)	0.691
2-3	40 (44.4)	12 (40.0)	
> 3	41 (45.6)	13 (43.33)	
Serum albumin (g/dL)			
> 3.5	9 (10.0)	3 (10.0)	0.005[1]
2.8-3.5	40 (44.4)	19 (63.3)	
< 2.8	41 (45.6)	8 (26.7)	
Prothrombin time INR			
< 1.7	26 (28.9)	8 (26.7)	0.029[1]
1.7-2.3	50 (55.6)	13 (43.3)	
> 2.3	14 (15.5)	9 (30.0)	
Hepatic encephalopathy			
None	90 (100.0)	30 (100.0)	NA
Ascites			
Absent	90 (100.0)	26 (87.4)	< 0.001[1]
Easily controlled	0 (0.0)	4 (13.3)	

[1]Indicates a significant difference between two groups. Age and baseline HCV RNA levels were normally distributed and are presented as mean and standard deviation. Baseline Child-Pugh scores were non-normally distributed and are presented as median and inter-quartile range (IQR). Other category variables are presented as number and percentage. HCV: Hepatitis C virus; MELD: Model for end-stage liver disease; INR: International normalised ratio.

respectively, after 2 wk. Treatment was discontinued if neutrophil count was $\leqslant 0.5 \times 10^9$/L or platelet count was $< 30 \times 10^9$/L. Patients tolerating the standard Peg-IFNα-2a dose of 180 μg/kg weekly were treated for 48 weeks. Patients who could not tolerate the standard dose were treated with the reduced dose of 90 μg/kg once weekly for up to 72 wk.

Patients with haemoglobin >100 g/L were initially treated with a standard dose of RBV (genotype 1: 1200 mg/d for patients with body weight > 75 kg and 1000 mg/d for patients with body weight ⩽ 75 kg; non-genotype 1: 1000 mg/d for patients with body weight >75 kg and 800 mg/d for patients with body weight ⩽ 75 kg). RBV dosage was reduced when haemoglobin levels decreased to ⩽ 100g/L after the dosage increase. RBV treatment was discontinued when haemoglobin levels were ⩽ 80 g/L. Patients tolerating the standard dose of RBV were treated for 48 wk. Patients developing cytopaenia during the treatment period were treated with cell growth-stimulating factor and/or erythropoietin. All patients were followed for 3 years.

31P MRS

A 3.0T MRI unit (Philips Medical Systems) was used[6]. All imaging was conducted after an overnight fast. An enveloping transmitter coil and a separate surface receiver coil were used. Both coils were double-tuned for protons at 64 MHz and phosphorus at 26 MHz. The proton signal was used to obtain a T1-weighted image (TR/TE, 800/16) in the axial plane to confirm patient positioning. The 31P MR spectra were localised to a centrally placed voxel within the liver by use of an image-selected *in vivo* spectroscopy sequence (voxel size, 70 mm × 70 mm × 70 mm; TR, 10000; number of signals averaged, 48). A voxel location within the right liver away from major vessels was used for each patient and was consistent for all baseline and follow-up images. The total examination time was 40 min with a 10-min acquisition time for the 31P MRS sequences. All patients underwent baseline 31P MRS before the start of antiviral treatment, and all underwent follow-up imaging 6 mo after the start of treatment.

Quantitation

Quantitation of the 31P signals was performed in the time domain with the advanced method for accurate, robust, and efficient spectral fitting (AMARES) algorithm included in the Magnetic Resonance User Interface (MRUI) software program (www.mrui.uab.es/mrui). Anonymity was assured and MR spectra were analysed by one blinded observer. The spectra were rechecked by another blinded observer. Peak areas for PME, PDE, inorganic phosphate, and the three nucleoside triphosphate moieties (γ, α, and β) were obtained with respect to the total phosphorus signal intensity. Because of previous findings highlighting the utility of the PME/PDE ratio, this index was used for further statistical analysis. Data from a bank of 15 age-matched healthy volunteers without a history of liver disease were used for comparison.

Statistical analysis

Age and baseline HCV RNA levels were normally distributed and presented as mean and standard deviation. Differences in age and baseline HCV RNA levels between the two groups were tested by the independent two-sample *t*-test. Child-Pugh scores were non-normally distributed and are presented as median and inter-quartile range. Differences in Child-Pugh scores between the two groups were tested by the non-parametric Mann-Whitney test. Other categorical variables are presented as number and percentage, and categorical variables were compared using the Fisher's exact test. Statistically significant variables from the univariate analyses were used in the multivariate analysis. All statistical tests were two-sided, and a *P*-value < 0.05 was considered statistically significant. All statistical analyses were performed using the SPSS 19.0 software (SPSS Inc, Chicago, IL, United States).

RESULTS

Patient demographics and baseline characteristics

As shown in Table 1, 120 patients who met the inclusion criteria were enrolled. Among them, 90 patients

Table 2 Changes in hepatic phosphomonoester to phosphodiester ratio before antiviral therapy and 6 mo after the start of antiviral therapy

	Child A	Child B	Child C
Before therapy	0.20 ± 0.17	0.27 ± 0.24	0.39 ± 0.18
Six mo after the start of therapy	0.16 ± 0.09	0.19 ± 0.12	0.22 ± 0.16
P	> 0.05	< 0.05	< 0.05

had sufficient blood cell counts for antiviral therapy. The remaining 30 patients, who refused antiviral therapy, were placed in the control group. Patients in the treatment group were significantly younger than those in the control group (mean age 52.7 *vs* 58.3 years, respectively, $P < 0.001$). There were no significant differences between the two groups in baseline HCV RNA levels. In addition, baseline MELD scores were not significantly different between the treatment and control groups (Table 1). Although baseline Child-Pugh scores, total bilirubin, and hepatic encephalopathy were not different between the two groups, significant differences in serum albumin, international normalised ratio (INR) for prothrombin time, and ascites were observed between the treatment and control groups ($P = 0.002$, $P = 0.018$, and $P < 0.001$, respectively).

Comparison of the PME/PDE ratio between before and after antiviral therapy

The PME/PDE ratios at 6 mo after the start of antiviral therapy in the Child B and C groups were significantly higher than those before therapy, but this was not seen in Child-Pugh A group (Table 2).

Changes in hepatic PME/PDE ratio in virological responders and nonresponders after antiviral treatment

Sixty-nine patients responded to antiviral treatment with a sustained viral response. In 54 of these patients, the PME/PDE ratio had decreased toward normal on follow-up MRS. Figure 1 is the graph of a responder whose spectra changed after treatment, showing a decrease in PME/PDE ratio. Fifteen of the 21 virological nonresponders had PME/PDE ratios on follow-up imaging similar to the baseline values. Another two nonresponders had an increase in the PME/PDE ratio on follow-up imaging (Table 3). An unchanged PME/PDE ratio was defined as a difference of not more than 0.03 in comparison with the baseline ratio. An increase was defined as a > 0.03 increase in PME/PDE ratio in comparison with the baseline value. A decrease in PME/PDE ratio was defined as a > 0.03 reduction in the ratio compared with the baseline value.

DISCUSSION

It is estimated that approximately 3% of the global population has chronic infection with the HCV and that approximately 4 million persons are newly infected each year[13]. In 55%-85% of patients, the infection develops into chronic liver disease, which in many cases remains asymptomatic. In approximately 20% of cases, fibrosis develops into cirrhosis, which leads to hepatocellular cancer in 5% of cases each year[14]. Liver biopsy is the reference standard for staging and grading chronic liver disease, but this invasive procedure is not without risk. There is a low mortality rate but a high error rate, predominantly owing to undersampling, whereby typically, less than 1/50000 of the liver volume is obtained for histological evaluation[2,15]. As a result of the problems associated with biopsy, a steady drive to find an effective noninvasive method for evaluating liver damage has led to developments both in testing with serological biomarkers of disease and in imaging. For ethical reasons and because most patients are unwilling to undergo repeated procedures, treatment algorithms in the United Kingdom rarely allow serial liver biopsy. Thus, the impetus to find a reliable and repeatable biomarker of disease activity and response to treatment has a renewed focus[6].

One particular noninvasive technique for characterising chronic liver disease is 31P MRS. Clinical (*in vivo*) 31P MRS is a noninvasive technique that can be used to provide direct localised biochemical information on hepatic metabolic processes. At present, many reports suggest that there is a clear correlation between 31P MR spectral classification and liver disease jurisprudence[6]. How-

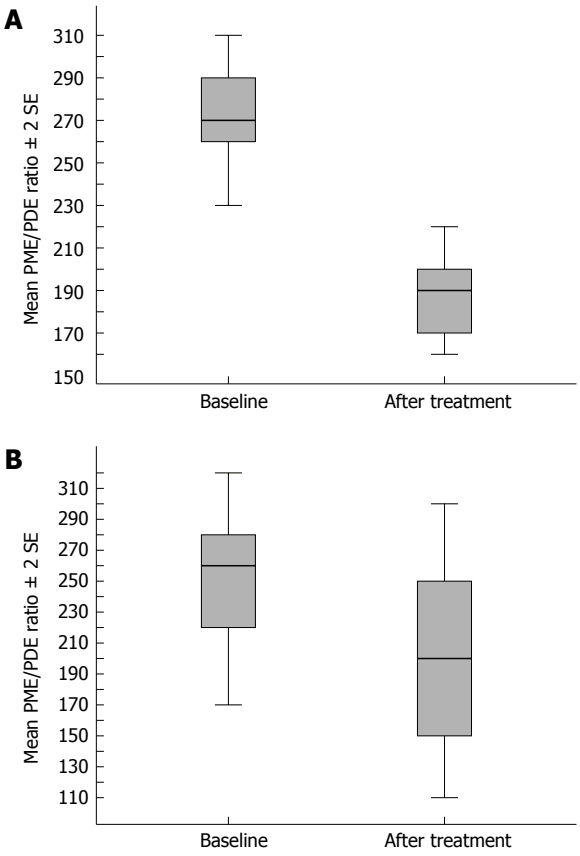

Figure 1 Change in phosphomonoester to phosphodiester ratio between baseline and after treatment in the responder group (A), and the nonresponder group (B). PME/PDE: Phosphomonoester/phosphodiester.

Table 3 Changes in hepatic phosphomonoester to phosphodiester ratio in virological responders and nonresponders after antiviral treatment *n* (%)

Patient group	PME/PDE decreased	PME/PDE unchanged	PME/PDE increased
Responders (n = 69)	54 (78)	9 (13)	6 (9)
Nonresponders (n = 21)	2 (10)	4 (20)	15 (70)
P	> 0.05	< 0.05	< 0.05

PME/PDE: Phosphomonoester to phosphodiester.

ever, because of the sensitivity and specificity, especially for chronic hepatitis C patients, it is needed to monitor changes of liver histology after antiviral treatment.

A typical 31P MR spectrum of the human liver *in vivo* contains resonances that can be assigned to PMEs, containing information from sugar phosphates in the glycolytic pathway and from cell membrane precursors such as phosphoethanolamine and phosphocholine; and to PDEs[7], containing information from the endoplasmic reticulum and from cell membrane degradation products such as glycerophosphorylcholine and glycerophosphorylethanolamine. In addition, in patients with precirrhotic liver disease, 31P MRS can be used in grading disease severity and compared with histology from liver biopsy. Research reports that 31P MRS PME is elevated in patients with cirrhosis and PDE is reduced. Thus, the PME/PDE ratio can be used as an indirect sign of liver disease at the metabolic level[16]. Some studies suggested that PME/PDE ratio increased with increasing severity of chronic liver disease and that this ratio was highly sensitive for the presence of cirrhosis[17]. With noninvasive imaging, we used the PME/PDE ratio to assess the severity of precirrhotic HCV-related liver disease[14].

In this study, the PME/PDE ratio was significantly decreased in the sustained virological responder group. This ratio remained the same or was increased in patients who were virological nonresponders (Figure 1). PME resonance contains contributions from cell membrane precursors and PDE resonance contains contributions from cell membrane degradation products[18,19]. The PME/PDE ratio thus gives information on cell turnover within the liver[20]. It has been shown that this ratio is reduced after effective viral eradication treatment[21,22]. It is also of interest that cirrhosis patients of the responders group also had a reduction in the PME/PDE ratio. Study findings of a good correlation between the PME/PDE ratio and degree of liver fibrosis[6] suggest that liver fibrosis can regress in patients with cirrhosis. The number of patients in our sample was too small for an absolute conclusion, but the findings fuel this controversial area. Overall, the results show that 31P MRS can be used as a completely noninvasive imaging indicator of response to treatment in a population of patients who may be undergoing imaging anyway, that is, patients with established cirrhosis undergoing screening for the development of hepatocellular carcinoma.

31P PME/PDE ratio is not 100% sensitive or specific. In our study, some patients who did not have a sustained response had a reduction in the PME/PDE ratio. Similarly, some patients in the sustained virological responder group had a worsening PME/PDE ratio but were subsequently found to be clear of the virus in longer-term virological follow-up studies. The PME/PDE ratios we obtained were at baseline and 6 mo after the start of antiviral therapy, but most patients should continue antiviral therapy for over 1 year, so repeating examination with 31P PME/PDE may bring higher sensitivity or specificity. On the other hand, the PME/PDE ratio could provide biochemical information on hepatic metabolic processes, which could indicate resolution of fibrosis.

This study indicates that the PME/PDE ratio can be used as an indicator of response to treatment. Most modern MR systems have the capability for MRS. 31P MRS is a noninvasive technique that can be used to provide direct localised biochemical information on hepatic metabolic processes. It is a useful technique for chronic hepatitis C patients on antiviral therapy.

COMMENTS

Background

Hepatitis C virus (HCV) is one of the leading causes of liver disease worldwide. Liver biopsy remains the gold standard for providing the stage (extent of fibrosis) and grade (degree of NI activity) of HCV-related liver disease, but this invasive procedure is not without risk. Thus, the impetus to find a reliable and repeatable biomarker of disease activity and response to treatment has a renewed focus.

Research frontiers

Clinical (*in vivo*) phosphorus-31 magnetic resonance spectroscopy (31P MRS) is the only noninvasive technique that can be used to provide direct localised biochemical information on hepatic metabolic processes.

Innovations and breakthroughs

This study was the first attempt to use 3.0T 31P MRS in assessment of response to antiviral therapy for chronic hepatitis C. It assessed the value of 3.0T 31P MRS, a noninvasive technique, in testing response to antiviral therapy for chronic hepatitis C. The technique could provide biochemical information on hepatic metabolic processes. The phosphomonoester (PME)/phosphodiester (PDE) ratio can be used as an indicator of response to antiviral treatment in chronic hepatitis C patients.

Applications

This study suggests that 31P MRS could provide biochemical information on hepatic metabolic processes. The PME/PDE ratio can be used as an indicator of response to antiviral treatment in chronic hepatitis C patients.

Terminology

Clinical (*in vivo*) 31P MRS is the only noninvasive technique that can be used to provide direct localised biochemical information on hepatic metabolic processes. A typical 31PMR spectrum of the human liver *in vivo* contains resonances that can be assigned to PMEs, containing information from sugar phosphates in the glycolytic pathway and from cell membrane precursors such as phosphoethanolamine and phosphocholine; and to PDEs, containing information from the endoplasmic reticulum and from cell membrane degradation products such as glycerophosphorylcholine and glycerophosphorylethanolamine, in addition to signals from inorganic phosphate and nucleotide triphosphates, including adenosine triphosphate. Many studies have reported a good correlation between elevated PME resonance and decreased PDE resonance in cirrhosis. The ratio of PME to PDE has traditionally been viewed as an index of cell membrane turnover and thus provides an indirect measure of grading of liver histology.

Peer review

This is a good descriptive study in which authors attempt to use 3.0T 31P MRS in assessment of response to antiviral therapy for chronic hepatitis C. 3.0T 31P

MRS represents a new noninvasive technique that provides biochemical information on hepatic metabolic processes and response to antiviral therapy for chronic hepatitis C.

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CASE REPORT

Pleomorphic hepatocellular carcinoma following consumption of hypericum perforatum in alcoholic cirrhosis

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Abstract

Hepatocellular carcinoma (HCC) often develops in patients with underlying liver disease, yet HCC with syncytial giant cells (SGCs) is extremely rare. Herein, we report a 55-year-old man with a 6-year history of alcoholic cirrhosis who during his regular checkup presented with marked elevation of alpha-fetoprotein. Clinical examination and imaging analyses revealed a tumor-like lesion in segment 4 of the liver, which was removed by limited wedge resection. Histological analysis by hematoxylin and eosin staining indicated pleomorphic and atypical nodules, with some SGCs, embedded within the boundaries of the neoplastic lesion. The adjacent liver parenchyma showed microvesicular steatosis, pericellular fibrosis, and moderate hemosiderin accumulation (grade 2, as determined by Prussian blue iron stain) in hepatocytes and Kupffer cells but no copper accumulation (as determined by orcein stain). Immunohistochemical analysis showed hepatocyte antigen-positive staining for the neoplastic cells and SGCs. The diagnosis was made for cirrhosis-related HCC with SGCs. The previous reports of pleomorphic HCC have featured osteoclast-like (*i.e.*, mesenchymal type) giant cells, making this case of epithelial type giant cells very rare. The patient's 6-month history of hypericum perforatum/St John's wort self-medication may have prompted the cirrhosis or HCC progression or the unusual SGC manifestation.

Key words: Hepatocellular carcinoma; Giant cell carcinoma; Hypericum perforatum; St John's wort; Syncytial giant cell; Pleomorphic liver tumor; Alcoholic cirrhosis

Core tip: This case report describes the clinicopathological-based finding of an unusual hepatocellular carcinoma (HCC) with syncytial giant cells (SGCs) in a 55-year-old man with a 6-year history of alcoholic cirrhosis. Unlike the previous case reports of these rare tumor types that have demonstrated the mesenchymal and non-neoplastic nature of the giant cells, the current case showed an epithelial and hepatocyte-originated neoplasmic nature. An intriguing feature of case is the patient's 6-month history of hypericum perforatum/St John's wort self-medication, which may have prompted the cirrhosis or HCC progression or the unusual SGC manifestation.

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INTRODUCTION

Hepatocellular carcinoma (HCC) often develops in patients with underlying liver disease, yet the HCC type with syncytial giant cells (SGC) is extremely rare. Multinucleated giant cells have been reported in a variety of neoplasms from many organs, including the liver, but these cells have featured an osteoclast phenotype[1-5]. The cellular origin of these osteoclast-like giant cells remains controversial, and various studies have provided evidence suggesting mesenchymal derivation as well as epithelial derivation[4-6].

Herein, we report the clinicopathological-based finding of cirrhosis-related HCC with SGC in an adult male with a long-term history of alcoholism and a short-term history of hypericum perforatum [commonly known as St John's wort (SJW)]. Immunohistochemical evidence indicated that the SGCs are of epithelial and not mesenchymal origin. Moreover, the patient's ingestion of SJW raises the intriguing possibility of its involvment with this unusual manifestation.

CASE REPORT

A 55-year-old man with a 6-year history of alcoholic cirrhosis diagnosis presented with marked elevation of alpha-fetoprotein (a-FP; 14000 ng/mL, reference range: 0-40 ng/mL) during his regular checkup. Magnetic resonance imaging revealed a tumor-like lesion on segment 4 of the liver (Figure 1). The patient was admitted to the Department of Surgery for treatment on 1 April 2008. During the initial interview, the patient self-described a long-term history of large-quantity daily alcohol intake (10 L of wine/d for 10 years) and a short-term history of unknown quantity daily intake of SJW (for the last few months). Hepatomegaly was found upon physical examination, but further serologic analyses revealed normal levels of ferritin (120 g/L, reference range: 24-248 g/L) and ceruloplasmin (32 g/L, reference range: 20-50 g/L) and negative results for autoimmune markers and hepatitis B- and C-specific antigens.

Fine needle aspiration of the tumor performed on 2 April 2008 revealed normal and atypical hepatocytes, nodular regenerative atypia, and transitional and differentiated bile duct epithelial cells. In addition, three distinct hepatocyte aggregations were observed, which were characterized by pleomorphism, non-uniform nuclear size, increased nucleocytoplasmic ratio, diffuse chromatin distribution, and large, prominent nucleoli. While the presence of these aggregates suggested a neoplastic character for the tumor-like lesion, atypia could not be excluded due to the presence of the regenerative nodules and cirrhotic features.

The patient underwent segment 4 resection on 4 April 2008. The procedure was modified perioperatively to a limited wedge resection with ethanol injection of the cut liver surface according to the observations of substantial ischemia in the left liver lobe, gross pathological features of cirrhosis in the right lobe, and presence of three small (about 2 mm each) satellite nodules. The resected specimen measured 3 cm × 2.5 cm × 1.5 cm. Sectioning revealed multiple white nodules throughout the tumor tissue, which itself was surrounded by normal liver tissue with a micronodular cirrhotic appearance.

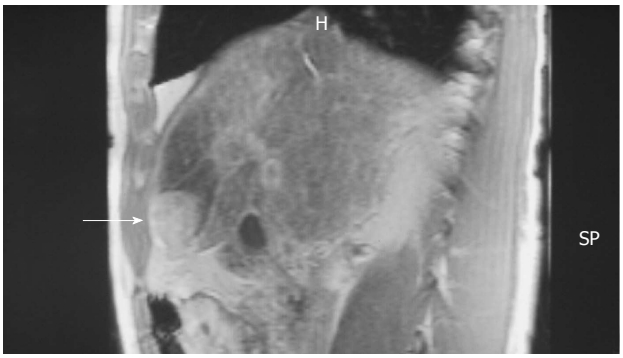

Figure 1 Magnetic resonance imaging showed a tumor-like lesion (arrow) and ischemia in segment 4 of the liver.

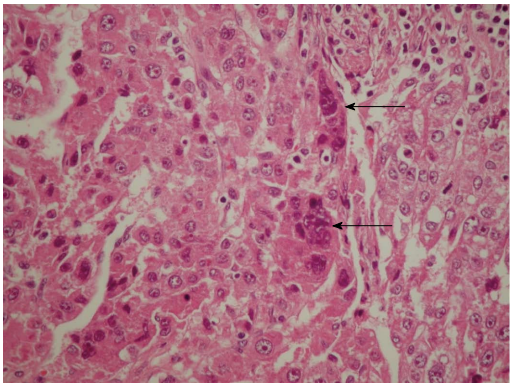

Figure 2 Hematoxylin and eosin-staining of a representative hepatocellular carcinoma section showing the multinuclear giant syncytial cells (arrows).

Subsequent microscopic analysis of the nodules revealed disordered architecture of the surrounding liver cell. The cells comprising the nodules showed pleomorphism and atypia, with some syncytial type multinuclear giant cells that had features similar to infantile giant cell hepatitis (Figure 2). Moreover, these giant cells also displayed the same nuclear atypia as the other HCC cells, which suggested that the cells were part of the tumor itself and not merely entrapped non-neoplastic cells; the marked lack of any giant cell changes in the adjacent liver tissue further reinforced this hypothesis.

A small number of mitotic figures, both typical and atypical and with or without apoptotic bodies, were observed in the tumor-like lesion, including the nodules, as well as beyond the lesion boundary, in the adjacent liver parenchyma. Inflammatory cells, such as lymphocytes and plasmatocytes, were present throughout the HCC and adjacent liver tissue, with greater incidence in the septa. Hyperplasia was observed in the marginal area of the nodules and small bile ducts. The adjacent liver parenchyma showed microvesicular steatosis, pericellular fibrosis, and moderate hemosiderin accumulation (grade 2, as determined by Prussian blue iron stain) in hepatocytes and Kupffer cells but no copper accumulation (as determined by orcein stain).

Immunohistochemical analysis showed hepatocyte

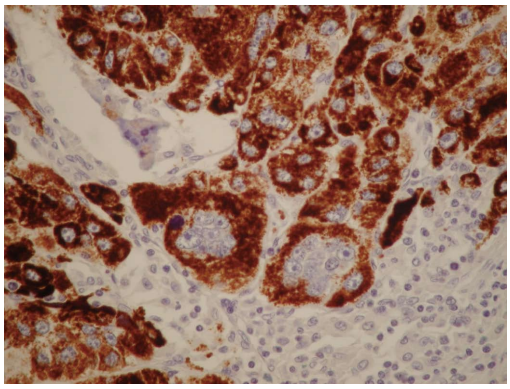

Figure 3 Mononuclear and multinuclear carcinoma cells showed reactivity for the hepatocyte antigen (brown). Magnification × 40.

antigen-positive staining for the neoplastic cells and SGCs (Figure 3). No cells showed immunoreactivity for macrophage-specific markers, such as CD68, but occasional cells showed immunoreactivity for the proliferation marker Ki-67/MIB-1 or the oncoprotein p53. The giant cells' nuclei showed a remarkable lack of immunoreactivity for all of the markers detected.

According to the collective histological findings, the diagnosis was made for cirrhosis-related HCC with SGCs. Up to the last follow-up examination, performed on 10 April 2013, the patient had maintained a-FP levels within the normal range and showed no signs of tumor recurrence by liver imaging.

DISCUSSION

While adult cases of giant cell HCC are relatively common, and its clinical and histologic features are well characterized, the case of giant cell HCC presented herein highlights the unusual histological manifestation of syncytial type cells[1-5]. Previous case reports of these rare tumor types have demonstrated the mesenchymal and non-neoplastic nature of the giant cells, leading to the suggested nomenclature of liver cell carcinoma with osteoclast-like giant cells[4-6]. However, the giant cells of the current case showed an epithelial and hepatocyte-originated neoplasmic nature, leading to the diagnosis of HCC with syncytial giant cells.

Another distinctive feature of this HCC case is its manifestation in cirrhotic liver. While cirrhosis was a likely etiology of the HCC, as suggested by the presence of nodular cirrhosis and microvesicular steatosis, the etiology of the cirrhosis was not obvious, and could have been long-term alcoholism or hemosiderosis. The patient's chronic consumption of SJW represents another potential complicating factor. The known hepatoxic properties of this herbal medicine may have contributed to or been the primary inducing factor of the syncytial phenotype of the giant cells.

The liver is among the first organs to experience toxic injury, as it is the main site of endo- and xenobiotic biotransformation. As such, a primary focus of drug development and clinical trials is determining the liver-related side effects of pharmaceutical and herbal medicines. Among the most common liver injuries are acute liver necrosis, hepatitis, cholestasis, fibrosis and cirrhosis; in addition, many of the medicines show either direct or indirect carcinogenic potential. Over the past few decades, the traditional herbal preparation of SJW extract has risen tremendously in popularity in the Western world, largely due to its observed benefits in treating mild to moderate depression[7-9], and bladder cancer[10,11].

Recent studies have indicated a potential clinical benefit of SJW for treating alcoholism, and implicated the bioactive phytochemical hyperforin as the constituent responsible for this response[12]. Interestingly, hyperforin has also been shown to have anti-microbial activity against Gram-positive bacteria and numerous viruses[13,14]. Studies of the molecular mechanisms underlying the bioactive activities of hyperforin have revealed a complex functional interaction with the other SJW constituents of essential oils, phloroglucinols, and flavonoids to induce a photoactivation process, possibly leading to production of singlet oxygen molecules[13,14]. Photoactivated-free radicals may exert the protective anti-infective or anti-tumor effects-for the latter, possibly through activation of apoptosis-related pathways[15,16].

In general, SJW is well tolerated as both topical and oral preparations. The most commonly reported adverse effects are gastrointestinal-related (*i.e.,* nausea, hypogastric pain, loss of appetite, diarrhea, gastric complaints[17] and sedative-related (*i.e.,* dizziness, confusion, and fatigue)[18]. Rare cases of photosensitivity development have also been reported, most often associated with systemic administration[19], and possible fertility risks have been suggested by some studies as well[20].

Another unknown mechanism of SJW is its ability to interact, either beneficially or detrimentally, with conventional drugs concomitantly-administered[21]. This possibly was highlighted by the observation of SJW-associated enhancement of P-glycoprotein, an intestinal multidrug transporter, and the cytochrome P450 (CYP) oxidative enzyme superfamily members CYP3A4 and CYP2C9. The induction of CYP3A4 represents a particularly troubling (and unresolved) clinical concern because of its abundance in the liver and intestine, both important sites of drug bio-transformation[22]. In addition, high-dosage SJW has been shown to activate the detoxification pathway mediated by the pregnane X receptor (PXR), a particularly promiscuous receptor; PXR overstimulation is detrimental and may result from SJW interactions with other drugs[23]. Finally, the non-standardized compositions of SJW extract preparations, available as over-the-counter medicines and often taken without clinical oversight, further complicate our ability to understand the benefits and risks of SJW, such as which may have contributed to the current case[24].

Despite these unknown features of SJW bioactivity, the significant anti-tumor effects of hyperforin in cell culture and animal systems have prompted continued efforts to investigate its potential as an efficacious cancer therapy that is less toxic than the current chemo- and radio-therapies. For example, *in vitro* studies of various rat and human cancers (*e.g.*, mammary carcinoma, squamous cell carcino-

ma, malignant melanoma, and lymphoma) showed equal or greater anti-tumor effects of hyperforin compared to the common cytostatic drugs camptothecin, paclitaxel, and vincristine[25]. Moreover, the molecular mechanism of hyperforin-mediated tumor cell apoptosis was shown to involve, at least partially, mitochondrial release of cytochrome c and caspase activation, thereby triggering cell death pathways[25,26]. However, as with any pharmacologic or herbal medicine, absolute anti-tumor activity is not guaranteed and inappropriate doses or concomitant drug administrations may create a pro-tumor situation.

For the case of cirrhosis-related HCC with SGCs described herein, we cannot conclude the role played by SJW with any certainty. The patient's ingestion of SJW may have created a hepatotoxic condition that promoted the progression of cirrhosis or HCC. Nonetheless, this case highlights the need for further studies of SJW to determine its side effects profile and safe dosages under various physiologic and pathogenic conditions; such knowledge is particularly important as the manufacture (*i.e.*, compositions) and use (*i.e.*, dose, duration, and in conjunction with other drugs) of SJW is non-uniform and widespread.

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CASE REPORT

Ileo-colonic intussusception secondary to small-bowel lipomatosis: A case report

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Abstract

Intestinal lipomatosis is a rare disease with an incidence at autopsy ranging from 0.04% to 4.5%. Because the lipomas are diffusely distributed in the intestine, most patients are symptom-free, and invasive intervention is not advised by most doctors. Here, we describe a case with intussusception due to small-bowel lipomatosis. Partial small bowel resection and anastomosis were performed because the intestinal wall was on the verge of perforation. This case indicates that regular follow-up is necessary and endoscopic treatment should be considered to avoid surgical procedures if the lipoma is large enough to cause intestinal obstruction.

Key words: Intussusception; Lipomatosis; Lipoma; Obstruction; Endoscopy

Core tip: Intestinal lipomatosis is a rare disease, obstruction due to a large lipoma may result in acute abdomen, and surgical intervention is applied infrequently. When patients are presented with symptoms, endoscopic treatment should be considered.

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INTRODUCTION

Intestinal lipomatosis is a rare disease with an incidence at autopsy ranging from 0.04% to 4.5% and most patients are symptom-free[1]. Symptomatic cases usually present as paroxysmal abdominal pain, hemorrhage of digestive tract, or abdominal masses. We here describe a case with ileo-colonic intussusception secondary to small-bowel lipomatosis.

CASE REPORT

A 52-year-old woman presented to our outpatient clinic in May 2013 with a history of abdominal pain for 21 d. The patient suffered from persistent abdominal cramps, accompanied by vomiting. The upright plain abdominal film revealed dilated small bowel loops with air-fluid levels. Intussusception was diagnosed by colonoscopy. Computed tomography (CT) scan showed multiple lipomas in small intestine (Figure 1A) and intussusception (Figure 1B). The patient was admitted to the hospital and emergency exploratory laparotomy was performed. During the operation, numerous lipomas 0.3-5 cm in diameter were found diffusely distributed in the intestine and ileo-colonic intussusception due to a large lipoma was confirmed (Figure 2). Partial small bowel resection and anastomosis were carried out because the intestinal

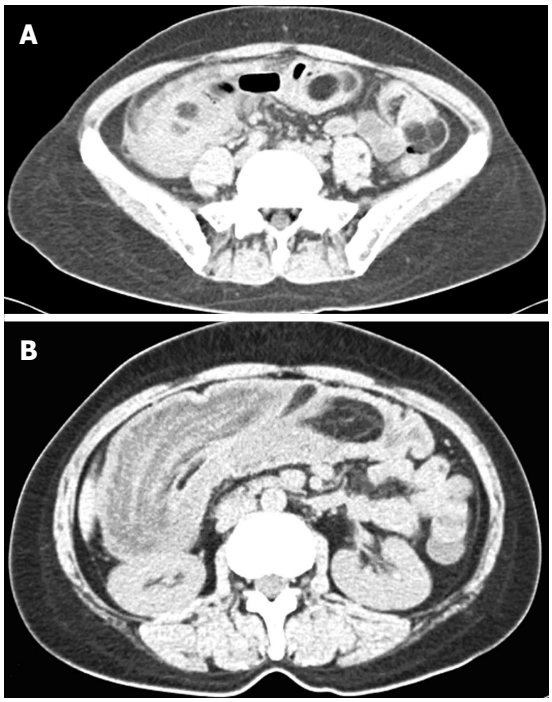

Figure 1 Abdominal computed tomography. A: Abdominal computed tomography (CT) (transverse view) revealed diffuse and multiple intramural fat density masses in the small intestine; B: Abdominal CT showed ileo-colonic intussusception.

wall was on the verge of perforation. Pathological examination confirmed a diagnosis of lipomatosis. Postoperative wound infection and cholecystitis occurred. After dressing change and anti-infection treatment, the patient was discharged in good condition one month later. The patient has remained symptom-free for 3 mo, although a small intestinal double contrast radiography revealed a filling defect in the duodenum and multiple submucosal masses in the small intestine (Figure 3).

DISCUSSION

Primary small intestinal tumors are uncommon, which account for about 1% of all gastrointestinal tumors, and primary lipomatosis of the small intestine is rare[2]. Diffuse and multiple lipomas in the intestine were first reported by Hellstrom[3] in 1906. CT and barium examination are useful techniques to confirm the diagnosis. The typical lipomas are usually seen as smooth, nonulcerated filling defects. Most lipomas are small and display no symptoms, and invasive treatment is unnecessary. When patients are presented with paroxysmal abdominal pain, hemorrhage of digestive tract and other symptoms, endoscopic treatment including endoscopic submucosal dissection, endoscopic snare resection and endoscopic unroofing techniques should be considered[4]. If the lipoma is large enough to cause intestinal obstruction or intestinal obstruction secondary to intussusception as shown in our patient, surgical intervention may be inevitable[5]. Because the lipomas are diffusely distributed in the intestine, the object of the surgery is not to remove

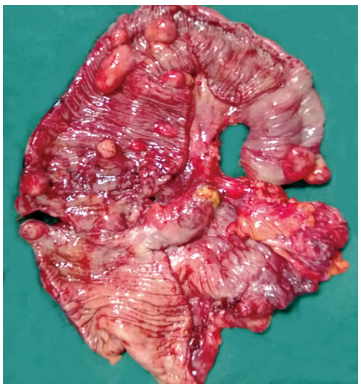

Figure 2 Multiple lipomas can be seen in the gross specimen.

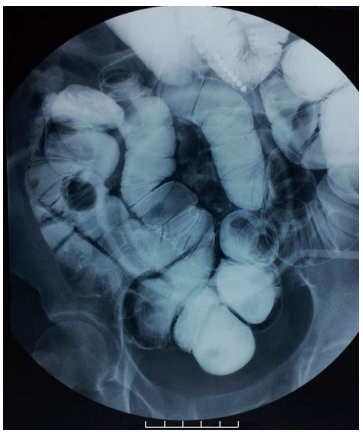

Figure 3 Small intestinal double contrast radiography revealed multiple submucosal masses in the small intestine.

all the lipomas but to relieve the obstruction. During the operation, large lipoma can be dislodged by local resection.

COMMENTS

Case characteristics

This condition may be asymptomatic and abdomimal pain is the most common symptom.

Clinical diagnosis

Symptomatic cases may present as abdominal pain, intestinal obstruction, intussusception, volvulus, or bleeding due to mucosal ulceration.

Differential diagnosis

With aid of computed tomography (CT), intestinal lipomatosis can be distinguished from liposarcoma by the homogeneity of its fatty content and absence of areas of increased density.

Laboratory diagnosis

Laboratory testing was not used for this condition.

Imaging diagnosis

Barium studies showed multiple submucosal masses in the small intestine and abdominal CT revealed multiple intramural fat density masses in the small intestine.

Pathological diagnosis

Under microscopy, the specimen showed multiple submucosal lipomatous nodules composed of abnormal collections of mature adipose tissue.

Treatment

If intestinal obstruction secondary to intussusception or volvulus occur, surgical intervention should be considered.

Related reports

Endoscopic treatment is recommended to remove the large lipomas to prevent obstruction.

Term explanation

Intestinal lipomatosis is characterized by diffuse and multiple lipomas in the intestine.

Experiences and lessons

Most patients with lipomatosis may be asymptomatic, but when intestinal obstruction secondary to intussusception or volvulus occur, surgical intervention may be inevitable. Endoscopic treatment is ideal in theory, but it is too difficult to perform in most hospitals.

Peer review

Lipomatosis is a rare disease, and intussusception secondary to lipomatosis is even rare. There are few literatures about this disease.

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CASE REPORT

Leptomeningeal carcinomatosis as the initial manifestation of gastric adenocarcinoma: A case report

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Abstract

Leptomeningeal involvement is usually reported as a secondary event in advanced gastric carcinoma. Leptomeningeal carcinomatosis (LMC), as the initial manifestation of asymptomatic gastric cancer, is exceedingly rare with only a few cases reported in recent years. The presenting neurologic symptoms include headache, vomiting and seizures and are usually clinically atypical. The diagnosis of LMC is made *via* identification of malignant cells that originate from epithelial cells in the cerebrospinal fluid by cytological examination and provides cues to track the primary tumor. Endoscopic examinations are crucial to confirm the presence of gastric cancer, and imaging studies, especially gadolinium-enhanced magnetic resonance imaging of the brain, are sometimes helpful in diagnosis. Thus far, there is no standard therapy for LMC, and despite all measures, the prognosis of the condition is extremely poor. Here, we report on the clinical features and diagnostic procedures for a patient with occult gastric cancer with Bormann type Ⅰ macroscopic appearance and poor differentiation in pathology, who presented with LMC-induced neurological symptoms as the initial clinical manifestation. Additionally, we review the similar cases reported over the past years, making comparison among cases in order to provide more information for the future diagnosis.

Key words: Meningeal carcinomatosis; Stomach neoplasms; Endoscopes; Gastrointestinal; Spinal puncture; Cytological techniques; Pathology; Clinical

Core tip: Here we report on a patient, initially and alone, presented with neurological symptoms and signs without any clues indicating gastric cancer. Evidence shows that the tumor directly spread solely to the brain without involvement of any other organs or tissues. How do we make an accurate and rapid diagnosis? Cerebral spinal fluid cytological studies play a key role in diagnosis, finding malignant cells that originate from epithelial cells. A gastroscopic examination was performed, revealing a tumor in the gastric antrum which was classified as Bormann type Ⅰ in macroscopic appearance, a rare type in Bormann classification, however, with poor differentiation in pathology. This patient survived for 4 mo without treatment. It is unclear whether this particular form of metastasis affects clinical outcomes.

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INTRODUCTION

Gastric cancer-induced leptomeningeal carcinomatosis (LMC) is less common; its incidence is only 0.14%-0.24% among all gastric cancers[1-3]. In very rare cases, LMC presents as the initial manifestation of an asymptomatic gastric adenocarcinoma where metastasis to the peritoneum and liver generally occur first. Herein, we report a case of LMC as the initial manifestation of a Bormann type I gastric adenocarcinoma with poor differentiation in pathology. The clinical features of this case and appropriate diagnostic procedures are discussed.

CASE REPORT

A 40-year-old woman, who had been diagnosed with adenomyosis and had undergone hysterectomy thereafter in December 2008, presented with complaints of a 2-mo headache and cervical pain. She was suspected to have an upper respiratory tract infection or to have caught a cold on July 21, 2010. At this time, she took a non-steroidal anti-inflammatory drug, and her symptoms were not alleviated. Her headache became increasingly severe 12 d after the onset of disease. On August 3, 2010, she visited a doctor due to a serious sustained sharp headache combined with projectile vomiting. A cranial computed tomography (CT) scan was performed, which indicated cerebral edema with mild ventricular dilatation; these findings were followed by administration of 20% mannitol to reduce intracranial pressure. Two days later, on August 5, 2010, she suffered a sudden loss of consciousness with limb twitching and an antiepileptic drug was administered. Afterwards, she was referred to another hospital due to the progression of the disease. Magnetic resonance imaging (MRI) revealed increased hydrocephalus and slight parenchymal swelling. Because meningeal irritation was noted, a lumbar puncture was performed and revealed that her cerebral spinal fluid (CSF) had an opening pressure of over 300 mmH_2O, glucose concentration of 4.13 mmol/L compared to a serum level of 5.5 mmol/L, protein content of 480 mg/L, chloride concentration of 110 mmol/L, and increased leukocyte levels (red blood cell count, 0 cells; white blood cell count, 8-10 $\times$ 10^6 cells/L; lymphocyte percentage, 74%). A repeat lumbar puncture revealed decreased glucose concentrations (CSF level of 2.79 mmol/L compared with serum level of 6.1 mmol/L), increased protein (790 mg/L), high intracranial pressure (over 300 mm H_2O), increased white blood cell count (25 $\times$ 10^6 cells/L) with a lymphocyte percentage of 75% and 0 counts of red blood cells, and a normal concentration of chloride (101.20 mmol/L, normal range 120-130 mmol/L).

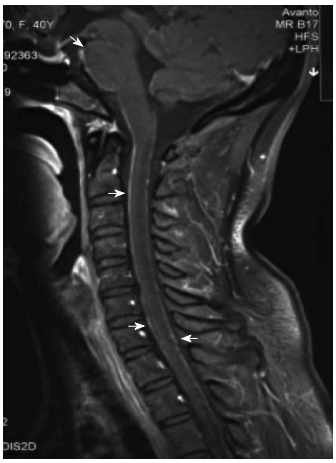

Figure 1 Sagittal magnetic resonance imaging with a T1-weighted gadolinium-enhanced sequence. Arrows show linear and punctiform contrast enhancement along the spine.

Cerebrospinal fluid culture was negative for acid-fast bacillus, *Cryptococcus neoformans* and bacterium. During her hospital stay, the onset of 3 epileptic seizures occurred, which were noticed by her husband or nurse. Although screening for tuberculosis infection was negative, she was suspected of having tuberculous meningitis and was started on tuberculostatic drugs, including rifampicin, isoniazid and pyrazinamide, as well as dehydration and diuretic drugs since August 7, 2010. Nevertheless, no improvement was observed after 7 d of treatment. Afterwards, on August 14, 2010, she was referred to the Brain Center of the Guangdong Province Hospital of Traditional Chinese Medicine.

On physical examination, the patient's body temperature was 36.3 ℃; her pulse was 60 beats/min, her respiratory rate 18 breaths/min and blood pressure 120/70 mmHg. Fundoscopic examination revealed bilateral papilledema (1PD each) and retinal edema was observed in the posterior pole.

The patient's neurological examination was notable for positive Kernig sign and Brudzinski's sign. The initial blood chemistry tests and metabolic screening (*e.g.*, electrolytes, kidney and liver function tests, blood glucose) were normal as were her routine stool and urination exams. The serum level of hepatitis B surface antigen was 287.1S/CO, and those of carcinoembryonic antigen (CEA), CA125, CA199, CA153 and alpha-fetoprotein in the serum were within normal limits. A repeat MRI scan was performed 1 week after admission, which indicated progressive linear and nodosity enhancement along the ventral surface of the brainstem, cerebellum and C1-T4 spinal cord (Figure 1). Expansion of the lateral ventricles, third ventricle and fourth ventricle was noted and communicating hydrocephalus was considered.

On August 12, 14, 17 and 18, 2010, additional lumber punctures were performed for a total of 4 taps during this period. The results of the CSF examination revealed

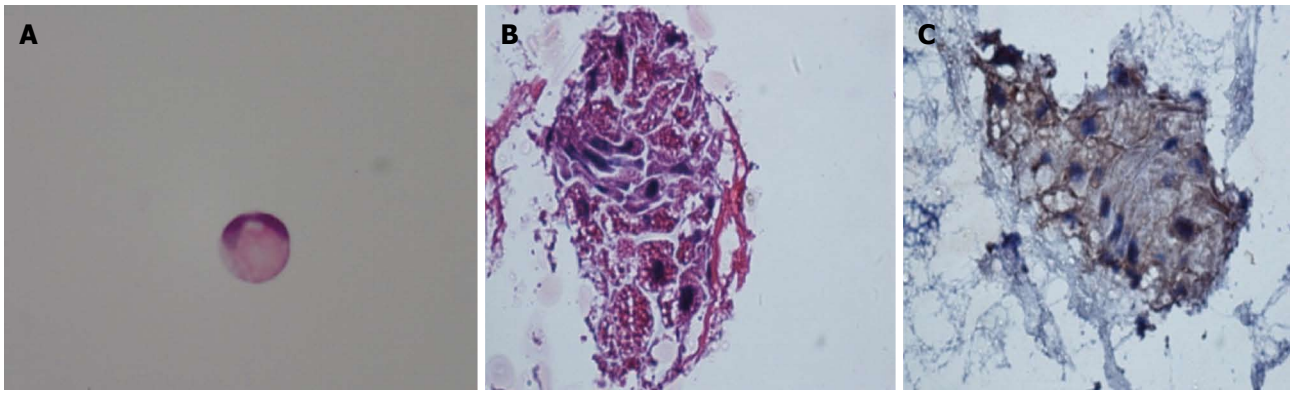

Figure 2 Detection of malignant cells and expression of creatine kinase in the patient's cerebral spinal fluid specimen. A: Signet-ring cells (hematoxylin and eosin staining, × 20); B: Malignant epithelial cells (hematoxylin and eosin staining, × 40); C: Positive expression of creatine kinase in carcinoma cells (immunohistochemistry staining, × 40).

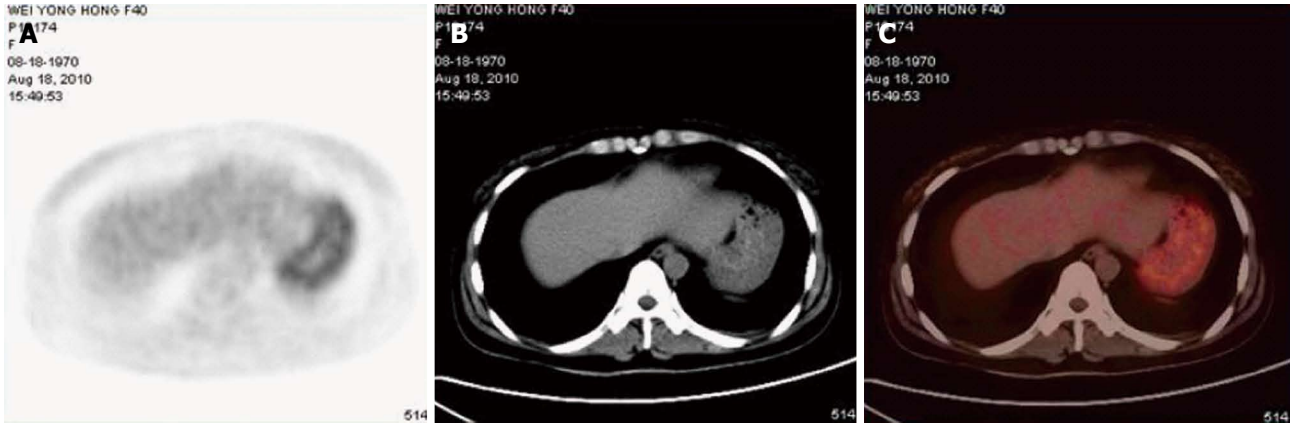

Figure 3 Position emission tomography/computed tomography finding. An increased 18F-FDG uptake in a diffuse manner, with a maximum standardized uptake value of 4.3 (mean 3.7) in the stomach. A: A cross-section image of position emission tomography (PET) scan; B: A cross-section image of computed tomography (CT) scan; C: PET/CT fusion image.

an increase in CSF opening pressure (> 330 mmH_2O for each tap), protein content (1470, 1140, 280 and 800 mg/L), WBC count (48, 7, 8, 7 × 10^6 cells/L) and CEA content (not determined on 12 August, 284.3, 351.9 and 347.0 ng/mL; serum level within normal limits), and a slight decrease in chloride concentration (108.4, 107.8, 109.5 and 108.9 mmol/L). Glucose levels were within or slightly lower than the lower limit of the normal range (3.06, 3.23, 2.14 and 3.13 mmol/L). CSF cytological analysis revealed malignant neoplastic cells (in particular, signet-ring cells, Figure 2) in the CSF specimen on the 14th and 17th of August, 2010. The diagnosis of LMC was made and the search for the primary tumor began. Immunohistochemical staining revealed that the CSF cells were positive for creatine kinase (CK) (Figure 2), which supports that the malignant neoplastic cells originated from the epithelium; this may be suggestive of the original tumor occurring in the esophagus and gastrointestinal tract, lungs or uterus. No expression of human epidermal receptor protein-2 (c-erbB-2; HER2) was detected *via* immunohistochemistry on de-stained CSF cytology slides.

On August 18, 2010, the patient's chest X-ray image was normal, and a whole-body position emission tomography (PET)/CT scan was conducted for further search for the original tumor. The PET/CT demonstrated that the stomach, which was not satisfactorily filled, had increased 18F-FDG uptake diffusely, with a maximum standardized uptake value (SUV_{max}) of 4.3 (mean 3.7) (Figure 3). This was subsequently explained by physiologic uptake. In addition to enlargement of the third and bilateral lateral ventricles and hydrocephalus, no abnormality was detected *via* PET/CT. Meanwhile, a gastroscopic examination was performed, revealing mass effect in the gastric antrum (Bormann class Ⅰ) (Figure 4). Because the endoscopic ultrasound and enhanced CT did not reveal infiltration of the tumor, and there were no positive findings of metastasis to the lymph nodes on PET/CT, the patient was roughly considered $T_xN_0M_1$ in clinical stage.

A subsequent histopathological examination of biopsies sampled during gastroscopy revealed poorly differentiated adenocarcinoma (Figure 4) with positive expression for CK and CEA (Figure 4), and partial positivity for CD68. An immunohistochemistry test was negative for HER2.

The patient was started on anti-tuberculostatic drugs, including isoniazid at a dose of 0.6 g per day, pyrazin-

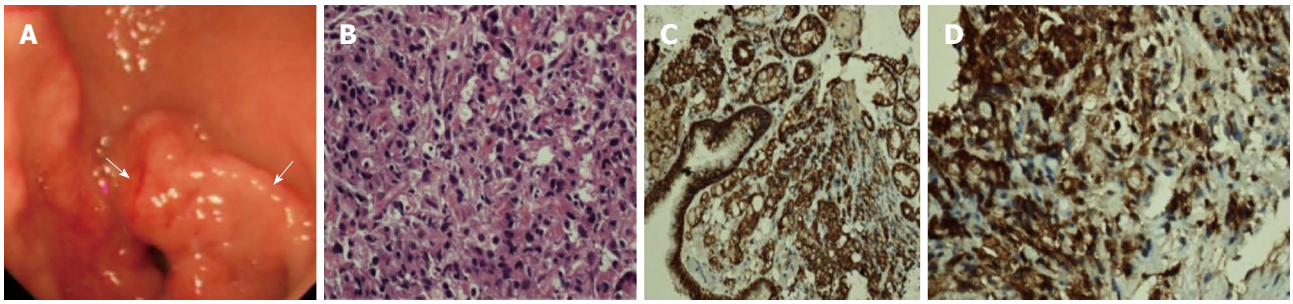

Figure 4 Gastroscopic view and histopathological findings of the tumor. A: 1/2 circle convex tumor (arrow) in the gastric antrum found by gastroscopy (Bormann class I); B: Poorly differentiated adenocarcinoma, diffused type, in biopsy of the gastric antrum sampled during gastroscopy (hematoxylin and eosin; × 40); C: Positive expression of creatine kinase; D: Carcinoembryonic antigen in biopsy specimen of gastric adenocarcinoma (immunohistochemical staining × 40).

amide at 1.5 g per day, and streptomycin at 0.5 g once, 2 times per day. The therapy was administered from August 7, 2010 until the day on which malignant neoplasm cells were found in the CSF. In deference to the patient's will, no further anti-tumor treatment was administered. During the hospital stay, the patient's condition worsened, and a neurological examination revealed nuchal rigidity and bilateral palsies of cranial nerves Ⅲ, Ⅴ, Ⅵ, Ⅶ, and XII. The patient was transferred to the hospital in her hometown, and after an unfortunate course of the disease, died on October 16, 2010, 4 mo after the initial presentation of her symptoms.

DISCUSSION

LMC is defined as diffuse spreading of malignant cells throughout the arachnoid membrane and the pia mater by propagation in the CSF. The majority of patients diagnosed with LMC have a prior cancer diagnosis. Meningeal involvement as a presenting symptom of malignancy is rare[4]. LMC is reportedly diagnosed clinically in 2%-4% of all cancer patients[5]; the prevalence of LMC in gastric cancer patients is as low as 0.14-0.24%[1-3], while the rate is 12%-14% in breast cancer, 10%-26% in lung cancer, and 17%-25% in melanoma[3].

To the best of our knowledge, the distinction and rarity of the present case are as follows: (1) this patient, initially and alone, presented with neurological symptoms and signs without any clues indicating gastric cancer, such as stomach ache, acid regurgitation, dyspepsia, anemia or occult fecal bleeding. This reminded us that the primary tumor may be occult without obvious symptoms or signs; this requires physicians to be prepared to consider alternative diagnostic decisions; (2) the gastroscopic examination identified the primary tumor as Bormann type Ⅰ, oddly, with poor differentiation in pathology. Traditionally, Bormann type Ⅰ gastric cancer has almost always been accompanied by high histopathological differentiation, while Bormann type Ⅲ or Ⅳ gastric cancer is poorly differentiated in pathology[6,7]. Moreover, the macroscopic appearance of Bormann type Ⅰ gastric cancer is less common than the other Bormann types; according to one large series of gastric cancer patients, the percentage of each Bormann classification was type Ⅰ in 7% of patients, type Ⅱ in 36% of patients, type Ⅲ in 25% of patients and type Ⅳ in 26% of patients[8]; and (3) the biological behavior of this patient's occult gastric adenocarcinoma is interesting. The tumor directly spread solely to the brain without involvement of any other organs or tissues such as the lungs, liver, bones or lymph nodes. It is unclear whether this particular form of metastasis affected the patient's clinical outcome; she survived for 4 mo without any anti-tumor therapy. The striking clinical picture that this LMC patient presented raises interesting questions regarding the biology and metastatic behavior of certain subclasses of gastric cancer.

Previously, only 7 similar cases have been documented in 6 reports[9-14], the detailed information of whom, as well as our present case, is summarized in Table 1. By reviewing the literature on these 7 cases and the disease progression of our case, we identified multiple relevant diagnostic issues that may be of clinical use to make prompt and accurate diagnoses when similar cases are encountered.

The clinical symptoms and signs of LMC are usually organized into 3 categories: cerebral, cranial nerve and spinal. Out of the 8 cases considered, 7, including our case, initially presented with headache, nausea and vomiting (cerebral symptoms and signs), 1 with back pain and weakness of the lower extremities (spinal symptoms and signs), 3 with diplopia, 1 with visual loss and 1 with hearing loss (cranial nerve symptoms and signs). Multifocal involvement of the meninges in LMC accounts for a great amount of variability in clinical presentations, thus making early diagnosis extremely challenging. Therefore, complicated and non-specified clinical features are those of which clinicians remain acutely aware to further track primary diseases.

As to the diagnosis of LMC, the presence of neoplastic cells in the CSF is diagnostic. A diagnosis of LMC may be supported by neuroimaging evidence, especially the results of gadolinium-enhanced MRI. Because cytological examination of the CSF is the *sine qua non* of diagnosis, CSF sampling procedures and analysis warrant close attention. Repeated CSF cytological examination is sometimes necessary. The presence of malignant cells was confirmed after the 2nd CSF examination in 2 cases reported in the literature[11,12], while our case required 4

Table 1 Reported cases of gastric cancer that initially presented with leptomeningeal carcinomatosis alone

Ref.	Case (age/sex)	Gastroscopic classification	Histopathological stage	Involvement of other organs/tissues	Treatment	Survival from onset of initial symptoms
Present case	40/F	Bormann Ⅰ	Adenocarcinoma	No	No	4 mo
Deeb *et al*[12], 1997	53/M	Not classified[2]	Poor differentiation	No	Intraventricular MTX	> 6 mo
Braeuninger *et al*[9], 2005	68/M	Not classified[2]	Signet cell type	Lymph nodes	Intrathecal chemotherapy	2 mo
Lee *et al*[10], 2007	49/F	Bormann Ⅳ		Lymphadenopathy	No	ND
Yamada *et al*[13], 2008	53/M	Bormann Ⅱ		ND	Radiotherapy	4.23 mo (127 d)
Gdovinova *et al*[11], 2009; Case 1	40/F	ND		Lymphatic node, ovary, peritoneum, leptomeninges	No	2 mo
Gdovinova *et al*[11], 2009; Case 2	49/F	Not classified[2]		ND	Yes[1]	2 mo
Ohno *et al*[14], 2010	62/M	Not classified[2]		ND	Radiotherapy	3 mo (12 wk)

[1]No further information was provided; [2]Detailed description; Deeb *et al*[12] (1997): multiple round, thickened, raised lesions, encroaching on the lumen with loss of vascular pattern in the distal esophagus just proximal to the gastroesophageal junction. The stomach was non-distensible and involved by diffuse marked nodularity with overlying normal mucosa and prominence of the areae gastricae, mostly in the proximal body with few ulcerations in the distal body; Braeuninger *et al*[9] (2005): the primary tumor site was disclosed by oesophagogastroduodenoscopy, where an ulcer of 15 mm in diameter was observed in the distal stomach; Gdovinova *et al*[11] (2009): gastroscopy revealed a callous mediogastric ulcus as well as peptic ulcerations in the duodenal bulb; Ohno *et al*[14] (2010): a large type 3 gastric cancer at the greater curvature side of the middle body. M: Male; F: Female; ND: Not determined; MTX: Methotrexate.

taps to confirm malignant cells. As previously reported, CSF cytological results are positive in 50% of all cases of meningeal metastasis from non-central nervous system neoplasms after a single lumbar tap and in 85% to 90% of cases after multiple taps[15]. Three simple measures can increase the likelihood of finding malignant cells in a CSF sample: (1) obtaining at least 10.5 mL of CSF for analysis[16]; (2) immediately processing the sample (within 1 h after collection[17]); and (3) obtaining CSF from a site adjacent to the affected central nervous system region[16].

Apart from cytological examination of the CSF, other CSF changes were observed in the 8 LMC cases described, such as increased opening pressure, increased protein content and white blood cell count and a glucose concentration within normal limits or slightly decreased (except that in 1 case with hypoglycorrhachia, glucose concentration was 0.6 mmol/L[11]; and our case with a glucose concentration of 2.79 mmol/L on the 1st CSF examination), which are supported throughout the literature[16]. Other notable CSF changes were described in these LMC cases, including increased lactate or CEA concentrations. Concerning the CSF markers of interest, their concentrations in CSF should be tested simultaneously with those in the serum to eliminate the possibility of passive diffusion[17]. As shown in our case, CSF CEA was within the range between 284.3 and 347.0 ng/mL, while that in the serum was within normal limits. Thus, when the CSF marker level is inappropriately high and the blood brain barrier is intact, these markers may be produced in the subarachnoid space by the tumor metastases.

With respect to clinical imaging techniques, as demonstrated in nearly all 8 LMC patients considered, brain CT and MRI without contrast yielded false negative results. However, 3 of the 8 patients obtained positive findings on gadolinium-enhanced MRI, such as nerve thickening and linear and punctiform enhancement of the leptomeninges. Leptomeningeal enhancement is suggestive of LMC, but not diagnostic. Therefore, one must consider other conditions that may produce similar imaging features, such as intracranial hypotension after craniotomy or lumber puncture, as well as infectious or inflammatory diseases. Although MRI is superior to CT with 1.5-2 times higher specificity and sensitivity[18,19], the rate of false negative MRI findings is approximately 30%; this could be further diminished by the use of greater amounts of gadolinium[20,21]. Additionally, it is important to perform MRI before CSF examination because spinal tap alone may induce long-lasting (weeks to months) diffuse meningeal enhancement[18]. When the primary cancer is unknown and CSF cytology is negative, MRI alone is not sufficient to establish an LMC diagnosis; histological confirmation is required before such a diagnosis can be made.

In the present case, whole-body PET/CT was performed to track the primary disease, which revealed increased glucose uptake in the stomach, with a SUV_{max} of 4.3. However, this abnormality was initially explained by physiological uptake, and close follow-up was suggested by the patient's radiologist. As indicated by Dassen AE[22], FDG-PET appears to have little or no value in the primary detection of gastric cancer. They suggested that there is a clear difference in the sensitivity of FDG-PET between different histological carcinoma subtypes, particularly in the non-intestinal (*i.e.*, diffuse) subtype. Carcinomas containing signet ring cells exhibit consistently low detectability by FDG-PET. Prior evidence indicates[23-28] that SUV counts between 7.7-13.2 were found in tubular gastric carcinoma and moderately differentiated carcinoma, which are significantly higher than those for mucinous adenocarcinoma and signet ring cell carcinoma (4.1-7.7).

Regarding the histopathological examination of primary gastric cancer, poorly differentiated adenocarcinoma was finally confirmed in all of the 8 LMC cases considered. This is consistent with previous reports in-

dicating that poorly differentiated adenocarcinoma with signet ring cell features is the most frequently occurring type of LMC associated with GC in Japan[29]; this is likely to be true in Korea[7].

By consensus, the prognosis of LMC is poor. The median survival in untreated patients is 4-6 wk, which may increase to 4-6 mo with aggressive treatment in some cases[30,31] Out of the 8 LMC cases described, 3[9-11] had metastasis to distant organs other than the meninges was found, such as in the lymph nodes, ovaries, and peritoneum. Five of these patients received anti-tumor therapy and their survival ranged from 2-6 mo[9,11-14]. This indicates that treatment was not associated with a clinically significant different prognosis than non-treatment (2 mo in 1 case in literature[11], and 4 mo in our case). Various relevant and concerning questions arise including those surrounding the clinical biological behavior of rare and odd adenocarcinomas, as in the present case with gastroscopic Bormann type I and poor differentiation, and whether this factor partially influences metastasis and patient survival.

In summary, to the best of our knowledge, this case of LMC originating from an occult gastric adenocarcinoma is the first ever reported with Bormann type I macroscopic appearance, but with poor differentiation in pathology. Its direct invasion to the meninges resulting in LMC as the initial presentation is very interesting, especially considering that there was no evidence of metastasis to other organs or tissues. It is unclear whether this particular form of metastasis affects clinical outcomes; our patient survived for 4 mo without treatment. Early diagnosis requires highly aware and vigilant physicians. CSF cytological studies play a key role in diagnosis and neuroimaging examinations are helpful; however, they present certain limitations.

COMMENTS

Case characteristics

The patient presented with a sustained headache, cervical pain, projectile vomiting, and a sudden loss of consciousness with limb twitching and epileptic seizures.

Clinical diagnosis

The main clinical findings include positive Kernig's sign and Brudzinski's sign in neurological examination.

Differential diagnosis

In this case, the differential diagnosis is tuberculous meningitis.

Laboratory diagnosis

The cerebral spinal fluid (CSF) examination revealed an increase in CSF opening pressure, protein content, WBC count and carcinoembryonic antigen (CEA) content, and CSF cytological analysis revealed malignant neoplastic cells.

Imaging diagnosis

Magnetic resonance imaging indicated progressive linear and nodosity enhancement along the ventral surface of the brainstem, cerebellum and C1-T4 spinal cord and position emission tomography/computed tomography demonstrated that the stomach had increased 18F-FDG uptake diffusely, with a maximum standardized uptake value of 4.3 (mean 3.7).

Pathological diagnosis

A histopathological examination of biopsies sampled during gastroscopy revealed poorly differentiated adenocarcinoma with positive expression for creatine kinase (CK), CEA and partial positivity for CD68, while the CSF cells was positive for CK.

Term explanation

Leptomeningeal carcinomatosis, also known as neoplastic meningitis, occurs when malignant cells enter the leptomeningeal space *via* hematogenous dissemination or direct extension. The malignant cells are spread throughout the neuraxis by the flow of the CSF, leading to disease throughout the central nervous system. Leptomeningeal carcinomatosis leads to substantial morbidity and mortality, and there are few, if any, effective treatments.

Experiences and lessons

The gastric cancer can metastasize to meninges alone without involvement of any other organs or tissues, and thus the patients may present with neurological abnormalities alone without any clue to the stomach. Early diagnosis requires highly aware and vigilant physicians. For diagnosis, CSF cytological studies play a key role and neuroimaging examinations are helpful; however, they present certain limitations.

Peer review

The strength of this report is that this case of leptomeningeal carcinomatosis (LMC) originating from an occult gastric adenocarcinoma is the first ever reported with Bormann type I in macroscopic appearance, but with poor differentiation in pathology. Its direct invasion to the meninges resulting in LMC as the initial presentation is very interesting, especially considering that there was no evidence of metastasis to other organs or tissues. It is unclear whether this particular form of metastasis affects clinical outcomes.

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Express *t* test as *t* (in italics), *F* test as *F* (in italics), chi square test as χ^2 (in Greek), related coefficient as *r* (in italics), degree of freedom as υ (in Greek), sample number as *n* (in italics), and probability as *P* (in italics).

Units

Use SI units. For example: body mass, m (B) = 78 kg; blood pressure, p (B) = 16.2/12.3 kPa; incubation time, t (incubation) = 96 h, blood glucose concentration, c (glucose) 6.4 ± 2.1 mmol/L; blood CEA mass concentration, p (CEA) = 8.6 24.5 μg/L; CO_2 volume fraction, 50 mL/L CO_2, not 5% CO_2; likewise for 40 g/L formaldehyde, not 10% formalin; and mass fraction, 8 ng/g, *etc.* Arabic numerals such as 23, 243, 641 should be read 23243641.

The format for how to accurately write common units and quantums can be found at: http://www.wjgnet.com/1007-9327/g_info_20100315223018.htm.

Abbreviations

Standard abbreviations should be defined in the abstract and on first mention in the text. In general, terms should not be abbreviated unless they are used repeatedly and the abbreviation is helpful to the reader. Permissible abbreviations are listed in Units, Symbols and Abbreviations: A Guide for Biological and Medical Editors and Authors (Ed. Baron DN, 1988) published by The Royal Society of Medicine, London. Certain commonly used abbreviations, such as DNA, RNA, HIV, LD50, PCR, HBV, ECG, WBC, RBC, CT, ESR, CSF, IgG, ELISA, PBS, ATP, EDTA, mAb, can be used directly without further explanation.

Italics

Quantities: *t* time or temperature, *c* concentration, *A* area, *l* length, *m* mass, *V* volume.
Genotypes: *gyrA*, *arg* 1, *c myc*, *c fos*, *etc.*
Restriction enzymes: *Eco*RI, *Hin*dI, *Bam*HI, *Kbo* I, *Kpn* I, *etc.*
Biology: *H. pylori*, *E coli*, *etc.*

Examples for paper writing

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