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The primary task of *WJG* is to rapidly publish high-quality original articles, reviews, and commentaries in the fields of gastroenterology, hepatology, gastrointestinal endoscopy, gastrointestinal surgery, hepatobiliary surgery, gastrointestinal oncology, gastrointestinal radiation oncology, gastrointestinal imaging, gastrointestinal interventional therapy, gastrointestinal infectious diseases, gastrointestinal pharmacology, gastrointestinal pathophysiology, gastrointestinal pathology, evidence-based medicine in gastroenterology, pancreatology, gastrointestinal laboratory medicine, gastrointestinal molecular biology, gastrointestinal immunology, gastrointestinal microbiology, gastrointestinal genetics, gastrointestinal translational medicine, gastrointestinal diagnostics, and gastrointestinal therapeutics. *WJG* is dedicated to become an influential and prestigious journal in gastroenterology and hepatology, to promote the development of above disciplines, and to improve the diagnostic and therapeutic skill and expertise of clinicians.

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EDITORIAL

Management of acute pancreatitis in Japan: Analysis of nationwide epidemiological survey

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Abstract

Acute pancreatitis (AP) is an acute inflammatory disease of the exocrine pancreas. In Japan, nationwide epidemiological surveys have been conducted every 4 to 5 years by the Research Committee of Intractable Pancreatic Diseases, under the support of the Ministry of Health, Labour, and Welfare of Japan. We reviewed the results of the nationwide surveys focusing on the severity assessment and changes in the therapeutic strategy for walled-off necrosis. The severity assessment system currently used in Japan consists of 9 prognostic factors and the imaging grade on contrast-enhanced computed tomography. By univariate analysis, all of the 9 prognostic factors were associated with AP-related death. A multivariate analysis identified 4 out of the 9 prognostic factors (base excess or shock, renal failure, systemic inflammatory response syndrome criteria, and age) that were associated with AP-related death. Receiver-operating characteristics curve analysis showed that the area under the curve was 0.82 for these 4 prognostic factors and 0.84 for the 9 prognostic factors, suggesting the comparable utility of these 4 factors in the severity assessment. We also examined the temporal changes in treatment strategy for walled-off necrosis in Japan according to the 2003, 2007, and 2011 surveys. Step-up approaches and less-invasive endoscopic therapies were uncommon in 2003 and 2007, but became popular in 2011. Mortality has been decreasing in patients who require intervention for walled-off necrosis. In conclusion, the nationwide survey revealed the comparable utility of 4 prognostic factors in the severity assessment and the increased use of less-invasive, step-up approaches with improved clinical outcomes in the management of walled-off necrosis.

Key words: Endoscopic necrosectomy; Diagnostic criteria; Epidemiology; Pancreatic pseudocyst; Systemic inflammatory response syndrome; Step-up approach;

Walled-off necrosis

Core tip: We analyzed the results of nationwide epidemiological surveys of acute pancreatitis in Japan to clarify the utility of the prognostic factor scores in the severity assessment and the trend in the treatment of walled-off necrosis. Among the 9 prognostic factors, 4 factors including base excess or shock, renal failure, systemic inflammatory response syndrome criteria, and age were associated with mortality by multivariate analysis. Receiver operating characteristics curve analysis demonstrated the comparable utility of these 4 factors to the 9 factors in the severity assessment. Less-invasive, step-up approaches with improved clinical outcomes have become popular in the management of walled-off necrosis.

Hamada S, Masamune A, Shimosegawa T. Management of acute pancreatitis in Japan: Analysis of nationwide epidemiological survey. *World J Gastroenterol* 2016; 22(28): 6335-6344 Available from: URL: http://www.wjgnet.com/1007-9327/full/v22/i28/6335.htm DOI: http://dx.doi.org/10.3748/wjg.v22.i28.6335

INTRODUCTION

Acute pancreatitis (AP) is an acute inflammatory disease of the pancreas characterized by the sudden onset of upper abdominal pain, nausea, emesis, and an increase of pancreatic digestive enzymes in the serum and urine[1-4]. Most patients with AP have a mild disease that only affects the pancreas and resolves spontaneously. However, 10%-20% of the patients develop necrosis of the pancreas and multiple organ failure, which may eventually lead to death[1-5]. AP is the most common digestive disease requiring hospitalization in the United States[6].

In Japan, nationwide epidemiological surveys of AP have been conducted every 4 to 5 years mainly by the Research Committee of Intractable Pancreatic Diseases, with the support of the Ministry of Health, Labour, and Welfare of Japan[5,7,8]. The latest survey was conducted targeting the AP patients treated in 2011[5]. A detailed analysis of the nationwide surveys would enable us to understand the current status of AP management and the issues that remain to be clarified. In this editorial, we review the results of the surveys focusing on the severity assessment and changes in the therapeutic strategy for walled-off necrosis (WON).

OVERVIEW OF THE NATIONWIDE SURVEY

The nationwide survey consisted of 2-staged postal surveys. The first survey aimed to estimate the number of patients with AP and the second survey aimed to elucidate the clinical-epidemiological characteristics of AP. The departments of internal medicine, gastroenterology, surgery, digestive surgery, and emergency all over Japan were listed and subjected to stratified random sampling. The sampling rates were 5%, 10%, 20%, 40%, 80%, 100%, and 100% for the stratum of hospitals with < 100 beds, ⩽ 100 to < 200 beds, ⩽ 200 to < 299 beds, ⩽ 300 to < 399 beds, ⩽ 400 to < 499 beds, ⩽ 500 beds, and the affiliated university hospitals, respectively. Several departments treating many pancreatic disease patients and emergency centers were classified as a special stratum, and all of them were selected. In the first survey, a questionnaire requesting a report of the number of patients with AP was sent. The second questionnaire regarding detailed clinicoepidemiological information was sent to departments reporting on the first questionnaire that they had seen AP patients. Clinical data of 2694 patients with AP were collected in the 2011 survey[5], of 2256 patients in the 2007 survey[7], and of 1779 patients in the 2003 survey[8]. In the 2011 survey, the second questionnaire included questions about etiology/symptoms, laboratory data, imaging findings, therapy, complications, and prognosis. The laboratory data and clinical symptoms that were included in the prognostic factor scores in addition to contrast-enhanced computed tomography (CECT) imaging grade were primarily assessed at admission[5].

EPIDEMIOLOGY OF AP IN JAPAN

The latest nationwide epidemiological survey estimated the total number of AP patients in Japan in 2011 as 63080, with an overall prevalence of 49.4 per 100000 persons[5]. Previous studies showed that the incidence of AP in the United States was 10.6 per 10000 person-years in 2009 and that it was 14.7 per 100000 person-years in the Netherlands in 2005[9,10]. These results suggest that the incidence of AP might vary among different populations. The estimated number of AP patients increased to 57560 in the 2007 survey[7] from 35300 in the 2003 survey[8]. AP mostly occurs in those middle-aged to elderly. In the 2011 survey, the mean age of the AP patients was 60.9, and the sex ratio (male to female) was 1.9. The most frequently affected ages were 60 to 69 years in men and 70 to 79 years in women. The three major

Table 1 The severity scoring criteria of acute pancreatitis defined by the Japanese Ministry of Health, Labor and Welfare (2008)

Prognostic factor	
Base excess ≤ -3 mEq/L or shock (systolic blood pressure < 80 mmHg)	
PaO_2 ≤ 60 mmHg or respiratory failure (needing respirator)	
BUN ≥ 40 mg/dL (or Cr ≥ 2 mg/dL) or	
oliguria (< 400 mL/d even after fluid therapy)	
Elevation of LDH twice or more than upper normal limit	
Platelet count ≤ 100000/μL	
Serum calcium ≤ 7.5 mg/dL	
CRP ≥ 15 mg/dL	
Meeting 3 or more SIRS criteria (body temperature > 38 ℃ or < 36 ℃, heart rate > 90/min, respiratory rate > 20/min or $PaCO_2$ < 32 torr, WBC > 12000/μL or < 4000/μL or > 10% immature leukocyte)	
Age ≥ 70 yr	
Classification of CT grade by contrast-enhanced CT	
Factor 1: Extent of extrapancreatic inflammation	
To the anterior pararenal extraperitoneal space	0 point
To the root of mesocolon	1 point
Further than inferior pole of kidney	2 points
Factor 2: Less-enhanced region of pancreas (divided into three segments; head, body and tail)	
Localized within one segment or limited to peripheral pancreas	0 point
Occupies two segments	1 point
Occupies more than two segments	2 points
Sum of factor 1 and factor 2 ≤ 1	grade 1
Sum of factor 1 and factor 2 = 2	grade 2
Sum of factor 1 and factor 2 ≥ 3	grade 3

Severe acute pancreatitis is defined as fulfilling 3 or more criteria of prognostic factors or revealing CT grade 2 or more. BUN: Blood urea nitrogen; Cr: Creatinine; CRP: C-reactive protein; CT: Computed tomography; LDH: Lactate dehydrogenase; SIRS: Systemic inflammatory response syndrome; WBC: White blood cells.

causes of AP are alcohol, biliary, and idiopathic. Alcohol was the leading cause (46.2%) in men, followed by biliary (19.7%), and idiopathic (13.4%). Biliary was the leading cause (40.3%) in women followed by idiopathic (22.8%) and alcohol (9.9%). The age distribution differed according to the etiology. Alcoholic pancreatitis was most frequently seen from ages 50 to 59, whereas biliary or idiopathic pancreatitis cases increased according to age. In cases of severe AP, the proportion of alcoholic cases increased from 30.9% in 2007 to 42.0% in 2011. A case control study in Japan showed that the risk of alcoholic AP increased as daily alcohol consumption increased[11]. The odds ratio (95%CI) for daily alcohol consumption of 20 ≤ to < 40 g, 40 ≤ to < 60 g, 60 ≤ to < 80 g, 80 ≤ to < 100 g, and ≥ 100 g were 1.7 (0.9-3.0), 3.1 (1.6-5.9), 4.2 (2.1-8.2), 5.3 (2.4-12.0), and 6.4 (3.4-12.4), respectively[11]. Another Japanese study showed that women developed alcohol-related AP 6.8 years earlier compared to men[12,13]. The duration of alcohol consumption was shorter, and the cumulative amounts of alcohol consumption before the development of alcoholic AP were smaller in women than in men. In 2011, the overall mortality of AP was found to be 2.6% and in severe AP, 10.1%[5].

DIAGNOSTIC CRITERIA OF SEVERE AP IN JAPAN

The severity assessment system for AP (2008) currently used in Japan consists of prognostic factor scores based on 9 clinical parameters and the CECT imaging grade (Table 1). If the total prognostic factor score is ≥ 3 or the CECT grade is ≥ 2, the patient is defined as having severe AP. The previous severity assessment system proposed in 2002 was more complicated than the 2008 system; it consisted of 5 clinical parameters, 10 blood test items, and CT findings. In cases with a severity score ≥ 2, systemic inflammatory response syndrome (SIRS) criteria and age should be considered in the severity score[14,15]. Several reports have shown that the severity scoring system of AP (2008) is more useful and easier for the prediction of prognosis than the previous one (2002)[14,15]. Of note, diagnosis of severe AP can be performed by CECT grade only, which enables diagnosis of AP with a low prognostic factor score. However, no previous large-scale multicenter studies have validated this system. To validate the prognostic factor score in the diagnosis of severe AP, we analyzed the nationwide survey in 2011. The outcome was AP-related hospital mortality assessed by a univariate logistic regression analysis. A predictive accuracy receiver-operating characteristics (ROC) curve was generated, and the area under the curve (AUC) was calculated. This study was approved by the Ethics Committee of Tohoku University Graduate School of Medicine (article#: 2015-1-519).

Data about the prognostic factor scores at admission and prognosis were available for 1990 cases with AP. The mortality increased according to the prognostic factor score (Figure 1A). In patients whose prognostic factor scores were ≥ 6, the mortality was as high as 43.5%. Data about the CT grades at admission and prognosis were available in 1618 cases with AP. The mortality increased according to the CT grade (Figure 1B). As shown in Figure 2, mortality was higher if any of the prognostic scores appeared. By univariate analysis, all of the prognostic factors were associated with AP-related death (Table 2). A multivariate analysis identified 4 out of the 9 prognostic factors (base excess or shock, renal failure, SIRS criteria, and age) that were associated with AP-related death. We also performed ROC curve analysis to evaluate the predictive accuracy of the prognostic factor scores for mortality. As shown in Figure 3A, the AUC of the prognostic factor score for predicting mortality was 0.84. If the cut-off point was set at a severity score of 3, as adopted currently, the sensitivity reached 0.53 with a specificity of 0.94. If the cut-off point was set at a severity score of 2, the sensitivity reached 0.75 with a specificity of 0.83. If we adopted the 4 prognostic factors found to be associated with AP-related death by a multivariate analysis, the AUC for predicting mortality was 0.82 (Figure 3B). The

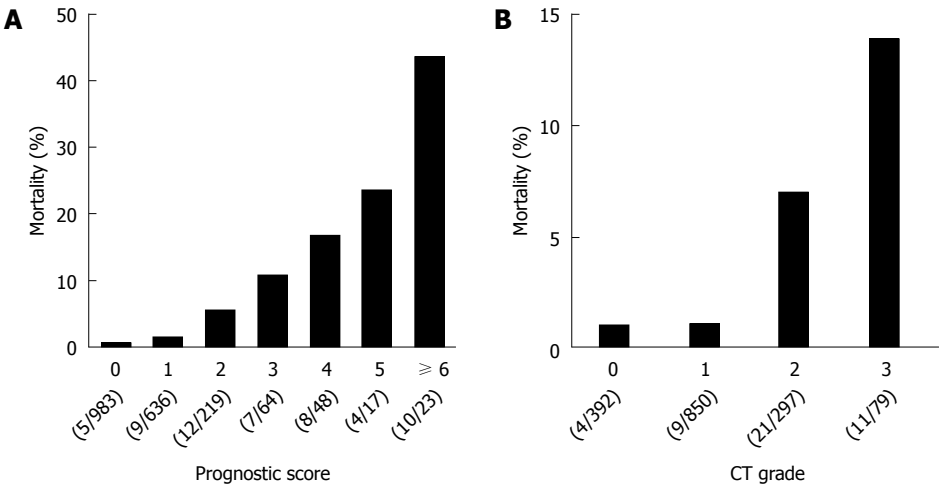

Figure 1 Mortality of the acute pancreatitis patients with the prognostic factor scores and computed tomography grades. A: Mortality of the 1990 AP patients stratified by the prognostic factor scores is shown. B: Mortality of the 1618 AP patients stratified by the CT grades is shown. AP: Acute pancreatitis; CT: Computed tomography.

Table 2 Univariate and multivariate analysis showing association of each prognostic factor with acute pancreatitis-related death

	OR (95%CI)	*P* value
Univariate analysis		
BE ⩽ -3 mEq/L or shock	12.1 (6.8-21.1)	< 0.0001
PaO_2 ⩽ 60 mmHg or respiratory failure	11.1 (5.7-20.9)	< 0.0001
BUN ⩾ 40 mg/dL (or Cr ⩾ 2 mg/dL or oliguria)	14.9 (8.3-26.4)	< 0.0001
Elevation of LDH (twice or more than UNL)	4.2 (2.3-7.4)	< 0.0001
Platelet count ⩽ 100000/μL	4.2 (1.6-9.5)	0.007
Serum calcium ⩽ 7.5 mg/dL	14.1 (7.2-26.8)	< 0.0001
CRP ⩾ 15 mg/dL	3.6 (1.9-6.7)	0.0003
Meeting 3 or more SIRS criteria	8.4 (4.7-14.8)	< 0.0001
Age ⩾ 70 yr	3.2 (1.8-5.5)	< 0.0001
Multivariate analysis		
BE ⩽ -3 mEq/L or shock	2.8 (1.3-6.1)	0.012
PaO_2 ⩽ 60 mmHg or respiratory failure	1.9 (0.8-4.4)	0.130
BUN ⩾ 40 mg/dL (or Cr ⩾ 2 mg/dL or oliguria)	3.8 (1.8-7.8)	0.0008
Elevation of LDH (twice or more than UNL)	1.5 (0.7-3.0)	0.280
Platelet count ⩽ 100000/μL	1.8 (0.6-4.9)	0.300
Serum calcium ⩽ 7.5 mg/dL	2.5 (0.99-6.1)	0.051
CRP ⩾ 15 mg/dL	1.0 (0.4-2.2)	0.980
Meeting 3 or more SIRS criteria	2.3 (1.0-4.7)	0.038
Age ⩾ 70 yr	3.5 (1.9-6.6)	< 0.0001

BE: Base excess; BUN: Blood urea nitrogen; CRP: C-reactive protein; LDH: Lactate dehydrogenase; SIRS: Systemic inflammatory response syndrome; UNL: Upper normal limit.

sensitivity reached 0.62 with a specificity of 0.92 if the cut-off point was set at score 2. These values were comparable or superior to those for the current severity assessment system using the 9 prognostic factor scores.

In the 2011 survey, the mortality of the patients defined as severe AP solely based on the prognostic factor scores was 7.5%[5]. The mortality of patients defined as severe AP based on the CT grade was 4.2%. The mortality was 25.9% in patients who were defined as severe for both prognostic factors and CT grade. In the revised Atlanta classification, severe AP is defined as the presence of persistent organ failure for more than 48 h[16]. In other words, using the Atlanta classification, severe AP cannot be diagnosed within 48 h of AP onset. CECT, especially used in combination with the prognostic factor score, could be useful to diagnose severe AP in patients at high risk of death in the early stages of AP.

FACTORS ASSOCIATED WITH SEVERITY AND PROGNOSIS

Because the nationwide epidemiological survey collected detailed laboratory data and information about the clinical course of patients, analysis of the nationwide survey data would be useful to identify and validate factors associated with the severity and prognosis of patients with AP. For example, Kikuta *et al*[17] reported that impaired glucose tolerance might have an impact on the development and clinical outcome of AP based on an analysis of the nationwide survey in 2007. They showed that idiopathic, but not alcoholic or biliary, AP patients with diabetes mellitus had higher mortality than those without diabetes mellitus.

Very recently, Nawaz *et al*[18] from the United States reported that elevated serum triglycerides (TGs) were associated with organ failure in AP. They showed that elevated serum TGs measured within 72 h of presentation were correlated with persistent organ failure. Because the body size and contribution of hypertriglyceridemia (HTG) to the etiology of AP vary among different populations[5,19], we validated the clinical impact of HTG in 998 AP patients using the 2011 survey data[20]. The frequencies of severe AP, persisting renal failure, and the necessity for intensive care unit

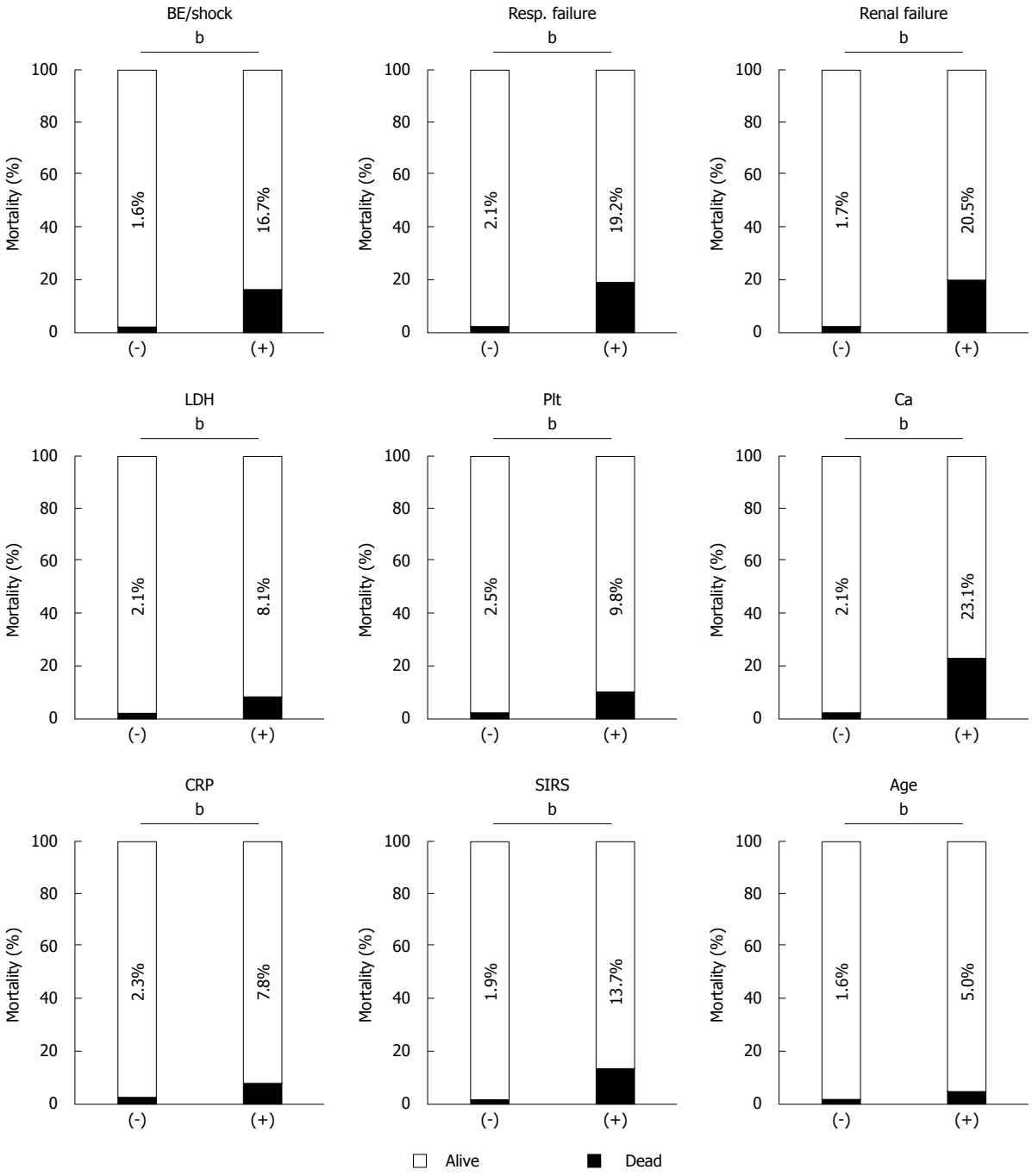

Figure 2 Mortality was higher if any of the prognostic factor items were positive. The mortality of the AP patients was assessed in the presence or absence of each prognostic factor items. $^{b}P < 0.01$ (χ^2 test). AP: Acute pancreatitis; BE: Base excess; BUN: Blood urea nitrogen; SIRS: Systemic inflammatory response syndrome; LDH: Lactate dehydrogenase.

treatment were higher in patients whose serum TG exceeded 200 mg/dL. The high-TG group patients were younger, predominantly male, obese, diabetic, and alcoholic.

However, the characteristics of the subjects were different between our study and the study by Nawaz *et al*[18]. The nationwide survey covered a wide range of hospitals and was not restricted to tertiary referral hospitals. The frequency of persistent organ failure was relatively low (4.9%) compared to the study by Nawaz *et al*[18] (26.9%). Subjects with a body mass index > 30% accounted for 43.8% of the patients in the study by Nawaz *et al*[18], whereas they accounted for only 4.8% in our study. Nevertheless, the nationwide survey confirmed that subjects with HTG are at high-risk for organ failure and require intensive care.

Age is also an important prognostic factor of patients with severe AP and it is included in the Japanese severity assessment system as well as in Ranson's criteria[21]. The mortality rate of severe AP patients younger than 30 years was 0%, but in those older than 80 years it exceeded 20%[5]. The high

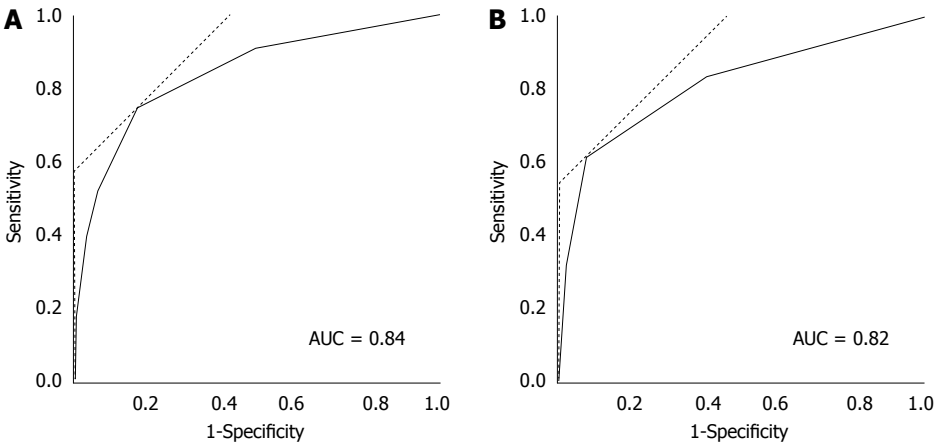

Figure 3 Receiver-operating characteristics curve analysis of the prognostic factor items. ROC curve analysis of the 9 (A) or 4 (B) prognostic factor items. AUC for predicting mortality was 0.84 for the 9 prognostic factor items and 0.82 for the 4 prognostic factors (base excess or shock, renal failure, SIRS criteria and age) and was found to be associated with AP-related death by a multivariate analysis. SIRS: Systemic inflammatory response syndrome; ROC: Receiver-operating characteristics curve; AUC: Area under the curve; AP: Acute pancreatitis.

Table 3 Comparison of the characteristics of patients with or without walled-off necrosis in 2011 survey *n* (%)

	WON (+), *n* = 124	WON (-), *n* = 226	*P* value
Median age (IQR)	59 (50-70)	62.5 (50-74)	0.190[1]
Male sex	102 (82.3)	164 (72.6)	0.042[2]
Severe pancreatitis	56 (45.2)	65 (28.8)	0.002[2]
Severity score ⩾ 3	19 (15.3)	23 (10.2)	0.160[2]
Severity score < 3	105 (84.7)	203 (89.8)	
CT grade ⩾ 2	51 (41.1)	54 (23.9)	0.001[2]
CT grade < 2	51 (41.1)	137 (60.6)	
CT grade unknown	22 (17.7)	35 (15.5)	
Etiology			
Biliary	23 (18.6)	42 (18.6)	0.980[2]
Alcohol	52 (41.9)	96 (42.5)	
Idiopathic	20 (16.1)	37 (16.4)	
Others	26 (21.0)	43 (19.0)	
Unknown	3 (2.4)	8 (3.5)	

[1]*t*-test; [2]χ^2 test. CT: Computed tomography; IQR: Interquartile range; WON: Walled-off necrosis.

mortality rate in aged patients was mainly due to organ failure, such as cardiovascular, respiratory, and renal failure. The higher mortality in aged patients will remain an important issue in the management of AP in aging countries like Japan.

MANAGEMENT OF WON IN JAPAN

The revised Atlanta classification for AP[16] defined WON, a disease entity previously known as pancreatic abscess or pseudocyst, as an encapsulated collection of necrotic tissue that develops later than 4 wk after the onset of AP. Infection of the necrotic tissue often requires prompt intervention, that had previously been performed primarily by open surgical approaches[22]. However, open surgical debridement of necrotizing pancreatitis is accompanied by a high hospital mortality of up to 23%[23].

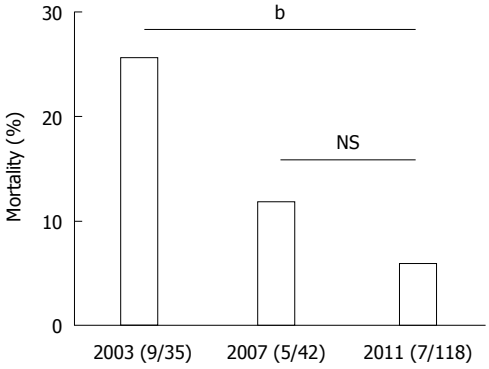

Figure 4 Temporal changes in mortality of patients with walled-off necrosis. Mortality of the patients with WON in the 2003, 2007, and 2011 surveys is shown. [b]$P < 0.01$ (χ^2 test). NS: Not significant; WON: Walled-off necrosis.

Recent advances in less-invasive, endoscopic approaches for WON treatment resulted in better clinical outcomes. A randomized, multicenter study clearly showed better clinical outcomes from the step-up approach over primary open necrosectomy in patients with necrotizing pancreatitis[24]. The management of WON has shifted from open surgical treatment to minimally invasive approaches. Due to its lower morbidity rate compared to surgical approaches, endoscopic treatment may be the preferred first-line approach for the treatment of WON[25].

To clarify the temporal changes in the treatment strategy and prognosis of WON in patients with AP in Japan, we analyzed the anonymous data of local complications collected by the 3 nationwide surveys in 2003, 2007, and 2011[5,7,8]. In the 2011 survey, information about the local complications was available for 350 patients. Because the term "pseudocyst" had been often used to describe a condition resembling WON in Japan, patients with pseudocysts later than 4 wk after the AP onset were included in this study.

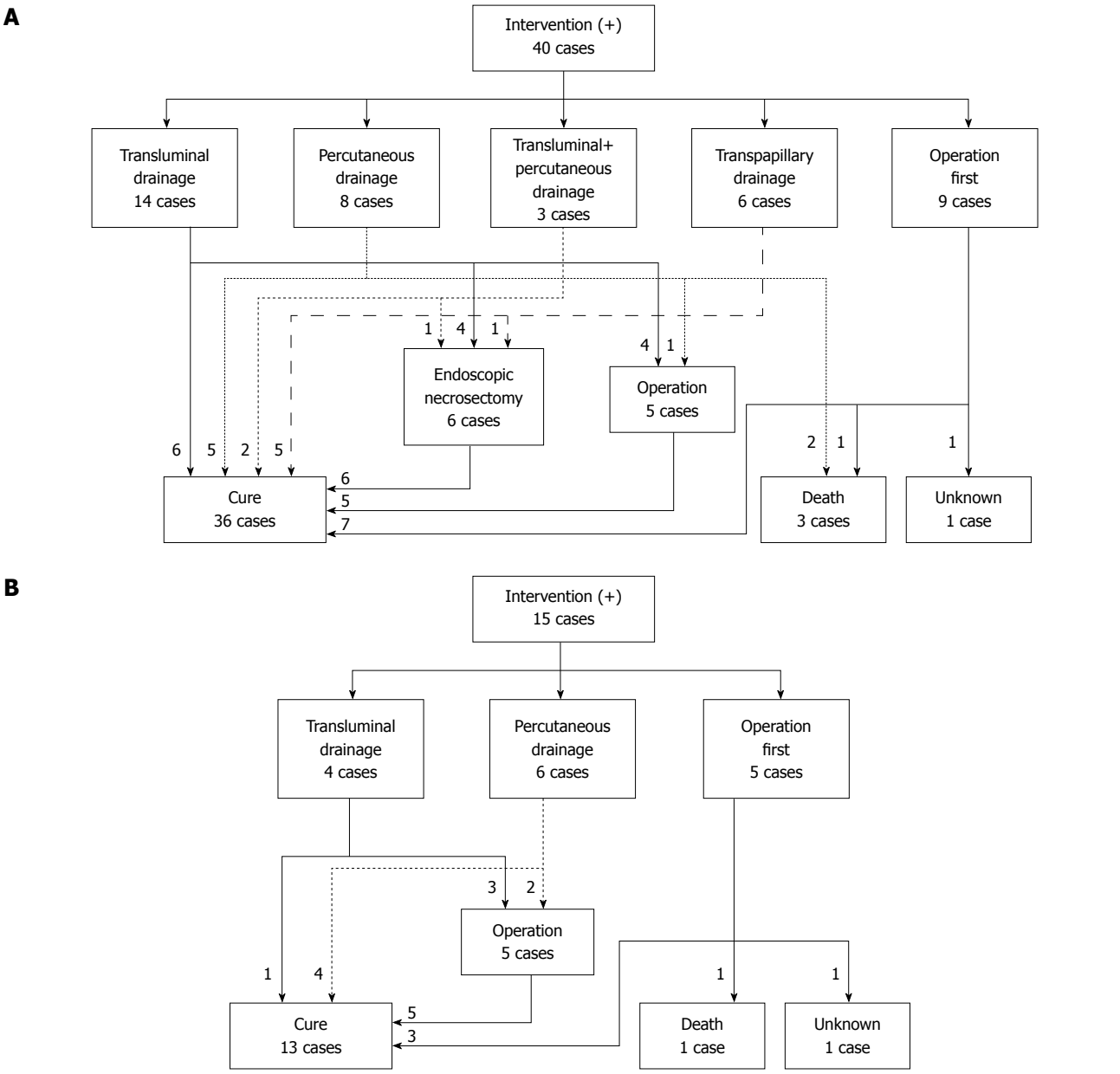

Figure 5 Flow-chart of treatment strategy for walled-off necrosis in the patients in the 2011 (A) and 2007 (B) surveys. A: The treatment strategy for the 40 patients who required intervention for WON in the 2011 survey is shown. B: The treatment strategy for the 15 patients who required intervention for WON in the 2007 survey is shown. WON: Walled-off necrosis.

At the time of the 2011 survey, the revised Atlanta classification for AP was not published yet and the term "WON" had not been well recognized in Japan.

The characteristics of the patients with WON (n = 124) were compared to those without WON (n = 226) (Table 3). Patients with WON were predominantly male, had more severe AP, and had higher CT imaging grades. In the 2003 and 2007 surveys, patients with pancreatic abscesses and/or intraabdominal abscesses were analyzed (n = 36 and n = 45, respectively). These patients were regarded herein as those with WON. The mortality of patients with WON due to AP-related events was 25.7% (9/35) in 2003, 11.9% (5/42) in 2007, and 5.9% (7/118) in 2011 (Figure 4). The mortality of AP patients with WON was significantly lower in 2011 compared to that in 2003 (P = 0.0008, χ^2 test).

Forty patients in the 2011 survey, 15 in the 2007 survey, and 25 patients in the 2003 survey required interventions for WON. In the 2011 survey, 9 of the 40 patients received open surgery as an initial treatment (Figure 5A). The other 31 patients received drainage therapies *via* transluminal, percutaneous, or transpapillary routes. Eighteen of the 31 patients were cured by drainage therapies alone. Endoscopic necrosectomy and surgery were performed in 6 and 5 patients, respectively. These 11 patients that received step-up approaches (drainage plus endoscopic necrosectomy or surgery) were cured. In the 2007 survey, 5 out of the 15 patients received surgery first

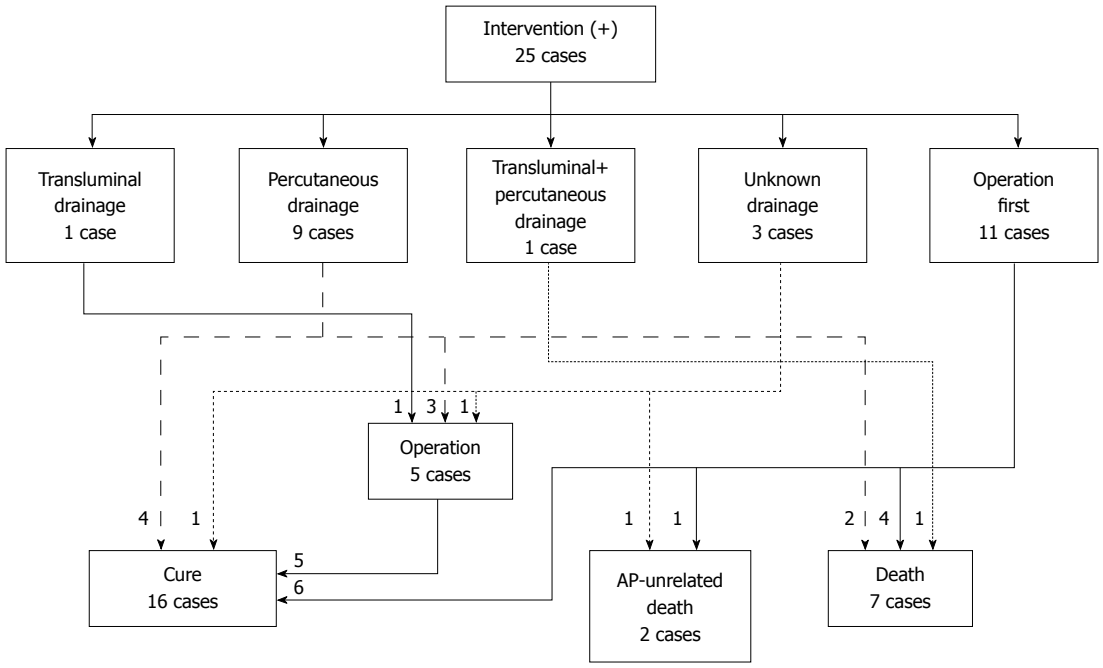

Figure 6 Flow-chart of treatment strategy for walled-off necrosis in the acute pancreatitis patients in the 2003 survey. The treatment strategy for the 25 patients who required intervention for WON in the 2003 survey is shown. AP: Acute pancreatitis WON: Walled-off necrosis.

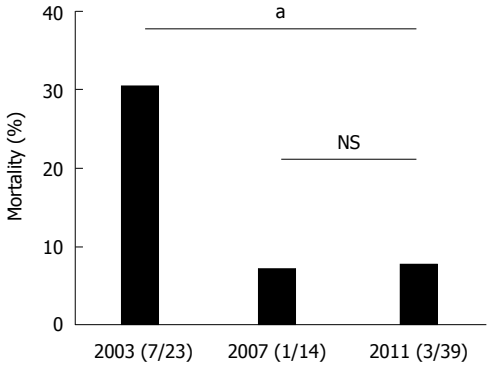

Figure 7 Temporal changes in mortality of patients with walled-off necrosis who received intervention. Mortality of the patients with WON in the 2003, 2007, and 2011 surveys is shown. $^{a}P < 0.05$ (χ^2 test). NS: Not significant; WON: Walled-off necrosis.

(Figure 5B). The other patients received drainage therapies and half of these patients were cured. The remaining 5 patients required surgery, and all of these patients were cured. In the 2003 survey, 11 out of 25 patients received surgery first (Figure 6). Among these patients, 4 patients died due to AP-related events. Other patients received drainage therapies, and 3 patients died. Five patients were cured by drainage therapies only and 5 patients required additional surgery (Figure 6).

The mortality of patients with WON receiving interventions was significantly lower in 2011 than that in 2003 (Figure 7). The mortality of the patients who required interventions was 30.4% (7/23) in 2003, 7.1% (1/14) in 2007, and 7.7% (3/39) in 2011 (excluding those with AP-unrelated deaths and unknown outcomes). In the 2003 survey, 44% (11/25) of the patients received surgery first treatment, and this ratio was reduced to 22.5% (9/40) in the 2011 survey. It is assumed that the proportion of patients receiving surgery first treatment has been decreasing further in recent years, because endoscopic necrosectomy has become popular since 2011[25]. Mortality would be further reduced by technical improvements in endoscopic interventions, such as balloon dilatation of a punctured tract, the placement of multiple plastic stents and a biflanged metal stent optimized for re-intervention[26,27].

Less-invasive endoscopic approaches for WON were accompanied by lower mortality, but several complications have been reported. Bleeding is the most common complication, followed by perforation and other rare complications[28]. Failure to control bleeding by an endoscopic approach will result in surgery or interventional radiology. A recent report described a standardized approach for endoscopic necrosectomy that could reduce the complication ratio, as defined by the assessment and management checklist for WON[29]. Such guidelines for the required equipment and backup preparations will further improve the clinical outcomes of WON treatment.

CONCLUSION

We reviewed the latest nationwide survey of AP in Japan. Nationwide surveys conducted regularly have provided us with updated information on the management of AP on a large, multicenter scale in Japan. Future studies on unsolved issues, including

the development of a more accurate and convenient severity assessment system and the optimization of therapeutic algorithms for the treatment of WON, would contribute to improved outcomes in this intractable disease.

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TOPIC HIGHLIGHT

2016 Colorectal Cancer: Global view

Mechanisms of resistance to anti-epidermal growth factor receptor inhibitors in metastatic colorectal cancer

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Abstract

The prognosis of patients with metastatic colorectal cancer (mCRC) remain poor despite the impressive improvement of treatments observed over the last 20 years that led to an increase in median overall survival from 6 mo, with the only best supportive care, to approximately 30 mo with the introduction of active chemotherapy drugs and targeted agents. The monoclonal antibodies (moAbs) cetuximab and panitumumab, directed against the epidermal growth factor receptor (EGFR), undoubtedly represent a major step forward in the treatment of mCRC, given the relevant efficacy in terms of progression-free survival, overall survival, response rate, and quality of life observed in several phase Ⅲ clinical trials among different lines of treatment. However, the anti-EGFR moAbs were shown only to be effective in a subset of patients. For instance, *KRAS* and *NRAS* mutations have been identified as biomarkers of resistance to these drugs, improving the selection of patients who might derive a benefit from these treatments. Nevertheless,

Author contributions: Sforza V performed research and wrote the paper; Martinelli E , Ciardiello F, Gambardella V, Napolitano S, Martini G, della Corte C, Cardone C, Ferrara ML, Reginelli A, Liguori G, Belli G and Troiani T contributed critical revision of the manuscript for important intellectual content.

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several other alterations might affect the response to these drugs, and unfortunately, even the responders eventually become resistant by developing secondary (or acquired) resistance in approximately 13-18 mo. Several studies highlighted that the landscape of responsible alterations of both primary and acquired resistance to anti-EGFR drugs biochemically converge into MEK-ERK and PIK3CA-AKT pathways. In this review, we describe the currently known mechanisms of primary and acquired resistance to anti-EGFR moAbs together with the various strategies evaluated to prevent, overcame or revert them.

Key words: Metastatic colorectal cancer; Epidermal growth factor receptor; Resistance; Mutation; *KRAS*; *NRAS*; *BRAF*; *PIK3CA*; *MET*; Monoclonal antibodies

Core tip: The treatment with anti-epidermal growth factor receptor (EGFR) monoclonal antibodies cetuximab and panitumumab in metastatic colorectal cancer is unfortunately burdened by the lack of clinical and molecular biomarkers that correlate with treatment response. Primary and acquired resistance have been shown to be the major culprits of the failure of anti-EGFR treatments. However, a deeper understanding of the molecular basis underlying both types of resistance has led to the proposal of several approaches designed to prevent, overcome or revert the drug resistance. Nevertheless, these approaches deserve further clinical investigation to allow us to use EGFR-targeted therapies more effectively in the correct population.

Sforza V, Martinelli E, Ciardiello F, Gambardella V, Napolitano S, Martini G, della Corte C, Cardone C, Ferrara ML, Reginelli A, Liguori G, Belli G, Troiani T. Mechanisms of resistance to anti-epidermal growth factor receptor inhibitors in metastatic colorectal cancer. *World J Gastroenterol* 2016; 22(28): 6345-6361 Available from: URL: http://www.wjgnet.com/1007-9327/full/v22/i28/6345.htm DOI: http://dx.doi.org/10.3748/wjg.v22.i28.6345

INTRODUCTION

Colorectal cancer (CRC) is considered the third most commonly diagnosed cancer in males and the second in females worldwide, with an estimated 1.4 million new cases in 2012. In the same year, CRC was responsible for 693900 deaths, making it the fourth leading cause of cancer-related death in men and the third in women[1]. Although the advances in screening and medical treatments have led a trend in reduction of both incidence and mortality, almost 20% of patients present metastases at the time of diagnosis, and approximately 35% of patients will subsequently develop a metastatic disease[2]. The prognosis of patients with metastatic colorectal cancer (mCRC) remains poor despite the impressive improvement observed over the last 20 years that led to an increase in median overall survival (OS) from 6 mo, with the only best supportive care (BSC), to approximately 30 mo with the introduction of active chemotherapy drugs, such as fluropyrimidines, oxaliplatin, irinotecan, TAS-102, and of targeted drugs, such as bevacizumab, cetuximab, panitumumab, aflibercept, and regorafenib[3,4]. However, despite these great advances, the efficacy of these treatments remains limited and substantially unpredictable. The reasons for this restricted success are firstly the development of various mechanisms of resistance to treatments and secondly the lack of clinical and molecular biomarkers that correlate with treatment response. This drug resistance is particularly relevant for drugs that target the epidermal growth factor receptor (EGFR).

EGFR is a tyrosine kinases receptor (RTK) and a member of the ErbB family to which HER2/neu (ERBB2), HER3 (ErbB3), and HER4 (ErbB4) also belong. EGFR is expressed in various cancers and in 60% to 80% of CRC, where it plays a key role in tumour development and progression. Various ligands (EGF, TGFα, amphiregulin, and epiregulin) bind specific extracellular domains (ECDs) of EGFR, inducing homo- and hetero-dimerization with other ErbB members and the subsequent activation of several pathways, including RAS-RAF-MEK-ERK and PIK3CA-AKT cascades, the SRC family kinases, PLCγ-PKC, and STATs. These pathways are involved in several cellular processes including tumour growth, inhibition of apoptosis, angiogenesis, invasion, and metastasis[5,6] (Figure 1).

For this reason, EGFR has been proposed as a target for anticancer therapies, and to date, two monoclonal antibodies (moAbs) against the EGFR, cetuximab and panitumumab, have been registered for the treatment of mCRC patients. Cetuximab, is a human-mouse chimeric monoclonal antibody (IgG1 subtype), whereas panitumumab is a fully human anti-EGFR monoclonal antibody (IgG2κ subtype). These antibodies bind the ECD of EGFR, blocking ligand-induced EGFR tyrosine kinase activation and causing EGFR cellular internalization and degradation[7,8]. Furthermore, cetuximab activates antibody-dependent cellular cytotoxicity. Cetuximab and panitumumab provided similar OS benefits in *KRAS* exon 2 wild-type (WT), chemotherapy-refractory mCRC in the ASPECCT trial[9].

EGFR-targeted therapies, both as single agents or in combination with chemotherapy, undoubtedly represent a major step forward in the treatment of mCRC, given the relevant efficacy in terms of progression-free survival (PFS), OS, response rate (RR), as well as quality of life, observed in several phase Ⅲ clinical trials among different lines of treatment[3]. However, not all patients will benefit from these treatments. Indeed, cetuximab and panitumumab

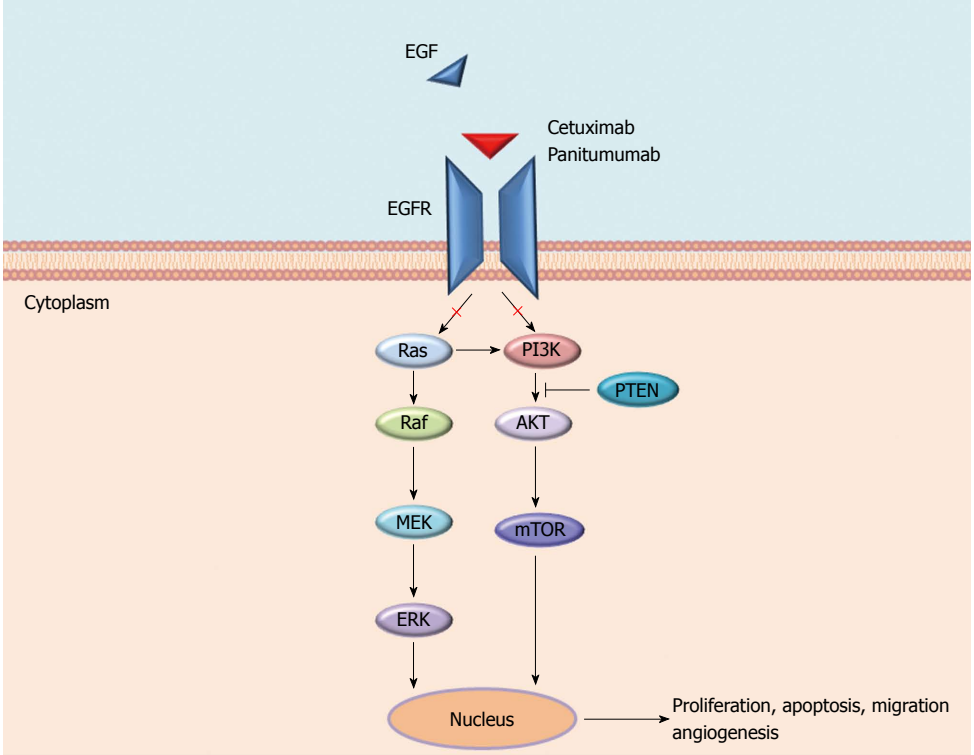

Figure 1 Epidermal growth factor receptor and its downstream signaling pathway. Binding of ligands such as epidermal growth factor (EGF) to Epidermal growth factor receptor (EGFR) activates downstream Ras/ERK and PI3K/Akt pathways and regulates various physiological processes. The anti-EGFR monoclonal antibodies (mAbs) cetuximab and panitumumab block the activation of these pathways.

when used as single agents in unselected patients with chemotherapy-refractory mCRC, achieved a RR of only 10%[10,11]. This low RR suggests that the majority of tumours harbour genetic alterations in proteins involved in EGFR pathway that impair the response to the anti-EGFR moAbs (intrinsic or primary resistance). Moreover, even the subset of patients who initially respond to these treatments will ultimately become refractory in approximately 3-18 mo by developing secondary (or acquired) resistance to anti-EGFR drugs[12]. These phenomenon might be explained if we consider that CRC, and in particular metastatic disease, is highly heterogeneous[13]. This heterogeneity implies that tumours from the same organ might have a completely different molecular landscape (inter-tumour heterogeneity) as well as different sensitivity to targeted agents, depending on which pathway is driving their growth. Furthermore, even in the same lesion, we might find clones with different sensitivity to drugs (intra-tumour heterogeneity) depending on the different molecular alterations harboured[14]. Unfortunately, to date, the molecular characteristics that allow the response to anti-EGFR moAbs are not yet completely understood, and the lack of predictive biomarkers do not permit the selection of patients who will potentially respond to these drugs. For instance, differently from other cancers, mutations in the EGFR or in downstream effectors of its signalling cascades (*e.g.*, KRAS, NRAS, BRAF, PIK3CA and PTEN loss) are not predictive of the efficacy of targeted agents[15,16].

In the era of targeted medicine, translational and clinical research efforts are being spent to better understand the complex molecular landscape of tumours to increasingly tailor the treatments to the molecular characteristics of the specific patient. The aim of this review is to provide an overview of the molecular mechanisms that underlie both primary and acquired resistance to anti-EGFR drugs in mCRC and to discuss possible future ways to circumvent them.

PRIMARY AND ACQUIRED RESISTANCE

Two sides of the same coin

The mechanisms of resistance to anti-EGFR moAbs can be categorized as primary or acquired according to the time of onset in respect to the treatment with these drugs, and also, although without a strict boundary, by the molecular alterations underlying them (Figure 2). Generally, the most frequent mechanisms of resistance are a result of genomic alterations in downstream effectors (*e.g.*, KRAS, NRAS, BRAF, and PIK3CA) of the EGFR signalling pathway, while the activation of other RTKs, such as MET or ERBB2 and their pathways, are more rare mechanisms[17-19]. In both cases, unless the EGFR continues to be pharmacologically blocked, an alternative signal transducer becomes activated, escaping the receptor inhibition. Notably, these genetic alterations have been identified as both mechanisms of primary and acquired resistance, and almost all of them biochemically converge on the activation of

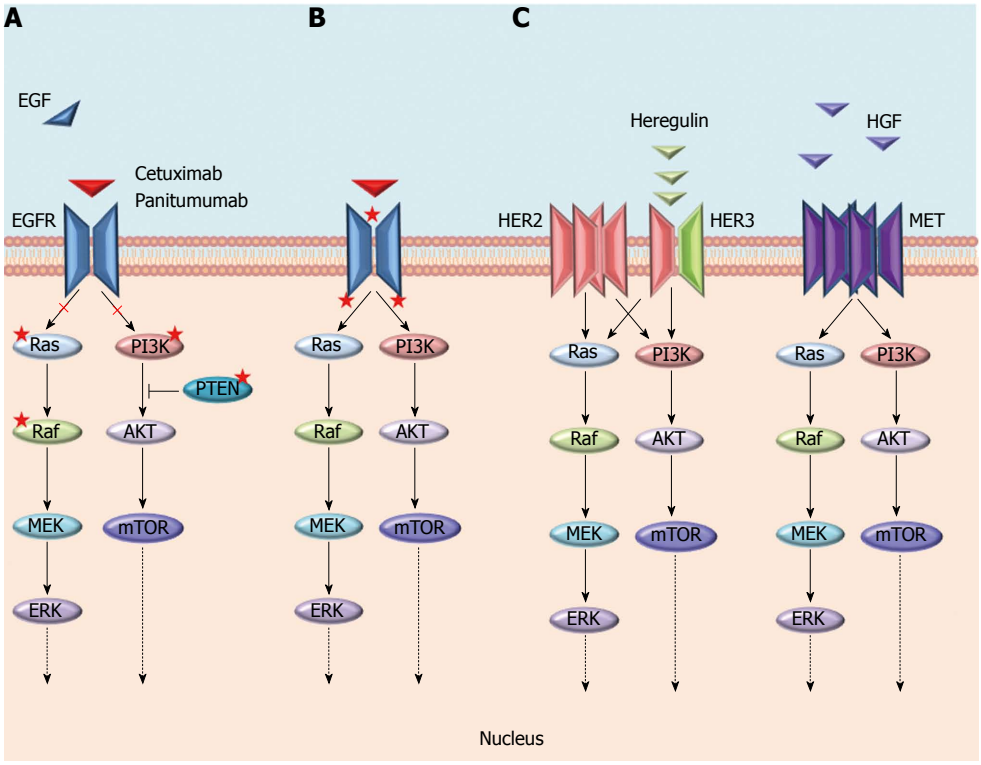

Figure 2 Mechanisms of resistance to anti-epidermal growth factor receptor monoclonal antibodies in metastatic colorectal cancer. A: Activating mutations of EGFR effectors, such as RAS, BRAF and PI3KCA, or PTEN loss of function, cause persistent activation of downstream signaling regardless of EGFR inhibition; B: Mutations in extracellular domain of EGFR inhibit cetuximab binding, but not panitumumab, mediating acquired resistance. Mutations in kinase domain of EGFR led pathways activation in the context of acquired resistance; C: Amplification/activation of alternative receptors such as HER2 or MET, can bypass the EGFR blockade and mediate pathways activation. EGFR: Epidermal growth factor receptor.

the MEK-ERK pathway[12]. The only exceptions are represented by rare mutations either in the ECD or in the tyrosine kinase domain of EGFR that have only been described as acquired mechanisms of resistance in patients treated with anti-EGFR moAbs[20-22]. Furthermore, different from primary resistance, acquired resistance is generally sustained by several genetic alterations that concomitantly emerge at treatment failure[22]. These aberrations may arise either as new genetic alterations, due to treatment-induced mutagenesis and tumour-intrinsic genomic instability (*e.g.*, mutations in ECD or tyrosine kinase domain of EGFR), or through the positive selection pressure of anti-EGFR therapies on a resistant subpopulation of cells already present in the original tumour[13]. Because of the overlapping of resistance mechanisms, the next chapters are focused on the description of single molecular alterations and whether the resulting mechanisms of resistance can be categorized as primary, secondary or both.

RAS

RAS is a family of three small GTPases (KRAS, NRAS, and HRAS) that work as downstream effectors within the mitogen-activated protein kinase (MAPK) pathway, coupling EGFR with intracellular signalling cascades[23]. The KRAS gene has been found mutated in approximately 40% of CRCs, mostly in exon 2 codons 12 (70%-80%) and 13 (15%-20%), whereas only a small percentage has been found in codons 61 (5%) and 146 (5%). These point mutations impair the intrinsic ATPase activity of RAS and cause the accumulation of mutant proteins in the active conformation (GTP-bound). The latter leads to constitutive MAPK pathway activation, regardless of the EGFR inhibition, that results in mitogenic and antiapoptotic signalling[17]. The mutational status of KRAS is highly concordant between the primary tumour and the metastasis, suggesting it has a role in the early processes of carcinogenesis[24].

In the early clinical trials in which cetuximab and panitumumab were used as monotherapies to treat patients with chemorefractory mCRC, an objective response rate (ORR) of only 10% was achieved[10,11]; these findings motivated researchers to elucidate the factors that were negatively impacting the efficacy of these drugs. In particular, retrospective analysis of KRAS mutational status from tumour samples of several randomized trials were able to strongly support the hypothesis that the *KRAS* mutations in codons 12 and 13 (exon 2) were associated with the lack of patient response to EGFR moAbs[17,25-27]. All together, the evidence led the American and European health authorities in 2009 to restrict the use of panitumumab and cetuximab only to the approximately 60% of patients with *KRAS* exon 2 WT tumours[26,28-31].

Nevertheless, because not all KRAS WT patients benefit from treatment with EGFR-directed therapy, researchers have tried to identify additional biomarkers of resistance that could explain this heterogeneity in clinical response. In particular, the retrospective analysis of the PRIME trial assessed the efficacy and safety of panitumumab plus oxaliplatin, fluorouracil, and leucovorin (FOLFOX4) compared with chemotherapy alone in first-line mCRC patients, according to RAS (*KRAS* or *NRAS*) mutation status. The results suggested that even mutations occurring in exon 3 (comprising codons 59 and 61), and exon 4 (which includes codons 117 and 146) of KRAS as well as mutations in exons 2, 3 and 4 of NRAS (all together called "expanded RAS mutations") can also be predictive of resistance to anti-EGFR treatments. These data were subsequently confirmed by the analyses of other phase Ⅱ and Ⅲ clinical studies[32-36]. In these trials, the prevalence of the expanded RAS mutations in patients defined as WT at exon 2 of KRAS ranged from 15% to 27%. Thus, considering all KRAS and NRAS mutations, approximately 53% of patients with mCRC are considered to have mutations in RAS and, therefore, to be refractory to EGFR blockade[32-36]. In line with these findings, our group published the results from the phase Ⅱ CAPRI trial, in which patients with *KRAS* exon 2 WT mCRC were treated with FOLFIRI plus cetuximab in first-line treatment and were then randomized at progression to receive FOLFOX alone or FOLFOX plus cetuximab. Our results confirmed the lack of benefit of cetuximab among the subset of patients harbouring KRAS or NRAS mutations[37]. Furthermore, in 2014, Sorich *et al*[16] published a meta-analysis of nine randomized controlled trials (RCTs) evaluating the role of EGFR antibodies in all lines of mCRC therapy. The meta-analysis revealed that treatment with anti-EGFR antibodies had superior efficacy in terms of PFS and OS for all *RAS* WT tumours compared with the expanded RAS mutant subgroup, and the efficacy was not significantly different between the expanded RAS mutant and *KRAS* exon 2 mutant subgroups. These results suggest that tumours with one of the new RAS mutations are more appropriately grouped with the tumours with a *KRAS* exon 2 mutation (forming the any *RAS* mutant group), rather than with tumours that do not have any RAS mutations[16]. These results demonstrated the prominent role of RAS mutations as biomarkers of primary resistance to anti-EGFR therapies. In response to the meta-analysis, the EMA and FDA have updated the prescribing indications for panitumumab and cetuximab, restricting their use to patients with *RAS* WT mCRC[38,39].

As said before, genetic alterations in RAS are even the most common molecular mechanisms that drive secondary resistance to anti-EGFR therapy in 50% to 80% of patients. These mutations may be present in a small fraction of cells within the tumour before treatment initiation and then may be selected by pressure from the anti-EGFR treatments or arise as a result of continued mutagenesis during the treatment[40,41]. Different from primary resistance, secondary resistance generally arises from more than one driver and arises at different frequencies. This pattern has been observed in both cell lines made resistant to cetuximab or panitumumab as well as in samples obtained from patients[42,43]. It is worth noting that the frequency of mutations at codon 61 of exon 3, in either *KRAS* or *NRAS* genes, is more prevalent in the acquired setting[22,42]. However, the KRAS gene has been found not only mutated but also amplified, although in a very small percentage of patients (0.7%), and this amplification has been observed as a mechanism in both primary and acquired resistance[43].

However, preclinical data and retrospective analysis from phase Ⅲ trials highlighted that not all *KRAS* mutations have the same role in mediating EGFR-resistance, and some patients with *KRAS* mutated tumours occasionally respond to anti-EGFR treatments. In particular, patients harbouring the *KRAS* G13D mutation have been found to achieve a benefit from cetuximab in both first-line and advanced lines of treatment[44,45]. A recently published meta-analysis of eight RCTs have assessed whether the efficacy of anti-EGFR mAbs for mCRC differs between tumours harbouring the *KRAS* G13D mutation and *KRAS* mutations other than the *KRAS* G13D mutation. The authors did not find any significant difference in terms of PFS or in OS between *KRAS* G13D and other *KRAS* mutations[46]. Schirripa *et al*[47] conducted a phase Ⅱ single-arm trial to provide prospective proof of the clinical benefit of cetuximab in *KRAS* G13D mutant mCRC patients. However, among 12 treated patients, no responses have been observed. Along the same lines, the Australasian Gastro-Intestinal Trials Group recently published the results of the phase Ⅱ ICE CREAM study (Irinotecan Cetuximab Evaluation and Cetuximab Response Evaluation Among Patients with a G13D Mutation), in which patients with G13D-mutated chemotherapy-refractory mCRC, who had progressed within 6 mo of irinotecan therapy, were randomly assigned to receive cetuximab with or without irinotecan. Among the 51 patients recruited, there was no statistically significant improvement in disease control at 6 mo with either cetuximab monotherapy or cetuximab plus irinotecan. Furthermore, no responses were observed with single-agent cetuximab[48].

The intra-tumour heterogeneity noted that CRC is often formed of clones with different mutational profiles and that in many tumours only a fraction of neoplastic cells carries the mutant allele. Given these considerations, Normanno *et al*[49] have proposed a quantitative assessment of *KRAS* mutation load as a tool to discriminate whether a low content of *KRAS* mutant alleles in mCRC cells may affect the response anti-EGFR moAbs. Although they found that patients with low *KRAS* mutation content responded to EGFR-based therapy, this benefit did not translate in a PFS advantage; indeed, PFS was similar to patients with

high KRAS tumours. The authors have suggested that it might be explained by either the expansion of a small fraction of cells carrying a resistance mutation or by the coexistence, in several low *KRAS* mutated tumours, of other mutations such as *BRAF* and *PIK3CA*. A quantitative assessment of mutational load might be useful to identify the priority target for therapeutic intervention; however, the complexity of tumour mutational profiles suggests that for many tumours combinations of target-based agents will likely to be necessary to control tumour growth[49].

Considering the key role of *RAS* mutations as mechanisms of resistance to anti-EGFR moAbs, many approaches have been investigated to target these mutations. One of the earlier approaches was the inhibition of RAS farnesylation that showed a potent antitumour activity in preclinical studies; however, this result was not confirmed in clinical experience. Several other approaches have been used to target RAS: blocking downstream effectors such as MEK and PIK3CA, identification of synthetic lethal (SL) interactions with mutant *KRAS*, or the use of small molecule inhibitors of KRAS[42,50-54]. Recently, a combination therapy co-targeting MEK and CDK4/6 with trametinib and palbociclib, respectively, was well tolerated and highly efficacious in *KRAS*-mutant CRC patient tumour-derived PDX[55,56]. However, a clinical evaluation of these agents is currently lacking. A biological strategy with Reovirus Serotype 3 - Dearing Strain (Reolysin®), a naturally occurring ubiquitous, non-enveloped human Reovirus, has been explored in *KRAS*-mutated mCRC because reovirus has been shown to replicate selectively in RAS mutant cells, causing cell lysis. A multicentre phase I study of Reolysin® in combination with FOLFIRI and bevacizumab in naïve patients with *KRAS*-mutated mCRC is ongoing[57]. To date, RAS remains the most elusive gene to target; thus, patients with *RAS* mutations are currently treated with chemotherapy with or without antiangiogenic drugs[3].

BRAF mutations

Mutation in other downstream effectors of the MAPK pathway beyond RAS may invalidate the effects of anti-EGFR moAbs, cetuximab and panitumumab. One example is BRAF, a serine/threonine protein kinase that is found mutated in approximately 10%-15% of CRCs[58]. The most common BRAF alteration is the point mutation V600E in the kinase domain, accounting for 80% of all *BRAF* mutations[59]. The BRAF V600E mutation is generally mutually exclusive with RAS mutations. However, recent data from the CAPRI trial demonstrated, in 12 out of 15 *BRAF* mutated tumour samples, the coexistence of *BRAF* mutations with other mutations, including *TP53, KRAS,* and *PIK3CA* exon 9 and exon 20[37,60].

The BRAF V600E mutation represents a biomarker of poor prognosis in the CRC population. This finding emerged from analyses of a large cohort of patients enrolled in several clinical trials that have consistently demonstrated the association of the *BRAF* V600E mutation with an increased colon cancer-specific mortality[61,62].

Although the prognostic role of *BRAF* mutation is well established, its role as a predictive biomarker to anti-EGFR treatments is not clearly understood because of both its low prevalence and the prominent negative prognostic role. Several studies have showed that patients harbouring a *BRAF* mutation do not achieve any benefit from anti-EGFR treatments in second-line or in later lines of therapy[62-64]. However, data from the first-line setting are less clear. For instance, in both CRYSTAL and OPUS studies, the addition of cetuximab to FOLFIRI and FOLFOX, respectively, slightly improved PFS and OS compared to chemotherapy alone[31,65]. On the contrary, recent studies in patients receiving first-line anti-EGFR moAbs in combination with chemotherapy did not show a statistically significant correlation between *BRAF* V600E mutation and response[32,34]. Also a recent meta-analysis of nine phase Ⅲ trials revealed that the addition of an anti-EGFR moAbs to first-line doublet chemotherapy for patients with *BRAF*-mutant disease was not associated with improved OS, PFS or ORR[66]. In conclusion, the small percentage of *BRAF* mutated patients, together with a lack of prospective studies, do not allow one to establish with certainty the predictive role of BRAF mutation for treatment with cetuximab and panitumumab.

BRAF mutation has been recognized, by circulating tumour DNA (ctDNA) analysis, also as a mechanism of acquired resistance in patients who first responded to anti-EGFR therapy[22].

In a phase Ⅱ trial, the BRAF inhibitor vemurafenib has been tested in previously treated patients with *BRAF*-mutated mCRC showing only a modest clinical activity (5% of RR) with respect to the impressive results (RR of 48% to 67%) obtained in *BRAF*-mutated melanoma[67]. In cell line models, it has been found that resistance to the BRAF-targeted approach seems to be caused by either persistent activation of the EGFR signalling pathway or the activation of other pathways such as PIK3CA/AKT[68,69]. These findings showed that the biology of BRAF activation is more heterogeneous in CRC than in other tumours and suggested a potential role for a combination approach. Indeed, the combination of dabrafenib and trametinib (BRAF/MEK inhibitor therapy) obtained better results than vemurafenib monotherapy in 43 patients with BRAF V600-mutant mCRC[70]. Furthermore, the first trials testing the double blockade with the BRAF inhibitor vemurafenib and either the EGFR inhibitor panitumumab or a PI3KCA/mTOR inhibitor demonstrated modest activity[71,72]. To date, several clinical trials are assessing the effects of BRAF inhibitors in combination with MEK, EGFR and PI3K inhibitors[73].

The use of ERK inhibitors to suppress MAPK activity is another potential strategy because it has been

observed that MAPK is usually upregulated in patients resistant to RAF inhibitors[74].

It is worth noting that the TRIBE study, comparing FOLFOXIRI plus bevacizumab *vs* FOLFOXIRI in first-line mCRC patients, showed a relevant hazard ratio for progression of 0.55 in favour of the combination with bevacizumab (interaction test $P = 0.320$), in the subgroup of 28 patients with tumours harbouring BRAF mutations[75].

PIK3CA mutations and loss of PTEN

The PIK3CA/AKT/mTOR signalling pathway is associated with several RTKs, including EGFR[76]. Alterations in genes that encode for these proteins play an important role in the development of malignant tumours and could impair the response to EGFR-targeted moAbs. In particular, activating mutations of *PIK3CA*, mostly occurring in exons 9 and 20, have been found in 10%-20% of CRCs, and in preclinical models, they were found to be predictive of a lack of benefit from cetuximab treatment[77-79]. In a retrospective analysis of 110 mCRC patients treated with anti-EGFR moAbs, a statistically significant association between *PIK3CA* mutations and primary resistance to treatment with cetuximab or panitumumab was reported in the population of patients with KRAS WT tumours[80]. A large retrospective analysis of 1022 tumour samples of patients treated with cetuximab described two important findings: (1) only *PIK3CA* exon 20 mutations was predictive of a lack of response to cetuximab in the *KRAS* WT subpopulation; and (2) *PIK3CA* exon 9 mutations and *KRAS* mutations were associated, suggesting a secondary role of *PIK3CA* exon 9 mutations on cetuximab efficacy[17]. Thereafter, two meta-analyses also described a negative predictive role of only *PIK3CA* mutations in exon 20 in terms of ORR, PFS, and OS in WT *KRAS* mCRC patients treated with anti-EGFR therapies[81,82]. Nevertheless, is difficult to evaluate the precise role of *PIK3CA* mutations with respect to anti-EGFR resistance because they are mostly found concurrently with *KRAS* or *BRAF* mutations and because of their low incidence, especially the exon 20 mutations. *PIK3CA* mutations have also been identified as mechanisms of secondary resistance in samples from patients who experienced relapse after treatment with EGFR-targeted moAbs[21].

Several data have also suggested a role of PIK3CA mutations as a prognostic biomarker. Indeed, an increased colon cancer-specific mortality has been found in patients with *PIK3CA*-mutated tumours, compared with WT ones. However, only the coexistence of *PIK3CA* exon 9 and 20 mutations has been reported to be associated with a worse prognosis than WT tumours[83]. Furthermore, *PIK3CA* mutations seem to predict a worse prognosis only in *KRAS* WT patients compared with *KRAS*-mutated[84].

PIK3CA signalling pathway may also be pathologically activated by the loss of PTEN, found in 30% of CRCs[85]. Firstly, Frattini *et al*[86] found in a cohort of patients treated with cetuximab and irinotecan that of 11 patients with lower PTEN expression in tumour samples at immunohistochemistry (IHC), none of the patients responded to treatment when compared with patients with normal PTEN expression who achieved a partial response. PTEN loss was also associated with shorter OS in patients with *KRAS* WT tumours treated with a cetuximab-based regimen[64]. In a retrospective analysis of a cohort of patients treated with cetuximab plus irinotecan, Loupakis *et al*[87] showed that PTEN IHC results were not completely concordant between primary tumours and metastases and that the PTEN status of primary tumours was not significantly predictive of cetuximab activity. Conversely, when PTEN IHC was performed on metastases, 36% of the patients with PTEN-positive samples responded to therapy compared with patients who harboured a PTEN-negative status. They also showed that patients who harboured both a KRAS WT tumour and PTEN-positive metastasis were much more likely to benefit from treatment in terms of RR, PFS, and OS. However, in the NCIC CTG/AGITG CO.17 trial, where 572 patients with pretreated mCRC were randomly assigned to receive cetuximab or BSC, no statistical significance was found with respect of the loss of PTEN and the clinical outcome of patients treated with cetuximab[88].

PTEN loss has been identified only as a primary mechanism of resistance to cetuximab and panitumumab. However, to date, *PIK3CA* mutations and PTEN expression have not been validated as predictive markers for EGFR moAbs therapy in mCRC for several key reasons. Firstly, PIK3CA and PTEN alterations mostly co-occur with *RAS* and/or *BRAF* mutations; secondly, the expression of PTEN protein by IHC is burdened by conflicting interpretations; and lastly, only PTEN expression in metastases, but not in primary tumours, has been associated with outcome[89].

Targeted treatments against PIK3CA or its downstream effectors such as mTOR or AKT in patients harbouring *PIK3CA* mutations or *PTEN* loss, although interesting, did not show a meaningful clinical activity[90,91]. A greater therapeutic effect has been observed when these drugs have been combined with RTK inhibitors in preclinical models; however, this benefit has not been confirmed in phase I trials[92]. Clinical trials evaluating the combination of the mTOR inhibitor everolimus with panitumumab and irinotecan in first-line mCRC patients are ongoing[93]. The combination of a PIK3CA inhibitor and a MEK inhibitor in preclinical models was more effective than MEK and PI3K/mTOR inhibition, and several clinical trials are exploiting this combination[94-96]. Furthermore, low-dose aspirin seems to improve survival in patients with *PIK3CA*-mutant tumours by aspirin-mediated COX2 inhibition, but this observation requires further prospective evaluation[97].

HER2/HER3

HER2 is a member of the ErbB family and is a recognized target in breast cancer[6]. This receptor does not

have any known ligand, and its activation is provided by the hetero-dimerization with other ligand-bound receptors of the same family. The preferred partner is HER3, and the heterodimer HER2-HER3 represents a powerful activator of intracellular signalling[98]. HER2 amplification allows the activation of MEK-ERK cascade regardless of the signalling of EGFR.

In two different studies, HER2 amplification has been highlighted as a predictor of lack of response to anti-EGFR antibodies[99,100]. In 2011, Bertotti *et al*[19] recognized HER2 amplification as a potential mechanism of primary resistance to cetuximab within a quadruple WT population (*KRAS*, *NRAS*, *BRAF*, and *PIK3CA* wild type) of immune-compromised mice (xenopatients) transplanted with fragments of CRC samples from patients. Firstly, the authors observed that HER2 was amplified only in a small percentage (2%-3%) of genetically unselected CRC patients. However, a greater frequency of HER2 amplification was observed in *KRAS* WT patients resistant to cetuximab (13.6%) and in up to 36% of xenopatients in the subset of quadruple WT, in which treatment with cetuximab was ineffective. The authors also envisioned a possible role of HER2 as a positive predictor of response to HER2-targeting agents in CRC. Hence, they performed a multiarm xenotrial demonstrating that the association of a dual EGFR/HER2 small molecule inhibitor (lapatinib) and cetuximab or pertuzumab, a monoclonal antibody directed against EGFR/HER2 heterodimer, was active in the subset of cetuximab-resistant, quadruple-negative, HER2-amplified metastatic CRC xenopatients and was a feasible combination for clinical trials[19]. Based on these findings, Siena and colleagues designed the HERACLES trial, a multicentre open-label phase Ⅱ trial, assessing the RR of trastuzumab combined with either lapatinib (cohort A) or pertuzumab (cohort B), in *KRAS* exon 2 (codons 12 and 13) WT and HER2 amplified mCRC patients who were resistant to standard therapies, including anti-EGFRs. To date, the results from cohort A have been recently published. The authors reported a frequency of 5% (48 patients) of HER2-positive tumours among 914 patients screened for the trial. Of the 27 patients enrolled, eight (30%, 95%CI: 14-50) achieved an overall objective response, and the median duration of the responses was 38 wk. Median PFS was 21 wk (95%CI: 16-32), while median OS calculated post hoc was 46 wk (95%CI: 33-68). The authors reported that responses were significantly more common in tumours with high *HER2* gene copy number. Additionally, the PFS was longer in this population. Finally, the combination was well tolerated, with most toxic effects being grade 1 or 2. To date, HER2 is the first druggable target in mCRC that has been shown to be a good predictor of response to targeted treatments[99].

Data published by Yonesaka *et al*[99] demonstrated the role of HER2 signalling in the context of acquired resistance to anti-EGFR moAbs. Moreover, they reported that hyper-activation of HER2 signalling was not only led by HER2 amplification but also through the overproduction of heregulin, an HER3 ligand. Using resistant clones from cetuximab-sensitive cell lines, as well as plasma and tissue samples from cetuximab-treated mCRC patients, the authors found that patients with acquired resistance to cetuximab had an increased percentage of HER2 amplification in post-treatment samples (71%) compared to the proportion present in pretreatment tumour cells (14%). In the same way, heregulin levels in plasma and tumour samples were significantly higher in patients who experienced a disease progression on anti-EGFR therapy. This indicates that enhanced HER2 signalling confers not only primary but also acquired resistance to anti-EGFR moAbs by leading to persistent activation of ERK signalling[99].

Additionally, HER3 has been described to have a role as a potential biomarker of resistance to anti-EGFR treatments. In a cohort of metastatic CRC patients treated in second- or third-line therapy with irinotecan and cetuximab, HER3 overexpression was associated with shorter PFS and OS[101]. Moreover, HER3 has been found mutated in approximately 11% of CRC patients, and these mutations, even if HER2 is not amplified, may limit the responsiveness to EGFR inhibitors[102].

MEHD7945A is a humanized IgG1 mAb with dual anti HER3/EGFR activity. In multiple xenograft models, MEHD7945A was demonstrated to be significantly superior with respect to the monospecific EGFR targeting agents[103]. In a phase I study, promising results have been achieved among patients with pretreated mCRC; however, no benefit for MEHD7945A + FOLFIRI *vs* cetuximab + FOLFIRI has been observed in a phase Ⅱ randomized trial in KRAS WT mCRC patients refractory to oxaliplatin[104,105].

EGFR mutations

EGFR mutations in CRC represent a mechanism of resistance described only in the acquired setting and might occur in approximately 20% of patients treated with cetuximab and in only 1% of patients treated with panitumumab[106]. In particular, Montagut *et al*[20] discovered a point mutation in the ECD of EGFR (S492R) in a CRC cell line made resistant to cetuximab and also confirmed these data in a small percentage of patients who relapsed after cetuximab treatment. This mutation does not allow the binding of cetuximab to the receptor, but it still allows the binding of panitumumab; indeed, the authors reported the experience of a patient with this mutation who was treated with panitumumab and who achieved a response from treatment[20]. Recently, Arena *et al*[21] discovered several other mutations in the EGFR ECD. They analysed tumour samples (pre- and post-treatment with cetuximab) obtained from 37 mCRC patients with acquired resistance to cetuximab and found two novel mutations, R451C and K467T, in two patients. The authors also discovered several other novel EGFR variants (S464L, G465R and I491M)

in CRC cell lines made resistant to cetuximab. These mutations are located in the cetuximab-binding region, with the exception of the *R451C* mutation. Functionally, all of these mutations prevent binding of cetuximab, and only *R451C* and *K467T* mutations are permissive for interaction with panitumumab[21].

Furthermore, Bettegowda *et al*[22] also described mutations in the EGFR kinase domain at codons 714 and 794 that were identified as circulating mutations by cell-free DNA analysis only in the setting of acquired resistance.

One possible strategy to overcome the resistance to anti-EGFR moAbs mediated by mutations in ECD of EGFR would be to create moAbs that bind to different epitopes located in this region. For instance, Sym004 is a mixture of two different mAbs directed against non-overlapping epitopes of domain Ⅲ of the EGFR. The antibodies bind simultaneously to the ECD of EGFR, inducing internalization and degradation of the receptor. In preclinical studies, Sym004 demonstrated a stronger tumour suppression compared to cetuximab and panitumumab[22].

Dienstmann and colleagues reported the results from the phase Ⅰ trial in which 42 patients with mCRC and acquired resistance to anti-EGFR inhibitors were enrolled to receive different doses (9 or 12 mg/kg weekly) of Sym004. All patients had a documented response to previous anti-EGFR mAb treatment followed by disease progression. Of the 39 patients evaluable for response, 17 (44%) had a different degree of tumour shrinkage, with an overall disease control rate (partial response and stable disease) of 67%; median PFS was 3.3 mo (95%CI: 2.6-4.9). Regarding toxicities, the most common drug-related adverse events of any grade were skin rash, dry skin, hypomagnesemia and pruritus[107,108]. Currently, Sym004 is under investigation in a phase Ⅱ clinical trial as a monotherapy in selected patients with *KRAS* WT CRC progressing to previous cetuximab or panitumumab-based therapy within 6 mo from trial enrolment.

Another drug, MM-151, is a third-generation EGFR inhibitor consisting of a mixture of three fully human IgG1 antibodies that bind distinct, non-overlapping epitopes on EGFR. In preclinical models, MM-151 showed an enhanced anticancer effect by improving the EGFR pathway inhibition, as well as inducing a more profound downregulation of the receptor and stimulating the innate immune responses[109]. The results of the phase I trial with MM-151 alone *vs* combination with irinotecan demonstrated the safety of the drug, and in particular, demonstrated an unusually long-lasting disease control in the combination arm.

MET

MET is a tyrosine kinase receptor involved in several cell processes, such as proliferation, apoptosis, invasion and angiogenesis. It is activated by its ligand, the hepatocyte growth factor (HGF). Several mechanisms may lead to an abnormal activation of MET, including MET amplification and/or increased expression of HGF[110].

In mCRC cancer, MET has been found amplified in approximately 2% of samples, where it has been associated with the development of distant metastases, and it has been significantly correlated with poor outcomes[111,112]. Furthermore, Bardelli *et al*[18] highlighted the role of MET amplification as a mechanism of both primary and acquired resistance to anti-EGFR moAbs in WT *KRAS* mCRC patients. They also showed an increased rate of amplification (12.5%) in a cetuximab-resistant xenopatients WT for *RAS*, *BRAF*, *PIK3CA*, and *HER2*[18]. However, only focal, high-grade amplification of the MET locus is associated with lack of response instead of modest gene copy number gains or polysomy of chromosome 7[113].

In preclinical models, Troiani *et al*[114] found that overexpression of TGF-alpha might contribute to cetuximab resistance in CRC cells through the induction of a EGFR-MET interaction; the treatment of these cells with a selective MET inhibitor restores cetuximab sensitivity, suggesting that the combined inhibition of both EGFR and MET pathways could represent a rational therapeutic strategy for preventing and/or overcoming cetuximab resistance in patients with mCRC.

In a randomized phase Ⅱ clinical trial of chemo-refractory, *KRAS* WT, anti-EGFR-naive mCRC, the combination of an anti-HGF moAb and panitumumab led to higher RR and a trend for a better outcome in the population with MET-overexpressed[115]. A phase I trial assessing the role of cabozantinib, a small molecule inhibitor of the tyrosine kinases c-Met and VEGFR2, plus panitumumab in chemo-refractory, KRAS WT patients is currently ongoing[116].

FUTURE DIRECTIONS

Treatment with anti-EGFR moAbs cetuximab and panitumumab might be limited by the presence of primary resistance or the emergence of multiple acquired mechanisms to escape from the EGFR blockade. To overcome these phenomena, several approaches have been proposed, and some of them have been anticipated above (Table 1).

To increase the efficacy of anti-EGFR mAbs, combinations of these drugs and vascular endothelial growth factor receptor (VEGFR) inhibitors were evaluated. However, the addition of cetuximab or panitumumab to bevacizumab and oxaliplatin-based chemotherapy in first-line *KRAS* WT mCRC patients, did not improve the outcomes[117,118].

Furthermore, although there were positive effects on both PFS and RR, the combination of cetuximab plus brivanib (a VEGFR multikinase inhibitor) increased toxicity and did not improve OS in patients with chemotherapy-refractory, *KRAS* WT mCRC[119].

One of the most promising approaches to circu-

Table 1 On-going clinical trials to overcome resistance to anti-epidermal growth factor receptor

Target	Drug	Study	ClinicalTrials.gov Identifier	Phase
EGFR	Afatinib Cetuximab	Afatinib and cetuximab combo *vs* cetuximab alone in treatment of patients with refractory *KRAS* WT mCRC	NCT01919879	Phase Ⅱ
EGFR	Sym004	Sym004 *vs* investigator's choice (best supportive care, capecitabine, 5-FU) in subjects with mCRC and acquired resistance to anti-EGFR moAbs	NCT02083653	Phase Ⅱ
EGFR, HER2, HER4	Neratinib Cetuximab	Neratinib and cetuximab in *KRAS*/*NRAS*/*BRAF*/*PIK3CA* WT mCRC patients resistant to cetuximab	NCT01960023	Phase Ⅰ/Ⅱ
EGFR	Cetuximab Irinotecan	Cetuximab plus irinotecan as rechallenge 3rd-line treatment of *KRAS*, *NRAS* and *BRAF* WT irinotecan-pretreated mCRC patients progressing after an initial response to a 1st-line cetuximab-containing therapy and a standard 2nd-line	NCT02296203	Phase Ⅱ
EGFR	Panitumumab FOLFIRI	FOLFIRI plus panitumumab in extended *RAS* WT and *BRAF* WT mCRC with acquired resistance to prior cetuximab (or Panitumumab) plus irinotecan-based therapy and who failed at least one subsequent non-anti-EGFR containing regimen	NCT02508077	Phase Ⅱ
ERBB2	Pertuzumab Trastuzumab	Pertuzumab and lapatinib in *KRAS* exon 2 WT and HER2-positive mCRC refractory to standard of care (including cetuximab or panitumumab)	-	Phase Ⅱ
MET	Cetuximab INC280	INC280 in combination with cetuximab in c-MET positive mCRC and HNSCC Patients who have progressed after anti-EGFR moAbs therapy	NCT02205398	Phase Ib
MET	Cabozantinib Panitumumab	Cabozantinib with panitumumab in subjects with *KRAS* WT refractory mCRC		Phase Ⅱ
BRAF/EGFR	Vemurafenib Cetuximab Irinotecan	Irinotecan and cetuximab with or without vemurafenib in *BRAF* mutant mCRC patients	NCT02164916	Phase Ⅱ
BRAF/PI3K/EGFR	LGX818 BYL719 Cetuximab	LGX818 and cetuximab or LGX818, BYL719, and cetuximab in *BRAF* mutant mCRC patients	NCT01719380	Phase Ⅰ/Ⅱ
BRAF/MEK/EGFR	Trametinib Dabrafenib Panitumumab	Trametinib and dabrafenib administered in combination with panitumumab in BRAF-V600E positive mCRC patients with secondary resistance to prior anti-EGFR therapy	NCT01750918	Phase Ⅰ/Ⅱ
PI3K-mTOR	Panitumumab Everolimus Irinotecan	Second line therapy with panitumumab, irinotecan and everolimus in *KRAS* WT mCRC patients	NCT01139138	Phase Ib/Ⅱ
PI3K/MEK	BKM120 MEK162	BKM120 plus MEK162 in adult patients with selected advanced solid tumors	NCT01363232	Phase Ib
Immune evasion	Pembrolizumab Cetuximab	Pembrolizumab plus cetuximab for *KRAS-NRAS-BRAF* WT mCRC patients	NCT02318901	Phase Ⅰ/Ⅱ

EGFR: Epidermal growth factor receptor; mCRC: Metastatic colorectal cancer; WT: Wild type; mAb: Monoclonal antibody; FOLFIRI: Fluorouracil, leucovorin, and irinotecan; PI3K: Phosphoinositide kinase-3; mTOR: Mammalian target of rapamycin.

mvent or reverse resistance to anti-EGFR moAbs is to target more than one downstream effector of the EGFR pathway. As reported above, their use as single agents did not prove clinical efficacy because of the activation of alternative pathways as a mechanism to escape the blockade. Because the alterations that confer resistance to the anti-EGFR moAbs biochemically converge to activate the MEK-ERK and AKT pathways, selective inhibitors of MEK kinases seemed an attractive target. In preclinical models, MEK inhibitors suppressed KRAS mutated cells resistant to cetuximab[120]. Several studies showed that activation of the PIK3CA pathway is a major mechanism of resistance that impairs the efficacy of MEK inhibitors in KRAS mutated cancers. Hence, dual inhibition with MEK and PIK3CA inhibitors resulted in a strong inhibition of tumour cell growth[121,122]. Troiani *et al*[123] demonstrated that combined inhibition of both EGFR and MEK had a synergistic antiproliferative and apoptotic effect in cells and xenografts with either primary or acquired resistance to cetuximab, representing a rational therapeutic strategy for preventing and/or overcoming cetuximab resistance in patients with mCRC. The same group also suggested that combining classical EGFR inhibitors with multitarget agents may circumvent primary and acquired resistance to EGFR inhibitors. For example, the combination of cetuximab and regorafenib, an oral multi-kinase inhibitor, could be an active combination and deserves further testing in a clinical setting[124].

Re-challenging patients with an alternative anti-EGFR moAb after failure with a drug of the same family has been tested. However, panitumumab has been demonstrated to provide minimal benefit in patients with *KRAS* WT mCRC who have experienced progression to cetuximab as prior therapy[125,126]. The hypotheses that pre-existing sensitive subclones may emerge after treatment breaks with anti-EGFR moAb has led the design of several clinical trials prospectively evaluating the rechallenge with anti-EGFR moAbs in the third-line setting after a response to the first-line therapy. Only patients who are quadruple WT are

being enrolled[127].

Interestingly, Ciardiello *et al*[128] recently presented the results from the second line of the CAPRI study and demonstrated that quadruple WT mCRC patients showed a significantly prolonged PFS as well as OS and RR from continuing cetuximab with a different chemotherapeutic agent beyond cancer progression after first-line chemotherapy plus the same anti-EGFR. These findings highlighted that molecularly selected mCRC patients have tumours that are highly dependent on EGFR signalling for their growth, despite the progression to the anti-EGFR drug[128]. These data are interesting and deserve further investigations.

Furthermore, as described above, new anti-EGFR such as Sym004 and MM-151 seem to be active but deserve further investigations[108,109]. Another new drug that has been proposed is GA201 (also known as RG7160, imgatuzumab), a humanized anti-EGFR IgG1 moAb that has showed an increased binding affinity for all FcgRIIIa variants expressed on immune effector cells, such as natural killer cells, leading to a significant improvement in terms of antibody-dependent cell mediated cytotoxicity-based cell killing. Encouraging results have been emerged from the phase I trial; however, a randomized phase Ⅱ trial assessing the role of GA201 in combination with FOLFIRI *vs* cetuximab plus FOLFIRI in second-line mCRC patients showed no PFS benefit for the experimental arm in both KRAS WT and KRAS mutant patients[129,130].

A promising approach is represented by the immune check point blockade with the antibodies against CTLA-4 (ipilimumab) or PD-1 (pembrolizumab, nivolumab) designed to interrupt the immune evasion strategies adopted by cancer cells. Mismatch-repair status has been found to be a useful biomarker in predicting the clinical benefit of immune checkpoint blockade with pembrolizumab. Indeed, a higher response has been achieved by patients with Microsatellite Instability High (MSI-High) tumours[131]. Several trials mainly targeting the PD-1/PDL-1 immune checkpoint pathway are ongoing[132]. Furthermore, preclinical and clinical evidence has suggested that the immune system contributes substantially to the therapeutic effects of mAbs *in vivo*[133]. The combination of immune modulators or checkpoint inhibitors with cetuximab is under evaluation as a first-line therapy of *KRAS* WT mCRC[134].

Early detection of resistance cell clones to anti-EGFR moAbs is another possible approach. However, the classical tumour biopsy might not be representative of tumour heterogeneity and is also an invasive procedure that is often not feasible due to the inaccessibility of metastatic lesions or due to the refusal of patients to be re-biopsied. However, liquid biopsies, *i.e.*, analysing ctDNA in blood samples, have been demonstrated to be useful tools for monitoring the emergence of drug resistance during the course of treatment. Indeed, different groups have demonstrated that analysis of ctDNA in plasma samples allowed detection of mutations predictive of EGFR moAbs resistance, approximately 10 mo before progression was assessed by radiological methods[22,41,135]. Nevertheless, larger and prospective trials are needed before this technique can enter in clinical routine.

CONCLUSION

CRC is still a leading cause of cancer-related mortality in the developed world. In recent years, remarkable advances in the genetic and biological understanding of cancer have led to the development of different targeted cancer therapies, such as the anti-EGFR moAbs cetuximab and panitumumab. However, the overall progress achieved with these drugs has been modest because they have been shown to be effective only in a subset of patients. Primary and acquired resistance have been shown to be the major culprits of the failure of anti-EGFR treatments. However, a deeper understanding of the molecular basis underlying both types of resistance has contributed to the proposal of several approaches to prevent, overcome or reverse drug resistance. Nevertheless, these approaches deserve further clinical investigation to allow us to use the EGFR-targeted therapies more effectively in the correct population.

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TOPIC HIGHLIGHT

2016 Colorectal Cancer: Global view

Immunotherapy in human colorectal cancer: Challenges and prospective

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Abstract

Human colorectal cancer (CRC) is the third most commonly diagnosed malignancies and the prognosis for patients with recurrent or metastatic disease is extremely poor. Although new chemotherapeutic regimen improves survival rates, therapy with better efficacy and less adverse effects is drastically needed. Immunotherapy has been investigated in human CRC for decades with limited success. However, recent developments of immunotherapy, particularly immune checkpoint inhibitor therapy, have achieved promising clinical benefits in many types of cancer and revived the hope for utilizing such therapy in human CRC. In this review, we will discuss important immunological landscape within the CRC microenvironment and introduce immunoscore system to better describe immunophenotyping in CRC. We will also discuss different immunotherapeutic approaches currently utilized in different phases of clinical trials. Some of those completed or ongoing trials are summarized. Finally, we provide a brief prospective on the future human CRC immunotherapy.

Key words: Immunotherapy; Human colorectal cancer; Adoptive cell therapy; Immune checkpoint inhibitor therapy; Immunosuppression

Core tip: Immunotherapy has recently achieved great clinical objective response in multiple cancer types. However, immunotherapy in human colorectal cancer (CRC) is still in its infancy. Identifying CRC patients who are responding to different forms of immunotherapy is drastically needed. In this review, we will discuss important immunological landscape within the CRC microenvironment and introduce immunoscore system

to better describe immunophenotyping in CRC. Knowledge gained from these studies may provide rational design for immunotherapy in human CRC patients.

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INTRODUCTION

Human colorectal cancer (CRC) is the third most commonly diagnosed malignancy and is the leading cause of death worldwide[1]. The projected global burden of CRC is expected to increase by 60% to more than 2.2 million new cases and 1.1 million deaths by 2030[2]. The combined use of irinotecan, oxaliplatin and oral form of 5-fluorouracil (5-FU) has shown significant therapeutic efficacy in human CRC. In addition, the neoadjuvant treatment of radiotherapy with/without chemotherapy and radical surgery with complete mesorectal excision has become the standard treatment for locally advanced rectal cancer. However, the toxicity of radiotherapy and chemotherapy such as actinic colitis is substantial and is associated with considerable morbidity and mortality. Adverse effects including chronic diarrhea and potential intestinal obstructions seriously impair the quality of life of the patients with CRC[3].

Tumor immunotherapy has gained momentum in recent years and shown significant clinical benefits for many types of cancer. For example, Ipilimumab, a specific antibody (Ab) for cytotoxic T lymphocyte associated antigen 4 (CTLA-4) has been used as the first or second line of immunotherapy for advanced melanoma patients[4]. In addition, anti-program death-1 (PD-1) Ab (nivolumab and pembrolizumab) was approved by the FDA in 2015 to treat patients with advanced non-small cell lung carcinoma (NSCLC) including squamous and non-squamous NSCLC[5]. Combined anti-CTLA-1 and anti-PD-1 mAbs to treat metastatic melanoma have shown remarkable clinical benefits[6,7]. The overall response rate reached over 60%. Therefore, tumor immunotherapy was proclaimed recently as "the Advance of the Year" by the American Society of Clinical Oncology (http://www.asco.org/press-center/asco-names-advance-year-cancer-immunotherapy). Despite great successes achieved in other cancer types, immunotherapy in human CRC is still in its infancy. This may be due to many factors such as lack of good ways to study CRC in humans and colorectal animal models are inadequate. In this review, we will examine an immunological landscape within the CRC microenvironment and summarize current major immunotherapies in human CRC. In addition, we will discuss potential challenges for CRC immunotherapies and offer our own prospective on this issue.

IMMUNOLOGICAL LANDSCAPE IN HUMAN CRC

The prognostic value of tumor infiltrating immune cells within the tumor microenvironment has been extensively studied and appreciated[8,9]. A molecular classification of human CRC has characterized the group of patients with microsatellite-instable (MSI) and microsatellite-stable (MSS) tumors. In CRC, MSI is due to a DNA mismatch repair deficiency leading to accumulation of insertions and deletions in DNA repeat sequence. Consequently, this defect results in many mutations that may generate potential immunogenic neoantigens which can be recognized by the immune system[10]. Studies analyzing patients with MSS tumors have shown associations of T cell subpopulations with prognosis. In addition, MSI CRC which comprises approximately 15% of sporadic CRC shows high levels of tumor-infiltrating T cells[11]. This is correlated with high mutational load in MSI tumors, typically 10-50 times more than those of MSS tumors. The high gene mutational load may lead to more tumor-specific neoantigens which may provoke potent anti-tumor T cell responses despite their inability to eradicate cancer naturally. Indeed, recent studies have shown that cancers with high gene mutational load have better therapeutic outcomes in response to immune checkpoint inhibitor therapy[12-14]. MSI tumors display more infiltrations of $CD8^+$ cytotoxic T lymphocytes (CTLs) as well as activated Th1 cells with more IFN-γ production and the Th1 transcription factor T-bet. In contrast, MSI tumors have less Th17 or Th2 cells. Therefore, in general, patients with MSI tumors have better survival than patients with MSS CRC. On the other hand, MSI CRC has high expression levels of immune checkpoint molecules including PD-1, PD-L1, CTLA-4, LAG-3, and IDO leading to an immunosuppressive microenvironment. This is also a good candidate for immune checkpoint inhibitor therapy[15]. In addition to DNA mismatch repair status, recent studies show that immunoscore is a better predictor for patient survival than microsatellite instability in human CRC[16,17]. Immunoscore is a scoring system based on the quantitated numbers of cytotoxic and memory T cells infiltrating in the core of the tumor and in the invasive margins of the tumor[8,18]. Previous studies have shown that immunoscore is the strongest survival prognostic factor in CRC[19]. More importantly, immunoscore may be used as a criteria parameter to choose patients with CRC for immunotherapy since a pre-existing anti-tumor T cells in the tumor is critical for tumor immunotherapy such as anti-PD-1 immune checkpoint inhibitor therapy. Indeed, it appears that patients with MSI CRC could be more responsiveness to such therapy than MSS patients[15]. However, a recent study using integrative

Table 1 Gene mutations in human colorectal cancer

Pathways	Cell cycle	WNT	MAPK	TGF	P53	PI3K-Akt	Apoptosis
Genes	MLH1[71] MSH2[71] MSH6[81] RAD54B[85] CCND1[88] BUB1B[90] AURKA[91] EP300[92]	APC[71] CTNNB1[77] MCC[82] AXIN2[86] ARID1A SOX9	KRAS[72] PLA2G2A[78] PTPN12[83]	SMAD4[73]	P53[74]	PTEN[75] NRAS[79] PIK3CA[84] FGFR3[87] AKT1[89]	DCC[76] BAX[80]

MLH1: MutL homolog 1; MSH2: MutS homolog 2; MSH6: MutS homolog 6; RAD54B: RAD54 homolog B (S. Cerevisiae); CCND1: Cyclin D1; BUB1B: BUB1 mitotic checkpoint serine/threonine kinase B; AURKA: Aurora kinase A; EP300: E1A binding protein P300; PC: Adenomatous polyposis coli; CTNNB1: Catenin (cadherin-associated protein), Beta 1, 88 kDa; MCC: Mutated in colorectal cancers; AXIN2: Axin 2; ARID1A: AT rich interactive domain 1A (SWI-Like); SOX9: SRY (sex determining region Y)-Box 9; KRAS: Kirsten rat sarcoma viral oncogene homolog; PLA2G2A: Phospholipase A2, group IIA (platelets, synovial fluid); PTPN12: Protein tyrosine phosphatase, non-receptor type 12; SMAD4: SMAD family member 4; P53: Tumor protein P53; PTEN: Phosphatase and tensin homolog; NRAS: Neuroblastoma RAS viral (V-Ras) oncogene homolog; PIK3CA: Phosphatidylinositol-4,5-bisphosphate 3-kinase, catalytic subunit alpha; FGFR3: Fibroblast growth factor receptor 3; AKT1: V-Akt murine thymoma viral oncogene homolog 1; DCC: DCC netrin 1 receptor; BAX: BCL2-associated X protein.

analyses in CRC found that patients with MSI CRC and a subgroup of MSS have high intratumoral immune gene expression which correlates with prolonged survival, suggesting that immunoscore may be a better indicator to predict patient survival[16].

GENETIC MUTATIONS IN HUMAN CRC

Genetic mutational loads have been linked to immune checkpoint inhibitors therapeutic efficacy[14,20]. In human NSCLC patients, anti-PD-1 therapeutic efficacy is associated with a higher number of mutational alterations in the tumors[12]. As described above, the MSI CRC microenvironment displays highly infiltrated activated $CD8^+$ cytotoxic T cells. In human CRC, it also shows that mismatch repair-deficient tumors are more responsive to PD-1 blockade treatment than mismatch repair-proficient tumors. This is correlated with mutational loads in the tumors as mismatch repair-deficient tumors have a mean of 1782 somatic mutations while 73 mutations in mismatch repair-proficient tumors[21]. Table 1 summarizes different gene mutations through different pathways in human CRC.

IMMUNOTHERAPY IN HUMAN CRC

Currently, there are many immunotherapies under clinical investigation in human CRC. Immunotherapy in CRC contains different approaches, including monoclonal antibody (mAb) therapy, cancer vaccines, chemoimmunotherapy, immune checkpoint inhibitors therapy, adoptive cell therapy, immune modulators, oncolytic virus therapy, adjuvant immunotherapy and cytokines treatment. Most immunotherapies are still in early-stage clinical development (phase Ⅰ and Ⅱ) for CRC treatment and some of these trials showed promising results. Until now, more than 15 immunotherapy clinical trials for human CRC have been completed and more than 20 clinical trials are recruiting or about to recruit patients (https://www.clinicaltrials.gov/ct2/results?term=colorectal+cancer+and+immunotherapy&no_unk=Y&pg=3). Some of those completed trials are summarized in Table 2 and ongoing trials are listed in Table 3. We will also briefly discuss five major immunotherapeutic approaches in CRC.

mAb therapy

Cetuximab and Panitumumab which target the epidermal growth factor receptor (EGFR) are humanized Ab and have been approved to treat metastatic CRC either alone or in combination with chemotherapeutic drugs[22]. However, approximately 4 out of 10 CRC patients have *KRAS* or *NRAS* mutations which make these Ab therapy ineffective. Therefore, testing for these gene mutations before treatment is necessary[23]. The mechanism of action for Cetuximab is mainly *via* blockade of EGFR signaling which is vital for tumor cell growth. In addition, Centuximab also engages immune mechanisms such as antibody-dependent cellular cytotoxicity and/or complement-dependent cytotoxicity for tumor killing[24,25]. Vascular endothelial growth factor Ab (Bevacizumab) was also approved initially for the first-line treatment of patients with metastatic CRC in combination with 5-FU-based chemotherapy[26]. However, the overall response rate is limited and adverse effects are substantial including increased risk for cardiac ischemic events. There are more humanized Abs currently in different phases of clinical trials such as adecatumumab against EpCAM, labetuzumab against carcinoembryonic antigen (CEA), and pemtumomab against Mucins.

IMMUNE CHECKPOINT INHIBITORS THERAPY

CTLA-4 is an immune checkpoint molecule that down-regulates T cell activation by binding to CD80/CD86

Table 2 Completed clinical trials of immunotherapy in human colorectal cancer

Ref.	Clinical phase	No. patients	Clinical setting	Immunotherapy	Comments
Morse *et al*[93]	Ⅱ	74	Liver or lung metastases from CRC removed by surgery	PANVAC-V + PANVAC-F + DC/ PANVAC-V + PANVAC-F + GM-CSF	Good safety record
Vermorken *et al*[44]	Ⅱ and Ⅲ	254	Colon cancer	Active specific immunotherapy (ASI) with an autologous tumor cell- bacillus Calmette-Guérin (BCG) vaccine with surgical resection/ resection alone	ASI gave significant clinical benefit in surgically resected patients in stage II colon cancer
	Dukes' stage B2-C3	80	Colon or rectal cancer	ASI with an autologous tumor cell-BCG vaccine	ASI may be beneficial to patients with colon cancer
Hanna *et al*[94]	Stage Ⅱ and Stage Ⅲ		Colon cancer	ASI consisting of autologous tumor cells mixed with BCG (OncoVAX™)	Increase 5-yr survival rate and 5-yr disease-free survival rate, reduce recurrence rate
Marshall *et al*[95]	Stage Ⅳ		Colon cancer	ALVAC-CEA	Safe and can elicit CEA-specific CTL responses
Harris *et al*[96]	Stage Ⅱ and stage Ⅲ	412	Colon cancer	Adjuvant active specific immunotherapy with an autologous tumor cell-BCG vaccine	More beneficial than resection alone

Table 3 Ongoing immunotherapy clinical trials in human colorectal cancer

Type	Cancer	Phase	Name	Identifier	Start time
Monoclonal antibodies	Untreated metastatic colorectal cancer	Ⅱ	RO5520985 (a bispecific anti-ANG-2/anti-VEGF-A	NCT02141295	5/15/2014
	Colorectal cancer	Ⅰ / Ⅱ	IMMU-132 (an Ab-drug conjugate targeting Trop-2)	NCT01631552	6/26/2012
	Metastatic colorectal cancer	Ⅰ / Ⅱ	IMMU-130 (an Ab-drug conjugate targeting CEACAM5)	NCT01605318	5/22/2012
	Metastatic colorectal carcinoma	Ⅰ	MGD007 (a dual-affinity re-targeting DART protein designed to target the glycoprotein A33)	NCT02248805	9/18/2014
	Metastatic colorectal cancer	Ⅰ	OMP-131R10 (an anti-RSPO3 Ab)	NCT02482441	6/19/2015
Immune checkpoint inhibitors	MSI positive/negative colorectal cancer	Ⅱ	MK-3475 (an Ab that blocks negative signals on T cells)	NCT01876511	6/10/2013
	MSI positive colorectal cancer	Ⅰ	Ipilimumab	NCT02060188	12/18/2013
	MSI negative colorectal cancer	Ⅱ	Nivolumab	NCT02060188	12/18/2013
	Colorectal cancer	Ⅰ	MED14736	NCT01975831	10/29/2013
	Colon cancer	Ⅰ / Ⅱ	Anti-CD27 (Varlilumab) and Nivolumab	NCT02335918	12/18/2014
Cancer vaccines	Metastatic colorectal cancer	Ⅱ	DC vaccine	NCT02615574	11/24/2015
	Colorectal cancer	Ⅰ	AVX701 (targets CEA)	NCT01890213	6/26/2013
	Metastatic colorectal carcinoma	Ⅰ	SGI-110 in combination with an allogeneic colon cancer cell vaccine (GVAX) and cyclophosphamide (CY)	NCT01966289	10/10/2013
	Colorectal cancer	Ⅰ	HER-2 vaccine	NCT01376505	6/9/2011
Adoptive cell therapy	Metastatic colorectal cancer	Ⅱ	TIL (tumor-infiltrating lymphocytes)	NCT01174121	7/31/2010
	Colorectal cancer	Ⅰ / Ⅱ	Anti-MAGE-A3-DP4 TCR	NCT02111850	4/9/2014
	Colorectal carcinoma	Ⅰ / Ⅱ	CAR T cells	NCT02617134	11/26/2015
	Colorectal cancer	Ⅰ	NK cells + CliniMACs CD3 and CD56 systems	NCT00720785	7/22/2008
Oncolytic virus therapies	KRAS mutant metastatic Colorectal cancer	Ⅰ	REOLYSIN in combination with FOLFIRI and Bevacizumab	NCT01274624	1/7/2011
Adjuvant immunotherapies	Recurrent colorectal cancer	Ⅰ / Ⅱ	Chemokine modulatory regimen	NCT01545141	2/29/2012
Cytokines	Colorectal carcinoma	Ⅰ	AM0010 (a recombinant human IL-10)	NCT02009449	12/2/2013
	Colorectal carcinoma	Ⅰ	Rh IL-15	NCT01572493	4/5/2012

molecules on antigen-presenting cells (APC). Programmed death receptor ligand 1/2 (PD-L1/L2) also negatively regulates effector T cell function by binding to PD-1 receptor on T cells. Generally induced by their respective ligands that are expressed on either tumor cells (*e.g.*, PDL1/ L2→PD-1) or APCs (*e.g.*, CD80/86→CTLA-4; PD-L1/L2→PD-1), activated CTLA-4 and PD-1 immune checkpoint pathways are potent inhibitors of tumor-reactive T cell activation, clonal expansion and subsequent tumor rejection[27,28]. Anti-CTLA-4 and anti-PD-1 mAbs effectively block these pathways resulting in the re-activation and clonal expansion of

tumor-reactive lymphocytes. Until recently, clinical trials with immune checkpoint inhibitors showed remarkable success in patients with different cancers, including metastatic melanoma and NSCLC patients[5,29]. Anti-CTLA mAb Yervoy (ipilimumab) was originally approved to treat non-resectable, late stage melanoma in 2011. Anti-PD-1 Ab (nivolumab and pembrolizumab) was approved by the FDA in 2015 to treat patients with advanced NSCLC including squamous and non-squamous NSCLC. Intriguingly, a new Phase 1/2 study (CheckMate 069) evaluating concurrent ipilimumab/nivolumab *vs* ipilimuab alone in chemotherapy naïve advanced melanoma patients reported a remarkable 61% objective response rate (34). Thus, combined ipilimumab and nivolumab was approved by the FDA for the frontline treatment of advanced melanoma in 2015.

In human CRC, a phase Ⅱ clinical trial was conducted to use pembrolizumab for the treatment of mismatch repair-deficient CRC and mismatch repair-proficient CRC[21]. Pembrolizumab was administered intravenously at a dose of 10 mg/kg every 14 d. A total of 32 patients with CRC were enrolled in this trial. Among them, 10 patients had mismatch repair-deficient tumors and 18 had mismatch repair-proficient tumors. In the patients with mismatch repair-deficient CRC, the immune-related objective response rate was 40% and the immune-related progression-free survival rate at 30 wk was 78%. In a sharp contrast, patients with mismatch repair-proficient tumors had 0% immune-related objective response rate and the immune-related progression free survival rate was 11%. More importantly, in the patients with mismatch repair-deficient CRC, the median progression-free survival and medial media overall survival were not reached while among the patients with mismatch repair-proficient tumors, the median progression-free survival was only 2.2 mo and the median overall survival was 5.0 mo. Although this trial has a relative small cohort of CRC patients, the findings are significant and further support the idea that mutation-associated neoantigen recognition is a critical component of the endogenous antitumor immune response. Thus patients with mismatch repair-deficient tumors may benefit greatly with anti-PD-1 immune checkpoint inhibitor therapy. In this trial, the investigators also analyzed the number of somatic mutations in tumors using whole-exome sequencing. They suggested that patients with mismatch repair-deficient tumors that have more than 20 times higher of mutation-associated neoantigens than in tumors without this deficiency should be the basis for the anti-PD-1 therapy. However, more studies need to be done to further support this conclusion.

Tremelimumab, another CTLA-4 inhibitor, is presently under clinical investigation in patients with advanced melanoma, hepatocellular carcinoma, non-small cell lung cancer and metastatic CRC[30-33]. However, the results of one study did not show clinical benefit from the single-agent administration to the patients with treatment-refractory CRC[33]. Previous studies also indicate that administrate anti-CTLA-4 Ab combined with other agents significantly improve the treatment effect in colon cancer[34-37]. However, CTLA-4 Ab treatment has reported previously that 43% of patients suffered from grades 3 to 4 autoimmune responses, such as enterocolitis, hypophysitis, dermatitis and hepatitis[38]. A phase Ⅱ clinical trial of Nivolumab and Nivolumab plus Ipilimumab in recurrent and metastatic microsatellite high colon cancer is underway (ClinicalTrials.gov Identifier: NCT02060188).

CANCER VACCINES

Cancer vaccines are designed to stimulate antigen-specific T-cell or B-cell response against cancer by providing antigens to professional APC such as dendritic cells (DCs). In addition, vaccines also include components intended to activate DCs pulsed with antigens and program them to migrate to a local lymph node.

DC vaccine

DC-based cancer vaccine has been previously approved by the FDA for the treatment of metastatic castrate-resistant prostate cancer[39]. Since most of CRCs express CEA which is a tumor-associated antigen (TAA), DCs can be pulsed with CEA mRNA[40] or CEA peptides[41]. In these early clinical trials, CEA-specific T cell immune responses were elicited in most of DC vaccinated CRC patients. The vaccines were well-tolerated and safe administration. Despite induced T cell responses, these clinical trials did not show significant objective tumor regression[42]. It may be rationale to combine DC-based vaccine with immune checkpoint inhibitor therapy to boost both endogenous and TAA-specific antitumor T cell response. In addition, some limitations of DC vaccines need to overcome, such as DC vaccine quality control, migration after vaccine injection, the specificity of tumor targets, and the expenses of clinical utilization[43].

OncoVAX

OncoVAX is designed to utilize patients' own cancer cells with an immunostimulating adjuvant to elicit antitumor immune responses against the recurrence of colon cancer after surgery. This patient-specific vaccine is composed of metabolically-active, sterile, irradiated, and non-tumorigenic autologous cancer cells, with or without fresh frozen Bacillus Calmette-Guerin (BCG) bacteria as an adjuvant. In this phase Ⅲ study, 254 patients were enrolled and randomized into two groups: active specific immunotherapy (ASI) with autologous tumor cell-BCG vaccine with tumor resection and resection alone. ASI was given three weekly vaccinations starting 4 wk after surgery. Patients were boosted at 6 mo with irradiated autologous tumor cells. It appears that OncoVAX has the major impact on stage Ⅱ disease with a significantly

longer recurrence-free period and 61% risk reduction for recurrences. Recurrence-free survival was also significantly longer with ASI. However, the overall survival was not significantly improved[44]. Longer follow-up studies reveal that a beneficial effect of OncoVAX is statistically significant for all endpoints including recurrence-free interval, overall survival, and recurrence-free survival but only in Stage Ⅱ colon cancer but not in Stage Ⅲ patients[45]. A recent meta-analysis also suggests that combined ASI therapy with surgery showed a significantly survival benefit[46]. However, it is unclear whether this benefit is associated with CRC patients with different stages.

ADOPTIVE T CELL THERAPY

Adoptive T cell therapy has the potential to enhance antitumor immunity and augment vaccine efficacy. Indeed adoptive cell immunotherapy based on the transfusion of genetically re-directed autologous T cells has demonstrated clinical promise for the treatment of both hematologic malignancies and solid tumors. Particularly, recent developments have been focused on gain-of-function strategy to endow effector T cells with desired antigen receptors, such as chimeric antigen receptor (CAR) T cells[47]. Interestingly, recent studies showed that modulating T cell metabolic pathways significantly augments effect T cell antitumor responses[48] and synergizes with immune checkpoint inhibitor therapy in cancer[49].

In a phase I clinical trial in CRC patients, genetically engineered T cells were adoptively transferred into three patients with metastatic CRC refractory to standard treatment. Those autologous T cells were genetically engineered to express murine TCR against human CEA[50]. Serum CEA levels dramatically decreased after treatment in all three patients (74%-99%) and one patient had an objective regression of tumor metastatic to the lung and liver. However, severe transient inflammatory colitis developed in all three patients. More clinical studies need to be done to demonstrate true benefit of CAR T cells in human CRC.

In addition to adaptive αβ T cell therapy in cancer, innate γδ T cells have also been shown to elicit potent antitumor immunity[51,52]. Innate γδ T cells have two major subsets with one predominately producing IFN-γ and another one secreting large amounts of IL-17. Although IL-17-producing γδ T cells play critical roles in cancer progression and metastasis[53-56], IFN-γ-producing γδ T cells have potent antitumor effect. Indeed, adoptive transfer of Vγ4 γδ T cells into tumor-bearing mice has shown a therapeutic effect[57]. In addition, we showed recently that *ex vivo* expanded human Vδ1 γδ T cells exhibited therapeutic effect in human colon cancer xenografted mouse model[58]. These expanded γδ T cells predominately express granzyme B, perforin, and IFN-γ. Adoptive transfer of γδ T cell therapy has been done in many types of cancer with varying efficacy[59].

CHEMOIMMUNOTHERAPY

Chemoimmunotherapy is chemotherapy combined with immunotherapy. Although chemotherapy and immunotherapy appear to be antagonistic as chemotherapeutic drugs not only kill tumor cells leading to dying tumor cells but also impact on immune cells, chemotherapy also depletes immune suppressive cells such as regulatory T cells, potentially enhancing antitumor immune response. In addition, lymphodepletion stimulates homeostatic T cell proliferation thus providing a unique opportunity for tumor immunotherapy. Chemoimmunotherapy has been widely used for hematological malignancy treatment[60]. The first chemoimmunotherapy clinical trial conducted in human colon cancer patients was reported in 2008[61]. Total 46 CRC patients with advanced disease were enrolled and received chemodrugs gemcitabine, oxaliplatin, levofolinic acid, and 5-FU followed by granulocyte macrophage colony-stimulating factor (GM-CSF) and IL-2 (GOLFIG-1 trial). In this trial, six patients showed a prolonged time to progression and survival. In addition, these patients had decreased Treg cells but increased central memory T cells as well as colon cancer-specific cytotoxic T cells. Interestingly, it appears that tumor-infiltrating Treg cells were associated with a better prognosis in patients received chemoimmunotherapy[62]. Recently, a multicenter open label phase Ⅲ trial was conducted as a frontline treatment of metastatic CRC[63]. Patients receiving chemoimmunotherapy (GOLFIG-2 trial) showed prolonged progression-free survival and increased overall response rate. However, this study was discontinued due to poor recruitment in the control arm. Thus it is too early to make any recommendation whether chemoimmunotherapy has any therapeutic efficacy for advanced CRC treatment. Nevertheless, these clinical studies provide "proof-of-principle" suggesting that GOLFIG chemoimmunotherapy may be used as a novel regimen for the first-line treatment of advanced CRC.

CONCLUSION

Immunotherapies have been investigated in human CRC for decades[64] although there is no approved immunotherapy for human CRC up to date. As described above, different immune-directed approaches have been developed such as DC-based vaccines and genetically engineered CAR T cells. However, these developments are still in the early stages. One promising approach for CRC immunotherapy may lie in immune checkpoint inhibitor therapy given the encouraging phase Ⅱ clinical trial data[21]. This is particularly important for patients harboring MSI tumors since more mutational loads lead to more neoantigens that can be recognized by effector T cells. Therefore, identifying proper criteria to choose eligible patients thus getting maximum therapeutic benefits is essential. It may be critical to assess immunological

profiles of human CRC. In this context, immunoscore system seems critical, which should be included in the eligibility criteria for patients receiving such therapy. In addition, more research needs to be done to identify more Ag targets that can be used for cancer vaccine development and/or CAR T cells. Increasing studies have identified new immune components which maybe the potential targets for CRC immunotherapy, such as GUCY2C, HHLA2, OR7C1, and MAGE-D4[53,65-70]. Furthermore, combined immunotherapies such as combined anti-CTLA-4 with anti-PD-1 or combined cancer vaccines with immune checkpoint inhibitor therapy may yield better therapeutic efficacy. Certainly, cautions are needed to avoid severe adverse effects that could be elicited by such combination therapy. Nevertheless, this is an exciting time for immunotherapy in human CRC.

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TOPIC HIGHLIGHT

2016 Gastric Cancer: Global view

Immunological battlefield in gastric cancer and role of immunotherapies

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Abstract

Like the wars predating the First World War where human foot soldiers were deemed tools in the battlefield against an enemy, so too are the host immune cells of a patient battling a malignant gastric cancer. Indeed, the tumour microenvironment resembles a battlefield, where the patient's immune cells are the defence against invading tumour cells. However, the relationship between different immune components of the host response to cancer is more complex than an "us against them" model. Components of the immune system inadvertently work against the interests of the host and become pro-tumourigenic while other components soldier on against the common enemy – the tumour cell.

Key words: Immune; Gastric cancer; Immune therapy; Immunology; Tumour microenvironment

Core tip: Many solid tumours are now being treated with immunotherapies and gastric cancer is no exception. Here we review the literature on molecular

subtypes of gastric cancer and how they each have different immunological responses and hence may be differentially responsive to these immunotherapies. We emphasise that while treatment of gastric cancer may be benefited by immunotherapy we should try to target this based on molecular and immunological signatures of the individual patient. This will match the ideal therapy to the specific patient and is a step forward on the pathos precision medicine.

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"Advances in medicine and agriculture have saved vastly more lives than have been lost in all the wars in history."

Carl Sagan

INTRODUCTION

Gastric cancer (GC) continues to be a significant cause of mortality globally, being the third leading cause of cancer-related death[1]. While there have been advances in the outcomes of many solid tumours[2-4], gastric adenocarcinoma, the predominant form of GC, has not shown the same degree of improvements in survival[5], despite aggressive multi-modality treatment[6]. Potent new immunotherapies induce host immune-mediated destruction of malignant cells and offer new hope in the battle against GC. Here we explore some of the positive and negative characteristics of the host immune response to the presence of a malignant cell.

It is incumbent on us to be aware that all cancers are not equal. The Cancer Genome Atlas (TCGA) Network has produced a landmark study using integrative genomics to molecularly phenotype four subtypes of GC[7] that are to some extent related to histological features of the disease. Previous studies suggest the histology of the tumour according to Lauren classification may explain some of the molecular heterogeneity of GC[8] but the host immune response to the cancer may also account for some of the differences. The TCGA study describes two particular subtypes both of which consist of predominantly intestinal type tumours that had a significant immunological association: the Epstein-Barr virus (EBV) subtype accounted for about 9%[7] of the GCs profiled and were characterised by a strong immune signature and; the MSI (Microsatellite Instability) subtype (22% of cancers in this study[7]), which had a high mutational load, also had a significant immune signature. While the other two GC subtypes [GS (Genomically Stable and predominantly diffuse) subtype and CIN (Chromosomal Instability subtype which are primarily intestinal in histology)] may have a host immunological response, this differed to the two immunogenic subtypes[7]. Here we explore some of the features of the immune response to GC to try and reconcile some of the clinical observations, such as differences in survival and also to explore the utility of immunotherapies for this particular cancer.

Currently, the immune context of GC comprises both anti- and pro-tumoural immune responses. The immune system includes inter-linked innate and adaptive arms, both have cellular and soluble effectors. The innate immune system cells respond to foreign antigens that are recognised *via* pathogen recognition receptors (PRR) for pathogen-associated molecular patterns (PAMPs) or danger associated molecular patterns (DAMPs)[9]. The PRR can recognise PAMPs or DAMPs derived from a diverse array of viruses, bacteria or tumour cells. The innate immune system is evolutionarily conserved and performs an immune surveillance role *via* cells [macrophages, dendritic cells (DCs), neutrophils and natural killer (NK) cells] and soluble factors such as, the complement system. There is considerable cross-talk between cells within the innate immune system as well as cross-talk with cells of the adaptive arm, for example, tissue resident DCs induce an adaptive immune response through antigen presentation[10]. The adaptive immune system recognizes and eliminates antigens; conventional T cells recognise antigen as peptide-major histocompatibility complex (MHC) on virus infected cells or tumour cells, whereas B cells recognise conformational antigen. Priming of naïve T and B cells to antigens occurs in the tissue draining lymph node of a particular organ. Effective antigen recognition and co-stimulation activates the antigen-specific T or B cell driving their proliferation and generation of effector and memory cells. Effector T cells traffic to the site of priming and participate in resolution of the threat/pathogen. Memory T cells reside in secondary lymphoid tissue (central memory), or the peripheral tissue (tissue resident memory cells) and can respond quickly to any future pathogen threat, termed "long term protective immunity". In healthy individuals the immune system is remarkably effective at responding to and eradicating a diverse array of pathogen threats; however the immune system can be a double-edged sword in cancer, which has the ability to shape the immune response to facilitate tumour cell growth and survival rather than eliminating the tumour[11].

THE IMMUNE SYSTEM AND CANCER

The immune system detects and eliminates tumour cells. This usually prevents cancer development through a process termed immune-surveillance[12,13]. Tumour-specific antigens (TSA) are antigens present only on tumour cells, while tumour-associated antigens (TAA) are antigens present on tumour cells as well as normal

cells. Expression of TSA and TAA generally results from tumour-associated genetic mutations. Tumour-resident DCs constantly sample the microenvironment *via* endocytosis, they process the TSA or TAA as peptides and assemble them on MHC, either in the endoplasmic reticulum for MHC class Ⅰ, or endosomes for MHC class Ⅱ. The DC requires an activation signal, such as a DAMP or PAMP, in order to mature and subsequently increase peptide MHC expression levels. Activated DCs change chemokine receptor and adhesion molecule expression making them responsive to chemokines emanating from the tumour draining lymph node (TDLN). Having migrated to the TDLN, the mature DC presents TSA/TAA on MHC class Ⅰ to $CD8^+$ T cell, or on MHC class Ⅱ to $CD4^+$ T cells, priming an antigen-specific T cell response[14]. For successful activation, Cytotoxic T cells (CTLs) require two signals from antigen processing cells (APCs); (1) antigen presentation, T-cell receptor (TCR) binding to peptide-MHC class Ⅰ molecules; and (2) co-stimulation, CD28 molecule on T cells binding to co-stimulatory molecules CD80 (B7-1) or CD86 (B7-2) on APCs. In the absence of signal 2, signal 1 induces immune tolerance to TAA/TSA. Signal 2 is only provided by mature DCs, as they express CD80/CD86 at higher levels. At this point, activated tumour-specific naïve T cells proliferate and form effector and memory T cells, as described for the pathogen response above. Tumour-specific $CD8^+$ effector T cells, also termed CTLs, traffic from the TDLN to the tumour and attack tumour cells presenting cognate antigen, with the help of $CD4^+$ helper T cells (Th cells), mainly Th1 cells. During the effector phase, T cells infiltrate the tumour (referred to as tumour infiltrating T lymphocytes or TILs) in response to chemokines, such as CX3CL1, CXCL9, CXCL10 and CCL5[15]. These TILs kill tumour cells by direct and indirect mechanisms. The direct mechanism utilises perforin and granzymes. Figure 1A outlines some of the aspects of antigen recognition, presentation and the effector immune cells (T cell and NK cell) killing of tumour cells. Tumour-specific CTL recognition of cognate antigen induces their activation and formation of an immune synapse (IS, a specialised molecular structure formed between a cytotoxic lymphocyte and a target cell) at the site of antigen recognition. Simultaneously, the CTL moves cytotoxic granules (containing perforin and granzymes) to the IS, these granules fuse with the CTL cell membrane and release their contents. Perforin polymerises and inserts into the tumour cell membrane forming a pore, this enables entry of granzyme B into the cytoplasm, which induces tumour cell apoptosis. Indirect mechanisms include secretion of cytokines including type Ⅰ IFN, IFN-γ and TNF[16,17]. After clearance, surviving $CD8^+$ T cells differentiate into T memory cells[18], which can retain anti-cancer properties and can enact faster and stronger anti-cancer immune response when they next encounter tumour cells.

Another cell type important in an early response to cancer is the NK cell. NK cells are part of the innate immune system that act non-specifically against tumour cells and can directly kill these cells. This type of anti-cancer immunity is reported in hematopoietic malignancies and solid tumours[19].

HOW CANCER ESCAPES FROM IMMUNE SYSTEM

The "immune-editing" paradigm was proposed to explain how tumour cells influence the behaviour of innate and adaptive immune cells through an immunosuppression process to finally present as a clinical tumour[20]. The immune-editing mechanism, which is the most important process during immunosuppression, consists of three sequential phases: elimination, equilibrium, and escape[20-22]. During the elimination phase, the immune system destroys developing tumour cells. In the equilibrium phase, sufficient tumour cells survive the immune attack to maintain tumour size, but there is no obvious tumour progression. During this phase, the immune system sculpts the immunogenicity of genetically unstable tumour clones. Finally, in the escape phase immune resistant tumour clones emerge, proliferate and spread either locally or to distant sites.

Precisely how tumour cells evade the immune system (in the escape phase), as summarised in Figure 1B, is an area of active research, and can be broadly grouped into three main mechanisms including:

Immune recognition/ignorance: where tumour cells can control immune recognition *via* down-regulation of antigens and MHC molecules on the cell surface[23-25].

Immune suppression/tolerance: Where tumour-derived suppression mechanisms are driven *via* tumour-derived cytokines and influence the differentiation of immune effectors driving their functional polarization to suppressors. Immune suppressors include tumour associate macrophages (TAMs), myeloid-derived suppressor cells (MDSCs) and regulatory $CD4^+$ T cells. Macrophages within the tumour microenvironment have been described as pro-tumourigenic as they support cancer initiation and progression, or anti-tumourigenic based on differentiation patterns into M1 or M2 subtypes[26,27]. M1 macrophages have a tumouricidal activity by producing pro-inflammatory cytokines, such as IL-1, IL-6, IL-23 and TNF. M2 macrophages possess a tumour-promoting capacity by producing IL-10 and TGF-β. TAMs frequently have a spectrum of differentiation and, through the balance of M1 and M2 macrophage subtypes in the tumour microenvironment, may influence aggressiveness of the tumour and prognosis of patients. There is generally a poor outcome if M2 macrophages predominate in the tumour microenvironment[28,29]. Tumour cells may

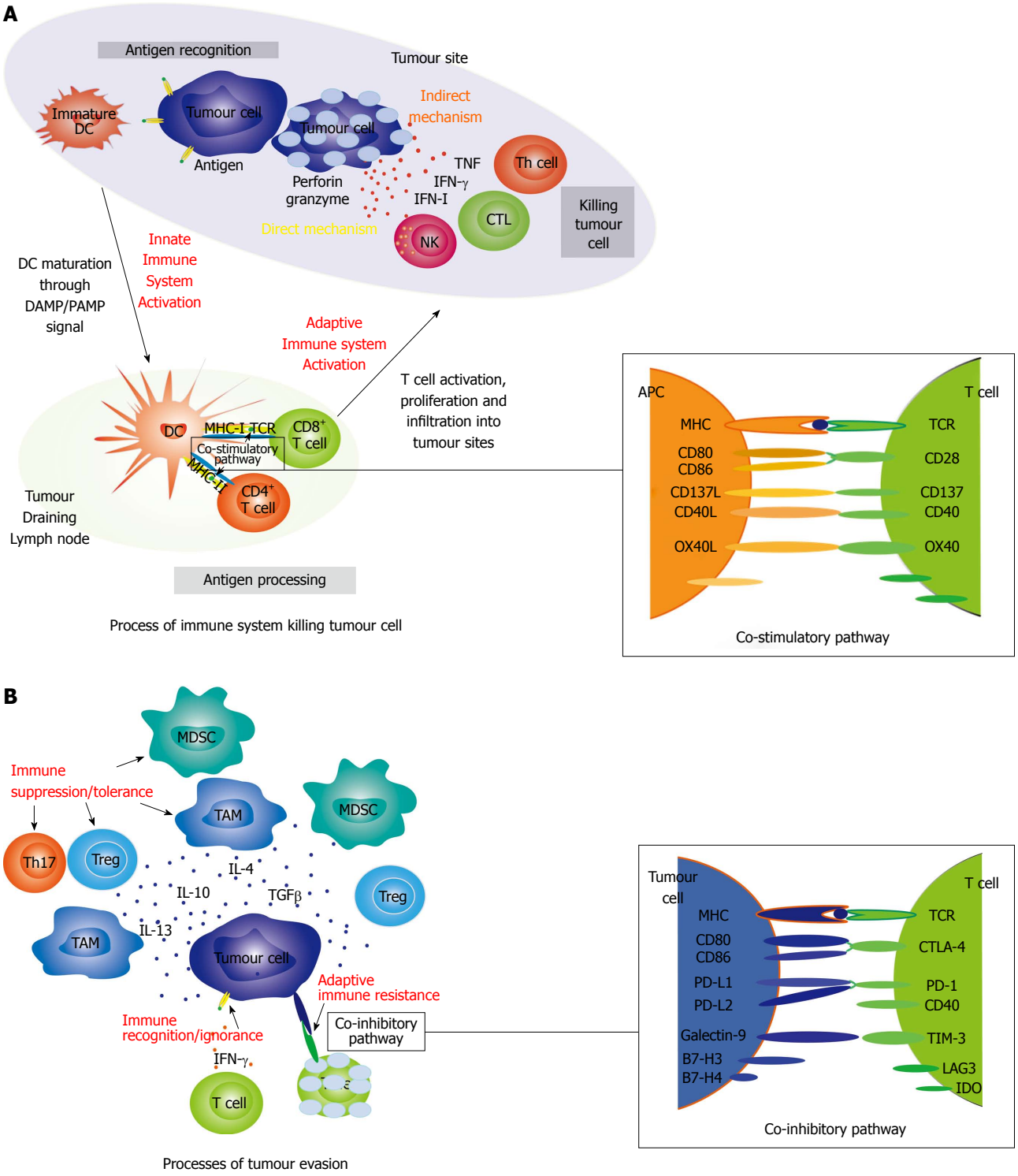

Figure 1 Process of immune system killing tumour and processes of tumour evasion. A: Outlines the process of immune system killing tumour cell. First, antigens (including neo-antigens) expressed by the tumour, are recognized by immature DC then the innate immune system is activated and the antigen is presented by APC to the T cell. The T cell becomes activated, then proliferates and infiltrates into tumour sites. The adaptive immune system is then responsible for activation of the effector immune cells (*e.g.*, CTL, Th cell or NK cell) that secrete cytokines to kill the tumour cell; B: Describes part of the processes of immune evasion. This is the mechanism by which the tumour cell evades the immune system (escape phase) through the processes of immune recognition/ignorance; immune suppression/tolerance and; adaptive immune resistance. DC: Dendritic cell; CTL: Cytotoxic T lymphocytes; Th cell: T helper cell; NK: Natural killer cell; APC: Antigen processing cell; MHC: Major histocompatibility complex; TCR: T-cell receptor.

also induce an M1 to M2 switch, mediated by TGF-β[30]. Several studies have identified an association with the density of TAMs in the microenvironment of GC and a poor outcome[31,32]. MDSCs are a group of activated but immature myeloid cells with strong immunosuppressive capacity that have been shown to support tumour cell growth, differentiation, and metastasis[33,34]. $CD4^+$ T cell response to tumour-derived antigen in the context

of TGF-β induces up-regulation of the key transcription factor (FoxP3) and regulatory T cells (Tregs) functional polarization. Tregs are powerful suppressors of the tumour-specific T cell responses (both CTL and effector $CD4^+$ T cells) and can be found at increased numbers in patient TILs, both at the tumour margin and inside the tumour itself[35].

Adaptive immune resistance: where tumour cells can induce T cell inactivation through a process described as "adaptive immune resistance"[36]. When CTL's recognise cognate antigens on tumour cells their effector mechanisms include secretion of IFN-γ, IFN-γ binding to tumour cell IFN-γR induces JAK-STAT signalling and up-regulation of tumour cell programmed death ligand-1 (PD-L1) expression. CTL recognition of antigen induces programmed death-1 (PD-1) expression. Binding of PD-L1 to its receptor PD-1 on T cells, delivers an inhibitory signal to the T cell IS and results in T cell paralysis. The over-arching result is tumour cell resistance to killing by T cells[36,37]. The molecules involved in the immune co-inhibitory pathways are called immune checkpoints. These molecules have an important normal physiological role, and are important in turning off the immune system once effective T cell effector function has been achieved (*i.e.*, once antigen has been cleared). The checkpoint inhibitors include: PD-1 (also known as CD279) and its ligands PD-L1 (B7-H1; also known as CD274) and PD-L2 (B7-DC; also known as CD273); Cytotoxic T-lymphocyte-associated protein 4 (CTLA-4, also known as CD152) and its ligands CD80 and CD86; T-cell immunoglobulin and mucin-domain containing-3 (TIM-3) and its ligand galectin-9; lymphocyte-activation gene 3 (LAG3, also known as CD223) and Indoleamine-pyrrole 2,3-dioxygenase (IDO). These pathways are discussed later as they have been transformed into successful immunotherapies in the war against cancer. Other components of the adaptive immune response are two subcategories of T cells, the Treg and Th17 cell. Tumour cells can induce Tregs, which in turn promote tumour progression by secreting TGF-β, as well as Th17 cells, which accelerate tumour progression by producing IL-17[38].

These are some of the physiological mechanisms that a tumour cell can exploit to survive, perpetuate and invade a host organism resulting in poor outcomes seen in many malignancies.

IMMUNOGENIC SUBTYPES OF GC

Integrated genomic analysis of GC showed that molecular subtypes have distinct signatures. The EBV and MSI subtypes have significant immune signatures (Figure 2A) compared to the CIN and GS subtypes (Figure 2B). It is recognised that MSI cancers result in increased tumour cell mutational load[39] and presentation of neo-antigens[40] resulting in an augmented host immune response with increased TILs[41-43], DCs and macrophages[44]. EBV-associated GC also has an increased density of TILs[41,45-47]. Despite a significant host immune response these tumours persist, likely due to immune escape mediated by over-expression of immune checkpoints, such as PD-L1 and PD-L2[7]. Llosa *et al*[48] found similar changes to the tumour microenvironment of MSI colorectal cancer. These MSI cancers showed up-regulation of immune checkpoints, such as PD-1, PD-L1, LAG-3, CTLA-4 and IDO. In colorectal cancer MSI-high specimens are often associated with high infiltration of $CD3^+$, $CD4^+$ and $CD8^+$ cells and show more frequent infiltration by PD-1 positive intraepithelial lymphocytes compared to microsatellite stability samples[49]. Indeed, colorectal cancer is a good example of how the immunological reaction to the tumour has been used as a prognostic marker and may identify a group of cancers that can be targeted with specific immunotherapies[50,51]. The mechanisms for the robust immune response in the EBV subtype remain unclear, but are likely due to long term inflammation induced by the infection in the stomach[52].

An association between lymphocytic infiltration and survival in GC was first proposed over 100 years ago[53]. Since then numerous studies have shown an increased density of TILs is associated with favourable clinical outcomes in a variety of solid tumours[54-57], including GC[58]. This holds true for intratumoural B cells[42] and NK cells[59] in GC. However, GC expression of the checkpoint inhibitor PD-L1 is associated with poor clinical outcomes[60-66]. Tumour expression of PD-L1 is not universally a negative predictor as patients with ovarian cancer expressing high PD-1 (generally thought to be expressed by the immune cells) and PD-L1 levels in tumour cells as well as TILs having favourable prognosis[67]. These contrasting results are partially explained by methodological differences in the studies where most investigators report on the intra-tumoural immune component only and ignore the peri-tumoural context of the cancer or focus on particular components of the immune response in isolation and ignore the dynamic environment that is the tumour microenvironment. Importantly, another variable that is not factored in the GC literature is the molecular characteristics of the tumour cell itself. As described in the Asian Cancer Research Group study from Cristescu *et al*[68], tumours of the MSI subtype (similar to TCGA) have the best prognosis of the subtypes described in their study. Marrelli *et al*[69] confirm the favourable prognosis of MSI in non-cardia intestinal gastric tumours. Kim *et al*[70] investigated the type and density of TILs and macrophages in the MSI-high subgroup of GC and found that increased density of intra-tumoural $CD8^+$ and $FoxP3^+$ TILs was associated with a good prognosis. It was further shown that the balance of TILs and TAMs (M2-polarized macrophages) also showed favourable prognostic significance[71].

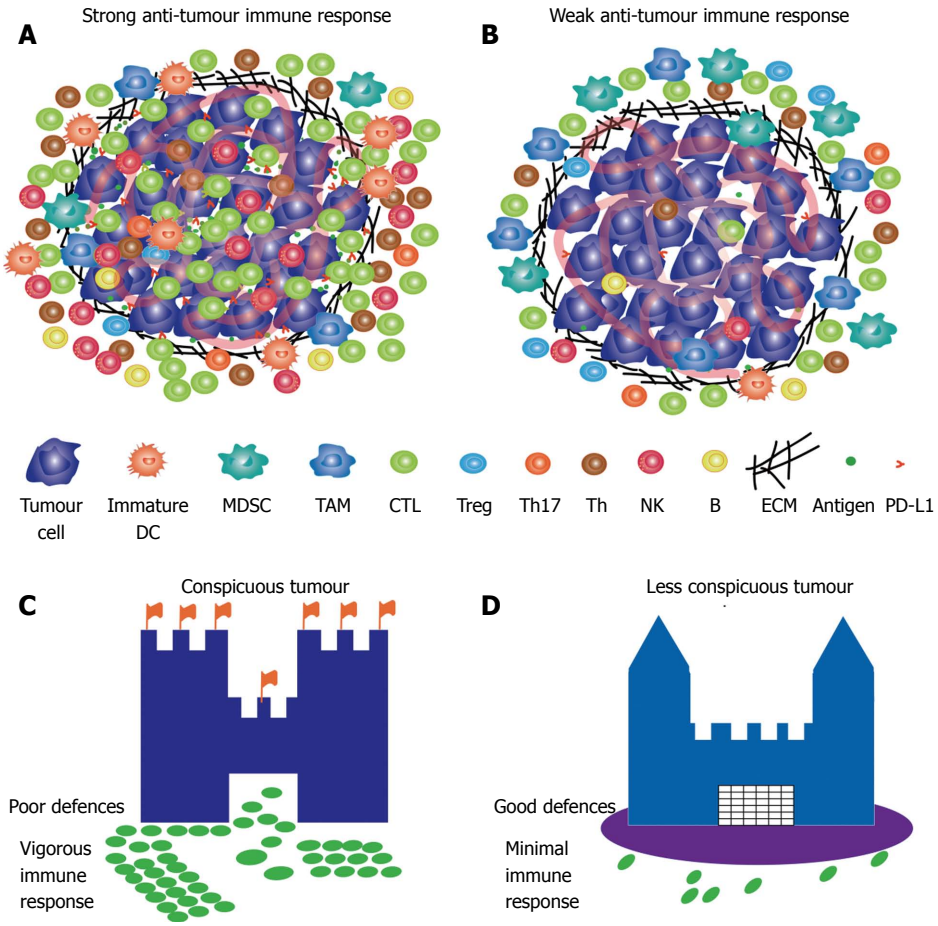

Figure 2 Two different tumour microenvironments of different molecular subgroups of gastric cancer. A: Example of a strong immunogenic tumour with increased antigen presentation and immune checkpoints expression, more TILs and other effector immune cells infiltration into the tumour; B: Example of a weak anti-tumour immune response with less antigen expression, less TILs and other effector immune cell infiltration into tumour and increased numbers of immunosuppressive cells; C: Schematic diagram representing poor defences from a conspicuous tumour with vigorous immune response; D: Schematic diagram representing good defences from a less conspicuous tumour with minimal immune response. MDSC: Myeloid-derived suppressor cell; TAM: Tumour associate macrophages; Treg: T regulatory cell; Th17: T helper cell-17; ECM: Extracellular matrix; MHC: Major histocompatibility complex; TCR: T-cell receptor.

IMMUNOTHERAPY OF CANCER

The immune system is an integral part of the tumour microenvironment and immune cell evasion by tumour cells has recently been highlighted as one of the hallmarks of cancer[11]. Promotion, or activation, of the immune system, referred to as "immunotherapy", has also been proposed as an option for targeted treatment. Unlike chemotherapy, which uses potent drugs to eliminate tumour cells or control their growth, cancer immunotherapy involves boosting the immune system of a patient to eliminate or control a malignancy. Using the immune checkpoint inhibitors (ICIs), T-cells can be re-activated or maintained in an active state allowing them to recognize and eliminate tumour cells. Documented clinical responses of ICIs in a number of cancer types, especially in solid tumours, including melanoma, non-small cell lung cancer, renal cell carcinoma[2-4] have been reported and provide us with new anti-cancer strategies. The use of immunotherapy, especially the ICIs, for treating GC is still in its infancy with several clinical trials underway.

CTLA-4, one of the immune checkpoints, is expressed on the surface of T cells following recognition of antigen. T cell CTLA-4 has a higher affinity than CD28 for APC CD80/CD86. This transduces an inhibitory signal to T cells serving as a "brake" on T cell activation. Ipilimumab, an IgG1 antibody which blocks CTLA-4 activity, allows ongoing APC priming of antigen-specific T cells in the TDLN (*i.e.*, brake removed). An additional proposed mechanism of ipilimumab action includes targeting of CTLA-4^{hi} intra-tumoural Tregs tipping the balance of effector T cells: Treg in favour of an anti-tumour response. Ipilimumab was the first approved immune checkpoint therapy and has shown a survival benefit in advanced stage melanoma patients[72,73]. The repercussions of meddling with the physiologic processes governing immunity is an increase in a variety of immune related side effects, including skin lesions (rash, pruritus, and vitiligo), colitis, thyroiditis, hypophysitis, and hepatitis[74]. A clinical trial in GC patients with unresectable, locally advanced or metastatic cancer following first line standard chemotherapy with a fluoropyrimidine/platinum combination (NCT01585987)

has recently been completed however initial results are not promising with poorer PFS (secondary endpoint) in the ipilimumab treated group[75]. Tremelimumab, another anti-CTLA-4 monoclonal antibody, was investigated as a second-line treatment for patients with unselected metastatic gastric and oesophageal adenocarcinomas. The results were disappointing and among 18 recruited patients only one patient achieved a partial response[76].

A second immune checkpoint target is the PD-1/PD-L1 axis. PD-1 is present on the surface of activated T-cells, B-cells and monocytes whilst PD-L1 is found on the surface of tumour cells and antigen presenting cells (macrophages and DCs)[2]. Similarly PD-L2, expressed exclusively on DCs, is also a ligand for PD-1 and has been shown to inhibit T-cell activation, proliferation and cytokine production[77]. Checkpoint inhibitor antibodies directed to the PD-1/PD-L1 pathway are thought to largely rescue function of pre-existing tumour-specific TILs.

Anti-PD-1 (nivolumab, pembrolizumab) or anti-PD-L1 (MSB0010718C, BMS936559, MPDL3280A, Medi4736) agents are humanized monoclonal antibodies, which inhibit binding of PD-1 to PD-L1 and restore T cell activity. Due to promising results from initial clinical trials utilising these antibodies in melanoma and in other cancer types, they are currently being explored in GC. A phase I clinical trial (NCT01928394) using nivolumab in GC patients has been completed and initial results showed objective responses occurred in patients irrespective of PD-L1 status[78].

Pembrolizumab has been tested in GC patients selected based on immunohistochemical staining of PD-L1 (NCT01848834)[79,80] and initial results reported at ESMO 2014[79] with updated results presented at ASCO 2015[80]. Eligible patients had PD-L1 positive staining in stromal or $\geqslant$ 1% tumour nest cells. Based on these criteria this study observed a 40% rate of PD-L1 positive cancers and demonstrated manageable toxicity and promising antitumour activity in advanced GC[80]. When used in melanoma patients, a positive response was associated with expression of four specific immune signatures (presented as an abstract)[81]. These findings were recapitulated in the GC[82] patients suggesting that screening for expression of these signatures could be used as a method to best select patients who might benefit from this treatment. A large number of clinical trials testing these drugs in combination with standard chemotherapies are currently underway and have been reviewed in detail elsewhere[83].

Several anti-PD-L1 monoclonal antibodies, including Avelumab (MSB0010718C), Durvalumab (Medi4736) and Atezolizumab (MPDL3280A) and BMS936559, are under evaluation in digestive cancers, including GC[84]. GCs comprise only a small minority of the patients recruited to the early phase clinical trials currently underway and as such only limited data on their efficacy is currently available[85]. It is worth noting that therapeutic strategies should be carefully considered. Whilst targeting the PD-1/PD-L1 + PD-L2 checkpoint pathways should increase anti-tumour efficacy, this may come at the cost of increased "off tumour target" toxicity. Therapies targeting only PD-L1 whilst maintaining PD-L2 activity may result in decreased anti-tumour effects coupled with decreased toxicity. There are currently no PD-L2 specific inhibitors available.

Therapeutic strategies targeting both CTLA-4 and PD-1 in combination are currently being tested in GC in the hope of identifying synergistic effects. The CheckMate032 (NCT01928394) trial testing the effects of nivolumab as a sole agent, or in combination with ipilimumab in a variety of solid cancers, including GC, and in a refractory setting is currently recruiting. This combination has previously showed successful tumour regression in the setting of melanoma[86].

The molecular, genetic and immunological heterogeneity described by the TCGA highlights a need to stratify patients based on their likelihood of responding to different treatment options including immunotherapy. Despite this, many of the clinical trials described above recruited GC patients of all subtypes which, unfortunately may dilute out the potential positive effects of these therapies. EBV and MSI subtypes of GC are associated with a vigorous immunological reaction, as well as over-expression of immune checkpoints, highlighting these two subtypes of GC as particularly attractive candidates for immune checkpoint blockade, and indeed trials in these particular GC subtypes are underway.

The EBV subtype described by the TCGA[7] is characterised by a high prevalence of mutations in the *PIK3CA* suggesting a possible therapeutic role for PI3K inhibitors. This subtype is also associated with a high prevalence of DNA hypermethylation and amplifications in the genes *CD274* and *PDCD1LG2* which encode the immunosuppressive proteins PD-L1 and PDL-2, which highlights this subtype as an ideal candidate for immunotherapy[7,83]. A clinical phase Ⅱ/Ⅲ trial (NCT02488759, CheckMate358) plans to test the efficacy of nivolumab in subjects with virus- associated tumours including EBV-positive GC. This trial is currently in the recruitment phase. Given that most of these patients have concurrent immune infiltrate and harbour mutations in targetable genes, an adjuvant approach including a targeted therapy in conjunction with a PD-1 inhibitor such as pembrolizumab may be warranted. Such a treatment combination would need to be evaluated to ensure that the targeted therapy doesn't directly inhibit immune effector cell signalling pathways.

The MSI TCGA GC subtype was characterised by high levels of microsatellite instability and elevated mutation rates[7]. Unsurprisingly gastrointestinal tumours that are MSI-H or mismatch repair deficiency, when compared to microsatellite stable tumours, have

shown promising immune-related objective response rates (ORR; 40% *vs* 0%) and progression-free survival (PFS; 78% *vs* 11%) when treated with PD-1 inhibitor, pembrolizumab[87]. This emphasizes this subtype as a potential candidate for immunotherapy.

Genomic amplifications in receptor tyrosine kinases were a distinguishing feature of the CIN subtype as defined by TCGA[7]. Many of these are candidates for treatment with molecular targeted therapies. A phase I clinical trial testing the effects of Pembrolizumab in combination with ramucirumab (NCT02443324) is currently recruiting and may be particularly effective in this subgroup. This group was also enriched for *TP53* mutations.

The GS TCGA subtype[7] (20% of all cases) comprised predominantly of tumours classified as diffuse GC, with poorer survival compared to the intestinal type GC, by the Lauren classification and was associated with mutations in *CDH1* and *RHOA* genes as well as aneuploidy. At this stage it is unclear whether this subtype would benefit from existing immunotherapies and warrants specific investigation.

With the significant clinical benefits from immune checkpoint blockade drugs, novel opportunities are emerging for GC treatment. To improve effectiveness of GC immunotherapy, novel criteria based on different molecular and immunological subtypes to predict potential response and prognosis are needed. Galon *et al*[88,89] have established an "immunoscore" in colorectal cancer based on the number and location of $CD3^+$ and $CD8^+$ cells[90]. This type of classification could be useful in GC. While we have focused on the immune component of the tumour microenvironment we must not lose sight that GC remains heterogeneous and while we may co-opt the immune system in destroying some cancers others may have mechanisms of resistance to avoid this form of killing. Therefore combination therapies may be the way of the future and we will need to be cognizant of the ensuing toxicities these therapies may invoke. It is important to also recognize the microenvironmental and immunological impact of the more traditional chemotherapeutics[91]. Examples include oxaliplatin, a platinum drug used often in GC chemotherapy which induces immunogenic cell death and provides a release of tumour antigens[92].

CONCLUSION

In most communities GC is diagnosed late and subsequently has poor prognosis. There are now exciting new therapies that utilise the host's immune system to fight back. However, data to date suggests we need to use these therapies judiciously to derive maximum benefit. GC is molecularly and immunologically heterogeneous, and this heterogeneity influences the tumour microenvironment in different ways. Returning to the battlefield analogy, the immunogenic or immune activating GC subtypes, EBV and MSI, are likely to be more conspicuous to the immune system by the expression of larger numbers of neo-antigens and other foreign epitopes that stimulate a vigorous immunological response that can be augmented by current therapies (Figure 2C), whereas the less immunogenic GCs, the CIN and GS subtypes, are more stealthy, with less antigen presentation providing a stronger defensive system against the host immune attack (Figure 2D). Like the battles in the wars of old, you need to choose your battlefield carefully and one of the key strategies, as enunciated by Sun Tzu, is to "know thy enemy", which translates to understanding the molecular nature of the cancer you are treating.

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TOPIC HIGHLIGHT

2016 Gastric Cancer: Global view

Benefits and harms of endoscopic screening for gastric cancer

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Abstract

Gastric cancer has remained a serious burden worldwide, particularly in East Asian countries. However, nationwide prevention and screening programs for gastric cancer have not yet been established in most countries except in South Korea and Japan. Although evidence regarding the effectiveness of endoscopic screening for gastric cancer has been increasingly accumulated, such evidence remains weak because it is based on results from studies other than randomized controlled trials. Specifically, evidence was mostly based on the results of cohort and case-control studies mainly conducted in South Korea and Japan. However, the consistent positive results from these studies suggest promising evidence of mortality reduction from gastric cancer by endoscopic screening. The major harms of endoscopic screening include infection, adverse effects, false-positive results, and overdiagnosis. Despite the possible harms of endoscopic screening, information regarding these harms remains insufficient. To provide appropriate cancer screening, a balance of benefits and harms should always be considered when cancer screening is introduced as a public policy. Quality assurance is very important for the implementation of cancer screening to provide high-quality and safe screening and minimize harms. Endoscopic screening for gastric cancer has shown promising results, and thus deserves further evaluation to reliably establish its effectiveness and optimal use.

Key words: Gastric cancer; Cancer screening; Upper gastrointestinal endoscopy; Mortality reduction; Cohort study; Case-control study; Harms

Core tip: Although evidence regarding the effectiveness

of endoscopic screening for gastric cancer has been increasingly accumulated based on consistent results, such evidence remains weak because it is based on the results of cohort and case-control studies mainly from South Korea and Japan. However, the consistent positive results suggest promising evidence of mortality reduction from gastric cancer by endoscopic screening. Despite the major harms of endoscopic screening, namely infection, adverse effects, false-positive results, and overdiagnosis, information regarding these harms remains insufficient. To provide appropriate cancer screening, a balance of benefits and harms should always be considered.

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INTRODUCTION

Gastric cancer has remained a serious burden worldwide, particularly in East Asian countries. In 2012, there was an estimated 1 million new cases of gastric cancer, with half of the world total occurring in Eastern Asia[1]. The highest mortality rates are observed in Eastern Asia, occurring at 24.0 per 100000 men and 9.8 per 100000 women. However, prevention and screening programs for gastric cancer particularly at the national level have not yet been established in most countries. The exceptions are South Korea and Japan where gastric cancer screening programs have already been introduced[2]. In Japan, gastric cancer screening using upper gastrointestinal series (radiographic screening) has been conducted as a national program since 1983, and it has been attributed to the decrease in gastric cancer mortality[3]. After the introduction of radiographic screening for gastric cancer, supporting evidence has been obtained from case-control and cohort studies mainly conducted in Japan[4].

Upper gastrointestinal endoscopy has been performed in clinical practice and is often introduced in opportunistic screening for gastric cancer in Asian countries[5]. Therefore, endoscopic screening has been anticipated to be introduced as a screening method in communities. Although South Korea was the first country to introduce endoscopic screening for gastric cancer, there was insufficient evidence of mortality reduction from gastric cancer when it was adopted as a national program[6]. Over the last decade, evidence regarding the effectiveness of endoscopic screening for gastric cancer has been increasingly accumulated. Recently in South Korea and Japan, cancer screening guidelines have been revised based on the new research results of endoscopic screening for gastric cancer[7,8]. In both guidelines, endoscopic screening takes an important position in gastric cancer screening. However, evidence regarding the effectiveness of endoscopic screening for gastric cancer remains controversial, and quality assurance needs to be established. Discussion related to its benefits and harms is needed to promote the establishment of endoscopic screening for gastric cancer based on current and reliable evidence.

EFFECTIVENESS OF ENDOSCOPIC SCREENING FOR GASTRIC CANCER

Evidence regarding the effectiveness of endoscopic screening for gastric cancer has been obtained from cohort and case-control studies mainly conducted in South Korea and Japan. Since these countries have already introduced endoscopic screening for gastric cancer, the study design is limited to observational studies. However, the consistent positive results from these studies suggest promising evidence of mortality reduction from gastric cancer by endoscopic screening.

Cohort studies

The results from 5 published cohort studies of endoscopic screening for gastric cancer conducted in China and Japan are shown in Table 1. The target population of these studies was limited to asymptomatic individuals in communities. For the first cohort study concluded in China, mortality reduction could not be shown[9]. In the area with a high incidence of gastric cancer, endoscopic screening was offered twice with a 5-year screening interval. The standard mortality ratio of participation in endoscopic screening was 1.01 (95%CI: 0.72-1.37) for men and 0.65 (95%CI: 0.26-1.32) for women.

Earlier studies conducted in Japan had several problems in that they included individuals aged over 70 years and ignored the screening history before the defined first screening[10,11]. Although the study by Hosokawa *et al*[11] had the largest sample size, the sample selection period was different between the radiographic screening group and the endoscopic screening: the radiographic screening group was selected from communities in 1995 whereas the endoscopic screening group was selected from screening center from 1986 to 1999. Therefore, the age distributions and backgrounds of individuals in both groups were different[11].

In Japan, although radiographic screening has been established as the standard method for the national gastric cancer screening program, some municipalities have now individually introduced endoscopic screening for gastric cancer. As an example, Niigata City has provided 3 types of gastric cancer screening since 2005: endoscopy, regular radiography, and photofluorography. After a 5-year follow-up period, standard mortality ratios (SMRs) were calculated and were

Table 1 Comparison of results from cohort studies of endoscopic screening for gastric cancer

Author	Riecken *et al*[9]	Matsumoto *et al*[10]	Hosokawa *et al*[11]	Hamashima *et al*[12]	Hamashima *et al*[13]
Publication	2002	2007	2011	2015	2015
Country	China	Japan	Japan	Japan	Japan
Target age, yr	35-64	⩾ 40	Average: 50.0	40-79	40-79
Follow-up, yr		9	5	5	6
Number of subjects	4364	7178	18011	16373	9950
Comparators	-	-	Radiographic screening	-	Radiographic screening
Number of comparators	-	-	36870	-	4324
Outcome indicators	SMR	SMR	Hazard ratio (HR)	SMR	Relative risk
Main results	1.01 (95%CI: 0.72-1.37)	Male: 0.71 (95%CI: 0.33-1.10) Female: 0.62 (95%CI: 0.19-1.05)	HR = 0.15 (95%CI: 0.05-0.50) Adjusted HR[1] = 0.23 (95%CI: 0.07-0.76)	0.43 (95%CI: 0.30-0.57)	Adjusted RR[2] 0.327 (95%CI: 0.118-0.908)

[1]Adjusted HR by sex and age; [2]Adjusted RR by sex, age group, and resident city. SMR: Standard mortality ratio; CI: Confidence interval; HR: Hazard ratio; RR: Relative risk.

Table 2 Comparison of results from case-control studies of endoscopic screening for gastric cancer

Author	Matsumoto *et al*[14]	Hamashima *et al*[15]	Cho[17]
Publication	2014	2013	2013
Country	Japan	Japan	South Korea
Target age, yr	54-91	40-79	⩾ 40
Number of cases	13	410	35457
Number of control	130	2292	141828
Comparator	Never-screened	Never-screened	Never-screened
Main results	0.206 (95%CI: 0.044-0.965)	0.695 (95%CI: 0.489-0.986)	0.430 (95%CI: 0.40-0.46)

referred to as cancer mortality rate of the population of Niigata City[12]. The SMRs of gastric cancer death were 0.43 (95%CI: 0.30-0.57) for the endoscopic screening group, 0.68 (95%CI: 0.55-0.79) for the regular radiographic screening group, and 0.85 (95%CI: 0.71-0.94) for the photofluorography screening group. The mortality reduction from gastric cancer was higher in the endoscopic screening group than in the regular radiographic screening group despite the nearly equal mortality rates of all cancers except gastric cancer. Tottori City and Yonago City have more than 10 years of history of conducting endoscopic screening for gastric cancer. Theses cities have also provided both endoscopic screening and radiographic screening. After 6 years of follow-up, the subjects screened by endoscopy showed a 67% reduction of gastric cancer compared with the subjects screened by radiography (adjusted relative risk by sex, age group, and resident city: 0.327, 95%CI: 0.118-0.908)[13].

Case-control studies

The results from case-control studies of endoscopic screening for gastric cancer conducted in South Korea and Japan are shown in Table 2. In previous Japanese guidelines, evidence regarding the effectiveness of radiographic screening for gastric cancer was based on the results of case-control studies[4]. Although these results suggest that gastric cancer mortality could be reduced by endoscopic screening, prudence must be observed in interpreting positive results because these case-control studies may have self-selection bias.

The results of community-based case-control studies of endoscopic screening in Japan have recently been reported by Matsumoto *et al*[14] and Hamashima *et al*[15]. Results of the larger case-control study of Hamashima *et al*[15] conducted in Tottori and Niigata prefectures showed a 30% reduction in gastric cancer mortality by participation in endoscopic screening at least once within 36 mo before the date of diagnosis of gastric cancer compared with never-screened individuals. Although the sample size was small in their Nagasaki study, Matusmoto *et al*[14] reported a higher mortality reduction from gastric cancer by 80%.

In South Korea, endoscopic screening has been performed together with radiographic screening, and the recent participation rate has exceeded that of radiographic screening[16]. Based on the national database, a nested case-control study from South Korea reported a 57% mortality reduction from gastric cancer by endoscopic screening[17]. Mortality reduction from gastric cancer by endoscopic screening was observed in the 40- to 79-year age group when participating in endoscopic screening within 1 year to 3 years before the date of gastric cancer diagnosis.

INDIRECT EVIDENCE REGARDING THE EFFCTIVENESS OF ENDOSCOPIC SCREENING

Mortality reduction from the target cancer should be evaluated as the most reliable evidence regarding the effectiveness of cancer screening. Sensitivity of the screening test, stage shift, and survival rate of detected cancers by screening are also occasionally considered as possible indicators showing indirect evidence regarding the effectiveness of endoscopic screening for

Table 3 Sensitivities and specificities of endoscopy and radiography for gastric cancer screening

Screening round	Method	Sensitivity by the detection method	Specificity by the detection method	Sensitivity by the incidence method
Prevalence screening	Endoscopic screening	0.955 (95%CI: 0.875-0.991)	0.851 (95%CI: 0.843-0.859)	0.886 (95%CI: 0.698-0.976)
	Radiographic screening	0.893 (95%CI: 0.718-0.977)	0.856 (95%CI: 0.846-0.865)	0.831 (95%CI: 0.586-0.964)
Incidence screening	Endoscopic screening	0.977 (95%CI: 0.919-0.997)	0.888 (95%CI: 0.883-0.892)	0.954 (95%CI: 0.842-0.994)
	Radiographic screening	0.885 (95%CI: 0.664-0.972)	0.891 (95%CI: 0.885-0.896)	0.855 (95%CI: 0.637-0.970)

Adapted from Hamashima *et al*[19].

gastric cancer. However, these three indicators are not valid for revealing evidence regarding the effectiveness of cancer screening because they include biases and require prudent interpretation.

Sensitivity of endoscopic screening

The sensitivity of endoscopic screening has recently been compared with that of radiographic screenings[18,19]. However, since the screening interval and sensitivity calculation method were different between the screening methods, a direct comparison of the results is not suitable. Although the definition of interval cancer was different between South Korea and Japan, the sensitivity of endoscopic screening was always higher than that of radiographic screening. However, there may be an increases frequency of overdiaganosis by endoscopic screening because it can detect cancer earlier and more than radiographic screening.

In a study conducted in South Korea, the sensitivity of endoscopic screening calculated by the detection method was 69.4% (95%CI: 66.4%-72.4%) for the first round of screening and 66.9% (95%CI: 59.8%-74.0%) for the subsequent round[18]. On the other hand, the sensitivity of radiographic screening was 38.2% (95%CI: 35.9%-40.5%) for the first round of screening and 27.3% (95%CI: 22.6%-32.0%) for the subsequent round[18]. In a study conducted in Japan, the sensitivity of prevalence screening for the first round was 0.955 (95%CI: 0.875-0.991) for endoscopic screening and 0.893 (95%CI: 0.718-0.977) for radiographic screening (Table 3)[19]. On the other hand, the sensitivity of incidence screening on the subsequent round was 0.977 (95%CI: 0.919-0.997) for endoscopic screening and 0.885 (95%CI: 0.664-0.972) for radiographic screening.

Stage shifts and survival rates of detected cancer by endoscopic screening

In South Korea, both endoscopic screening and radiographic screening have been provided in the national screening programs[6]. Among cancers detected from 2002 to 2007 based on the national cancer registry, localized gastric cancers were more frequently recorded in endoscopic ever-screened patients than in radiographic ever-screened patients and never-screened patients[20]. Compared with never-screened patients, the odds ratio for being diagnosed with localized gastric cancer in endoscopic-screened patients was 2.10 (95%CI: 1.90-2.33). Stage shifts by endoscopic screening could lead to improvement of the survival rate of the detected cancer by endoscopic screening. In a study conducted in Japan, the 5-year survival rates were 91.2% ± 1.5% (95%CI: 87.5%-93.8%) for the endoscopic screening group, 84.3% ± 2.9% (95%CI: 87.5%-93.8%) for the radiographic screening group, and 66.0% ± 1.6% (95%CI: 62.8%-68.9%) for the outpatient group[21].

HARMS OF ENDOSCOPIC SCREENING

The major harms of endoscopic screening include infection, adverse effects, false-positive results, and overdiagnosis. Infection and adverse effects are original risks of endoscopic screening for gastric cancer, but false-positive results and overdiagnosis are characteristics common in cancer screening.

As everyone is a potential source of infection, all endoscopy procedures can be contaminated[22]. Hepatitis B infection caused by endoscopy was reported in the 1980s in Japan[23,24]. *Helicobacter pylori* (*H. pylori*) infection was reportedly caused by upper intestinal endoscopy and induced acute gastric mucosal lesions[25,26]. The Japan Gastrointestinal Endoscopy Society has published guidelines and manuals for the proper cleaning and disinfection of endoscopes, and had also promoted appropriate methods of cleaning and disinfection of endoscopes according to the standard guidelines set by the World Gastroenterology Organization[27].

Over the last 3 years, the Japanese Association of Gastroenterological Cancer Screening has recorded the number of adverse effects of endoscopic screening for gastric cancer during latest 3 years[28-30]. Of the 740245 endoscopic examinations conducted, the rate of adverse effects was 78 per 100000 participants in endoscopic screening for gastric cancer. The most common adverse effects were nasal bleeding and gastric mucosal laceration. The number of bleeding cases after biopsy was 21, with 4 cases requiring admission. However, the association between bleeding and anticoagulant use was unclear. Although endo-

scopic examination is often performed after the temporary stoppage of anticoagulants, there are risks of thrombosis during drug holidays[31-33] and bleeding after retaking anticoagulants[34,35]. However, regardless of taking anticoagulants, there is always a possibility of bleeding to occur[36,37]. Although serious adverse effects including anaphylactic shock and respiratory depression have been reported, there was no case leading to death in any of the reports of the Japanese Association of Gastroenterological Cancer Screening. In a survey conducted by the Japanese Gastrointestinal Endoscopy Society, cases of death caused by sedation for endoscopic examination have been reported[38].

A false-positive result is a common harm in cancer screening and requires further examination to definitively diagnose gastric cancer. In breast cancer screening, it has been suggested that a false-positive result induces psychological anxiety[39]. Although the rate of endoscopic screening for gastric cancer has been reported to be 14.9% for prevalence screening and 11.2% for subsequent screening[19], there have been no reports related to psychological burden from endoscopic screening of gastric cancer.

Overdiagnosis is the most serious harm of cancer screening[40]. Apparently, there is still no study estimating the number of overdiagnosis of gastric cancer by endoscopic screening. Based on the results of endoscopic screening for gastric cancer, the observed number of detected cancer was twice compared with the expected number in the target group of endoscopic screening for gastric cancer[41]. The excess cancers included not only overdiagnosis cases but also early cancers which have the actual possibility of progressing into advanced cancers that lead to death.

Sensitivity is affected by overdiagnosis and it is often overestimated. The detection method is the most common and simplest procedure of calculating sensitivity wherein the number of detected cancers is used as the numerator and the sum of detected cancers and interval cancers is used as the denominator. Although the detection method is commonly used for measuring the sensitivity of the screening method, it cannot exclude cases of overdiagnosis. Notably, the incidence method was developed to avoid cases of overdiaganosis during sensitivity calculations[42]. Breast, lung, and colorectal cancer screenings have been evaluated using the incidence method[43-45]. In prevalence screening, the sensitivity was reportedly 0.955 (95%CI: 0.875-0.991) by the detection method and 0.886 (95%CI: 0.698-0.976) by the incidence method (Table 3)[19]. In incidence screening, the sensitivity was reportedly 0.977 (95%CI: 0.919-0.997) by the detection method and 0.954 (95%CI: 0.842-0.994) by the incidence method[19]. The discrepancy between the results calculated by the detection method and the incident method was small, suggesting the negligible effect of overdiagnosis on endoscopic screening for gastric cancer.

DISCUSSION

To effectively introduce a new cancer screening method, mortality reduction from the target cancers must be carefully evaluated based on appropriate and reliable studies. However, since randomized controlled trials related to gastric cancer screening are lacking, observational studies have played as a central role in providing evidence regarding mortality reduction from gastric cancer. Importantly, evidence obtained from observational studies has limitations because such evidence cannot exclude serious biases, particularly selection bias. On the other hand, the results of observational studies can show the actual effectiveness in real settings. As South Korea and Japan have already introduced gastric cancer screening, planning a new randomized controlled trial of endoscopic screening for gastric cancer is difficult. Although lines of evidence regarding the effectiveness of endoscopic screening have been accumulated, information on harms remains insufficient. This becomes a barrier for estimating the net benefits of endoscopic screening for gastric cancer.

The adverse effects of endoscopic screening cannot be ignored because the participants of gastric cancer screening are asymptomatic and healthy people who have not yet experienced adverse effects following their participation in cancer screening. However, as upper gastrointestinal endoscopy is an invasive technology, adverse effects cannot be avoided. Bleeding is a common adverse effect and it can occur regardless of whether a patient is taking anticoagulants or not[36,37]. Moreover, respiration depression can lead to death when sedation is used in endoscopic examination[38]. On the other hand, endoscopy-induced infection becomes a serious problem with the widespread use of endoscopic examinations. Also, there is a risk of transmitting any infection *via* endoscopy if endoscope is not property cleaned and disinfected. These adverse effects and infection can be reduced by appropriate management. This is the basic requirement of quality assurance of cancer screening. In European countries, quality assurance guidelines for cervical, breast, and colorectal cancers have been published and they have become standards for the management of these programs[46-48]. Since 2000, South Korea has introduced endoscopic screening for gastric cancer as one of its national cancer screening programs and has developed quality assurance guidelines[6,49]. In Japan, an academic society has developed a quality assurance manual for endoscopic screening of gastric cancer and has recommended the appropriate management[50-52].

False-positive result and overdiagnosis are common harms of all cancer screenings. Both harms lead to unnecessary further examinations and additional burden for participants in cancer screenings. When cancer screening starts, these harms cannot be avoided[53]. Recently, a value framework has been suggested as a new concept of cancer screening[53,54]. In this concept,

providing the appropriate number of cancer screening is recommended to minimize the harms and maximize the screening value. The Korean guidelines for gastric cancer screening defined the target age group from 40 to 69 years[8]. The Japanese guidelines for gastric cancer screening set the starting age from 50 years with no upper age limit[7]. Both guidelines have recommended a 2-year screening interval. Based on a comparison of the stage distribution of detected cancers by endoscopic screening, a 2-year screening interval was suggested in a Korean study[55]. However, in a Korean case-control study, mortality reduction was shown even if the screening interval was extended until 3 years[17]. To minimize harms, additional studies are needed to determine the appropriate target age group and screening interval.

Although the burden of gastric cancer has not been ignored worldwide, gastric cancer screening programs using endoscopy are currently limited to South Korea and Japan. *H. pylori* is one of the main causes of gastric cancer, and 78% of all gastric cancer cases are estimated to be attributed to chronic *H. pylori* infection[56]. IARC has recommended *H. pylori* screening and treatment strategies considering the disease burden and local context[56]. Although risk stratification can be carried out using *H. pylori* antibody and serum pepsinogen tests[57], it is difficult to predict individuals who will not have gastric cancer in the future because of low predictive specificity of these tests. On the other hand, it is possible to diagnose *H. pylori* infection by endoscopy based on a specific feature in the gastric mucosa[58]. Although the discrimination ability to predict the development of gastric cancer by biomarkers and endoscopy is insufficient, considerations should be given on how to use biomarkers in combination with endoscopic screening, for example, adaptation to expand the screening interval. Further study is needed regarding the combination of endoscopic screening with these biomarkers.

CONCLUSION

Lines of evidence regarding the effectiveness of endoscopic screening have been steadily accumulated showing consistent results. However, these lines of evidence remain weak because they are based on the results of studies other than randomized controlled trials. Moreover, even if possible harms of endoscopic screening can be ascertained, specific information regarding these harms is still insufficient. To provide appropriate cancer screening, a balance of benefits and harms should always be considered when cancer screening is introduced as a public policy. Quality assurance is very important for the implementation of cancer screening to provide high-quality and safe screening and minimize harms. Endoscopic screening for gastric cancer has clearly shown promising results, and thus warrants confirmatory evaluation to reliably establish its effectiveness and optimal use.

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TOPIC HIGHLIGHT

2016 Hepatitis C virus: Global view

Hepatitis C virus: Promising discoveries and new treatments

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Abstract

Despite advances in therapy, hepatitis C virus (HCV) infection remains an important global health issue. It is estimated that a significant part of the world population is chronically infected with the virus, and many of those affected may develop cirrhosis or liver cancer. The virus shows considerable variability, a characteristic that directly interferes with disease treatment. The response to treatment varies according to HCV genotype and subtype. The continuous generation of variants (quasispecies) allows the virus to escape control by antivirals. Historically, the combination of ribavirin and interferon therapy has represented the only treatment option for the disease. Currently, several new treatment options are emerging and are available to a large part of the affected population. In addition, the search for new substances with antiviral activity against HCV continues, promising future improvements in treatment. Researchers should consider the mutation capacity of the virus and the other variables that affect treatment success.

Key words: Hepatitis C infection; Hepatitis C virus; Treatments; Antiviral research

Core tip: In recent years, new treatments for hepatitis C have been approved and represent a major advancement in this field. However, there are limitations that should be considered, and research for new treatments must continue. The objective of this review is to demonstrate the breakthroughs that have occurred and to discuss future developments.

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INTRODUCTION

Hepatitis C virus (HCV) infection is an important cause of cirrhosis and hepatocellular carcinoma worldwide[1-3]. It is very dangerous due to the breakthrough of long-term asymptomatic HCV[4].

HCV is transmitted through exposure to infected blood and blood products. HCV infection can be spread through blood transfusion, injection drug use, sexual intercourse, surgery, and tattooing[2,5]. HCV infection is defined as the presence of HCV RNA and anti-HCV antibodies in the serum or plasma; a positive HCV antibody test indicates exposure to HCV and could represent a current or past infection. A positive HCV RNA test indicates a current HCV infection[2].

It is estimated that 130-150 million people globally have chronic hepatitis C infection, and a significant number of those who are chronically infected will develop liver cirrhosis or liver cancer. According to the World Health Organization, 350000 to 500000 people die each year from hepatitis C-related liver diseases. The most affected regions are Central and East Asia and North Africa, although the virus is found worldwide[6].

The natural history of HCV is influenced by a wide variety of factors. Host factors include age at infection, gender, race, obesity, steatosis, insulin resistance/diabetes, genetics, alanine aminotransferase levels and exercise. Viral factors include HCV RNA level, quasispecies/genotype, coinfection with hepatitis B virus and coinfection with human immunodeficiency virus. Environmental factors include alcohol use, cigarette use, cannabis use, caffeine consumption and herbal product use[1].

Approximately 15%-45% of infected persons spontaneously clear the virus within 6 mo of infection without any treatment, and the remaining 55%-85% of persons may progress to persistent chronic infection. It is estimated that the risk of developing cirrhosis of the liver within 20 years is 15%-30% in those with chronic HCV infection[6], and the risk of developing hepatocellular carcinoma is 1%-4% per year[1].

Acute HCV hepatitis in immunocompetent individuals is generally asymptomatic, but immunocompromised hosts (HIV infection) experience lymphoplasmatic portal inflammation, interface hepatitis, necroinflammatory lobular changes, moderately advanced fibrosis or rapid progression to fibrosis over a period of time[7].

Chronic hepatitis is defined as the persistence of infection for at least 6 mo after the onset of infection and is characterized by necroinflammation accompanied by a variable degree of fibrosis[7], end-stage liver disease and hepatocellular carcinoma[1].

The main extrahepatic manifestations in patients with HCV infection are immune- and inflammatory-related. Immune-related extrahepatic manifestations include mixed cryoglobulinemia, cryoglobulinemic vasculitis, B-cell NHL, Sicca syndrome, arthralgia/myalgia, autoantibody production (*i.e.*, cryoglobulins, rheumatoid factor, and antinuclear, anticardiolipin, antithyroid and anti-smooth muscle antibodies), polyarthritis nodosa, monoclonal gammopathies, and immune thrombocytopenia. Inflammatory-related extrahepatic manifestations consist of type 2 diabetes mellitus, insulin resistance, glomerulonephritis, renal insufficiency, fatigue, cognitive impairment, depression, impaired quality of life, polyarthritis/fibromyalgia, and cardiovascular disorders (*i.e.*, stroke and ischemic heart disease)[8]. Recent studies have suggested that HCV infection leads to increased risk of developing cardiovascular diseases and has been linked to increased risk of mortality caused by these diseases[9].

Several studies have considered the existence of occult HCV infection (viral RNA identified in hepatocytes but absent in serum). Although occult HCV infection is challenged by some researchers and is characterized by others as a milder condition than chronic hepatitis C, it is necessary to consider its existence because some studies have shown links between occult HCV infection and liver cirrhosis and hepatocellular carcinoma. In addition, due to the emergence of new treatment options, all possibilities should be considered, and special attention should be given to transfusion centers and patient risk groups[10,11].

HCV

HCV, classified in the family *Flaviviridae*, is an enveloped, single stranded positive sense RNA virus with a genome approximately 9600 nucleotides in length[12], encoding approximately 3000 amino acids[13]. Most of the genome is composed of a single open reading frame that encodes ten proteins: three structural proteins (core, E1, E2) and seven non-structural proteins (p7, NS2, NS3, NS4A, NS4B, NS5A, NS5B)[13]. Most recently, an HCV protein named F was reported[14,15].

The most variable region of the genome is the region that codes the membrane glycoproteins E1 and E2[16]. Within the E2 gene, two hypervariable regions (HVR1 and HVR2) are described that show less sequence homology between isolates, with 50% identity[17]. Non-structural protein genes, such as the core gene, are some of the most conserved on the genome[12]. Within the 5'-NCR region, the most distant related isolates present 90% sequence identity[18,19].

The host immune system, large population sizes, short generation times and high replication rates are the factors that lead to the genetic variability

of HCV[20]. Seven genetic lineages (genotypes 1 to 7) are recognized. These genotypes are subdivided into closely related sub-types differing from each other by 15% in nucleotide sequences[21]. Differences between the complete genomes occur at 31%-33% of nucleotide sites[22]. In Western countries, subtypes 1a, 1b and 3a are believed to cause the majority of HCV infections and are widely distributed[12].

For example, only 10%-20% of patients chronically infected with HCV genotype 1 show complete and permanent disappearance of the virus when treated with IFN-α alone, and 40%-50% experience successful treatment when treated with a combined therapy (INF-α/RBV). When patients infected with genotypes 2 or 3 are treated with monotherapy or combined therapy, higher successful treatment rates are observed (50% and 70%-80%, respectively)[23,24].

The mutation rate of HCV is estimated to be 2.5×10^{-5} mutations per nucleotide per genome replication[25], which is one of the highest rates for RNA viruses, including retroviruses[26]. Recombination also increases the genetic variability of HCV. Inter and intra genotypic recombinations have already been reported in various geographic locations, and the existence of intergenotypic recombination forms has also been reported[27,28]. Genotype 2 is considered to be present in most of the cases of recombinant forms, but other genotypes, except for genotype 4 and 7, also exhibit recombinant forms[29]. At the same time, the mutation rate may negatively affect the viability of viral populations. In a situation called lethal mutagenesis, a nucleotide sequence loses its information when the error rate transcends a tolerable limit[30].

Due to the high replication rate of HCV, an extensive number of variants are continuously produced during infection. These variants are closely related to each other but differ in nucleotide sequence, circulating as a complex population known as quasispecies. This population is able to rapidly adapt to a constantly changing environment[31,32]. This adaptation could lead to the coexistence of diverse variants in infected patients, creating an environment for intra and inter quasispecies interactions[33]. As a result of the continuous generation of variants, some of the variants may adapt to this changing environment and escape control by antiviral drugs[34]. Predominant and minor quasispecies can be transmitted in humans[35,36] and experimentally infected chimpanzees[37,38]. It is important to highlight that the HCV population fluctuates in patients during therapy, suggesting that HCV quasispecies may follow different evolution paths in different patients[39].

Host genetics are also indicated as a factor that could influence HCV evolution and treatment response[40]. For example, single nucleotide polymorphisms near the IL-28B gene appear to be associated with a low genetic variability in the NS3 coding region of the HCV genome[41].

EVOLUTION OF HCV TREATMENT

Since HCV treatment began in the early 1990s, treatment options have improved. Interferon alpha (IFN-α) was the first pan-genotypic option, with sustained virologic response (SVR) rates of 8%-21%[42]. Subsequently, the guanosine analogue ribavirin (RBV) was combined with IFN-α, which enhanced SVR rates to 40%. Then, pegylated IFN-α (PEG-IFN-α) associated with RBV improved SVR rates from 42% to 52%[43-45]. In 2011, the first wave of direct-acting antiviral agents (DAAs), NS3/4A protease inhibitors telaprevir (TVR) and boceprevir (BOC), became available. The association of these protease inhibitors with PEG-IFN-α/RBV improved SVR rates among patients with HCV genotype 1 infection. In treatment-naïve patients, the addition of TVR or BOC to PEG/RBV leads to an SVR increase of approximately 30%. Despite this improvement in SVR, these drugs were associated with serious adverse events (AEs) and low tolerance. More recently, a second wave of new DAAs allowed IFN-free, highly effective regimens. SVR can be achieved in more than 90% of treated patients *via* IFN-free regimens, with minimal AEs and high tolerability[46-49]. Unlike the nonspecific IFN-α-based and PEG-IFN-α-based therapies, DAAs target various proteins involved in HCV replication. In addition, most of these agents are specific to one or more genotypes. The classes of DAAs include NS3/4A protease inhibitors, NS5A inhibitors (nucleotides and non-nucleotide analogues), and NS5B polymerase inhibitors. During HCV replication, NS3/4A serine protease is required for self-cleavage, the NS5A region plays an important role in viral replication and assembly, and the NS5B region encodes RNA polymerase. Some of these drugs were initially combined with PEG-IFN-α and RBV, including the protease inhibitors TVR, BOC and simeprevir (SMV) and the NS5B polymerase inhibitor sofosbuvir (SOF). However, these regimens were still associated with PEG-IFN-α and RBV AEs and low tolerability[50-52].

All-oral treatment options followed this development, some associated with RBV and some not, including the combinations of SMV and SOF and SOF and NS5A inhibitor daclatasvir, the 3D regimen (paritaprevir/ritonavir/ombitasvir, co-administered with dasabuvir), and SOF plus ledipasvir. In recent years, more drugs have been designed, including grazoprevir, elbasvir, asunaprevir, beclabuvir, faldaprevir, and deleobuvir[52,53]. Drug options and their respective mechanisms and genotype sensitivity are shown in Table 1. The combined therapies increased SVR rates and tolerability and shortened treatment duration[52]. Treatment options for HCV infection, ranging from IFN-based regimens to new all-oral combinations, are presented in Table 2.

Although HCV treatment has clearly improved since the introduction of DAAs, some challenges remain. Some populations remain difficult to treat, such as cirrhotic patients, prior non-responders, genotype 3-infected patients and patients with renal

Table 1 Drugs for hepatitis C virus treatment

Mechanism of action	Drug	Genotype
Protease inhibitor	Telaprevir	1
	Boceprevir	1
	Simeprevir	1
	Paritaprevir	1, 4
	Grazoprevir	1, 4
	Asunaprevir[1]	1
	ABT-450[1]	1
	Faldaprevir[1]	1
NS5A inhibitors	Daclatasvir	1, 3
	Ombitasvir	1, 4
	Ledipasvir	1, 4, 5, 6
	Elbasvir	1, 4
	Velpatasvir[1]	4
NS5B inhibitors		
Nucleotide-analogue	Sofosbuvir	1, 2, 3, 4, 5, 6
Non-nucleoside analogue	Dasabuvir	1
	Beclabuvir[1]	1

[1]Non-FDA approved drugs.

impairment[52,60]. In this group, the combination of grazoprevir and elbasvir appears to result in high SVR rates in the short-term[72]. Most of the DAAs have drug-to-drug interactions, requiring the substitution or suspension of some medications during HCV treatment[9]. The development of viral drug-induced resistance may compromise actual and future treatment options. Finally, some DAA regimens have significant cost barriers and are not fully available worldwide[52,73].

DAAs are currently too expensive for governments worldwide, especially in some low- and middle-income countries. Nevertheless, price decreases have already been announced for some of these drugs. As patents expire, high drug costs will be reduced, although this process will take several decades. This prospect offers some hope that universal access to the treatment might be possible and that DAAs will be optimally used to reduce HCV-related mortality and incidence in low- and middle-income countries[74,75].

ANTIVIRAL RESEARCH

Despite the progress of current DAAs in terms of SVR and treatment tolerability, difficult to treat populations and the emergence of resistant virus species indicate the continued need for research into new treatment options.

Some studies indicate that caffeine has the potential to improve liver function in patients chronically infected with HCV. Through *in vitro* research, it was possible to verify that caffeine may be an important new agent for anti-HCV therapies due to its efficient inhibition of HCV replication at non-toxic concentrations[76].

Many researchers use the bovine viral diarrhea virus (BVDV), another *Flavivirus* member, as a surrogate model in HCV studies because propagation *in vitro* is difficult, and HCV and BVDV share similarities with respect to replication cycle, biology, and genetic organization[77]. Many studies have sought new compounds with antiviral activity from natural products. For example, a study performed in Brazil[78] investigated the antiviral activity of several marine invertebrates and the microorganisms isolated from them. This study showed that an extract produced from the *Bacillus* genus, isolated from the sponge *Petromica citrina*, has potential antiviral activity and demonstrated an inhibition of 98% and a high selectivity index during viral adsorption. Another study performed in Brazil[79] described the antiviral activity, with 99% of inhibition and a selectivity index greater than 200 μmol/L, of compounds produced from *Streptomyces chartreusis*, a termite-associated bacterium.

Extracts from plants have also been investigated as potential producers of novel compounds that could be used to treat HCV. For example, one study[80] that screened Brazilian plant species described four compounds-a natural alkaloid isolated from *Maytrenus ilicifolia* and three other compounds from *Peperomia blanda*-that could drastically reduce RNA levels and viral protein levels during HCV replication.

In addition to the importance of screening, and due to the number of extracts that could be evaluated, others research studies have already identified molecules from natural sources belonging to different chemical families that have antiviral activity that affects different stages of the HCV life cycle[81]. Some active molecules cited in this review[81] include naringenin, extracted from grapefruit, quercetin, extracted from *Embelia ribes*, honokiol, extracted from *Magnolia grandiflora*; and others.

The polyphenols excoecariphenol D and corilagin, both isolated from the Chinese mangrove plant (*Excoecaria agallocha*), were shown to potentially inhibit HCV RNA replication in cells[82]. The monoterpene camphor, isolated from *Ocimun basilicum*, was identified as a potential virucidal, suggesting that the mechanism of action of this compound acts directly on the viral particle[83].

Another compound known as silymarin, a flavonoid extract from the milk thistle of *Silybum marianum*, showed promising antiviral activity, both *in vitro* and *in vivo* at different stages of HCV replication: entry of the HCV into the cell hosts, RNA and protein expression and in the secretion of infectious viral particles[84]. This compound has also been reported[85] to potently reduce HCV RNA levels *in vivo* when administered intravenously, and to dose-dependently inhibit the HCV 3a core gene[86].

CONCLUSION

In recent years, treatment for hepatitis C has made considerable advances, represented by an increase in SVR rates, reduction of treatment duration and reduction of AEs. However, despite these advances, the

Table 2 Treatments for hepatitis C virus infection

Ref.	Treatment, wk	Genotype	SVR (total, cirrhotic, non-cirrhotic)	Previously treated Patients included
Davis *et al*[53]	IFN, 24	1	3; NA; NA	Yes
	IFN + RBV, 24		30; NA; NA	
IDEAL Study Team[54]	PEG-IFN/RBV, 48	1	39.8-40.9; 42.1-43.6; 20.7-23.6	No
ADVANCE Study Team[48]	TVR + PEG-IFN/RBV	1	75; 62-81; 62	No
REALIZE Study Team[49]	TVR + PEG-IFN/RBV, 48	1	63-64; 28-84; NA	Yes
SPRINT-2 Investigators[47]	BOC + PEG-IFN/RBV, 28	1	67; 70; 50	No
	BOC + PEG-IFN/RBV, 48		68; 70; 50	
RESPOND-2 Investigators[46]	BOC + PEG-IFN/RBV, 36	1	59; 35; 64	Yes
	BOC + PEG-IFN/RBV, 48		66; 77; 66	
PILLAR[55]	SMP + PEG-IFN/RBV, 12	1	80.5; NA; 80.5	No
	SMP + PEG-IFN/RBV, 24		86.1; NA; 86.1	
Gane *et al*[56]	SOF + RBV, 12	2 or 3	100; NA; 100	No
	SOF + PEG-IFN/RBV, 8		100; NA; 100	
COSMOS[57]	SMP + SOF + RBV, 24	1	NA; 93; 79	Yes
	SMP + SOF, 24		NA; 93; 100	
	SMP + SOF + RBV, 12		NA; 93; 93	
	SMP + SOF, 12		NA; 90; 94	
OPTIMIST-1[58]	SMP + SOF, 8	1	83; NA; 83	Yes
	SMP + SOF, 12		97; NA; 97	
OPTIMIST-2[59]	SMP + SOF, 12	1	83; 83; NA	Yes
AI444040 Study group[60]	DCV + SOF, 24	2 or 3	100; NA; NA	No
	DCV + SOF + RBV, 24		86; NA; NA	No
	DCV + SOF, 24	1	100; NA; NA	Yes
	DCV + SOF + RBV, 24		95-100; NA; NA	
ALLY-3 Study Team[61]	DCV + SOF, 12	3	86-91; 63; 96	Yes
ION-2 Investigators[62]	LDV + SOF, 12	1	94; 86; 97	Yes
	LDV + SOF, 24		99; 100; 99	
	LDV + SOF + RBV, 12		96; 87; 100	
	LDV + SOF + RBV, 24		99; 100; 99	
ION-3 Investigators[63]	LDV + SOF, 8	1	94; NA; 94	No
	LDV + SOF + RBV, 8		93; NA; 93	
	LDV + SOF, 12		95; NA; 95	
MALACHITE- Ⅰ / Ⅱ[64]	OBV + PTV/r + DSV + RBV, 12	1	97-99; NA; 97-99	Yes
PEARL- Ⅰ[65]	OBV + PTV/r	4	91; NA; 91	No
	OBV + PTV/r + RBV		100; NA; 100	Yes
SAPPHIRE- Ⅱ[66]	ABT-450/r + OBV + DSV + RBV, 12	1	96.3; NA; 93.6	Yes
TURQUOISE- Ⅱ[67]	ABT-450/r + OBV + DSV + RBV, 12	1	91.8; 91.8; NA	Yes
	ABT-450/r + OBV + DSV + RBV, 24		95.9; 95.9; NA	
ASTRAL-2 and ASTRAL-3[68]	SOF + VEL, 12	2	99; 100; 99	Yes
		3	95; 93; 98	
C-WORTHY[69]	GZR + EBR, 12	1	91-97; 91-97; NA	Yes
	GZR + EBR + RBV, 12		90-94; 90-94; NA	
	GZR + EBR, 18		94-97; NA; NA	
	GZR + EBR + RBV, 18		97-100; NA; NA	
Everson *et al*[70]	DCV + ASV + BCV, 12	1	88.8-89.5; 71.4-100; 87.5-91.1	No
	DCV + ASV + BCV + RBV, 12		85.7; 100; 85	
SOUND-2[71]	FDV + deleobuvir + RBV, 16	1	59; NA; NA	No
	FDV + deleobuvir + RBV, 28		59-69; NA; NA	
	FDV + deleobuvir + RBV, 40		52; NA; NA	
	FDV + deleobuvir, 28		39; NA; NA	

SVR: Sustained virological response; IFN: Interferon alfa; RBV: Ribavirin; PEG-IFN: Peginterferon alfa; TPV: Telaprevir; BOC: Boceprevir; SMP: Simeprevir; SOF: Sofosbuvir; DCV: Daclatasvir; LDV: Ledipasvir; OBV: Ombitasvir; PTV/r: Paritaprevir/ritonavir; DSV: Dasabuvir; VEL: Veltapasvir; GZR: Grazobuvir; EBR: Elbasvir; ASV: Asunaprevir; BCV: Beclabuvir; FDV: Faldaprevir.

limitations of available treatments must be addressed. The difficult to treat populations and the emergence of resistant virus species indicate the continued need for research into new treatment options. Therefore, research must continue so that new substances with potential antiviral activity against this virus are identified and the limitations of existing treatments can be overcome. In addition to treatment, we must always consider the importance of prevention and the need for global pacts that will allow these treatments to be made available throughout the world.

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TOPIC HIGHLIGHT

2016 Irritable Bowel Syndrome: Global view

Personality traits and emotional patterns in irritable bowel syndrome

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Abstract

The review focuses on those personality traits (neuroticism, extraversion, openness to experience, agreeableness, and conscientiousness), constructs (alexithymia and distressed - Type D personality) and emotional patterns (negative and positive) that are of particular concern in health psychology, with the aim to highlight their potential role on the pathogenesis, onset, symptom clusters, clinical course, and outcome of irritable bowel syndrome (IBS). Personality traits and emotional patterns play key roles in affecting autonomic, immune, inflammatory, and endocrine functions, thus contributing not only to IBS clinical expression and symptomatic burden, but also to disease physiopathology. In this sense, psychological treatments should address those personality traits and emotional features that are constitutive of, and integral to IBS. The biopsychosocial model of illness applied to IBS acknowledges the interaction between biological, psychological, environmental, and social factors in relation to pain and functional disability. A holistic approach to IBS should take into account the heterogeneous nature of the disorder, and differentiate treatments for different types of IBS, also considering the marked individual differences in prevalent personality traits and emotional patterns. Beyond medications, and lifestyle/dietary interventions, psychological and educational treatments may provide the optimal chance of addressing clinical symptoms, comorbid conditions, and quality of life in IBS patients.

Key words: Alexithymia; Depression; Anxiety; Anger; Conscientiousness; Irritable bowel syndrome; Emotions; Personality; Neuroticism; Extraversion

Core tip: The complex nature of irritable bowel syndrome (IBS) requires a multidisciplinary approach

from different fields of scientific knowledge. This review examines the contribution of personality traits and emotional patterns to pathophysiology, clinical expression, and outcome of IBS. Several personality traits and constructs, such as neuroticism, conscientiousness, and alexithymia, are closely associated with IBS. Negative emotions, which are probably more entangled with neurobiological substrates, seem to have a key role in the brain-gut axis dysfunction which characterizes IBS. Based on the reviewed evidence, effective treatments for IBS should also address personality traits and emotions to improve outcomes in IBS patients.

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INTRODUCTION

Irritable bowel syndrome (IBS) is a functional disorder of the lower gastrointestinal (GI) tract characterized by abdominal pain or discomfort, alterations in bowel habits (constipation and/or diarrhoea), and changes in stool frequency and/or form[1].

IBS has a great impact on socio-relational and work functioning, and patients usually report significant reduction of health-related quality of life, work productivity, and general distress in everyday life activities including diet, travel, physical appearance, family, education, and physical and sexual relationships[2]. In addition, IBS presents a primary healthcare burden accounting for the majority of the total health costs associated with general practitioners andgastroenterologists consultations, hospital admissions, and pharmacotherapy prescriptions.

The pathogenesis of IBS is heterogeneous and complex, probably resulting from interactions of several factors. Traditionally, pathophysiological explanations of IBS have focused on host-related factors such as abnormal motility, visceral hypersensitivity, and enhanced pain perception[3,4], although none of these factors can account for symptoms burden in the majority of IBS patients. Research has also focused on dietary intolerance[5], low grade inflammation and altered gut immune activation[6], intestinal permeability and alteration of microbiota[7], abnormalities in the autonomic nervous system[8], and stress[9].

More recently, the relationship among central nervous system (CNS), autonomic nervous system (ANS), and enteric nervous system (ENS), and the bi-directional communication between the neural and immunological networks within the gut, related to as the brain-gut axis, has provided a major contribution to the understanding of IBS pathogenesis[10]. The dysregulation and/or the hyperreactivity of the brain-gut axis involves neural, immune and endocrine pathways that are affected by environmental, biological and psychosocial stressors[11,12].

Basic and applied research across a range of clinical areas has established the importance of the biopsychosocial model of illness[13] that has provided a valid framework for understanding the bi-directional relationships between mind and body. Differently from prior paradigms which have offered purely biological or entirely psychosocial explanations, such perspective, integrating biological and clinical sciences with individual features, has postulated how heterogeneous factors and environmental processes interact together to affect physical health and illness onset, course, and outcome[14]. It has been suggested that psychosocial factors may modify the course of illness, and that their relative weight is quite variable, depending on individual differences, on illness typology, and also, in the same individual, that their contribution may even vary between different episodes of the same illness[13].

Actually, although the dominant model of disease still remains biomedical, a large amount of research have highlighted the role of psychological factors, stressful life events, and environmental demands in modulating individual vulnerability to diseases, whereas psychological well-being seems to be a protective factor in the dynamic interplay between health and illness[14]. Thus, taking into account general factors such as quality of life, daily functioning, productivity and social performances, cognitive abilities and emotional stability should be an essential part of diagnostic and clinical processes and patient care[15].

Within a holistic, person-centred perspective, there is a growing need to include patients related outcomes (PROs), such as self-reported symptoms, psychological well-being, patients' satisfaction with treatments, and functional status in the context of clinical care and in treatment decision making, with the aim to provide subjective indicators of the impact of disease and of treatment efficacy, and a more extensive knowledge of clinical outcomes[16].

Within the biopsychosocial framework, IBS pathophysiology can be viewed as resulting from multiple interactions between biological mechanisms and psychosocial factors: external stressors, including life events, may affect and disrupt the regulation and activity of the brain-gut axis, therefore contributing to IBS onset, symptoms recrudescence, treatment response, and outcome[17]. Beyond psychosocial stressors, also genetic predisposition and early-life stress, both physiologic or psychological, may affect the bidirectional pathways between gut and brain, thus influencing individual vulnerability to develop IBS later in life, with each successive exposure to stressors possibly triggering or exacerbating IBS symptoms[18].

The role of negative effects, autonomic nervous system, stress-hormone and immune systems, along

with psychiatric comorbidity and subclinical variations in depression, anxiety, and anger in relation to pathophysiology and clinical expression of IBS have been highlighted in our previous review on these issues[19].

The present review was aimed to examine the role of personality traits and prevalent emotional patterns on the pathogenesis, onset, symptom clusters, clinical course, and outcome of IBS within the biopsychosocial model of illness and disease.

PERSONALITY: CONCEPTUAL ISSUES AND TAXONOMY

The term personality refers to regularities and consistencies in behavior and forms of experience, including relatively consistent pattern of thoughts, feelings, and perceptions[20].

Although personality is actually an all-encompassing concept making other terms almost unnecessary, constructs as "temperament" and "character" are still used, and they offer a main contribute for a deep understanding of personality formation.

From a developmental perspective, individual differences appear early in life as simple, stylistic features of personality grounded on a biological substratum of relatively heritable differences in emotional reactivity and regulation, and in withdrawal/approach behaviors towards environmental stimuli; these early individual differences are encompassed under the term temperament[21]. Character refers to those aspects of personality that are shaped by learning and interaction with the environment; however, the distinction between temperament and character traits is not so clear cut, considering that each personality trait is virtually heritable, and its full expression will be ultimately determined by environmental influences, also mediated by epigenetic mechanisms altering genome function under exogenous stimuli[22].

One of the most interesting developments in personality research has been the emergence of the five-factor model accounting for the trait structure of personality[23]. The model identifies five broad personality dimensions that are assumed to have a biological origin, and that have demonstrated remarkable stability across cultures and, in the same individuals, for up to 45-year intervals[24].

The identified dimensions are neuroticism, extraversion, openness to experience, agreeableness, and conscientiousness. Neuroticism refers to a tendency toward negative emotions (anxiety, hostility, depression) with high reactivity to physiological changes, emotional instability, vulnerability to stress, and an inclination toward impulsive behaviors. Extraversion refers to the attitude to experience positive emotions, warmth, excitement seeking, and activity. Openness to experience describes tendencies toward imagination and fantasy, aesthetics, creativity, ideas and values, and thought flexibility. Agreeableness involves a pro-social, altruistic orientation towards others, trust, straightforwardness, and tender-mindedness. Finally, Conscientiousness includes competence, order, self-discipline, and achievement striving.

There is a general agreement that the trait remains the best construct for conceptualizing individual differences; further consensus lies upon the hierarchical organization of traits, with a larger number of lower-ord er traits combined to form fewer higher-order traits. However, the nature of some of the putative traits is still a matter of debate within different frameworks; neuroticism and extraversion are clearly associated to the response to negative and positive emotions, respectively.

Thus, emotionality may be considered as a relatively stable individual trait, so that subjects characterized as highly emotional will strongly react to emotional stimuli, particularly negative ones.

Beyond the five-factor model, other personality constructs seem relevant in health research: alexithymia and the distressed (Type D) personality.

Alexithymia, one of the personality traits that has received higher attention in the psychosomatic literature, involves a reduced ability to identify, describe and discern subjective emotions and feelings, poor imaginative thought and introspection, and a cognitive style that is concrete and externally oriented[25].

Type D personality consists of two stable general traits: Negative affectivity, and social inhibition, and it is characterized by the tendency to experience negative emotions across a wide range of daily situations, and to suppress and inhibit emotional expression[26,27]. Table 1 highlights main personality models.

PERSONALITY AND HEALTH

The old-fashioned assumption which has dominated earliest psychosomatic research was that specific personality profiles were associated with specific somatic illnesses; however, no evidence has been reached by this line of research. More recently, a large amount of research has examined the relationships between personality traits and health, starting from the hypothesis that personality traits could be distal predictors of health outcomes, influencing health outcomes either directly[28] or *via* a number of mechanisms. Three main mechanisms have been identified: pathogenesis, in which traits may result in various physiological reactions both to external and internal stimuli, leading to susceptibility to illness, health behaviors, and coping with illness[29]. Furthermore, personality traits may also influence health *via* social cognitions and associative processes, whereby environments become associated with symptoms and illness behaviors, acting as triggers to illness presentation[30], and, finally, communication with health professionals. Regarding pathogenesis,

Table 1 Personality models

Model	Features
Biosocial[21]	Temperament: heritable differences in emotional reactivity and regulation, and in withdrawal/approach behaviors towards environmental stimuli. Character: aspects of personality that are shaped by learning and interaction with the environment.
Five factor[23]	Neuroticism: tendency toward negative emotions (anxiety, hostility, depression) with high reactivity to physiological changes, emotional instability, vulnerability to stress, and an inclination toward impulsive behaviors. Extraversion: attitude to experience positive emotions, warmth, excitement seeking, and activity. Openness to experience: tendencies toward imagination and fantasy, aesthetics, creativity, ideas and values, and thought flexibility. Agreeableness: pro-social, altruistic orientation towards others, trust, straightforwardness, and tender-mindedness. Conscientiousness: competence, order, self-discipline, and achievement striving.
Alexithymia[25]	A reduced ability to identify, describe and discern subjective emotions and feelings, poor imaginative thought and introspection, and a cognitive style that is concrete and externally oriented.
Type D[26]	Negative affectivity: stable tendency to experience negative emotions (feelings of dysphoria and tension, negative view of self, somatic symptoms, attention bias towards adverse stimuli). Social inhibition: stable tendency to inhibit the expression of emotions and behaviors in social interaction (feeling to be inhibited, tense and insecure when with others).

consistent individual differences in stress reactivity as documented by changes in the hypothalamic-pituitary-adrenal (HPA) axis, measured by cortisol levels, and in the ANS system, as indexed by cardiovascular activity, may reflect consistent variations in basic personality traits. It is almost convincing that variations in reactivity to stressors are to some extent built on individual differences in personality traits; beyond stress perception and biological reactions to stressors[31], personality traits have an acknowledged influence on stressful life event exposure, a process known as stress generation[32]. Higher neuroticism and impulsivity, along with lower agreeableness, directly predicted the occurrence of dependent stressful life events, those events which are partly attributable to the person's own behavior, and indirect effects linked these personality traits to new health problems, suggesting that personality-related stress generation contributes to the decline of physical health in late mid-life[33].

More directly, neuroticism has been also associated with reduced cellular immune activity[34], increased pro-inflammatory cytokine levels[35], and lower cortisol stress reactivity[36]; a negative constellation of personality traits involving higher neuroticism, lower agreeableness and openness was associated with diminished stress reactions both of the cardiovascular system and the HPA axis[37]. The association between neuroticism and blunted physiological stress responses has not been extensively replicated, since a number of studies reported no association between neuroticism and cortisol changes during exposure to stressors[38,39].

Other personality traits of the five-factor model have received less attention in the context of health studies. Extraversion was related to reduced cytokine levels, increased cortisol levels, and, along with high levels of Openness and Conscientiousness, to slow disease progression[40]; moreover, low conscientiousness was associated with higher cumulative illness burden in later life[41], whereas high conscientiousness was a reliable predictor of longevity[42]. No relationships were found among agreeableness, cortisol levels, and cardiovascular stress reactivity, whereas conflicting results have been reported for openness[43,44].

Moving towards other personality constructs, it has been proposed that alexithymia could affect health through a number of pathways, directly influencing autonomic, immune and endocrine activities leading to tissue damage and to the increased vulnerability to illnesses, or indirectly, by somatosensory amplification that causes low tolerance to painful stimuli[45]. Alexithymic trait resulted associated with increased mortality[46], worse physical health outcomes[47], increased risk taking, internet addiction, and negative health and sexual behaviors[48-50]. High prevalence of alexithymia was found in major diseases, such as cancer[51], type 1 diabetes[52], and systemic lupus erythematosus[53].

Finally, Type D personality seems to confer a specific vulnerability to chronic stress, and it has been associated with increased pro-inflammatory cytokine levels[54], higher risk of morbidity, mortality, low subjective and physician-assessed health ratings, lesser health behaviors[41], and worse illness perceptions in cancer survivors[55].

Overall, findings from health research highlight the potential role of personality, conceived as built up from broad and stable traits, as a unifying structure embracing heterogeneous psychosocial factors which tend to cluster together, and contribute to raise the risk for illness onset, course, and outcome.

PERSONALITY AND IBS

The large amount of findings reporting high rates of psychological symptoms and psychiatric comorbidity in IBS seems to indicate that affective and psychosocial symptoms may be specific and basic to the syndrome; nevertheless, research on personality structure and traits distribution in IBS subjects is still questionable.

Early studies on personality factors in IBS patients were performed by using the Eysenck Personality Inventory (EPI)[56] which measures two pervasive, independent, and opposite dimensions of personality, the polarities extraversion-introversion and neuroticism-stability; these dimensions, according to the

Authors, should account for most of the variance in the personality domain. Neuroticism scores on the EPI resulted significantly higher only in diarrhoea-predominant IBS (IBS-D) subjects when compared with patients affected by ulcerative colitis and general medical patients[57]. Palmer *et al*[58] evidenced that IBS patients were significantly more neurotic and less extroverted than general population (established normative data), but significantly less neurotic and more extroverted than patients affected by psycho-neurotic disorders (neurotic depression, anxiety phobic state, obsessional illness, hysterical disorder, or a combination of these). However, it should be taken into account that the cited studies are characterized by small samples, low statistical power, restricted range, and arbitrary categorization of psychiatric disorders.

The shared agreement reached by the five-factor model[23] as a useful theoretical framework for understanding and describing basic personality dimensions, and the development of reliable questionnaires, such as the NEO Five-Factor Inventory (NEO-FFI)[59] for the assessment of the factor structure of personality descriptors, have led to a renewed interest in exploring personality factors in functional GI disorders (FGIDs).

As reviewed in the previous section, among the five factors neuroticism has been identified as the personality dimension more closely associated to health and illness processes, and this evidence is also supported in IBS research[60]. Neuroticism, which confers a marked vulnerability to negative emotions, is one of the main features of patients with FGIDs also when controlling for psychiatric comorbidity[61], and it has also been identified as a risk factor for the development of IBS[62].

A community study evidenced an association between neuroticism and a past history of sexual, physical, and emotional/verbal abuse in IBS patients, suggesting that neuroticism could predispose to the reporting or development of IBS symptoms by a subset of subjects[63]. The link between neuroticism and abuse has been further supported by a more recent, longitudinal study by the same research group: abuse during childhood was significantly associated with elevated levels of neuroticism which was strongly related with baseline prevalence of depression and anxiety, and with moderately elevated scores on interference with life and activities, but not pain. Based on their findings, the Authors suggested a conceptual model for IBS characterized by a "vicious circle" between mood disorders and bowel symptoms in adulthood, with initial input from early life factors[64].

Regarding the evaluation of the five personality factors in prevalent IBS subtypes IBS-D, constipation-predominant (IBS-C), and alternating diarrhoea and constipation (IBS-A), two studies have reported conflicting results.

A first study in non-psychiatric IBS subjects showed that IBS-C patients were characterized by higher levels of neuroticism and conscientiousness, as documented by higher mean scores on the NEO-FFI[65]. Opposed findings have been reported by a cross-sectional study on a sample of Japanese university students, since IBS-D patients showed higher levels of neuroticism than IBS-C subjects and healthy controls; moreover, neuroticism was positively correlated with the severity of IBS symptoms[66]. Such conflicting results reflect the heterogeneity of findings from research assessing negative emotions and psychological distress in IBS subtypes, with studies reporting more psychological symptoms in IBS-C patients[67,68], and others documenting higher rates of psychiatric comorbidity and psychological distress in IBS-D patients[69]. It is well-known that negative emotionality, a feature of neuroticism, can increase colonic motility, and this effect is more evident in IBS patients[70]; high levels of neuroticism, anxiety sensitivity, trait worry, and increased vigilance toward visceral sensations are common features of IBS patients and reliable predictors of IBS symptoms[71]. However, it seems quite difficult to interpret these findings, and it remains unclear to draw univocal conclusions from research on personality and psychological differences among IBS subtypes, as documented by the mixed results reported in a recent meta-analysis[72].

Beyond neuroticism, another personality dimension, conscientiousness, resulted sporadically related with IBS in previous studies[65]. More recently, complaint severity in IBS patients was found negatively associated with conscientiousness and agreeableness, and positively associated with neuroticism and anxiety; the relationship between complaint severity reports and conscientiousness was modified by genetic variation in catechol-O-methyltransferase (COMT) which is involved in mediating sympathetic and dopaminergic tone through catecholamines degradation, thus participating in the complex affective, personality, and cognitive networks also involved in IBS pathophysiology and clinical expression[73].

A quite consistent amount of research has examined the role of alexithymia in IBS. Alexithymia has been found significantly associated with IBS and others FGIDs with variable prevalence rates (up to 66%), probably depending on methodological differences in diagnostic and assessment criteria[74,75]; moreover, alexithymia seems to affect responsiveness to treatment[75,76].

A study aimed at evaluating potential psychosocial predictors of IBS diagnosis and severity has identified six factors that significantly predicted IBS symptom severity, accounting for 61.3% of the variance: two alexithymia features (difficulty in identifying feelings and in describing feelings), gender, maladaptive cognitive schemas (defectiveness/shame and entitlement), and general psychological distress[77]. Similar findings emerged from a recent study that assessed alexithymic

Table 2 Personality and irritable bowel syndrome

-Several personality traits and constructs, such as neuroticism, conscientiousness, and alexithymia, are closely associated with irritable bowel syndrome (IBS).
-Negative emotionality, a feature of neuroticism, can increase colonic motility; high levels of neuroticism, anxiety sensitivity, trait worry, and increased vigilance toward visceral sensations are common features of IBS patients and reliable predictors of IBS symptoms.
-The relationship between complaint severity reports and conscientiousness was modified by genetic variation in catechol-O-methyltransferase (COMT) which is involved in mediating sympathetic and dopaminergic tone through catecholamines degradation, thus participating in the complex affective, personality, and cognitive networks also involved in IBS pathophysiology and clinical expression.
-Potential mechanisms by which alexithymia could affect IBS severity include the core features of this personality construct, such as the tendency to focus on, intensify, and misinterpret bodily sensations and somatic sensations triggered by states of emotional arousal; moreover, higher pain intensity to rectal distension in alexithymic IBS patients than in non-alexithymic controls has been documented.

features and gastrointestinal-specific anxiety (GSA), which refers to worry, hypervigilance to, fear, and avoidance of GI sensations and contexts in IBS patients; results showed the association among alexithymia, GSA, and severity of IBS symptoms, with alexithymia explaining much more unique variance in IBS severity as compared with GSA[78]. Potential mechanisms by which alexithymia could affect IBS severity include the core features of this personality construct, such as the tendency to focus on, intensify, and misinterpret bodily sensations and somatic sensations triggered by states of emotional arousal; moreover, higher pain intensity to rectal distension in alexithymic IBS patients than in non-alexithymic controls has been documented[79].

Type D personality, the latest personality construct taken into account for the purposes of the present review, has not received the same attention as other personality factors in IBS research.

One study[80] evidenced that Type D personality, whose prevalence reached 40.6% in the examined sample, significantly decreased health-related quality of life (HRQoL); this finding was congruent with the extensive evidence of impaired HRQoL in several clinical samples affected by diseases other than GI disorders, mainly in coronary heart disease patients[54]. However, regression analyses showed that only the dimension negative affect of Type D personality, along with severity of symptoms and duration of treatment, remained strong independent determinant of HRQoL, whereas no significant associations between social inhibition and HRQoL were found.

Yet, caution is needed in interpreting such findings, since the established association between Type D personality and quality of life exclusively relies on negative affectivity which could affect HRQoL independently from the personality construct. A further study reported significantly higher prevalence rates of Type D personality in IBS patients than in healthy controls (45.4% *vs* 12.8%, respectively); Type D personality was significantly correlated with poor self-reported sleep quality, and it remained an independent predictor of impaired sleep also when controlling for the confounding influence of socio-demographic and clinical variables, including anxiety, and depression symptoms[81].

Taken together, the available findings do not allow drawing firm conclusions on the contribution of personality traits and constructs to IBS patho-physiology, clinical presentation, and severity of symptoms. This may be due to the complex nature of personality dimensions that are formed by higher order-traits comprising simpler, basic dimensions, such as emotional features, which are probably more connected to neurobiological substrates. Table 2 summarizes the association between personality and IBS.

In the following sections we review main emotional patterns and their possible implications in health and IBS.

EMOTIONAL PATTERNS: POSITIVE AND NEGATIVE EMOTIONS

Emotional patterns may be conceived as individual differences in emotional reactivity, processing and regulation; more specifically, they involve detection and appraisal of emotionally salient stimuli, and regulatory processes that can be automatic or controlled, conscious or unconscious, occurring at one or more points in the emotion generative process and final expression.

Two broad dimensions of emotional patterns, accounting for the most part of basic individual differences in affective processes and emotional responses, and involved in psychological well being and distress, have been identified: positive affect (PA), or pleasantness, and negative affect (NA), or unpleasantness[82] (Figure 1). Although PA and NA, for their seemingly opposite nature, could be considered as two basic, separate poles of the same affective continuum, they are rather discrete dimensions describing not only positive *vs* negative valence (*e.g.*, happy *vs* sad), but also involving activation levels (*e.g.*, aroused *vs* unaroused)[83]. This approach is particularly functional to health research for connecting affective activation with physiological arousal, which is thought to be a primary pathway through which emotions may influence health[84].

PA is a dimension reflecting pleasurable engagement with the environment, and characterized by the prevalence of positive feelings and mood states such as interest, enthusiasm, happiness, motivation, high energy levels, mental alertness[85]; high PA levels translate into high energy, activity and concentration, whereas low PA is a state of fatigue and poor energy.

NA is a pervasive disposition to experience aversive

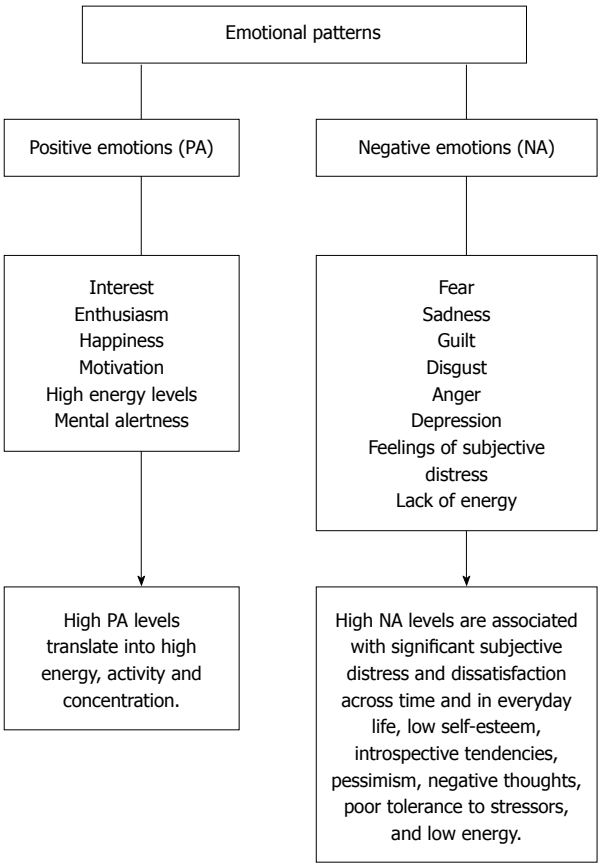

Figure 1 Emotional patterns: Positive and negative emotions.

emotions, such as fear, sadness, guilt, disgust, anger, depression, feelings of subjective distress, and lack of energy; high NA levels are associated with significant subjective distress and dissatisfaction across time and in everyday life, low self-esteem, introspective tendencies, pessimism, negative thoughts, poor tolerance to stressors, and low energy[86].

In an evolutionary sense, quite discordantly from the assumptions of folk psychology that postulate that negative emotions are worse than positive ones, negative emotions are evolved adaptations which served the purpose of surviving in life-threatening situations, and their adaptive value lies in their ability to elicit specific action tendencies[87,88]. Anger and fear, for instance, evoke the "fight or flight" response, the urges to attack, also in defensive terms, or to escape, mobilizing optimal physiological support for the action called forth, and requiring substantial physical energy also through heightened cardiovascular reactivity that redistributes blood flow to relevant motor districts, and through specific neuroendocrine pathways that sustain the stress reactions[89].

On the other hand, positive emotions evoke nonspecific action tendencies (*e.g.*, approach behaviors) and, secondarily, they are characterized by a relative lack of autonomic reactivity[89].

It has also been suggested that, whereas most negative emotions narrow individuals' thought-action repertoires, according to the purpose of generating specific and targeted action tendencies, many positive emotions broaden individuals' behavioral repertoires, prompting them to pursue a broader range of actions such as explore, play, approach, and building vital physical, cognitive, and social resources[90]. Furthermore, positive emotions lead to more prosocial and affiliative behaviors, and facilitate cognitive flexibility by shifting selective attention to reward stimuli in the environment[91].

PA and NA can be brief and transient, longer lasting, or trait-like features; within the research area on the impact of emotions on general health, each of these possibilities has been taken into account[92]; for the purposes of this review, in the next section we summarize research that has evaluated stable emotional patterns as trait attributes of individuals.

EMOTIONAL PATTERNS AND HEALTH

A growing evidence indicates that emotional states are associated with health. Whether the chronic experience of negative emotions can influence the development of illness, or trigger and exacerbate disease episodes, is still a matter of debate. Furthermore, it must also be taken into account that the effects of emotions in a healthy biological system may be rather different from effects in an impaired organism[93]. When exploring the possible influence of emotions on health, both the nature of the emotional experience, including valence (positive *vs* negative), frequency, intensity, and duration, and the type of the disease and course features, such as stage, progression, and severity, must be considered.

From a longitudinal point of view, and within a developmental framework, it has been shown that patterns of emotional functioning that emerge in childhood are almost maintained into adulthood; thus, emotional functioning in childhood may provide an early indicator of adult health risk[94]. High chronic distress in childhood has been associated with a range of adult physical health outcomes such as number of physical illnesses[95], inflammatory diseases[96], and obesity[97].

Positive emotions are generally associated with improved physical and mental health[98], reduced risk of stroke[99], and lower mortality in both older groups and chronically ill samples[100].

However, it has also been shown that high levels of PA may be detrimental to end-stage or high short-term mortality diseases, whereas diseases with longer term expectations for living, in which adherence to medical regimens and diverse behavioral factors (*e.g.*, healthy lifestyles) could play a role, were benefited or unaffected by PA[92].

Most research have focused on the harmful impact of NA on health: to the extent that negative emotions generate cardiovascular reactivity, higher NA has been

mainly related to heart disease[101]; further lines of research have examined NA role in cancer[102], and in chronic illnesses, such as arthritis and diabetes[93].

Two sets of candidate mechanisms by which emotions may influence physical health have been highlighted. The first approach involves the impact of emotional states on thoughts and conducts that may potentially influence health-related behaviors, such as perceptions of risks, decisions to seek medical screenings or treatment, adherence to exercise and dietary regimens[103], strength and quality of social support, and interpersonal relationships driven by the predominant emotional pattern[104]. In a broad sense, positive affectivity and well-being are associated with healthy habits and lifestyles, as documented by the inverse relationships among depression, anxiety, and leisure-time physical activity[105]. On the other hand, negative effects are related to unhealthy patterns of functioning, poorer social networks, higher frequency of stressful events, and negative social interactions[92].

A more intriguing hypothesis that has received support by a growing body of research[106,107] suggests that emotions have the potential to directly influence health through psychobiological processes, defined as the physiological consequences associated with emotional arousal and subsequent changes in multiple systems (*e.g.*, cardiac functioning, blood pressure, inflammation and immune responses, neuroendocrine pathways), thus leading to increased vulnerability to illness, or modifying illness course and progression[108]. It is acknowledged that negative emotions confer increased risk for disorders with an inflammatory and immune aetiology[109]: depression, anxiety, and anger have been linked with higher levels of pro-inflammatory cytokines such as interleukin IL-6 and other inflammation mediators, including C-reactive protein, and cellular adhesion molecules[110-112].

EMOTIONAL PATTERNS AND IBS

Research that has examined negative emotions in relation to the main pathophysiological and symptomatic correlates of IBS has most commonly considered anger, anxiety, and depression[67,113,114].

Such discrete emotions have been consistently associated to visceral and pain hypersensitivity. In the presence of negative emotions, visceral sensations tend to be more noticeable and labeled as painful[115]. IBS patients, but also subjects with mild GI symptoms, presented attentional biases to GI pain-related symptoms and social threats words[116], a higher tendency to scan the body for symptoms, and a greater burden of abdominal pain[117] than healthy controls.

Also in IBS children and their parents negative emotions and multiple somatic complaints have been consistently reported[118]; furthermore, up to 45% of children with functional abdominal pain (FAP) displayed clinically elevated anxiety[119], whereas adolescents with frequent abdominal pain resulted at increased risk for depression, social isolation, and impairment in school functioning[120]. A study on a small sample of IBS children (n = 10; mean age 10.5 ± 2.2 years) has demonstrated a significant correlation between emotional instability and indexes of visceral hypersensitivity; emotional instability involved negative emotions, impulsiveness and impatience, all features associated with less effective ability to manage stressful life events[121].

In adults, evidence is more controversial: depression and anxiety were positively related with abdominal pain and pain duration[117], anxiety, depression, and the recall of painful memories were associated with a greater perception of visceral pain[122], depression levels were higher in those patients who reported lowered rectal pain threshold, but only in the alternating IBS subtype[123], whereas other studies did not find significant differences in negative emotions and pain thresholds according to IBS subtypes[124].

Regarding intestinal motility patterns, emotional arousal can augment colonic motility and diarrhoea also in healthy subjects, but this effect is mostly pronounced in IBS patients[70]; laboratory studies have provided evidence that anger-provoking conditions significantly increased colon motility in IBS patients, whereas anger suppression was associated with prolonged gastric emptying and delayed gut transit[125-127].

A role for negative emotions in low-grade inflammation and altered immune activity in IBS has garnered support from studies demonstrating alterations on several inflammatory and immune parameters resulting in an imbalance of the proinflammatory and anti-inflammatory cytokines: elevated peripheral levels of the proinflammatory cytokines interleukine (IL)-1β and tumor necrosis factor α, and decreased levels of IL-10, an anti-inflammatory cytokine, differentiated IBS patients with anxiety and depression from IBS subjects without negative emotions, and from healthy controls[128,129].

Finally, negative emotional patterns seem to be involved in another clinical feature of IBS: health-care seeking behavior. Anxiety has been identified as one of the main reason of medical consultation among IBS subjects[130]. However, the opposite is also true; the co-occurring experience of life events eliciting emotional arousal can lead individuals to attribute IBS symptoms to the stressful situation[112]. A recent study on a community sample of university students with IBS-like symptoms compared with a non-IBS reference group showed that health care utilization was mainly associated with IBS symptom severity, and not with emotional distress[131]. On the other hand, health care-seeking behavior is a non-specific feature of IBS patients, and it depends on many factors, such as the presence of pain, the severity of bowel symptoms, and duration of illness.

Overall, in interpreting findings from studies of negative emotions and IBS it seems important to bear in mind the utility of a dimensional method based

Table 3 Emotional patterns and irritable bowel syndrome

-Negative emotions, which are probably more entangled with neurobiological substrates, seem to have a key role in the brain-gut axis dysfunction which characterizes irritable bowel syndrome (IBS).
-Anger, anxiety, and depression have been consistently associated to visceral and pain hypersensitivity. In the presence of negative emotions, visceral sensations tend to be more noticeable and labeled as painful.
-Emotional arousal can augment colonic motility and diarrhoea; laboratory studies have provided evidence that anger-provoking conditions significantly increased colon motility in IBS patients, whereas anger suppression was associated with prolonged gastric emptying and delayed gut transit.
-A role for negative emotions in low-grade inflammation and altered immune activity in IBS has garnered support from studies demonstrating alterations on several inflammatory and immune parameters resulting in an imbalance of the proinflammatory and anti-inflammatory cytokines.

on the assumption that emotions rarely occur in isolation; thus, considering emotional patterns rather than each emotion in isolation could lead to a more realistic approach to the pathophysiology and clinical expression of IBS.

A recent systematic review and meta-analysis has suggested that neither psychological markers nor symptom-based criteria and/or biomarkers alone performed well in diagnosing IBS, whereas combining symptoms, biomarkers and/or psychological markers seemed to perform better. The Authors suggested that one possible reason for the poor performance of psychological markers was that the reviewed studies predominantly used markers of anxiety and depression to predict the presence of IBS, whereas it would be more useful to include other emotional dimensions beyond anxiety and depression for improving the accuracy of diagnosing IBS[132]. Table 3 shows main findings on emotional patterns and IBS.

TREATMENT IMPLICATIONS: FOCUS ON PERSONALITY AND EMOTIONAL PATTERNS

The biopsychosocial model applied to IBS acknowledges and highlights the interaction between biological, psychological, environmental, and social factors in relation to pain and functional disability. Within this framework, and also considering the bidirectional communications within the brain-gut axis, a holistic approach involving medications, lifestyle changes, dietary interventions, psychopharmacological and psychological treatments, and educational and behavioral strategies should provide the optimal chance of addressing clinical symptoms, comorbid conditions, and quality of life in IBS patients.

Psychological and psychosocial treatments involving interactions between body and mind could be effective treatment strategies in reducing GI symptoms in adults with IBS; psychological interventions were significantly effective at first post-treatment assessment and at both short-term and long-term follow-up[133].

Various models of psychotherapy have been used in IBS with promising results; cognitive behavioral therapy, gut-directed hypnotherapy, interpersonal psychotherapy, mindfulness, body awareness psychotherapy, relaxation/stress management, and meditation have been proven effective on gastrointestinal symptoms and quality of life in IBS patients[134,135].

As reviewed in the previous sections, personality traits and emotional patterns play key roles in affecting autonomic, immune, inflammatory, and endocrine functions, thus contributing not only to IBS clinical expression and symptomatic burden, but also to disease physiopathology. In this sense, psychological treatments should address those personality and emotional features that are constitutive of and integral to IBS.

The choice of a specific psychological model should take into account individual differences in clinical symptoms and personality features, after a careful assessment including a broad evaluation of gastrointestinal and pain symptoms, hyperarousal, emotional patterns, personality traits and profile, psychosocial aspects, and quality of life.

It has been shown that cognitively-focused therapies, such as comprehensive self-management, were less effective in reducing clinical and pain symptoms in IBS patients with higher sympathetic tone[136]. IBS patients characterized by high levels of negative emotions could benefit of treatments aimed at activating inhibitory processes through the utilization of emotion regulation techniques to down-regulate emotional arousal, and to enhance self-regulation of affective reactions to internal and environmental stimuli[137,138]. Beyond negative emotions, the trait of neuroticism is characterized by dysfunctional cognitions, worries, negative appraisals and catastrophizing, defined as the attitude to put emphasis on the threat value of painful stimuli[139]; accordingly, IBS patients with accentuated neuroticism as the prevalent personality trait could ameliorate on cognitive treatments targeting dysfunctional beliefs, automatic thought processes, and cognitive biases.

Impaired body awareness has been reported in IBS-D patients[69] and in alexithymia[140]; particularly when an alexithymic component is present, body awareness techniques and psychoeducational strategies oriented to reduce misinterpretation of bodily sensations could be useful for helping patients to identify and express emotions, and to disentangle somatic symptoms from signs of emotional arousal[141].

Based on the reviewed evidence, multi-symptomatic IBS patients requires a complex, multidisciplinary approach. The process of prescribing pharmacological and non-pharmacological treatments should take into account the heterogeneous nature and clinical presentation of the disorder, attempting to differentiate the treatment for different types of IBS, also considering the profound individual differences in

prevalent personality traits and emotional patterns.

CONCLUSION

A large amount of research has provided evidence that personality traits and emotional patterns influence health, disease, and quality of life through a range of biological and behavioral pathways, including physiological reactions to stimuli, reactivity to stressors, health behaviors, and coping with illness. This evidence also extends to IBS: personality and affective features are central components of the biopsychosocial model of IBS, being involved in functioning and dysregulation of the brain-gut axis, and contributing to the onset, recurrence and recrudescence of IBS. From early developmental stages, both genetic and environmental factors interact to shape emotional arousal and regulation, vulnerability to stressors, effective or maladaptive coping strategies, visceral and pain sensitivity, and thus symptom perception, illness behavior, daily functioning, quality of life and, finally, health outcomes.

Given the relationships above, it is increasingly evident that a complex and heterogeneous disease such as IBS requires a multidisciplinary, integrated approach coming from different, although complementary disciplines. Further insights on vulnerability factors and pathophysiological pathways leading to symptoms clusters and clinical expression of IBS should be addressed in order to promote better health, quality of life, and effective treatments for IBS patients.

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TOPIC HIGHLIGHT

2016 Liver Transplantation: Global view

Metabolic complications in liver transplant recipients

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Abstract

The metabolic syndrome (MS), which includes obesity, dyslipidaemia, hypertension and hyperglycaemia according to the most widely accepted definitions now used, is one of the most common post-transplant complications, with a prevalence of 44%-58%. The MS, together with the immunosuppression, is considered the main risk factor for the development of cardiovascular disease (CVD) in transplant recipients, which in turn accounts for 19%-42% of all deaths unrelated to the graft. The presence of MS represents a relative risk for the development of CVD and death of 1.78. On the other hand, non-alcoholic fatty liver disease (NAFLD), considered as the manifestation of the MS in the liver, is now the second leading reason for liver transplantation in the United States after hepatitis C and alcohol. NAFLD has a high rate of recurrence in the liver graft and a direct relation with the worsening of other metabolic disorders, such as insulin resistance or diabetes mellitus. Consequently, it is vitally important to identify and treat as soon as possible such modifiable factors as hypertension, overweight, hyperlipidaemia or diabetes in transplanted patients to thus minimise the impact on patient survival. Additionally, steroid-free regimens are favoured, with minimal immunosuppression to limit the possible effects on the development of the MS.

Key words: Metabolic syndrome; Liver transplantation; Immunosuppressions; Risk factors; Non-alcoholic fatty liver disease

Core tip: The metabolic syndrome is a very frequent complication after liver transplantation; indeed, over half transplant patients will eventually develop it. It is also a risk factor for the development of cardiovascular disease, one of the main causes of long-term death after transplantation. The identification and early treatment of such factors as hypertension, dyslipidaemia, obesity and diabetes is crucial to

achieve a positive impact on long-term survival of liver transplant patients.

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INTRODUCTION

The optimization over recent years of the surgical technique and immunosuppressive therapy has led to excellent survival rates after liver transplantation, reaching 90% at one year and 80% at five years[1-3]. This greater survival has, however, been accompanied by an increase in medical complications derived from the transplant, such as the development of de novo malignancies, recurrence of the underlying disease, metabolic complications and cardiovascular diseases, which today constitute the main causes of death unrelated to the graft[4-6]. The metabolic syndrome (MS), which includes obesity, dyslipidaemia, hypertension and hyperglycaemia according to the most widely accepted definitions now used (Table 1)[7,8], is one of the most common post-transplant complications, with a prevalence ranging from 44%-58% in different studies[9-13]. The MS, together with the immunosuppression, is considered the main risk factor for the development of cardiovascular disease (CVD) in transplant recipients, which in turn accounts for 19%-42% of all deaths unrelated to the graft[10,14]. The presence of the MS represents a relative risk for the development of CVD and death of 1.78[15]. On the other hand, non-alcoholic fatty liver disease (NAFLD), considered as the manifestation of the MS in the liver, is now the second leading reason for liver transplantation in the US after hepatitis C and alcohol[16,17]. NAFLD has a high rate of recurrence in the liver graft and a direct relation with the worsening of other metabolic disorders, such as insulin resistance or diabetes mellitus[18]. Consequently, it is vitally important to identify and treat as soon as possible such modifiable factors as hypertension, overweight, hyperlipidaemia or diabetes in transplanted patients to thus minimise the impact on patient survival. Additionally, steroid-free regimens are preferred, with minimal immunosuppression to limit the possible effects on the development of the MS.

Table 1 Definition of the metabolic syndrome by the National Cholesterol Education Program, Adult Treatment Panel Ⅲ adapted by the National Heart, Lung and Blood Institute/American Heart Association, and the International Diabetes Federation

American heart association	International diabetes federation
At least 3 of the following criteria: Waist circumference > 88 cm for women and > 102 cm for men Fasting glucose > 100 mg/dL Systolic blood pressure > 130 mmHg and/or diastolic blood pressure > 85 mmHg HDL < 50 mg/dL for women and < 40 mg/dL for men Triglycerides > 150 mg/dL	Abdominal obesity according to gender and ethnicity specific values (*i.e.*, waist circumference > 80 cm for women and > 90 cm for men if they are American or European) and at least 2 the following criteria: Fasting glucose > 100 mg/dL Systolic blood pressure > 130 mmHg and/or diastolic blood pressure > 85 mmHg HDL < 50 mg/dL for women and < 40 mg/dL for men Triglycerides >150 mg/dL

HDL: High density lipoprotein.

COMPONENTS OF POST-LIVER TRANSPLANT METABOLIC SYNDROME

Obesity

According to the World Health Organisation obesity is determined from the body mass index (BMI) as: overweight BMI: 25-29.9 kg/m^2, class Ⅰ: 30-34.9 kg/m^2, class Ⅱ: 35-39.9 kg/m^2 and class Ⅲ: > 40 kg/m^2. Central obesity seems to confer more risk of developing the MS and CVD than peripheral obesity[19,20].

Two considerations concerning obesity and its impact on the results of liver transplantation should be considered; the presence of obesity at the time of transplantation and the development of obesity after transplantation.

Patients who are overweight or obese before the transplant remain overweight or obese after the transplant[9]. Over 15% of patients who are of normal weight when they receive their liver transplant become obese within one year and over 25% within 3 years[9,21]. This can be explained by the correction of the catabolic state induced by the cirrhosis and which disappears after transplantation, as well as the increased appetite due to the absence of chronic disease and the use of drugs like steroids. Weight gain after a transplant is associated with an increased risk for the MS and its complications, such as CVD, kidney disease or NAFLD/non-alcoholic steatohepatitis (NASH) on the liver graft[22,23].

Another point is whether the presence of overweight at the time of transplantation impacts on the short- and long-term results post-transplant. One study found that at 5 years post-transplant there was greater mortality among patients who had a BMI > 35 (class Ⅰ) and BMI > 40 (class Ⅱ) when they received their transplant as compared with non-obese patients, though this study did not consider the possible influence of the presence of ascites[24]. This may, therefore, be a confounding factor, as it could have been the presence of ascites at the time of transplantation that was associated with greater post-transplant mortality and not the greater BMI of the patients. Other studies, however,

that considered obesity but corrected for ascites found no differences in survival between obese and non-obese patients, probably due to the more exhaustive control of cardiovascular risk factors undergone by obese patients during the pre-transplant period. This, therefore, highlights the need to consider the presence of ascites at the time of transplantation and its association with worse results when interpreting the impact of the BMI on post-transplant results[25]. Even in cases of morbid obesity excellent survival rates can be achieved, both for the graft and the patient, provided there is adequate selection[25,26]. A study by the United Network of Organ Sharing found a lower survival rate among patients with a BMI > 40 and a high MELD score[27]. Nevertheless, adoption of vigorous measures to prevent and correct overweight must be taken from before the time of transplant.

Hypertension

Although the incidence of hypertension before transplantation is low it can still reach 40%-85% afterwards[28-30]. The immunosuppressive drugs, either alone or combined with other factors, are the main cause of the onset of hypertension, due mainly to the renal and systemic haemodynamic changes they induce. Steroids can also induce hypertension due to their mineralocorticoid effect as well as the increase in vascular resistance and cardiac contractility. Calcineurin inhibitors, mainly cyclosporine rather than tacrolimus[31,32], produce hypertension due to vasoconstriction of the afferent renal arteriole, which in turn induces reabsorption of sodium and water and volume expansion, with the resulting increase in blood pressure[33]. Mammalian target of rapamycin (mTOR) inhibitors, when combined with calcineurin inhibitors, can also cause hypertension[34].

Salt-restriction diets and the correction of other associated risk factors accompanied by physical activity are determinant for the prevention and control of hypertension. If drugs are required to control the hypertension, calcium antagonists are considered the first choice as they act directly on the pathophysiological mechanism producing hypertension. In liver transplant patients the recommended drugs are amlodipine/felodipine because they do not interfere with the hepatic metabolism of calcineurin, unlike diltiazem, verapamil or nifedipine, which interfere with the cytochrome P450 and can increase the levels of calcineurin inhibitors and thus their possible toxicity. Angiotensin-converting enzyme inhibitors and angiotensin-receptor blockers have a limited effect when used as single therapy during the early post-transplant period because the activity of the plasma renin system is low during this period, so that these drugs are more useful at later stages after the transplant in which plasma renin activity is greater[33]. Specific beta blockers are considered second-line therapy. Although the ideal blood pressure level in transplant patients has not been established, the suggested levels are < 130/80; for which up to 30% of patients would require at least two drugs[35,36].

New-onset diabetes

In cirrhotic patients the prevalence of glucose intolerance is 60%-80% and that of diabetes is 10%-15%. The incidence of new-onset diabetes after liver transplant (NODALT) ranges from 14%-44%, similar to that seen after other solid-organ transplants (kidney, lung, heart)[37-39]. Pre-transplant diabetes and the BMI have been found in one study to be factors predicting the development of NODALT[9]. NODALT is associated with a high risk of developing CVD and post-transplant mortality[38,40].

The predominant role played by the liver in the regulation of carbohydrate, protein, lipid and drug metabolism makes it the main organ responsible for the maintenance of glucose homeostasis. This has led some authors[41,42] to suggest that it is the liver graft itself that causes the metabolic disorders that occur after the transplant. A high incidence of NODALT has been observed in patients who receive a graft with steatosis, which is associated with insulin resistance[37]. Likewise, grafts from donors after circulatory death have a greater incidence of NODALT, probably in relation to the damage derived from the warm ischemia on the development of insulin resistance[43]. However, in comparison to patients who receive a liver transplant from a deceased donor, patients who receive a liver transplant from a living donor (LDLT) have a lower incidence of NODALT, probably because LDLT livers have more favourable characteristics regarding such factors as age, BMI, or liver function state[43].

Certain genotype characteristics of the graft are also considered to be determinant in the metabolic status after liver transplantation[44]. Various gene polymorphisms have been associated with metabolic disorders and a particular response to immunosuppressive drugs. This would explain the inter-individual, and even the intra-individual variability of certain drugs like tacrolimus concerning their pharmacokinetic characteristics or dose individualisation[45-47].

Recurrence of the underlying liver disease can also influence the appearance of NODALT. A strong association has been found between early recurrence of hepatitis C and NODALT[48], probably related with the damage to the beta cells induced by the hepatitis C virus (HCV). The recurrence of steatosis/steatohepatitis, which can reach around 60% in patients who receive their transplants for this reason, has also been strongly associated with the development of the MS, as well as diabetes[49]. The association between insulin resistance and beta-cell dysfunction is well known in cirrhosis caused by such agents as alcohol, HCV, or NASH, which all damage beta cells and alter glucose regulation. This results in over 90% of cirrhotic patients becoming intolerant to glucose during the final stages of the disease, with up to 30% developing diabetes[50,51]. Various studies suggest that liver transplantation can

resolve up to 70% of cases of pre-transplant diabetes as a result of improving insulin resistance, with the other cases that fail to resolve possibly being due to the persistence of beta-cell injury[52-54]. Nevertheless, these patients all remain exposed to factors associated with the development of diabetes after transplantation, such as immunosuppression, the presence of HCV, or age.

The use of immunosuppressive drugs after liver transplantation plays a crucial role in NODALT. Steroids cause increased insulin resistance and reduced beta-cell secretion. Likewise, calcineurin inhibitors, mainly tacrolimus, have been considered the inducers of NODALT, principally *via* reduction of insulin secretion by the beta cells through several pathways[55,56]. The mTORs everolimus and sirolimus have not, however, been found to be more effective than tacrolimus in post-transplant blood glucose control[57]; with one study even finding that mTORs reduce beta-cell mass and increase insulin resistance[58]. This has all led to current immunosuppressive regimens tending to use steroid-free protocols and minimisation of the immunosuppression.

Recent studies have also shown the role of the intestinal microbiota in the regulation of carbohydrate metabolism, as well as its influence on the pathogenesis of glucose metabolism disorders. The intestinal microbiota could be affected by liver transplantation through multiple factors, including immunosuppression. Some authors have found an association between the dysbiosis produced by tacrolimus, insulin levels and the insulin resistance index[44].

Dyslipidaemia

Although the prevalence of dyslipidaemia in cirrhotic patients is low, due to the alteration in hepatic synthesis, it can nevertheless reach 70% in liver transplant recipients[59-62]. As with other components of the MS, immunosuppression also plays a fundamental role in dyslipidaemia. Steroids produce hypercholesterolaemia and hypertriglyceridaemia due to stimulation of the activity of acetyl-CoA carboxylase and fatty acid synthesis[63,64]. Calcineurin inhibitors can also induce dyslipidaemia, more often cyclosporine than tacrolimus[65-67]. Cyclosporine produces a reduction in biliary cholesterol excretion and blocks the LDL-cholesterol receptors, with the resulting increase in blood levels[36]. mTOR inhibitors induce hypertriglyceridaemia by increasing the activity of adipose tissue lipase and reducing lipoprotein lipase, especially if combined with cyclosporine[68,69].

The treatment of dyslipidaemia should be oriented towards dietary measures, steroid withdrawal and minimisation of immunosuppression. The treatment of post-transplant hypercholesterolaemia generally necessitates the use of drugs since dietary measures alone are not usually effective. Statins are the drugs of choice for the treatment of hypercholesterolaemia. Pravastatin is most recommended because it is not metabolised by the P450 cytochrome and does not interact with the immunosuppression, unlike other statins like simvastatin, fluvastatin, atorvastatin or lovastatin, though these are widely used in transplant recipients with no great problems. Special care is required with the use of ion exchange resins given their effect on the enterohepatic circulation and their repercussion on the absorption of calcineurin inhibitors, particularly cyclosporine. Hypertriglyceridaemia with normal cholesterol concentrations is also usual in liver transplant recipients. It responds best to dietary treatment, particularly the use of omega 3 fatty acids. Drug therapy with fibrates (gemfibrozil) is reserved for severe cases, and is generally well tolerated[33,36,70].

PREDICTORS OF POST-LIVER TRANSPLANT METABOLIC SYNDROME

The prevalence of the MS after liver transplantation is around 50%, depending on the criteria used[9]. The factors most consistently related with the risk of developing post-liver transplant MS are a high recipient age at the time of transplant, the presence of diabetes mellitus before transplantation, an increase in BMI after transplantation, smoking and the indication for the transplant (hepatitis C, alcohol or cryptogenic cirrhosis)[9,10,13,71,72]. Some studies have found the use of cyclosporine as an immunosuppressive agent to be a risk factor. In addition, recent studies provide increasing evidence for certain gene polymorphisms as independent risk factors for the development of the MS[39,45,73].

REPERCUSSION OF THE METABOLIC SYNDROME AFTER LIVER TRANSPLANTATION

The MS after a liver transplant can have a significant negative impact on post-transplant morbidity and mortality due to its involvement in the development of different clinical aspects directly related with post-transplant survival.

Cardiovascular risk

All the components of the MS are considered cardiovascular risk factors. The higher prevalence of the MS in liver transplant recipients is associated with a higher incidence of cardiovascular events than in the general population[74], though the risk is lower than in recipients of a kidney or heart transplant. This is because patients with chronic liver disease experience haemodynamic and metabolic changes resulting from peripheral vasodilation, as well as having low blood pressure and cholesterol levels, which all make these patients less liable to develop cardiovascular events, unlike the situation in kidney or heart transplant recipients. In addition, liver transplant recipients require

Table 2 Recommendations and treatment aims in patients with the metabolic syndrome

Maintain a healthy lifestyle
Stop smoking
Perform regular physical exercise
Weight control
Blood pressure
< 140/90 mmHg (if there are no other associated risk factors)
< 130/80 mmHg
(if diabetes, kidney failure or established cardiovascular disease)
Fasting blood glucose levels: 80-130 mg/dL
Post-prandial levels:140-180 mg/dL
Glycated haemoglobin < 6.5%-7.0%
LDL cholesterol level
< 130 mg/dL (if no cardiovascular risk)
< 115 mg/dL (if moderate cardiovascular risk)
< 110 mg/dL (if high cardiovascular risk)

HDL: High density lipoprotein.

lower immunosuppression than patients who receive a kidney or heart transplant[75].

The incidence of cardiovascular events after liver transplantation is around 10% at 3 years[30]. Diabetes mellitus, hypertension and having received the transplant due to NAFLD are the risk factors most associated with cardiovascular events[76]. It is important to note that the risk of experiencing a cardiovascular event is 4 times greater in liver-transplant recipients who have the MS than those who do not have it[75]. Around 20% of non-hepatic causes of death in liver transplant patients are due to CVD, which are one of the main causes of death unrelated with the graft[14,77]. Factors predicting cardiovascular events are an older recipient age (OR = 1.2), male gender (OR = 2), NODALT (OR = 2), post-transplant hypertension (OR = 1.8) and the use of mycophenolate mofetil (OR = 2)[30].

In general, prevention measures and treatment aims in CVD based on studies in the general population are also applicable to liver transplant patients, as no specific studies have yet been undertaken of the impact of these measures in transplant recipients. These recommendations are outlined in Table 2[78].

Kidney failure

The presence of the MS in both the general population and in liver transplant recipients is associated with a higher incidence of kidney failure[78]. The reduction in glomerular filtration and the microalbuminuria associated with hypertension or diabetes and the resulting structural damage in the kidney can be increased by the effect of immunosuppressive drugs, which in turn leads to the higher incidence of chronic kidney disease in transplant patients with the MS[79].

Recurrence of hepatitis C

There is a bidirectional relationship between hepatitis C and insulin resistance and diabetes, these latter two being recognised as risk factors for the progression of the fibrosis in patients with hepatitis C, whether or not they have received a transplant[80,81]. Additionally, the recurrence of hepatitis C is also recognised as a risk factor for the development of NODALT[60,82], which in turn is related with greater progression of the fibrosis.

Hepatitis C also affects lipid metabolism, producing a reduction in serum lipid concentrations. Though this could potentially be beneficial, reducing the cardiovascular risk, it has also been related with alterations in the intracellular lipid balance, which could increase the hepatic steatosis[83].

The use of current direct-action antiviral agents against HCV and their high rate of efficacy has led to the recurrence of HCV becoming much less prevalent, with the resulting lower rate of possible effects on metabolic factors.

NAFLD and NASH

NAFLD and NASH can be considered hepatic events of the MS[23]. Around 20% of patients with NASH can eventually develop cirrhosis and require liver transplantation. Over 60% of the patients who receive a transplant due to NASH experience a recurrence during the first year, with the main risk factor being the presence of the MS[84]. One study found that around 20% of liver transplant patients who did not previously have fatty liver developed NAFLD afterwards and around 10% developed NASH post-transplant[85]. On the other hand, various studies have identified a 10% increase in the BMI as the main risk factor for the development of NAFLD[49,74,86]. Nonetheless, the true impact of the presence of NAFLD and NASH after transplantation still remains unclear.

CONCLUSION

The high prevalence of the MS after a liver transplant and its relation with the development of cardiovascular events, as well as its involvement in other clinical aspects after the transplant that can seriously influence morbidity and mortality, necessitates the early identification of these factors to achieve adequate management of the risks, thereby minimising their impact on patient survival. Other aspects in post-transplant MS, such as the role of gene polymorphisms or the gut microbiota require much greater study.

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TOPIC HIGHLIGHT

2016 Pancreatic Cancer: Global view

Re-evaluation of classical prognostic factors in resectable ductal adenocarcinoma of the pancreas

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Abstract

Pancreatic ductal adenocarcinoma carries a poor prognosis with annual deaths almost matching the reported incidence rates. Surgical resection offers the only potential cure. Yet, even among patients that undergo tumor resection, recurrence rates are high and long-term survival is scarce. Various tumor-related factors have been identified as predictors of survival after potentially curative resection. These factors include tumor size, lymph node disease, tumor grade, vascular invasion, perineural invasion and surgical resection margin. This article will re-evaluate the importance of these factors based on recent publications on the topic, with potential implications for treatment and outcome in patients with pancreatic cancer.

Key words: Pancreatic cancer; Survival; Prognostic factors

Core tip: Many studies have investigated morphological indicators of survival in patients with resectable pancreatic cancer. This article scrutinizes the recent literature related to these classical prognostic factors and examines whether these factors still are able to influence patients' outcomes in the era of multimodal treatment.

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INTRODUCTION

Pancreatic cancer has a devastatingly poor prognosis.

The overall survival remains unchanged over a wide period of time with at a 5-year survival of 5%-10%[1]. Furthermore, according to recent data, the incidence of pancreatic cancer is increasing[2-4]. Pancreatic ductal adenocarcinoma constitutes the most common histopathological subtype of pancreatic cancer originating from the ductal cells of the exocrine pancreas. Surgery remains the only cure for pancreatic cancer, but long-term survival is uncommon despite the addition of oncological treatments.

Over the years, several important prognostic factors for resectable pancreatic cancer have been identified. Among these prognostic factors are tumor size, presence or absence of lymph node metastases (loco-regional and/or distant), vascular tumor involvement (*e.g.*, portal vein, mesenteric vein and/or artery), perineural tumor growth and resection margin status (from tumor to resection line)[5-9].

Many studies have been conducted to elucidate the relative importance of these prognostic factors in terms of recurrence-free survival and long-term cure. Despite this information, limited progress has been made regarding survival aspects. Pancreatic surgery is associated with high morbidity and potentially also immune suppression. Therefore, it is of vital importance to accurately determine which patients that really derive benefit from surgery in order to avoid unnecessary surgical intervention, as well as facilitate treatment planning and guide neo- and adjuvant treatments. The aim of this review was to re-investigate classical morphological prognostic factors in resectable pancreatic cancer by summarizing the recent literature on the topic published since the year 2000.

TUMOR SIZE

Tumor size is a morphological variable that can be determined preoperatively and as such as carries much important information for treatment planning. Being part of the current TNM staging system of pancreatic cancer, tumor size is the only discriminant between T1 and T2 tumors (cut-off 2 cm). However, the size at which point a pancreatic tumor becomes associated with aggressive features remains undetermined. This is why most studies that have evaluated the effects of tumor size on survival have not used uniform definitions (Table 1). In general, larger tumors > 2-3 cm have worse prognosis compared to smaller tumors. Tumor size has also been linked to other adverse prognostic factors. For example, tumors with a diameter above 2 cm have been found to have an increased risk of lymph node metastases[10].

Tumor size may also affect the rates of margin positivity following pancreatic surgery. It has been found that larger tumors increase the risk of tumor deposits being harbored in the resection line[11]. Larger tumors generally have a greater malignant potential, increasing the risk of tumor involvement of peripancreatic structures[12,13].

However, although most studies indicate impaired survival for larger tumors, a small tumor diameter does not exclude poor prognosis[14,15]. Interestingly, it has been found that tumors with a diameter less than 1 cm may also be associated with malignant potential and rapid disease progression[16,17]. Histopathological data obtained from analysis of "early" pancreatic cancer show that small tumor may display the same microvascular invasiveness pattern (*i.e.*, a poor prognostic factor) as larger tumors[18,19].

This, somewhat, unclear relationship between tumor size and prognosis may reflect different tumor biology and invasiveness, independent of tumor size[20], regulating outcome.

LYMPH NODE STATUS

Dissemination into the lymphatic system is a major route for pancreatic cancer metastasis. Lymph node status has been demonstrated to be one of the most potent predictors of survival. Several recent studies (Table 2) have proposed that the ratio of metastatic to examined lymph nodes (LNR) may be more powerful predictors of survival than the mere dichotomization into positive or negative lymph nodes. LNR also seems to have negative impact on the long-term survival ($\geqslant$ 60 mo)[21]. Poor prognosis has been observed with an LNR over 0.3-0.4.

There is a wide time span in terms of median survival between studies evaluating the impact of lymph node status on survival after resection for pancreatic cancer. Patients with N0 tumors displayed a superior median survival of up to 40 mo and patients with LNR > 0.3 displayed a median survival of 6 mo at the lowest. The wide range in median survival may reflect different biology in those tumors that spread to lymph nodes and those that do not. It may also be speculated that there is a biological difference in the tumor invasiveness both regarding loco-regional and distant lymph node metastases. In general, lymph node metastases can be correlated to increasing tumor size but not in all of the cases.

It is recommended to sample at least 12 lymph nodes for histopathological diagnosis and staging[22]. Standard lymphadenectomy for pancreatic head tumors include resection of lymph node stations 5, 6, 8a, 12b1, 12b2, 12c, 13a, 13b, 14a, 14b, 17a and 17b, while tumors of the body and tail of the pancreas require removal of stations 10, 11, and 18. Extended lymphadenectomy during Whipple's procedure include resection of lymph nodes along the left side of the superior mesenteric artery and around the celiac trunk, splenic artery or left gastric artery. There is no convincing evidence that extensive lymph node resection in conjunction to pancreatic surgery increases the overall survival and is therefore not recommended[23].

Lymph node status may also be defined based

Table 1 Tumor size and survival for resectable pancreatic cancer

Ref.	Year	*n*	Tumor size, cut-off	Median OS	Statistical model	HR (95%CI)	*P* value
Matsumoto *et al*[38]	2015	968	⩾ 3 cm	NR	Multivariable	1.72 (1.16-2.56)	0.006
Jeong *et al*[39]	2015	276	⩽ 2.5 cm	13 mo	Multivariable	1.65 (1.17–2.32)	0.004
			> 2.5 cm	25 mo			
Hur *et al*[17]	2015	18338	*In situ*	120 mo	Multivariable	1	
			⩽ 1.0 cm	27 mo		3.09 (2.35-4.07)	< 0.001
			1.1-2.0 cm	17 mo		4.35 (3.42-5.53)	< 0.001
			> 2.0 cm	11 mo		5.69 (4.49-7.21)	< 0.001
Yamamoto *et al*[40]	2015	195	⩽ 2 cm	29 mo (mean)	Multivariable	0.40 (0.17-0.83)	0.012
Elberm *et al*[41]	2015	1070	< 2 cm	19 mo	Multivariable	0.63 (0.50-0.78)	< 0.001
Liu *et al*[31]	2015	411	> 2 cm	NR	Univariable	1.46 (1.14-1.88)	0.003
Okada *et al*[42]	2014	200	⩾ 3 cm	NR	Multivariable	3.26 (1.52–7.00)	0.002
Dusch *et al*[21]	2014	415	< 3 cm	16 mo	Univariable	-	< 0.03
Shin *et al*[15]	2014	537	⩽ 3 cm	22 mo	Multivariable	1.4 (1.13-1.73)	0.002
			> 3 cm	14 mo			
Kooby *et al*[37]	2014	1399	Continuous	20 mo	Multivariable	1.12 (1.06-1.18)	< 0.001
Petermann *et al*[10]	2013	114	⩽ 2 cm	35 mo	Multivariable	0.52 (0.25–1.05)	0.071
			2.1–3.4 cm	16 mo			
			3.5–4.5 cm	20 mo			
			> 4.5 cm	8 mo			
Jamieson *et al*[43]	2013	217	⩽ 3 cm	23 mo	Multivariable	1.28 (0.93–1.76)	0.13
			> 3 cm	16 mo			
Franko *et al*[14]	2013	7135	⩽ 1 cm	N0/N1: 38/18 mo	Multivariable	1	
			1.1–2 cm	N0/N1: 26/19 mo		1.18 (0.94-1.48)	0.152
			> 2 cm	N0/N1: 19/14 mo		1.67 (1.34-2.07)	< 0.001
Lad *et al*[44]	2013	382	Continuous	16 mo	Multivariable	1.23 (1.07–1.41)	0.003
Sugiura *et al*[45]	2013	208	⩾ 3 cm	NR	Univariable	NR	0.014
Hong *et al*[18]	2012	209	< 3 cm	20 mo	Univariable	NR	0.06
			⩾ 3 cm	15 mo			
Bhatti *et al*[46]	2010	84	⩽ 2 cm	34 mo	Univariable	NR	0.017
			> 2 cm, invasion-	27 mo			
			> 2 cm, invasion+	11 mo			
Yamada *et al*[47]	2009	335	> 4 cm	NR	Multivariable	2.69 (1.30-5.56)	< 0.05
Ueda *et al*[48]	2009	140	< 3 cm	22 mo	Multivariable	1.85 (1.14-3.10)	0.013
			⩾ 3 cm	11 mo			
Kaneoka *et al*[49]	2009	84	< 2 cm	NR	Multivariable	1	
			2–4 cm	20 mo		1.7 (1.0–3.1)	0.031
			> 4 cm	11 mo		2.3 (1.3–4.5)	0.003
Campbell *et al*[50]	2009	163	Continuous	14 mo	Multivariable	1.02 (1.00-1.03)	0.049
Doi *et al*[51]	2007	133	⩽ 4 cm	45% dead within 1 yr	Univariable	1.55 (1.06–2.24)	0.02
			> 4 cm	67% dead within 1 yr			
Zacharias *et al*[52]	2007	81	⩽ 3 cm	28 mo	Multivariable	1.9 (1.1–3.1)	0.018
			> 3 cm	14 mo			
Pawlik *et al*[6]	2007	905	⩾ 2 cm	17 mo	Multivariable	1.24 (1.01-0.51)	0.04
Moon *et al*[53]	2006	94	< 3 cm	25 mo	Multivariable	0.46 (0.27-0.78)	0.004
Cleary *et al*[20]	2004	123	Continuous	14 mo	Univariable	1.2 (1.0–1.4)	0.01
Ahmad *et al*[54]	2001	125	< 2 cm	16 mo	Univariable	1	
			2-4 cm			1.05 (0.58-1.89)	0.87
			> 4 cm			1.15 (0.61-2.15)	0.66
Sohn *et al*[55]	2000	616	< 3 cm	21 mo	Multivariable	0.72 (0.57-0.90)	0.004
			⩾ 3 cm	14 mo			
Meyer *et al*[56]	2000	113	⩽ 2 cm	28 mo	Multivariable	2.27	< 0.006
			> 2 cm	13 mo			

OS: Overall survival.

on the location of the lymph nodes involved. Several studies describe lymph node involvement as N1 (metastases in loco-regional lymph nodes) or N2-3 (metastases in distant lymph nodes). A few studies evaluated para-aortic lymph denoted as N3. There is a clear survival difference between local or distant lymph nodes metastases (*e.g.*, para-aortic lymph node metastases). Most studies, but not all, report that para-aortic lymph node involvement is associated with a poor prognosis, being an independent factor of adverse outcome (Table 3). Frozen-section examination of para-aortic nodes accurately detects distant lymphatic involvement reliably and should be routinely performed[24]. The presence of metastases is considered a contraindication to proceed with pancreatic resection.

Table 2 Prognostic relevance of lymph node ratio

Ref.	Year	*n*	Cut-off	Detection rate (%)	Median OS with highest LNR (mo)	Survival model	HR (95%CI)	*P* value
Fouquet *et al*[34]	2014	166	0.2	76 (46)	NR	Multivariable	2.38 (1.4-4.0)	0.001
Dusch *et al*[21]	2014	415	Continuous	0.10 (0-0.81)[1]	NR	Multivariable	1.73 (NR)	0.002
Valsangkar *et al*[57]	2013	14907	0.3	4038 (27)	NR	Multivariable	2.27 (1.67-3.08)	< 0.001
Lewis *et al*[58]	2013	424	0.3	83 (20)	16	Multivariable	1.97 (NR)	< 0.001
Robinson *et al*[59]	2012	131	0.15	70 (53)	NR	Multivariable	4.14 (NR)	< 0.010
La Torre *et al*[60]	2011	101	0.2	30 (30)	13	Multivariable	4.88 (1.07-22.2)	0.008
Bhatti *et al*[46]	2010	84	0.3	26 (31)	6	Multivariable	2.7 (1.6–4.4)	0.010
Riediger *et al*[61]	2009	182	0.3	32 (18)	NR	Multivariable	2.2 (1.4-3.6)	< 0.001
Slidell *et al*[22]	2008	3868	0.4	602 (16)	10	Multivariable	1.82 (1.59-2.07)	< 0.001
Pawlik *et al*[6]	2007	905	0.4	154 (17)	12	Multivariable	2.55 (1.75-2.70)	0.001

[1]Mean (range). LNR: Lymph node ratio; OS: Overall survival.

Table 3 Para-aortic lymph node involvement and survival

Ref.	Year	*n*	Detection rate (%)	Median OS with PALN (mo)	Survival model	HR (95%CI)	*P* value
Sho *et al*[62]	2015	882	102 (12)	17	Multivariable	1.15 (0.87-1.50)	0.335
Schwarz *et al*[24]	2014	111	17 (15)	16	Univariable	NR	0.038
Kanda *et al*[63]	2011	429	49 (11)	8	Univariable	NR	0.006
Murakami *et al*[64]	2010	103	18 (17)	12	Multivariable	1.84 (0.28-1.07)	0.078
Doi *et al*[51]	2007	133	19 (14)	5	Multivariable	2.90 (1.60-5.02)	0.001
Shimada *et al*[65]	2006	133	29 (22)	13	Univariable	NR	< 0.001

OS: Overall survival; PALN: Para-aortic lymph node involvement.

TUMOR GRADE

The WHO classification of tumor grade in pancreatic cancer is based on the original proposal of Klöppel *et al*[25] and takes into consideration mucin production, glandular differentiation, mitotic activity and nuclear atypia. Several studies have shown that tumor grade is an important prognostic indicator after resection of pancreatic cancer[26-28]. Wasif *et al*[29] analyzed 8082 patients with resected pancreatic cancer. This study found that high tumor grade had a larger impact on survival than both tumor size and lymph node metastases, both of which are part of the current TNM staging system. This observation lead the authors to conclude that the inclusion of tumor grade into the TNM staging for pancreatic cancer would improve prognostic stratification and better reflect the aggressive prognosis of poorly differentiated tumors.

VASCULAR INVASION AND PORTO-MESENTERIC VEIN RESECTION

Pancreatic tumors may occlude peripancreatic vascular structures, either partly or totally, and in the latter case are categorized as unresectable. The extent of vessel wall involvement, *i.e.*, involvement of tunica adventitia, media or intima, has been correlated with outcome[30]. Invasion of major retroperitoneal blood vessels has been found to be an independent predictor of poor survival[11,30,31]. Vascular resections should be performed if it is possible to obtain R0 resections, which is supported by previous histopathological data[32]. In the situation with positive para-aortic lymph nodes, however, venous and arterial tumor involvement may not be a prognostic factor for survival. It has been speculated that para-aortic lymph node involvement might be a stronger prognostic factor than vascular involvement.

Portal vein and superior mesenteric vein invasion often occurs due to the anatomic location of the tumor. Venous invasion was for a long time considered a contraindication to surgery. Today, vascular resection has become more common. Most studies indicate that vascular resection has similar survival rates as standard resection (Table 4), but it should be performed in carefully selected cases, given the slightly increased perioperative mortality rate and some reports that indicate worse survival, likely due to more advanced disease. Arterial resection during pancreatic resection is associated with poor short- and long-term outcome and is not recommended outside of clinical trials[33].

PERINEURAL INVASION

Perineural invasion (PNI) is a common way of pancreatic tumor growth. Several studies have revealed that PNI in specimens from patients who underwent surgical resection was associated with worse survival (Table 5). Data also indicate that perineural involvement is a predictor for early cancer recurrence[34]. The lack of perineural involvement may be a good prognostic

Table 4 Porto-mesenteric vein resection and survival

Ref.	Year	*n*	VR rate (%)	Median OS without VR/with VR (mo)	Survival model	HR (95%CI)	*P* value
Jeong *et al*[39]	2015	276	46 (17)	16/12	Multivariable	1.15 (0.78–1.71)	0.474
Murakami *et al*[66]	2015	937	435 (46)	26/19	Multivariable	1.16 (0.89-1.53)	0.268
Wang *et al*[67]	2014	122	64 (53)	31/18	Multivariable	NR	NS
Kelly *et al*[68]	2013	492	70 (14)	19/12	Multivariable	1.14 (0.83-1.57)	0.410
Gong *et al*[69]	2013	566	119 (21)	20/13	Univariable	NR	< 0.050
Castleberry *et al*[70]	2012	3582	281 (8)	Increased 30-d postoperative mortality, 2.9% *vs* 5.7%	Multivariable	2.1 (1.22-3.73)	0.008
Chakravarty *et al*[71]	2010	87	12 (14)	10/9	Multivariable	NR	0.591
Ouaissi *et al*[72]	2010	149	59 (40)	19/18	Multivariable	DFS: 0.43 (0.22-0.82)	0.011
Kaneoka *et al*[49]	2009	84	42 (50)	26/12	Multivariable	NR	NS
Kurosaki *et al*[73]	2008	77	35 (45)	20/20	Univariable	NR	0.330
Fukuda *et al*[30]	2007	121	37 (31)	NR	Univariable	NR	0.550
Carrère *et al*[74]	2006	133	45 (34)	19/15	Univariable	NR	0.690
Shimada *et al*[75]	2006	149	86 (58)	35/14	Multivariable	2.25 (1.09–3.62)	NR
Tseng *et al*[76]	2004	291	110 (38)	27/23	Multivariable	1.13 (0.79-1.63)	0.500
Poon *et al*[77]	2004	50	12 (24)	21/20	Univariable	NR	0.769
Nakagohri *et al*[78]	2003	81	33 (41)	10/15	Univariable	NR	NS
Bachellier *et al*[79]	2001	87	31 (36)	12/12	Univariable	NR	0.480

OS: Overall survival; PALN: Para-aortic lymph node involvement; NS: Not significant.

Table 5 Prognostic role of perineural invasion

Ref.	Year	*n*	Detection rate (%)	Median OS with PNI (mo)	Survival model	HR (95%CI)	*P* value
Kondo *et al*[36]	2015	209	197 (94)	15	Multivariable	No of PNIs: < 14: 1 14-40: 1.96 (1.01-3.93) > 40: 5.81 (3.17-11.35)	< 0.001
Fouquet *et al*[34]	2014	166	133 (81)	NR	Multivariable	2.77 (1.40-5.26)	0.001
Chatterjee *et al*[80]	2012	212	123 (58)	29	Multivariable	1.70 (1.18-2.45)	0.005
Takahashi *et al*[81]	2012	110	56 (51)	NR	Multivariable	2.48 (1.11-5.52)	0.026
Sahin *et al*[82]	2012	544	473 (87)	29	Multivariable	1.60 (1.08-2.36)	0.019
Robinson *et al*[59]	2012	134	128 (96)	NR	Multivariable	5.52 (NR)	< 0.050
Kanda *et al*[63]	2011	429	148 (34)	NR	Multivariable	1.72 (1.15-2.58)	< 0.001
Shimada *et al*[35]	2011	153	146 (94)	7 (DFS)	Multivariable	2.19 (1.36-3.52)	0.001
Murakami *et al*[64]	2010	103	31 (30)	NR	Multivariable	1.93 (1.03–3.62)	0.041
Kazanjan *et al*[83]	2008	182	112 (62)	20	Multivariable	2.66 (1.74-4.06)	< 0.001

OS: Overall survival; PNI: Perineural invasion.

marker of disease free survival[35]. However, various criteria for the diagnosis of PNI have been used, and the frequency of PNI in pancreatic cancer varies widely among the previous reports, between 30%-96%. According to a recent study the severity of PNI can be correlated with survival[36]. This study demonstrated that the number of PNIs was a potent predictor of survival in patients with resectable pancreatic cancer.

RESECTION MARGINS

The R classification for pancreatic cancer entails estimation of the radicality of resection. R0 denotes complete microscopic tumor removal. R1 indicates microscopic residual tumor, while R2 indicates macroscopic residual tumor. The influence of margin status on outcomes in pancreatic cancer remains controversial (Table 6). This is largely due to lack of standardization of margin definitions and reporting. Most studies show a worsened prognosis of R1 compared to R0 resection.

The poor prognosis in R1 resections is underscored by the observation that once tumor cell deposits have reached beyond the resected pancreatic surface area, it is difficult to improve survival by trying to convert R1 to R0 resections. Data show that the overall survival of turning R1 to R0 resections is still not convincingly high and once a positive intraoperative resection margin is discovered on frozen section it is doubtful whether the conversion to R0 resection is a "true R0" since the overall survival is not changed[37].

The definition of margin clearance is still under debate. However, nowadays most studies now use a margin clearance over 1 mm to define R1 resection.

CONCLUSION

Despite technical advances in the field of pancreatic

Table 6 Radicality of resection and survival

Ref.	Year	*n*	R1 definition	R1 rate (%)	Median OS R0/R1 (mo)	Survival model	HR (95% CI)	*P* value
Kooby *et al*[37]	2014	1399	1 mm	R0 (86)	21	Multivariable	1	0.009
				R1→R0 (5)	12		1.55 (1.11-2.16)	0.004
				R1 (9)	14		1.46 (1.13-1.90)	
Konstantinidis *et al*[84]	2013	554	1 mm	157 (28)	35/14	Univariable	NR	< 0.001
Kimbrough *et al*[85]	2013	283	0 mm	76 (27)	22/15	Multivariable	NR	NS
Gnerlich *et al*[86]	2012	285	1 mm	97 (34)	22/16	Univariable	NR	0.010
Jamieson *et al*[11]	2010	148	1 mm	110 (74)	27/15	Multivariable	1.76 (1.15–2.28)	0.009
Van den Broeck *et al*[87]	2009	144	1 mm	48 (33)	24/12	Univariable	NR	< 0.001
Chang *et al*[88]	2009	365	0 mm	131 (36)	20/13	Multivariable	1.48 (1.15–1.89)	0.002
Campbell *et al*[50]	2009	163	1 mm	128 (79)	25/13	Multivariable	1.44 (0.90-2.32)	0.132
Westgaard *et al*[89]	2008	40	1 mm	18 (45)	16/11	Univariable	NR	0.300
Esposito *et al*[90]	2008	111	1 mm	84 (76)	NR	Univariable	NR	0.370
Raut *et al*[91]	2007	360	0 mm	61 (17)	28/22	Multivariable	NR	NS
Verbeke *et al*[92]	2006	26	1 mm	22 (85)	37/11	Multivariable	NR	0.790
Howard *et al*[93]	2006	226	0 mm	68 (30)	NR	Multivariable	1.39 (1.02-1.90)	0.030

NS: Not significant; OS: Overall survival.

surgery, the long-term prognosis of pancreatic cancer still remains dismal. This article updates the role of established prognostic factors after curatively intended surgery for pancreatic cancer.

Tumor size is a prognostic factor, with survival decreasing in parallel to increased tumor size. However, small tumors (< 2 cm) may still metastasize and be associated with a poor outcome.

Lymph node involvement is associated with poor survival. A LNR of > 0.3 is a strong prognostic determinant. Para-aortic node sampling with frozen-section examination detects distant lymphatic involvement reliably and should be performed routinely. Metastatic deposits in para-aortic lymph nodes indicate distant disease, and should be considered a contraindication for surgical resection.

Tumor grade may be as powerful a prognostic factor as tumor size and lymph node status.

Invasion of major retroperitoneal blood vessels predicts poor outcome. The extent of vessel wall involvement is correlated with survival. Patients undergoing portal vein resection for pancreatic cancer have a similar long-term prognosis to patients undergoing standard resection.

PNI is present in most pancreatic tumors. The severity of PNI is a novel prognostic factor.

Tumor cells in the resection margin increase the risk of early deaths. A margin clearance over 1 mm should be achieved. However, additional resection to achieve a negative neck margin after positive frozen section is not recommended due to lack of survival advantage.

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REVIEW

Pathophysiological role of guanylate-binding proteins in gastrointestinal diseases

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Abstract

Guanylate-binding proteins (GBPs) are interferon-stimulated factors involved in the defense against cellular pathogens and inflammation. These proteins, particularly GBP-1, the most prominent member of the family, have been established as reliable markers of interferon-γ-activated cells in various diseases, including colorectal carcinoma (CRC) and inflammatory bowel diseases (IBDs). In CRC, GBP-1 expression is associated with a Th1-dominated angiostatic micromilieu and is correlated with a better outcome. Inhibition of tumor growth by GBP-1 is the result of its strong anti-angiogenic activity as well as its direct anti-tumorigenic effect on tumor cells. In IBD, GBP-1 mediates the anti-proliferative effects of interferon-γ on intestinal epithelial cells. In addition, it plays a protective role on the mucosa by preventing cell apoptosis, by inhibiting angiogenesis and by regulating the T-cell receptor signaling. These functions rely to a large extent on the ability of GBP-1 to interact with and remodel the actin cytoskeleton.

Key words: Guanylate-binding proteins; Colorectal carcinoma; Inflammatory bowel diseases; Interferon

Core tip: Guanylate-binding proteins (GBPs) are interferon-stimulated factors involved in the defense against cellular pathogens and inflammation. In addition, guanylate-binding proteins have been established as reliable markers of interferon-γ-activated cells in various diseases including colorectal carcinoma and inflammatory bowel diseases. The GBP-1 is the best characterized member of the family. For instance, the expression of GBP-1 has been associated with a better outcome in colorectal carcinoma. The inhibition of tumor growth by GBP-1 is due to its strong anti-angiogenic activity as well as its direct anti-tumorigenic effect on tumor cells. In inflammatory bowel diseases, on the one hand GBP-1 mediates the anti-proliferative effects of interferon-γ on intestinal epithelial cells, and on the other hand, it protects the mucosa by preventing cell apoptosis, by inhibiting angiogenesis and by regulating the T-cell receptor signaling. These functions rely to a large extent on the ability of GBP-1 to interact with and remodel the actin cytoskeleton.

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INTRODUCTION

Guanylate-binding proteins (GBPs) belong to the dynamin family of large GTPases and display two main particularities that distinguish them from small GTPases of the Ras superfamily. First, they hydrolyze GTP upon self-activation by oligomerization and therefore do not require the presence of GTPase-activating proteins (GAPs) or guanine nucleotide exchange factors (GEFs)[1-3]. Second, they are inducible proteins expressed in response of various stimuli, mainly to type 1 and type 2 interferons (IFNs) and, to a lower extent, to the inflammatory cytokines interleukin (IL)-1β and tumor necrosis factor (TNF)-α[4,5]. Up to now, seven human GBPs (GBP-1 to -7) and eleven mouse GBPs (mGBP-1 to -11) have been described[6,7]. Most of the available knowledge is related to human GBP-1. The structure of GBP-1 has been resolved and comprises two domains: (1) a large globular α/β domain displaying the canonical GTPase sequences at the N-terminus and (2) a long C-terminal part organized in an index finger-like domain composed exclusively of α-helices (7-13, Figure 1)[8]. Because of the high sequence homology between all GBPs, their respective structures are considered to be similar. In addition, GBP-1, GBP-2 and GBP-5 are C-terminally prenylated, allowing their attachment to cellular membranes[9].

To date, biochemical as well as structural investigations have led to deeper insights into the molecular structure and enzymatic mechanism of human GBP-1. Dimer formation is induced by GTP binding leading to reorientation of a catalytic arginine side chain, thereby accelerating hydrolytic activity[1,10]. Moreover, the same catalytic machinery is employed to catalyze a second step of phosphate cleavage resulting in GMP as the major product[11,12]. While this is a unique feature among GTPases, another intriguing observation is the impact of this catalytic activity on changes in the C-terminal helical domain of GBP-1[2,13]. A salt bridge contact between the GTPase domain and the helical domain is switched such that a major reorientation of α-helices 12 and 13 allows for formation of a second contact area on the GBP-1 dimer resulting in association of the two α13 helices and thereby juxtaposition of the farnesyl groups attached to the C-termini[14]. Further biochemical experiments with farnesylated GBP-1 are required to disclose the role of this nucleotide hydrolysis-driven rearrangement for interaction with other GBP isoforms (see *e.g.,* ref. 9) and with membranous compartments.

Initially, GBPs were shown to be among the most highly induced proteins in human fibroblasts exposed to IFN-γ[4]. Subsequently, it was reported that GBP-1 expression can be induced *in vitro* by treatment with IFN-γ in many cell types including primary cells (endothelial cells, fibroblasts, keratinocytes, B-cells, T-cells and peripheral blood mononuclear cells), immortalized intestinal epithelial cells and several tumor cell lines[5,15]. In agreement with its induction by IFNs and inflammatory cytokines, GBP-1 has predominantly been detected *in vivo* in inflamed tissues associated with various diseases such as psoriasis, lupus erythematosus, adverse drug reactions and Kaposi's sarcoma[5,16,17]. Consequently, GBP-1 has been established as a robust marker of inflammation, specifically to detect activation of cells by IFN-γ at the single cell level in tissues[16].

In addition to their association with inflammation, GBPs participate in cell-autonomous defense against bacteria, protozoa and viruses[18]. Moreover, elevated expression of GBP-1, together with other GBPs, has been observed in the colons of patients with inflammatory bowel disease (IBD) or in colorectal carcinoma (CRC)[15,19-21]. This review will focus on the pathophysiological roles of GBPs in CRC and IBD, and especially on GBP-1, the best described member of the GBP family to date.

CLINICAL RELEVANCE OF GBP EXPRESSION IN CRC AND IBD

GBP-1 is an independent positive prognostic factor in CRC

CRC is the third most frequent type of cancer in developed countries. The development of sporadic CRC has been considered the archetype of multistep carcinogenesis. Over the past two decades, major progress has been made in the characterization of CRC. New types of classifications have been proposed,

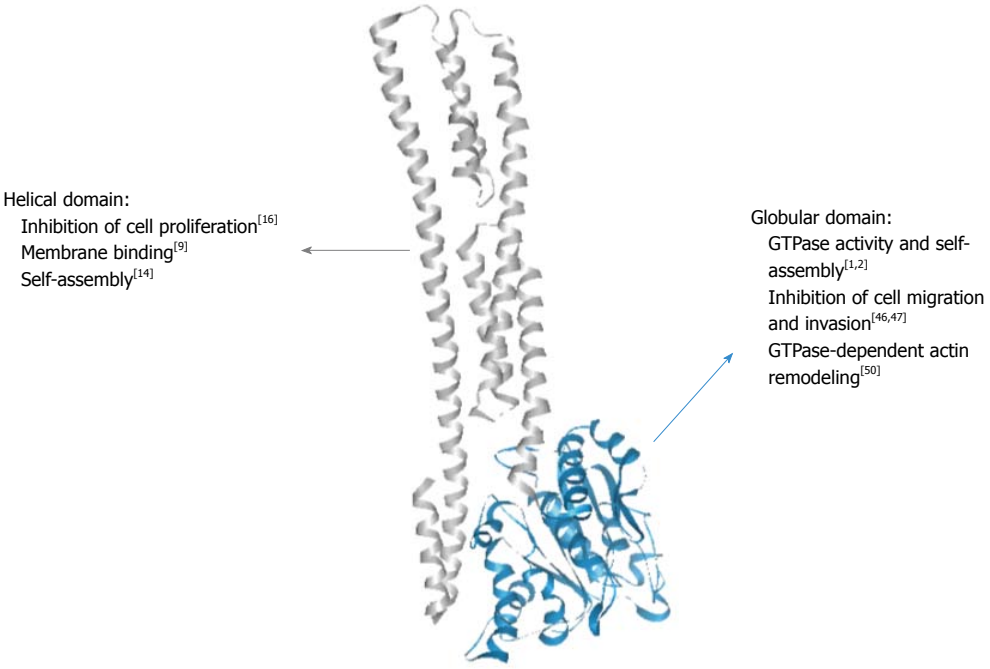

Figure 1 Structure-function relationships of guanylate-binding protein-1. The guanylate binding protein-1 can be roughly divided into two domains: a compact globular domain carrying the GTPase activity at the N-terminus (blue) and a long helical domain comprised of α-helices at the C-terminus (grey), which are responsible for different protein functions.

which have allowed prognoses to be refined, and have opened new perspectives for improving therapeutic decisions. Various kinds of molecular subtypes have been described including chromosomal instability, microsatellite instability and CpG island methylator phenotype[22-24]. In addition, gene expression profiles that correlate with prognosis have been identified. For instance, CRCs with a stem cell gene expression signature are associated with a poor prognosis[24,25]. Besides molecular classifications, the host's immune response has emerged as a powerful prognostic factor in CRC. Additionally, immune cell infiltration into the tumor and a polarized immune response have been shown to be of prognostic relevance. In particular, the presence of an active Th1 adaptive immune response in CRC correlates with a better outcome[26]. More precisely, the presence of cytotoxic and memory T-cells, together with the expression of Th1-associated factors (IFN-γ, IL-12 or IRF1), has been associated with a better prognosis in CRC[26,27]. In the meanwhile, such associations have been observed in a variety of solid tumors including breast, lung, ovarian and liver cancers[28]. Interestingly, molecular studies of CRCs also revealed the presence of an "inflammatory" gene expression signature enriched with interferon-stimulated genes (ISGs) and associated with improved prognosis[24,25]. Overall, the so-called Immunoscore has been shown to be better than the standard histo-pathological classification or the presence of microsatellite instability as a predictor of outcome for patients with CRC[26,29,30]. Expression of GBP-1, along with several other ISGs, has been detected in CRC[19,31]. Furthermore, a study involving 388 patients showed GBP-1 to be an independent prognostic marker in CRC, which correlates with prolonged 5-year cancer-specific survival[19]. This has subsequently been confirmed in a study of The Cancer Genome Atlas (TCGA) network, which also found that the expression of GBP-1 and GBP-4 in CRC was associated with a less aggressive phenotype, including tumor stage, lymph node invasion and metastasis[32]. Of note, an association has been found between expressions of GBP-1 and GBP-2 and the regression of melanoma metastasis after immunotherapy[33]. Furthermore, GBP-1 participates in a signature of immune function genes associated with recurrence-free survival in breast cancer patients[34]. In addition, GBP-2 has been shown to be expressed in esophagus squamous cell carcinomas and in breast cancer, where it is associated with T-cell infiltration and a better prognosis[35,36]. Hence, expression of GBPs appears to be generally associated with a prognostically favorable immunological response to cancer. Specific evidence has been obtained that GBP-1 mediates the anti-tumorigenic effects induced by a Th1-dominated micromilieu.

GBP-1 is a marker for IFN-γ-induced cell activation in IBDs

IBDs comprise two main forms of chronic relapsing disorders, namely ulcerative colitis (UC) and Crohn's disease (CD)[37]. The pathogenesis of IBD is not yet fully understood, but it is generally believed to involve dysregulation of intestinal homeostasis, characterized by the loss of barrier function and an aberrant immune response leading to the loss of tolerance of enteric bacterial flora accompanied by acute inflammation[37,38]. Active IBD displays infiltration of the lamina propria by

Table 1 Expression and functions of guanylate-binding protein-1

GBP-1 expression	
Inflammatory bowel diseases	Expressed in UC and CD[15,20,21]
	Expression in epithelial cells, endothelial cells and immune cells[15,21]
Colorectal carcinoma	Expression in the stroma and partially in tumor cells[15,19]
	Associated with Th1-dominated angiostatic micromilieu and improved survival[19,31]
Functions of GBP-1	
Endothelial cells	Anti-angiogenic, inhibits proliferation, migration, invasion and spreading; protection against apoptosis[16,44,46,47,50]
Intestinal epithelial cells	Inhibits proliferation; protection against apoptosis[20,43]
Colon tumor cells	Inhibits proliferation, migration, invasion and tumor growth *in vivo*; no induction of apoptosis (but partially required)[9]
T-cells	Inhibition of spreading and early T-cell receptor signaling[54]

GBP: Guanylate-binding protein; UC: Ulcerative colitis; CD: Crohn's disease.

both innate and adaptive immune cells and increased local levels of cytokines[39-42]. Compared to healthy controls, specimens with active CD or UC show increased expression of GBP-1 at the RNA and protein levels[15,21,43]. In addition, GBP-1 mRNA expression correlated with the expression of IFN-γ in whole tissue samples, and GBP-1 expressing cells were found in the vicinity of IFN-γ-producing cells[21]. While UC has been considered as a Th2-mediated condition mostly driven by IL-13, Th1 cytokines such as IL-12 or IFN-γ have been described as primarily involved in CD. However, this paradigm has been revised and cytokines such as TNF-α, IFN-γ, IL-1, -6, -12 and -17 are considered to be involved in the pathogenesis of both diseases[42]. Accordingly, GBP-1 expression is also increased in both, and at similar levels (Table 1)[21]. In agreement with human data, elevated expression of GBP-1 was also detected in colonic tissues of mice undergoing colitis after treatment with dextran sodium sulfate (DSS)[21]. This increase was observed in both, acute and chronic DSS models and was associated with an elevation in IFN-γ tissue expression[21]. Hence, GBP-1 expression seems to be a useful marker for inflammation and IFN-γ-induced cell activation in IBD. Cytokines such as IFN-γ, TNF-α and IL-1β are not only involved in the recruitment and activation of immune cells but can also affect epithelial or endothelial cells, leading to an amplification of tissue damage and inflammation[39-42]. In IBD samples, GBP-1 expression was observed in infiltrating immune cells, in endothelial cells and in epithelial cells, confirming that IFN-γ indeed acts *in vivo* on both mesenchymal and epithelial cells[15,21].

GBP-1 FUNCTIONS IN STROMAL CELLS

GBP-1 mediates the anti-angiogenic effects of IFNs in CRC

Expression of GBP-1 has been detected in the stroma of CRCs[15,19]. In the course of tumorigenesis, the angiogenic switch allows tumors to grow beyond a critical size. In the tumor micro-environment, pro-angiogenic and anti-angiogenic factors co-exist and the resulting angiogenic balance determines the state of the vasculature. Based on the observation that the inflammatory cytokines IL-1β, TNF-α and IFN-γ inhibit proliferation and migration of endothelial cells, GBP-1 was identified by differential display RT-PCR as a gene induced by these angiostatic cytokines and repressed by the angiogenic factors VEGF and bFGF[16]. Subsequently, it has be shown that GBP-1 expression in tissues is closely associated with blood vessel endothelial cells that are exposed to these cytokines[5,16]. In agreement with these findings, in CRC, the expression of GBP-1 in vessels correlated with significantly repressed angiogenic activity and the presence of an angiostatic micro-environment, indicated by co-expression of the anti-angiogenic cytokines CXCL9, -10 and -11[19]. Because of its association with immune angiostasis in tissues *in vivo*, GBP-1 was investigated for directly exerting angiostatic activity. Indeed, it was shown to inhibit the proliferation of human umbilical vein endothelial cells (HUVECs) through its helical domain (Figure 1)[16]. It was also found to mediate the effects of IFN-α by protecting HUVECs from serum starvation-induced apoptosis[44]. Of note, in the same study, continuous exposure to IFN-α led to senescence of HUVECs. Unexpectedly, GBP-1 was shown to be secreted, however exclusively by endothelial cells[45]. As yet, the role of extracellular GBP-1 remains unknown. Interestingly, GBP-1 has been shown to inhibit the invasiveness and tube-forming capabilities of HUVECs by down-regulating MMP-1 expression[46]. This effect was found to be dependent on the GTPase activity of the protein. Furthermore, GBP-1 is able to inhibit migration and spreading of endothelial cells on a fibronectin matrix through expression of integrin-α4, indicating that its effect might depend on the extracelluar matrix composition of the surrounding micromilieu[47]. Mouse GBP-2 has similarly been shown to inhibit endothelial cell spreading[48,49]. Interestingly, GBP-1 has been shown to interact with other GBPs (GBP-2 to -5) and to recruit them in its own cellular compartment, suggesting that GBPs act in a cooperative manner and that GBP-1 is dominant over the other family members[9]. The effects of GBP-1 on cell migration, invasion and spreading can be explained by the fact that GBP-1 was found to bind β-actin[50]. More precisely, it has been found to mediate the IFN-γ-induced disruption of actin fibers in various tumor cell

lines and in endothelial cells[50]. The disintegration of actin fibers by GBP-1 occurred through direct binding between both proteins, both *in vivo* and *in vitro* using purified recombinant GBP-1 and actin[50]. The interaction with β-actin required both self-assembly and the GTPase activity of GBP-1[50]. Taken together, it has been shown that GBP-1 exerts a powerful anti-angiogenic role by directly inhibiting endothelial cell proliferation, migration, invasion and spreading, together with protecting cells against apoptosis, thus creating an angiostatic state for the vessels (Table 1 and Figure 2). Thereby, GBP-1 links anti-tumor immune response and inhibition of angiogenesis in CRC as a major mediator of the anti-angiogenic effect of IFNs.

Role of GBP-1 in IFN-induced vascular remodeling in IBD

As chronic IBD is established, the vascular system undergoes profound reorganization[51]. On the one hand, angiogenesis is induced through the production of VEGF. On the other hand, inflammatory stimuli activate endothelial cells, enhancing leukocyte binding, tissue infiltration and ultimately, chronic inflammation[52,53]. However, the resulting vasculature is dysfunctional. Expression of GBP-1 was observed in vascular cells and was associated with *in situ* IFN-γ expression in human IBD tissues and in DSS-induced mouse colitis samples, indicating that those vessels were in an anti-angiogenic state[21]. This was confirmed by the fact that neutralization of IFN-γ resulted in an increase in blood vessel density[21]. Hence, the angiostatic effects of IFN-γ mediated by GBP-1 seem to be a more general mechanism, because it also occurs in colitis. Of note, IFN-γ was found to increase vascular permeability in the same mouse colitis model. Thereby, IFN-γ is involved in establishing dysfunctional vasculature, potentially participating in the aggravation of tissue damage and in the increase of immune cell infiltration[21].

GBP-1 regulates T-cell receptor signaling

Expression of GBP-1 has been detected *in vitro* and *in vivo* in monocytes/macrophages and T-cells in response to IFN-γ[5,19,54]. In T-cells, GBP-1 has been found to mediate the interaction between early T-cell receptor signaling and the cytoskeleton[54]. More precisely, it was required for inhibiting T-cell spreading, down-regulating CD3 and CD45 at the cell surface, and decreasing IL-2 release. Its function in T-cells was mediated by interactions with the actin-regulating proteins plastin-2 and βII-spectrin[54]. Hence, GBP-1 might participate in a negative feedback loop in T-cells activated by IFN-γ resulting in reduced IL-2 expression during Th1 differentiation (Figure 2). Therefore, GBP-1 might prevent T-cells from over-activation, leading to a protective effect during IBD. It cannot be excluded that GBP-1 might also participate in T-cell anergy, which would result in a negative impact on the anti-tumor immune response in CRC. However, the overall impact of GBP-1 expression remains positive, which indicates that its effects on endothelial and tumor cells overcome the inhibitory functions in T-cells.

GBP-1 FUNCTIONS IN COLON EPITHELIAL AND TUMOR CELLS

GBP-1 exerts anti-tumorigenic effects in colon tumor cells

In CRC, GBP-1 expression is predominantly detected in the desmoplastic stroma. In about half the cases, GBP-1 expression is also detected in tumor cells[15]. This suggests that the stimulus responsible for GBP-1 expression, for instance IFN-γ, originates from infiltrating cells and that the tumor cells might have lost responsiveness to the respective factor and concomitantly the ability to express GBP-1. This suggests that the loss of GBP-1 expression may be a tumor escape mechanism fostered by the genetic and epigenetic instability of the cancer cells, which does not affect the adjacent stromal cells. In this framework, it is important to note that GBP-1, as shown for endothelial cells, can also inhibit proliferation, migration and invasion of CRC cell lines (Table 1, Figures 1 and 2)[15]. In addition, GBP-1 was able to reduce anchorage-independent growth and tumor growth in a mouse xenograft model[15]. Using RNA interference, it was confirmed that GBP-1 mediates the anti-proliferative, anti-migratory and anti-invasive effects of IFN-γ in CRC cell lines[15]. However, it did not induce apoptosis by itself, even if it was at least partially required for IFN-γ-induced apoptosis[15]. In this context GBP-1 must be regarded as a bona fide tumor suppressor gene. Nevertheless, its effects on tumor cells seem to be highly tumor type specific[55]. In accordance with its functions in CRC cells, GBP-1 has also been shown to inhibit mammary tumor growth in mice[56]. In contrast, GBP-1 expression has been associated with Paclitaxel resistance in ovarian cancer cell lines[57,58], docetaxel resistance of prostate cancer cells[59], and with radioresistance[60]. Other reports have shown that GBP-1 induces glioblastoma growth in mice through increased invasiveness but not proliferation of glioblastoma cells[61,62]. Similarly, it was found to induce invasion of oral squamous cell carcinoma cells[55]. Based on these findings it is clear that GBP-1 has anti-tumor functions in CRC but its activity may be converted to pro-tumorigenic functions in other tumor types. It remains to be determined in future studies whether this is dependent on specific cellular or micromilieu-derived co-factors or is partly the result of different experimental setups. Overall, GBP-1 has been shown to have a pleiotropic role in CRC, by mediating both the anti-angiogenic and anti-tumorigenic effects of IFN-γ.

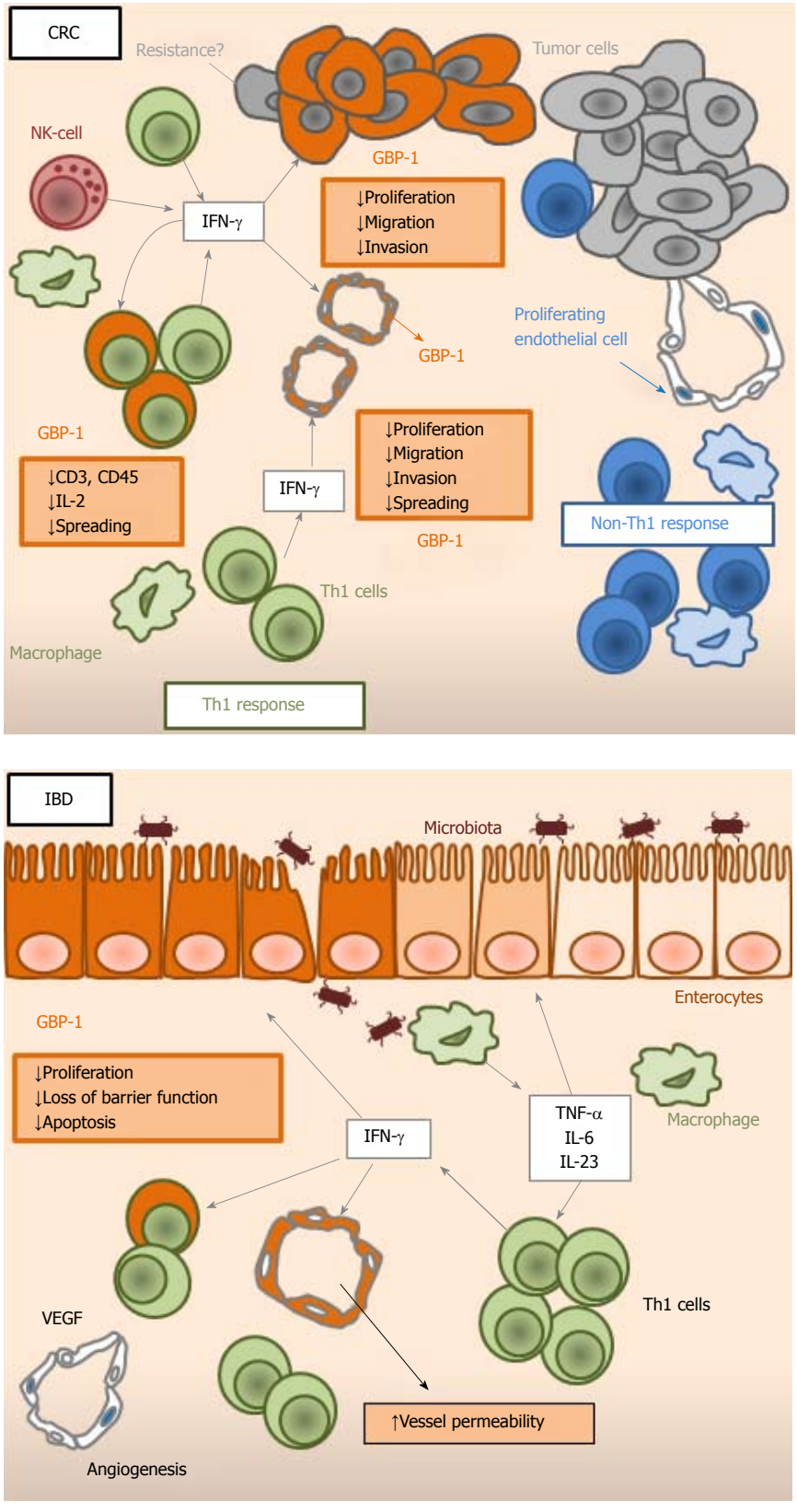

Figure 2 Role of guanylate-binding protein-1 in colorectal carcinoma and inflammatory bowel diseases. In colorectal carcinoma (CRC), guanylate-binding protein (GBP)-1 expression is associated with a Th1-dominated micromileu and results in an angiostatic vasculature. In CRC tumor cells, expression of GBP-1 induces an anti-tumorigenic phenotype. Absence of expression in tumor cells in a GBP-1-positive context indicates a mechanism of resistance. In T-cells, GBP-1 participates in the modulation of the T-cell receptor signaling pathway. In inflammatory bowel disease (IBD), GBP-1 expression is elevated in active disease and inhibits the proliferation of intestinal epithelial cells, thereby exerting a protective effect against the loss of barrier function and apoptosis. Here again, GBP-1 is associated with angiostasis and T-cell regulation. INF: Interferon; IL: Interleukin.

GBP-1 inhibits the proliferation of intestinal epithelial cells

Expression of GBP-1 in intestinal epithelial cells has been observed in human colon specimens of IBD and in the inflamed colonic mucosa of DSS-treated mice[15,21,43]. In addition, immortalized human primary colon epithelial cells are able to express GBP-1 *in vitro* after treatment with IFN-γ, IL-1β or TNF-α[15]. When induced by IFN-γ/TNF-α, GBP-1 was described as inhibiting cell proliferation of the colonic epithelial cell lines SKCO15 and T84, which harbor an enterocyte-stereotypic differentiation, are polarized and therefore are considered valuable models for studying intestinal epithelial cells[43]. In these cell lines, GBP-1 was able to reduce β-catenin protein levels and β-catenin serine 552 phosphorylation in a proteasome-independent mechanism[43]. On the other hand, it was found to have a protective effect against IFN-γ-induced apoptosis and loss of barrier function in the colonic epithelium (Figure 2)[20].

GBPS AND CELLULAR RESPONSE TO INTESTINAL PATHOGENS

Several human and mouse GBPs have been shown to be involved in the response against intracellular pathogens including viruses such as vesicular stomatitis virus, encephalomyocarditis virus, hepatitis C virus, influenza A virus, dengue virus, swine fever virus and human immunodeficiency virus-1[63-69]. GBPs have also been shown to mediate host's defense against bacterial and mycobacterial infectious agents such as *Listeria monocytogenes*, *Chlamydia trachomatis, Salmonella typhimurium*, and *Mycobacterium bovis*[70-72]. Finally, GBPs are involved in intracellular immunity against *Toxoplasma gondii*[73]. In particular, GBP-1 has been shown to be up-regulated in a colon cell line after rotavirus infection[74]. Furthermore, GBP-1 was found to be up-regulated differently in two mouse strains after colonization with commensal bacteria, and it is thought to participate, together with other interferon-stimulated genes, to host-specific responses to commensal gut bacteria, thereby shaping the microbiota[75]. Actually, IFN-α expression induced by commensal nonpathogenic *Escherichia coli* in the intestine of newborn mice was shown to mediate GBP-1 expression[76]. Furthermore, GBP-1 was required for the anti-apoptotic effects of IFN-α in immature human colon epithelia treated with staurosporine, indicating that it might play a role in the prevention of intestinal epithelial apoptosis induced by commensal bacteria[76].

CONCLUSION

Overall, expression of GBPs and in particular GBP-1 has been shown to be associated with infection by intracellular pathogens, Th1-driven inflammation during IBD and the Th1-dominated anti-tumor immune response in CRC. Additionally, GBP-1 exerts strong anti-proliferative, anti-migratory and anti-invasive effects on various cell types, resulting in the creation of angiostatic vasculature in IBD and CRC and in direct anti-tumorigenic effects on CRC cells.

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REVIEW

Management of psoriasis patients with hepatitis B or hepatitis C virus infection

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Abstract

The systemic therapies available for the management of Psoriasis (PsO) patients who cannot be treated with more conservative options, such as topical agents and/or phototherapy, with the exception of acitretin, can worsen or reactivate a chronic infection. Therefore, before administering immunosuppressive therapies with either conventional disease-modifying drugs (cDMARDs) or biological ones (bDMARDs) it is mandatory to screen patients for some infections, including hepatitis B virus (HBV) and hepatitis C virus (HCV). In particular, the patients eligible to receive an immunosuppressive drug must be screened for the following markers: antibody to hepatitis B core, antibody to hepatitis B surface antigen (anti-HBsAg), HBsAg, and antibody to HCV (anti-HCV). In case HBV or HCV infection is diagnosed, a close collaboration with a consultant hepatologist is needed before and during an immunosuppressive therapy. Concerning therapy with immunosuppressive drugs in PsO patients with HBV or HCV infection, data exist mainly for cyclosporine a (CyA) or bDMARDs (etanercept, adalimumab, infliximab, ustekinumab). The natural history of HBV and HCV infection differs significantly as well as the effect of immunosuppression on the aforementioned infectious diseases. As a rule, in the case of active HBV infection, systemic immunosuppressive antipsoriatic therapies must be deferred until the infection is controlled with an adequate antiviral treatment. Inactive carriers need to receive antiviral prophylaxis 2-4 wk before starting immunosuppressive therapy, to be continued after 6-12 mo from its suspension. Due to the risk of HBV reactivation, these patients should be monitored monthly for the first 3 mo and then every 3 mo for HBV DNA load together with transaminases levels. Concerning the patients who are occult HBV carriers, the risk of HBV reactivation is very low. Therefore, these patients generally do not need antiviral prophylaxis and the sera HBsAg and transaminases dosing can be monitored every 3 mo. Concerning PsO patients with chronic HCV infection their management with immunosuppressive drugs is less problematic as compared to those infected by HBV.

In fact, HCV reactivation is an extremely rare event after administration of drugs such as CyA or tumor necrosis factor-α inhibitors. As a rule, these patients can be monitored measuring HCV RNA load, and ALT, aspartate transaminase, gamma-glutamyl-transferase, bilirubin, alkaline phosphatase, albumin and platelet every 3-6 mo. The present article provides an updated overview based on more recently reported data on monitoring and managing PsO patients who need systemic antipsoriatic treatment and have HBV or HCV infection as comorbidity.

Key words: Psoriasis; Therapy; Conventional disease-modifying drugs; Biological disease-modifying drugs; Hepatitis B virus infection; Hepatitis C virus infection

Core tip: At present, no guidelines give clear indications regarding the management of psoriasis patients with concomitant hepatitis B or hepatitis C virus infection who need a systemic treatment. On the basis of the available literature data, this paper provides an overview in this field from a practical point of view. A particular emphasis is given, with regard to the use of biological drugs, in the aforementioned patients.

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Table 1 Therapies approved by European Medicines Agency for the treatment of psoriasis

	Recommended doses for adult patients
Conventional DMARDs	
Acitretine	0.25-1 mg/kg per day
Cyclosporin a	2-5 mg/kg per day
Methotrxate	10 mg to 25 mg per week
Biologic DMARDs	
Infliximab	5 mg/kg at 0, 2 and 6 wk followed by a maintenance regimen of 5 mg/kg every 8 wk
Adalimumab	80 mg initially, 40 mg on day 8, and 40 mg every other week thereafter
Etanercept	50 mg subcutaneously 2 times a week for 3 mo; (starting doses of 50 mg once a week have been shown to be effective); maintenance: 50 mg subcutaneously once a week
Golimumab[1,2]	50 mg once a month
Certolizumab pegol[1,2]	400 mg at 0, 2 and 4 wk followed by a maintenance regimen of 200 mg every other week
Ustekinumab[3]	45 mg initially, 45 mg at 4 wk, followed by a maintenance regimen of 45 mg every 12 wk[3]
Secukinumab[2]	300 mg at 0, 1, 2, 3, and 4 wk followed by a maintenance regimen of 300 mg every 4 wk. For some patients, a dose of 150 mg may be acceptable

[1]Approved for adults with active psoriatic arthritis; [2]No data available regarding the administration of patients with HBV or HCV; [3]For patients weighing > 100 kg (220 lbs), the recommended dose is 90 mg initially, 90 mg at 4 wk, followed by a maintenance regimen of 90 mg every 12 wk. HBV: Hepatitis B virus; HCV: Hepatitis C virus.

INTRODUCTION

Psoriasis (PsO) is a frequent inflammatory immune-mediated disease affecting approximately 2% of the population[1]. Various clinical types of psoriasis exist. The plaque-type, also known as psoriasis vulgaris (PV), is the most common form (80%-90% of the cases)[2]. Typical lesions of PV are represented by monomorphic, sharply demarcated erythematous plaques covered by silvery lamellar scales. From 70% to 80% of patients are affected by limited forms of PsO and need to be treated only with topical and or photo-therapy[2]. Patients with more extensive PsO (> 10% of the body surface area) or psoriatic arthritis (PsA) are in greater need of treatment. For these patients prolonged systemic therapies are often necessary[2-4].

The therapeutic armamentarium available for the cure of PsO encompasses the conventional disease-modifying drugs (cDMARDs) and biological DMARDs (bDMARDs) (Table 1).

cDMARDS represent the first line of therapies in high-need psoriatic patients, while bDMARDs are for those subjects in whom cDMARDs have either failed, were not tolerated, or were contraindicated[5-8].

The choice of a systemic treatment depends upon several variables linked to both the characteristics of a given patient and those of the drug administered.

Regarding the systemic treatments currently available for PsO, with the exception of acitretin, all the other drugs listed in Table 1 are immunosuppressive[9-11]. Therefore, the guidelines presently available, although with some differences among them, recommend screening PsO patients for some common infectious diseases [human immunodeficiency virus, latent tuberculosis, hepatitis B virus (HBV) and hepatitis C virus (HCV)] before starting an immunosuppressive treatment[12-18].

In particular, for HBV and HCV infection, screening for the following serologic markers should be evaluated: antibody to hepatitis B core (anti-HBc), antibody to hepatitis B surface antigen (anti-HBsAg), hepatitis B surface antigen (HBsAg) and antibody to HCV (anti-HCV)[12,15,18,19]. In case of detection of one or more markers of HBV infection the patients must be evaluated for the presence of HBV DNA in the sera[20]. If anti-HCV serum is detected, HCV RNA should be searched for by a sensitive method[20]. The existence of either HBV or HCV infection or both in PsO patients eligible

for a systemic therapy poses a series of challenging problems. In particular, the administration of an immunosuppressive drug can alter the relationship between the host and the virus and worsen a coexisting chronic infection. Moreover, cDMARDs have different degrees of hepatic toxicity[9,11] therefore increasing the risk of worsening an already compromised liver as a consequence of the HBV or HCV infection.

PsO patients with infectious diseases such as HBV or HCV are excluded by the randomized controlled clinical trial. Therefore, data available on PsO patients with HBV or HCV infection, treated with systemic drugs, rely mainly on the reports of single cases or analyses of small groups of patients.

The aim of the present article is to describe how to manage and monitor PsO patients with HBV or HCV infection who need systemic antipsoriatic drugs.

MONITORING AND MANAGEMENT OF PsO PATIENTS TREATED WITH cDMARDs OR bDMARDs AND CONCOMITANT HBV INFECTION

The first aspect to consider in patients with severe PsO and concomitant HBV infection, is to define the phase of the latter disease[18-20] and the degree of possible liver damage. To do so, a close collaboration between dermatologist and hepatologist is needed[18-20].

As a rule, during the active phases of HBV infection, systemic anti-psoriatic therapies should be deferred. After an adequate control of infection, by means of anti HBV drugs is obtained[18-21], therapies should be started. Active HBV infection includes different phases[22-24] not necessarily sequential, such as: (1) acute infection (defined as new-onset HBV infection that may or may not be icteric or symptomatic); and (2) chronic (defined as the persistence of HBsAg for six months or more) and encompassing different phases (immune-tolerant, HbeAg-positive immune-active, HBeAg-negative immune reactivation).

Acitretin is the only drug that could be administered during the active phases of HBV infection. However, the administration of said drug should be reserved only for selected cases without a severe impairment of liver function.

In daily clinical practice the more frequent scenarios that can be encountered are represented by patients with serological markers indicative of a previous exposure to HBV, with low or undetectable viral load. In particular, these subjects can be in one of the following infectious phases: (1) inactive HBV infection [serum HBV DNA < 2000 IU/mL, normal alanin aminotransferase (ALT) levels, HBsAg present, antibody to hepatitis B envelope antigen (anti-HBeAg)] present, minimal liver necroinflammation but variable fibrosis); (2) occult HBV infection (serum HBV DNA < 200 IU/mL or undetectable, HbsAg negative, anti-HBc positive, anti-HBs negative); and (3) resolved HBV infection [(rHBV); anti-HBs positive ± anti-HBc]. Some confusion exists regarding the terms "occult carrier" and "rHBV". In fact, some Authors define as "occult carriers" (or "potential occult carriers") those patients who are indicated by other authors as rHBV and vice versa[22-27]. In this paper, the terms will be used following the above reported classification. The patients, whether inactive or occult carriers or with rHBV, can be at risk of HBV reactivation (defined as the sudden increase in HBV replication) after starting an immunosuppressive therapy[28]. As expected, the risk is significantly greater in the inactive carriers as compared to occult ones. Even more rare is the case of viral reactivation in rHBV[29]. The possible occurrence of one of the three previously mentioned infectious phases in a given PsO patient, raises some problems regarding their therapeutic management. In particular: (1) which drug can be safely administered? (2) which patients need anti HBV prophylaxis to prevent HBV reactivation? And (3) which serological markers should be monitored after an antipsoriatic therapy is started?

To answer these questions the cDMARDs and bDMARDs must be analyzed separately.

cDMARDs

Acitretin: Acitretin administration is considered, due to its potential hepatotoxicity, a relative contraindication in hepatitis resulting from viral infections[18]. However, acitretin (preferably in association with ultraviolet B therapy) can be a possible option in those subjects without significant signs of liver damage as revealed by serological [ALT, aspartate transaminase (AST), gamma-glutamyl-transferase (GGT), bilirubin, alkaline phosphatase] and instrumental (ultrasonography, fibroscan) methods. Unfortunately, the effectiveness of acitretin as monotherapy and its side-effects (teratogenicity, mucosal dryness, hypertriglyceridemia) other than the potential hepatic toxicity[30] limits its use in many patients.

Cyclosporin a: The current dermatologic guidelines on the management of psoriatic disease do not give clear indications regarding the use of cyclosporin a (CyA) in patients with PsO and concomitant HBV infection[18]. The only reports of HBV reactivation concern severe immunosuppressed subjects such as renal transplant recipients and hematological patients[31,32].

In a cohort of patients with rheumatoid arthritis (RA) who were anti-HBc positive and/or anti-HBs positive (defined by the Authors as resolved HBV infection) treated with either cDMARDs and or bDMARDs, CyA did not result associated with an activation of HBV infection[33]. However, the potential risks of HBV reactivation should not be underestimated also using the relatively low doses of CyA as those usually given to PsO patients. Lacking clear indications, and on the

Table 2 Diagnosis and distribution of patients treated with tumor necrosis factor-α inhibitors according to the hepatitis B virus serological profile

Ref.	Diagnosis, *n*	Inactive carriers (HBsAg$^+$), *n*	Occult carriers or resolved HBV (anti-HBc$^+$, anti-HBs$^-$ or anti-HBs ± anti-HBc), *n*	Prophylaxis[2], *n*	Reactivation, *n*
Charpin *et al*[42]	PsA, 5	0	5	0	0
Prestinari *et al*[43]	PsO, 1	0	1	0	0
Nosotti *et al*[44]	PsO, 4; PsA, 3	1	6	1 (Lamivudine)	0
Caporali *et al*[45]	PsA, 4	0	4	0	0
Kim *et al*[46]	PsA, 2	0	2	0	0
Fotadiou *et al*[47]	PsO, 7	7	0	7 (Lamivudine)	0
Prignano *et al*[48]	PsO, 12	0	12	0	0
Cassano *et al*[49]	PsO, 28; PsA, 34	0	62	0	0
Cho *et al*[50]	PsA, 2	2	0	0	1
Navarro *et al*[51]	PsO, 13	0	13	0	0
Laurenti *et al*[52]	PsA, 8	1	7	1 (Lamivudine)	0
Navarro *et al*[53]	PsO, 4	4	0	3 (Lamivudine); 1 (adefovir[2] entecavir)	0
[1]Sanz-Bueno *et al*[54]	PsO, 20	0	20	0	0

[1]This group also includes 6 patients who received UTK; [2]Only inactive carriers. PsO: Psoriasis; PsA: Psoriatic arthritis.

basis of our personal experience, we believe that CyA could be administered to patients who are HBV occult carriers or with rHBV if adequately monitored (see below).

Methotrexate: Data available on the use of methotrexate (MTX) in patients with HBV infection were gathered from patients with rheumatologic or inflammatory bowel disease[34,35]. Whether or not MTX can be safely administered to patients with a history of HBV infection is not clear[34,36].

Basically, in the dermatological field, the different guidelines agree on avoiding MTX therapy in all patients whose seromarkers indicate an exposition to HBV[12,18,37].

bDMARDs

TNF inhibitors: There is evidence from experimental models that TNF plays a critical role in HBV clearance from infected hepatocytes[38]. Therefore, a detrimental effect is expected in terms of worsening or reactivation of HBV infection in subjects treated with TNF inhibitors (TNFis).

Over the past years several cases of HBV reactivation have been reported with either infliximab (IFX), adalimumab (ADA) or etanercept (ETA) therapy, mainly in patients with rheumatologic inflammatory diseases or inflammatory bowel disease[21,39,40].

Whether or not different TNFis carry a different risk of HBV reactivation is currently not clear[41]. Some studies suggest a major risk of HBV reactivation for IFX as compared to other TNFis[21].

Presently, literature data regarding the administration of TNFis in PsO or PsA subjects with concomitant HBV infection is available for 200 patients[42-56]. The majority of subjects reported are inactive or occult carriers, or with rHBV.

On the whole, patients were treated for a period ranging from 24 wk[43] to 6 years[52]. The TNFi more frequently administered was ETA, followed by ADA and finally IFX.

In Table 2 are summarized the cases of PsO and/or PsA grouped in: (1) inactive carriers; and (2) occult carriers or rHBv. The cases shown in Table 2, are limited to those for whom there was sufficient information in each report to permit their inclusion in one of the two above cited groups.

As shown in Table 2, none of the patients who were occult carriers or with rHBV experienced an HBV reactivation during TNFis therapy. None of the above cited subjects received antiviral prophylaxis.

Concerning patients who were inactive carriers, none of those who received antiviral prophylaxis experienced an HBV reactivation. On the contrary, HBV reactivation has been reported only in one case of an inactive carrier who did not receive antiviral prophylaxis (Table 2). In addition, two other patients of the same series with chronic HBV, who did not take prophylaxis, had viral reactivation[50].

Regarding the use of TNFis in PsO with active HBV infection only one case has been reported so far[57]. Said subject received lamivudine therapy one month before starting IFX. This therapy was continued during treatment with TNFi for 6 mo with a significant improvement of PsO and a decrease of both viral load and transaminases. Thereafter, to obtain a better control of viral replication, lamivudine was substituted with enetecavir and at the 9th month a further decrease of viral load was recorded, transaminases levels being within normal range and PsO under control.

Ustekinumab: Ustekinumab (UTK) is a fully human immunoglobulin G1k monoclonal antibody, anti-IL12p40, which binds to the shared p40 subunit of

Table 3 Diagnosis and distribution of patients treated with ustekinumab according to the hepatitis B virus serological profile

Ref.	Diagnosis, *n*	Inactive carriers (HBsAg$^+$), *n*	Occult carriers or resolved HBV (anti-HBc$^+$, anti-HBs$^-$ or anti-HBs ± anti-HBc), *n*	Prophylaxis[1], *n*	Reactivation, *n*
Navarro *et al*[53]	PsO 1	1	0	1 (Entecavir)	0
Chiu *et al*[60]	PsO 14	11[2]	3	4 (Entecavir)	2
Hayashi *et al*[61]	PsO 5	0	5	0	0
Koskinas *et al*[62]	PsO 1	0	1	0	1
Steglich *et al*[63]	PsO 1	0	1	1 (Lamivudine)	0

[1]Only inactive carriers; [2]Six with diagnosis of chronic hepatitis. PsO: Psoriasis; HBsAg: Hepatitis B surface antigen; HBV: Hepatitis B virus; HBc: Hepatitis B core.

IL-12 and IL-23 with high affinity and specificity[8]. Because IL-12 plays a key role in triggering an effective cellular immune response directed towards the elimination of intracellular pathogens[58,59] its inhibition can contribute to HBV reactivation.

From 2013, 28 cases of PsO with concomitant HBV infection have been treated with UTK[53,54,60-62]. The duration of treatments ranged from 4 mo[60] to 3 years[63].

In Table 3, 22 out of the 28 above cited patients are shown, since 6 subjects belong to the case series reported by Sanz-Bueno *et al*[54] and included in Table 2.

At present the lack of data does not permit us to draw any conclusions about the safety of UTK in patients with HBV infection.

However, as shown in Table 3, 2 of the 11 inactive carriers and one patient with rHBV experienced a reactivation of HBV infection after 4 mo, 7 mo and 16 wk, respectively[60,62]. Antiviral prophylaxis was not administered to said subjects.

Antiviral prophylaxis

On the basis of the above reported data, it is clear that HBV infection does not represent a barrier to the administration of an immunosuppressive therapy in patients with severe PsO. However, when a patient is eligible to a long-lasting immunosuppressive therapy such as bDMARDs, the risk of HBV reactivation must be taken into account.

It is widely accepted that all subjects who are inactive carriers need an antiviral prophylaxis[10,38-40]. The latter should be started 2-4 wk before a bDMARD is given and continued for 6-12 mo after its suspension[10,38-40]. Concerning the type of antiviral drug to be administered, the American Gastroenterology Association (AGA) suggests a third generation nucleos(t)ide (entecavir or tenofovir) due to their high resistance to lamivudine[64].

Whether or not patients who are occult carriers or with rHBV infection should receive antiviral prophylaxis is a debatable issue[10,38-40,64]. The recent AGA guidelines suggest administering antiviral prophylaxis also in patients who are HbsAg-negative/anti-HBc positive (whether or not anti-HBs positive) treated with either TNFis or UTK[64]. However, in the context of PsO treatment with bDMARDs patients do not seem to carry a concrete risk of HBV reactivation. Therefore, in the above mentioned category of patients prophylaxis against HBV reactivation is probably not necessary[10,44].

Monitoring HBV reactivation

Regarding patients who are inactive carriers, considering the risk of HBV reactivation during therapy with a bDMARD, HBV DNA, ALT and AST, serum levels should be monitored monthly for the first 3 mo, then quarterly and continued after 6-12 mo of discontinuation of the aforementioned treatment[10,44]. More controversial is whether or not to measure HBV DNA serum levels in those PsO or PsA patients who are occult carriers or with rHBV treated withimmunosuppressive drugs (CyA or bDMARDs)[10,40,44]. As already stated above, said patients do not seem to run a concrete risk of HBV reactivation. Moreover, the measurement of viral load is an expensive test. Therefore, occult carriers as well as patients with rHBV, can be monitored every 3 mo, checking for the presence in the serum of HBsAg in conjunction with the measurement of ALT and AST levels[10,44]. Monitoring should be continued with the same above cited timing after 6-12 mo from the discontinuation of the immunosuppressive therapy.

MONITORING AND MANAGEMENT OF PsO PATIENTS TREATED WITH cDMARDs OR bDMARDs AND CONCOMITANT HCV INFECTION

After acute HCV infection occurs, from 15% to 25% of subjects spontaneously clear viremia while 75% to 85% of individuals develop chronic HCV infection[65]. The diagnosis of this last condition is based on the detection of both HCV antibodies and HCV RNA in the presence of signs of chronic hepatitis, either by elevated aminotransferases or by histology[66]. For the majority of patients the course of chronic HCV infections is benign. However, from 10% to 20% of subjects develop cirrhosis, generally in a time gap from 20 to 30 years[65]. It has been estimated that each year 1% to 4% of patients with HCV related cirrhosis will develop hepatocellular carcinoma (HCC), and 20%

will further progress to decompensated cirrhosis[65].

In contrast to HVB, HCV reactivation is not very common[67]. However, when HCV reactivation occurs, its morbidity and mortality rates are similar to those of HBV reactivation[20].

Concerning the treatment of PsO patients with concomitant HCV infection, a relatively limited number of data are available.

Among cDMARDs, acitretin and CyA could be two possible options while MTX is in general contraindicated for its substantial risk of hepatic toxicity[9,12,17,18]. Among bDMARDs, TNFis seems to be a relatively safe option (see below).

Very few data are at present available concerning the management with UTK of HCV infected patients since this biologic was introduced on the market several years after TNFis. Moreover, the use of UTK is presently approved only for PsO and PsA patients.

In the past, CyA has been considered by some Authors as third line as compared to TNFis in PsO subjects with HCV infection[67].

However, several data indicate that both CyA and TNFis do not seem to cause different risks in worsening the chronic course of HCV infection[68-72].

CyA and bDMARDs are probably the more frequently administered drugs for high-need PsO patients with concomitant HCV infection.

No data are currently available concerning the concomitant administration of systemic therapies for PsO and the new direct-acting antiviral medications approved for the treatment of chronic HCV infection.

In the following paragraphs treatment with CyA or bDMARDs of PsO patients with HCV infection are described.

cDMARDs

CyA: Due to immunosuppresive activity, a detrimental effect of CyA in individuals with a chronic infection such as HCV, could be expected. However, experimental and clinical data indicate that CyA, in addition to its anti-inflammatory activity, can inhibit HCV viral replication[73]. In particular, this last effect has been found in patients carrying genotype 1 or 4 HCV[71,72]. However, also in patients carrying genotype 2, which is scarcely sensitive to antiviral effect of CyA, no worsening of HCV infection has been observed in spite of a favourable clinical course of their immunomediated diseases[72]. Said data clearly indicate that immunosuppressive and antiviral activity of CyA follow different pathways[73].

Current available literature data on PsO patients with concomitant HCV infection treated with low dose of CyA include 11 patients, 7 of whom affected by PsA[69-72,74]. These patients received CyA for a period ranging from 16 to 38 mo and none experienced a worsening of their HCV infection. In some patients a lowering of HCV RNA serum levels has been reported[69].

bDMARDs

TNFis: Data available concerning the management with TNFis of PsO patients with HCV infection are increasing.

TNF-α is a key cytokine in stimulating immunomediated response to infections, especially against intracellular pathogens. Therefore, after inhibition of TNF-α, a worsening of a viral infection such as HCV could be expected. However, experimental and clinical data suggest that increased levels of TNF-α can have a detrimental effect on HCV infection[75-77]. In particular, the aforementioned cytokine seems to reduce cell capabilityto respond to interferon (IFN) signalling and, consequently, impair viral clearance[76]. Moreover, a direct correlation between elevated levels of TNF-α and those of ALT has been reported, with increased TNF-α levels having more severe histological activity[75,77]. A further indication that decreasing TNF-α concentrations can play a favourable effect on HCV infection comes from a study in which etanercept was administered as adjuvant to IFN and ribavirin in a group of HCV infected patients[78]. These subjects showed a higher decline of viral load and ALT as compared to the placebo group.

In addition to the data above reported, further observations indicate that TNFis can be safely administered to patients with different immune-mediated diseases and concomitant HCV infection[39,40,79]. In particular, in most of said patients viral load remained stable or decreased[39,40,79]. Serum HCV RNA increase > 1 log above baseline was rarely recorded and could not be confidently attributed to TNFis.

At present, 45 PsO and 33 PsA patients with concomitant HCV infection have been treated with TNFis[48,53,80-106]. Diagnosis, ALT and viral load outcomes at the last follow-up of the mentioned subjects are shown in Table 4. Of all the patients reported, 66 received a single TNFi (56 ETA, 9 ADA, 1 IFX). Six patients were treated sequentially with 2 TNFIs (1 with IFX and then ADA, 4 with ETA and then ADA and 1 with ETA and then IFX). One patient was treated sequentially with 3 TNFis (ETA followed by IFXand then ADA).

The duration of TNFis was highly variable ranging from 1 to 48 mo[97,105].

At baseline, liver biopsy specimens were available from 16 patients which revealed various grades of fibrosis ranging from F0 to F4[81-85,87,95,96,99,101-103]. Of said subjects only 2 underwent a second liver biopsy control which showed no significant histological changes compared to pretreatment findings[95].

As shown in Table 4, in the majority of the patients described, ALT and HCV RNA viral load remained unchanged or declined during TNFis therapy.

On the whole, the safety profile of TNFis appears to be reasonably good inPsO patients with concomitant HCV infection even if 4 cases of hepatocelluar carcinoma (HCC) were recorded[53,105]. However, 3 out of

Table 4 Diagnosis and laboratory characteristics of reported psoriatic patients with chronic hepatitis B virus infection treated with tumor necrosis factor-α inhibitors

Ref.	Diagnosis, *n*	Concomitant HCV therapy, *n*	ALT outcomes at last follow-up compared to baseline, *n*	HCV viral load outcome at last follow-up compared to baseline, *n*
Khanna *et al*[80]	PsA, 1	1 (N/A)	1 (N/A)	1 (N/A)
Magliocco *et al*[81]	PsA, 3	0	3 (=)	2 (↓); 1 (N/A)
Cecchi *et al*[82]	PsO, 1	0	1 (=)	1 (=)
De Simone *et al*[83]	PsO, 2	0	2 (=)	2 (↓)
Asladinis *et al*[84]	PsA, 1	0	1 (=)	1(↓)
Rokshar *et al*[85]	PsO, 1	0	1 (N/A)	1 (=)
Pitarch *et al*[86]	PsA, 1	1 (N/A)	1 (=)	1 (N/A)
Linadarki *et al*[87]	PsA, 1	0	1 (=)	1 (=)
Alcaide *et al*[88]	PsO, 1	0	1 (=)	1 (=)
Piccolo *et al*[89]	PsO, 1	0	1 (↑)	1 (↓)
Collazzo *et al*[90]	PsO, 1	0	1 (=)	1 (N/A)
Cassano *et al*[91]	PsO, 1	0	1 (=)	1 (=)
Cavazzana *et al*[92]	PsA, 1	0	1 (=)	1 (=)
Behnam *et al*[93]	PsO, 1	1 (IFN + Rib)	1 (↓)	1 (↓)
Ventura *et al*[94]	PsO, 1; PsA, 1	0	2 (↓)	1 (↑); 1 (↓)
Paradisi *et al*[95]	PsA, 2	0	2 (=)	2 (=)
Prignano *et al*[96]	PsO, 1	0	1 (N/A)	1 (=)
Richetta *et al*[97]	PsO, 1[1]	0	1 (N/A)	1 (N/A)
Garavaglia *et al*[98]	PsO, 3; PsA, 2	1 (IFN + Rib)	1 (=); 4 (↓)	3 (=); 1 (↓)
Gandhi *et al*[99]	PsO, 1	0	1 (↓)	1 (↓)
Di Lernia *et al*[100]	PsO, 1[3]	0	1 (=)	1 (=)
Zanni *et al*[101]	PsA, 3	0	3 (=)[4]	3 (=)
Prignano *et al*[48]	PsO, 6	0	6 (=)	6 (=)
Mederacke *et al*[102]	PsA, 1	1 (IFN + Rib)	1 (N/A)	1 (↓)
Navarro *et al*[53]	PsO, 20[2]	3 (IFN + Rib)	See references	See references
Bartalesi *et al*[103]	PsO, 1	1 (IFN + Rib)	1 (↓)	1 (↓)
Costa L *et al*[104]	PsA, 15	0	13 (=); 2 (↓)	14 (=); 1 (↓)
Di Nuzzo *et al*[105]	PsA, 2	1 (IFN + Rib)	1 (=); 1 (↓)	2 (↓)
Salvi *et al*[106]	PsO, 1	0	1 (↑)	1 (=)

[1]1 erythrodermic psoriasis; [2]1 erythrodermic psoriasis, 2 palmoplantar psoriasis; [3]1 palmoplantar psoriasis; [4]1 patient with concomitant alcoholic hepatitis. PsO: Psoriasis; PsA: Psoriatic arthritis; =: No significant change; ↓: Decreased; ↑: Increased; N/A: Not available; IFN: Interferon; Rib: Ribavirine.

4 said patients were affected by cirrhosis and it is impossible to draw any conclusion regarding a possible role of TNF inhibition in the appearance of the above cited HCC.

UTK: Concerning IL 12 and IL 23, both targeted by UTK, little is known about their activity in HCV infection. Presently available data suggest that IL-12 can have a relevant role against HCV in either acute or chronic phases of infection[57,107,108].

At present, seven patients with PsO and HCV infection have been treated with UTK. Abuchar *et al*[109] in 2013, described the case of one patient with erythrodermic PsO and HCV infection who, after 2 mo of UTK therapy, experienced a very good response regarding his PsO without worsening of HCV infection.

Chiu *et al*[60] reported their experience on 4 PsO patients with HCV infection who received UTK for a period ranging from 5 to 11 mo. During the follow-up period, HCV viral copy numbers increased in 3 cases with only one subject meeting the criterion of HCV reactivation, recorded after 1 mo of UTK therapy. This last patient, who at baseline had a diagnosis of cirrhosis and previous HCC experienced a recurrence of HCC after 4 mo of UTK treatment.

Navarro *et al*[53], in a study published in 2013, reported that two PsO patients with concomitant HCV infection received UTK for 16 and 12 mo, respectively. The first patient, at the end of follow-up, showed a decrease of viral load and slight increase of both ALT and AST. In the second patient at the end of follow-up, viral load remained unchanged with a slight decrease of both ALT and AST serum levels.

More data are needed to better define whether or not UTK can have a role in the treatment of PsO patients with HCV infection.

Monitoring and managing of HCV infection

No guidelines are currently available on how to monitor PsO patients with HCV infection during treatment with CyA or bDMARDs.

Monitoring liver function tests (ALT, AST, alkaline phosphatase, bilirubin, albumin and platelet) and HCV-RNA load every 3-6 mo could be a valuable option for the majority of the patients.

However, it is important to point out that an optimal follow-up of the above-cited patients requires a strict collaboration with a consultant hepatologist. This latter will decide the timing of instrumental examinations, whether or not to perform a liver biopsy and whether

or not to offer a prophylactic anti-viral treatment.

CONCLUSION

HBV and HCV are the pathogens which more frequently cause chronic hepatitis[110].

Only few studies have evaluated the prevalence of the above-cited infectious disease in the PsO population. In 2011, Yang *et al*[111] reported an increased prevalence of HBV and HCV in PsO Taiwanese patients. In 2010, Cohen *et al*[112] in their study performed in Israel found an increased prevalence of HBC but not of HBV in PsO compared to control. The different frequency of HBV and HCV infection, as well as of PsO worldwide, can account for the discrepancies among the data published so far.

The chance to encounter in clinical practice PsO patients in high need of treatment who are HBV or HCV infected, should not be underestimated. Concerning said issue all current guidelines recommend screening PsO patients eligible for an immunosuppressive therapy due to the presence of HBV and HCV infection. However, no guidelines presently available give indications on how to manage and monitor these patients. Notwithstanding the above-cited limitations, immunosuppressive therapies should not be an insurmountable barrier for subjects with severe PsO and concomitant chronic HBV or HCV infection.

As already stated in this paper, it is essential that the aforementioned patients be referred to a hepatologist for expert clinical management.

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REVIEW

Enhanced recovery pathways in pancreatic surgery: State of the art

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Abstract

Pancreatic surgery is being offered to an increasing number of patients every year. Although postoperative outcomes have significantly improved in the last decades, even in high-volume centers patients still experience significant postoperative morbidity and full recovery after surgery takes longer than we think. In recent years, enhanced recovery pathways incorporating a large number of evidence-based perioperative interventions have proved to be beneficial in terms of improved postoperative outcomes, and accelerated patient recovery in the context of gastrointestinal, genitourinary and orthopedic surgery. The role of these pathways for pancreatic surgery is still unclear as high-quality randomized controlled trials are lacking. To date, non-randomized studies have shown that care pathways for pancreaticoduodenectomy and distal pancreatectomy are safe with no difference in postoperative morbidity, leading to early discharge and no increase in hospital readmissions. Hospital costs are reduced due to better organization of care and resource utilization. However, further research is needed to clarify the effect of enhanced recovery pathways on patient recovery and post-discharge outcomes following pancreatic resection. Future studies should be prospective and follow recent recommendations for the design and reporting of enhanced recovery pathways.

Key words: Pancreas surgery; Perioperative care; Length of stay; Postoperative complications; Pancreatic neoplasms; Evidence-based medicine

Core tip: In this study, we reviewed the available literature for enhanced recovery pathways in pancreatic surgery with a special focus on the evidence underlying specific perioperative interventions implemented in this surgical subspecialty and on postoperative outcomes. Although the quality of available studies is suboptimal, enhanced recovery proved to be safe and has the potential to reduce postoperative length of stay and costs after pancreatic resection. No evidence is available regarding post-discharge outcomes and patient functional recovery. Further research is needed to clarify the impact of care pathways on patient recovery after pancreatic surgery.

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INTRODUCTION

The number of patients undergoing pancreatic surgery has increased dramatically in the last decades[1]. At the same time, outcomes have improved. Postoperative mortality rates have decreased from nearly 20% in the 1980s[2] to 1%-2% thanks to centralization policies[3], advances in surgical technique, perioperative care and multidisciplinary management of complications[4]. However, even in high-volume centers (*i.e.*, institutions performing more than 15-20 pancreatic resections per year)[3] patients still experience significant post-operative morbidity[5] and full recovery after surgery takes longer than we think[6]. Recent studies found that patients undergoing pancreatic cancer resection take around 6 mo to return to their preoperative quality of life[7]. This finding suggests that an effort should be made to improve perioperative care and support patients in their recovery with the aim to reduce postoperative disability. In addition, considering that most of these patients receive surgery for cancer, it should be kept in mind that returning to a valid functional capacity status is also an essential pre-requisite to face adjuvant chemotherapy, which is now a mainstay of pancreatic cancer treatment.

Around 20 years ago, a Danish group lead by Henrik Kehlet reported on a series of nine colonic surgery patients that were treated with a multimodal intervention program including epidural analgesia, early oral nutrition and mobilization[8]. This represented the first step in the development of so-called fast-track programs, which later evolved in what are currently known as enhanced recovery pathways (ERPs). ERPs are standardized, multimodal, multidisciplinary care plans that integrate various evidence-based interventions in the perioperative period. Their main goal is to facilitate recovery by attenuating the metabolic surgical stress response and limiting postoperative organ dysfunction through multiple pharmacological, nutritional and physical approaches[9]. Moreover, ERPs aim to better organize care for patients undergoing a particular procedure, and thereby contribute to reducing unwanted variability in care processes and outcomes. A meta-analysis of 38 randomized trials across multiple specialties concluded that ERPs reduced the risk of complications by about 30% and were associated with reduced hospital stay by about 1 d overall[10]. The impact was consistent across specialties, which included colorectal, upper GI, genitourinary, thoracic and joint surgery.

In this review we will first discuss the specific elements included in ERPs for pancreatic surgery. We will then describe the evidence accumulated so far on the effect of enhanced recovery on postoperative outcomes following pancreatectomy, and finally we will suggest future directions in this field of research.

CHARACTERISTICS OF AN ENHANCED RECOVERY PATHWAY FOR PANCREATIC SURGERY

In 2012 the Enhanced Recovery After Surgery (ERAS®) Society drafted guidelines for perioperative care for patients undergoing pancreaticoduodenectomy (PD)[11]. The body of evidence for many ERP interventions was in large part extrapolated from studies in other fields of gastrointestinal surgery, mainly colorectal. Following the GRADE Working Group guidelines[12], recommendations were based on quality of evidence but also on the balance between desirable and undesirable effects; and on values and preferences. Accordingly, in certain cases, strong recommendations were reached from low-quality data and *vice versa*. Table 1 summarizes the specific elements that should be included in ERPs for PD according to the ERAS® Society guidelines. We will now provide an explanation for specific items of interest in the preoperative, intraoperative and postoperative settings.

Patient education and engagement

Preparing patients and their caregivers before surgery is fundamental. Patient should be informed about how they should get ready for surgery, what to expect on the day of surgery, and they should receive objectives for each postoperative day including instructions about their drains, infusions, diet and mobilization. Patients should be encouraged to play an active role in their recovery. In fact, patient knowledge and engagement have the potential to improve adherence to ERP elements, and it has been shown to reduce hospital stay, improve pain control and increase patient satisfaction[13]. The format and the way education is delivered

Table 1 Enhanced recovery pathway interventions for pancreatic surgery

Element	Description
Preoperative	
Patient education	Dedicated counseling providing patients with information and goals for recovery
Optimization of organ dysfunction	Optimization of patient comorbidities and patient conditioning
Oral immunonutrition	Oral immunonutrients should be taken for 5-7 d prior to surgery
Selective biliary drainage	Endoscopic biliary drainage only indicated if serum bilirubin > 14.5 mg/dL, in case of cholangitis or planned neoadjuvant treatment
Avoid mechanical bowel preparation	Oral bowel preparation should not be used
Minimize fasting	Intake of clear fluids up to 2 h before anesthesia, and solid food until 6 h before.
Carbohydrate loading	A carbohydrate drink should be given the morning before surgery
Intraoperative	
Thromboembolic disease prophylaxis	Low molecular weight heparin should be administered
Antimicrobial prophylaxis	Antibiotic prophylaxis should start 30-60 min before incision
Epidural and opioid sparing analgesia	Avoid opioids. Multimodal analgesia including thoracic epidural analgesia, acetaminophen, NSAIDs. Early transition to oral analgesics
PONV prophylaxis	Multimodal nausea and vomit prophylaxis
Avoid hypothermia	Active cutaneous warming
Balanced intravenous infusions	Avoid fluid overload. Maintain near-zero fluid balance. Potential benefit in the use of goal directed fluid therapy.
Postoperative	
Avoid nasogastric intubation	Nasogastric tube should be removed at the end of surgery
Glycemic control	Avoid hyperglycemia with frequent blood sugar monitoring and insulin infusion when necessary
Early removal of urinary drainage	Bladder catheter should be removed within postoperative day 2
Early removal of perianastomotic drain	Early drain removal in patients at low risk for pancreatic fistula
Early oral feeding	Patients should be allowed a normal diet without restrictions as tolerated
Gastrointestinal stimulation	Oral laxative and chewing-gum should be started early after surgery
Early stop of intravenous infusions	Intravenous fluids should be stopped as soon as patients are able to tolerate oral liquids
Early mobilization	Scheduled active mobilization should start from postoperative day 1
Audit	Systematic audit on care processes and outcomes

NSAID: Non-steroidal anti-inflammatory drugs; PONV: Postoperative nausea and vomit.

can influence the patient's ability to retain information and act accordingly[14]. Written or multimedia information have been shown to have a significant advantage on oral communication alone as information are often forgotten by patients[15].

Optimization of organ dysfunction

Preoperative optimization aims at improving patient physiologic reserve to better tolerate the incoming stress of surgery. Patients at higher risk for postoperative morbidity such as elderly, frail and patients with severe comorbidities should be evaluated in a multidisciplinary setting[16]. There is preliminary evidence in colorectal surgery that patients may benefit from prehabilitation programs focusing on improving co-existing morbid conditions and delivering effective nutritional therapy and physical exercise[17]. A relevant proportion of patients undergoing pancreatic resection are elderly cancer patients with multiple comorbidities. Thus it can be speculated that this specific surgical population may particularly benefit from this approach.

Preoperative biliary drainage

Biliary drainage in patients with jaundice should not be routinely performed as it may increase the risk of serious adverse events, which are related to the drainage procedure[18]. In a recent RCT including patients with serum bilirubin concentrations < 14.6 mg/dL, preoperative endoscopic biliary drainage showed higher morbidity compared to patients undergoing upfront surgery[19]. Patients candidate to neoadjuvant treatment or experiencing a cholangitis represent an exception. It is still unclear if patients with higher bilirubin levels actually benefit from preoperative drainage.

Preoperative fasting and carbohydrate loading

Traditionally patients have been kept fasting from midnight to prevent the risk of aspiration of gastric contents at induction of anesthesia. This leads to dehydration and increases insulin resistance triggering a catabolic state[20], which is one of the main mechanisms responsible for poor surgical outcomes[21]. It has been almost 20 years that anesthesia societies worldwide started to recommend a 6 h fast for solids, and allow oral intake of clear fluids up to 2 h before surgery, as it does not increase the risk of aspiration in healthy adults undergoing elective procedures[22]. The administration of a carbohydrate-rich drink before surgery (50 g, 2-3 h preoperatively) can increase insulin sensitivity[23], and shift cellular metabolism to a more anabolic state[20]. A recent Cochrane review found only a slight reduction of postoperative hospital stay but no difference in complications or other outcomes when compared to placebo fluids[24]. In patients undergoing PD, a double-blind placebo-controlled randomized clinical trial showed that carbohydrate-rich drinks can be safely administered,

as the residual gastric volume at anesthesia induction was similar between the carbohydrate drink and placebo groups[25].

Multimodal analgesia

A multimodal approach for analgesia is the best strategy to obtain optimal pain control and enable patient recovery. A key element is neuraxial blockade *via* thoracic epidural analgesia, which provides excellent analgesia and is associated with reduced surgical stress response[26]. Epidurals in major open surgery have been shown to reduce morbidity, especially respiratory complications, and facilitate recovery of bowel function compared to systemic opioids[27]. A retrospective study by Pratt *et al*[28] raised concern that epidural in pancreatic surgery may be associated with increased major complications linked to the common occurrence of postoperative hypotension that may lead to anastomotic failure and increased fluid administration in response. No other study has confirmed these findings and epidurals are still considered the "gold standard". In the event of epidural-related hypotension due to vasodilatation, vasopressors should be considered to avoid fluid overload[29]. In addition, acetaminophen and nonsteroidal anti-inflammatory drugs (NSAIDs) should be prescribed routinely to aid in epidural analgesia withdrawal and early transition to oral analgesics to promote patient mobilization. Opioids are given only if adequate analgesia (Visual Analogue Scale for pain < 4/10) is not obtained with all of the above.

Postoperative nausea and vomiting prophylaxis

Although no specific trial has specifically investigated postoperative nausea and vomiting (PONV) prophylaxis strategies in pancreatic surgery, it is evident that nausea and vomit early after surgery impair the return to oral nutrition, and mobilization. Ideally, patients should be screened for PONV risk factors using a simple risk calculator like the Apfel score[30]. PONV prophylaxis begins during surgery and continues in the first hours postoperatively. A tailored strategy with multiple modalities and agents should be used in patients at high and moderate risk[31]. Common prophylactic interventions during surgery include the use of total intravenous anesthesia (TIVA) with minimization of volatile anesthetics, and the use of loco-regional anesthesia techniques to spare systemic opioids. In addition, prophylaxis protocols include the administration of corticosteroids (*e.g.*, dexamethasone) after induction of general anesthesia, and 5-HT_3 receptor antagonists (*e.g.*, Ondansetron) or butyrophenones (*e.g.*, Droperidol) at the end of surgery.

Balanced intravenous infusions

Administering the optimal amount of fluids during and after surgery has the goal to maintain euvolemia and avoid both organ hypo- and hyper-perfusion. Salt and water overload significantly increase the complication rate and delay bowel function[32]. However, it is not the restriction in fluid administration but the "near zero" fluid balance that should be achieved, as this has been showed to improve outcomes in major open abdominal surgery[33]. Patients in ERPs usually require less maintenance fluids as they avoid prolonged fasting and bowel preparation thus have minimal deficits to be replaced[34]. Pancreatectomy is usually associated with large volume depletion and considerable blood loss, which makes it more difficult to achieve a correct balance. Several noninvasive devices providing continuous cardiac performance measures are available and can be used to tailor fluid management. Overall, their use reduces complications after major surgery[35]. Cannesson *et al*[36] recently implemented a goal directed fluid therapy (GDFT) algorithm in major surgery patients including 94 undergoing pancreatectomy, and found that GDFT significantly decreased complications and reduced length of hospital stay (LOS) by 18%. Although evidence for this approach is still preliminary, the use of monitoring devices to tailor fluid therapy should be encouraged particularly in high-risk patients (*e.g.*, patients with multiple comorbid diseases or candidate to multivisceral resection or complex vascular reconstructions).

Postoperatively, intravenous fluids should be discontinued when patients are able to drink enough liquids. After pancreatectomy in an ERP, oral fluid intake is usually started on the first day after surgery and the drip may be heplocked within 48-72 h after surgery depending on patient symptoms and tolerance of oral intake.

Perianastomotic drain

The use of an abdominal drainage in pancreatic surgery has been traditionally advocated as it is thought to minimize the consequences of a pancreatic fistula and allow a conservative management of this complication. In contrast, in other abdominal surgery contexts, drains have been abandoned as they have been associated with increased risk of drain-related infectious complications and prolonged hospital stay[37]. Currently, the routine use of a perianastomotic drain during pancreatic resection has been challenged[38]. However, a recent multicenter randomized controlled trial investigating the use of a closed-suction intraperitoneal drain *vs* no drain was stopped early because of an increase in 90-d mortality in the group of PD patients without drain[39]. Thus, it clearly provided level I evidence that a concept of routine non-drainage in all cases after PD is not safe and should be abandoned. Nonetheless, further research is warranted to assess the possibility of selective drainage only in patients with a high-risk of developing pancreatic leak (*e.g.*, soft pancreatic texture and small duct). Evidence for distal pancreatectomy is currently lacking.

Concerning the timing of removal of the periana-

stomotic drain, a randomized trial supports its early removal (*i.e*., on postoperative day 3) in patients at low risk of pancreatic fistula and low drain amylase value on postoperative day 1[40]. In this subgroup of patients, early removal was associated with a significantly decreased rate of pancreatic fistula, abdominal and pulmonary complications compared to patients with prolonged drainage. Until further data are available, systematic postoperative drainage and early removal in patients at low risk of pancreatic fistula (firm pancreas, wide pancreatic duct) is recommended[41].

Early oral nutrition

Allowing normal food at will from the first day after surgery has been shown to be feasible and safe after pancreatic surgery[42]. A large RCT including nearly one hundred patients who underwent pancreatectomy found no advantage to withholding feeding compared to normal food[43]. Furthermore, in 2014 Gerritsen *et al*[44] showed that allowing early oral feeding immediately after PD compared to prolonged naso-jejunal tube feeding is associated with reduced time to adequate oral intake and shortened LOS. Patients should be informed that early satiety, decreased appetite, and ultimately delayed gastric emptying (DGE) are common symptoms after pancreatic surgery. Thus they should be advised to gradually increase the amount of food intake as tolerated. Abdominal complications including pancreatic fistula are very common after pancreatic resection, and they may impair oral intake for long. In this event, enteral tube feeding through naso-jejunal catheters or jejunostomy should be preferred to parenteral nutrition[45].

DGE prevention and gastrointestinal stimulation

No prokinetic agent has been shown to successfully prevent delayed gastric emptying, which is defined as a functional gastroparesis causing the inability to tolerate oral diet and requiring nasogastric tube decompression[46]. Chewing sugar-free gum is a simple low cost intervention that decreases time to recovery of gastrointestinal function after colorectal surgery as part of an ERP[47]. A recent small-size RCT carried out in PD patients found that chewing gum slightly accelerates the return to bowel function and to oral intake, but the data were not significant[48]. Other authors have proposed the use of magnesium sulphate or lactulose in pancreatic surgery but few results other than safety have been reported[42]. For pylorus-preserving PD, there is preliminary data suggesting that constructing the duodenojejunostomy in an antecolic (as opposed to a retrocolic) fashion reduces DGE[49].

Early mobilization

It is well known that staying in bed leads to deconditioning that can largely be prevented by physical activity[50]. However, there is little evidence that the implementation of specific interventions to increase mobilization improves outcomes[51]. In the context of ERPs for colorectal surgery, being out of bed on the first postoperative days is an independent predictor of shorter hospital stay[52]. After pancreatic resection, due to the extent of surgical trauma, patients experience a prolonged recovery compared to other abdominal procedures. In addition, epidural-related hypotension is a common symptom limiting postoperative mobilization. However, mobilization out of bed should be scheduled early and adequate pain control provided to facilitate it.

OUTCOMES OF ENHANCED RECOVERY IN PANCREATIC SURGERY

In recent years, reporting of clinical pathways for patients undergoing pancreatic resection has progressively increased. Early reports included retrospective single cohort studies[42] and retrospective studies comparing newly implemented ERPs with historical cohorts[53-55]. They all focused on the feasibility and safety of implementing care pathways that included a limited number of perioperative interventions. Most of these studies only featured postoperative care elements such as multimodal analgesia, early return to oral diet and removal of tubes and drains, and scheduled mobilization. Compliance with the pathway seemed adequate, morbidity and readmission rates were low, and the authors concluded that this approach was safe, feasible, and promoted earlier discharge. In the following years, the number of publications on this topic, thus the number of patients and the experience with this type of approach increased but the quality of studies remained suboptimal. To date, there is still no report of a randomized clinical study, and no clinical study has been prospectively registered in an international trial registry.

Overall, seventeen trials[53-69] comparing ERPs to usual perioperative care in pancreatic surgery were published between 2000 and 2015. Table 2 reports the study design and characteristics of studies analyzed in this review. Only one study by Joliat *et al*[67] included a prospective ERP cohort, while all others performed a retrospective review comparing patients treated with a recently implemented ERP to historical controls. In a study from the Netherlands[64], the Authors also included an ERP-like group in which only a limited number of enhanced recovery elements were included.

The total number of patients included in the analyzed studies was 3220 (1576 ERP *vs* 1644 usual care). Study sample size ranged from 41 to 635 patients. Fourteen studies included PD patients, two of which also included total pancreatectomy patients. Only three studies investigated ERPs in the context of left pancreatectomy. This focus on PD is probably not only related to the greater proportion of patients who undergo this procedure compared

Table 2 Study design and characteristics

Study	Year	Design	Sample size		Type of resection
			ERP	Control	
Porter *et al*[53]	2000	Retrospective cohort	80	68	PD, TP
Vanounou *et al*[54]	2007	Retrospective cohort	145	64	PD
Kennedy *et al*[55]	2007	Retrospective cohort	92	44	PD, TP
Balzano *et al*[56]	2008	Retrospective cohort	252	252	PD
Kennedy *et al*[57]	2009	Retrospective cohort	71	40	LP
Nikfarjam *et al*[58]	2013	Retrospective cohort	20	21	PD
Abu Hilal *et al*[59]	2013	Retrospective cohort	24	20	PD
Braga *et al*[60]	2014	Retrospective cohort	115	115	PD
Kobayashi *et al*[61]	2014	Retrospective cohort	100	142	PD
Nussbaum *et al*[62]	2014	Retrospective cohort	50	100	LP
Nussbaum *et al*[63]	2014	Retrospective cohort	100	142	PD
Coolsen *et al*[64]	2014	Retrospective cohort	144[1]	86	PD
Shao *et al*[65]	2015	Retrospective cohort	325	310	PD
Sutcliffe *et al*[66]	2015	Retrospective cohort	65	65	PD
Joliat *et al*[67]	2015	Prospective cohort[2]	74	87	PD
Morales Soriano *et al*[68]	2015	Retrospective cohort	41	44	PD
Richardson *et al*[69]	2015	Retrospective cohort	22	44	LP

[1]Includes 47 patients treated with an enhanced recovery-like pathway; [2]Only the ERP group was prospective. PD: Pancreaticoduodenectomy; TP: Total pancreatectomy; LP: Left pancreatectomy.

to distal pancreatectomy, but also to the greater impact that this procedure has on patient recovery. In fact, PD is characterized by lengthy operative times, and extensive fluid and protein losses. At least three visceral anastomoses are fashioned leading to slower recovery of bowel function and obviously a greater chance of major complications compared to distal pancreatectomy. Laparoscopy was included in two out of three ERPs for patients undergoing distal pancreatectomy. In the context of enhanced recovery, laparoscopy has the potential to further accelerate recovery, as it has been shown for colorectal surgery[70], but conclusive evidence is lacking on the outcomes following laparoscopic distal pancreatectomy compared to the open approach. However, several comparative nonrandomized studies have found that laparoscopy can shorten postoperative recovery compared to open surgery in terms of accelerated return to oral intake, recovery of bowel function and reduced LOS[71]. The minimally invasive approach for PD, mainly laparoscopy, is slowly becoming more popular. However, this procedure is technically demanding requiring long operative times and a steep learning curve[72-74]. Single-centre studies from high-volume institutions have demonstrated that laparoscopic PD is feasible and safe in patients with benign and malignant pancreatic lesions[75,76]. In their meta-analysis including only comparative cohort studies, de Rooij *et al*[77] found no difference in postoperative mortality, morbidity and pancreatic fistula rates. They also reported quicker postoperative recovery resulting in reduced LOS. Nonetheless, the level of evidence supporting laparoscopic PD remains low and limited to small nonrandomized series. Even fewer evidence is available for robotic surgery, which may potentially facilitate the transition from open surgery and shorten the learning curve. In addition, to our knowledge there is no series evaluating the results of minimally invasive PD in the context of an ERP.

The role of adherence to ERP elements

In comparative studies analyzed in this review, a total of 17 individual elements aiming to enhance recovery after pancreatic resection were identified (Table 3). The number of elements used within each study ranged from 4 to 17 (median 9). The elements most frequently included were mostly part of postoperative care: standardized perianastomotic drain management (n = 17), omission or early removal of the nasogastric tube (n = 16), early oral feeding (n = 16), thromboembolic disease prophylaxis (n = 13), and early mobilization (n = 13).

Collecting information about adherence to the different care processes included in the ERP is important in order to understand outcomes and how to improve care. However, only a few studies reported the adherence to the individual ERP elements[59,60,67]. This confirms recent findings from Day *et al*[78] showing that the current standard of reporting in enhanced recovery trials is frequently incomplete, suggesting the need for guidelines for the design and reporting of such studies.

It is still unclear if there is an ideal combination of ERP items that should be implemented, what is the impact of overall adherence to the ERP and the relative contribution of each element included. Studies in colorectal surgery suggest that there is a dose-effect relationship between adherence and postoperative outcomes[79]. In pancreatic surgery patients, Braga *et al*[60] found that adherence was suboptimal for most of the postoperative interventions, especially perioperative fluid management and achievement of daily mobilization milestones. Notably, patients who experienced postoperative complications had a poor compliance early in the ERP pathway, suggesting that early low adherence may be associated with occurrence of postoperative complications. Sutcliffe *et al*[66] used a drain amylase cutoff of 350 U/L on the first day after surgery to stratify high- from low-risk PD patients and chose to implement only a limited

Table 3 Enhanced recovery pathway elements used in comparative studies

Study	Preoperative				Intraoperative					Postoperative								Total number of ERP elements
	Patient education and counselling	No mechanical bowel preparation	Shorter pre-operative fasting	Carbohydrate loading	Prophylactic antibiotics	Thromboembolic disease prophylaxis	Epidural/ multimodal analgesia	Prevention of nausea and vomiting	Prevention of hypothermia	Early nasogastric tube removal	Early removal of urinary catheter	Early discontinuation of IV fluids	Glycemic control	Standardized perianastomotic drain management	Early oral feeding	Early mobilization	Stimulation of GI function	
Porter *et al*[53]										√	√			√	√			4
Vanounou *et al*[54]	√				√	√	√			√	√			√				7
Kennedy *et al*[55]					√	√	√			√	√	√		√	√	√		9
Balzano *et al*[56]	√				√	√	√			√				√	√	√		8
Kennedy *et al*[57]					√	√	√			√	√	√		√	√	√		9
Nikfarjam *et al*[58]	√		√		√	√	√			√	√	√		√	√	√	√	12
Abu Hilal *et al*[59]			√			√						√		√	√	√	√	7
Braga *et al*[60]	√	√	√		√	√	√	√	√	√		√		√	√	√		13
Kobayashi *et al*[61]	√	√	√							√				√	√			6
Nussbaum *et al*[62]					√	√				√	√	√		√	√	√		8
Nussbaum *et al*[63]					√	√	√			√	√	√		√	√	√		9
Coolsen *et al*[64]	√		√	√	√	√	√		√	√	√	√		√	√	√	√	14
Shao *et al*[65]							√			√				√	√			4
Sutcliffe *et al*[66]	√	√	√	√	√	√	√	√	√	√				√	√	√		13
Joliat *et al*[67]	√	√	√	√	√	√	√	√	√	√	√	√	√	√	√	√	√	17
Morales Soriano *et al*[68]		√	√		√		√	√	√	√	√			√	√	√		11
Richardson *et al*[69]	√		√	√		√				√	√	√		√	√	√		10

Table 4 Postoperative length of stay and readmission rates

Study	Postoperative length of stay (d)			Readmission rates		
	ERP	Usual care	*P* value	ERP	Usual care	*P* value
Porter *et al*[53]	12	15	0.001	10 (15)	9 (11)	0.620
Vanounou *et al*[54]	8	8	0.357	13 (9)	4 (6)	0.508
Kennedy *et al*[55]	7	13	< 0.001	7 (8)	3 (7)	> 0.05
Balzano *et al*[56]	13 (7-110)	15 (7-102)	< 0.001	18 (7)	16 (6)	0.865
Kennedy *et al*[57]	Mean 7	Mean 10	0.037	5 (7)	10 (25)	0.027
Nikfarjam *et al*[58]	8 (7-16)	14 (8-29)	< 0.001	3 (15)	0	0.107
Abu Hilal *et al*[59]	8 (7-13)	13 (10-20)	0.015	1 (1)	2 (8)	0.583
Braga *et al*[60]	11 (5-51)	13 (8-54)	0.226	14 (12)	12 (10)	0.835
Kobayashi *et al*[61]	22 ± 12	36 ± 24	< 0.001	2 (2)	2 (2)	0.689
Nussbaum *et al*[62]	6 (5-9)	7 (5-9)	0.026	15 (30)	20 (20)	0.219
Nussbaum *et al*[63]	11 (8-18)	13 (10-18)	0.015	31 (31)	36 (25)	0.850
Coolsen *et al*[64]	14 (7-83)	20 (9-132)	< 0.050	11 (13)	14 (14)	NR
Shao *et al*[65]	14 ± 7	18 ± 8	< 0.001	43 (13)	44 (14)	0.725
Sutcliffe *et al*[66]	9 (4-70)	10 (4-114)	0.160	9 (15)	5 (8)	0.260
Joliat *et al*[67]	15 (11-24)	19 (14-29)	0.029	NR	NR	NR
Morales Soriano *et al*[68]	14 ± 1.3	19 ± 2	0.014	9 (10)	4 (9)	> 0.05
Richardson *et al*[69]	3 (3-4)	6 (5-10)	< 0.001	2 (9)	8 (18)	0.476

Data for length of stay are reported as median (range or interquartile range) or mean ± SD. Data for readmission are reported as number of patients (%). ERP: Enhanced recovery pathway.

number of ERP elements in the high-risk group. Early oral intake, avoidance of nasogastric drainage, and early perianastomotic drain removal were only applied to low-risk patients. Although it is intuitive that high-risk patients will be more likely to develop postoperative morbidity and experience a slower recovery, with holding oral diet has been repeatedly shown to be unnecessary and to delay recovery after pancreatic surgery[43,44], while prolonging postoperative nasogastric drainage after elective abdominal surgery is a known risk factor for pulmonary complications and delayed return to bowel function[80]. Additionally, di Sebastiano *et al*[81] found that tolerating oral diet is an independent factor predicting early discharge within and ERP for PD. Studies published so far have shown that ERPs are safe and feasible in all elective patients undergoing pancreatic resection, including the elderly[82]. Thus it is not recommended to select patients for being treated within an ERP. Further studies are warranted to clarify the impact of adherence to the pathway and identify key elements associated with improved outcomes.

Postoperative outcomes

The most common postoperative outcome considered in studies evaluating enhanced recovery in pancreatic surgery was LOS. Despite being influenced by many non-clinical factors such as surgeon's preference, social situation, caregiver availability as well as distance from the hospital[83], LOS is an easy way to monitor outcomes within an institution as it relates to recovery, complications and costs. In addition, it is important to monitor hospital readmissions as discharging patients early may lead to misdiagnosed complications and increase the risk of patients returning to the emergency room and being readmitted early after discharge[84].

Table 4 shows LOS and readmissions for the studies analyzed in this review. The majority of the studies reported that primary LOS was significantly shorter when patients undergoing pancreatic resection were treated within an ERP with no increase in hospital readmissions. This corroborates with the results observed in other surgical populations[10]. Differences in LOS between control and ERP groups varied from 1 to 14 d. It is important to observe that in studies where this difference was not significant[60,66], subgroup analyses showed that in uncomplicated patients and patients experiencing minor complications, LOS was significantly shorter in the ERP compared to the usual care group.

Table 5 reports morbidity and mortality for the comparative studies analyzed. Complication rates ranged from 16% to 70%. Five of the sixteen studies reporting postoperative morbidity rates showed differences in overall complications when the control and ERP groups were compared. No difference was found in postoperative mortality. None of the studies reported differences in surgical complications between groups, while three studies[56,61,65] found a significant reduction in the occurrence of delayed gastric emptying. A meta-analysis of randomized controlled trials in colorectal surgery found that ERPs had a protective effect only on medical complications whereas surgical complications were similar to the usual care[85]. Compared to colectomy, pancreatectomy is associated with a higher rate of postoperative complications, often exceeding 50%. Most of these are surgical complications related to pancreatic fistula, which are unlikely to be influenced by the implementation of a care pathway as this does not modify relevant prognostic factors such as pancreatic

Table 5 Morbidity and mortality rates

Study	Complication rates			Mortality rates		
	ERP	Usual care	*P* value	ERP	Usual care	*P* value
Porter *et al*[53]	56 (70)	52 (76)	0.210	2 (3)	1 (1)	0.870
Vanounou *et al*[54]	77 (54)	40 (62)	0.207	2 (1)	1 (2)	0.918
Kennedy *et al*[55]	34 (37)	19 (44)	> 0.05	1 (1)	1 (2)	> 0.05
Balzano *et al*[56]	119 (47)	148 (59)	0.014	9 (4)	7 (3)	0.798
Kennedy *et al*[57]	11 (16)	15 (38)	> 0.05	1 (1)	1 (2)	> 0.05
Nikfarjam *et al*[58]	NR	NR		NR	NR	-
Abu Hilal *et al*[59]	8 (40)	6 (67)	0.077	0	0	-
Braga *et al*[60]	69 (60)	76 (66)	0.339	4 (4)	4 (4)	1
Kobayashi *et al*[61]	39 (39)	54 (60)	0.004	0	1.1	0.957
Nussbaum *et al*[62]	13 (26)	24 (24)	0.842	0	0	-
Nussbaum *et al*[63]	43 (43)	53 (41)	0.792	1 (1)	4 (3)	0.651
Coolsen *et al*[64]	46 (53)	48 (49)	> 0.05	4 (5)	6 (6)	> 0.05
Shao *et al*[65]	127 (39)	173 (55.8)	< 0.001	40 (12)	53 (17)	NR
Sutcliffe *et al*[66]	15 (34)	15 (41)	0.650	2 (3)	2 (3)	1
Joliat *et al*[67]	50 (68)	71 (82)	0.046	3 (4)	4 (5)	1
Morales Soriano *et al*[68]	12 (30)	24 (55)	0.029	0	2 (2)	> 0.05
Richardson *et al*[69]	6 (27)	17 (39)	0.421	0	0	-

Data are reported as number of patients (%). ERP: Enhanced recovery pathway.

texture and duct diameter, and intraoperative blood loss[86].

Costs

Six studies analyzed hospital costs after the implementation of an ERP for pancreatic surgery[53,55,57,67,69]. Three of them found a significant decrease in cost following the implementation of an ERP[53,55,69]. All analyses were limited to in-hospital resources, and the most significant savings were due to a reduction in board and room costs because of reduction in LOS. Notably, Joliat *et al*[67] performed a cost minimization analysis where they also took into account the fixed costs for the implementation of the pathway including a full-time dedicated ERP nurse manager and the use of a specific ERP database. No significant difference was found in overall costs but savings occurred for anesthesia, operating room, medication and laboratory costs. It should be noted that no study compared indirect costs between patients treated within an ERP and usual care. As care pathways aim at improving patient recovery and they result in reduced LOS, we may hypothesize that societal costs including time spent away from work and need for prolonged home support may be reduced as well. In colorectal surgery patients, a prospective study found that patients managed with an ERP incurred in lower societal costs compared to a conventional care strategy[87]. After discharge, patients managed in the ERP experienced less productivity loss, had less caregiver burden and made fewer visits to outpatient health centers.

Other postoperative outcomes

In the context of ERPs for pancreatic surgery there is no study reporting recovery outcomes other than traditional short-term measures such as hospital LOS and complication rates, which are of interest for clinicians but do not reflect the complexity of the recovery process and fail to capture patient's perspective. An alternative measure of in-hospital recovery may be obtained by assessing the time to achieve specific discharge criteria ("time to readiness for discharge")[88]. The main advantage of this measure is that only factors related to physiological recovery are considered, without the influence of organizational and personal factors that affect LOS. Moreover, in line with the principles of patient-centered care[89], recent literature has advocated that postoperative recovery be measured using patient-reported outcomes (reports of health coming directly from the patient without interpretation by others)[90]. The main advantage of using patient reported outcomes in the context of recovery is that they allow a broad assessment of health across various domains, engaging patients as the key stakeholders in the recovery process.

FUTURE DIRECTIONS

We suggest that future research in this context should move in two directions: (1) designing studies with higher methodological quality to determine the impact of ERPs on postoperative outcomes and patient recovery after pancreatic resection; and (2) exploring the role of prehabilitation to optimize patients at high-risk of major complications.

Although non-randomized studies can yield relevant information when RCT data is not available, randomization is still the best approach to prevent selection bias in intervention studies. Therefore, it is our opinion that RCTs should be encouraged to provide convincing evidence about the role of ERPs in pancreatic resection patients. We do recognize that conducting RCTs to study complex interventions such as ERPs is challenging[91], and that a relevant number of ERP

interventions are now considered standard of care even in institutions where a formal ERP has not been implemented, but examples of well-conducted trials in other surgical populations show that it is possible[70,92]. In addition, all studies even if non-randomized, should be prospective and follow a structured reporting platform for enhanced recovery pathways as recently proposed[78]. Moreover, more relevant recovery outcomes including physiological variables (*e.g.*, postoperative stress response markers), long-term results and patient-reported outcomes should be investigated.

Recent literature reports scoring systems to predict patients at higher risk for major complications after pancreatic resection[5,86]. In addition, research advocates the assessment of body composition measures such as abdominal muscle area and visceral adiposity in cancer patients undergoing PD, as sarcopenic and visceral obese patients are at higher risk for pancreatic fistula and postoperative mortality[93,94]. High-risk patients could benefit from a prehabilitation program, which aims at improving patient's coexisting chronic disease therapy, nutritional status, and physical function through a multidisciplinary counseling involving multiple medical specialists, nutritionists and physiotherapists[16,17]. Considering that systemic treatment with chemotherapy is virtually recommended at any stage of pancreatic cancer, patients at very high risk for postoperative mortality could even be shifted from upfront surgery to a tailored preoperative pathway including neoadjuvant chemotherapy[95] and a prehabilitation program. This would allow a greater proportion of patients to receive treatment compared to an adjuvant setting where about a quarter of patients are unable to undergo chemotherapy due to surgical complications, poor performance status, or comorbidity[96]. According to West *et al*[97], a structured preoperative exercise program in patients undergoing neoadjuvant treatment for rectal cancer can improve patient physical fitness and reduce surgical risk. In the context of pancreatic surgery, the use of neoadjuvant therapy would potentially buy the time needed to carry out prehabilitation eventually leading to improved surgical and oncologic outcomes. Future studies are needed to verify the clinical effectiveness of prehabilitation in pancreatic cancer patients at high risk for postoperative morbidity and mortality, and test the feasibility of a combination of neoadjuvant treatment and a physical intervention in this setting.

CONCLUSION

This review analyzed the state of the art of enhanced recovery pathways in pancreatic surgery. Although the amount of literature has grown exponentially in the last decade, the methodological quality of available studies is suboptimal. Most of the studies suggested that the use of ERP is safe and has the potential to reduce primary LOS and hospitalization costs. Well-designed trials are needed to provide conclusive evidence about the role of ERPs in pancreatic surgery. Future studies should be prospective, follow recent recommendations for the reporting of enhanced recovery pathways and should also take into account more specific recovery outcomes such as physiological and patient reported outcomes.

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REVIEW

Specific CD8$^+$ T cell response immunotherapy for hepatocellular carcinoma and viral hepatitis

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Abstract

Hepatocellular carcinoma (HCC), chronic hepatitis B (CHB) and chronic hepatitis C (CHC) are characterized by exhaustion of the specific CD8$^+$ T cell response. This process involves enhancement of negative co-stimulatory molecules, such as programmed cell death protein-1 (PD-1), cytotoxic T-lymphocyte antigen-4 (CTLA-4), 2B4, Tim-3, CD160 and LAG-3, which is linked to intrahepatic overexpression of some of the cognate ligands, such as PD-L1, on antigen presenting cells and thereby favouring a tolerogenic environment. Therapies that disrupt these negative signalling mechanisms represent promising therapeutic tools with the potential to restore reactivity of the specific CD8$^+$ T cell response. In this review we discuss the impressive *in vitro* and *in vivo* results that have been recently achieved in HCC, CHB and CHC by blocking these negative receptors with monoclonal antibodies against these immune checkpoint modulators. The article mainly focuses on the role of CTLA-4 and PD-1 blocking monoclonal antibodies, the first ones to have reached clinical practice. The humanized monoclonal antibodies against CTLA-4 (tremelimumab and ipilimumab) and PD-1 (nivolumab and pembrolizumab) have yielded good results in testing of HCC and chronic viral hepatitis patients. Trelimumab, in particular, has shown a significant increase in the time to progression in HCC, while nivolumab has shown a remarkable effect on hepatitis C viral load reduction. The research on the role of ipilimumab, nivolumab and pembrolizumab on HCC is currently underway.

Key words: Hepatocellular carcinoma; CD8$^+$ T cells;

Immune checkpoint modulation; Chronic viral hepatitis; Cytotoxic T-lymphocyte antigen-4; Programmed cell death protein-1

Core tip: In certain types of chronic diseases, such as hepatocellular carcinoma and chronic viral hepatitis, disease curation involves restoration of the specific cytotoxic T cell response. Chronic hepatotropic viruses and tumoural cells develop mechanisms to induce exhaustion of the specific CD8$^+$ T cells in order to escape immune destruction. One hallmark of this dysfunction is the overexpression of negative co-stimulatory molecules. Blockade of these negative co-stimulatory pathways, a process known as immune checkpoint modulation, is a promising novel therapy that could improve the treatment of liver diseases that feature T cell exhaustion.

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INTRODUCTION

Specific CD8$^+$ T cells have a central role in pathogenesis of hepatocellular carcinoma (HCC) as well as control of infection with hepatitis B virus (HBV) and hepatitis C virus (HCV) because these cells are able to recognise infected/tumoural cells and destroy them[1-11]. Nevertheless, in chronic viral infections and tumoural diseases that feature high-grade and persistent antigenemia, the adaptive immune system has to surrender in order to diminish tissue damage[12-16]. This is the case for HCC and chronic viral hepatitis, wherein tumoural cells and HBV/HCV viruses modulate common mechanisms to induce specific T cell exhaustion. Among such viral and tumoural strategies, the induction of negative co-stimulatory molecules stands out.

Unfortunately, the on-going lack of effective treatments for HCC[17], for achieving complete HBV clearance[18] and for preventing HCV relapse after direct-acting antiviral (DAA) agent failure[19] has led to an urgent need for developing new therapeutic approaches, such as immunotherapy focused on specific cytotoxic T cell restoration[20]. Modulation of negative co-stimulatory signalling molecules expressed on these cells could have a substantial impact when developed as a therapeutic tool. In this review we discuss the specific CD8$^+$ T cell response during HCC and chronic hepatitis B and C (commonly known as CHB and CHC respectively), focusing on the disease mechanisms used by tumoural cells and hepatotropic viruses to induce T cell exhaustion and on the potential therapeutic strategies to modulate co-stimulatory pathways in order to restore specific T cell reactivity.

T CELL EXHAUSTION

CD8$^+$ T cell activation depends on physical interaction between the T cell receptor and the major histocompatibility complex I (MHC I)/epitope complex, as well as that between co-stimulatory molecules with their ligands in an adequate cytokine milieu[21]. Upon completion of their effector tasks, primed specific T cells switch-off their effector activity by expressing negative co-stimulatory molecules, generating a sustained memory T cell population[22]. Thus, the balance between positive and negative co-stimulation determines the status of CD8$^+$ T cell activation and the intensity of the accompanying immune response[23].

During tumoural and persistent viral infections - characterized by high-grade and persistent antigenemia - the adaptive immune system is tuned down in order to avoid host-induced tissue damage. Tumoural cells and persistent viruses have developed mechanisms that induce early expression of negative co-stimulatory molecules so as to favour T cell exhaustion before the effector T cells are able to control the disease[24-26]. This phenomenon could represent an evolutionary advantage for the chronic persistence of these diseases.

T cell exhaustion is characterized by a lack of effector cell capacity that is linked to overexpression of negative co-stimulatory pathways. Upon binding to their respective ligands, these negative co-stimulatory molecules act to disrupt the processes of T cell proliferation as well as secretion of type-Ⅰ cytokines and development of cytolytic functions, creating an environment that allows for tumour persistence and virus evasion[13,27-29]. Among these negative co-stimulatory pathways, the most advanced in the pipeline for clinical use are cytotoxic T-lymphocyte antigen-4 (CTLA-4) and programmed cell death protein-1 (PD-1), as will be discussed later[30,31].

Exhausted CD8$^+$ T cells were first identified during chronic lymphocoriomeningitis virus (LCMV) infection and defined as virus-specific CD8$^+$ T cells that did not produce antiviral cytokines and were ineffective at controlling the infection[32]. Since that time, subsequent research has provided descriptions of T cell exhaustion in different human chronic infections and cancers[33-35]. Loss of functionality occurs in a hierarchical manner throughout the process of exhaustion. It has been noted that the greater the antigen load or the duration of the disease, the greater the extent of exhaustion. Usually, functions such as interleukin (IL)-2 production, high expansion ability and *ex vivo* killing are lost first; this stage is named "partial exhaustion Ⅰ". In the next stage of exhaustion, "partial exhaustion Ⅱ", these cells lose their ability to produce tumour necrosis factor (TNF)-α, and their expansion ability and antigen-induced production of interferon (IFN)-γ

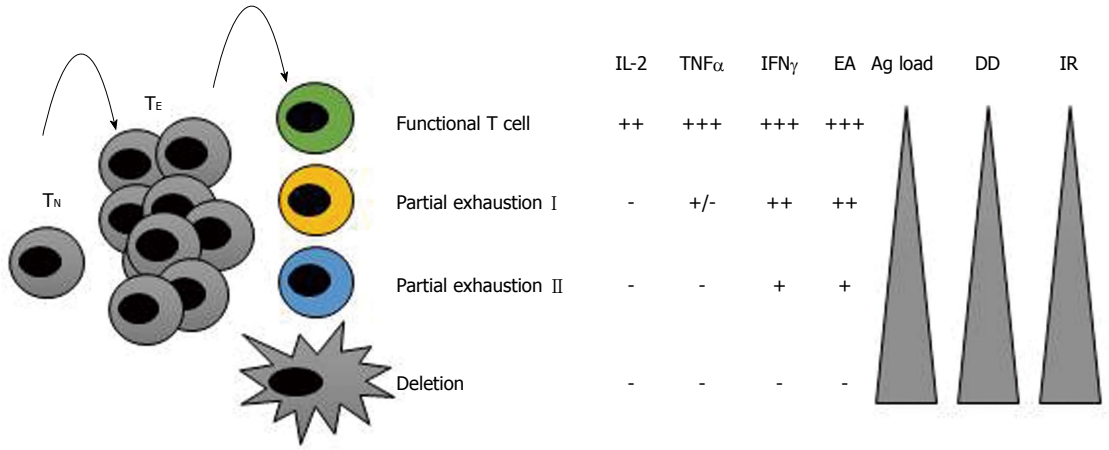


Figure 1 T cell exhaustion during diseases with persistent and high antigenemia. At the beginning of an infection, naïve T cells (T_N) are primed and differentiate into effector T cells (T_E). During acute infections, T_E are completely functional and control the pathogen/tumoural cell. After clearing the antigen, these cells are then deleted by apoptosis and a memory population is generated and maintained. Nevertheless, in conditions of chronic infections or tumours, these cells gradually loss their effector capacity, becoming exhausted. The greater the antigen load or duration of the infection, the more exhausted the cells become. The steps of exhaustion are summarised here. In "partial exhaustion I" IL-2 production, high expansion ability and *ex vivo* killing are lost. In "partial exhaustion II", the more advanced stage of exhaustion, these cells lose their capacity to produce tumour necrosis factor (TNF)-α, produce less interferon-γ and proliferate less. In the final stage of exhaustion, these cells are deleted by apoptosis. Ag: Antigen; DD: Duration of the disease; EA: Expansion ability; IR: Inhibitory receptors' expression.

become impaired. The final stage of exhaustion is the deletion of these cells by apoptosis[32,36,37] (Figure 1). A detailed understanding of the mechanism underlying this process may aid in development of efficacious therapies that restore the function of these cells and - from a practical point of view - the modulation of negative co-stimulatory pathways.

LIVER AS A TOLEROGENIC ORGAN

As previously described, one reason why specific cytotoxic T cells become exhausted in HCC, CHB and CHC is related to the strategies developed by the pathogen/tumour itself; yet, the host contributes to the exhaustion process as well, due to the particular liver features that are described below.

Bowen *et al*[38] elegantly showed that activation of primary CD8$^+$ T cells within the lymph nodes leads to an efficient response, whereas activation of primary CD8$^+$ T cells within the liver commits T cells to the development of an immunotolerant state. This divergent response is related to the liver's intense tolerogenic properties, which are in line with this organ's role in dealing with a massive load of foreign antigens from the gastrointestinal tract. For this reason, in order to develop new immunotherapeutic approaches to treat viral hepatitis and HCC it is first necessary to understand how intrahepatic immunity is regulated. An important feature to consider is that liver can support primary T cell activation independently of secondary lymphoid tissues and involvement of dendritic cells (DCs). Moreover, the ligands expressed by resident liver cells could favour exhaustion of specific liver-infiltrating T cells after antigen recognition. These two conditions could definitely impair the quality of T cell response[39,40].

Several liver cell types (listed below) can work as antigen-presenting cells (APCs) to activate naïve CD8$^+$ T cells.

Hepatocytes

Hepatocytes represent about two-thirds of the total cell population in the liver. Antigen presentation by hepatocytes is the most relevant mechanism of infection with hepatotropic viruses. Naïve CD8$^+$ T cells can directly interact with hepatocytes *via* liver sinusoidal endothelial cell (LSEC) fenestrations[41]. Although hepatocytes have been demonstrated as capable of promoting rapid activation and proliferation of CD8$^+$ T cells *in vivo*[39,40], they do not express positive co-stimulatory molecules, such as CD80 and CD86; therefore, because of this they could fail to induce functional CD8$^+$ T cells in *in vivo* conditions[42,43]. Besides, one of the ligands of the negative co-stimulatory molecule PD-1 (PD-L1) can be expressed by hepatocytes[44], and its interaction with PD-1 on the hepatocyte-activated CD8$^+$ T cell contributes to its functional suppression[45].

Kupffer cells

Kupffer cells (KCs) are the resident macrophages in the liver and represent the largest population of resident tissue macrophages in the entire body[46]. KCs are localized mainly in the periportal area, where they serve to clear endotoxins and phagocytose debris and microorganisms. These cells can also pass through the space of Dissé, coming into contact with hepatocytes and phagocytosing any with apoptotic features[47,48]. KCs express Fas-ligand[49] and PD-L1[50], leading to apoptosis and functional exhaustion of CD8$^+$ T cells respectively. In addition, the KCs can secrete immunosuppressive cytokines, such as IL-10

and tumour growth factor (TGF)-β, both of which can contribute to T cell exhaustion[51].

LSECs

LSECs can express MHC and co-stimulatory molecules and are capable of presenting antigen to CD8$^+$ T cells by at least two pathways, thereby promoting tolerance. Firstly, these cells express PD-L1 even at low antigen concentration[52] and, secondly, they can secrete IL-10 and TGF-β[53], which could impair CD8$^+$ T cell activation, as previously commented on.

Hepatic stellate cells

Hepatic stellate cells (HSCs), located at the space of Dissé, represent the major cell type involved in liver fibrosis, but they are also involved in antigen presentation[54]. TGF-β secreted by the HSCs contributes both to liver fibrosis and to the exhaustion of CD8$^+$ T cells[55].

DCs

Resident hepatic DCs are predominantly immature cells, prone to capturing and processing of antigens[56]. Because IL-10 and TGF-β are secreted by KCs and LSECs, the uninfected liver provides a unique cytokine environment that may render a tolerogenic state for the resident DCs[56-58]. Moreover, resting DCs can induce peripheral CD8$^+$ T cell tolerance through up-regulation of PD-1 and CTLA-4[59].

Consequently, CD8$^+$ T cells that are activated by these liver APCs are not optimally primed and fail to exert effector functions, thus promoting tolerance and T cell exhaustion. This situation can represent a survival advantage for hepatotropic viruses and HCC, since the specific cytotoxic T cells that are capable of recognising viral and tumoural antigens can become exhausted easily, due to the tolerogenic liver status. Such an environment features high-level expression of negative co-stimulatory ligands on the resident liver cells as well as induction of negative co-stimulatory receptors on the specific T cells, as related to the liver cytokine milieu.

Following our above introduction of the concept of T cell exhaustion in HCC, CHB and CHC, as well as of the mechanisms involved in this process, we will next highlight the current evidence showing why specific cytotoxic T cell response restoration could impact HCC, HCV and HBV treatment.

HCC

Worldwide rates of liver cancer classify it as the fifth most common cancer in men and the seventh in women. Infection with HBV and HCV, chronic alcoholism and fatty liver disease, among others, are major risk factors for HCC[60]. Once diagnosed, HCC usually has a poor prognosis, due to lack of efficacy of the available treatments. Therefore, novel effective therapies are urgently needed to treat patients with this type of tumour, particularly for those in advanced stages for whom the most efficacious of the current treatments are still only suboptimal.

Immune evasion is a general strategy of cancers, but much is still unknown about it. Most of the research on this phenomenon has focused on devising ways to directly destroy the tumoural cell, while the role that immune system restoration may play in resisting or eradicating the tumour formation and its progression has been largely, if not completely, overlooked[61]. Adaptive immune response, especially the cytotoxic response, is known to play a crucial role in the control of solid tumours[8]. Several lines of evidence have been reported that support the importance of CD8$^+$ T cells during HCC. Firstly, the presence of a high number of tumour-infiltrating T cells in HCC tissue suggests a role in HCC pathogenesis[9]. Secondly, the quantity of tumour-infiltrating T cells is considered a good prognosis marker of HCC[10]. Finally, adoptive immunotherapy could protect against HCC, diminishing the recurrence risk after surgical treatment[11].

Mizukoshi *et al*[62] analysed immune responses against various HCC epitopes in peripheral blood mononuclear cells from patients with HCC. After radiofrequency ablation (RFA), the authors noted an improvement of these responses in two-thirds of the patients; interestingly, those patients with a detectable response also experienced longer survival[62]. Flecken *et al*[12] recently described some tumour-associated antigen (TAA)-specific CD8$^+$ T cell responses in HCC. In that elegant study, the authors applied overlapping peptides to a large cohort of HCC patients and showed that a variety of TAAs can induce CD8$^+$ responses against α-fetoprotein (AFP), glypican-3 (GPC-3), melanoma-associated antigen-1 (MAGE-1) and New York-oesophageal squamous cell carcinoma-1 (NY-ESO-1). The authors also showed a positive correlation between either the quantity of TAA-specific CD8$^+$ T cells or the number of TAA targets and the survival of these patients. Finally, they also demonstrated that TAA-specific CD8$^+$ T cells were able to proliferate, but not able to produce IFN-γ after antigen encounter[12]. Therefore, HCC features CD8$^+$ T cells that are able to recognise tumoural neo-antigens; although, these cells display an exhausted behaviour. Interestingly, PD-1 was found to be up-regulated in these cells, a feature which could represent a base for immunotherapy by blocking this negative co-stimulatory molecule. Consequently, one possible approach for HCC treatment could be to restore the effector capabilities of these cells and one option towards achieving this end could be the modulation of negative co-stimulatory pathways, such as PD-1, as will be discussed below.

HCV

HCV was first cloned in 1989 as a non-A non-B he-

patitis virus[63]. Since then, substantial progress has been made in our understanding of both the virus and its interactions with the host system. The final result of this intense research effort has been the generation of DAAs that show curative effect on HCV infection in approximately 95% of the CHC patients[64-66].

In the last two decades, we have learnt several important lessons about the strategies that the HCV employs to avoid the immune system in order to persist in the host. HCV-specific CD8$^+$ T cells play an essential role in controlling HCV during acute infection[1-3], based upon their abilities to both recognize and destroy the infected cell through cytolytic and non-cytolitic mechanisms[4,67]. However, in approximately 70% of primo-infections, the virus is able to persist in the host, leading to chronic infection[68,69]. Viral escape mutations are the first mechanism used by HCV to avoid immune control, exploiting the lack of a proofreading function by the viral polymerase[70]. The second mechanism involves overwhelming the immune response. Because of the persistent antigenemia that accompanies HCV infection, the HCV-specific CD8$^+$ T cell response becomes exhausted and fails to control infection, featuring loss of effector capacities and overexpression of negative-regulation pathways[13,14].

Thus, although DAAs are very effective treatments, continued research in HCV immunotherapy is still necessary because of the existence of DAA non-responders and to develop it as a strategy to boost other anti-viral treatments (both established and new) and to support development of a therapeutic vaccine. Moreover, since these HCV-specific CD8$^+$ T cells up-regulate negative co-stimulatory molecules, blocking the interaction of these receptors with their ligands could be considered as a potential therapeutic strategy.

HBV

HBV is a hepatotropic non-cytopathic DNA virus and member of the family Hepadnaviridae[71]. Approximately 2 billion people worldwide have been infected by HBV, and it is estimated that more than 350 million of these individuals are persistent carriers of the virus. Most HBV infections occur *via* vertical transmission. Around 5%-10% of patients infected during adulthood develop CHB, with 10%-30% of those patients progressing to liver cirrhosis and/or HCC. Ultimately, however, 1-2 million HBV-related deaths are reported annually[72].

The primary treatment of CHB is based on two kinds of drugs currently: pegylated IFN-α and nucleoside/nucleotide analogues[73]. Nevertheless, complete HBV eradication, with clearance of the covalently closed circular DNA, is rarely achieved[18], making it necessary to develop new effective therapies for this major public health problem. The potential benefit of immunotherapy in HBV is highlighted by several important aspects of the infection itself. Firstly, in subjects who spontaneously clear the HBV infection, viral control is determined by the development of a strong, polyclonal and multi-specific CD8$^+$ T cell response[5-7]; this usually happens during adulthood, when nearly 90% of the infected subjects are able to control the virus and in contrast to individuals (children) who obtain the virus through vertical transmission and in who the HBV persists due to immunotolerance induction[74]. Secondly, CHB resolution occurs in some bone marrow transplantation recipients who received tissue from a donor with natural immunity to HBV[75]. Finally, those individuals with natural immunity to HBV maintain immunological memory through HBV-specific CD8$^+$ T cells that can last decades after the primo-infection that is capable of controlling virus at trace amounts[76]. Nevertheless, in chronically infected patients, the HBV-specific CD8$^+$ T cell response is weak (barely detectable) and exhausted in both the peripheral blood and the liver[15,16], and this feature is accompanied by up-regulated expression of negative co-stimulatory molecules, as will be discussed in the following paragraphs. Thus, taking into account these facts, immunotherapy based on modulation of the co-stimulatory pathway could be a promising approach to improve HBV chronic infection treatment.

IMMUNE CHECKPOINTS

The cytotoxic T cell response is essential to eradication of tumoural and virus-infected cells. In patients with chronic viral hepatitis and HCC this response is impaired and, theoretically, its restoration could help in disease control. As previously commented, one of the strategies used by tumoural and virus-infected cells to induce exhaustion of the CD8$^+$ T cells is up-regulation of negative co-stimulatory molecules. Therefore, therapeutic blockade of various inhibitory receptors, a process also referred to as "checkpoint blockade", has begun to provide very promising results in the treatment of different diseases; these will be summarised hereafter.

The first proof of concept of the efficacy of this kind of treatment was reported for "ipilimumab", a human monoclonal antibody against the negative co-stimulatory molecule CTLA-4 that was approved by the United States' Food and Drug Administration in 2011; this drug is currently in clinical use for treating metastatic melanoma[77]. Since its introduction, this antibody (and others) against different co-stimulatory molecules has entered testing for other malignancies and various viral infections. There are several completed, on-going and planned clinical trials for investigating treatment of chronic hepatitis and HCC with single-agent inhibitors, as well as with combinations of inhibitors targeting multiple checkpoints or adding other therapies to this blockade. In the next lines, we will review the mechanism of action of these immune checkpoints, and the effect of blockade as determined in pre-clinical and clinical studies.

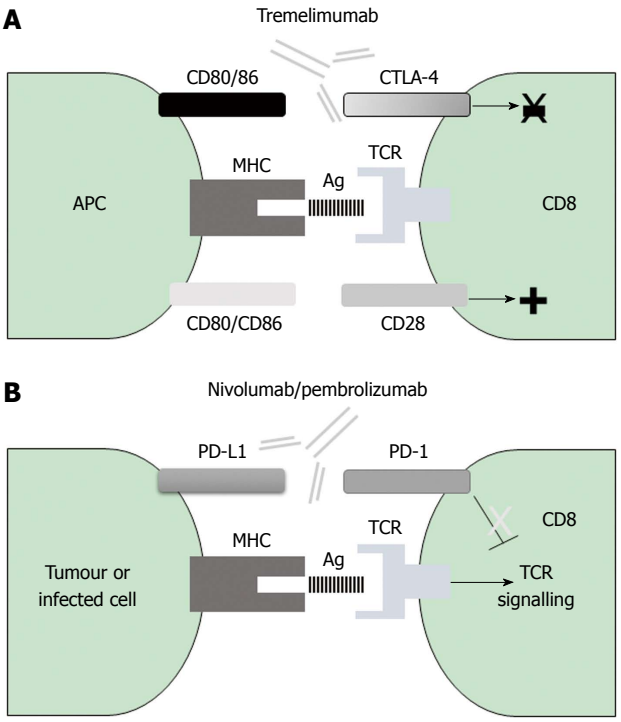


Figure 2 Mechanisms of action of negative co-stimulatory pathways involved in current immunotherapy for cytotoxic T cells. A: The CTLA-4 immune checkpoint impairs early T cell activation; the inhibitory CTLA-4 molecule binds with higher affinity to the CD80/CD86 ligands on antigen presenting cells and prevents their binding to the positive co-stimulatory molecule CD28, with the consequence being a decrease in T cell activation. B: The PD-1 negative receptor on T cells interacts with either PD-L1 or PD-L2 on the surface of the infected or tumoural cell, thereby promoting T cell exhaustion; blockade of either CTLA-4 or PD-1 with humanized monoclonal antibodies releases the break that is exerted by these molecules and results in T cell activation. CTLA-4: Cytotoxic T-lymphocyte antigen-4; MHC: Major histocompatibility complex; PD-1: Programmed cell death protein-1; TCR: T cell receptor.

Table 1 Clinical trials of immune checkpoint inhibitors in patients with chronic hepatitis and hepatocellular carcinoma

NCT	Status	Disease	Agent	Result	Ref.
01008358	Completed	HCC and CHC	Tremelimumab	TTP: 6.48 m	[30]
01853618	Recruiting	HCC	Tremelimumab	TTP: 7.4 m	[111]
00703469	Completed	CHC	Nivolumab	15% significant reduction of viral load	[31]
02658019	Not yet recruiting	HCC	Pembrolizumab	NA	[112]
01658878	Recruiting	HCC	Nivolumab Ipilimumab	NA	[113]

Tremelimumab and ipilimumab are humanized monoclonal antibodies against CTLA-4; Nivolumab and pembrolizumab are humanized monoclonal antibodies against PD-1. HCC: Hepatocellular carcinoma; NA: Not available; NCT: Clinical trial number at clinicaltrials.gov; TTP: Time to progression.

CTLA-4

Mechanism of action of CTLA-4: Since its discovery in 1987[78], research has determined that CTLA-4 is expressed only on T cells, where it regulates early immune activation. This negative co-stimulatory molecule counteracts the activity of the positive co-stimulatory molecule CD28[79-82]. After antigen encounter, the CD28 co-signal triggers the T cell receptor (TCR) signal that activates T cells[83]. CTLA-4 and CD28 share the same APC-expressed ligands, namely CD80 and CD86. CTLA-4 displays at least two different ways by which it can inhibit T cell activation; in the first, it inhibits positive signalling of CD28 according to the feature that CTLA-4 has more affinity for CD80 and CD86 than the positive co-stimulatory molecule CD28[84] and in the second, CTLA-4 directly inhibits TCR signalling[85,86] (Figure 2).

The central function of CTLA-4 is regulation of the access of CD28 to its shared ligands in order to protect against autoimmunity and to switch-off a normal immune response after antigen control has been achieved. The vital importance of CTLA-4 was demonstrated in two different studies that were based on Ctla-4 knock-out mice. The CTLA-4-deficient mice present a profound immune dysregulation and autoimmune disease that leads to massive lympho-proliferation and fatal multi-organic tissue destruction[87,88]. Nevertheless, the inadequate induction of CTLA-4 under viral infection and tumour conditions and disrupted effects on specific CD8$^+$ T cells could favour early exhaustion of these cells and consequently allow persistence of the disease.

Thus, considering the known mechanism of action of CTLA-4, enhancement of the CD8$^+$ T cell response by CTLA-4 blockade could represent a satisfactory approach to treating diseases that feature persistent antigenemia, such as viral hepatitis and HCC.

CTLA-4 blockade (pre-clinical): Nakamoto *et al*[27] discovered that CTLA-4 is overexpressed in PD-1$^+$ intrahepatic mononuclear cells of patients with CHC (Table 1). In addition, when the authors blockaded these inhibitory receptors individually they found no restoration of intrahepatic HCV-specific CD8$^+$ T cell response. Surprisingly, however, when they blocked both inhibitory receptors simultaneously, the effector ability of these cells was restored, indicating the existence of a synergic effect between both receptors[27]. CTLA-4 blockade alone, however, could be sufficient to restore specific cytotoxic T cell response in persistent HBV infection. Schurich *et al*[28] showed that CTLA-4 blockade is able to restore the expansion ability of HBV-specific CD8$^+$ T cells in both the intrahepatic and peripheral compartments of patients with CHB. It is unfortunate, though, that to date the research in HCC has only involved *in vitro* investigations of the blockade of CTLA-4 in peripheral blood mononuclear cells from HCC patients and that the results have shown no restoration of the ability of IFN-γ secretion by GPC-3-specific CD8$^+$ T cells[29]. However, a study of a mouse model carried out by

Leach *et al*[89] showed that *in vivo* administration of antibodies against CTLA-4 can result in regression of certain types of tumours, specifically those that are more immunogenic. These last data are consistent with the results obtained in a recent clinical trial that will be commented on later in this review.

PD-1

Mechanism of action of PD-1: PD-1 was first identified in 1992 by Ishida *et al*[90] as a negative co-stimulatory molecule that belongs to the CD28 immunoglobulin superfamily of transmembrane proteins[91]. PD-1 is inducibly expressed on T cells, B cells and monocytes, upon their activation[92]. The PD-1 ligands, PD-L1 and PD-L2, are members of the B7 co-stimulatory molecules family[93,94]. APCs and non-lymphoid tissues, including the liver, express PD-L1, while DCs and macrophages can up-regulate PD-L2 expression[34,44,94-97] (Table 1). Interaction of PD-1 with its ligands leads to inhibition of proliferation through a cell cycle arrest at G_0/G_1 and also impairs IL-2 secretion by T cells[98]. In addition, interaction between PD-1 and PD-L1 or PD-L2 promotes apoptosis and secretion of the immunosuppressive cytokine IL-10[99-101] (Figure 2).

The immunoregulatory properties of PD-1 are reflected by the Pdl-1 knockout mouse model, which presents with severe autoimmune disease[102]. Thus, PD-1 is considered to play an important role in controlling the cellular immune response and in switching-off cells after they have completed their tasks in order to avoid autoimmune disorders; yet, its early expression can induce T cell exhaustion. Several studies have reported a positive correlation between exhaustion and PD-1 up-regulation[24,103,104]. Therefore, the blockade of PD-1 and its ligands could represent an efficient therapeutic approach by which to restore an effector T cell response against HCC and viral hepatitis.

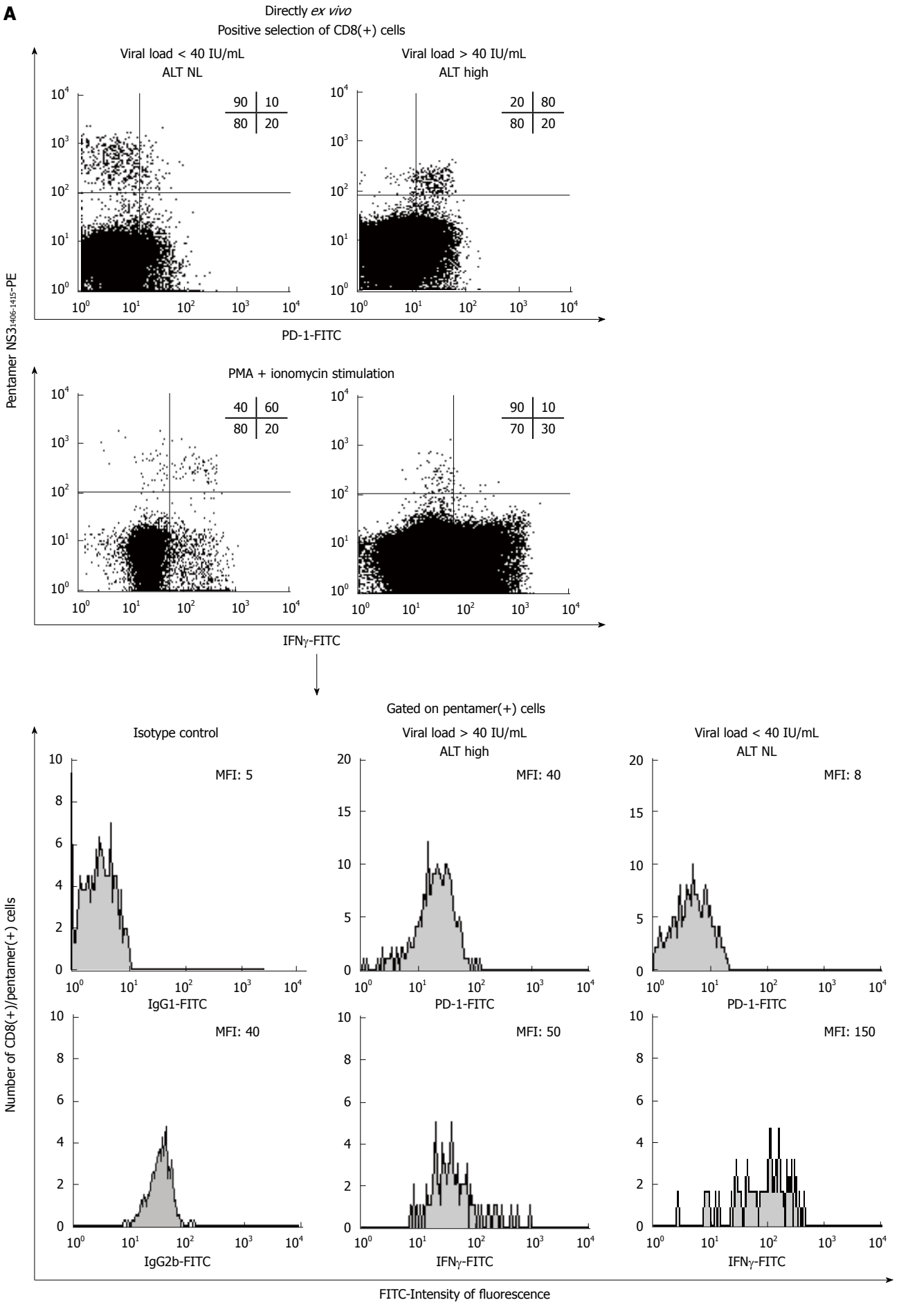
PD-1/PD-L1 blockade (pre-clinical): Several studies have been carried out to evaluate the effect of blocking the PD-1/PD-L1 pathway under conditions of viral hepatitis and HCC. Studies focusing on CHC, by Penna *et al*[105] and Radziewicz *et al*[106], along with data obtained by our own group, have shown that up-regulation of PD-1 affects HCV-specific CD8$^+$ T cells in peripheral blood and in the intrahepatic compartment during chronic HCV infection (Table 1). Besides, blockade of the PD-1/PD-L1 interaction was shown to improve the expansion ability of and IFN-γ secretion from HCV-specific CD8$^+$ T cells[24,105,106]. Moreover, Fuller *et al*[107] elegantly showed how PD-1 blockade could control HCV replication in a chimpanzee model of CHC. Interestingly, the chimpanzee with controlled infection also presented a broader base-line immunity response than the cohort animals that were non-responders, suggesting that anti-PD-1 treatment may be useful in only those cases with a critical threshold of pre-existing HCV-specific CD8$^+$ T cells.

Figure 3 shows an example of HCV-specific CD8$^+$ T cell restoration achieved by use of anti-PD-1 monoclonal antibody. In a study of CHB infection carried out by Peng *et al*[25], PD-1 up-regulation on HBV-specific CD8$^+$ T cells was shown in blood samples of patients with chronic infection *vs* the controls (Table 1); moreover, blockade of the PD-1/PD-L1 pathway was shown to significantly enhance the expansion ability of and the IFN-γ production from HBV-specific CD8$^+$ T cells after antigen encounter[25]. Fisicaro *et al*[108] further demonstrated that HBV-specific CD8$^+$ T cells from the mononuclear cell population of livers infected with hepatitis virus express higher levels of PD-1 than those in the peripheral compartment; additionally, blockade of the PD-1/PD-L1 pathway was shown to improve the effector capacity of these intrahepatic cells, as evidenced by measure of their expansion ability and production of the Tc1 cytokines, such as IL-2 and IFN-γ[108]. Tzeng *et al*[109] studied T cell exhaustion affecting the intrahepatic infiltrating T cells, using a mouse model of persistent HBV infection; the authors discovered that PD-1 was up-regulated on the HBV-specific CD8$^+$ T cells and that blockade of the interaction between PD-1 and PD-L1 results in restoration of the capacity of these cells to produce IFN-γ[29]. Furthermore, Gao *et al*[26] showed that overexpression of the PD-1 ligand, PD-L1, in HCC is associated with tumour aggressiveness, providing the rationale for developing a new therapy based on blockade of the PD-1/PD-L1 pathway (Table 1). Finally, Shi *et al*[110] demonstrated that the HCC-infiltrating CD8$^+$ T cells have a drastic increase in PD-1 expression. Taken together, these data support the rationale to set-up clinical trials to analyse the usefulness of blocking the PD-1/PD-L1 pathway in HCC and HBV/HCV chronic infections.

Checkpoint inhibitors blockade: Clinical trials

Taking into account all the previously discussed known characteristics and features of the specific CD8$^+$ T cell response in HCC and chronic viral hepatitis, a number of clinical trials have been designed to analyse the effect of PD-1 and CTLA-4 pathway blocking (Table 2). Here, we will describe the completed, on-going and planned clinical trials investigating checkpoint modulation as a therapeutic approach for treating HCC and chronic viral hepatitis.

Regarding CTLA-4, Sangro *et al*[30] performed the first pilot clinical trial to address the potential anti-tumour and anti-viral effects of a monoclonal anti-CTLA-4 antibody in CHC and HCC. For this purpose, the authors used "tremelimumab", a fully humanized IgG2 monoclonal antibody that blocks CTLA-4. The study cohort was a small population of patients with HCC superimposed on CHC. Tremelimumab showed an acceptable safety profile, as well as both anti-tumour and anti-viral activities. The anti-tumour effect was particularly encouraging as it was characterized by a


A
Directly *ex vivo*
Positive selection of CD8(+) cells
Viral load < 40 IU/mL
ALT NL
Viral load > 40 IU/mL
ALT high
90 10
80 20
20 80
80 20
Pentamer NS3$_{1406-1415}$-PE
PD-1-FITC
PMA + ionomycin stimulation
40 60
80 20
90 10
70 30
IFNγ-FITC
Gated on pentamer(+) cells
Isotype control
MFI: 5
Viral load > 40 IU/mL
ALT high
MFI: 40
Viral load < 40 IU/mL
ALT NL
MFI: 8
IgG1-FITC
PD-1-FITC
PD-1-FITC
MFI: 40
MFI: 50
MFI: 150
IgG2b-FITC
IFNγ-FITC
IFNγ-FITC
Number of CD8(+)/pentamer(+) cells
FITC-Intensity of fluorescence

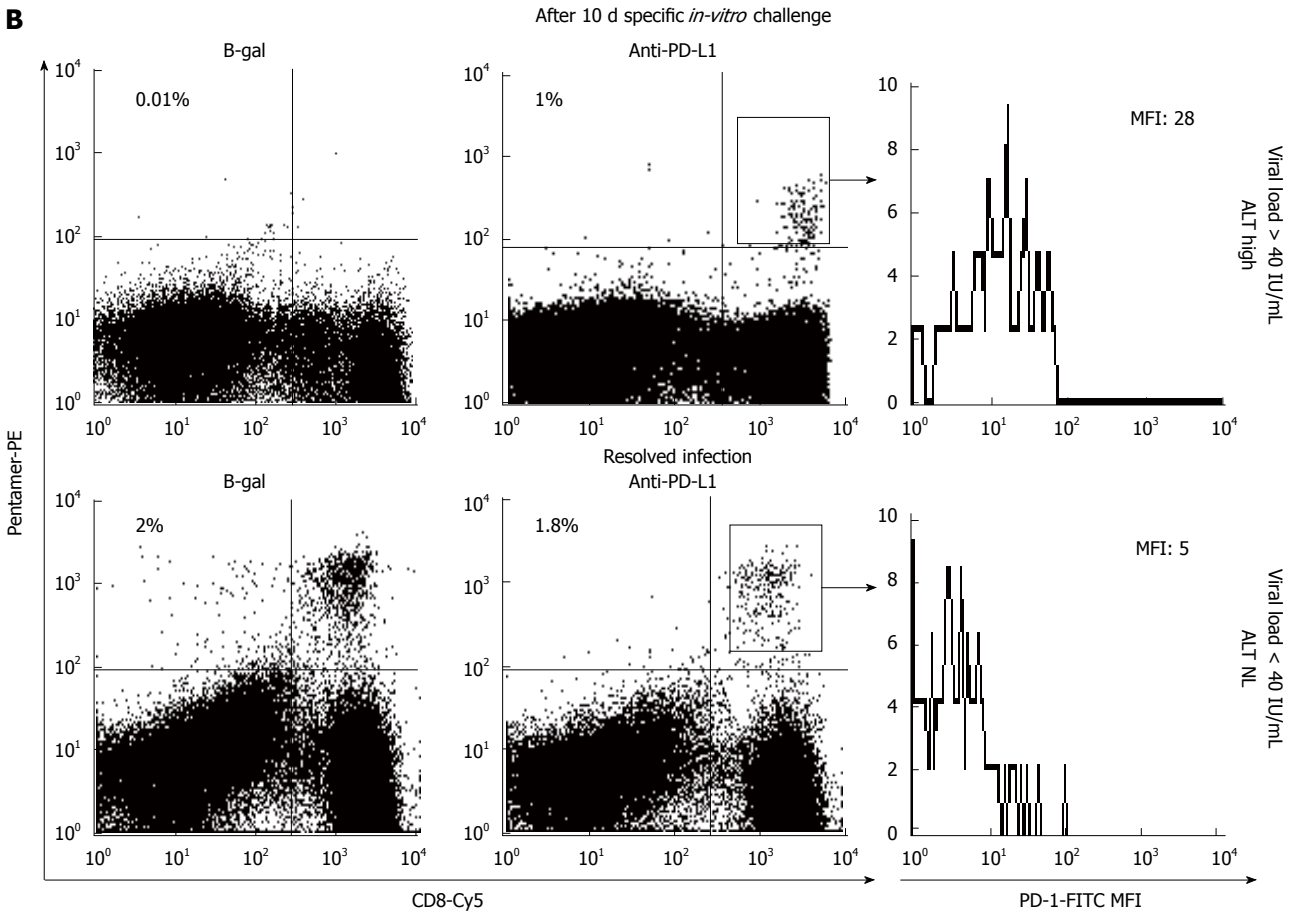


Figure 3 PD-1/PD-L1 blockade-induced restoration *in vitro* of hepatitis C virus-specific CD8$^+$ T cells from a patient with chronic hepatitis C virus infection. A: FACScan® dot-plots directly gated on *ex vivo* CD8$^+$ T cells showing the PD-1 phenotype and the interferon (IFN)-γ secretion of HCV-NS3$_{1406}$-pentamer-binding CD8$^+$ T cells from a patient with chronic HCV infection and a patient who was able to resolve the infection; B: FACScan® dot-plots showing the frequency of HCV-NS3$_{1406}$-pentamer-binding CD8$^+$ T cells after a 10-d specific *in vitro* challenge in the presence and absence of anti-PD-L1 to block the interaction between PD-1 and its ligand in the patients from (A) with chronic HCV infection and who resolved the infection; PD-1 expression on gated HCV-NS3$_{1406}$-pentamer-binding CD8$^+$ T cells after expansion is also shown. ALT: Alanine aminotransferase; HCV: Hepatitis C virus.

Table 2 Overexpressed negative pathways in liver diseases

Disease	Overexpressed NR	Ligand for the overexpressed NR
HCC	CTLA-4[29], PD-1[12]	PD-L1[26], CD48[135]
CHB	CTLA-4[28], PD-1[25], LAG-3[117], Tim-3[129]	PD-L1[136]
CHC	CTLA-4[27], PD-1[24,105,106], LAG-3[116], 2B4[13,122], Tim-3[128]	PD-L1[44]

Overexpression of negative receptors on specific CD8$^+$ T cells in different malignancies and overexpression of their cognate ligands in the liver tissue. CHB: Chronic hepatitis B; CHC: Chronic hepatitis C; HCC: Hepatocellular carcinoma; NR: Negative receptor.

significant increase in the time to progression (TTP), up to 6.48 mo, with respect to the control group. For its anti-viral activity, the drug was shown to induce a significant decrease in viral load, with 15% of the patients achieving sustained virological response without any other anti-HCV treatment. This anti-viral efficacy may appear low, but it could represent a difference in patients who are non-responders to DAA, in who a DAA + anti-PD-1 treatment combination could improve the sustained virologic response rate. Moreover, these outcomes may be improved by use of a higher dose of the drug[30].

Other studies with the same anti-CTLA-4 molecule are currently in progress. Greten *et al*[111], for example, are currently recruiting patients to participate in their clinical trial to test the safety and effectiveness of tremelimumab in combination with transarterial chemoembolization or RFA in advanced liver cancer. These authors presented their preliminary results during the 2015 American Society of Clinical Oncology Annual Meeting, stating that the combination was safe, feasible and effective, increasing TTP up to 7.4 mo[18].

Similar to the results of the studies on CTLA-4, the current research data from the studies of PD-1/PD-L1 blockade are encouraging. Gardiner *et al*[31] described, for the first time and as proof-of-concept, their findings from an evaluation of anti-PD-1 antibody treatment in patients with CHC, in which the fully human anti-PD-1 monoclonal IgG4 antibody "nivolumab" was administered as a single dose. The treatment did not produce any significant side effects, indicating a suffi-

cient safety, and one-third of the patients experienced significant reductions in viral load. Perhaps, if the authors had combined blockade of the PD-1 pathway with modulation of other co-stimulatory molecules, they could have achieved better results. However, the importance of this pilot clinical trial is that it highlights the significant role of the PD-1 pathway during CHC[31] and shows that it could represent an adjuvant treatment for DAA regimes.

Feun *et al*[112] have already designed a clinical trial to determine the anti-tumour effect of anti-PD-1 antibody treatment in patients with advanced, unresectable HCC using another humanized monoclonal IgG4 antibody that binds to PD-1, which is called "pembrolizumab". The primary objective of this study is to assess therapeutic efficacy, but the researchers also plan to evaluate the expression of PD-L1 in tumour tissue, with the aim of gaining insights into which cases may benefit most from this type of treatment.

After realising that blocking either CTLA-4 or PD-1 pathways could be useful to treat HCC, it becomes apparent that one option to increase anti-tumoural effectiveness could be a treatment combination. A clinical trial is currently in progress to study such an alternative, namely the role of combining the fully humanized anti-CTLA-4 IgG1 antibody called "ipilimumab" in conjunction with the previously introduced anti-PD-1 "nivolumab"[113]. The first part of that study will evaluate the safety profile of nivolumab in HCC patients, after which the efficacy of nivolumab will be compared with that of sorafenib. Finally, the researchers plan to address the safety and efficacy profiles of the combination nivolumab and ipilimumab for treatment of advanced HCC[86]. In the near future, hepatologists and oncologists should know if this hopeful combination represents a *bona fide* treatment option for patients with advanced HCC.

Other inhibitory receptors

Although CTLA-4 and PD-1 are the best characterized inhibitory receptors to date, there are other negative co-stimulatory molecules involved in CD8$^+$ T cell exhaustion that deserve to be studied. Most of the work carried out on the role of these other co-stimulatory molecules has been done in the conditions of HVB and HCV infections, while information in the condition of HCC remains scarce.

LAG-3

LAG-3 was identified in 1990 by Triebel *et al*[114]. Even though its main ligand is MHC class Ⅱ, it also helps PD-1 to maintain CD8$^+$ T cell exhaustion during chronic viral infections[115]. Chen *et al*[116] demonstrated that LAG-3 was overexpressed on HCV-specific CD8$^+$ T cells in patients with CHC and that LAG-3 blockade restored effector capacity of these cells, as evidenced by measure of their expansion ability and cytokine production (Table 1). Kennedy *et al*[117] demonstrated different expression patterns of LAG-3 on HBV-specific CD8$^+$ T cells during the natural history of CHB, whereby LAG-3 up-regulation occurs progressively, suggesting that HBV infection induces a progressive status of T cell exhaustion over time. Li *et al*[118] studied LAG-3 expression in patients with HBV-related HCC and discovered an overexpression of LAG-3 on the HBV-specific CD8$^+$ T cells from liver tissue, as compared with those in the peripheral blood. Taking these collective data into account, LAG-3 appears to be another immune checkpoint to bear in mind during consideration of diseases affected by T cell exhaustion.

Natural killer cell receptor (2B4)

The 2B4 was first identified in 1993 by Valiante *et al*[119]. Its ligand, CD48, has 5-10 times stronger affinity for 2B4 than CD2, a molecule necessary for T cell activation[120,121]. This competitive advantage of 2B4 for binding to CD48 could impair T cell activation if 2B4 is induced early. Bengsch *et al*[13] and Schlaphoff *et al*[122] studied 2B4 expression on HCV-specific CD8$^+$ T cells in the blood of patients with chronic hepatitis infections and demonstrated overexpression in PD-1$^+$-HCV-specific CD8$^+$ T cells, which was further linked to an exhausted behaviour. Similarly, Kroy *et al*[123] showed an up-regulation of 2B4 in HCV-specific CD8$^+$ intrahepatic lymphocytes, as compared with peripheral lymphocytes, during CHC. Bengsch *et al*[13] studied the effect of blockade of several inhibitory receptors, including 2B4, in the functionality of HBV-specific CD8$^+$ T cells during CHB and found that the response to blockade was primarily mediated by PD-1 (Table 1). Considering all of these data, 2B4 up-regulation seems to be linked with PD-1 overexpression, and its modulation could have a less robust effect on T cell restoration than other negative co-stimulatory molecules.

T-cell immunoglobulin and mucin-domain containing-3

T-cell immunoglobulin and mucin-domain containing (Tim)-3 was first identified in 2002 by Monney *et al*[124]. Tim-3 is another immune checkpoint receptor that limits the duration and magnitude of T cell responses[125-127]. McMahan *et al*[128] demonstrated that Tim-3 was up-regulated on HCV-specific CD8$^+$ T cells in patients with chronic hepatitis infection, as compared to those who had been able to control the infection; in addition, remarkably, Tim-3 blockade restored the expansion ability of these cells. Wu *et al*[129] studied Tim-3 expression on HBV-specific CD8$^+$ T cells during CHB and discovered that Tim-3 overexpression was related to disease progression; the authors also demonstrated that blockade of the Tim-3 pathway restored the effector capacity of the HBV-specific CD8$^+$ T cells[130] (Table 1). Interestingly, a lack of Tim-3 has thus far not been found to be associated with autoimmune diseases[131], making Tim-3 a very attractive therapeutic target.

CD160

CD160 was discovered in 1998 by Anumanthan *et al*[132] and is another negative co-stimulatory molecule associated with T cell exhaustion[133]. Bengsch *et al*[13] and Schlaphoff *et al*[122] also studied CD160 expression on HCV-specific CD8$^+$ T cells obtained from patients with chronic hepatitis infections and demonstrated an overexpression of this protein that was related with exhaustion. Nevertheless, Kroy *et al*[123] showed that there was no overexpression of this molecule in intrahepatic HCV-specific CD8$^+$ T cells in CHC, as compared to the peripheral cells. Another study from Viganò *et al*[134] investigated the expression of CD160 on HCV-specific CD8$^+$ T cells during CHC and found functional impairment in these cells, which was independent of PD-1 expression; moreover, the blockade of CD160/CD160L in this study restored the expansion ability of the HCV-specific CD8$^+$ T cells[134] (Table 1). Nevertheless, due to the current contradictory data more studies are needed to gain an adequate understanding of this pathway before it can be considered as a potential therapeutic target.

CONCLUSION

Immune checkpoint modulation can resolve CD8$^+$ T cell exhaustion *in vitro* and *in vivo* in chronic infections with hepatoviruses and in tumoural diseases. This therapy could represent a promising treatment option for patients with advanced HCC and chronic viral hepatitis who are non-responders to the current standard of care. The use of humanized monoclonal antibodies against negative co-stimulatory molecules can reverse the exhaustion state of CD8$^+$ T cells, thereby making them able to control either the tumour or the virus. In the upcoming years, we will likely see the results of combining these agents with current HCC and anti-viral therapies. While the current results from clinical studies remain modest, they could be the base for encouraging development and implementation of novel therapeutic strategies. Clearly, we must expand our basic and clinical knowledge about how modulating and finely tuning these receptors will help to avoid adverse events, and how appropriate patient selection will help to define the groups most likely to respond to this kind of treatment. We must also learn how to best use these drugs in combination with other therapies in order to obtain the maximum clinical benefit. Fortunately, the field of immunotherapy highlights the fact that translational medicine can improve the health of our patients, bringing the results obtained by basic research to the bedside in a short period.

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REVIEW

Prevention and management of hepatitis B virus reactivation in patients with hematological malignancies treated with anticancer therapy

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Abstract

Hepatitis due to hepatitis B virus (HBV) reactivation can be severe and potentially fatal, but is preventable. HBV reactivation is most commonly reported in patients receiving cancer chemotherapy, especially rituximab-containing therapy for hematological malignancies and those receiving stem cell transplantation. All patients with hematological malignancies receiving anticancer therapy should be screened for active or resolved HBV infection by blood tests for hepatitis B surface antigen (HBsAg) and antibody to hepatitis B core antigen (anti-HBc). Patients found to be positive for HBsAg should be given prophylactic antiviral therapy to prevent HBV reactivation. For patients with resolved HBV infection, no standard strategy has yet been established to prevent HBV reactivation. There are usually two options. One is pre-emptive therapy guided by serial HBV DNA monitoring, whereby antiviral therapy is given as soon as HBV DNA becomes detectable. However, there is little evidence regarding the optimal interval and period of monitoring. An alternative approach is prophylactic antiviral therapy, especially for patients receiving high-risk therapy such as rituximab, newer generation of anti-CD20 monoclonal antibody, obinutuzumab or hematopoietic stem cell transplantation. This strategy may effectively prevent HBV reactivation and avoid the inconvenience of repeated HBV DNA monitoring. Entecavir or tenofovir are preferred over lamivudine as prophylactic therapy. Although there is no well-defined guideline on the optimal duration of prophylactic therapy, there is growing evidence to recommend continuing prophylactic antiviral therapy for at least 12 mo after cessation of chemotherapy, and even longer for those who receive rituximab or who had high serum HBV DNA levels before the start of immunosuppressive

therapy. Many novel agents have recently become available for the treatment of hematological malignancies, and these agents may be associated with HBV reactivation. Although there is currently limited evidence to guide the optimal preventive measures, we recommend antiviral prophylaxis in HBsAg-positive patients receiving novel treatments, especially the Bruton tyrosine kinase inhibitors and the phosphatidylinositol 3-kinase inhibitors, which are B-cell receptor signaling modulators and reduce proliferation of malignant B-cells. Further studies are needed to clarify the risk of HBV reactivation with these agents and the best prophylactic strategy in the era of targeted therapy for hematological malignancies.

Key words: Hepatitis B virus reactivation; Hematological malignancies; Rituximab; Hematopoietic stem cell transplant; Prophylactic antiviral therapy

Core tip: Hepatitis due to hepatitis B virus (HBV) reactivation can be severe and potentially fatal. All patients with hematological malignancies receiving anticancer therapy should be screened for hepatitis B surface antigen (HBsAg) and antibody to hepatitis B core antigen. Patients found to be positive for HBsAg should be given prophylactic antiviral therapy. For patients with resolved HBV infection, either preemptive therapy guided by serial HBV DNA monitoring or prophylactic antiviral therapy, especially for patients receiving high-risk therapy are reasonable options. Further studies are needed to find out the best prophylactic strategy in the era of targeted therapy for hematological malignancies.

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INTRODUCTION

Patients infected with hepatitis B virus (HBV) are at risk of reactivation of the virus if they receive chemotherapy, even if they have chronic or resolved HBV infection. HBV reactivation is most commonly reported in patients receiving cancer chemotherapy for haematological malignancies or haemopoietic stem cell transplantation (HSCT) recipients, although it has also been reported in patients receiving treatment for solid tumours like breast cancer.

The first few reports of hepatitis B reactivation were in patients with lymphoma[1], but the highest risk of HBV reactivation is seen with the potent anti-CD20 monoclonal antibody, rituximab, which came into use in the last two decades and results in profound B-cell depletion.

The newer generation of anti-CD20 monoclonal antibody, obinutuzumab, will induce a more profound B-cell depletion than rituximab does, and the risk of HBV reactivation is expected to be high. Similarly, the newer targeted therapies, such as the Bruton's tyrosine kinase (BTK) inhibitors and phosphatidylinositol 3-kinase (PI3K) inhibitors, which have recently proven to be successful in a number of hematological malignancies[2-12], may also potentially cause HBV reactivation because they block B-cell antigen receptor signalling and reduce malignant proliferation of B-cells. The increasing use of proteasome inhibitors in multiple myeloma and hypomethylating agents in elderly patients with acute myeloid leukemia should arouse caution on the risk of HBV reactivation in these hematological malignancies.

The initiating factor of HBV reactivation is thought to be loss of immune control over viral replication. During chemotherapy, when the immune system is suppressed, HBV replicates dramatically and the viral load increases causing widespread infection of hepatocytes. When chemotherapy is stopped and immune function is restored, liver cells containing HBV may produce a strong immune-mediated reaction causing liver damage[13].

The clinical manifestations of HBV reactivation range from asymptomatic self-limiting anicteric hepatitis to potentially fatal, severe liver failure. Deranged liver function due to HBV reactivation may also lead to a delay or interruption of the chemotherapy regimen, which is likely to increase the risk of morbidity and mortality associated with the underlying malignancy.

Most of the data of HBV reactivation in cancer patients come from lymphoma patients receiving chemotherapy and rituximab. This article describes the risk and clinical course of HBV reactivation in patients with lymphoma, as well as those with other hematological malignancies such as multiple myeloma or acute leukemia, or receiving the novel agents used in hematological malignancies. We will also discuss the choice of antiviral agent for the prevention of HBV reactivation and duration of antiviral prophylactic therapy and management of HBV reactivation.

DEFINITIONS OF HBV REACTIVATION AND CLINICAL MANIFESTATIONS

Approximately 350 million people worldwide are chronically infected with HBV. All patients with HBV infection develop anti-HBc and the antibody persists after clearance of HBsAg. Therefore, anti-HBc is a good marker of current as well as past infection with HBV. Anti-HBs can be present due to previous infection or successful hepatitis B vaccination. The presence of anti-HBc without HBsAg or anti-HBs indicates resolved

HBV infection, but patients with this marker are still at risk of HBV reactivation and a fatal outcome. Although HBV DNA is rarely found in peripheral blood after the HBV infection has resolved, trace amounts are often found within the liver and can be activated when the immune response is suppressed.

HBV reactivation is diagnosed by a marked increase or de novo appearance of HBV DNA in serum[14]. Reactivation of HBV replication is defined as a marked increase in HBV replication [⩾ 2log increase (100 fold) from baseline levels or a new appearance of HBV DNA to a level of ⩾ 100 IU/mL] in a person with previously stable or undetectable levels of HBV DNA[15]. Newer and more sensitive HBV DNA assays have increased the number of patients who can be diagnosed with HBV reactivation[16].

HBV reactivation can be described as exacerbation of chronic hepatitis B or reactivation of past hepatitis B infection. The latter can be further defined as reverse HBsAg seroconversion (reappearance of HBsAg), or appearance of HBV DNA in serum in the absence of HBsAg.

The course of HBV reactivation has three phases[15,17]. During the first phase, there is an increase in levels of HBV DNA in the serum of an HBsAg-positive person or a reappearance of HBsAg or HBV DNA in serum in a person who was previously HBsAg-negative or had undetectable serum HBV DNA, respectively. Symptoms of hepatitis are usually absent and levels of liver enzymes, *e.g.,* alanine aminotransferase (ALT), are not elevated.

During the second phase, serum HBV DNA levels continue to increase, and there is an increase in ALT levels, with or without symptoms of acute hepatitis. Hepatic injury may progress in some patients, causing liver failure and even death. These changes in the second phase may occur between chemotherapy administrations or after cessation of chemotherapy, and may result from reconstitution of the host immune response[18].

In the third phase, hepatic injury resolves either spontaneously or as a result of withholding immunosuppressive therapy or initiation of antiviral therapy.

HBV reactivation can occur at any time during or after chemotherapy. It typically occurs after the second or third courses of chemotherapy for patients undergoing treatment for lymphoma. In patients receiving rituximab for the treatment of non-Hodgkin's lymphoma, HBV reactivation may occur after a median of six doses and up to 12 mo after the last dose of rituximab[19].

HBsAg-negative/anti-HBc-positive patients who receive allogeneic HSCT are at risk of developing HBV reactivation for several years after transplantation because of the long delay in reconstituting the recipient's immune response to HBV[20].

RISK FACTORS FOR HBV REACTIVATION

Underlying diseases

HBV reactivation is most commonly reported in patients with lymphoma, but it is unclear whether lymphoma itself increases the risk of HBV reactivation because there are no studies comparing the risk in patients with other diseases receiving similar chemotherapeutic regimens. The frequent association between lymphoma and HBV reactivation might be related to the intensity of the chemotherapy regimen, resulting in marked immunosuppression. Alternatively, it might also be related to a higher prevalence of HBV infection among lymphoma patients[21-25].

Deng *et al*[26] recently showed that in HBsAg-positive diffuse large B-cell lymphoma (DLBCL) patients, the majority (96%) of patients' amino acid sequences of heavy- and light-chain complementarity-determining region 3 exhibited a high homology to antibodies specific for HBsAg, and 90% of *IgHV* and *IgLV* genes were mutated. This suggests that HBV-associated DLBCL might arise from HBV antigen-selected B-cells.

Although most early reports of HBV reactivation were in patients with lymphoma, more data on HBV reactivation have recently emerged in patients with other hematological diseases like multiple myeloma. Multiple myeloma is the second most common hematological malignancy. HBV reactivation has been reported in patients who are HBsAg-positive and in those who are HBsAg-negative/anti-HBc-positive[27-30]. Moreover, severe immune dysfunction associated with advanced myeloma may also predispose myeloma patients to virus reactivation[31].

Mya *et al*[27] investigated the incidence of hepatitis B reactivation in 273 patients with multiple myeloma undergoing high-dose therapy followed by autologous stem cell transplant (HDT-ASCT) and treatment with novel agents. Patients were screened for the presence of HBsAg and anti-HBc. The prevalence of HBV infection was 5.5%, including three cases of HBV reactivation despite lamivudine prophylaxis. Of the three patients with HBV reactivation, two developed reactivation 3 to 5 mo after HDT-ASCT while receiving thalidomide maintenance, and one reactivated 3 years after HDT-ASCT followed by bortezomib salvage therapy.

Another study by Li *et al*[30] analyzed 139 myeloma patients. HBsAg-positive patients underwent prophylactic therapy before starting immunosuppressive therapy, and the incidence of HBV reactivation was 22.1%. This high incidence of HBV reactivation is believed to be due to the use of bortezomib and/or treatment with ASCT.

The risk of HBV reactivation is significant in patients with acute myeloid leukemia (AML) receiving chemotherapy, with an incidence similar to that in

patients with lymphoma. A recent study by Chen *et al*[32] analysed 490 AML patients and found that the incidence of HBV reactivation and HBV-related hepatitis were 9.5 and 8.3 per 100 person-years, respectively, in AML patients who are also chronic hepatitis B carriers. This is similar to the incidence of HBV reactivation in lymphoma patients. Prophylaxis with anti-HBV agents significantly decreased the risk of hepatitis B reactivation among HBV carriers (13% *vs* 61%, $P < 0.001$). Since fulminant hepatitis B is a catastrophic event for AML patients infected with HBV[33-35], periodic assessment of liver function and HBV serological status or prophylactic antiviral therapy is important during chemotherapy. Further prospective studies of patients with AML would be useful to assess the true incidence of HBV flare-ups and the best prophylactic strategy.

HBV reactivation is common in the setting of HSCT as a result of profound immunosuppression, the use of multiple immunosuppressive agents for allogeneic transplantations and substitution of the preexisting immune system with one that has not been exposed to HBV in the past[14].

The risk is greatest among patients undergoing allogeneic HSCT because of the requirement for high-dose conditioning chemotherapy, and the profound immunosuppression and prolonged use of immunosuppressive agents to prevent the development of graft-*vs*-host-disease after HSCT. Patients undergoing autologous HSCT receive high-dose chemotherapy like high-dose melphalan in multiple myeloma or BEAM [BCNU (carmustine), etoposide, cytarabine, melphalan] in relapsed or refractory lymphoma. The risk of HBV reactivation in autologous HSCT is similar to that in patients undergoing intensive chemotherapy.

HBV reactivation is not uncommon in HBsAg-negative/anti-HBc positive patients undergoing HSCT, with a rate of HBsAg seroconversion (reappearance of HBsAg in a person who was HBsAg-negative, anti-HBc-positive before HSCT) of 20% according to a retrospective study[20]. The cumulative probability of HBsAg seroconversion was 42.9% at 4 years after HSCT.

Gender

Male sex is a consistent host factor shown to be associated with an increased risk of HBV reactivation[1,36]. In one study of 78 HBsAg-positive patients with various types of cancer, 29% of the male patients had HBV reactivation compared with 10% of the female patients[36].

HBV serologic status

HBsAg-positive patients have a higher risk of HBV reactivation than patients who are HBsAg-negative-anti-HBc-positive. HBsAg positivity is associated with a 5- to 8-fold increase in the risk for HBV reactivation[37]. Moreover, those with detectable or high levels of serum HBV DNA prior to start of immunosuppressive therapy have a higher risk of HBV reactivation than do those with undetectable or low levels of HBV DNA[38-40].

HBV reactivation in HBsAg-negative patients is also increasingly being encountered nowadays because of the use of B-cell-depleting agents[16,41,42]. Most HBsAg-negative-anti-HBc-positive patients have undetectable serum HBV DNA levels, but the risk of HBV reactivation is higher in the minority with detectable serum HBV DNA at baseline. Moreover, in HBsAg-negative-anti-HBc-positive patients, those who have an undetectable or low titre of anti-HBs level at the onset of immunosuppressive therapy or have a loss of anti-HBs during immunosuppressive therapy have an increased risk of HBV reactivation[43-47].

Types of anticancer therapies

Steroids are a common component of treatment for hematological malignancies, especially lymphoid diseases, and are combined with chemotherapy in most regimens used to treat lymphoma and multiple myeloma. It was shown that long-term treatment with prednisolone increases HBsAg levels and HBV DNA in hepatocytes, with rebound immune T-cell function and subsequent hepatocyte destruction upon withdrawal of the corticosteroid[48]. High-dose steroid (> 20 mg/d of prednisolone) and long duration of therapy (> 4 wk) increases the risk of HBV reactivation.

Anthracyclines, such as doxorubicin or daunorubicin, are commonly used to treat hematological malignancies such as lymphoma and acute leukemia. There is a significant risk of HBV reactivation in patients receiving doxorubicin as part of the chemotherapeutic regimens[49,50].

B-cell depleting agents, like anti-CD20 monoclonal antibodies, are used in various hematological malignancies, including lymphoma and chronic lymphocytic leukemia (CLL). These monoclonal antibodies (including rituximab, ofatumumab) act against B-lymphocyte antigen CD20, resulting in profound depletion of the B-cells involved in priming specific cytotoxic T-cells[51]. Rituximab was the first monoclonal antibody approved by the Food and Drug Administration (FDA), and is the most widely used treatment for CD20-positive B-cell lymphoma patients. Rituximab is a chimeric type 1 antibody that kills CLL cells primarily by means of complement-dependent and antibody-dependent cellular cytotoxicity after binding to CD20. The use of rituximab is associated with a more than 5-fold increase in the risk of HBV reactivation[19]. In 2013, the United States FDA issued a black box warning concerning the risk of HBV reactivation in patients receiving anti-CD20 monoclonal antibodies[52].

The profound B-cell depletion induced by rituximab interferes with the generation of anti-HBs, which can neutralize circulating HBsAg. Rituximab worsens the impairment of antigen-presenting B-cells that is seen with chronic HBV, leading to insufficient induction of CD4 T-cell activation and proliferation and a T-cell

Table 1 Drug classes and corresponding risks of HBV reactivation[49,67]

Drug class	Drug	Risk of HBV reactivation for HBsAg-positive patients	Risk of HBV reactivation for HBsAg-negative/anti-HBc-positive patients
Monoclonal antibody	Rituximab Ofatumumab Obinutuzumab	High (30%-60%)	High (> 10%)
Anthracycline chemotherapy	Doxorubicin Epirubicin Daunorubicin	High (15%-30%)	High (> 10%)
Corticosteroids	High dose, *e.g.*, Prednisolone ⩾ 20 mg for ⩾ 4 wk	High (> 10%)	Not available
	Moderate dose, *e.g.*, Prednisolone < 20 mg for ⩾ 4 wk	Moderate (1%-10%)	Moderate (1%-10%)
	Low dose, *e.g.*, Prednisolone < 1 wk	Low (< 1%)	Low (< 1%)
Tyrosine kinase inhibitors	Imatinib, nilotinib	Moderate (1%-10%)	Moderate (1%-10%)
Traditional immunosuppressive agents	Methotrexate, azathioprine, 6-mercaptopurine	Low (< 1%)	Low (< 1%)

HBV: Hepatitis B virus.

hyporesponsive state[53].

Alemtuzumab is an anti-CD52 monoclonal antibody that is useful in refractory chronic lymphocytic leukemia patients and certain types of T-cell lymphoma. HBV reactivation has also been reported to occur with alemtuzumab therapy[54-57].

Tyrosine kinase inhibitors (*e.g.*, imatinib, dasatinib, nilotinib) are currently the standard treatment for all phases of chronic myeloid leukemia. There have been some reports of HBV reactivation in patients who have been given these agents[58-64]. The exact mechanism for HBV reactivation is not clear with tyrosine kinase inhibitors, but it may be related to immune restoration. Clinicians need to give prophylactic antiviral therapy or regularly monitor HBV DNA and liver enzymes of HBV-infected patients during imatinib treatment.

The use of hypomethylating agents including decitabine and azacitidine has increased in recent years, especially in the elderly acute myeloid leukemia (AML) patients. AML is common in elderly patients and the median age of onset is 67 years[65,66]. To date, there has been no reported case of HBV reactivation in patients using hypomethylating agents, possibly because the degree of myelosuppression with these agents is not as severe as it is with cytotoxic chemotherapy for AML. There is no recommended prophylactic strategy for HBV reactivation in these patients using hypomethylating agents. We would recommend consideration of antiviral prophylaxis for HBsAg positive patients using hypomethylating agents for the treatment of AML, since myelosuppression is a side effect of these agents. Table 1 summarizes the drug classes and corresponding risks of HBV reactivation[49,67].

Newer monoclonal antibodies

Adult T-cell leukemia/lymphoma (ATLL) is a rare but aggressive subtype of T-cell lymphoma with a higher prevalence in Japan and South America. Mogamulizumab is a humanized monoclonal antibody targeting the C-C chemokine receptor 4 that was developed and introduced into the management of ATLL. However, there have been reports of HBV reactivation in Japanese patients treated with this agent, affecting both HBsAg-positive and HBsAg-negative/anti-HBc patients[68-70]. One of these cases of HBV reactivation with mogamulizumab in an ATLL patient was fatal[68]. Further prospective studies are needed to estimate the risk and incidence of HBV reactivation in patients using this agent and to establish regular HBV DNA monitoring-guided preemptive antiviral therapy for such patients.

Brentuximab vedotin is an anti-CD30 drug-conjugated antibody used in the treatment of relapsed or refractory Hodgkin lymphoma and CD30 positive T-cell lymphoma[71-73]. CD30 can be found on malignant lymphoid cells and activated T-cells. CD30 has an important role in developing memory and effector $CD4^+$ T-cells, but its effects on B-cells are controversial. Following binding to CD30, brentuximab vedotin will be internalized rapidly and cause cell cycle arrest and apoptosis. There has been a case report from China of HBV reactivation in one patient given brentuximab vedotin[74].

Obinutuzumab is a newer generation of anti-CD20 monoclonal antibody. Similar to rituximab, it is a humanized, glycol-engineered type 2 antibody also targeted against CD20[75]. Based on the risk of HBV reactivation with rituximab, the FDA has mandated a warning about risk of HBV reactivation with obinutuzumab, although no specific events of reactivation have been reported with this agent. Obinutuzumab showed higher efficacy than rituximab, by inducing direct cell death and enhanced antibody-dependent cellular cytotoxicity (with less complement-dependent cytotoxicity). It can cause more profound suppression of CD20 than rituximab. A comparison of obinutuzumab with rituximab showed better complete response rate and longer progression-free survival with obinutuzumab than with rituximab,

when both were given in combination with chlorambucil in the treatment of CLL[76]. The use of this agent would be expected to increase in the treatment of CLL and other lymphoproliferative diseases.

Other novel agents

There are many new targeted agents for hematological malignancies, including the BTK ibrutinib and the PI3K delta inhibitor idelalisib, which are both B-cell receptor signaling modulators. They have been approved by FDA for the treatment of CLL and certain low-grade non-Hodgkin's lymphomas (NHL). These oral compounds are currently in various clinical trials in patients with different subtypes of aggressive and low-grade B-cell NHLs and they will be used more clinically in the future.

There is already a case report concerning the HBV reactivation in a CLL patient receiving ibrutinib[77]. This patient with relapsed CLL had previously resolved hepatitis B infection and was negative for HBsAg and positive for anti-HBc Ab. HBV reactivation occurred five months after starting ibrutinib treatment. The patient recovered from HBV reactivation after receiving entecavir therapy. Ibrutinib acts by blocking B-cell antigen receptor signalling, thereby reducing malignant proliferation of B-cells and inducing cell death[4]. Ibrutinib irreversibly binds IL-2-inducible kinase and inhibits activation of Th2 cells after T-cell receptor stimulation. This inhibition is specific to Th2-polarized CD4 T-cells, because redundant resting lymphocyte kinase remains functional, thus providing a compensatory platform for activation of Th1 and CD8 T-cells[78].

Physicians should be vigilant about the possibility of HBV reactivation when using these agents. We recommend screening patients for HBV antigens before using the BTK or PI3K blockers, and prescribing prophylactic antiviral therapy in HBsAg-positive patients. Further studies are required to assess the risk of HBV reactivation in patients receiving small molecules in the treatment of lymphoma and leukemia.

Myelofibrosis is a myeloproliferative disease characterized by excessive production of reticulin and collagen fibers, hepatosplenomegaly, anemia and a tendency to transform to acute leukemia. About 50% of patients present the JAK2V617F mutation, resulting in a constitutively activated Janus-activated kinase (JAK) signal and increased production of cytokines. Ruxolitinib is a novel inhibitor JAK1 and JAK2 that has been approved for the treatment of patients with myelofibrosis. Clinical trials have shown that ruxolitinib can effectively reduce the size of the spleen and liver and improve myelofibrosis-related symptoms[79,80]. However, there are also reports of HBV reactivation in patients with myelofibrosis following ruxolitinib treatment[81,82].

The approval of the proteasome inhibitor bortezomib for newly diagnosed and relapsed or refractory multiple myeloma patients marked a revolutionary change in the drug treatment of multiple myeloma[83-85]. Bortezomib is also the backbone of induction therapy for transplant-eligible patients prior to stem cell harvest. It inhibits T-cell function[86,87], which could promote viral replication in patients with multiple myeloma. Bortezomib has been shown to be associated with HBV reactivation in both HBsAg positive patients and HBsAg negative/anti-HBc positive patients with or without stem cell transplant[29,30,88]. HBV reactivation has been reported 1 to 3 years after stem cell transplantation in some patients and it may be due to immune dysfunction in patients with multiple myeloma and factors associated with bortezomib-containing chemotherapy and autologous stem cell transplant.

Multiple myeloma cells uniformly overexpress CD38. Daratumumab, a CD38-targeting, human IgG1κ monoclonal antibody, was shown to have a favorable safety profile and encouraging efficacy in heavily pretreated patients with refractory myeloma[89]. Daratumumab can potentially increase the risk of HBV reactivation because CD38 is expressed in B-cell hematological malignancies. There is no reported case so far and further studies are needed to assess the risk of HBV reactivation with this monoclonal antibody.

There has been a report of HBV reactivation occurring after six cycles of pomalidomide/doxorubicin/dexamethasone treatment in a myeloma patient with an initially unknown HBV carrier status[90]. This had not been observed after previous administration of highly immunosuppressive high-dose melphalan prior to autologous stem cell transplantation, which may indicate that the immunomodulatory agent (pomalidomide) was the cause of the HBV reactivation. More data are needed to determine whether immunomodulators affect the risk of HBV reactivation.

PREVENTION OF HBV REACTIVATION

HBV screening

The key to prevention of HBV reactivation is the identification of patients with HBV infection before initiation of chemotherapy. Patients at risk of HBV reactivation are easily identified by testing HBsAg and anti-HBc. The European Association for the Study of the Liver and Asian-Pacific Association for the Study of the Liver recommend universal HBV screening prior to initiation of immunosuppressive therapy while the American Association for the Study of Liver Diseases, American Gastroenterological Association and American Society of Clinical Oncology recommend screening patients with risk factors[49,91-94]. We recommend screening all patients with hematological malignancies for active or prior HBV infection by testing for HBsAg and anti-HBc in serum.

HBsAg positive patients

The risk of HBV reactivation is high in HBsAg-positive patients receiving chemotherapy. The risk is

even higher in patients with high HBV replication at baseline with high serum HBV DNA level, receiving rituximab-containing chemotherapy or a stem cell transplant. A systematic review of 14 studies showed that, in HBsAg-positive patients who were not given prophylaxis with the antiviral agent lamivudine during chemotherapy, the rate of HBV reactivation, liver failure, and death were 32%, 13%, and 7%, respectively[95]. Furthermore, lamivudine prophylaxis could decrease HBV reactivation and HBV hepatitis by 80% to 100% and eliminate HBV liver failure, as well as reducing the delay or interruption of chemotherapy due to HBV reactivation[96].

Therefore, once a patient is identified as being seropositive for HBsAg, the patient will benefit from receiving prophylactic antiviral treatment for hepatitis B before or concomitantly with starting chemotherapy, irrespective of the HBV DNA level. The number needed to treat to prevent one reactivation was 3 patients[97]. Antiviral therapy started after HBV reactivation may not be able to prevent hepatitis or hepatitis flares[16], because antiviral therapy usually takes a few weeks to months to reduce viral loads and control the disease, during which there is ongoing inflammation and necrosis of the liver[14]. Moreover, randomized trials have shown that prophylactic antiviral therapy is more effective than a pre-emptive strategy in HBsAg-positive patients[98,99]. Another major limitation of pre-emptive antiviral therapy is its reliance on close monitoring of HBV DNA levels.

HBsAg-negative/anti-HBc-positive patients

Patients with resolved HBV infection may have occult HBV infection with persistent detectable viremia despite the absence of detectable circulating HBsAg[14]. The HBV reactivation rate ranged from 8.9% to 41.5% in HBsAg-negative/anti-HBc-positive patients receiving rituximab-containing chemotherapy in different studies with different definitions of HBV reactivation, using either HBsAg seroconversion or detectable HBV DNA[16,41,42,45,99]. A meta-analysis of 15 studies (6 prospective and 9 retrospective) involving 578 patients gave a pooled risk estimation of HBV reactivation (ALT more than 3 times the upper limit of normal and either HBsAg seroconversion or an increase in serum HBV DNA) of 6.3% in patients exposed to rituximab[100]. Most of the reported cases of HBV reactivation in patients with resolved HBV infection were self-limited, but fulminant hepatitis and hepatitis-induced mortality were sometimes reported[99,101-105].

For patients with resolved HBV infection, no standard strategy has yet been established to prevent HBV reactivation. There are usually two options. One is pre-emptive therapy guided by serial HBV DNA monitoring, whereby the antiviral drug is given as soon as HBV DNA becomes detectable. Pre-emptive antiviral therapy may be a reasonable strategy, but there is limited evidence regarding the optimal interval and period of monitoring[16,106]. An alternative approach is prophylactic antiviral therapy, especially for high-risk chemotherapy such as rituximab-containing chemotherapy. This strategy may effectively prevent HBV reactivation and avoid the inconvenience of repeated HBV DNA monitoring[107]. Moreover, antiviral therapy may be less effective once high titers of viremia are attained, and the same medication could be more effective if given earlier, when viremia is minimal.

Prophylactic antiviral therapy is more effective than pre-emptive antiviral therapy in preventing HBV reactivation in high-risk patients, such as those receiving rituximab or undergoing HSCT. Whether pre-emptive antiviral therapy is more cost-effective in clinical settings associated with moderate risk of HBV reactivation (for example, HBsAg-negative-anti-HBc-positive patients receiving chemotherapy for lymphoma that does not include anti-CD20 therapy) is unclear. A major limitation of pre-emptive antiviral therapy is its reliance on close monitoring of HBV DNA levels and prompt initiation of antiviral therapy when HBV reactivation is detected. Table 2 summarizes the prospective studies in HBsAg negative/anti-HBc positive patients with hematological malignancies who received pre-emptive or prophylactic treatment.

More reports of resolved HBV infection in other hematological malignancies like multiple myeloma have been published in recent few years. In a recently published study of 230 HBsAg-negative/anti-HBc-positive multiple myeloma patients, 12 (5.2%) displayed HBV reactivation, which was defined as the reappearance of HBsAg with or without de novo detection of HBV DNA in the blood[29]. The cumulative rate of HBV reactivation was 5% at 2 years and 8% at 5 years[29]. Absence of anti-HBs and high-dose therapy and then autologous stem cell transplant were found to be risk factors associated with HBV reactivation. The cumulative incidence rate of HBV reactivation rate at 2 years was significantly higher in patients who were anti-HBs negative than anti-HBs positive at baseline (9% *vs* 3%, $P = 0.033$) patients, and was also significantly higher for patients who had received high-dose therapy and then autologous stem cell transplant than for patients who had not (7% *vs* 0%, $P = 0.025$). These high-risk patients may benefit from regular monitoring of HBV DNA levels.

Future prospective studies are needed on cost-effective strategies for the optimal timing and duration for the regular monitoring of HBV DNA and indication of prophylactic antiviral therapy in multiple myeloma patients for avoiding HBV-related liver complications and mortality.

Another recent study included 315 HBsAg-negative, anti-HBc positive patients who received autologous or allogenic stem cell transplantation. The primary endpoint was the incidence of HBV reactivation. With a median follow-up duration was 21.4 mo, antiviral prophylaxis was not given to 219 patients, and 96 received

Table 2 Prospective studies on HBV reactivation in haematology patients with resolved hepatitis B virus infection (HBsAg negative anti-HBc positive patients)

Author/year	Disease	Study design	Sample size	Definition of HBV reactivation	Rate of HBV reactivation	Risk factor(s) identified for HBV reactivation	Death from HBV reactivation
Yeo *et al*[99] 2009	Diffuse large B-cell lymphoma	All patients were observed every 2-3 wk during anticancer therapy and every 6-8 wk for 9 mo after anticancer therapy Start lamivudine upon HBV reactivation	46	HBsAg seroconversion (the reappearance of HBsAg) with an increase in HBV DNA levels when compared with baseline HBV DNA levels, in the absence of acute infection with HAV, HCV, or other systemic infections	25% in patients receiving R-CHOP	Absence of anti-HBs, use of rituximab and male sex	20% died of HBV reactivation
Huang *et al*[107] 2013	B-cell lymphoma	Entecavir prophylaxis (continued until 3 mo after completing chemotherapy) *vs* preemptive treatment	80	Elevation of HBV viral load to 2000 IU/mL with two consecutive determinations (2 wk apart)	Incidence was 4.3% in entecavir group and 25.9% in the control group at 1.5 yr	Lack of entecavir prophylaxis	Nil
Seto *et al*[45] 2014	CD20 positive B-cell lymphoma	HBV DNA monitoring every 4 wk Start entecavir upon detection of HBV reactivation	63	HBV DNA ≥ 10 IU/mL	2-yr cumulative rate 41.5%	Anti-HBs < 10 mIU/mL	Nil
Hsu *et al*[16] 2014	CD20 positive B-cell lymphoma	HBV DNA monitoring every 4 wk Start entecavir upon detection of HBV reactivation	150	> 10-fold increase in HBV DNA, compared with previous nadir levels	Incidence was 10.4%	Absence of anti-HBs	Nil
Kusumoto *et al*[46] 2015	CD20 positive B-cell lymphoma	HBV DNA monitoring every 4 wk Start entecavir upon detection of HBV reactivation	269	HBV DNA ≥ 11 IU/mL	Incidence was 8.3% at 1.5 yr	Anti-HBs < 10 mIU/mL and baseline HBV DNA below level of quantification	Nil

HBV: Hepatitis B virus; HBsAg: Hepatitis B surface antigen; HBc: Hepatitis B core antigen.

prophylaxis. The median duration of prophylaxis was 7.0 mo. HBV reactivated in 12 patients (4 in the group receiving prophylaxis and 8 in the non-prophylactic group). The median time to reactivation was 20.5 mo after starting chemotherapy[108]. All patients who developed HBV reactivation were treated with antiviral agents. The risk of reactivation was increased significantly in patients who had an allogeneic HSCT and those with loss of anti-HBs. This study showed that short-term antiviral prophylaxis may not be able to decrease the risk of HBV reactivation. This suggests that prophylaxis should be used for longer than 24 mo or careful monitoring of HBV DNA should be combined with on-demand antiviral treatment to prevent hepatitis flares in post-transplant patients.

HBsAg negative/anti-HBc negative/anti-HBs positive patients

HBV reactivation in patients with isolated anti-HBs is extremely rare. In general, individuals who are seropositive only for anti-HBs (HBsAg negative and anti-HBc negative) are usually considered as having a history of HBV vaccination and are at no risk for HBV reactivation. Routine monitoring is usually not recommended. However, HBV reactivation has been reported to occur in patients who were seropositive for anti-HBs alone[109,110].

A patient with follicular lymphoma without record of hepatitis B vaccination had only anti-HBs but no anti-HBc before chemotherapy. He later developed high viremia with an HBV escape mutant, which was difficult to detect by HBsAg assays[109]. The escape mutants of HBV carry mutations in the major antigenic region of HBsAg. They are able to grow in the presence of anti-HBs. Anti-HBc may appear very late.

Another patient with diffuse large B-cell lymphoma without record of vaccination against HBV was negative for HBsAg and anti-HBc, and positive for anti-HBs (127 IU/mL) before chemotherapy. She had HBV reactivation after finishing rituximab-containing chemotherapy. She died of liver failure although antiviral treatment was started after detection of HBV reactivation[110].

Therefore, clinicians should be aware of this possible complication when they give chemotherapy to hematology patients who are HBsAg negative/anti-HBc negative/anti-HBs positive. When the patient has unexplained deranged liver function, HBV DNA should

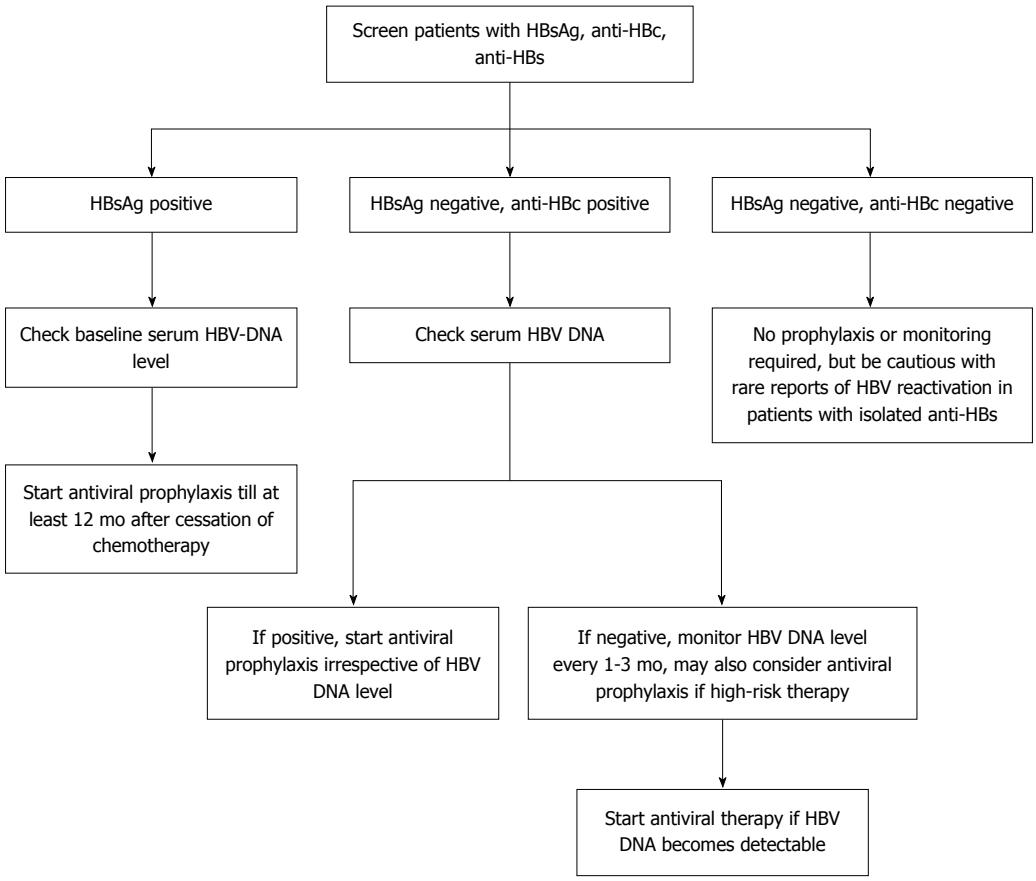

Figure 1 Recommended algorithm for hepatitis B virus testing and treatment in patients with hematological malignancies receiving anti-cancer therapy. HBV: Hepatitis B virus; HBsAg: Hepatitis B surface antigen; HBc: Hepatitis B core antigen.

be checked to look for HBV reactivation in this setting.

Figure 1 is a suggested algorithm for testing of HBV and management of patients with hematological malignancies receiving anticancer therapy. Table 3 summarizes the recent international guidelines on the management of patients with HBV infection receiving chemotherapy.

CHOICE OF ANTIVIRAL THERAPIES

Lamivudine was the first oral antiviral agent used to treat chronic HBV infection, and has been studied in randomized controlled trials for its efficacy to prevent HBV reactivation in patients receiving immunosuppressive therapies[85,98]. Lamivudine is generally safe and well tolerated, but it is also associated with a high incidence of viral resistance after prolonged use for more than 6 mo due to the emergence of YMDD mutations[93]. Lamivudine has been shown to have a low barrier to resistance with rates of antiviral resistance of 14%-32% after 1 year of treatment and 60%-70% after 5 years of treatment in clinical trials in patients with chronic hepatitis B[93]. Emergence of drug resistance mutations may outweigh the benefits of preventative antiviral therapy and can lead to hepatitis flares, liver failure and death.

Antiviral agents with high genetic barriers to resistance (*i.e.*, entecavir or tenofovir) are preferred to lamivudine in the treatment of chronic HBV infection[79]. A prospective randomized study by Huang *et al*[111] recruited 121 patients seropositive for the HBsAg with untreated DLBCL (61 received entecavir prophylaxis and 60 received lamivudine prophylaxis). It showed that the rate of various endpoints were significantly lower in the entecavir than the lamivudine group: for HBV-related hepatitis (0% *vs* 13.3%, P = 0.003), HBV reactivation (6.6% *vs* 30%, P = 0.001), and chemotherapy disruption (1.6% *vs* 18.3%, P = 0.002)[111].

A recent retrospective study in 213 patients (entecavir group, 70 patients; lamivudine group, 143 patients) found a lower incidence of HBV reactivation in the entecavir group than the lamivudine group (0% *vs* 7.0%, P = 0.02). No HBV reactivation was noted in the patients with a baseline HBV DNA level < 2000 IU/mL. A baseline HBV DNA level > 2000 IU/mL, HBeAg, and lamivudine were significantly associated with HBV reactivation[40]. Another recent study also showed that, compared with lamivudine, entecavir has more potent antiviral efficacy and may be a better choice for prophylaxis of HBV reactivation in HBsAg-positive allogenic HSCT recipients[112].

There are also recent data suggesting that tenofovir may help to prevent HBV reactivation. During routine

Table 3 Comparison of international guidelines on the management of patients with hepatitis B virus infection receiving chemotherapy

Association guidelines	HBV screening	Screening tests	HBsAg positive patients	HBsAg negative, anti-HBc positive patients	Antiviral drug recommended	Duration of antiviral therapy
EASL 2012[91]	All candidates for chemo- and immunosuppressive therapy	HBsAg, anti-HBc, HBV DNA if serology positive	Prophylactic antiviral therapy, test for HBV-DNA level	Test for HBV DNA; provide prophylactic antiviral therapy if detectable HBV DNA; monitor ALT and HBV DNA levels closely if no detectable HBV DNA	Lamivudine if HBV DNA < 2000 IU/mL and the treatment duration is finite and short, Entecavir or tenofovir if HBV DNA is high, and/or lengthy and repeated cycles of immunosuppression	12 mo after cessation of therapy
APASLD 2012[92]	All patients prior to receiving immunosuppression or chemotherapy	HBsAg, anti-HBc	Prophylactic antiviral therapy	Monitor HBV-DNA	Lamivudine or entecavir or tenofovir	Continue until 6 mo after end of chemotherapy
AGA 2015[49]	High risk of HBV reactivation (> 10%) and moderate risk of HBV reactivation (1%-10%) Routine screening not recommended for low risk of HBV reactivation (< 1%)	HBsAg, anti-HBc , HBV DNA if serology positive	Prophylactic antiviral therapy	Antiviral prophylaxis over monitoring for patients if the chemotherapy is associated with high or moderate risk of HBV reactivation	Drug with high barrier to resistance is favored over lamivudine	6 mo after discontinuation of therapy and at least 12 mo for B-cell depleting agents
AASLD 2009[93], no update in the version in 2016[126]	Anyone at high risk of HBV infection	HBsAg and anti-HBc	Prophylactic antiviral therapy	No recommendation	Lamivudine or telbivudine if the anticipated treatment duration is < 12 mo and baseline HBV DNA is not detectable Tenofovir or entecavir if anticipated treatment duration > 12 mo	Maintain for 6 mo after completion of chemotherapy
ASCO 2015[94]	Risk-adapted HBV screening strategy	HBsAg, anti-HBc , HBV DNA if serology positive	Prophylactic antiviral therapy	Consider Antiviral prophylaxis if the systemic cancer therapy is associated with high risk of HBV reactivation	Entecavir, tenofovir	Minimum of 6 mo after stopping therapy, longer than 12 mo for patients receiving anti-CD20 monoclonal antibodies

HBV: Hepatitis B virus; EASL: European Association for the Study of the Liver; APASLD: Asian Pacific Association for the Study of the Liver; AGA: American Gastroenterology Association; AASLD: American Association for the Study of Liver Diseases; ASCO: American Society of Clinical Oncology.

clinical practice, none of the 25 patients who received prophylactic treatment with tenofovir had HBV flares and all achieved undetectable serum HBV DNA during a mean follow-up period of 17.2 mo. One patient even had HBsAg seroconversion[113].

Entecavir will be a better option in the case of significant renal impairment as tenofovir has a small risk of inducing proximal tubular dysfunction and renal insufficiency[114-116]. On the other hand, tenofovir is preferred to entecavir if patients have received lamivudine therapy previously because of a high rate of resistance (genotypic resistance of 51% and virologic breakthrough of 43% within 5 years)[117]. Prophylaxis with telbivudine or adefovir is not recommended because of development of drug resistance and there are limited data from clinical trials using these agents[49].

A recent study showed that concurrent telbivudine treatment with initial chemotherapy can reduce HBV reactivation in HBsAg-positive lymphoma patients, and the efficacy is independent of the baseline HBV viral loads[118]. This study in 60 HBsAg-positive patients showed that the rate of HBV reactivation was 11.7% (7/60), and the median time to HBV reactivation was 228 d (range 113-699 d). The fulminant hepatitis rate was 6.6%. The rates of undetectable HBV DNA and ALT normalization were 61.7% and 83.3%, respectively[118].

Duration of antiviral therapies

There are few data available in the literature to guide physicians on when antiviral therapy can be stopped. For patients receiving B cell-depleting agents, the duration of antiviral therapy should be extended to at least 12 mo after cessation of the B-cell depleting agent, because there is profound suppression of B-cell function[14,19,107]. The duration of nucleoside analog therapy also depends on the patient's baseline serum HBV DNA level and the degree of fibrosis/cirrhosis

present. A previous study showed that high levels of serum HBV DNA (≥ $4\log_{10}$ copies/mL) before chemotherapy was the most important predictor of HBV reactivation after withdrawal of prophylactic antiviral therapy[119].

There is growing evidence that HBsAg-positive patients with lymphoma can develop hepatitis B flares due to HBV reactivation more than 6 mo after cessation of chemotherapy[21,120]. It is reasonable to consider extending the duration of prophylactic antiviral therapy to at least one year after completion of chemotherapy.

Kim *et al*[121] also reported the importance of HBV DNA levels and consolidation period as predictors of HBV reactivation after withdrawal of prophylactic antiviral therapy. Their study enrolled 95 HBsAg-positive patients who were analyzed for sustained off-treatment virological response, defined as HBV DNA levels below 2000 IU/mL for at least 12 mo after the end of therapy. The baseline HBV DNA level was shown to be an independent factor associated with sustained off-treatment virological response. The rate of HBV reactivation was 72.1% and 23.5% for those with HBV DNA < 2000 IU/mL and ≥ 2000 IU/mL, respectively ($P < 0.001$). Consolidation treatment duration showed association with sustained off-treatment virological response only for those with low baseline HBV DNA levels. The sustained off-treatment virological response rates were 54.5%, 71.4%, 73.9%, and 100% for consolidation treatment durations of < 3, 3-6, 6-12, and ≥ 12 mo, respectively, among those with baseline HBV DNA < 2000 IU/mL[121].

Liu *et al*[122] recently analyzed 107 newly diagnosed DLBCL patients with HBV infection who received chemotherapy. The median time from the cessation of antitumor therapy to the withdrawal of prophylactic antiviral therapy was 6.1 mo. Ten of the 46 patients in the HBsAg-positive group (21.7%) experienced delayed HBV reactivation, whereas none in the HBsAg-negative/anti-HBc-positive group exhibited delayed HBV reactivation.

HBV reactivation can occur many years after HSCT, so antiviral prophylaxis is required for a longer duration. The optimal duration of therapy is not well defined in HSCT patients with occult infection. HBV reactivation can occur months or even years (up to 6 years) after transplantation[20, 123].

Management of Hepatitis B virus reactivation

Any adverse change in liver function in a patient undergoing chemotherapy needs to be thoroughly investigated, to distinguish HBV reactivation from other potential causes, including infections by other hepatitis virus (A, C, D, E) or opportunistic infections (*e.g.,* cytomegalovirus, herpes viruses) and hepatotoxic drugs. Once HBV reactivation is detected, patients should receive antiviral therapy with either entecavir or tenofovir[49]. Severe flares can be accompanied by an increase in IgM hepatitis B core antibody, which may be misdiagnosed as acute HBV infection.

Hepatitis B flare-ups are generally rare when patients are receiving anti-HBV prophylaxis with potent antivirals. When patients are receiving lamivudine as a prophylaxis, they may develop resistance and may benefit from rescue therapy such as entecavir or tenofovir. We need to watch out for resistance when using low genetic barrier drug such as lamivudine. A recent study also showed that some patients could recover virologically and biochemically after combination regimens with lamivudine plus adefovir or entecavir plus adefovir, respectively[124].

The goal of treatment is to prevent the development of severe hepatitis, hepatic failure and even mortality. Close monitoring of liver enzymes, bilirubin levels and the clotting profile is essential. Occasionally, patients progress to hepatic failure despite nucleoside therapy[16], especially if they already have jaundice or a marked increase in liver enzymes. Although there have been a few reports of successful liver transplantation in patients who develop liver failure, most of those with hematological malignancy will die because their underlying disease will preclude them from being candidates for liver transplantation[125].

CONCLUSION

Hepatitis due to HBV reactivation is a common and important complication in patients receiving cancer chemotherapy, especially for hematological malignancies. Although potentially fatal, HBV reactivation is preventable through screening with blood tests and administration of prophylactic antiviral therapy for patients with moderate or high risk of HBV reactivation.

We recommend undertaking screening tests for HBsAg, anti-HBc and anti-HBs in all hematology patients about to start anticancer therapy. Risk stratification based on their serologic status and the types of therapies would be the next step. For HBsAg-positive patients, prophylactic antiviral therapy is essential. There are two options for HBsAg-negative/anti-HBc-positive patients. One is pre-emptive therapy guided by serial HBV DNA monitoring, whereby the antiviral drug is as soon as HBV DNA becomes detectable. The other approach is routine prophylactic antiviral therapy. We recommend prophylactic therapy in patients receiving high-risk therapies like anti-CD20 monoclonal antibodies or HSCT recipients.

Entecavir or tenofovir are preferred over lamivudine as prophylactic therapy. We recommend continuing preventative antiviral therapy for at least 12 mo after the completion of chemotherapy and even longer for those who receive rituximab or who had high serum HBV DNA levels before the start of chemotherapy. Screening for HBV before the start of chemotherapy is the key to preventing HBV reactivation.

In the era of targeted therapy for hematological

malignancies, further studies are needed to estimate the risk of HBV infection and the optimal prophylactic strategy in patients receiving new targeted therapies. Although there is currently limited evidence to guide the optimal preventive measures, we recommend consideration of antiviral prophylaxis in HBsAg-positive patients to minimize the risk of HBV reactivation, especially with the BTK and PI3K inhibitors, which are B-cell receptor signaling modulators and reduce proliferation of malignant B-cells.

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Polyethylene glycols: An effective strategy for limiting liver ischemia reperfusion injury

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Abstract

Liver ischemia-reperfusion injury (IRI) is an inherent feature of liver surgery and liver transplantation in which damage to a hypoxic organ (ischemia) is exacerbated following the return of oxygen delivery (reperfusion). IRI is a major cause of primary non-function after transplantation and may lead to graft rejection, regardless of immunological considerations. The immediate response involves the disruption of cellular mitochondrial oxidative phosphorylation and the accumulation of metabolic intermediates during the ischemic period, and oxidative stress during blood flow restoration. Moreover, a complex cascade of inflammatory mediators is generated during reperfusion, contributing to the extension of the damage and finally to organ failure. A variety of pharmacological interventions (antioxidants, anti-cytokines, *etc.*) have been proposed to alleviate graft injury but their usefulness is limited by the local and specific action of the drugs and by their potential undesirable toxic effects. Polyethylene glycols (PEGs), which are non-toxic water-soluble compounds approved by the FDA, have been widely used as a vehicle or a base in food, cosmetics and pharmaceuticals, and also

as adjuvants for ameliorating drug pharmacokinetics. Some PEGs are also currently used as additives in organ preservation solutions prior to transplantation in order to limit the damage associated with cold ischemia reperfusion. More recently, the administration of PEGs of different molecular weights by intravenous injection has emerged as a new therapeutic tool to protect liver grafts from IRI. In this review, we summarize the current knowledge concerning the use of PEGs as a useful target for limiting liver IRI.

Key words: Ischemia reperfusion injury; Polyethylene glycol; Liver preconditioning; Liver transplantation; UW solution; IGL-1 solution; SCOT solution; PEG rinse solution; Machine perfusion

Core tip: Pharmacological treatments for preventing liver ischemia reperfusion injury are limited, due to the complex pathophysiology of this condition. The drugs currently used for preventing ischemia-reperfusion injury (IRI) all have local and specific activity with potentially damaging side effects. This review focuses on the current understanding of polyethylene glycols, which are non-toxic polymers, as new emerging agents for limiting liver IRI, and proposes directions for future investigations.

Pasut G, Panisello A, Folch-Puy E, Lopez A, Castro-Benítez C, Calvo M, Carbonell T, García-Gil A, Adam R, Roselló-Catafau J. Polyethylene glycols: An effective strategy for limiting liver ischemia reperfusion injury. *World J Gastroenterol* 2016; 22(28): 6501-6508 Available from: URL: http://www.wjgnet.com/1007-9327/full/v22/i28/6501.htm DOI: http://dx.doi.org/10.3748/wjg.v22.i28.6501

INTRODUCTION

It is well known that the interruption of blood to an organ (ischemia) and its subsequent restoration (reperfusion) leads to irreversible damage which is termed ischemia reperfusion injury (IRI). IRI is inherent to liver surgical procedures such as hepatic resection and liver transplantation[1-3]. During liver resections, the damage is commonly a consequence of the vascular occlusion of the liver hilum (Pringle' s maneuver) under normothermic conditions[1]. In the case of transplantation, the damage is sustained during cold storage of the liver graft (at 4 ℃) in preservation solution following explantation from the donor, and during subsequent warm reperfusion and implantation into the recipient[4].

There are several steps between organ recovery and transplantation that can exacerbate the damage to the graft. The most important are organ procurement (pre-preservation), conservation in preservation solution (cold storage), and rewarming (graft washout) before transplant (reperfusion). The cumulative injuries due to each step are determinant for the successful graft outcome after transplantation but the most significant lesions occur during cold ischemia, graft rewarming and normothemic reperfusion after transplantation.

At the cellular level, prolonged ischemia leads to ATP breakdown and provokes the accumulation of hypoxanthine, mitochondrial de-energization and ionic alterations which finally lead to liver cell necrosis. Upon oxygenation during reperfusion, reactive oxygen species (ROS) generation by uncoupled mitochondria promotes oxidative stress and a complex cascade of inflammatory mediators (nitric oxide, cytokines, adhesion molecules, chemokines, *etc.*) which all contribute to the spread of the damage and finally to cell death[4].

Because of the range of mechanisms involved in hepatic IRI, the choice of preventive or therapeutic strategies is very difficult[5]. Pharmacological strategies for preventing IRI focus on the use of specific agents, but the benefits of these drugs are limited because of their local actions, side effects and potential toxicity. In this situation, there is a clear need to test the use of non-toxic, water-soluble and protective agents for tissues such as PEGs as "preconditioning agents" for preventing IRI and also as potential targets for therapeutic interventions in organ transplantation.

This review is an update of the most significant advances in the use of polyethylene glycols (PEGs) as therapeutic tools for protecting the liver against IRI, placing specific emphasis on future perspectives in liver graft preservation and transplantation.

PEGS: CHEMICAL STRUCTURE AND MEDICAL USE

PEGs are non-immunogenic, non-toxic and water-soluble polymers which show no electric charge and no affinity for any specific organ. They are composed of repeating units of ethylene glycol which form polymers with a linear shape of different molecular weight[6-9]. PEGs with different shapes can be obtained by using different initiator molecules during the polymerization reaction (*e.g.,* hexa-glycerin instead of methanol to form a tri PEG) or by joining different linear PEGs to create different structures, as shown in Figure 1.

PEGs are negligibly metabolized *in vivo* and are mainly unaltered when eliminated from the body either by the kidneys (for PEGs < 30 kDa, slowly for 30 kDa < PEGs < 40 kDa) or in the faeces (for PEGs > 20 kDa)[10]. PEGs are generally considered to have low toxicity *via* all routes of administration, as demonstrated by tests in many animals[11]. Due to their high flexibility, hydrophilicity, and the large number of water molecules coordinated by their chains, PEGs present a greater hydrodynamic volume than would be expected from their molecular weight, and they show

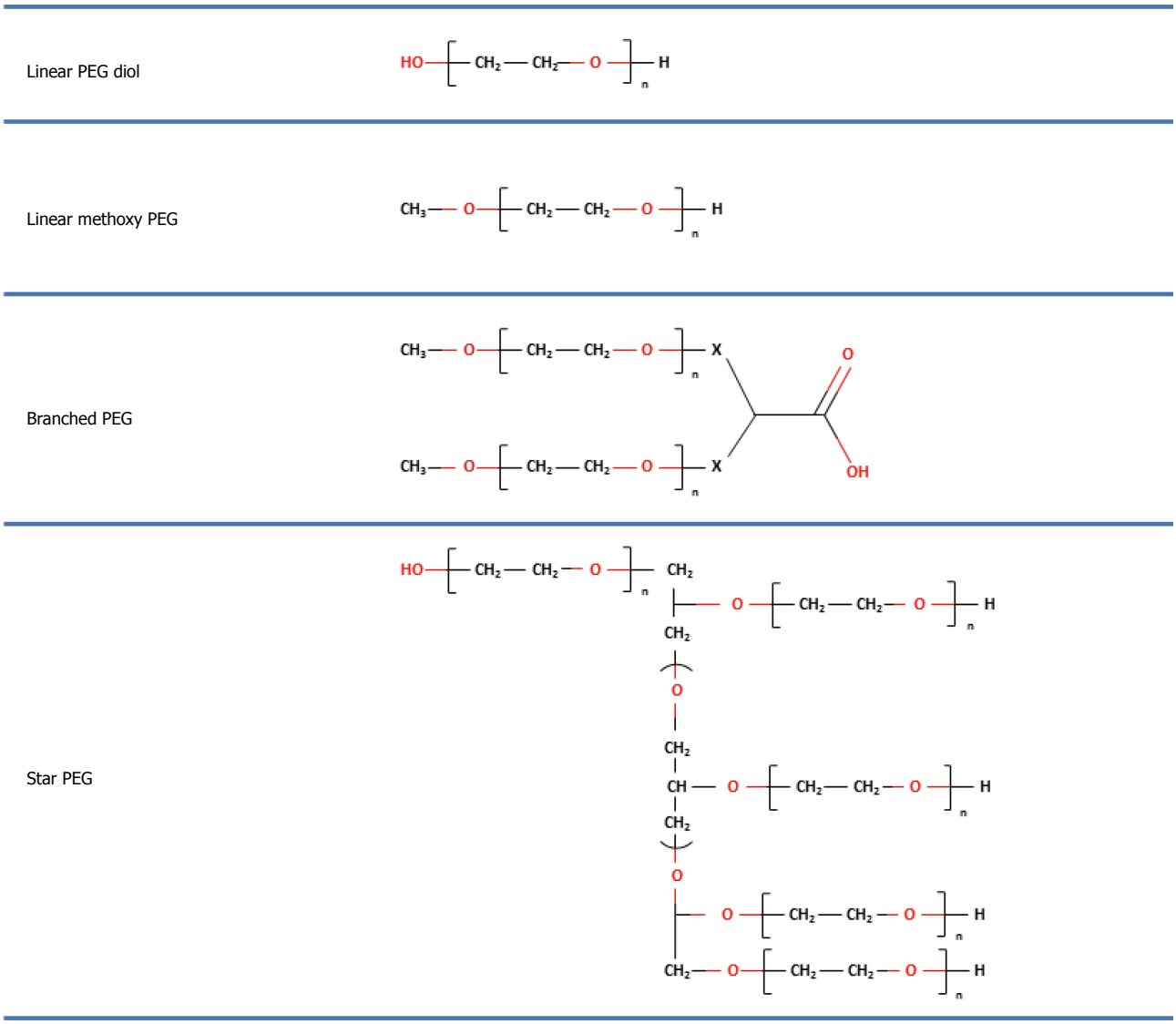

Figure 1 Schematic composition of linear, branched and star polyethylene glycols. Polyethylene glycols (PEGs) are synthesized by a process of linking repeating units of ethylene glycol. The reaction gives products with one or two end chain hydroxyl groups termed methoxy-PEG or diol-PEG, respectively. Then, the linear PEG with branches at irregular intervals along the polymer chain forms branched PEGs. Star-shaped PEGs are the simplest class of branched PEGs with a general structure consisting of several (more than three) linear chains connected to a central core.

high protein-rejecting properties[12].

PEGs have an apparent molecular weight which, depending on the molecular weight of the polymer, can be 5-10 times higher than a corresponding soluble protein of similar molecular mass, as shown by gel permeation chromatography[13]. The Food and Drug Administration (FDA) has approved the use of PEGs as a vehicle or a base in food, cosmetics and pharmaceuticals, including injectable and bowel solutions[14,15]. For example, low molecular weight PEGs are widely used as the basis of a number of laxatives and soft capsules, and high molecular weight PEGs have been used as components in organ preservation solutions to attenuate injury from cold perfusion in animal organs such as pancreas[16], small bowel[17], kidney[18] and liver[19]. In addition, the attachment of PEG (PEGylation) to drugs, peptides, proteins, nanoparticles, micelles, and liposomes is spreading as a technology for enhancing the bioavailability, stability, safety, and efficacy of a wide range of therapeutic agents[20,21]. Examples include PEGylated interferon alpha, which is used to treat hepatitis C[22], or PEGylated antihuman TNF-alpha for rheumatoid arthritis[23].

PEG STRATEGIES TO PREVENT ISCHEMIA-REPERFUSION INJURY: AN APPROACH TO THE LIVER

The use of PEGs to minimize the deleterious effects of IRI has not been studied in depth. However, PEGs have been shown to be effective in cell protection against hypoxia/oxygenation, as additives in preservation and perfusion solutions for organ transplantation and, more

recently, as "preconditioning agents" for preventing IRI in heart and liver. As a result, PEGs may offer new therapeutic strategies for applications in clinical liver surgery and transplantation, as indicated below.

PEG and cryopreservation ("supercooling")

Current technologies can preserve livers outside the body for about 12 h using a combination of cold temperatures and a preservation solution. This has helped increase the number of successful liver transplants, but extending the time a liver can survive outside the body even further would provide many extra benefits.

The presence of PEGs has been shown to be determinant for hepatocyte preservation in hypothermic conditions[24-26]. The addition of PEG8 to the preservation solution suppressed cell swelling in cultured hepatocytes, keeping them relatively well-preserved and restoring membrane integrity[24-26]. This is consistent with the further development of a slow-cooling method that first chills rat livers at 4 ℃ and then drops the temperature to below freezing (named "supercooling"), allowing them to be stored in a "supercooled" but non-frozen state[27]. In this connection, a recent study by Berendsen *et al*[28] presented a method for extended liver storage combining supercooling and machine perfusion. An essential step in this method was the addition of PEG35 to the preservation solution. Similar results were found by the same researchers with "supercooled" hepatocytes: this addition of 5% PEG35 to the storage solution prevented cold-induced lipid peroxidation and maintained hepatocyte viability and functionality during supercooling[25-27].

PEGs in organ preservation solutions

The cold static preservation of solid organs using preservation solutions is the gold standard in clinical organ transplantation today. PEG35 and PEG20 have been used as oncotic agents in IGL-1 and SCOT 20 preservation solutions respectively to prevent cell swelling[29-32]. The presence of PEG35 in IGL-1 makes this solution a good alternative to UW solution (the standard goal for liver transplantation), especially in the presence of moderate to severe hepatic steatosis[33,34]. PEG20 is the basic component of the SCOT solution, which furthermore contains low K+/high Na+ concentrations. PEG20 at 15 g/L has been found to reduce alloantigen recognition after liver reperfusion in comparison to UW solution[35]. However, the use of this PEG20 in preservation solutions has not shown a greater benefit than PEG35[35].

PEG35 (at 1 g/L) plays a key role in reducing the higher vulnerability of fatty livers to IRI[33]. This is mainly due to the production of nitric oxide (NO), whose vasodilatory properties contribute to counteracting the exacerbated alterations of microcirculation in steatotic livers due to the accumulation of fat in the sinusoids, which makes it more difficult to obtain an adequate hepatic revascularization after transplantation. Moreover, the NO generated may act as a suitable scavenger for preventing the impairment of lipid peroxidation in fatty livers against reperfusion[36].

It has also been demonstrated that PEG interferes with the coagulation system and reduces platelet adhesion *in vitro* and *in vivo*[37,38] by forming a molecular barrier on the glycocalyx. This PEG barrier prevents acute platelet deposition on damaged arteries. When a relatively low molecular weight PEG (< 10 kDa) was conjugated to pericardium, it reduced the deposits of calcium and decreased platelet and leukocyte surface attachment[39,40]. Therefore, the conjugation of PEG to the surface of endothelial cells seemed to reduce inflammation and control water content. Longer PEG chains, such as that of PEG35, might be expected to interact with the surface of endothelial cells of blood vessels and/or remain in the interstitial fluid of transplanted liver, thus promoting the above mentioned beneficial effects even after the washout of the organ graft.

The presence of PEG35 in IGL-1 solution also promotes the activation of several protective cell signaling pathways during liver cold storage, as a self-response of the organ to oxygen deprivation. This leads to the induction of cytoprotective factors such as adenosine monophosphate protein kinase (AMPK), an enzyme which is involved in the glucose metabolism breakdown and modulates the energy balance towards an energy preserving state. PEG35 also activates other protective factors associated with the deprivation of oxygen supply to the organ such as the hypoxia inducible factor HIF alpha[41].

PEG in perfusion solutions (graft washout and machine perfusion)

Rinse solutions help to wash the liver graft preserved in organ preservation solutions by avoiding air emboli and the secondary effects of the remnants of preservation solution, such as the excessively high concentration of intravascular potassium and metabolic waste during cold storage[42,43]. Although there is no consensus among physicians on how the graft flushes should be carried out, the most widely used solutions are Ringer Lactate and 5% human albumin[42].

PEG35 is a suitable additive in rinse solution for an efficient liver graft washout, and also ensures additional protection against reperfusion injury[44]. Protective mechanisms induced by PEG35 against liver reperfusion injury mainly involve the preservation of liver mitochondrial status[44], as shown in Figure 2.

Rhodamine 123 cell viability marker (in green) shows the preserved membrane potential of liver mitochondria in liver grafts preserved in UW and then rinsed with PEG35 solution when compared to livers rinsed with Ringer lactate. In this case, Evans Blue labeling (in red) shows the albumin content and the disrupted mitochondrial membranes. Thus,

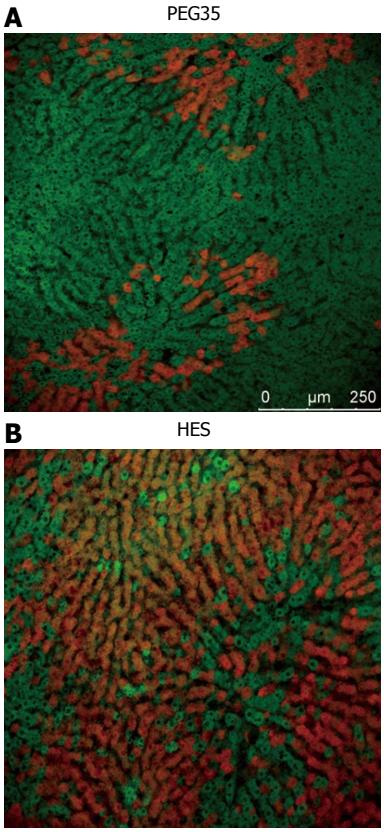

Figure 2 PEG35 preserves liver graft function. Confocal microscopy images showing green fluorescence of rhodamine 123 cell viability marker. Liver grafts were better preserved when they were rinsed with a solution containing PEG35 (A) rather than Ringer lactate solution (B).

PEG35 preserves the cytoskeleton structure and cell morphology against the effects of IRI[44,45].

Static cold preservation remains the gold standard, but the growing needs of liver transplantation oblige physicians to use machine perfusion (MP) techniques in different temperature conditions: hypothermia (HMP), normothermia (NMP) and subnormothermia (SNMP) for graft preservation purposes[46-48]. The use of PEG in MP solutions is very limited in contrast to UW-gluconate and KPS solutions[46-48]. Bessems *et al*[49,50] have shown that substitution of hydroxyethyl starch by PEG in Polysol perfusion solution achieved equal or better function and less damage in rat liver after 24 h of HMP, and that this Polysol-PEG solution was more efficient than UW-Gluconate perfusion solution. In rat steatotic livers, cold storage using Polysol resulted in significantly better integrity and functions of the liver[51] and thus improved the preservation quality of partial liver transplantation[52]. More recently, it was shown that addition of PEG35 to SNMP at 5 g/L using the "supercooling" technique was necessary to achieve successful liver transplantation after six days' preservation[28,53]. Thus, PEG contributes to the rapid extension of cooling and the lower temperatures attained also contributed to preserving the membrane and cytoskeletal structure of hepatocytes during HMP[25].

PEGs as "preconditioning" agents for IRI prevention

Recent investigations in the heart have found that high molecular weight PEG (15-20 kDa) protected cardiac myocytes from hypoxia reoxygenation[54]. More recently, these cardioprotective benefits for PEG 15-20 were observed when it was administered just before reperfusion[55].

With this in mind, our group explored the benefits of using PEG35 to limit IRI in different experimental models of cold ischemia and warm ischemia reperfusion in rats[56,57]. Intravenous administration of PEG35 to rats before the induction of cold ischemia-reperfusion insult (a single 10 mg/kg dose) protected fatty livers from the lesions associated with IRI[56]. The prevention of liver damage was accompanied by a high protection of liver cytoskeleton and mitochondria, which was concomitant with increased phosphorylation of pro-survival protein kinase b (AKT) and the activation of cyto-protective factors such as e-NOS and AMPK respectively[56].

These investigations reveal that *in vivo* PEGs improve the initial conditions of organs against the cold ischemia reperfusion insult. This PEG strategy can be considered as a useful tool for multi-organ preconditioning before organ recovery and then static cold storage/machine perfusion preservation.

These protective mechanisms of PEG were also corroborated in a warm ischemia-reperfusion model in the rat[57]. Intravenous administration of PEG35 at 10 mg/kg was protective against IRI. In this case, PEG35 not only prevented mitochondria damage, but also promoted the activation of prosurvival pathways (AKT, AMPK), and reinforced the cytoskeleton structure and preservation of the hepatocytes' morphological features[44,56]. However, the precise mechanisms by which PEGs interact with the cytoskeleton remains to be elucidated.

FUTURE PERSPECTIVES

Many studies have been designed to prevent mitochondrial dysfunction and to increase endothelial NO generation as a tool for favoring a rapid recovery of liver graft viability after reperfusion. The use of new NO-releasing molecules covalently linked to PEG, as oncotic agents for fatty liver preservation, could help to prevent exacerbated microcirculation in steatotic liver grafts. This practice has been extended to the pharmacological preconditioning of fatty livers, with very promising results (unpublished data).

Moreover, the use of another alternative molecule similar to butanediol mononitrate, conjugated to the carboxylic groups of PEG derivatives by an ester linkage, may provide a new kind of PEG derivative obtained by preparation of PEG-dendron polymers. This may be useful for defining new PEG molecules for supercooling purposes either in combination with MP or not.

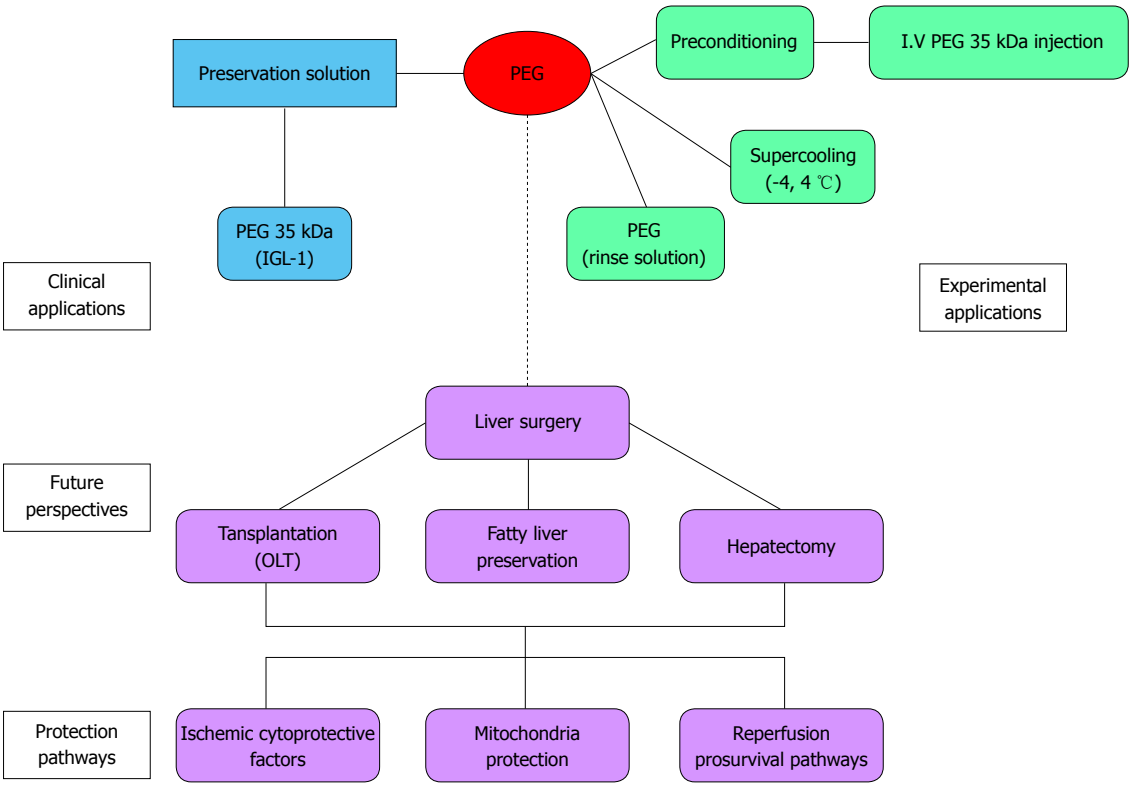

Figure 3 State of the art and future perspectives of polyethylene glycols as effective tool for limiting hepatic ischemia-reperfusion injury. Currently, the clinical applications of polyethylene glycols (PEGs) focus on their use as oncotic agents in IGL-1 solution for liver transplantation (in blue). Recent experimental investigations confirm the value of PEG35 for "supercooling" strategies combined with machine perfusion, as well as its use as a component in rinse solution for liver graft washout. Intravenous PEG35 treatment has also been shown to induce liver protection against cold and warm ischemia-reperfusion injury (IRI) (in green). Future clinical applications should be investigated (in violet). PEG protection mechanisms are characterized by a prevention of mitochondrial damage and by the promotion of several cyto-protective factors during IRI (in violet).

On the other hand, given the potential of PEGs as therapeutic targets for liver protection against IRI, it will be important to identify new PEG derivatives for use in liver transplantation as preconditioning agents. PEG treatment in donors and during reperfusion could minimize the deleterious effects of IRI, such as oxidative stress, cytoskeleton disruption and apoptosis. Moreover, in reduced orthotopic liver transplantation, the prophylactic administration of PEG to donor or/and recipient could contribute to a better liver regeneration of the implanted reduced graft and thus contribute to preventing the small-for-size syndrome present in living-living donor transplantation. In this particular case, the use of new derivatives based on growth factors releasing molecules covalently linked to PEG could offer potential advantages for rapid liver regeneration. Hepatic Growth Factor may be a suitable candidate. The benefits and perspectives of PEGs for limiting liver IRI are summarized in Figure 3.

CONCLUSION

The use of PEG may improve the initial conditions of organs available for transplantation, especially in the case of the most vulnerable ones such as steatotic livers. PEG is a very promising tool for limiting IRI in liver surgery (hepatectomy and transplantation) but further investigation in clinical trials is needed.

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ORIGINAL ARTICLE

Basic Study

c-Jun N-terminal kinase-mediated Rubicon expression enhances hepatocyte lipoapoptosis and promotes hepatocyte ballooning

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Abstract

AIM: To clarify the relationship between autophagy and lipotoxicity-induced apoptosis, which is termed "lipoapoptosis," in non-alcoholic steatohepatitis.

METHODS: Male C57BL/6J mice were fed a high-fat diet (HFD) for 12 wk, after which the liver histology and expression of proteins such as p62 or LC3 were evaluated. Alpha mouse liver 12 (AML12) cells treated with palmitate (PA) were used as an *in vitro* model.

RESULTS: LC3-Ⅱ, p62, and Run domain Beclin-1 interacting and cysteine-rich containing (Rubicon) proteins increased in both the HFD mice and in AML12 cells in response to PA treatment. Rubicon expression was decreased upon c-Jun N-terminal kinase (JNK) inhibition at both the mRNA and the protein level in AML12 cells. Rubicon knockdown in AML12 cells with PA decreased the protein levels of both LC3-Ⅱ and p62. Rubicon expression peaked at 4 h of PA treatment in AML12, and then decreased. Treatment with caspase-9 inhibitor ameliorated the decrease in Rubicon protein expression at 10 h of PA and resulted in enlarged AML12 cells under PA treatment. The enlargement of AML12 cells by PA with caspase-9 inhibition was canceled by Rubicon knockdown.

CONCLUSION: The JNK-Rubicon axis enhanced

lipoapoptosis, and caspase-9 inhibition and Rubicon had effects that were cytologically similar to hepatocyte ballooning. As ballooned hepatocytes secrete fibrogenic signals and thus might promote fibrosis in the liver, the inhibition of hepatocyte ballooning might provide anti-fibrosis in the NASH liver.

Key words: Ballooned hepatocyte; Caspase 9; c-Jun N-terminal kinase; Rubicon; SP600125

Core tip: Autophagy is interrupted in both *in vivo* and *in vitro* non-alcoholic steatohepatitis (NASH) models, and impaired autophagy is mediated by Run domain Beclin-1 interacting and cysteine-rich containing (Rubicon) protein expression *via* c-Jun N-terminal kinase phosphorylation. Rubicon expression appears prior to apoptosis and enhances palmitate toxicity in the hepatocytes, and caspase-9 decreases Rubicon at the protein level during lipoapoptosis. Caspase-9 inhibition with Rubicon expression induces both hepatocyte enlargement and endoplasmic reticulum stress accumulation. The present study extends our knowledge on the precise balance between lipoapoptosis and autophagy *via* Rubicon expression in NASH and reveals a possible pathophysiology of ballooned hepatocytes in NASH.

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INTRODUCTION

The prevalence of non-alcoholic fatty liver disease (NAFLD) is drastically increasing in the Western countries[1]. In some NAFLD patients, persistent inflammation and progressive fibrosis develop in the liver, a condition termed non-alcoholic steatohepatitis (NASH)[2]. As NASH will progress to liver cirrhosis and end-stage liver disease, its pathogenesis needs to be clarified[3]. NASH is histologically characterized by lipid accumulation with inflammation in the liver, which is considered to be a result of lipotoxicity-induced hepatocyte apoptosis[2]. Infiltration of immune cells following hepatocyte death activates hepatic stellate cells, inducing the generation of collagen fiber and the development of liver fibrosis[2]. Therefore, hepatocyte apoptosis is considered to be an important step in development of NASH[4,5].

Lipid-induced apoptosis, which is termed "lipoapoptosis," has been established to cause NASH[6,7]. Saturated free fatty acids (FFAs), such as palmitate (PA), induce hepatocyte lipoapoptosis *via* endoplasmic reticulum (ER) stress, c-Jun N-terminal kinase (JNK) phosphorylation, and mitochondrial dysfunction[8,9]. Recently, it has been reported that saturated FFAs can inhibit autophagy[10] which is a process of cellular self-digestion[11]. In addition, it has been demonstrated that autophagy is inhibited in the NASH liver[12]. As autophagy removes both aggregated proteins and damaged organelles, it maintains organelle quality and prevents apoptosis[11]. It has been reported that impaired autophagy is associated with lipoapoptosis and that chemical agent-induced apoptosis inhibits autophagy[10,13]. Accumulating evidence suggests that inhibition of autophagy is associated with hepatocyte apoptosis in the NASH liver. However, the underlying mechanism remains unclear, and gaining a clearer understanding of the inhibition of autophagy by lipoapoptosis may lead to the development of novel therapeutic strategies for NASH. In line with the above concept, we focused this study on a molecule associated with autophagy inhibition: Run domain Beclin-1 interacting and cysteine-rich containing (Rubicon)[14]. Rubicon has been found to inhibit the fusion of lysosomes to autophagosomes. However, whether Rubicon is expressed in the NASH liver and which role it would play in NASH has not been elucidated yet.

Another histopathological hallmark of NASH is the presence of ballooned hepatocytes. The number of ballooned hepatocytes generally correlates with the severity of liver inflammation and fibrosis in NASH[15-17]. Therefore, the prevalence of ballooned hepatocytes is considered to be a marker of NASH activity. Ballooned hepatocytes can be histologically identified on the basis of several features, such as swelling, central nuclei, rarefied cytoplasm, and Mallory-Denk bodies[18]. Mallory-Denk bodies contain several proteins, such as keratin, chaperones, kinases, and protein degradation machinery[19]. Although proteins in Mallory-Denk bodies should be decomposed *via* the protein degradation pathway, they are accumulated in ballooned hepatocytes. These findings indicate that the protein degradation pathway related to autophagy may be impaired in ballooned hepatocytes.

The aims of the present study were as follows: (1) to confirm the presence of an autophagic state in an *in vivo* NASH model using mice fed a high-fat diet (HFD); (2) to evaluate the intracellular signaling associated with both autophagy and lipoapoptosis; and (3) to clarify the relation between autophagy inhibition and ballooning of hepatocytes during lipoapoptosis.

MATERIALS AND METHODS

Animals

Male 5-wk-old C57BL/6J mice were obtained from Charles River Laboratories (Charles River, Yokohama, Japan) and were maintained on a 12-h light/12-dark cycle in humidity-controlled rooms at 22 ℃ with *ad libitum* access to drinking water. After 1 wk of

habitation, 5 mice were assigned to each of normal chow and HFD (HFD-60, Oriental Yeast CO., Tokyo, Japan) groups and were fed their respective diets for 12 wk. All of the mice were sacrificed using isoflurane anesthesia after overnight fasting at 20 wk of age. All of the animal experiments were approved by the Animal Care and Use Committee of Iwate Medical University (Morioka, Japan; 25-025).

Cells

Alpha mouse liver 12 (AML 12) cells, a hepatocyte cell line from a mouse transgenic for human transforming growth factor α, were kindly supplied by Professor Itaru Kojima, Gunma University. Because autophagic status was confirmed in the NASH mouse model, we employed a cell line generated from the same species, AML 12, to investigate autophagic status in NASH in detail.

Antibodies and reagents: The antibodies used in this study were obtained from the following sources: anti-p62 (1:1000; #5114), anti-LC3 (1:1000; #4108), anti-Rubicon (1:1000; #8465), anti-cleaved caspase-3 (1:1000; #9661), and rabbit anti-phospho-JNK (1:1000; #9251) were obtained from Cell Signaling Technology, Tokyo, Japan; mouse anti-C/EBP homologous protein (CHOP) (1:500; sc-575), mouse anti-phospho-c-Jun (1:1000; sc-822), and goat anti-β-actin (1:1000; sc-1616) were obtained from Santa Cruz Biotechnology, Santa Cruz, CA, United States). Alexa Fluor 488-conjugated IgG was from Life Technologies (Tokyo, Japan). The JNK inhibitor SP600125 (#420119) was obtained from Calbiochem (San Diego, CA, United States). Caspase-9 inhibitor Z-LEHD-FMK (ab142026), and pan-caspase inhibitor QVD-OPh (ab141421) were obtained from Abcam Biochemicals (Tokyo, Japan).

Histological analysis: Liver tissues were collected from the mice, fixed in 10% neutral buffered formalin, paraffin-embedded, and sectioned. Liver specimens were stained with hematoxylin and eosin according to standard procedures. All samples were evaluated in a blinded manner by single pathologist, who scored inflammation, steatosis, and ballooning using non-alcoholic steatohepatitis activity scores[20].

Hepatocyte treatments: Palmitate (PA; #P5585, Sigma Aldrich, Tokyo, Japan), dissolved in isopropanol at a stock concentration of 160 mmol/L, was used to investigate signal transduction during lipoapoptosis. AML12 cells were treated for the indicated time periods or with the indicated concentrations of PA. For inhibition studies, AML12 cells were treated with PA (400 μmol/L or 800 μmol/L) for 4 h or 10 h in the presence or absence of 30 μmol/L SP600125, 20 mol/L z-LEHD-fmk, or 20 μmol/L QVD-OPh. All inhibitors simultaneously added with PA treatment.

Biochemical analysis of the *in vivo* NASH model: Blood samples were obtained from the mice by cardiac puncture. Serum was obtained from the blood by centrifugation at 3000 × *g* for 10 min. The serum levels of alanine transaminase (ALT) were analyzed using an autoanalyzer (JCA-BM2250; JEOL, Tokyo, Japan).

Cell proliferation assay

For AML12 proliferation studies, WST-8 [2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium monosodium salt] (Nacalai Tesque, Kyoto, Japan) incorporation experiments were performed using a microplate reader (Multiskan FC; Thermo Fisher Scientific, Yokohama, Japan). All proliferation assays were performed at least 4 times for each group. The results are presented as the ratio of incorporation in the cells that received the indicated treatment to that in vehicle-treated cells.

Immunocytochemistry for p62

AML12 cells seeded in 6-well plates were fixed with 4% paraformaldehyde in PBS and permeabilized with 0.0125% (w/v) CHAPS in PBS. The primary antibody for anti-p62 was used at a dilution of 1:500. The secondary antibody was Alexa Fluor 488-conjugated IgG, and ProLong Antifade with DAPI (Molecular Probes, Eugene, OR, United States) was used as the mounting medium. Images were acquired using an EVOS microscope (AMF 4300; Life Technologies) with excitation and emission wavelengths of 488 nm and 507 nm, respectively. The experiments were repeated three times.

Measurement of cell size

AML12 cells seeded in 6-well plates received the indicated treatments. Images were acquired with the AMF 4300 microscope. Individual cells were identified and the sizes of 25 randomly selected cells were measured using the Image J software program ver. 1.47 (NIH, Bethesda, MD, United States). The results were presented as the ratio of the size of treated cells to that of control cells.

Quantitative real-time PCR

Total cellular RNA of AML 12 cells was extracted using an RNeasy Mini Kit (Qiagen, Tokyo, Japan) and was reverse-transcribed into complementary (c) DNA with Moloney murine leukemia virus reverse transcriptase (Invitrogen, Camarillo, CA, United States) and random primers (Invitrogen, Camarillo, CA, United States) as described previously[21]. Quantification of the cDNA template was conducted on a 7500 Real-Time PCR system (Applied Biosystems, Waltham, MA, United States) and analyzed using the 7500 Software program. The PCR primers were as follows: for mouse p62 (NM_011018): forward 5'-GAAGCTGCCCTATACCCACA-3' and reverse

5′-TGGGAGAGGGACTCAATCAG-3′ (65 bp); for mouse Rubicon (NM_001200038): forward 5′-GATGGGGAGCGTCTGCTA-3′ and reverse 5′-TCCACAGTCGTCTTCAAATTACC-3′ (74 bp). Mouse Actin (NM_007393.4), amplified with the following primers: forward 5′-TAAGGCCAACCGTGAAAAG-3′ and reverse 5′-ACCAGAGGCATAGGGACA-3′ (104 bp), was used as an internal control. The target mRNA expression levels were expressed relative to Actin for each sample as described previously[21]. The experiment was repeated three times.

Small interfering RNA transfection

The small interfering RNAs (siRNAs) used for the knockdown of endogenous Rubicon protein and the negative control siRNA were purchased from Life Technologies. siRNA (5 pmol) was transfected into cells using Lipofectamine RNAiMAX (Life Technologies), according to manufacturer's instructions. The examinations were performed after 30 h of transfection.

Immunoblotting analysis

Whole cell lysates were prepared as described previously[21]. Equal amounts of protein (10-50 μg) were resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis on 4%-12% acrylamide gels, transferred to polyvinylidene difluoride membranes, and incubated with primary antibodies. Then, the membranes were incubated with the appropriate horseradish peroxidase-conjugated secondary antibodies (BioSource International, Camarillo, CA, United States). Bound antibody was visualized using a chemiluminescent substrate (ECL Prime; Amersham, Buckinghamshire, United Kingdom).

Statistical analysis

All data represent the results of at least three independent experiments and are expressed as the mean ± SD. The differences between the groups were compared using Student's *t*-test and one-way analysis of variance with a *post-hoc* Dunnett's test. *P*-values < 0.05 were considered to be statistically significant.

RESULTS

Autophagic process is inhibited in mice with steatohepatitis

The mice were divided into 2 groups: those fed a normal chow diet (CT, $n = 5$) and those fed an HFD diet (HFD, $n = 5$). After 12 wk, all mice were sacrificed, and liver histology, serum ALT levels, and protein expression in the liver were examined. Body weight, ALT measurement, and histological scores were evaluated in 5 mice per group. Protein expression was evaluated in 3 mice per group. The body weight of the HFD mice was significantly higher than that of the CT mice (Figure 1A). The ALT level was significantly higher in the HFD mice than in the CT mice (Figure 1A; 77 ± 11.0 IU/mL *vs* 25 ± 1.4 IU/mL). Histological analysis revealed the accumulation of lipid droplets in the liver (Figure 1B and C) and an increase in the number of ballooned hepatocytes (Figure 1C) in HFD mice as compared to CT mice. These findings confirmed that the HFD mice in the present study were compatible with a NASH model.

To confirm impairment of the autophagic process in this model, we evaluated autophagic marker protein expression in the liver by immunoblotting analysis. When protein was decomposed through autophagy, p62 was induced, and both p62 and LC3-Ⅱ were subsequently degraded *via* the autophagic process. The expression of p62 in the livers of the HFD mice was higher than in those of CT mice (Figure 1D). However, the expression of both LC3-I and LC3-Ⅱ was also increased in the HFD mice (Figure 1D). Expression of the autophagy inhibitor Rubicon was significantly higher in the HFD mice than in the CT mice (Figure 1D). These findings indicated that autophagy was impaired in the HFD mice. Additionally, we evaluated JNK phosphorylation, which is a key mediator of hepatocyte lipoapoptosis. Protein expression of phosphorylated JNK was increased in the HFD mice as compared to the CT mice.

PA induces cell death in a dose- and time-dependent manner

To investigate the detailed mechanism of the impaired autophagic process, we employed the mouse hepatocyte cell line AML12 in an *in vitro* study. A cell proliferation assay revealed that PA induced cell death in a dose- and a time-dependent manner, confirming PA cytotoxicity to AML12 cells (Figure 2A and C). JNK phosphorylation, ER stress, and cleaved caspase-3 were increased by PA in both a dose- and a time-dependent manner (Figure 2B and D). Therefore, we considered that PA induced apoptosis in AML12 cells.

PA inhibits the autophagic process in AML12 cells and induces Rubicon expression

Next, we evaluated autophagy-related protein expression in PA-treated AML12 cells. PA induced p62 expression after 10 h of incubation with 800 μmol/L of PA in a dose- and a time-dependent manner (Figure 2B and D). The increased p62 level indicated induction of autophagy. If the autophagic process would progress normally, LC3-Ⅱ would be expected to decrease with the decomposition of p62-associated protein aggregates. However, LC3-Ⅱ expression peaked at 6 h of incubation with 800 μmol/L of PA. These findings indicated that PA induced autophagy, but that the autophagic process was interrupted around 6 h of incubation. Since LC3-Ⅱ expression was further decreased at 10 h of incubation (Figure 2D), autophagy progressed with longer incubation times.

In the *in vitro* model, 600 μmol/L of PA and 4 h of incubation with PA increased Rubicon expression, while

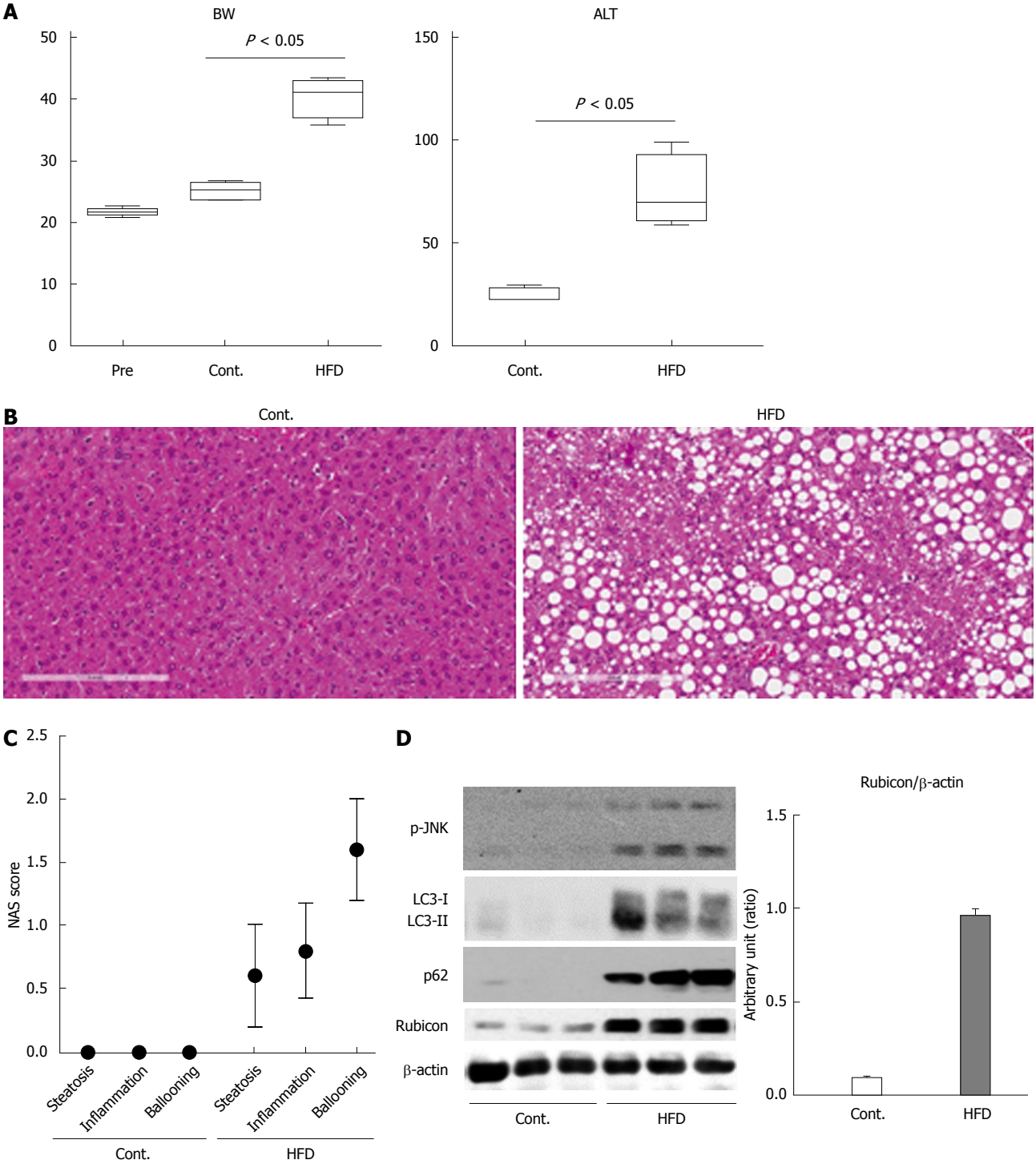

Figure 1 Mice fed high-fat diet show steatosis and inflammation of the liver, and impairment of the autophagic process. A: The body weights of mice at the start of feeding or at 12 wk after feeding with normal chow (Cont.) or high-fat diet (HFD) are presented in the left panel. The serum ALT levels after 12 wk of feeding are shown in the right panel; B: Histology of the liver of control and HFD mice is shown in the left and the right panel, respectively (Hematoxylin and Eosin staining); C: Non-alcoholic steatohepatitis activity scores (NAS) of control and HFD mice; D: Immunoblotting analyses of phosphorylated JNK, p62, LC3, Rubicon, and β-actin. Protein samples were prepared from the liver tissue of each of the control and HFD mice. All of the above experiments were repeated three times and representative results are shown. The quantitative data are presented as the means ± SD.

10 h of incubation with 800 μmol/L of PA decreased Rubicon expression in AML12 cells (Figure 2B and D). Considering the progress of apoptosis at higher dose and longer incubation time of PA, Rubicon expression would be negatively related with apoptotic signals (Figure 2B and D).

PA-induced Rubicon expression inhibits autophagy, resulting in enhanced lipotoxicity in AML12 cells

To check whether PA-induced Rubicon expression was associated with lipoapoptosis, we knocked down Rubicon in AML12 cells using siRNA (siRubicon). After incubation with 800 μmol/L of PA for 4 h, the number

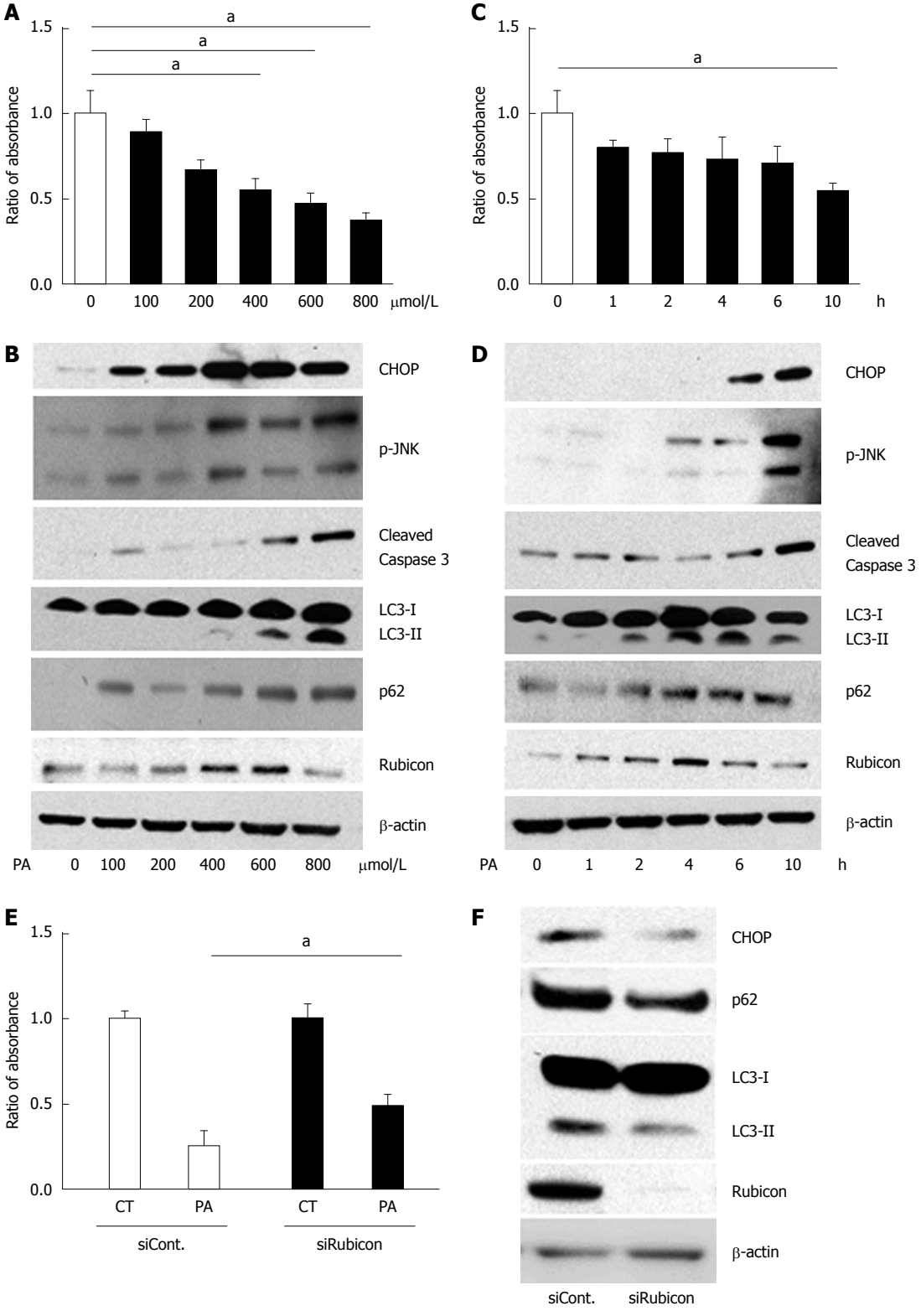

Figure 2 Treatment with palmitate induces apoptosis in a dose- and a time-dependent manner, and initiates but impairs the autophagic process in AML12 cells. A and B: AML12 cells were incubated with PA at the indicated concentrations for 10 h. Untreated AML12 cells were used as the control. A: PA cytotoxicity as evaluated by cell proliferation assay. Living cells are presented as the ratio of absorbance of cells treated with indicated conditions to that of untreated AML12 cells; B: Immunoblotting analyses of CHOP, phosphorylated JNK, cleaved Caspase-3, LC3, p62, Rubicon, and actin. Whole cell lysates were prepared from AML12 cells incubated at the indicated concentrations for 10 h; C and D: AML12 cells were incubated with 800 μmol/L of PA for the indicated periods. Untreated AML12 cells (0 h) were used as the control; C: PA cytotoxicity as evaluated by cell proliferation assay. Living cells are presented as the ratio of absorbance at the indicated incubation time to the absorbance at 0 h; D: Whole cell lysate was prepared from the AML12 cells with 800 μmol/L PA for the indicated incubation times; E and F: AML12 cells were incubated with 800 μmol/L PA for 4 h after transfection with the indicated siRNA: control siRNA (siCont) or Rubicon siRNA (siRubicon); E: PA cytotoxicity as evaluated by cell proliferation assay. Living cells are presented as the ratio of absorbance in AML12 cells with 800 μmol/L PA for 10 h (PA) to that in AML12 control (CT) cells; F: Whole cell lysate was prepared from AML12 cells treated with 800 μmol/L PA with the indicated siRNA. All of the above experiments were repeated three times and representative results are shown. The quantitative data are presented as the mean ± SD; $^{a}P < 0.05$.

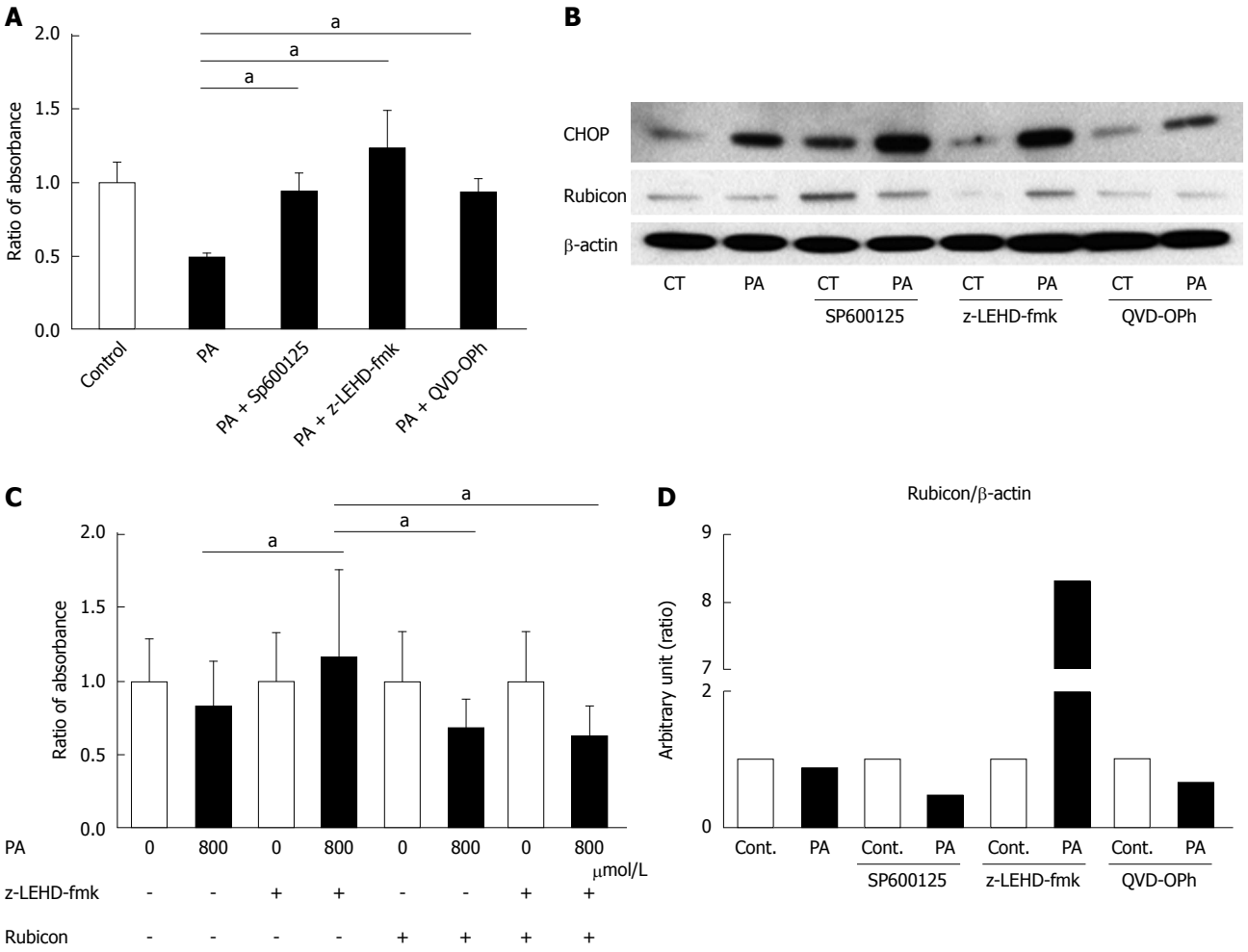

Figure 3 c-Jun N-terminal kinase inhibitor, Caspase-9 inhibitor, and Pan-Caspase inhibitor ameliorate palmitate-induced cell death, and Caspase-9 inhibition attenuates the decrease of Rubicon protein. A: AML12 cells were incubated with 800 μmol/L PA for 10 h in the presence or absence of either 30 μmol/L SP600125, 20 μmol/L LEHD-fmk, or 20 μmol/L QVD-OPh. Untreated AML12 cells were used as the control. PA cytotoxicity was evaluated by cell proliferation assay. Living cells are presented as the ratio of the absorbance at the indicated conditions to that of AML12 without PA treatment; B: Whole cell lysates were prepared from AML12 cells treated with PA (800 μmol/L) for 10 h in the presence or absence of either 30 μmol/L SP600125, 20 μmol/L z-LEHD-fmk, or 20 μmol/L QVD-OPh. Immunoblot analysis of CHOP and Rubicon. β-actin was used as the loading control; C: The size of the AML12 cells after PA treatment (800 μmol/L) for 10 h was compared to that of untreated AML12 cells using the Image J software program. z-LEHD-fmk and/or siRubicon were used for Caspase-9 inhibition or Rubicon knockdown. The difference in cell size under each treatment condition is presented as the ratio to the cell size of the respective controls. All of the above experiments were repeated three times and representative results are shown. The quantitative data are presented as the mean ± SD; $^{a}P < 0.05$.

of viable cells was significantly higher in siRubicon-treated than in control siRNA (siCont)-treated cells (Figure 2E). Expression of both p62 and LC3-Ⅱ was lower in siRubicon- than in siCont-treated cells. Furthermore, PA-induced expression of the ER stress-related transcription factor CHOP was lower in Rubicon-silenced than in control cells (Figure 2F). These results indicated that PA-induced Rubicon expression inhibited autophagy, and that Rubicon enhanced lipotoxicity *via* the impairment of autophagy.

PA-induced Rubicon expression is mediated by JNK signaling

To evaluate the mechanism of PA-induced Rubicon expression, we used pharmacological inhibitors of lipoapoptosis-related molecules: JNK inhibitor (SP600125), caspase-9 inhibitor (Z-LEHD-FMK), and pan-caspase inhibitor (QVD-OPh). All of the inhibitors ameliorated lipotoxicity in the AML12 cells (Figure 3A). Interestingly, SP600125 decreased Rubicon expression in response to PA in comparison to the control (Figures 3B and 4A). The results confirmed that PA induced Rubicon expression and indicated that Rubicon expression was mediated by JNK signaling in both AML12 cells and the human hepatoma cell line HepG2 (Figures 3B, 4A, 4C and 5). JNK inhibition ameliorated PA-induced c-jun phosphorylation and decreased PA-induced protein expression of both p62 and LC3-Ⅱ (Figure 4A). Immunohistochemical analysis revealed that p62 expression was diffuse in the cytoplasm of AML12 cells that were treated with PA, and was repressed by SP600125 inhibitor (Figure 4B). p62 mRNA expression in cells treated with PA and SP600125 was higher than that in cells treated with SP600125 alone, while it was lower than in cells treated with PA alone, as indicated by

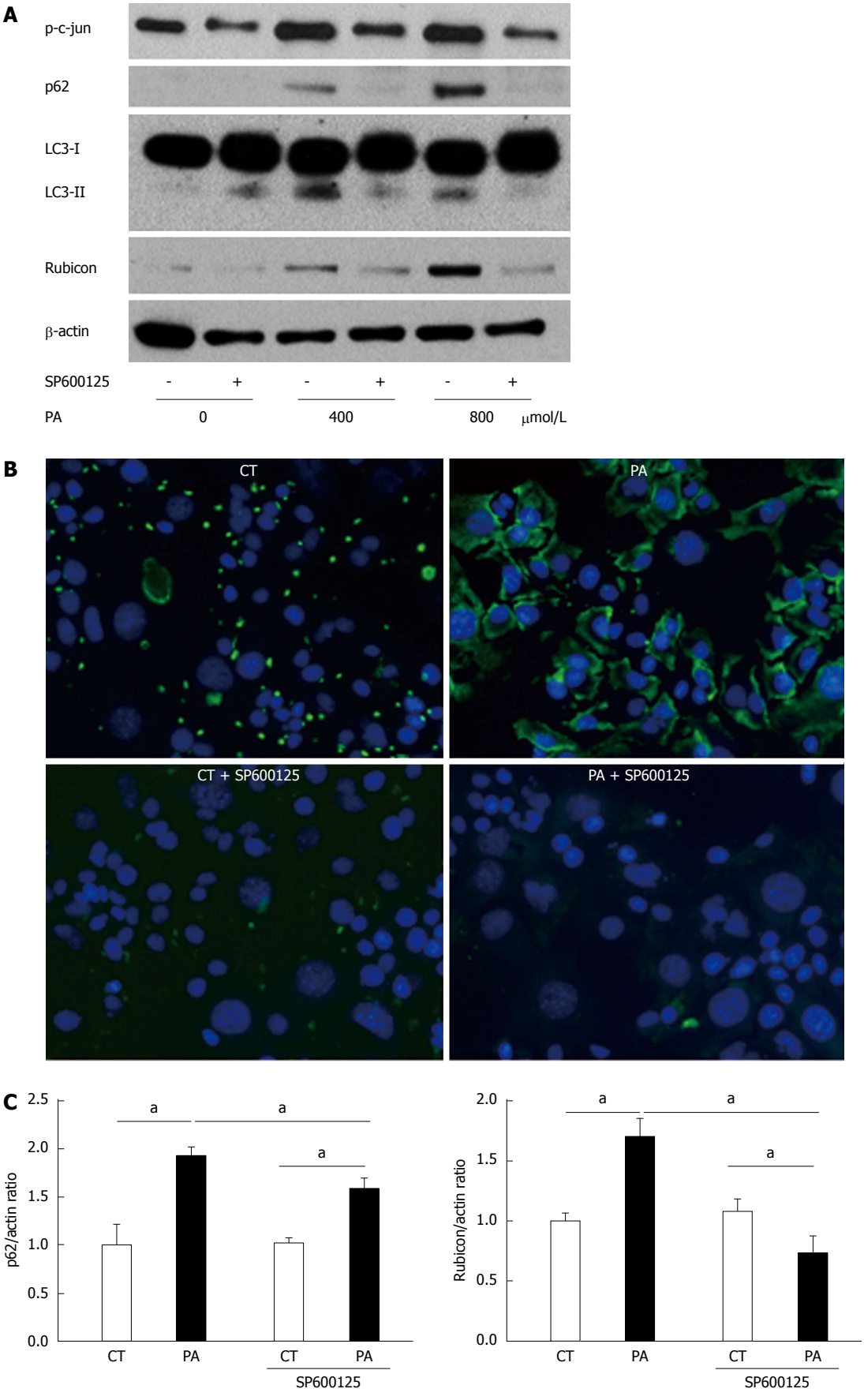

Figure 4 Palmitate induces expression of both p62 and Rubicon, and PA-induced Rubicon expression is mediated by c-Jun N-terminal kinase phosphorylation. A: Whole cell lysates were prepared from AML12 cells treated with PA (400 or 800 μmol/L) for 4 h in the presence or absence of SP600125. Immunoblotting analyses were performed for p62, LC3, and Rubicon. β-actin was used as the loading control; B: AML12 cells were incubated with 800 μmol/L PA for

4 h in the presence or absence of 30 μmol/L SP600125. p62 antibody was used as the primary antibody, and Alexa Fluor® 488-labeled secondary antibody was used for detecting p62 antibody. Samples were visualized using an EVOS microscope; C: Total RNA was prepared from AML12 cells treated with PA (800 μmol/L) for 4 h in the presence or absence of 30 μmol/L SP600125. Vehicle-treated cells were used as the control (CT). Both p62 and Rubicon mRNA were quantified by RT-qPCR, normalized to Actin, and expressed as the fold-change *vs* control cells without SP600125. All of the above experiments were repeated three times and representative results are shown. The quantitative data are presented as the mean ± SD; $^{a}P < 0.05$ *vs* control.

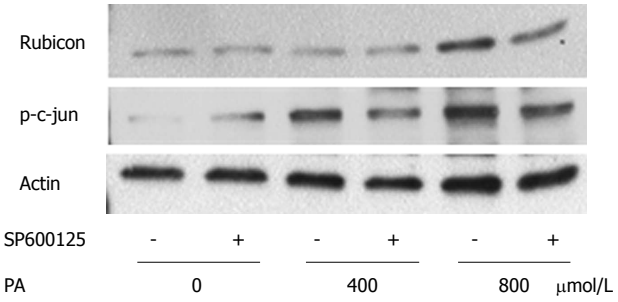

Figure 5 PA-induced Rubicon expression was mediated by c-Jun N-terminal kinase phosphorylation. Whole cell lysates were prepared from HepG2 cells treated with PA (400 or 800 μmol/L) for 4 h in the presence or absence of SP600125. Immunoblotting analyses were performed for p-c-jun and Rubicon. Actin was used as the loading control.

RT-qPCR (Figure 4C). These data indicated that p62 expression was mediated by both JNK-independent and JNK-dependent signals. When PA-induced Rubicon expression was inhibited by siRNA or JNK inhibitor, both PA-induced LC3-Ⅱ expression and p62 expression decreased (Figures 2F and 4A).

PA-induced Rubicon expression decreases with progression of lipoapoptosis, which might be affected by caspase-9 activation

We observed that Rubicon expression decreased at the protein level under the condition of lipotoxicity (Figure 2B and D). Because apoptosis was correlated with autophagy, we hypothesized that the lipoapoptosis signaling would affect Rubicon expression. When inhibitors of lipoapoptosis were used in AML12 cells that were treated with PA, Z-LEHD-fmk maintained a greater amount of Rubicon protein than both AML12 with PA alone and AML12 with other inhibitors (Figure 3B). Because the pan-caspase inhibitor QVD-OPh did not ameliorate the decreased Rubicon expression during lipoapoptosis, the cause of the decreased Rubicon expression was upstream of the executioner caspases (caspase-3 and -7). We considered that caspase-9 activation was associated with inhibition of Rubicon expression during lipoapoptosis.

Caspase-9 inhibition induces ER stress accumulation and hepatocyte enlargement, which is required for Rubicon expression

Both caspase-9 deletion and JNK phosphorylation have been previously reported to be associated with hepatocyte ballooning[22]. Therefore, we suspected that Rubicon also affects hepatocyte morphology. To test this hypothesis, the effect of lipotoxicity on cell size was evaluated under the conditions of caspase-9 inhibition and/or Rubicon knockdown. The size of PA-treated AML12 cells was significantly larger in the z-LEHD-fmk group than in the PA-alone group (Figure 3C). In contrast, Rubicon knockdown ameliorated hepatocyte enlargement in the PA-treated z-LEHD-fmk group (Figure 3C). In addition, caspase-9 inhibition induced accumulation of CHOP protein (Figure 3B).

DISCUSSION

NASH is characterized by both inflammation and fibrosis in the liver. Lipotoxic insults lead to persistent hepatocyte apoptosis, resulting in fibrosis in the liver. During these processes, ER stresses, such as the unfolding of proteins, accumulate in the hepatocytes as a result of apoptotic insults[23,24]. Autophagy can inhibit the accumulation of these insults by decomposing the aggregated proteins and removing the damaged organelles[11,25]. Therefore, autophagy can be considered not only a process of energy supply but also a survival mechanism that protects against lipoapoptosis. When the autophagic process is interrupted, insults accumulate and damaged cells are led to lipoapoptosis (Figure 2B, D and F).

JNK phosphorylation is a key signal of hepatocyte lipoapoptosis[5,9,21]. Previous studies have shown that a JNK-dependent pathway leads to inflammation and fibrosis in the NASH liver[26], and that JNK phosphorylation is associated with hepatocyte ballooning, which is a hallmark of NASH[22]. The present study demonstrated that JNK phosphorylation was involved in the induction of lipoapoptosis as well as in the inhibition of autophagy *via* Rubicon expression, revealing a new role of JNK signaling in hepatocyte lipoapoptosis. When Rubicon was knocked down, ER stress-induced lipoapoptosis was decreased. Impaired autophagy *via* Rubicon induced the accumulation of ER stress, consequently enhancing lipotoxicity. Indeed, Rubicon expression appeared before apoptosis (Figure 2B, D and F). These data suggest that impairment of autophagy occurs before lipoapoptosis. Thus, decreasing the expression of Rubicon could be an important early intervention to prevent NASH development.

As lipoapoptosis progressed, Rubicon expression decreased (Figure 2B and D). The pan-caspase inhibitor did not ameliorate the decrease of Rubicon protein (Figure 3B). In contrast, caspase-9 inhibitor Z-LEHD-fmk attenuated the decrease of Rubicon expression (Figure 3B). Thus, we speculate that caspase-9 might decompose Rubicon. Interestingly, caspase-9 has been suspected to be a key molecule in hepatocyte

ballooning. Levels of caspase-9 were found to be lower in ballooned than in normal hepatocytes in the NASH liver[22]. Furthermore, caspase-9-knockdown Huh-7 cells showed biological similarities with ballooned hepatocytes, such as intracellular lipid accumulation, accumulation of ER stress, and lipotoxic insult-induced sonic hedgehog signaling *via* JNK phosphorylation[22]. PA treatment upon caspase-9 inhibition in AML12 induced the accumulation of CHOP protein (Figure 3B). In contrast, a recent report demonstrated that pan-caspase inhibitor improved inflammation and fibrosis in the liver, and decreased the number of ballooned hepatocytes in HFD mice[27]. These data indicate that caspase-9 might play an important role in the formation of ballooned hepatocytes. According to the present study, caspase-9 inhibition has cytological effects reminiscent of hepatocyte ballooning, such as hepatocyte enlargement and accumulation of ER stress. Interestingly, knockdown of Rubicon counteracted the caspase-9 inhibition-induced hepatocyte enlargement. These data indicate that Rubicon expression and caspase-9 inhibition are required for the enlargement of hepatocytes. We hypothesize that caspase-9, JNK, and Rubicon are key molecules for hepatocyte ballooning in the NASH liver.

The present study revealed a number of significant findings: (1) autophagy is interrupted in both *in vivo* and *in vitro* NASH models; (2) PA-induced lipoapoptosis inhibits autophagy by Rubicon expression *via* JNK phosphorylation; (3) Rubicon expression appears prior to apoptosis and enhances PA toxicity in the hepatocytes; (4) Caspase-9 decreases Rubicon at the protein level during lipoapoptosis; and (5) Caspase-9 inhibition with Rubicon expression induces hepatocyte enlargement. We conclude that PA-induced JNK phosphorylation directly induced apoptosis and indirectly enhanced apoptosis *via* the inhibition of autophagy by Rubicon expression, and that Rubicon, caspase-9, and JNK are key molecules for lipotoxic insult-induced hepatocyte ballooning.

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COMMENTS

Background

Lipoapoptosis in hepatocytes leads to infiltration of immune cells, activation of hepatic stellate cells, and generation of collagen fibers in the liver. Thus, prevention of hepatocyte apoptosis is considered to be a therapeutic strategy for non-alcoholic steatohepatitis (NASH). As apoptosis and autophagy negatively interact with each other, we focused this study on the Rubicon protein, a negative regulator of autophagy, in the NASH liver.

Background

Ballooned hepatocytes, which are a well-known hallmark of NASH disease severity, accumulate toxic insult. Hepatocytes in which caspase-9 was knocked down with lipotoxicity demonstrated a number of similarities with ballooned hepatocytes, such as accumulation of protein degradation machinery or secretion of fibrogenic signal. The interaction between exceeded apoptosis, impaired autophagy, and ballooning of hepatocytes has never been elucidated. The detailed interaction between these signals may be a therapeutic target of the NASH liver.

Innovations and breakthroughs

This is the first report to show that Rubicon is overexpressed in hepatocyte lipoapoptosis, Rubicon enhances lipotoxicity, caspase-9 decomposes Rubicon protein, and caspase-9 inhibition leads to ballooning of hepatocytes. Although interactions between apoptosis and autophagy have been previously reported elsewhere, the present study demonstrates the role of each of Rubicon and caspase-9 during hepatocyte lipoapoptosis.

Applications

Rubicon is induced during lipoapoptosis by c-Jun N-terminal kinase phosphorylation and enhances apoptosis *via* autophagy inhibition. Since caspase-9 inhibition and Rubicon overexpression led to hepatocyte ballooning, Rubicon may be associated with ballooning in hepatocytes. As the prevalence of ballooned hepatocytes correlates with disease severity in the NASH liver, control of Rubicon has potential for NASH therapy.

Terminology

Lipoapoptosis is a term for lipotoxicity-induced apoptosis. Lipoapoptosis in hepatocytes induces the infiltration of immune cells into the NASH liver, which leads to the generation of collagen fibers. Thus, lipoapoptosis is considered a leading cause of NASH development.

Peer-review

This manuscript contains fascinating and novel data for understanding of pathophysiology of NASH.

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ORIGINAL ARTICLE

Case Control Study

TCF7L2 rs7903146 polymorphism is associated with gastric cancer: A case-control study in the Venezuelan population

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Author contributions: Chiurillo MA and Valderrama E designed the research; Valderrama E collected material and clinical data from patients; Torres K and Labrador L performed the assays; Chiurillo MA, Torres K and Labrador L analyzed the data; Chiurillo MA wrote the paper.

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Abstract

AIM: To explore the association between *TCF7L2* rs12255372 and rs7903146 single nucleotide polymorphisms (SNPs) and gastric cancer risk in Venezuelan patients.

METHODS: We performed a case-control study including 122 paraffin-embedded archived intestinal-type gastric cancer samples and 129 biopsies obtained by superior endoscopy from chronic gastritis patients. Gastric cancer samples were classified according the degree of carcinoma differentiation. Genomic DNA was extracted from tissues, and the two SNPs of *TCF7L2* gene (rs12255372 and rs7903146) were genotyped by polymerase chain reaction-restriction fragment length polymorphism reactions. Multiple regression analysis with adjustments for age and gender were performed and best-fitting models of inheritance were determined.

Statistic powers were post-hoc calculated.

RESULTS: After adjusting for age and sex the *TCF7L2* rs7903146 TT genotype was associated with gastric cancer risk under the recessive genetic model (OR = 3.11, 95%CI: 1.22-7.92, *P* = 0.017). We further investigated the distribution of rs12255372 and rs7903146 genotypes according gastric cancer stratified by degree of differentiation, and we observed that carriers of rs7903146 T allele (CT + TT *vs* CC) had a significantly increased risk of moderate/well differentiated gastric cancer (dominant model, OR = 2.55, 95%CI: 1.35-4.80, *P* = 0.004), whereas the rs7903146 TT genotype was associated with poorly differentiated gastric cancer in the recessive model (OR = 3.65, 95%CI: 1.25-10.62, *P* = 0.018). We did not find association between rs12255372 SNP and the susceptibility of developing gastric cancer.

CONCLUSION: *TCF7L2* rs7903146 polymorphism is associated with gastric cancer risk in the Venezuelan population, and could be related to determine the degree of differentiation of tumor cells.

Key words: Gastric cancer; Wnt/β-catenin pathway; *TCF7L2*; Single nucleotide polymorphism; Genetic susceptibility

Core tip: TCF7L2 transcription factor plays an important role in transcriptional activation induced by the Wnt/β-catenin pathway, which is reported to be associated with human carcinogenesis and it is found activated in 30%-50% of gastric cancers. *TCF7L2* polymorphisms rs12255372 and rs7903146 are associated with a significant risk of type 2 diabetes and in the development of several types of cancer. This is the first report of association of these *TCF7L2* variants with the risk of gastric cancer. We conducted a case-control study including samples of Venezuelan patients in which the rs7903146 T allele was found associated with the risk of gastric cancer, suggesting its use as potential diagnosis biomarker in patients with this malignance.

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INTRODUCTION

The transcription factor 7-like 2 (*TCF7L2* or *TCF-4*) gene, is located on the long arm of chromosome 10q25.3[1]. Moreover, the TCF7L2 protein is a high mobility group box-containing transcription factor, which acts as an effector of the Wnt/β-catenin signaling pathway, therefore playing a pivotal role in cell development and growth regulation[2-4].

The TCF7L2 protein is also involved in blood glucose homeostasis, and their gene variants rs7903146 (C>T) and rs12255372 (G>T) [it is in high linkage disequilibrium (LD) with rs7903146] are among the most significant genetic factors influencing the risk for type 2 diabetes (T2DM)[5-7]. Although the specific role of TCF7L2 in the development of T2DM is still being investigated, evidence indicates that alterations in the Wnt signaling pathway affect insulin secretion through the reduction of the GLP-1 production[8,9]. Moreover, aberrant Wnt signaling is involved in the pathogenesis of numerous types of human cancers[10], and particularly to the development and progression of gastric cancer[11].

Although with contradictory conclusions, several studies have studied the association between *TCF7L2* rs7903146 and rs12255372 single nucleotide polymorphisms (SNP) with susceptibility to several types of cancer, including in the prostate, breast, colon, rectum, lung and ovary[11-21]. However, to the best of our knowledge, the participation of these SNPs in the susceptibility of gastric cancer has not been evaluated yet.

Here, we present a case-control study carried out to evaluate the role of rs7903146 and rs12255372 polymorphisms in the risk of gastric cancer in the Venezuelan population where gastric cancer is the leading cause of death due to cancer (http://www.mpps.gob.ve/).

MATERIALS AND METHODS

Subjects

A total number of 122 gastric cancer cases and 129 controls were included in this study. The group of cases consisted of paraffin-embedded intestinal-type gastric cancer samples according to Laurén's classification, which were obtained from the Pathology Department Service of the Hospital Antonio María Pineda (HAMP), Barquisimeto, Venezuela. Tumor samples were classified into well differentiated, moderately differentiated and poorly differentiated cancer depending on the degree of differentiation of the cancerous cells[22].

Patients diagnosed with chronic gastritis without evidence of gastric cancer constituted the control group. Chronic gastritis samples obtained from patients with criteria for indication of endoscopy (Gastroenterology Service of the HAMP) were evaluated according to the Sydney classification system in regard to the presence and degree of atrophic gastritis, granulocytic infiltration and lymphocytic infiltration. Two independent experts in pathology from the Department of Pathology (HAMP) evaluated all biopsies. The Bioethics Committee of the School of Health Sciences, Universidad Centroccidental

Table 1 Characteristics of the study population *n* (%)

Variables	Controls	Gastric cancer
Overall	129	122
Sex[a]		
Male	63 (48.8)	88 (72.1)
Female	66 (51.2)	34 (27.9)
Age[a], mean ± SD (yr)	58.81 ± 9.99	62.32 ± 14.29
Histological differentiation		
Well		14 (11.5)
Moderated		56 (45.9)
Poor		52 (42.6)

[a]$P < 0.05$ *vs* control.

Lisandro Alvarado (UCLA) approved this study, and all patients gave their written informed consent to participate in the study.

Genotyping

Genomic DNA was extracted from paraffin sections of patients' tissues by MagneSil® Genomic Fixed Tissue System (Promega, United States), and from endoscopic biopsies using the Wizard Genomic DNA Purification kit (Promega, United States) following the manufacturer's instructions.

SNPs genotyping was achieved by polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) method. We designed primers to detect both polymorphisms using software DNAMAN version 7.212 (Lynnon Corporation) in order to amplify shorter DNA fragment, which resulted more efficient when DNA extracted from paraffin-embedded tissues was used as template. The primer sets included in the reactions to amplify the rs7903146 and rs12255372 polymorphisms were: forward 5'-ACAATTAGAGAGCTAAGCACTTTTTAGGTA-3'[23], reverse 5'-CTAAGTTACTTGCCTTCCCTG-3, and forward 5'-GAAAGTGTATTGCTATGTCCAG-3', reverse 5'-CAGAGGCCTGAGTCATTATCA-3', respectively.

PCR reactions were performed in 25 μL reaction volume containing 1-2 μL of genomic DNA, 1 × Green GoTaq® Flexi Buffer, 1.5 μmol/L $MgCl_2$, 0.2 μmol/L dNTPs, 0.6 μmol/L of each primer and 1.25 U of GoTaq DNA Polymerase (Promega, United States). The amplification conditions were: 3 min at 95 ℃; then 35 cycles of 20 s at 95 ℃, 30 s at 59 ℃ (for rs12255372)/ 57 ℃ (for rs7903146), and 30 s at 72 ℃; followed by a final extension cycle of 5 min at 72 ℃. To perform the allelic assignment PCR products were incubated at 37 ℃ overnight with a restriction enzyme, *Rsa*I (New England Biolabs, United States) for rs7903146 and *Tsp*509I (New England Biolabs, United States) for rs12255372. For rs7903146, the *Rsa*I enzyme produces two fragments of 81-bp and 29-bp with the C allele, whereas the T allele is not cleaved, and its PCR products remains of 110-bp. Fragments with the rs12255372 G allele are not cleaved and remains of the original size (119-bp), moreover, the T allele PCR product results in two fragments of 85-bp and 34-bp after incubating with *Tsp*509I enzyme. PCR products and restriction fragments were analyzed on 3% agarose gel electrophoresis with ethidium bromide staining. To validate the RFLP-PCR assays we randomly select 20% of the samples to carry out DNA nucleotide sequencing. Furthermore, 30 samples of each genotype were re-genotyped and a concordance of 100 was observed.

Statistical analysis

P values and ORs with 95%CI were calculated using multiple regression analysis adjusted by age and gender. A *P* value of < 0.05 was considered statistically significant when comparing differences among groups, and the analyses were carried out using the SPSS 11.0 package software (SPSS Inc., United States). We used two-sided χ^2 test to determine if genetic distributions were in Hardy-Weinberg equilibrium. The analysis for LD was estimated using the Arlequin software version 3.5.1.2. The comparisons of genotype distributions of polymorphisms were performed following the codominant, dominant and recessive inheritance models, taking into account known risk alleles. To determine the best-fitting models we used the Akaike information criterion (AIC). Post-hoc power analyses were calculated using G*Power software (version 3.1). A biomedical statistician from the UCLA performed statistical review of the study. All authors accessed the data of the study and agreed final version of the manuscript.

RESULTS

The characteristics of the cases and controls are summarized in Table 1. Gastric cancer tissues included 14 well- (11.5%), 56 moderately (45.9%), and 52 poorly (42.6%) differentiated carcinoma. As shown in Table 2, the genotype distributions of rs7903146 and rs12255372 SNPs in the control group were in Hardy-Weinberg equilibrium ($P > 0.05$). The differences between the groups with respect to the distribution by age and sex were significant; therefore we adjusted for these variables in the subsequent analyses of the relationship between polymorphisms and gastric cancer susceptibility. Rs7903146 and rs12255372 SNPs were in moderate LD ($D' < 0.644$; $r^2 < 0.33$). Among gastric cancer cases five samples did not amplify with the rs12255372 primer set.

The rs7903146 TT genotype was significantly associated with increased risk of gastric cancer under both the codominant (OR = 3.61, 95%CI: 1.36-9.61, $P = 0.01$) and the recessive model (OR = 3.11, 95%CI: 1.22-7.92, $P = 0.017$), after adjustment for age and gender (Table 2). However, the recessive model of inheritance was suggested as the best-fitting one by the AIC score.

Furthermore, we evaluated the genotype distribution of rs7903146 and rs12255372 SNPs in the

Table 2 Association of *TCF7L2* rs7903146 and rs12255372 polymorphisms with gastric cancer *n* (%)

SNP	Risk allele	HWE (control), *P* value	Inheritance model	Genotype	Control	Gastric cancer	OR (95%CI)[1]	*P* value[1]
rs7903146	T	0.741			*n* = 129	*n* = 122		
				CC	73 (56.6)	56 (45.9)	1	
			Codominant	CT	49 (38.0)	48 (39.3)	1.30 (0.75–2.25)	0.345
				TT	7 (5.4)	18 (14.8)	3.61 (1.36–9.61)	0.010
			Dominant	CC	73 (56.6)	56 (45.9)	1	
				CT + TT	56 (43.4)	66 (54.1)	1.58 (0.94–2.64)	0.082
			Recessive	CC + CT	122 (94.6)	104 (85.2)	1	
				TT	7 (5.4)	18 (14.8)	3.11 (1.22–7.92)	0.017
rs12255372	T	0.053			*n* = 129	*n* = 117		
				GG	85 (65.9)	78 (66.7)	1	
			Codominant	GT	35 (27.1)	32 (27.3)	1.06 (0.59–1.92)	0.841
				TT	9 (7.0)	7 (6.0)	1.11 (0.38–2.25)	0.849
			Dominant	GG	85 (65.9)	78 (66.7)	1	
				GT + TT	44 (34.1)	39 (33.3)	1.06 (0.61–1.83)	0.839
			Recessive	GG + GT	120 (93.0)	110 (94.0)	1	
				TT	9 (7.0)	7 (6.0)	1.02 (0.36–2.90)	0.972

[1]Adjusted by age and gender; Statistical power (1-β) was calculated for all observed *P* values. HWE: Hardy-Weinberg equilibrium; SNP: Single nucleotide polymorphism.

Table 3 Distribution of *TCF7L2* rs7903146 and rs12255372 single nucleotide polymorphisms according to the degree of histological differentiation of gastric cancer *n* (%)

SNP	Inheritance model	Genotype	Control	M/W GC	OR (95%CI)[1]	*P* value[1]	P GC	OR (95%CI)[1]	*P* value[1]
rs7903146			*n* = 129	*n* = 70			*n* = 52		
		CC	73 (56.6)	25 (35.7)	1		31 (59.6)	1	
	Codominant	CT	49 (38.0)	36 (51.4)	2.33 (1.20–4.51)	0.012	12 (23.1)	0.57 (0.26–1.23)	0.152
		TT	7 (5.4)	9 (12.9)	5.70 (1.60–20.3)	0.007	9 (17.3)	2.99 (0.99–8.92)	0.050
	Dominant	CC	73 (56.6)	25 (35.7)	1		31 (59.6)	1	
		CT + TT	56 (43.4)	45 (64.3)	2.55 (1.35–4.80)	0.004	21 (40.4)	0.88 (0.45–1.71)	0.708
	Recessive	CC + CT	122 (94.6)	61 (87.1)	1		43 (82.7)	1	
		TT	7 (5.4)	9 (12.9)	2.93 (0.99–8.68)	0.053	9 (17.3)	3.65 (1.25–10.6)	0.018
rs12255372			*n* = 129	*n* = 66			*n* = 51		
		GG	85 (65.9)	38 (57.6)	1		40 (78.4)	1	
	Codominant	GT	35 (27.1)	24 (36.3)	1.44 (0.39–5.36)	0.589	8 (15.7)	0.51 (0.21–1.21)	0.129
		TT	9 (7.0)	4 (6.1)	1.63 (0.83–3.21)	0.156	3 (5.9)	0.81 (0.20–3.26)	0.770
	Dominant	GG	85 (65.9)	38 (57.6)	1		40 (78.4)	1	
		GT + TT	44 (34.1)	28 (42.4)	1.58 (0.84–3.00)	0.158	11 (21.6)	0.56 (0.26–1.22)	0.145
	Recessive	GG + GT	120 (93.0)	62 (93.9)	1		48 (94.1)	1	
		TT	9 (7.0)	4 (6.1)	1.15 (0.33–4.05)	0.823	3 (5.9)	0.87 (0.22–3.43)	0.844

[1]Adjusted by age and gender; Statistical power (1-β) was calculated for all observed *P* values. W/M: Well and moderately differentiated gastric cancer; P GC: Poorly differentiated gastric cancer; SNP: Single nucleotide polymorphism.

gastric cancer samples divided according to the degree of histological differentiation of tumors (Table 3). To conduct these analyses, samples of well and moderately differentiated gastric carcinoma were gathered in a single group (57.4%; 70/122). Compared with CC genotype, rs7903146 CT heterozygous and TT homozygous genotypes, as well as the combined genotype CT + TT, had a significantly increased risk for moderate/well differentiated gastric cancer (ORs = 2.33, 5.70 and 2.55, respectively), adjusted by age and gender (Table 3).

Moreover, rs7903146 TT genotype was associated with poorly differentiated gastric cancer in the recessive model (OR = 3.65, 95%CI: 1.25-10.62, *P* = 0.018). However, in these analyses the AIC score suggested the dominant model (CT + TT *vs* CC) as the best-fitting one in the comparisons of gastritis samples with both groups of gastric cancer. Importantly, the post-hoc analysis revealed that the study has acceptable statistical power (1 - β > 0.80 at type I level of 0.05) to support the observed significant associations for rs7903146 genotypes. Finally, we did not identify any significant difference in genotype frequencies of rs12255372 SNP between gastric cancer and gastritis groups, even taking into account the degree of tumor differentiation (Tables 2 and 3).

DISCUSSION

Gastric cancer is a multifactorial disease that results

from the complex interplay of several host, bacterial, and environmental factors acting at gastric mucosa, that lead to the deregulation of many oncogenic signaling pathways[24]. Among them, the Wnt/β-catenin pathway is observed active in 30% to 50% of gastric cancer tissues and in several types of gastric cancer cell lines[25-27].

Available data confirmed that gain-of-function mutations in Wnt activators, as *CTNNB1* (the gene that encodes β-catenin protein), and/or inactivating mutations and promoter hypermethylation in tumor suppressor genes (*e.g.*, *APC*) lead to nuclear β-catenin accumulation and constitutive activation of the Wnt pathway in gastric cancer[11]. In the nucleus, free β-catenin binds TCF7L2 transcription factors, thereby modulating expression of genes (*e.g.*, *c-myc*) implicated in proliferation, inhibition of apoptosis, tissue invasion and metastasis[28]. It is known that alterations in *TCF7L2* gene and its expression, which also have a role in T2DM susceptibility, mediate carcinogenic effects through increased expression of *c-myc* and *cyclin D*[12,29]. Moreover, while several mutations in Wnt pathway components, such as *APC*, *CTNNB1*, *β-TrCP*, *Axin1* and *Axin2* have been implicated in gastric cancer[11,30], the only *TCF7L2* alterations so far reported in gastric tumors are somatic frameshift mutations in the exon 14 of the gene[31,32].

In our work the rs7903146 TT genotype was related with the risk of gastric cancer in the codominant and recessive models (OR = 3.61 and 3.11, respectively). Interestingly, the T allele at rs7903146 *TCF7L2* is the most correlated genetic variant with T2DM susceptibility, which has also been associated with the risk for several types of cancer.

Although, case-control studies involving *TCF7L2* rs7903146 and rs12255372 polymorphisms and cancer susceptibility have shown contradictory results, recent meta-analyses revealed that the *TCF7L2* rs7903146 SNP is associated significantly with the risk of breast, prostate and colon cancer, as well as between the rs12255372 polymorphism and the susceptibility of breast cancer[33-36]. Moreover, the rs12255372 SNP was not found associated with gastric cancer risk in this work.

The mechanism involving *TCF7L2* gene polymorphisms with cancer risk remains unclear, however the fact that *TCF7L2* gene product participates in Wnt/β-catenin signaling pathway allows to envisage their participation in carcinogenesis. Moreover, recent evidence suggests that *TCF7L2* polymorphisms may be related with changes in expression levels of its gene product. Gaulton *et al*[37] showed that the *TCF7L2* intronic SNP rs7903146 is located in an islet FAIRE (Formaldehyde-Assisted Isolation of Regulatory Elements)-enriched site and affects chromatin state and enhancer function. Furthermore, *TCF7L2* rs7094463, rs10749127, and rs11196224 SNPs, which correlate with recurrence of prostate cancer in patients that were treated with radical prostatectomy, are located in potential transcriptional regulatory regions[38]. It is suggested that these DNA polymorphisms can alter the transcription factor binding sites and thus affect the *TCF7L2* expression level.

Our results also suggest that the rs7903146 polymorphism (T allele) may be involved in defining the degree of differentiation of tumor cells. However, we cannot rule out that the small number of gastric adenocarcinoma samples with the *TCF7L2* rs7903146 TT genotype could drive the observed association with the degree of differentiation of tumor cells when it was used codominant and recessive models. The association of genetic and epigenetic alterations with subtypes of gastric carcinoma suggests particular interactions for the development of a gastric tumor with specific degree of differentiation[39-41]. Furthermore, due to the aggressiveness of gastric cancer has been associated with the degree of differentiation of tumor cells, the evaluation of this aspect should be considered in the management of gastric cancer[22,42].

In conclusion, this is the first study that examines the role of *TCF7L2* rs7903146 and rs12255372 SNPs related to susceptibility of gastric cancer in a Venezuelan high-risk population. Moreover, after adjustment for age and gender, we found that the rs7903146 polymorphism was significantly associated with the genetic susceptibility to gastric cancer in the Venezuelan population. This work gives additional support to understanding the participation of alterations in the Wnt/β-catenin pathway in the gastric carcinogenesis, and could represent a contribution to the identification of novel biomarkers for detection and/or monitoring progression or recurrence of gastric cancer. However, although the post-hoc analysis indicates that there was enough statistical power to support the observed associations, analysis of a larger sample size is needed to corroborate the participation of the *TCF7L2* polymorphisms in the risk of gastric cancer.

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COMMENTS

Background

TCF7L2 is an effector of the Wnt/β-catenin signaling pathway, whose deregulation can result in human carcinogenesis. *TCF7L2* variants rs12255372 and rs7903146 besides being associated with risk of type 2 diabetes have been involved in the development of several cancers.

Research frontiers

Gastric cancer continues being one of the leading causes of cancer-related death in the world. *TCF7L2* variants rs12255372 and rs7903146 have been

related to the development of some types of cancer, but their participation in the susceptibility of gastric cancer has not been evaluated yet.

Innovations and breakthroughs

Its results indicate that the rs7903146 T allele is associated with gastric cancer risk in Venezuelan population, suggesting its use as potential diagnosis biomarker in patients with this malignance.

Applications

Potential use of rs7903146 as diagnosis biomarker in patients with this gastric cancer.

Peer-review

The paper is well organized and the results are very straightforward and clear.

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ORIGINAL ARTICLE

Observational Study

Trends and predictions for gastric cancer mortality in Brazil

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Abstract

AIM: To analyze the effect of age-period and birth cohort on gastric cancer mortality, in Brazil and across its five geographic regions, by sex, in the population over 20 years of age, as well as make projections for the period 2010-2029.

METHODS: An ecological study is presented herein,

which distributed gastric cancer-related deaths in Brazil and its geographic regions. The effects of age-period and birth cohort were calculated by the Poisson regression model and projections were made with the age-period-cohort model in the statistical program R.

RESULTS: Progressive reduction of mortality rates was observed in the 1980's, and then higher and lower mortality rates were verified in the 2000's, for both sexes, in Brazil and for the South, Southeast and Midwest regions. A progressive decrease in mortality rates was observed for the Northeast (both sexes) and North (men only) regions within the period 1995-1999, followed by rising rates.

CONCLUSION: Regional differences were demonstrated in the mortality rates for gastric cancer in Brazil, and the least developed regions of the country will present increases in projected mortality rates.

Key words: Gastric neoplasms; Brazil; Projections; Mortality

Core tip: Currently there are no detailed predictions in Brazil per geographic region and this study will provide the means for the elaboration of public health actions. This study presents a high citation potential, due to the innovative methodology and to the scientific development of Brazil, which is among the countries with most publications nowadays.

de Souza Giusti ACB, de Oliveira Salvado PTC, dos Santos J, Meira KC, Camacho AR, Guimarães RM, Souza DLB. Trends and predictions for gastric cancer mortality in Brazil. *World J Gastroenterol* 2016; 22(28): 6527-6538 Available from: URL: http://www.wjgnet.com/1007-9327/full/v22/i28/6527.htm DOI: http://dx.doi.org/10.3748/wjg.v22.i28.6527

INTRODUCTION

Gastric cancer incidence has been decreasing globally since 1950[1], however its aggressiveness, malignity and, consequently, its prognosis, remain unaltered. Gastric cancer is currently one of the main causes of cancer-related deaths worldwide, and appears as the fourth most frequent cancer in men and sixth in women, being the fifth cancer-related cause of death for both sexes in the world[1].

Regarding risk factors for the development of gastric cancer, a higher incidence is verified in men than in women, in a 2:1 proportion, approximately[2]. Also, researchers remark that the main etiological factors related to the development of gastric cancer are infection by *Helicobacter pylori* (*H. pylori*) and diet, as the elevated consumption of salted foods (preserved or smoked), meat, and refined carbohydrates is directly associated with the risk of developing this neoplasm, while a diet based on fiber, vegetables and fresh fruit presents the inverse association[3,4].

Gastric cancer is usually also associated with low socioeconomic conditions[5], an aspect that highlights the importance of analyzing mortality along with the geographic distribution of the population, especially in countries characterized by high socioeconomic inequalities.

Given the epidemiological importance of gastric cancer, essential measures to support public policy actions include the analysis of the age-period and birth cohort on the distribution of mortality and mortality rate projections. These analyses enable the evaluation of the role of risk factors as well as modifications in therapeutics and diagnosis methods in the evolution of mortality rates[6,7].

Brazil presents demographic and socioeconomic heterogeneity across its five geographic regions, which translates to different mortality and morbidity patterns due to non-transmissible chronic diseases. Addressing cancer, both in prevention and attention to patients, requires differentiated responses that should be adapted to each specific region. Monitoring the trends of incidence and mortality rates, as well as risk factor prevalence, is paramount for vigilance actions and planning of prevention and treatment policies. The objectives of this manuscript are to analyze the effect of age-period and birth cohort in gastric cancer mortality, for Brazil and its five geographic regions, by sex, for the population over the age of 20, and make projections for the period 2010-2029.

MATERIALS AND METHODS

Study design and population

An ecological study is presented herein, on the distribution of deaths by gastric cancer, in Brazil and its five geographic regions, per sex. The study included deaths classified as 151 (stomach neoplasm) and C16 (malignant stomach neoplasm) in the 9th and 10th edition of the International Classification of Diseases (ICD), respectively. The study population included Brazilian men and women over 20 years of age. Mortality data were obtained from the Mortality Information System (MIS/DATASUS). Population data were obtained from the Informatics Department of the Unified Health System (DATASUS), based on the population censuses of 1980, 1991, 2000 and 2010. Inter-census projections for populations on July, 1st of the inter-census years were estimated by the Brazilian Institute of Geography and Statistics (BIGS).

Study variables

After extraction, data were corrected, redistributing 50.0% of the registries classified as ill-defined causes (codes 780-789 in ICD-9, and R00-R99 in ICD-10),

utilizing the redistribution methodology of the World Health Organization (WHO)[8]. After redistribution of ill-defined registries, deaths with incomplete diagnosis were redistributed proportionally by year and age group[9] (Mello *et al*[8], 2002). Herein gastric cancer deaths were corrected by two death groups with incomplete diagnosis: incomplete diagnosis for general cancer and incomplete diagnosis for gastric cancer. The following codes were considered as incomplete diagnosis for general cancer: C-77 to C-80 and C-97 in ICD-10; and codes 195, 197 to 199, 238 to 239 in ICD-9. For incomplete diagnosis of gastric cancer, the following codes were considered: 150 in ICD-9 and C-26 in ICD-10.

Once death data were corrected, mortality rates for gastric cancer were calculated per 100000 inhabitants, adjusted by the world population[10]. Age groups, periods and birth cohorts were grouped in five-year intervals, totaling 13 age groups (20-24 years of age to over 80 years of age), six periods (1980-1984 to 2005-2009) and 20 birth cohorts (1895-1899 to 2005-2009).

Data projections were made by sex for the periods 2010-2014, 2015-2019, 2020-2024 and 2025-2029 based on the three observed periods (1995-1999, 2000-2004 and 2005-2009), with results being presented in three age groups (0-39, 40-59 and ⩾ 60 years of age) as well as the total result.

Statistical analysis

Age-period and birth cohort (APC) effects were calculated by the Poisson regression method. In this model, effects act in a multiplicative manner on the rates and the logarithm of the expected rate value is a linear function of the effect of age, period and cohort[6,7].

$ln(E[r_{ij}]) = ln(\theta_{ij}/N_{ij}) = \mu + \alpha_i + \beta_j + \gamma_k$

Where is the mortality rate expected for age i and period j, θ_{ij} is the number of deaths for age i and period j, and N_{ij} expresses the population under risk of death in age i and period j; μ represents the average of the effect, α_i represents the effect of group age i, β_j represents the effect of period j, and γ_k is the effect of cohort k.

The greatest limitation with the estimation of APC effect parameters is the linear relationship between the factors age, period and cohort, which hinders the estimation of the complete model. Methodologies have been proposed to address this issue; however, there is still no consensus in literature[6,7]. The APC effect parameters were estimated by estimable functions: deviations, curvatures, and *drift*, a method proposed by Holford[6,7].

A reference age group was selected (50-54 years of age), along with a reference period (1990-1994), and a reference cohort (which was the median value, as central cohorts are more stable). This manuscript utilized the 1930-1934[6,7] cohort as reference. The adjustment of the model was evaluated by the statistical function deviance, defined as twice the logarithm of the likelihood of the complete model in relation to the logarithm of the likelihood function of the estimated model. The contribution of effects was evaluated by the comparison between the deviance of the model with the specific effect in relation to the complete model (age-period-cohort). The results with $P \leqslant 0.05$ were considered statistically significant.

The association measurements, respective confidence intervals (95%CI), and the adjustments of the models were calculated by statistics program R version 3.2.1, with the Epi 1.1.18 library (R Foundation for Computational Statistics, Viena, Austria http://www.r-project.org).

Projections were made for each period utilizing the age-period-cohort model of the Norpred program, inscribed within the program R. Data were compiled in 5-year blocks and the limit age group considered for analysis was the first with more than 10 cases for the combined period. The results of the projections are presented by the observed and expected deaths for each period, for each Brazilian state. Also, for each period, adjusted mortality rates were calculated based on the standard world population to enable comparison with international data, expressed per 100000 inhabitants per year (ASW/100000 inhab). Variations between the number of cases in the last projected year (2025-2029) and the last observed period (2005-2009) were calculated, considering the proportion of variation that occurred in terms of changes in risk or demographics (size and structure of population). Both components can be different from zero and present a positive or negative direction. Calculation is expressed as follows[11]:

Δ tot = Δ risk + Δ pop= (Nfff - Noff) + (Noff - Nooo)

Where Δ tot is the total change, Δ risk is change in function of risk, Δ pop is change in function of population, Nooo is the number of observed cases, Nfff is the number of projected cases, and Noff is the number of expected cases when mortality rates increase throughout the observed period.

RESULTS

Within the period 1980-2009, there were 314445 deaths registered in Brazil, corresponding to an average standardized mortality rate of 11.71 deaths per 100000 inhabitants. After correction of the number of deaths, there was an increase of 30.8% in the number of deaths (411558), representing an average mortality rate of 15.32 deaths per 100000 inhabitants for gastric cancer, in individuals over 20 years of age. It must be highlighted that, throughout the country, the highest proportion of deaths occurred in the male sex. Also, the highest mortality rates for gastric cancer were observed for the male sex in Brazil and in all geographic regions of the country.

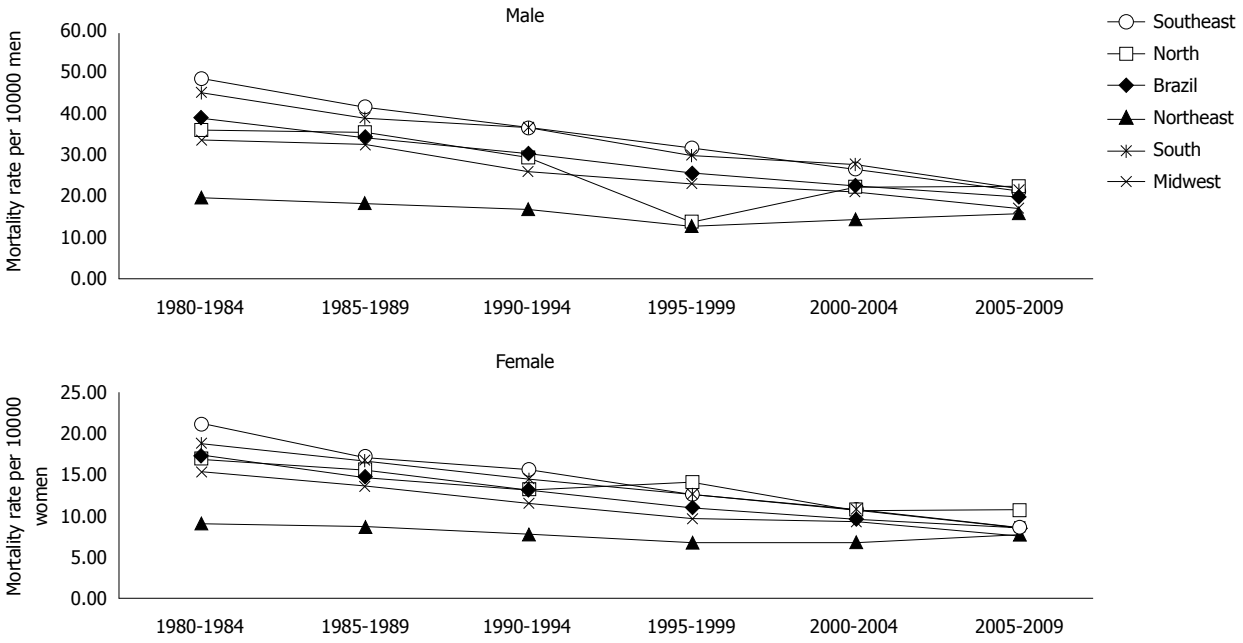

Figure 1 Mortality from gastric cancer according to sex and geographical regions, in Brazil, 1980 to 2009.

Analysis of the evolution of gastric cancer mortality rates for the last 30 years indicated a progressive decrease in rates in the 1980's, with higher and lower rates in the 2000's for both sexes in Brazil and the South, Southeast and Midwest regions. In the Northeast region, for both sexes, and in the North region for the male sex, there was a progressive decrease in mortality rates until 1995-1999, followed by rising rates (Figure 1).

In Brazil, as well as in all geographic regions and both sexes, the mortality rates for gastric cancer increased considerably after the age group 55-59 years of age. The highest mortality rates were verified in the age group over 80 years of age. Analysis of mortality according to study periods evidenced decreasing trends for both sexes in the South, Southeast and Midwest regions.

Regarding the mortality rates per age group according to birth cohorts, decreasing trends were observed in the evolution of mortality rates, for both sexes, in Brazil and in the Midwest, Southeast and South regions. This reduction occurred after the 1920 birth cohort, and was observed in all age groups. However, in the North region, increasing mortality rates were identified, for both sexes, in the age groups 75-79 years of age and over 80 years of age, starting from the 1919-1924 birth cohort. A similar profile was verified in the Northeast region, for both sexes, in individuals born after the 1910-1914 cohort for age groups after 65-69 years of age.

Regarding death risk in the analyzed periods, Brazil presented a protection risk (relative risk, RR, under 1) when compared to the reference period 1990-1994. Analysis of death risk per geographic region pointed towards disparities between the Brazilian geographic regions. The South and Southeast regions showed decreases in death risks due to this neoplasm, with a protection effect (RR $\leqslant$ 1) especially in the period 2005-2009 for both sexes. The Midwest region presented a very specific profile, as despite RR was above 1 in all periods in relation to the reference period, there was a progressive reduction in death risk due to gastric cancer in the analyzed periods (Figure 1). The North and Northeast regions presented decreases in death risks for the period 1995-1999, but in the following periods there was an increase in risk (RR $\geqslant$ 1), for both sexes (Figure 2).

Brazil and the Midwest, North, Southeast and South regions presented reductions in death risks for gastric cancer for both sexes, with a protection effect for birth cohorts after 1940-1944, when compared to the reference cohort. This reduction was more expressive in the South and Southeast regions (Figure 2).

Table 1 shows the deviance changes in the sequential construction of APC models. In the evolution of rates for both sexes, in Brazil and its geographic regions, the model with three factors (APC) presented best fit, except for the Midwest region and female sex, for which the most explanatory model was age-cohort (AC).

When comparing the evolution of standardized mortality rates for gastric cancer, increases were observed for the male sex in the North and Northeast regions, and in the female sex in the Northeast region. The projections for the Midwest, South and Southeast regions as well as the pooled analysis for Brazil indicated a reduction in mortality rates for the male sex and stability for the female sex. The number of cases per region, the adjusted rates for the observed period, and projections per sex are presented in Table 2.

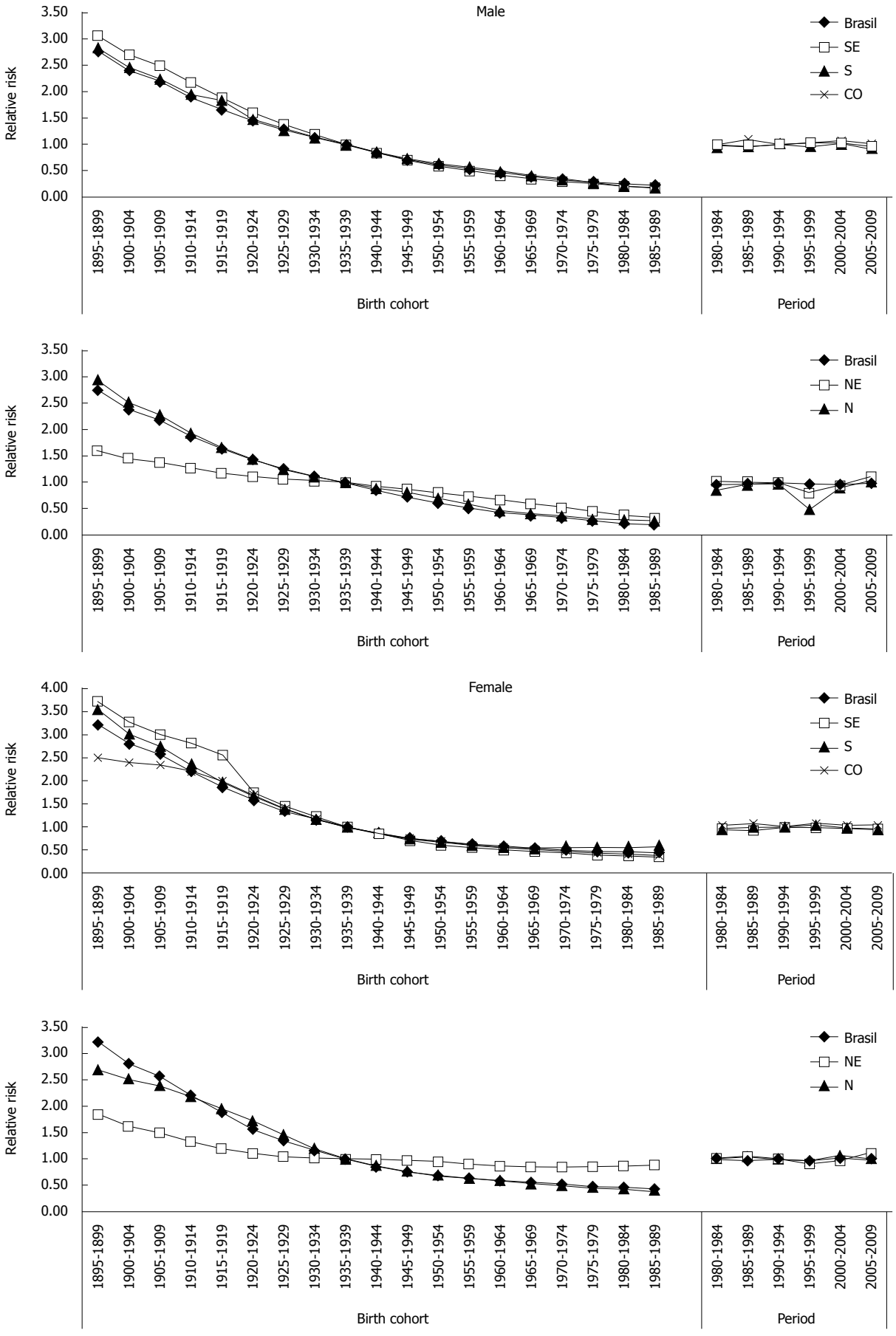

Figure 2 Results of the Age-period-cohort model, adjusted for gastric cancer mortality, according to to sex and geographical regions, in Brazil, 1980 to 2009.

Table 1 Deviance changes in the sequential construction of age-period and birth cohort models

Models	Brazil					
	Female			Male		
	Resid.DF	Res.Dev	*P* value	Resid.DF	Res.Dev	*P* value
Age	72	9069.3		72	15752.0	
Age-drift	71	661.6	< 0.00001	71	515.1	< 0.00001
Age-cohort	67	390.5	< 0.00001	67	403.0	< 0.00001
Age-period-cohort	64	372.5	0.001	64	370.6	< 0.00001
Age-period	68	642.8	< 0.00001	68	485.9	< 0.00001
Age-drift	71	661.6	0.001	71	515.1	< 0.00001
			Midwest Models			
Age	72	399.77		72	899.91	
Age-drift	71	88.88	< 0.00001	71	116.80	< 0.00001
Age-cohort	67	72.14	0.002	67	111.94	0.302
Age-period-cohort	64	66.18	0.200	64	95.83	0.003
Age-period	68	83.03	0.002	68	100.13	0.366
Age-drift	71	88.88	0.210	71	116.80	0.002
			North Models			
Age	72	325.04		72	1310.75	
Age-drift	71	140.37	< 0.00001	71	779.28	< 0.00001
Age-cohort	67	129.65	0.030	67	751.44	< 0.00001
Age-period-cohort	64	110.22	0.001	64	178.57	0.003
Age-period	68	121.64	0.022	68	194.68	< 0.00001
Age-drift	71	140.37	0.001	71	779.28	< 0.00001
			Northeast Models			
Age	72	566.75		72	1038.52	
Age-drift	71	405.46	< 0.00001	71	656.22	< 0.00001
Age-cohort	67	269.65	< 0.00001	67	597.78	< 0.00001
Age-period-cohort	64	142.85	< 0.00001	64	155.70	< 0.00001
Age-period	68	230.83	< 0.00001	68	208.92	< 0.00001
Age-drift	71	405.46	< 0.00001	71	656.22	< 0.00001
			South Models			
Age	72	1903.85		72	3090.98	
Age-drift	71	219.90	< 0.00001	71	207.96	< 0.00001
Age-cohort	67	152.41	< 0.00001	67	189.07	0.001
Age-period-cohort	64	124.92	< 0.00001	64	129.74	< 0.00001
Age-period	68	200.58	< 0.00001	68	143.44	< 0.00001
Age-drift	71	219.90	0.001	71	207.96	< 0.00001
			Southeast Models			
Age	72	6993.8		72	11604.0	
Age-drift	71	447.6	< 0.00001	71	399.5	< 0.00001
Age-cohort	67	278.8	< 0.00001	67	290.5	< 0.00001
Age-period-cohort	64	217.6	< 0.00001	64	209.5	< 0.00001
Age-period	68	389.4	< 0.00001	68	284.7	< 0.00001
Age-drift	71	447.6	< 0.00001	71	399.5	< 0.00001

For Brazilian men, an increase is expected in the number of deaths (30203) when comparing the last observed period with the last projected period, representing a 62% growth, of which 101% is due to population increase and -39% is due to reduction in risk. For women, the expected increase in the number of deaths is 20308, with 99% growth due to changes in population and -23% in reduction of risk. Figure 3 shows the changes in the number of deaths, in function of risk and population increase, when comparing the last observed period and the last projected period for gastric cancer in the Brazilian regions.

DISCUSSION

This study evidenced disparities in the evolution of mortality rates due to gastric cancer in Brazilian geographic regions, within the analyzed periods. There was an increase in death risk by this neoplasm in the North and Northeast regions starting from the 2000's, when compared to the reference period.

Gastric cancer, despite the decrease presented in incidence and mortality rates, is still one of the main cancer-related causes of death globally[12]. This disease presents a bad prognosis, with 5-year survival rates

Table 2 Observed and predicted number of deaths by age and world age-standardized rates in Brazil

Males			Observed			Predicted			
			1995-1999	2000-2004	2005-2009	2010-2014	2015-2019	2020-2024	2025-2029
Males									
North	Age	0-39	99	126	156	200	243	277	296
		40-59	700	818	929	1119	1247	1478	1670
		⩾ 60	1287	1597	2151	2792	3579	4332	5195
	Total		2086	2541	3236	4110	5069	6087	7160
	ASW		13.6	13.3	14.3	15.0	15.2	14.8	14.2
Northeast	Age	0-39	310	332	405	388	377	366	365
		40-59	1662	2041	2633	3226	3745	4135	4155
		⩾ 60	4086	5624	7020	9070	11415	14018	16827
	Total		6058	7997	10058	12685	15537	18519	21347
	ASW		7.5	8.7	9.8	10.9	11.4	11.6	11.4
Midwest	Age	0-39	94	98	102	102	105	97	92
		40-59	684	707	753	779	813	941	1126
		⩾ 60	1404	1705	1827	2039	2371	2784	3362
	Total		2182	2510	2682	2920	3289	3821	4580
	ASW		13.5	12.2	10.6	9.3	8.4	7.9	7.8
Southeast	Age	0-39	801	616	660	563	576	619	524
		40-59	6255	6404	6304	5748	5629	6052	7537
		⩾ 60	16611	16790	14648	14316	15277	17650	21598
	Total		23667	23810	21612	20627	21482	24320	29658
	ASW		18.7	15.5	12.0	9.6	8.3	7.9	8.3
South	Age	0-39	250	235	241	188	137	120	108
		40-59	2245	2356	2403	2361	2410	2444	2649
		⩾ 60	5515	6052	6062	6392	7041	8199	9745
	Total		8010	8643	8706	8941	9588	10762	12502
	ASW		18	16.2	13.6	11.5	10.3	9.7	9.5
Brazil	Age	0-39	1660	1403	1567	1796	2105	2502	2736
		40-59	13382	12196	12944	13909	15114	16962	19978
		⩾ 60	32344	31439	34145	37297	41994	48255	56146
	Total		47386	45038	48656	53002	59213	67718	78861
	ASW		16.7	13.3	12.2	11.2	10.4	10.0	10.0
Females									
North	Age	0-39	110	122	127	159	163	174	180
		40-59	324	375	460	557	659	790	909
		⩾ 60	678	792	1040	1327	1739	2244	2870
	Total		1112	1289	1627	2044	2561	3208	3959
	ASW		7.1	6.4	6.8	6.9	7.0	7.1	7.0
Northeast	Age	0-39	274	314	380	460	554	527	524
		40-59	943	1114	1394	1773	2105	2490	2754
		⩾ 60	2551	3152	4318	5723	7392	9309	11348
	Total		3768	4580	6092	7955	10051	12326	14626
	ASW		4	4	4.7	5.2	5.6	5.8	5.9
Midwest	Age	0-39	82	92	96	93	87	82	79
		40-59	277	333	382	442	547	634	717
		⩾ 60	603	771	875	1028	1217	1515	1947
	Total		962	1196	1353	1562	1850	2232	2743
	ASW		5.8	5.3	4.7	4.3	4.0	3.9	3.9
Southeast	Age	0-39	576	652	675	674	685	627	562
		40-59	2413	2778	3008	2983	3140	3569	4168
		⩾ 60	8058	9652	9619	9950	10667	12047	14398
	Total		11047	13082	13302	13606	14493	16243	19127
	ASW		6.8	6.4	5.4	4.6	4.2	4.1	4.2
South	Age	0-39	215	217	258	257	280	238	220
		40-59	984	949	1052	1150	1283	1546	1857
		⩾ 60	3005	3106	3143	3268	3578	4111	4939
	Total		4204	4272	4453	4675	5142	5895	7016
	ASW		7.6	6.2	5.3	4.7	4.4	4.4	4.6
Brazil	Age	0-39	1313	1376	1511	1811	2107	2268	2515
		40-59	5540	5458	6274	7075	8168	9879	11902
		⩾ 60	16991	17450	18982	20635	23267	27116	32678
	Total		23844	24284	26767	29522	33542	39263	47095
	ASW		6.9	5.6	5.2	4.8	4.7	4.7	4.9

ASW: Age-standardized rates.

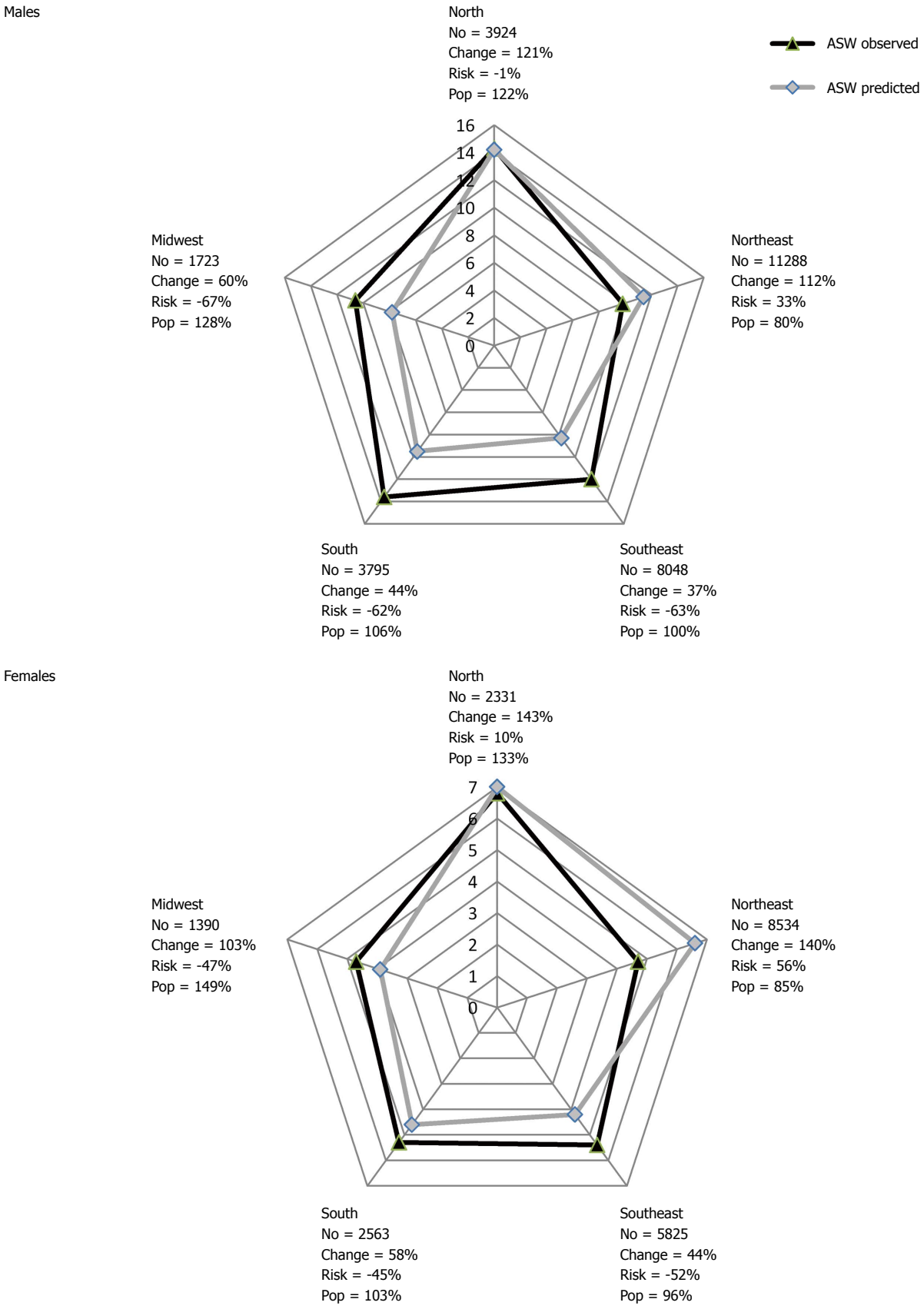

Figure 3 World age-standardized rates, changes in numbers of deaths relative change due to risk and changed population, between 2005-2009 (observed) and 2025–2029 (predicted) of gastric cancer mortality in Brazil. ASW: Age-standardized rates; Pop: Population.

under 20%[13].

The mortality rates presented in Brazil, in all regions and for both sexes, are located at intermediate levels, similar to the rates of Venezuela and Argentina, but still superior to those of developed countries such as the United States, Spain, France and Canada. However, Brazilian rates are lower than the mortality rates verified for China, Japan and South Korea[12-20].

This neoplasm can be classified according to its location, in cardia and non-cardia gastric cancer. These diseases present different risk factors and population distributions. While non-cardia gastric cancer presents main risk factors such as *H. pylori* infection, consumption of salt-preserved food, low consumption of fruit and vegetables, consumption of alcohol and use of tobacco, cardia gastric cancer (esophagogastric junction adenocarcinoma) is a pathology related to obesity, gastroesophageal reflux and Barrett esophagus. Besides, this disease is more common in developed countries and white race individuals, while non-cardia gastric cancer is more incident in Afro-Americans and in developing countries[19,21,22]. There have been important global reductions in incidence and mortality rates for non-cardia gastric cancer; however, an increasing trends have been observed for esophagogastric junction adenocarcinoma, which represents currently 50% of new diagnosed cases of gastric cancer. The prognosis of this type of cancer is worst than non-cardia gastric cancer, as esophagogastric junction adenocarcinoma can disseminate to the abdominal region as well as to the lymph nodes of the thoracic region. Due to the absence of symptoms at the beginning of the disease, esophagogastric junction adenocarcinoma is usually diagnosed in advanced stages[23].

As in other parts of the world, the Brazilian and per region rates for the male sex were approximately twice higher than mortality rates for the female sex[19,21,22]. Some authors believe that this difference is due to the co-existence of other risk factors and that there is unequal exposition according to sex[5,21]. Other authors mention that this reality is related to the fact that women are more aware of health issues than men[18].

Mortality due to this neoplasm in Brazil and in the Midwest, South and Southeast regions presented decreasing trends, along with France, Italy, Spain, Germany, South Korea, Japan and China[1,14]. In the United States and United Kingdom, half of gastric cancers are located in the cardia area, and in the last 30 years, globally, incidence of this disease has increased between 5 and 6 times[22].

A reduction in death risk was evidenced in Brazil and in the South and Southeast regions, starting from the 1990's, and increased risk was observed in the North and Northeast regions after the 2000's. Changes in mortality rates of diseases can reflect changes in exposition to risk factors (environmental and/or associated with lifestyle), as well as improvements in diagnosis, treatment, verification and certification of deaths. The differences presented between the North-Northeast regions and other regions of Brazil, especially in the 2000's, can be a result of the period effect, due to improvement in death registry data and better access to health services. Due to an increase in the possibilities of diagnosing this neoplasm, even if in advanced stages of the disease, these regions presented higher mortality rates in the 2000's.

Regarding birth cohorts, a progressive reduction in death risk was verified starting in the XX century, in all regions and for both sexes. This result was similar to the results of South Korea, Japan, United States, and Spain[15,17,24,25]. This reality can be explained by the prevalence reduction of *H. pylori* infection, use of refrigerators (which increased the consumption of fruit and vegetables)[26-29], besides the reduction in the consumption of salt-preserved foods[30] and better sewage collection and treatment, which contributes to reducing the transmission of *H. pylori*, especially in children and teenagers[19,22]. Reduction of mortality can also be related to new therapeutic strategies implemented within the last decade for the treatment of gastric cancer. These are based on neoadjuvant/adjuvant chemotherapy treatment, which can be associated with radiotherapy[31]. A literature review study with meta-analysis evidenced that patients treated with adjuvant chemotherapy presented better global survival (HR = 0.82; 95%CI: 0.76-0.90; $P < 0.001$) and disease-free survival (HR = 0.82; 95%CI: 0.75-0.90, $P < 0.001$) when compared to patients submitted to surgical treatment only[32]. However, there is still no consensus within literature on the best treatment to be utilized, and no studies were found in Brazil that evaluated the implementation of these new therapeutic measures on the survival of patients.

Survival rates are affected by early diagnosis, standardized surgery techniques, nutritional therapy, availability of beds in intensive care units, and the existence of specialized health teams for cancer treatment[13,17]. In this way, the pronounced inequity between Brazilian geographic regions (large urban centers *vs* the interior) regarding access to cancer diagnosis and treatment services can influence the evolution of mortality rates for this disease. The study by Oliveira *et al*[33] evidenced sanitary gaps related to breast cancer treatment, especially in North Brazil, with half of health assistance concentrated in few capital cities. The cities of Rio de Janeiro and São Paulo (Southeast Brazil) were responsible for one fifth of attendances, mostly for the resident population. Brazil has a public and universal health system that faces limitations regarding funding and access to diagnosis and treatment services. This reality should not be exclusive to breast cancer treatment and highlights the difficult access to health services that most cancer patients suffer in Brazil[34].

The projections indicated that the least developed

regions of Brazil (North and Northeast) will present increments in gastric cancer mortality rates. These regions present structural challenges in oncology services[34-36]. Also in the North and Northeast regions of the country, the increased mortality can be explained by higher difficulty in the access to diagnosis and treatment services. The ratio medical doctor/inhabitant is lower in the North and Northeast regions (in 2010, approximately 1 doctor per 1000 inhabitants) than in the South and Southeast regions (respectively 2.5 and 2.0 doctors per 1000 inhabitants). The distribution of oncology specialists and oncology hospitals is also unbalanced[37]. Also, there is concentration of oncology services in large urban centers, as a consequence of internal migration and development of these areas. The search for appointments with specialists and access to diagnosis and treatment services generates dislocation from rural areas to large urban centers, which delays diagnosis and therefore, entail in worst prognosis for these patients[32].

A limitation to be considered in the study is the impossibility of separating cardia and non-cardia locations for gastric cancer, which seem to present different behaviors according to most recent studies, as the Brazilian Mortality Information System does not differentiate gastric cancers according to histological type[21]. Another important fact to consider is the lack of historical series for risk factor prevalence, which could aid in the analysis of the observed changes. Finally, it must be highlighted that this is an ecological population study, and intra-regional differences can be found in Brazil due to its large continental dimensions, especially regarding the quality of death registries, which the authors attenuated after correcting death data. Limitations related to the APC models must also be mentioned, as these are still under development and there is no consensus in literature on the best methodology to correct the issue of non-identification of the complete model[6,7].

The projections made must be interpreted with caution, as the diagnostic and therapeutic conditions can change in the future, and consequently the mortality trends could be slightly modified. The projection of mortality rates is very important to support the planning of public health measures, as well as to control modifiable risk factors at short and long terms, on the burden of the disease to the population[33]. The main objective of the projections made herein was to provide the required information for health planning purposes, aiming at the selection of vigilance actions for gastric cancer.

COMMENTS

Background

The evolution of gastric cancer mortality has evidenced decreasing trends for Brazil in the South, Southeast and Midwest regions of the country, for both genders. However, after the 1990's, increasing trends were observed in the Northeast (both genders) and North (in men only) regions. After the 1940-1944 birth cohort, reduction in death risk was observed for this neoplasm, in both genders, for Brazil and its South, Southeast and Midwest regions. The opposite situation was verified for the Northeast region, which presented a progressive death risk for cohorts born from the 1940-1944 cohort. Regarding mortality projections until 2030, increased mortality rates were evidenced for both genders, in the North and Northeast regions of the country.

Research frontiers

The study evidenced a progressive reduction in risk of death from gastric cancer in birth cohorts after the 1940's, in the most developed regions of Brazil, with the opposite occurring in the poorer geographic regions. These findings are similar to those in studies carried out in South Korea, Denmark, Japan, United States, England, Italy, Switzerland and Spain, and evidences the important role played by basic sanitation and access to health services in gastric cancer mortality.

Innovations and breakthroughs

In most epidemiological studies, trend analysis of mortality rates is based on the evaluation of mortality by age group and death date. The present study analyzed the effect of age-period-birth cohort on the evolution of gastric cancer mortality rates and enabled the evaluation of factors related to age and period as well as whether modifications in mortality trends for this disease were associated to changes in exposition to long-term risk factors (birth cohort effect).

Applications

The results suggest flaws in gastric cancer prevention and control measures in the North and Northeast regions of Brazil. The findings will help plan Brazilian public policies directed to the promotion of primary, secondary and tertiary prevention, to reduce mortality rates for gastric cancer in Brazil and its geographic regions.

Terminology

South region: this Brazilian geographic region presents the best human development indices. This region includes the states of Rio Grande do Sul, Santa Catarina and Paraná. Southeast region: this is the most populous and rich region of the country, and 85% of industry-related jobs are located in this region. This region comprehends São Paulo, Minas Gerais, Rio de Janeiro and Espírito Santo. Midwest region: the main economic activity of this region is farming and livestock. This region presents the second lowest demographic density of the country, and is constituted by Mato Grosso, Mato Grosso do Sul, Goiás and Distrito Federal. Northeast region: this Brazilian region presents one of the worst human development indices, and is characterized by the presence of a semi-arid region within its territory, and is one of the poorer areas of the country. This region comprises nine states: Alagoas, Bahia, Ceará, Maranhão, Paraíba, Pernambuco, Piauí, Rio Grande do Norte and Sergipe. North region: this Brazilian region presents the lowest demographic density, as well as the second worst human development index. This region spans the Amazon Forest, which is an important ecosystem for the world, and includes the states of Amazonas, Amapá, Pará, Tocantins, Roraima and Rondônia. Age effect: evaluates the influence of age on the evolution of mortality rates. Period effect: evaluates the impacts of changes in diagnostic methods, treatment protocols, as well as changes in death certification and improvement of mortality information systems on the evolution of mortality trends. Birth cohort effect: analyzes whether modifications in mortality trends were associated with changes in exposition to long-term risk factors.

Peer-review

In this study the authors report trends in the incidence and mortality of gastric cancer over the last decades and make a prediction on the mortality in the upcoming years. All in all the study is well conducted and has interesting results, needs some minor revisions.

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ORIGINAL ARTICLE

Prospective Study

Can optical diagnosis of small colon polyps be accurate? Comparing standard scope without narrow banding to high definition scope with narrow banding

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Abstract

AIM: To study the accuracy of using high definition (HD) scope with narrow band imaging (NBI) *vs* standard white light colonoscope without NBI (ST), to predict the histology of the colon polyps, particularly those < 1 cm.

METHODS: A total of 147 African Americans patients who were referred to Howard University Hospital for screening or, diagnostic or follow up colonoscopy, during a 12-mo period in 2012 were prospectively recruited. Some patients had multiple polyps and total number of polyps was 179. Their colonoscopies were performed by 3 experienced endoscopists who determined the size and stated whether the polyps being removed were hyperplastic or adenomatous polyps using standard colonoscopes or high definition colonoscopes with NBI. The histopathologic diagnosis was reported by pathologists as part of routine care.

RESULTS: Of participants in the study, 55 (37%) were male and median (interquartile range) of age was 56 (19-80). Demographic, clinical characteristics, past medical history of patients, and the data obtained by two instruments were not significantly different and two methods detected similar number of polyps. In ST scope 89% of polyps were < 1 cm *vs* 87% in HD scope (P = 0.7). The ST scope had a positive predictive value (PPV) and positive likelihood ratio (PLR) of 86% and 4.0 for adenoma compared to 74% and 2.6 for HD scope. There was a trend of higher sensitivity for HD scope (68%) compare to ST scope (53%) with almost the same specificity. The ST scope had a PPV and PLR of 38% and 1.8 for hyperplastic polyp (HPP) compared to 42% and 2.2 for HD scope. The sensitivity and

specificity of two instruments for HPP diagnosis were similar.

CONCLUSION: Our results indicated that HD scope was more sensitive in diagnosis of adenoma than ST scope. Clinical diagnosis of HPP with either scope is less accurate compared to adenoma. Colonoscopy diagnosis is not yet fully matched with pathologic diagnosis of colon polyp. However with the advancement of both imaging and training, it may be possible to increase the sensitivity and specificity of the scopes and hence save money for eliminating time and the cost of Immunohistochemistry/pathology.

Key words: High definition colonoscopy; Narrow band imaging; Polyp detection; Colon cancer screening

Core tip: This study analyzed the size of polyps and stated whether the polyps being removed were hyperplastic or adenomatous polyps using standard colonoscopes or high definition colonoscopes with narrow band imaging (NBI), suggests that high definition scope was more sensitive in diagnosis of adenoma than standard white light colonoscope without NBI scope. Hence we save money for eliminating time and the cost of immunohistochemistry/pathology.

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INTRODUCTION

Colorectal cancer is one of the most common cancers in the United States[1]. Early detection of colon cancer by colonoscopy and polyp removal is likely to decrease mortality from the disease. Colonoscopy is now established as the gold standard for the identification of both colorectal cancer and polyps[2]. It is estimated that up to 15 million colonoscopies are performed annually in the United States[3,4].

On the other hand, most polyps, which are either biopsied or removed, are non-neoplastic in nature, which provide additional burden to the pathologist[5] as well as the cost associated with unnecessary biopsies and the risk with polypectomies[6]. Also the colonoscopic miss rate of adenomas, which are considered to be precursors of colorectal cancer, is as high as 24%[7]. Therefore, the distinction between non-neoplastic and neoplastic colorectal polyps *in vivo* with a suitable technique can improve the accuracy of colonoscopy, particularly as a higher adenoma detection rate, could be cost saving by eliminating the need for routine pathology on every polyp < 1 cm removed during colonoscopy.

Improvements in the resolution of imaging techniques in colonoscopy over the years have resulted in a substantial increase in the polyp detection rate in the colon. One of these new imaging techniques is narrow band imaging (NBI). NBI is a relatively new endoscopic technique that increases the accuracy of diagnosis using narrow-band width filters in a red-green-blue (RGB) sequential illumination system[6]. This results in enhancement of the surface mucosal morphology, so improves the detailed visualization of the micro vascular and micro structural pit patterns[7].

A number of randomized trials comparing narrow band imaging colonoscopy with white light colonoscopy for detection of colorectal polyps reported variable results. This discrepancy in results is related to inadequately powered studies due to difference in the number and experience of endoscopists involved in the studies, as well as small sample size[8].

The first study was from Japan[9]. They examined thirty four patients and they found statistically significant difference between NBI (sensitivity 100%, specificity 75%) compared to standard scope (sensitivity 83%, specificity 44%, $P < 0.05$ for specificity). In the current study, we present a comparison of polyp detection rate and accuracy, using standard scope without NBI and high definition scope with NBI.

MATERIALS AND METHODS

Patients

A total of one hundred forty seven African Americans (AAs) patients who were referred to Howard University Hospital (HUH) for screening or, diagnostic or follow up colonoscopy, during a 12-mo period in 2012, were prospectively recruited. All patients were consented based on approved HUH IRB. Their colonoscopies were performed by 3 experienced endoscopists (more than 2000 colonoscopies each) at the same endoscopy center, who determined the size and stated whether the polyps being removed were hyperplasic or adenomatous polyps, using standard colonoscope or high definition colonoscope with NBI. Patients were assigned to undergo colonoscopy using either standard scope without NBI or high definition (HD) scope with NBI.

Data collection

Data collected for this survey include: Date of procedure, patient's date of birth, gender, and race, height (Ht), weight (Wt), education, associated condition, reason for colonoscopy, past history of colon polyps, family history of colon cancer, smoking, alcohol consumption, colon preparation quality, number of polyps, polyp size, polyp

Table 1 Comparing the characteristics of patients underwent standard scope *vs* high definition scope with narrow band imaging *n* (%)

Parameters	Standard scope (1) (*n* = 68)	HD scope (*n* = 72)	*P* value
Female	41 (60)	48 (67)	0.40
Age (yr), median (IQR)	56 (52-61)	57 (53-64)	0.70
Education			0.40
High school and lower	31 (46)	38 (54)	
> High school	37 (54)	34 (47)	
H/o previous colonoscopy	25 (37)	19 (26)	0.20
H/o previous colon polyp	6 (9)	7 (10)	0.80
Family h/o colon cancer	22 (22)	12 (17)	0.40
Indication			0.20
Screening	34 (50)	45 (63)	
Diagnostic	18 (26)	19 (26)	
Follow up	16 (24)	8 (11)	
Colon preparation			0.08
Good	63 (93)	71 (99)	
Moderate	5 (7)	1 (1)	
Number of patient with polyp diagnosis	41 (60)	49 (68)	0.30
Total number of polyps detected, median (IQR)	1 (1-2)[1]	2 (1-3)[2]	0.20
Adenoma detection rate	23 (34)	32 (44)	0.20
Advanced adenoma detection rate	7 (10)	8 (11)	0.90
Hyperplastic polyp detection rate	19 (28)	21 (29)	0.90
Proportion of patients with multiple polyps	17 (41)[1]	28 (57)[2]	0.10

[1]*n* = 41; [2]*n* = 49. HD: High definition.

location, type of scope, endoscopist name, duration of colonoscopy, colonoscopy diagnosis, pathologist name and histology diagnosis. Adenomatous polyps with tubulovillous histology or size > 1 cm or with high grade dysplasia were define as advance adenoma.

Endoscopy procedure

Among 140 patients with recorded endoscopy type, 49% of patients had colonoscopy with standard scope. All three endoscopists performed procedure using both scopes at the same rate (9 min median normal withdraw time as quality standard). Data and the predicted diagnosis were collected from patients who had colonoscopy by the same three endoscopists. Bowel preparation was good and moderate in 95% and 5% of patients, respectively. The procedures were performed under a nurse administered standard sedation with Fentanyl and Midazolam. Colonoscopy withdrawal times were recorded by the nursing staff. Polyps were removed using forceps biopsy, and sent for histological analysis by the pathologist who was not aware of the endoscopic diagnosis.

Statistical analysis

We compared the demographic and clinical characteristics between a group of patients who underwent standard colonoscopy *vs* NBI by Student's *t*-test to χ^2 whichever was appropriate. For each method sensitivity, specificity, positive predictive value (PPV), negative predictive value (NPV) and, positive likelihood ratio (PLR) of colonoscopy diagnosis was calculated with comparison to pathologic diagnosis as gold standard. Calculation of 95% confidence interval and statistical comparison between two instruments was performed by established methods. All statistical analyses were performed using Stata 12.0 (Stata Corp., College park, TX, United States).

RESULTS

Patients and endoscopy

One hundred and forty seven adult patients were recruited and underwent colonoscopy during a one year period. Among them 55 (37%) were male and median (range) of age was 56 (19-80). Among the patients "screened with" underwent standard scope, 31 (46%) had high school or lower education, while 37 (54%) had higher education, compared to 38 (54%) and 34 (46%) who underwent HD scope, respectively. Among the patients who underwent standard scope, 25 (37%) had previous colonoscopy, which 6 of them (9%) had previous history of polyp, while these numbers for the patients underwent HD scope were 19 (26%) and 7 (10%) respectively. Also 22 patients (22%) underwent standard scope had a family history of colon cancer, while this number for HD scope with NBI was 12 (17%). The most common reason for colonoscopy with both scopes was screening, 50% of the patients underwent standard scope and 62% of the patients underwent HD scope. Table 1 compares the characteristics of patients underwent standard scope *vs* HD scope with NBI.

Colonoscopies were done by standard (49%) and HD (51%) scopes. Among 147 patients, 57 patients (39%) had normal colonoscopy. Number of patients diagnosed with any type of polyp were 41(60%) using standard scope and 49 (68%) using HD scope (P = 0.3). Among all 90 patients with polyps, 179 polyps were removed. The median (range) of polyp number in a patient was 2 (1-5) and was not significantly different between two scopes (P = 0.2). Among the polyps removed by standard scope, 89% were < 10 mm, compare to 87% for HD scope with NBI (P = 0.7). In lesions, the most frequent anatomic location was ascending colon (29%), followed by descending colon (18) and rectum (each 16%). This distribution was not different between both scopes (P = 0.5). The most frequent clinical diagnoses were hyper plastic polyp (HPP; 46%), adenoma (43%) and diminutive (11%). Tables 2 and 3 indicate the clinical value of colonoscopy diagnosis for adenoma and HPP when compared to the corresponding pathologic diagnosis.

Diagnostic ability of adenoma by the type of scope

Adenoma detection rate and advanced adenoma

Table 2 Clinical diagnostic value of adenoma by scope

	Sensitivity (%)	Specificity (%)	PPV (%) (true positive/all positive)	NPV (%) (true negative/all negative)	PLR (%) (true positive/false positive)
ALL	61 (51-70)	79 (69-86)	78 (68-86)	62 (53-71)	2.9 (1.8-4.5)
Scope 1	53 (39-67)	87 (70-95)	86 (69-94)	55 (41-69)	4.0 (1.5-10.4)
Scope 2	68 (55-79)	74 (60-84)	74 (60-84)	69 (55-79)	2.6 (1.6-4.3)
P value for two scopes	0.1	0.2	0.2	0.1	

Numbers in parentheses indicate 95%CI. PPV: Positive predictive value; NPV: Negative predictive value; PLR: Positive likelihood ratio.

Table 3 Clinical diagnostic value of hyper plastic polyp by scope

	Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)	PLR (%)
All	73 (59-84)	63 (55-71)	40 (30-51)	88 (79-93)	2.0 (1.5-2.6)
Scope 1	74 (51-88)	59 (46-71)	38 (24-54)	87 (73-94)	1.8 (1.2-2.7)
Scope 2	74 (54-86)	66 (55-76)	42 (29-57)	88 (77-94)	2.2 (1.5-3.2)
P value for two scopes	0.9	0.4	0.7	0.9	

Numbers in parentheses indicate 95%CI. PPV: Positive predictive value; NPV: Negative predictive value; PLR: Positive likelihood ratio.

Table 4 Comparison of clinical characteristics of all polyps in standard scope vs high definition scope with narrow band imaging *n* (%)

Parameters	Standard scope (1) (*n* = 75)	HD scope (2) (*n* = 103)	*P* value
Polyps < 10 mm in size	67 (89)	89 (87)	0.7
Adenoma detection rate	45 (60)	53 (51)	0.3
Hyperplastic polyp detection rate	19 (25)	26 (25)	0.9

HD: High definition.

detection rate were not significantly different between two scopes (Table 1). Standard scope has a sensitivity of 53% and specificity of 87% in detecting adenoma, compared to 68%, 74% for HD scope with NBI, respectively. Standard scope has a higher specificity, but HD scope with NBI has a higher sensitivity in detecting adenoma. Positive likelihood ratio for standard scope is higher than HD scope with NBI (4 compare to 2.6; Table 2).

Diagnostic ability of HPP by the type of scope

HPP detection rate for standard scope and HD was 28% and 21%, respectively (*P* = 0.9, Table 1). Standard scope has a sensitivity of 74% and specificity of 59% in detecting HPP, compared to 74% and 66% for HD scope, respectively. Both scopes don`t show significant difference in detecting HPP. Positive likelihood ratio for HD scope with NBI is slightly higher than standard scope (2.2 compare to 1.8; Table 4).

Diagnostic ability of adenoma by endoscopists

Endoscopist 1 has a higher sensitivity in detecting adenoma (70%) followed by endoscopist 2 (60%) and endoscopoist 3 (52%) regardless of the type of scope. Endoscopist 1 also has a higher accuracy in detecting adenoma (PLR = 6.3%) compared to the other two endoscopists (2.8 and 1.8 for endoscopists 2 and 3 respectively) regardless of the type of scope (Table 5).

Diagnostic ability of HPP by endoscopists

Endoscopist 1 has lower sensitivity (53%) but higher accuracy (PLR = 3.4%) in detecting HPP compared to the other two endoscopists, regardless of the type of scope. Endoscopist 2 has the highest sensitivity (91%) in detecting HPP, regardless of the type of scope (Table 6).

Polyp detection rate using standard scope without NBI

Out of 28 adenoma "diagnosed in real time" pre-diagnosis using standard scope without NBI, 24 were matched to the histology report (PPV = 86%). For HPP, these numbers were 37, with 14 matched to the histology report (PPV = 38%; Figure 1).

Polyp detection rate using standard scope with NBI

Out of 49 adenoma prediagnosis using HD scope with NBI, 36 were matched to the histology report (PPV = 74%). For HPP, these numbers were 45, with 19 matched to the histology report (PPV = 42%; Figure 2).

DISCUSSION

The standard white light colonoscopy does not have the ability to accurately distinguish between adenomatous and hyperplastic polyps[10]. This distinction has an important clinical impact as adenomatous polyps are considered neoplastic whereas hyperplastic polyps are benign and don't have a malignant potential. Although the removal of adenomatous polyps is recommended since it disrupts the adenoma-carcinoma sequence and prevents from the development of colorectal cancer, hyperplastic polyps can safely be left behind without significant consequences[10]. The removal of

Table 5 Comparasion of adenoma detection between endoscopists

Parameter	Sensitivity	Specificity	PPV	NPV	PLR
Endoscopist 1	75 (55-88)	88 (70-96)	86 (65-95)	79 (61-90)	6.3 (2.1-18.5)
Endoscopist 2	60 (46-74)	78 (58-90)	84 (67-93)	51 (34-67)	2.8 (1.2-6.3)
Endoscopist 3	52 (35-68)	72 (55-84)	64 (45-80)	61 (45-74)	1.8 (0.4-1.0)
P value for three endoscopists	0.2	0.3	0.1	0.1	

PPV: Positive predictive value; NPV: Negative predictive value; PLR: Positive likelihood ratio.

Table 6 Comparison of hyper plastic polyp detection between endoscopists

Parameter	Sensitivity	Specificity	PPV	NPV	PLR
Endoscopist 1	53 (32-73)	83 (66-93)	67 (42-85)	74 (57-85)	3.2 (1.3-7.8)
Endoscopist 2	91 (62-98)	56 (43-69)	29 (17-46)	97 (84-99)	2.1 (1.5-3.0)
Endoscopist 3	87 (62-96)	58 (44-71)	39 (25-56)	93 (79-98)	2.1 (1.4-3.1)
P value for three endoscopists	0.026	0.033	0.049	0.008	

PPV: Positive predictive value; NPV: Negative predictive value; PLR: Positive likelihood ratio.

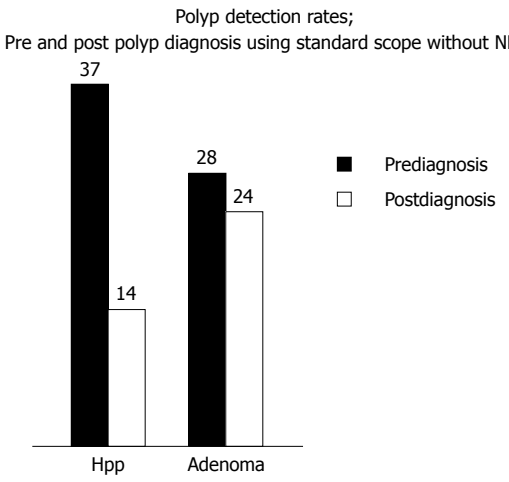

Figure 1 Pre- and post-polyp diagnosis using standard scope without narrow band imaging. HPP: Hyper plastic polyp.

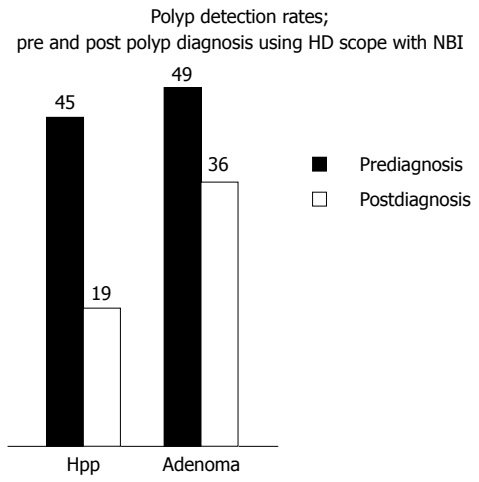

Figure 2 Pre- and post-polyp diagnosis using high definition scope with narrow band imaging. HPP: Hyper plastic polyp.

hyperplastic polyps could be avoided with real-time identification of polyp type during colonoscopy, leading to a decrease of the procedure duration, costs and risk of complications[10]. Studies found a conflicting results comparing the accuracy of standard scope *vs* NBI scope in detection of polyps and prediction of histology in real time[8,11-13]. Some trials findings favored the standard scope, others the NBI scope whereas some studies did not reveal any difference between both scopes.

Sabbagh *et al*[11] conducted a randomized controlled trial and meta-analysis of published studies comparing the narrow-band imaging to conventional colonoscopy in detection of colorectal polyps. A total of 482 patients were included, 241 into the intervention (NBI) colonoscopy and 241 into the conventional colonoscopy group[11]. No significant difference was found in the mean number of polyps when comparing the conventional procedure to the NBI system (0.41 *vs* 0.29). The overall detection rate of lesions (*n* = 174) and polyps (*n* = 169) by histological examination per patient in the entire study group were 36.1% and 35.1% respectively, with adenomas and hyperplastic polyps found, respectively, in 55.0% (*n* = 93/169) and 37.9% (*n* = 64/169) of all patients. In this study[11], the overall rate of polyp detection was significantly higher in the conventional group compared to the NBI group (RR = 0.75, 95%CI: 0.60-0.96). The results of Sabbagh *et al*[11] are different than our results. In our study, there was no difference in the rate of polyps detection between the standard scope and NBI with 41 (60%) patients diagnosed with any type of polyp using standard scope and 49 (68%) using HD/NBI scope (*P* = 0.3). The median (range) of polyp number in a patient was 2 (1-5) and was not significantly different between two scopes (*P* = 0.2), this difference could be attributed to the sample size and study population. In our trial included a total of 147 patient and all were African Americans, also the white-light group, in Sabbagh *et al*[11]'s study, could have had better

mucosal visualization during the withdrawal phase compared to that of the NBI group because of the darkening of the image associated with the use of NBI. This may have led to the finding of significantly greater number of polyps found in the white-light group. In addition, one third of patients had less than excellent colon preparation, which may have contributed to the poorer performance of the NBI visualization. Sabbagh *et al*[11] also performed a systemic review of the current evidence including 7 randomized control trials which showed no significant differences among groups in the mean number of polyps, the mean number of adenomas, and the rates of patients with at least one polyp or one adenoma. Two randomized control trials revealed a significant difference in the mean rate of adenomas detection in favor of the NBI group[14,15]. One trial reported a significantly higher detection in the mean number of flat adenomas in the NBI group[16], while other study demonstrated the opposite[17] and two other trials did not find any significant difference between the standard scope and the NBI[18,19].

Another metanalysis of 9 randomized control trials compared the yield and miss rate of narrow band imaging and white light endoscopy in patients undergoing screening or surveillance colonoscopy[8]. There was no significant difference between high-definition narrow-band imaging (HD-NBI) and high-definition white light endoscopy (HD-WLE) for the detection of adenomas (OR = 1.01, 95%CI: 0.74-1.37; I^2 = 60%; six RCTs) or for the detection of patients with polyps, patients with adenomas, the detection of adenomas over 10 mm, flat adenomas and flat adenomas per patient[8]. There was no significant difference for HD-NBI *vs* HD-WLE in polyp miss rate (OR = 1.17, 95%CI: 0.80-1.71; I^2 = 50%; three randomized control trials) or adenoma miss rate (OR = 0.65, 95%CI: 0.40-1.06; I^2 = 10%; three randomized control trials)[8]. These results are consistent with ours, however, our study did not evaluate the miss rate.

A prospective trial of 302 patients compared standard broadband white light colonoscopy with narrow-band imaging for the differentiation of colorectal polyps during real-time colonoscopy by using a modified Kudo pit pattern classification and vascular color intensity grading[20]. Overall, NBI accuracy was 80% compared with 77% for white light alone[20]. NBI performed significantly better than white light in diagnosing adenomas (sensitivity 80% *vs* 69%), particularly for adenomas ≤ 5 mm (75% *vs* 60%)[20]. There was no difference between NBI and white light for nonadenomatous polyps[20]. These findings are consistent with our results (sensitivity 68% *vs* 53% for NBI and Standard scope respectively). The diagnostic accuracies in this study[20] were better for larger polyps. Compared with white light, however, NBI did not significantly improve accuracy in any size or shape category, nor for any segment of the colon. In our study the most frequent anatomic location of the lesions was ascending colon (29%), followed by descending colon (18%) and rectum (each 16%). This distribution was not different between two scopes as well (P = 0.5). The researchers in this study[20] repeated the trial after training the endoscopists in detection and differentiation between different types of polyps. An equal number of polyps were analyzed in each of the two study periods (133 and 132, respectively). NBI accuracies significantly improved from 74% to 87% between the two study periods however, white light accuracies did not change (78% first half and 79% second half). After the learning curve was reached, NBI was significantly more accurate than white light[20]. The studies demonstrated that there is a learning curve with regard to NBI assessment of colorectal polyps, and that NBI performs better than the ordinary broadband white light once this "learning" is achieved[20].

A recent systematic review and meta-analysis on the real-time diagnostic operating characteristics of NBI colonoscopy included 28 studies with a total of 6280 polyps diagnosed in 4053 patients[13]. Endoscopic diagnosis of colorectal polyps with NBI showed highly accurate diagnostic performance, the area under the HSROC curve was 0.92 (95%CI: 0.90-0.94). The overall sensitivity of NBI diagnosis was 91.0% (95%CI: 87.6%-93.5%) and specificity was 82.6% (95%CI: 79.0%-85.7%) compared with histology[13]. The sensitivity and specificity of diagnosis of diminutive polyps, made with high confidence was 93.4% (95%CI: 87.4%-96.7%) and 84.0% (95%CI: 76.6%-89.3%), respectively[13]. The findings of this meta-analysis suggest that real-time endoscopic diagnosis of colorectal polyps performed using NBI has a high diagnostic performance[13]. Endoscopic diagnosis correctly characterized 91% of neoplasms and 83% of non-neoplastic polyps. This study addressed the standards set forth by the American Society of Gastrointestinal Endoscopy (ASGE) for the "resect and discard" strategy[12]. The summary agreement was 92.6% in this study[13], supporting the clinical use of such a strategy. In our study the sensitivity 68% and specificity 74% of the NBI were much lower as compared to the findings of McGill *et al*[13] meta analysis. This discrepancy could be related to the size of the population studied, however our project studied only a minority population African Americans.

The findings of our study have some limitations: the total number of polyps was relatively small 179 as compared to higher number in other studies. A sub group analysis for polyps ≤ 5 mm was not performed, the "resect and discard" strategy has been proposed by the ASGE for adenomas ≤ 5 mm in size without pathologic assessment when the endoscopic diagnosis provides a ≥ 90% agreement in assignment of postpolypectomy surveillance intervals compared with decisions based on pathology[12]. Interobserver variations were also noted, a possible training of the endoscopists prior to the use of the NBI could have improved the outcome. The cost saving for

this approaches is substantial and was confirmed by Kessler *et al*[21]'s study. Based on the annual volume of colonoscopy in the United States, the annual up-front cost savings of forgoing the pathologic assessment would exceed a billion dollars[21].

Overall, the colonoscopy diagnosis is not yet fully matched with pathologic diagnosis of colon polyp. However with the advancement of both imaging and training, it may be possible to increase the sensitivity and specificity of the scopes and hence save money for eliminating time and the cost of Immunohistochemistry/pathology.

COMMENTS

Background

High definition colonoscopy (HD) with narrow band imaging (NBI) scope has advantage to detect vascular or mucosal characteristics so that any abnormal growth could be better visualized and diagnosed than standard colonoscopy.

Innovations and breakthroughs

Diagnostic accuracy using HD scope with NBI is cost saving as it eliminates and/or decreases the volume of specimens that need pathology assessment and diagnosis, especially for polyps < 1 cm.

Applications

The results of present study indicate that HD-NBI scope more sensitive for adenoma diagnosis than standard scope colonoscopy.

Terminology

High definition with narrow band imaging scope correspond to improvements in the resolution of imaging techniques in colonoscopy, that increases the accuracy of diagnosis using narrow-band width filters in a red-green-blue sequential illumination system. It improves the detailed visualization of the micro vascular and micro structural colon pit in patterns.

Peer-review

The present study presents the differences between two advanced explorations with different levels of viewing the pathological target, which are colon polyps. The exposure mode, of the differences between the two techniques is in accordance with the standards of a well written article.

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Epidemiology of functional gastrointestinal disorders in infants and toddlers: A systematic review

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Abstract

AIM: To assess the functional gastrointestinal disorders (FGID) prevalence in infants and toddlers.

METHODS: PubMed, EMBASE, and Scopus were searched for original articles from inception to February 2016. The literature search was made in accordance with Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA). For inclusion, each study had to report epidemiological data of FGID on children up to 4 years old and contain standardized outcome Rome Ⅱ or Ⅲ criteria. The overall quality of included epidemiological studies was evaluated in accordance to Loney's proposal for prevalence studies of health literature. Two reviewers assessed each study for inclusion and extracted data. Discrepancies were reconciled through discussion.

RESULTS: It was identified a total of 101 articles through the databases and two through the manual search. A total of 28 articles fulfilled the eligibility criteria. After reading the full articles, 13 of them were included in the present review. Twelve studies were written in English and one in Chinese, and published between 2004 and 2015. Eight articles (61.5%) were performed in Europe, three (23.1%) in America and two (15.4%) in Asia. Sample size varied between 45 and 9660 subjects. Cross-sectional frequency was reported in majority of studies (k = 9) and four studies prospectively followed the subjects. 27.1% to 38% of participants have met any of Rome's criteria for gastrointestinal syndromes, of those 20.8% presented two or more FGID. Infant regurgitation and functional constipation were the most common FGID, ranging from less than 1% to 25.9% and less than 1% to 31%, respectively. Most included studies were of moderate to poor data quality with respect to absence of confidential interval for prevalence rate and inadequate sampling methods.

CONCLUSION: The scarcity and heterogeneity of FGID data call for the necessity of well-designed epidemiological research in different levels of pediatric practice and refinement of diagnostic.

Key words: Infant; Functional gastrointestinal disorders; Epidemiology; Prevalence; Toddler

Core tip: Epidemiological studies on functional gastrointestinal disorders in infants and toddlers provide variable prevalence rates in both pediatric outpatient and inpatient practice. A number of investigations and reviews have been conducted using Rome's criteria for functional disorders to depict the magnitude of the problem, however, few investigations have reported meaningful results with adequate methodology. The current literature review suggested higher impact of pediatric feeding and defecation problems that affect very young children, respectively infant regurgitation and functional constipation.

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INTRODUCTION

Functional symptoms are corporal manifestations that arise in the absence of anatomic abnormality, inflammation, or tissue damage. Historical descriptions and theories on complex interaction between biological, psychological and social factors that predispose, precipitate and/or perpetuate brain-gut axis disorders are accounted in psychosomatic and neuroscience literature[1]. Common complaints that often determine visits to pediatric practices are feeding and eating symptoms and elimination problems in infants and toddlers, such as: regurgitation, nausea, vomiting, heartburn, abdominal pain and bellyaches, abdominal distension, bloating, belching, chronic diarrhea or constipation, fecal soiling, retching, or food refusal. Most of them lack biological ground to guide the recovery process.

Gastrointestinal (GI) disorders in childhood are major reasons that drive caregivers to consult healthcare settings[2-5]. As such, functional gastrointestinal disorders (FGID) stand as a group of conditions that include a mixture of age-dependent, chronic or recurrent symptoms without evident structural or biochemical abnormalities affecting GI functioning. Recent report of Kids' Inpatient Sample Database on hospitalization and cost has indicated the increasing burden of childhood FGID in the United States from 1997 to 2009[6]. Abdominal pain and constipation in childhood were the most common discharge diagnoses. However, good quality epidemiological data on FGID in infants and toddlers to guide the reorganization of pediatric practice are scant.

Many pediatric caregivers lead to misuse primary care and GI specialized care, driving to unnecessary investigations and pharmacological treatments. While several GI symptoms without obvious causal explanation are distressing for small children and their caregivers, these burdensome conditions are not life threatening when parental concerns are properly addressed. Therefore, accurate estimates of prevalence and consequences in agreed description of GI syndromes are required for defining the need for treatment in overloaded healthcare settings.

Purposing public health policy and resource provision, epidemiological studies might show similar frequency for common GI conditions across countries in varied levels of development. Projected proportion of pediatric FGID cases in the community, and different levels of healthcare setting, would help a better allocation of financial support and organize health service delivery.

The aim of this literature review is to critically examine current evidence of knowledge on FGID in infants and toddlers, through systematic search of prevalence data on common functional GI problems.

MATERIALS AND METHODS

Search strategies

A literature search was conducted in following databases: PubMed, EMBASE, and Scopus in accordance with Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA)[7]. The search terms were "functional gastrointestinal disorder" OR "functional gastrointestinal symptoms" AND "epidemiology" OR "prevalence" OR "incidence". In addition, for each seven specific category of FGID in infants and toddlers, new search was performed with the disorder's nomenclature and equivalent synonyms. For example, "infant regurgitation" AND "gastroesophageal reflux" were combined with epidemiological terms (Appendix in the Supplementary materials).

There was no language restriction and the period covered was from inception to February 22, 2016. For inclusion, each study had to report epidemiological data on children up to 4 years old and contain standardized outcome criteria (Rome Ⅱ or Ⅲ)[8,9]. Case report, letter, editorial, intervention studies, case-control studies, treatment guidelines, review, and duplicate articles were eliminated. "Similar articles" option and manual search of reference list of review articles, book chapter, and gray literature completed the investigation. Experts in pediatric gastroenterology

Table 1 Classification of functional gastrointestinal disorders in infants and toddlers

	Rome Ⅲ nomenclature	Age	Frequency	Duration	Synonym or approximate terms
G1	Infant regurgitation	3 wk-12 mo	⩾ 2 regurgitations/d	⩾ 3 wk	Gastroesophageal reflux
G2	Infant rumination syndrome	3-8 mo[1]		⩾ 3 mo	Childhood feeding and eating disorders[2]
G3	Cyclic vomiting syndrome		⩾ 2 vomiting episodes	hours - days	Periodic vomiting
G4	Infant colic	0-4 mo	⩾ 3 d/wk for ⩾ 1 wk	⩾ 3 h/d	Infantile colic; abdominal pain syndrome Wessel's criteria: irritability, fussing or crying
G5	Functional diarrhea	6-36 mo[1]	⩾ 3 painless stools/d	⩾ 4 wk	Infantile diarrhea
G6	Infant dyschezia	< 6 mo	⩾ 10 min straining		Straining and crying
G7	Functional constipation	⩽ 4 yr	⩽ 2 defecations/wk ⩾ 1 incontinence/wk	⩾ 4 wk	Classic Iowa criteria Functional defecation disorder Functional fecal retention[3]

[1]Onset of symptoms; [2]International Classification of Disease and Diagnostic and Statistical Manual systems; [3]Rome Ⅱ nomenclature.

were contacted to request full text or unpublished data. Two reviewers (Ferreira-Maia AP, Wang YP) assessed each study for inclusion and extracted data. Discrepancies were reconciled through discussion.

Afterward data extraction from studies included in qualitative synthesis, a review summarized main epidemiological findings in two broad sections to cover main Rome foundation's categories (Table 1): (1) feeding and eating disorders; and (2) colic and defecation problems.

Major measurement instruments for assessment of FGID, such as Questionnaire on Pediatric Gastrointestinal Symptoms (QPGS)[10,11] and Pediatric Quality of Life (PedsQL) Inventory[12] were discussed.

Critical appraisal of research literature on childhood GI problems

The overall quality of included epidemiological studies was evaluated in accordance to Loney's proposal for prevalence studies of health literature[13]. All studies were scored on eight criteria: (1) sample size; (2) sampling adequacy; (3) unbiased sampling frame; (4) measures of outcomes; (5) unbiased assessors; (6) response rate with refusals described; (7) prevalence with confidence intervals and by relevant subgroups; and (8) appropriate description of study subjects for the research question. The study receives one point for each criterion met, which possible score ranges from zero to eight. The higher score indicates better study quality.

The sample size criterion was not used to exclude studies. However, we considered an appropriate sample size if it was based on local population estimates or if it was higher than 500. This minimum sample size was calculated to permit outcome assessment using simple random sampling, with a conservative estimate of distinct FGID of 20% in the age bracket of infant and toddler[14], confidence level of 95%, and precision of 1.8%, resulting in a sample of 494 subjects.

Two reviewers (Ferreira-Maia AP and Wang YP) performed the evaluation and final results were discussed one by one.

Methodological issues

In evaluating studies of the epidemiology of FGID in infants and toddlers, the following methodological questions must be considered: (1) the case definition and evaluation of FGID conditions; (2) the comparability of FGID assessments across studies; (3) the assessment of FGID prevalence and incidence; and (4) the comparability of FGID data drawn in different settings.

What is a case of FGID?

Current diagnoses of FGID are symptom-based criteria defined by a mixture of chronic or recurring GI symptoms with no structural or biochemical explanation. The increasing recognition of FGID has been ascribed to the efforts to operationalize clear-cut categories by Rome foundation (http://www.romecriteria.org/). The accomplishment to legitimize and update knowledge on FGID has subjected scientists and clinicians around the world to classify and appraise the science of GI function and dysfunction. Bringing together extensive consensus debates and field studies, this foundation has included a pediatric committee since 1999[15]. Nonetheless, the classification of FGID in infants and toddlers remains largely unexplored and poorly understood.

For Rome Ⅲ, published in 2006, a working team committed to discuss the case definition of a FGID occurring in children up to 4 years[16]. Under chapter G, four Rome Ⅱ criteria remained the same in Rome Ⅲ - regurgitation, rumination, diarrhea, and dyschezia. By consensus, infant colic (G4) was included in Rome Ⅲ and broader criteria for cyclic vomiting syndrome (G3) and functional constipation (G7) were amended (Table 1). Some duration and frequency requirements were relaxed.

The infant regurgitation (G1) is a common feeding manifestation in infants below the age of 1 year and the frequent reasons of counseling to general practitioners and pediatricians[16]. Whereas common complaints include overfeeding, air swallow during feeding, crying or coughing, physical exam generally reveals no abnormality or weight gain delay.

The infant rumination (G2) is an uncommon feeding

disorder and difficult to differentiate from commoner conditions causing vomiting and weight loss. In general, the symptoms start between 3 and 8 mo of age[16]. In infancy or early childhood, avoidance of food or restricted food intake, can include a wide spectrum of feeding disorder, for example pica, rumination disorder, and other presentations that are listed in International Classification of Disease (ICD)[17] and Diagnostic and Statistical Manual (DSM) system[18].

The cyclic vomiting syndrome (CVS; G3) is described as recurrent, stereotypic episodes of severe nausea and vomiting lasting hours to days that are separated by symptom-free intervals[19]. The CVS is most observed in toddlers than infants[20,21]. The attacks can occur at regular intervals or sporadically in typical episodes that begin at the same time of day, commonly during early morning or night. Thereafter, vomiting tends to wane, although complaint of nausea persists until the end of episode. In order to facilitate early recognition and mitigate patient suffering, the working committee of Rome Ⅲ has modified the number of vomit episodes to two for the diagnosis of CVS, instead of three episodes in Rome Ⅱ[16].

The infant colic (G4) is a condition that usually prompts parents to seek medical care. Classical Wessel's criteria defined fussing or colic as "crying for three hours or more on at least three days in at least three weeks"[22]. Since infants with colic are often referred to pediatric gastroenterologists, the Rome Ⅲ working group achieved consensus to include infant colic as FGID. "Paroxysms of irritability, fussing or crying lasting three hours per day and occurring three days each week" is the amended symptomatic description of colic[16]. Although this condition improves with time (for most infants, crying and irritability begin to decrease by four months of age), it causes significant parental distress, involving long crying bouts and hard-to-soothe behavior[23].

The functional diarrhea (G5) is defined by daily painless, recurrent passage of three or more unformed and large amount of stools for more than four weeks, and with onset between ages of 6 and 36 mo. The important point is no failure to thrive neither inadequate diet. The child feel unworried about the loose stools, and the symptom remit spontaneously by school age[16].

The infant dyschezia (G6) is categorized by a minimum 10 min of straining and crying before successful passage of soft stools in an otherwise healthy infant under 6 mo of age[16]. When occurring several times daily, these episodes are exhausting for the infant and anxiety provoking for the caregivers. The parents usually seek medical help for their child during the first 2 to 3 mo of life with concerns that their child is constipated. In general, the parents describe a healthy infant who cries for 20 to 30 min, turns red in the face, and screams, seemingly in pain, before defecation takes place[24].

The functional constipation (G7) is one of most common reasons that lead parents to visit gastroenterological services[4,14]. Rome Ⅱ criteria for functional constipation were modified in Rome Ⅲ, in terms of symptom duration and frequency: from 12 wk to 1 mo and from ⩽ three defecations to ⩽ two defecations per week[16]. This change was based on data showing that the longer functional constipation goes unrecognized, the less successful treatment is[25].

Generally, along with frequency and duration of the symptoms for diagnosis of a separate syndrome, the Rome criteria operationalized the age range according to the probable occurrence of functional symptoms. Almost all FGID listed in Rome Ⅲ use monothetic classification rule, which is definition terms of characteristics are both necessary and sufficient to identify members of that condition. One exception is the functional constipation (G7) that is polythetic rule, applied to minimal number of two out of six defining characteristics, *i.e*., none of the features has to be found in the category in terms of a set of criteria that are neither necessary nor sufficient. For instance, this prototypical description requires that each case of constipation only must possess certain characteristics, allowing identification of broad varieties of the syndrome.

Most exclusionary requirements of Rome Ⅲ emphasize the benign nature of the FGID in infants and toddlers, with inter-episodic normal health or no failure in thrive. Hierarchical rules of organic exclusion are not always specified, in order to attain the stringent definition of "functional" disorders, where structural or biochemical abnormalities affecting GI functioning must be ruled out. Since around 5% of colic symptoms[26] and 7.7% of constipation symptoms[27] may be consequence of organic etiology, some heterogeneous conditions may be mislabeled as FGID.

Despite of mounting interest for searching causal explanations and applicability of these criteria in pediatric settings, few epidemiological data have strengthened the face validity and clinical utility of symptom-based Rome Ⅲ criteria. Lack of sensitivity for constipation in Rome Ⅱ[25,28] and high restrictiveness for dyschezia in Rome Ⅲ[24] were claims raised by researchers. While some researchers regard Rome Ⅲ functional constipation more sensitive than Rome Ⅱ in population under 18 years[29], its applicability in infants and toddlers remains dubious. Similar low rates of constipation in children fewer than 5 years were reported by a study comparing these two criteria in Thailand[30].

Recent efforts to build a multidimensional strategy for establishing the diagnosis of FGID following the approach of DSM for psychiatric syndromes[18] may provide a more thorough description of GI syndromes. Psychosocial and behavioral aspects of the GI problems should be included in future criteria to supplement the complexity of childhood FGID. Although the successive editions of DSM may serve as roadmap to construct more robust categories of FGID, available studies using reliable Rome Ⅲ criteria have insufficient data to move

forward. Large and well-designed epidemiological studies with unbiased sampling are required to document all aspects of natural history of FGID, such as clinical manifestations, pattern of comorbidity, impairment, disability, service use, laboratory study and exclusion of organic disorders, parent-child interaction, family study of genetic aspects, and therapeutic response to different types of intervention. Systematic assessments with validated standardized inventories or structured interviews can improve the nosological validity of FGID in childhood.

Comparability of assessment of FGID in infants and toddlers across the studies

Subjective appraisals of GI symptoms through parental reports are unavoidable in small children, because complaints that occur among infants and toddlers depend largely on observation of their caregivers, as well as the decision to set medical appointment. As such, the tendency for FGID to cluster within families was highlighted in previous researches[3,5]. Potential psychosocial mechanisms that may contribute to the intergenerational transmission of illness behaviors were case-control tested in mothers of irritable bowel syndrome children[31]. Parental reinforcement or modeling of symptoms, coping, psychological distress, and exposure to stress were adult responses to child symptoms that might influence the reporting of children's GI and non-GI symptoms[32].

The implications of inheritance or social learning in the family of FGID are unclear[33]. Studies in children aged 4 or more years have demonstrated that the parent-child concordance was largely poor[11]. In addition, the increased frequency of psychopathology in parents who reported data for their children and overlap with FGID should emerge as potential threat to information bias[3,34].

The QPGS was originally proposed as both a parent report and child self-report for the assessment of child and adolescent GI symptoms[10,11]. Recently, the group of University of North Carolina at Chapel Hill has translated the Rome Ⅲ FGID diagnostic criteria for infant and toddler into a symptomatic questionnaire (QPGS-RIII) to be answered by their parents or guardians, showing satisfactory face and content validity[34]. For instance, this tool was tested among 332 respondents under 4 years who attended a gastroenterology clinic and agreement between parent and physician was poor to acceptable (κ = 0.18-0.76).

Infant rumination and functional diarrhea were the two categories with poor overlap between parents and physicians. Since over half of new pediatric GI clinic patients met Rome Ⅲ criteria for one or more FGID[4], a standardized questionnaire with high specificity that satisfy explicit criteria may enhance diagnosis before specialized gastroenterological care appointment.

The impact of pediatric diseases and treatments is increasingly assessed from the perspective of quality of life. In accordance with World Health Organization, health-related quality of life should measure dimensions of physical, emotional (including psychological and cognitive), social, and school/day care functioning. Either pediatric patients or their parents should be asked to report validated measure of quality of life. The Pediatric Quality of Life Inventory (PedsQL; http://www.pedsql.org/) 4.0 Generic Core Scales has been used to assess children with several health conditions, as well as healthy populations[35,36]. The respondents who met Rome's criteria for FGID presented worse quality of life and more likely have used health care service[5]. Recently, the PedsQL4.0 has been intensively used to investigate FGID[35-38]. The development of the PedsQL GI symptoms was reported[39] and examined in cross-cultural settings[40]. Psychometric properties and construct validity for its applicability in infants up to 24 mo have been accepted[41].

Comparability of prevalence and incidence rates of FGID

Great variation of prevalence rates can be attributable to sampling methods and population frame. Many of studies on FGID have selected children of different or large age range, from early childhood to late adolescence, hampering comparisons between similar FGID occurring below 4 years. Recruitment methods and settings were varied, but most investigations used data on consecutive treatment-seeking samples in tertiary care, threatening the validity of estimates due to selection bias of hospital-based data[4]. Representativeness of recruited sample and comparability of estimated prevalences are issues of upmost concern. Prospective assessment for organic exclusion was not always reported in studies.

A study to determine the incidence of functional diseases must have a prospective or longitudinal design, and should include children known not to have the disease, who may be observed over an appropriate time period. This type of study is onerous and long time consuming, demanding intense planning and large number of trained researchers. Only two incidence studies were identified for FGID[42,43], their data were drawn from Irish Pediatric Surveillance Unit for CVS in Ireland and Danish National Patient Registry for eating disorders in Denmark. Respectively, case definition has used criteria from the First International Symposium on CVS and the ICD-10 classification[17] of childhood feeding and eating disorders (F98.2 and F50.8).

After recognizing the scarcity of epidemiological data, a recent worldwide Delphi consensus study was conducted to gather data from pediatric workers and scientists to achieve a convergent expert opinion on real-world magnitude of FGID in infants less than 12 mo[14]. In general, the expert panel agreed that good quality data in pediatric gastroenterology were lacking, but the most common FGID in this age range were regurgitation, colic, and constipation, with expected prevalence around 30%, 20%, and 15%, respectively.

Functional diarrhea and dyschezia were under-investigated conditions and the likely prevalence was estimated as around 5% for both FGID.

Comparability of FGID data drawn in different settings

The appropriateness of study design and sampling method for the research question is crucial to determine prevalence of a particular health outcome. An observational study (survey) is the appropriate study design to determine the prevalence of a target condition, by covering the whole population of interest. When the specific population is small, *e.g*., single pediatric service or private practice, some studies survey the whole population and cannot generalize the results to other populations. Notably, potential selection bias may emerge in accordance with the level of health care specialization, producing overestimated proportion of GI diseases[4,28]. Characteristics of children attending well-baby clinics, immunization center and primary care may differ greatly from the population who visit pediatric gastroenterologist or are referred to a tertiary care for further investigation. In addition to presence of real distressing FGID, this differential probability of service contact depends of caregiver's own characteristics (*e.g*., previous history of GI disorders) and socio-economic features, as well as the availability of healthcare services. Taking all bias into account, the comparability of frequency of health outcome diagnosed in different levels of pediatric practice should be avoided.

Since service use sample was referred from lower level of pediatric healthcare, the prevalence rates were likely higher than the sample recruited from community. The appropriate type of sampling frame or list for study recruitment from which subjects are selected is critical for the sample representativeness. Studies of whole, narrowly defined communities are usually done as door-to-door surveys, but this limits the generalizability of the findings outside that community. In large surveys, groups of individuals (*e.g*., families or children living in defined geographical areas) are selected as the survey units.

Census data can provide data sets from which one can draw a sample that is deemed to have minimal bias, since specific groups of persons are not excluded as they might be in a healthcare service, Internet use or telephone list[4,5,28,44]. When it is not possible to cover the total population, the best sampling technique is probability (simple random) sampling of persons from a defined subset of the population.

In some instances, stratification may be necessary to correctly represent subgroups, in order to ensure representativeness of the population and validate the generalizability of the results for the entire population. For example, exclusive school lists may underrepresent the institutionalized children (hospital, orphanage, correctional communities) or those who are homeless. In relation to the aim of reviewing FGID prevalence studies, the "convenience" sample could be importantly biased in the way that sizable proportion of children with FGID could be missed (because the "cases" of diseases were hospitalized or in home), thus reducing the prevalence of GI diseases in the sample. Population-based surveys offer superior epidemiological data than studies that have recruited children from community, school, well-baby clinic, primary care or specialty care.

RESULTS

Literature search and general description of included studies

The search flow diagram is displayed in Figure 1. It was identified a total of 101 articles through the three databases and two through the manual search. Of these, after removing duplicate records and reading the title and abstract, a total of 28 articles fulfilled the eligibility criteria. After reading the full articles, 13 of them were included in the present review. The 15 studies excluded were listed in the Supplementary materials (Appendix).

Regarding to 13 included articles, 12 studies were written in English and one in Chinese, and published between 2004 and 2015. Eight articles (61.5%) were performed in Europe (Italy, the Netherlands and Turkey), three (23.1%) in America (United States and Brazil) and two (15.4%) in Asia (China and Thailand).

The total sample size varied between 45 and 9660 subjects. In cross-sectional studies, the sample size ranged from 264 to 5030 participants[3,5] among community dweller or attenders of primary care level (*e.g*., well-baby clinic, immunization center). In Italian formal primary care service, the surveyed population ranged from 2642 to 9660 children[45-47], all enrolled in National Health Service. No routine indication of loss or exclusion was reported in treatment seeking or referred sample to specialty gastroenterological clinic, and the number of participants ranged between 45 and 402[4,44].

Cross-sectional frequency was reported in majority of studies (k = 9), although several investigations followed the recruited sample to confirm diagnosis or organic exclusion. Only four studies prospectively followed infants and toddlers for describing the change of prevalence rate of target GI syndrome at different ages.

Most investigations were conducted with questionable sampling frame, in specialty gastroenterological clinic or university-based hospital (k = 4), primary care (k = 3) or well-baby clinic (k = 2). Further studies in community (k = 4), two of them reported data with debatable methodology (*e.g*., quota sampling, online panel, non-random recruitment), and only two studies were population-based prospective cohorts[48,49] and provided generalizable data of prevalence of functional constipation, respectively in Brazil and the

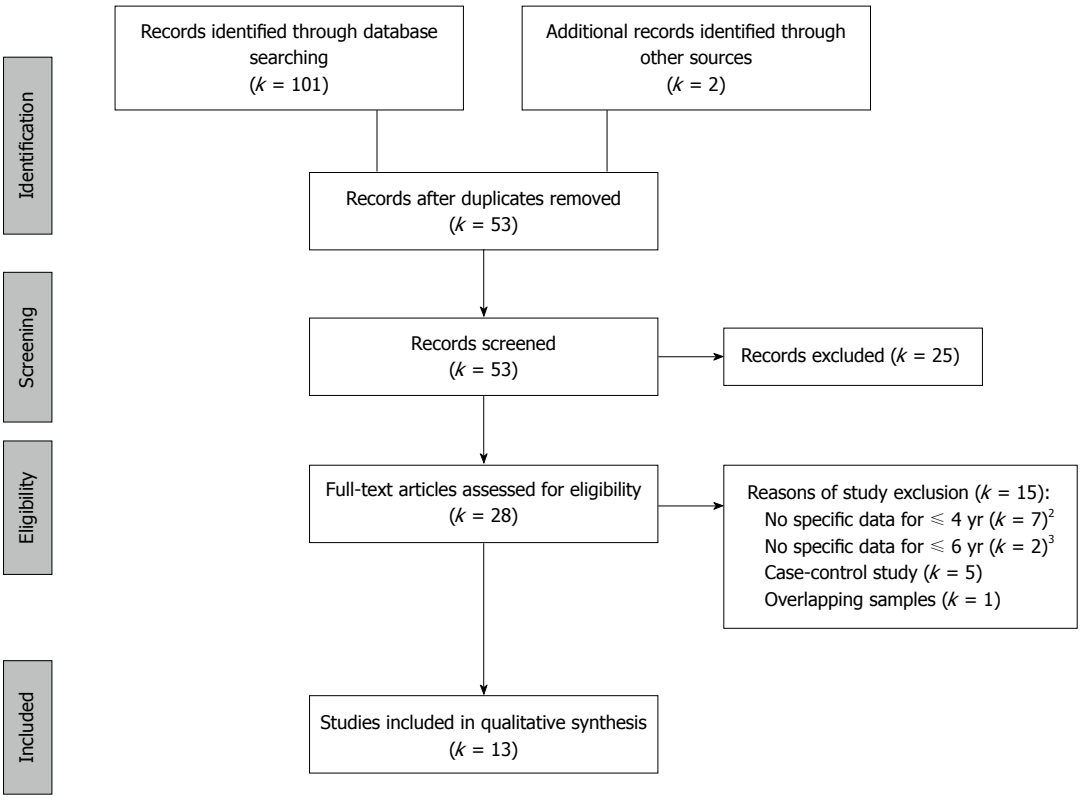

Figure 1 Flow diagram for identifying eligible articles[1]. [1]Flow diagram according to PRISMA (www.prisma-statement.org); [2]Rome III nomenclature; [3]Rome II nomenclature.

Netherlands.

Independent assessors applied the Rome foundation's criteria, either version Ⅱ or Ⅲ. Two studies[24,48] modified the Rome criteria and one study[30] compared the two versions.

No age, sex, or race differences were found in FGID diagnoses. 27.1% to 38.0% of infants and toddlers have met any of Rome's criteria for GI syndromes[3,5], of those 20.8% presented two or more FGID[5]. In pediatric specialty gastroenterological care, FGID was confirmed in 52% of treatment-seeking subjects under to 4 years, around 18% of them presented two or more FGID[4]. In comparison with those who did not meet Rome criteria, subjects with FGID had lower quality of life, delayed thrive, increased medical visits, mental health visits, and hospital stays[3,5].

The critical appraisal of 13 included studies indicated that good quality researches reporting main categories of FGID in infants and toddlers were scarce. Only five articles obtained scores seven or six according to Loney's proposal[3,24,30,48,49]. The most common quality problem was prevalence rates without confidence interval and/or no detail by subgroup (k = 12), inadequate sampling method (k = 9), inappropriate sampling frame (k = 8), refusers not described (k = 7) and/or insufficient sample size (k = 5) (Supplementary Table 1).

Below, we discussed main epidemiological investigations associated to feeding and eating problems (Table 2), and colic and elimination problems (Table 3).

Feeding and eating problems in infants and toddlers

In total, there were six studies on feeding and eating problems. All of them used questionnaire on FGID to assess the symptoms and half of them were performed in Italy[45-47].

All studies had information about infant regurgitation. The rates ranged from less than 1% in primary care[45,47] to over 25% by online panel in community[5]. Further rate of 13.2% was described in specialty care in United States[4] and 17.9% in Chinese community[3].

Only two studies reported frequency of infant rumination, both from United States, as 4.3% by online panel in community[5] and 3% in specialty care[4].

Based on Rome Ⅲ, a rate of online parental report of 3.4% was estimated for cyclic vomiting syndrome among children between 1 and 3 years[5] and 10.2% for children under 4 years in a single tertiary care[4], both studies from United States.

Colic and defecation problems in infants and toddlers

Eleven studies described data about colic and defecation problems. Six of them used questionnaire on FGID to assess the symptoms. Three studies were conducted in the Netherlands; two in Italy; two in United States; and the remaining from Thailand, Turkey, China and Brazil.

Three studies had information about frequency of infant colic in children between zero and 4 years. One study reported 4.2% in a specialty care[4], and the

Table 2 Prevalence or frequency of functional gastrointestinal disorders: Eating and feeding problems in infants and toddlers

Author, year, country	Study design, setting	Sample size (participation %)	Age bracket	Case definition	Case ascertainment	Score	FGID prevalence %		
							Regurgitation	Rumination	Cyclic vomiting
Van Tilburg, 2015, United States	Cross-sectional, community	264 (82.5)	0-3 yr	Rome Ⅲ	Online panel w/mothers QPGS-RIII PedsQL4.0	2	25.9	4.3	3.4
Rouster, 2015, United States	Cross-sectional, specialty care	332 (91.0)	0-4 yr	Rome Ⅲ	Parental interviews QPGS-RIII	4	13.2	3.0	10.2
Liu, 2009, China	Cross-sectional, community	5030 (99.4)	0-2 yr	Rome Ⅲ	Parental interviews Parental questionnaire on GI symptoms	6	17.9		
Primavera, 2009, Italy	Cross-sectional, primary care	9291 (NR)	0-14 yr	Rome Ⅱ	Interviews w/parents and children FGID questionnaire	4	0.44		
Campanozzi, 2009, Italy	Cross-sectional, primary care	2642 (NR)	1-12 mo	Rome Ⅱ	Interviews w/parents and children I-GERQ	4	12.0		
Miele, 2004, Italy	Cross-sectional, primary care	9660 (NR)	1-12 mo	Rome Ⅱ	Interviews w/parents and children FGID questionnaires	4	0.74		

Score = Methodological strength of study (maximum 8) by Loney's criteria. PedsQL4.0: Pediatric Quality of Life version Inventory 4.0; I-GERQ: Infant Gastroesophageal Reflux Questionnaire; QPGS-RIII: Questionnaire on Pediatric Gastrointestinal Symptoms - Rome Ⅲ; FGD: Functional gastrointestinal disorders; NR: Not reported; w: With; GI: Gastrointestinal.

others reported 5.9%[5] and 1.4%[3], both carried out in community sample.

The overall rates of functional diarrhea across four studies ranged from 0.07% in primary care[45] to 12.3% in community[3]. The only that used Rome Ⅱ criteria was also the one with the lowest rate (0.07%).

Concerning to frequency of infant dyschezia through three studies: two cross-sectional studies, with children aged until 4, reported frequency of 2.4% in community[5] and 3.3% in specialty care[4]; one prospective cohort study in well-baby clinics, with children between 1 and 9 mo of age, reported rates 3.9% (Rome Ⅲ) and 17.3% [Modified-Rome Ⅲ (M-Rome Ⅲ)] at age of 1 mo, and 0.9% (Rome Ⅲ) and 6.5% (M-Rome Ⅲ) at age of 3 mo[24].

Ten studies reported data about infant constipation. To definition of cases: four articles used Rome Ⅲ criteria; other four Rome Ⅱ; one M-Rome Ⅱ; and one study compared Rome Ⅱ and Rome Ⅲ.

For those articles that used Rome Ⅲ criteria, the frequency of constipation varied between 11.6% at age of 3 mo in a healthy newborns sample[44] to 29.2% in children under 4 years from a specialty care[4].

Rates from 64% in children below 6 years from a tertiary care[28] to 0.68% in infants aged less than 12 mo from a primary care[45] were reported through the studies that used Rome Ⅱ criteria.

There was only one population-based birth-cohort study[49] that used Rome Ⅱ criteria; it reported prevalence rates of 11.2%, 15.7% and 14.2% at age of 2, 3 and 4 years respectively.

The Brazilian population-based birth-cohort study[48] used M-Rome Ⅱ criteria and described prevalence rates of 27.3% at age of 2 years and 31% at age of 4 years.

The last study[30], reported frequencies of 1.9% and 1.6%, respectively for Rome Ⅱ and Ⅲ criteria in children under 5 years.

DISCUSSION

This review, relied on data from 13 articles, showed a vast variation in the FGID prevalence in infants and toddlers. The majority of studies (k = 7) recruited participants from primary care or specialty care. Further studies were 4 cross-sectional ones in community and 2 in well-baby clinics. Good quality epidemiological data reporting main categories of FGID in infants and toddlers were limited. Based on current review, it is suggested that there is an increasing burden attributed to FGID in pediatric practices. However, generalizable information is restricted in terms of community studies and well-assessed diagnosis that might support observed differences across pediatric care levels.

Infant regurgitation and functional constipation are the most investigated disorders in FGID and might have public health impact.

More conclusive recommendation should be avoided as the rates vary greatly across studies. Although the broad variation in the infant regurgitation prevalence may reflect the poor quality of epidemiological data (recruitment and sampling frame), expert respondents to a recent consensus survey according to Rome Ⅲ criteria reported an average worldwide prevalence of 29%[14]. This rate is similar to the rate reported in the included study with a community sample. For instance,

Table 3 Prevalence or frequency of functional gastrointestinal disorders: Colic and defecation problems in infants and toddlers

Author, year, country	Study design, setting	Sample size (participation %)	Age bracket	Case definition	Case ascertainment	Score	FGID prevalence %			
							Colic	Diarrhea	Dyschezia	Constipation
Tharner, 2015, the Netherlands	Population-based birth-cohort, community	4823 (61)	2-4 yr	Rome Ⅱ	Interviews w/parents and children, CEBQ - parental	7				2 yr: 11.2 3 yr: 15.7 4 yr: 14.2
Van Tilburg, 2015, United States	Cross-sectional, community	264 (82)	0-3 yr	Rome Ⅲ	Online panel w/mothers QPGS-RIII PedsQL4.0	2	5.9	8.8	2.4	14.1
Kramer, 2015, the Netherlands	Prospective cohort, well-baby clinics	1292 (87)	1-9 mo	Rome Ⅲ M-Rome Ⅲ	Interviews w/parents and children Parental questionnaire	6			1 mo: 3.9/17.31 3 mo: 0.9/6.51	
Rouster, 2015, United States	Cross-sectional, specialty care	332 (91)	0–4 yr	Rome Ⅲ	Parental interviews QPGS-RIII	4	4.2	0.3	3.3	29.2
Osakatul, 2014, Thailand	Cross-sectional, hospital's catchment area	3010 (97.1)	4 mo-5 yr	Rome Ⅱ Rome Ⅲ	Parental interviews Laboratory exams	7				1.9 (Rome Ⅱ) 1.6 (Rome Ⅲ)
Turco, 2014, Italy	Prospective birth-cohort, university-based hospitals	402 (86.4)	0-12 mo	Rome Ⅲ	Telephone interview w/parents	4				3 mo: 11.6 6 mo: 13.7 12 mo: 10.7
Mota, 2012, Brazil	Population-based birth-cohort, community	3799 (92)	0-4 yr	M-Rome Ⅱ	Interviews w/mothers	7				2 yr: 27.31 4 yr: 31.01
Liu, 2009, China	Cross-sectional, community	5030 (99.4)	0-2 yr	Rome Ⅲ	Parental interviews Parental questionnaire on GI symptoms	6	1.4	12.3		13.7
Aydoğdu, 2009, Turkey	Cross-sectional (retrospective), university-based hospital and specialty care unit	193 (NR)	0-5 yr	Rome Ⅱ Iowa criteria	Medical records Laboratory exams	3				51.9
Miele, 2004, Italy	Cross-sectional, primary care	9660 (NR)	1-12 mo	Rome Ⅱ	Interviews w/parents and children FGID questionnaires	4		0.07		0.68
Voskuijl, 2004, the Netherlands	Cross-sectional, tertiary care	45 (65.7)	≤ 6 yr	Rome Ⅱ Iowa criteria	Patient diary Medical interviews w/ parents and children Physical examination	3				64.0

Score = Methodological strength of study (maximum 8) by Loney's criteria. CEBQ: Child Eating Behavior Questionnaire; QPGS-RIII: Questionnaire on Pediatric Gastrointestinal Symptoms - Rome Ⅲ; PedsQL4.0 Pediatric Quality of Life version Inventory 4.0; I-GERQ: Infant Gastroesophageal Reflux Questionnaire; NR: Not reported; w: With; M-Rome: Modified Rome criteria.

three surveys from Italy used the National Health Service registry in different regions of the country and reported frequencies as low as less than 1% to 12% of children in primary care.

As a pathology liable to affect all ages, most of included studies provided data on functional constipation. The Dutch Generation R study and the Brazilian study investigated the entire pediatric population and provided unbiased information on functional constipation of higher external validity. The first study reported prevalence similar to that estimated by experts (around 15%)[14], but the second study described a two times larger prevalence. This finding might be due the use of modified Rome Ⅱ, with broader criteria.

The colic symptom is common and leads one in six families (17%) with children to seek a health professional[50], although there was a wide variation in the frequency of functional infant colic in community and specialty setting, highlighting the necessity of studies with rigorous design and diagnostic criteria to promote a adequate prevalence.

The true prevalence related to infant rumination, CVS, diarrhea and dyschezia remains changeable and uncertain. In view of scarce data on these FGID, it is hard to know how they develop in infants and toddlers. Therefore, better training of pediatricians and investigators and clearer descriptions of disorders may refine clinical utility and endorse research of Rome Ⅲ category.

Limitation

The lack of well-conducted studies has imposed us to compare existing studies that have consistently used reliable criteria for functional disorders. Therefore, the epidemiological knowledge on FGID in infants and toddlers still has to resort to few surveys that have ensured the reproducibility of case definition. In this direction, summary meta-analysis is not performed due to insufficient studies conducted with similar methodology.

The observed heterogeneity of frequency rates is likely beyond those features attributable to chance. The difference has arisen because of clinical dissimilarities between studies (for example, setting, types and age of participants, respondents or assessors) or methodological differences (such as diagnostic criteria, assessment tool, sampling frame). Our qualitative appraisal revealed that most included studies were of moderate or below average standard of generalizability. Notwithstanding, non-eligible articles (without data on FGID, non-reliable Rome criteria, non-specific data to infants and toddlers) were excluded to avoid spuriously inflated frequencies of FGID.

Reporting bias in cross-sectional data is most related to delay in publication (file drawer bias) and selective language bias. However, we have actively contacted experts to inquire on non-published data (such as poster presentation, conference paper, local journals) and non-English surveys. We were able to get access to one additional study not covered in initial search[48] and another non-English study[3]. In summary, it is judicious to state that the abovementioned heterogeneity must be more related to the quality of existing investigations than to publication bias.

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COMMENTS

Background

Unremitting gastrointestinal symptoms wane and wax in a significant proportion of infants younger than 48 mo. The pathophysiology of the functional gastrointestinal disorders (FGID) remains unclear, but likely involves a complex interplay of autonomic, psychosocial, dietary, microbial, and gastric sensorimotor disturbances. FGID in infants and toddlers seem to be related to an increasing healthcare burden, though, valid epidemiological data are scarce. The aim of this review is to examine current evidence of knowledge on FGID in infants and toddlers, through systematic search of prevalence data on common functional GI problems.

Research frontiers

Until nowadays, there is no laboratorial test for diagnoses of FGID and clinicians must rely on the patients' symptoms for identification of the clinical pictures. In the case of infants and toddlers, careful assessment to establish threshold of the parental/caregiver report is required. The validity of explicit diagnostic criteria and reliability of psychometric tools is still limited. Strategies to avoid excessive hospitalizations and unnecessary investigative testing are needed. While global recognition and legitimization of FGID can expand the pathophysiological understanding of brain-gut axis dysfunction to further optimize clinical management for these patients, adequate epidemiological studies - with reliable and valid case definition, appropriate design, sufficient sample size, correct sampling, and data collection - can shed light from the black box of FGID in infants and toddlers.

Innovations and breakthroughs

FGID in infants and toddlers seem to be common in pediatric outpatient and inpatient practice, mainly infant regurgitation and functional constipation. Conversely, few population-based studies on epidemiology issue were conducted so far, and, good quality epidemiological data to support diagnostic criteria are lacking. The update of Rome criteria (Rome Ⅳ) is launched in 2016, but few reformulations for FGID in infants and toddlers are made. Nevertheless, the important advantage of this new version is to merge scientific investigation and clinical practice to improve the diagnostic classification system. For this purpose, predefined fields of interest have been created since 2013 such as gut microflora, the role of food and diet, the nature of severity for FGID, the development and validation of Rome Ⅳ questionnaire, the management of FGID in primary care setting and the multi-dimensional clinical profile.

Applications

This review highlights future directions for research: (1) well-designed epidemiological studies should be conducted in different levels of pediatric practice, in terms of sample recruitment, representativeness, sample size, and clinical assessment; (2) a new classification system of early childhood FGID must be simple, easy to understand (especially by primary care physicians and nurses), and must include age-dependent gastroenterological features recognizable by parents; (3) research agenda of comprehensive studies on course and associated disability of FGID should be set to refine its definition and classification; and (4) multidimensional approach can improve the current symptom-based classification of Rome criteria.

Terminology

FGID comprise chronic or recurrent symptoms that arise in the absence of anatomic abnormality, inflammation, or tissue damage. The symptoms are variable and age-dependent.

Peer-review

In this systematic review, the authors have presented a carefully designed study on the epidemiology of FGID in infants and toddlers. Based on the overall data the authors indicated future directions in the field of epidemiological studies concerning FGID.

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CASE REPORT

Systemic mastocytosis: A rare cause of non-cirrhotic portal hypertension

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

Informed consent statement: The informed consent statement was waived because the patient has died.

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Abstract

Mastocytosis is a clonal neoplastic disorder of the mast cells (MC) that can be limited to the skin (cutaneous mastocytosis) or involve one or more extracutaneous organs (systemic mastocytosis). The clinical manifestations of mastocytosis are heterogeneous ranging from indolent disease with a long-term survival to a highly aggressive neoplasm with survival of about 6 mo. Although liver involvement in aggressive systemic mastocytosis (ASM) is relatively common, the development of portal hypertension with or without cirrhosis is rare. We report a case of ASM without skin involvement in a 72-year-old caucasian male who presented with non-cirrhotic portal hypertension based on clinical, analytical, imagiological and endoscopic findings. Given the hematological picture, the correct diagnosis was established based on ancillary tests for MC using bone marrow aspirates and biopsy. Extensive involvement of the liver and gastrointestinal tract was histologically documented. The disease progressed rapidly and severe pancytopenia and recurrent upper gastrointestinal bleeding became the dominant problem. This case illustrates the challenge

in establishing a diagnosis of ASM especially when the clinical picture is atypical and without skin involvement. Gastroenterologists should consider infiltrative disease, particularly systemic mastocytosis, as a differential diagnosis in a clinical case of portal hypertension of unknown etiology.

Key words: Systemic mastocytosis; Mast cells; Non-cirrhotic portal hypertension; Upper gastrointestinal bleeding; Cladribine

Core tip: This clinical case describes an interesting and uncommon case of aggressive systemic mastocytosis with hepatic and gastrointestinal involvement. It illustrates not only a rare cause of non-cirrhotic portal hypertension, but also an atypical gastrointestinal involvement. The aim of this case report is to demonstrate the challenge in establishing a diagnosis of systemic mastocytosis especially when the clinical picture is atypical and without skin involvement, and alert Gastroenterologists to consider infiltrative diseases, as differential diagnosis in a clinical case of portal hypertension of unknown etiology.

Martins C, Teixeira C, Ribeiro S, Trabulo D, Cardoso C, Mangualde J, Freire R, Gamito É, Alves AL, Cremers I, Alves C, Neves A, Oliveira AP. Systemic mastocytosis: A rare cause of non-cirrhotic portal hypertension. *World J Gastroenterol* 2016; 22(28): 6559-6564 Available from: URL: http://www.wjgnet.com/1007-9327/full/v22/i28/6559.htm DOI: http://dx.doi.org/10.3748/wjg.v22.i28.6559

INTRODUCTION

Mastocytosis, a myeloproliferative neoplasm characterized by an abnormal proliferation of MC, is an uncommon, heterogeneous and progressive disease[1].

The vast majority of cases are confined to the skin (urticaria pigmentosa), but approximately 10% involve extracutaneous sites, most commonly bone, liver, spleen, gastrointestinal tract and lymph nodes[1,2]. The clinical manifestations are related to the release of MC mediators (*e.g.,* facial flushing, urticaria, itching, diarrhea) and to uncontrolled growth and infiltration of clonal MC in different organs (*e.g.,* hepatomegaly, splenomegaly, pancreatic insufficiency). Although most of the symptoms are not life-threatening, they may induce a significant impairment of quality of life. Nevertheless, when infiltration results in organ function impairment, the disease is considered aggressive and decrease life expectancy.

A diagnosis of mastocytosis has to fulfill the World Health Organization (WHO) diagnostic criteria[3] in which the demonstration of neoplastic MC infiltrates is the *sine qua non* condition. SM is diagnosed when the major and at least one minor or three minor criteria are satisfied (Table 1). Its signs and symptoms are divided into two groups: *B symptoms* ("borderline benign - be watchful") and *C symptoms* ("consider cytoreductive therapy"). The diagnosis of ASM can be made when one or more *"C"* findings are present. *"C"* findings include anemia (Hb < 10 g/dL), thrombocytopenia (< 100000/mm^3), neutropenia, hepatopathy with portal hypertension or ascitis, splenomegaly with hypersplenism, malabsorption with weight loss and osteolysis with pathological bone fractures.

Table 1 World Health Organization criteria for SM (adapted from Valent *et al* 2001)[4]

Major Criteria
Multifocal dense infiltrates of mast cells (> 15 mast cells in aggregates) at bone marrow biopsy and/or in sections of other extracutaneous organ(s)
Minor Criteria
> 25% of mast cells in bone marrow are atypical cells or spindle-shaped mast cells in infiltrates detected on sections of extracutaneous organ(s)
c-kit point mutation at codon 816 in the bone marrow or in another extracutaneous organ(s)
Mast cells in the bone marrow or in the another extracutaneous organ(s) expressing CD2 and/or CD25 with CD117
Serum tryptase levels > 200 ng/mL

Overlapping symptoms and heterogeneous clinical scenarios make early diagnosis extremely difficult. An absence of skin lesions at the time of diagnosis has been reported in up to 40%-50% of patients with ASM[5]. The liver is frequently involved, but only a minor percentage of ASM patients develop portal hypertension and/or cirrhosis.

In this paper we report a rare case of ASM without skin lesions who presented with non-cirrhotic portal hypertension. Bone, liver and gastrointestinal involvements were observed and histologically documented. The extensive bone marrow and gastrointestinal infiltration with the development of severe pancytopenia and recurrent upper gastrointestinal bleeding, respectively, were responsible for the poor prognosis and fatal outcome.

CASE REPORT

We report a 72-year-old caucasian male referred to our hospital due to severe anemia. The patient presented with a 3-mo clinical picture of significant involuntary weight loss, anorexy, astenia, night sweats, low-grade fever and, more recently, melena. The remaining patient's history was uneventful. He reported no history of smoking, alcohol or drugs consumption. Physical examination showed pallor, painless hepatomegaly and splenomegaly. Skin lesions, superficial lymphadenopathy, ascitis and jaundice were absent.

Initial laboratory tests showed normocytic and normochromic anemia (hemoglobin 6.1 g/dL),

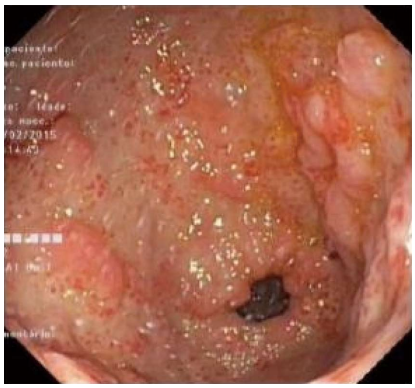

Figure 1 Gastric antral vascular ectasia observed on upper gastrointestinal endoscopy.

normal total white blood cells count (7.1 × 10^9/L) but with monocytosis (18%) and eosinophilia (10%), mild thrombocytopenia (141 × 10^9/L) and normal prothrombin time. Liver tests were normal except for high alkaline phospatase (274 U/L). Laboratorial study of anemia revealed a multifactorial etiology with ferropenic and non-autoimmune hemolytic components.

Abdominal ultrassound showed homogeneous hepatosplenomegaly. *Doppler* ultrassonography of the liver revealed portal vein dilation (14 mm), decreased portal flow velocity, hepatofugal portal flow and high hepatic artery resistance indexes. Upper gastrointestinal endoscopy showed small esophageal varices and gastric antral vascular ectasia (GAVE) treated with argon plasma coagulation (Figure 1). Total colonoscopy was normal.

In order to clarify the etiology of portal hypertension additional investigations were performed. Serum protein electrophoresis revealed low albumin but normal gammaglobulin levels. Serological testing for hepatitis B and C virus, HIV and autoimmune markers were negative. Serum copper, ceruloplasmin and alpha-fetoprotein were normal. Ferritin was moderately high (497 ng/mL).

Given the hematologic changes in a patient with wasting syndrome and a non-cirrhotic portal hypertension, to exclude hematologic disease, he underwent a thoraco-abdominopelvic computed tomography (CT) scan which confirmed homogeneous hepatosplenomegaly and showed no lymphadenopathy (Figure 2); a bone marrow aspiration and biopsy, which revealed multifocal infiltration of atypical MC (> 15 aggregates) with a spindle-shape, representing 31% of total cellularity; immunohistochemistry study identified positive staining for CD117 and CD25 (Figure 3). Serum tryptase levels were high (200 mcg/L). Liver biopsy confirmed massive infiltration by atypical MC and mild portal fibrosis but without evidence of cirrhosis (Figure 4).

These findings were fully consistent with SM fulfilling the WHO diagnostic criteria for this entity, namely the presence of the major and three minor

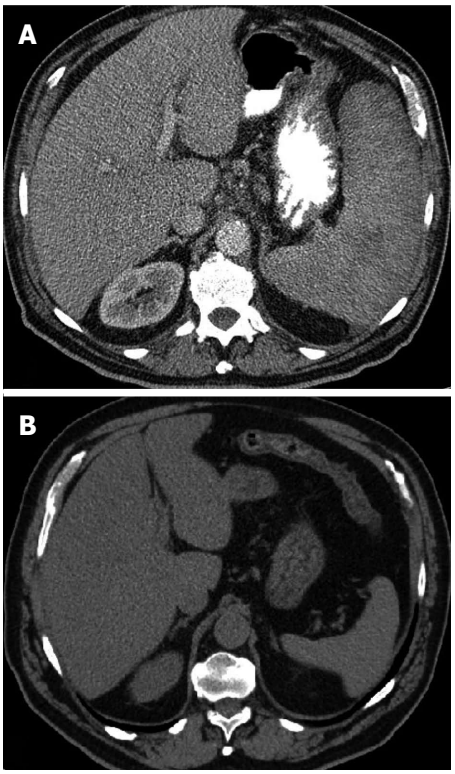

Figure 2 Images on abdominal computed tomography scan. A: Homogeneous hepatosplenomegaly; B: Normal spleen on computed tomography scan performed two years earlier.

criteria: > 25% of atypical MC in bone marrow and liver, coexpression of CD25 and CD117 by atypical MC, and high serum tryptase levels.

The patient was referred to the Hematology Department and began chemotherapy with cladribine with initial partial response. However, the disease became non-responsive to cladribine and progressed, with development of severe pancytopenia and recurrent upper gastrointestinal bleeding due to extensive involvement of the stomach (Figure 5), refractory to medical and endoscopic treatment. The patient died after one year.

DISCUSSION

Mastocytosis is a rare disorder with several studies reporting an incidence of 5-10 cases/10^6 people/year. It is recognized as a myeloproliferative neoplasm by the 2008 WHO Classification of Tumours of Haematopoietic and Lymphoid Tissues and is defined as an accumulation of clonal MC in one or more organs. The skin is involved in most patients (80%-90%), commonly presented as pigmentosa urticaria. SM is characterized by clonal MC accumulation in bone marrow and other extracutaneous organs.

ASM is an uncommon subtype of SM (12%) and occurs generally in adults. It is defined by the presence of at least one *"C"* finding, associated with constitutional symptoms and organomegaly, particularly of

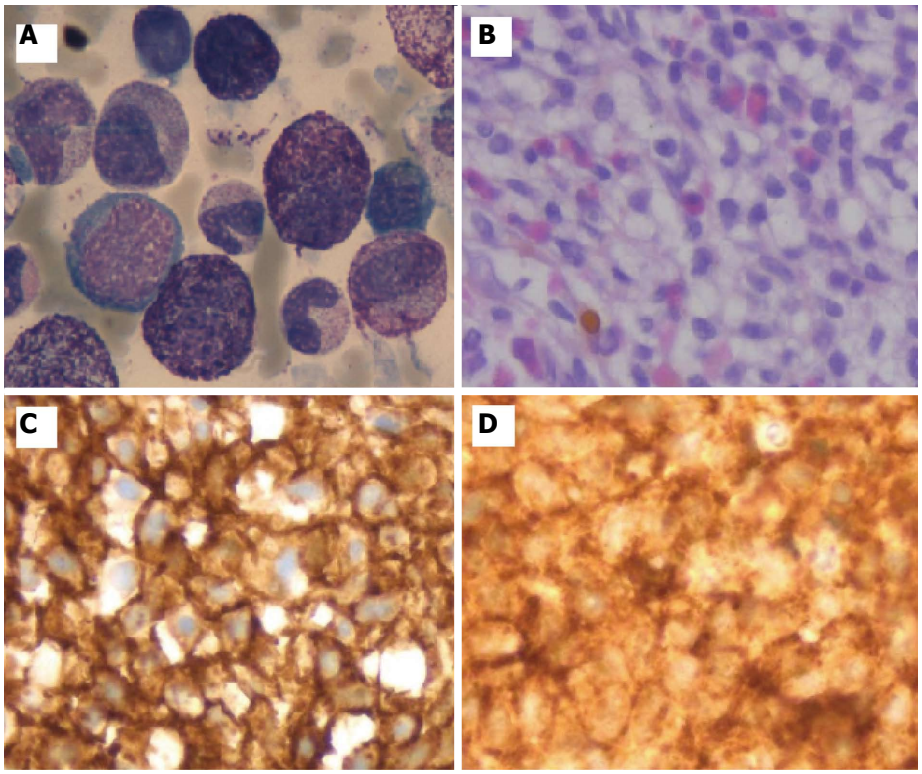

Figure 3 Immunohistochemistry study identified positive staining for CD117 and CD25. A: Atypical spindle-shaped MC on bone marrow aspirate (HE × 100); B: Hypercellularity due to infiltration by atypical MC on bone biopsy (HE × 100); C: MC were strongly positive for CD25; and D: CD117 (D) (IHC × 100).

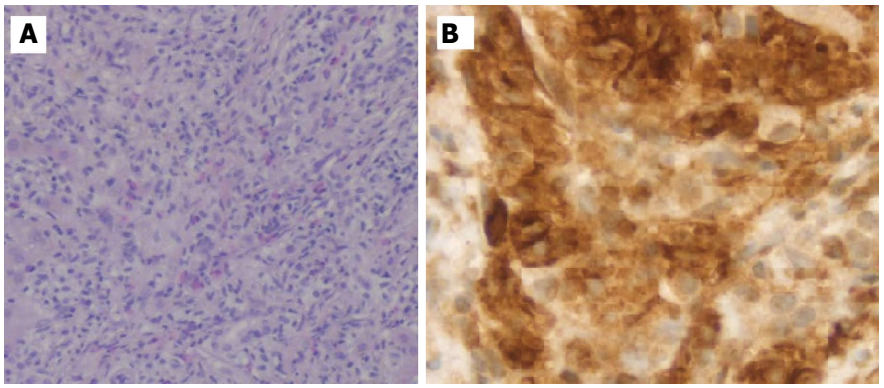

Figure 4 Microscopic images of the liver. A: Liver biopsy showed an increased number of spindled MC (HE × 40); B: The MC are highlighted by positive CD117 staining (IHC × 100)

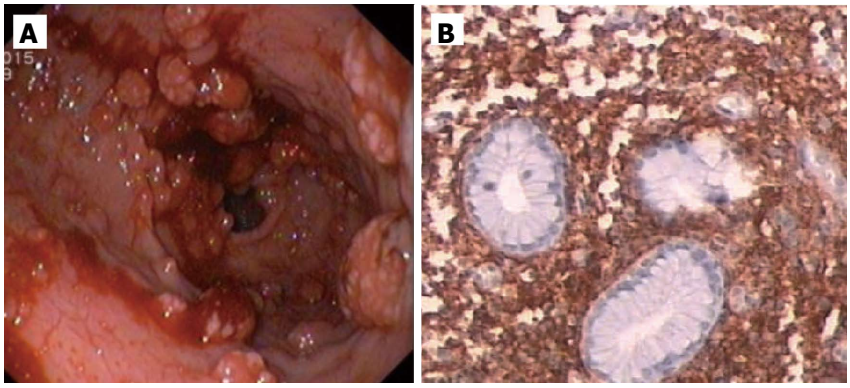

Figure 5 Extensive involvement of the stomach. A: Endoscopic image of the stomach showed diffuse polypoid lesions with active bleeding; B: Microscopic appearance of the gastric mucosa showed infiltration by atypical MC strongly positive for CD117 (IHC × 100).

liver, spleen and lymph nodes.

Regarding hematologic abnormalities in SM, the most common is mild-to-moderate anemia, which occurs in up to 50% of patients[6]. Normally, MC is not found in the circulation except in those with mast cell leukemia. Eosinophilia occurs in 25% of patients and monocytosis, when present in patients with ASM, represent a signal of advanced disease[7].

Liver involvement in ASM is common and may include abnormal liver tests, hepatomegaly, portal hypertension, fibrosis, cirrhosis and liver failure. Hepatomegaly is found in 41%-72% of patients. MC infiltrate the liver diffusely with or without portal fibrosis but rarely with cirrhosis or portal hypertension (4%). The development of portal hypertension and the lack of skin involvement are poor prognosis factors. Yoshida *et al*[8] reported a case of mast cell leukemia, a rare form of SM, complicated with rapidly progressing non-cirrhotic portal hypertension due to blocking of sinusoidal and venous flow. Despite significant hepatic involvement, liver tests are usually normal, except for alkaline phosphatase which is increased due to bone involvement[9]. Furthermore, hepatic lesion severity is not correlated with the size or the results of liver function tests[9].

Gastrointestinal involvement is seen in 70%-80% of patients with SM. Abdominal pain (51%), diarrhea (43%) and nausea or vomiting (28%) are the most common GI complaints[10]. Gastrointestinal bleeding, usually due to peptic ulcer disease, occurs in 11% of SM[10]. Other causes of upper gastrointestinal bleeding, such as GAVE, portal hypertensive gastropathy and esophageal varices, are very rare. The most common upper endoscopic features in SM patients are peptic esophagitis, peptic ulcers, thickened gastric or duodenal folds and nodular mucosa[10].

Patients with ASM are candidates for MC cytoreductive therapies, which may include interferon-alpha-2b, cladribine, glucocorticoides, tyrosine kinase inhibitors or hydroxyurea[11]. The clinical course of patients with ASM is variable, with some experiencing a rapid decline over 12 to 24 mo, while others follow a slower course with several years of survival[12].

In this case, the initial approach revealed a clinical picture of non-cirrhotic portal hypertension. The lack of skin manifestations delayed the diagnosis. The high suspicion of hematological disease due to wasting syndrome, *B* symptoms, hepatosplenomegaly and bicytopenia, led to the diagnosis, based on bone marrow aspirate and biopsy findings, which revealed infiltration by atypical MC with positive staining for CD117 and CD25. The liver biopsy confirmed massive infiltration by atypical MC but without evidence of cirrhosis. The diagnosis of ASM was established based on WHO 2008 criteria and the presence of "C" findings, particularly, anemia, hepatopathy with portal hypertension and splenomegaly with hypersplenism.

Besides the rare clinical presentation, this report describes an uncommon form of gastrointestinal involvement in SM. Unlike peptic ulcer disease, this patient showed atypical upper endoscopic findings, namely, esophageal varices, GAVE and diffuses gastric polypoid lesions. The last two were responsible for recurrent and refractory upper gastrointestinal bleeding.

In this patient, there were several poor prognosis factors, such as, older age at onset, weight loss, lack of skin involvement, severe anemia, trombocytopenia, monocytosis, hepatosplenomegaly, portal hypertension and gastrointestinal bleeding.

This report describes an interesting case of SM as a rare cause of non-cirrhotic portal hypertension with an atypical gastrointestinal involvement. Needless to say, patients with obvious dermatological findings are diagnosed by clinicians more quickly as having SM. Unfortunately, patients lacking skin manifestations have a delayed diagnosis even though they often have more aggressive disease. Therefore, Gastroenterologists should consider infiltrative disease, particularly ASM, as differential diagnosis in a clinical case of portal hypertension of unknown etiology. Presently there is no cure for SM and cytoreductive therapy and a multidisciplinary team approach are recommended.

COMMENTS

Case characteristics

A 72-year-old male presented with a clinical picture of non-cirrhotic portal hypertension.

Clinical diagnosis

Aggressive systemic mastocytosis with liver and gastrointestinal involvement.

Differential diagnosis

Viral hepatitis, alcoholic liver disease, infiltrative diseases (lymphoma, other myeloprolipherative disorders and amyloidosis) granulomatous diseases (sarcoidosis and tuberculosis), genetic disorders (Wilson disease and hemochromatosis) and vascular diseases such as portal vein thrombosis, among others.

Laboratory diagnosis

Normocytic and normochromic anemia, monocytosis, eosinophilia, thrombocytopenia, high alkaline phospatase and high serum tryptase levels.

Imaging diagnosis

Abdominal ultrasound with *Doppler* ultrassonography of the liver revealed hepatosplenomegaly, portal vein dilation, decreased portal flow velocity, hepatofugal portal flow and high hepatic artery resistance indexes. Upper endoscopy showed esophageal varices and gastric antral vascular ectasia.

Pathological diagnosis

A bone marrow aspiration and biopsy revealed multifocal infiltration of atypical mast cells (> 15 aggregates) with positive staining for CD117 and CD25; liver biopsy confirmed massive infiltration by atypical MC but without cirrhosis.

Treatment

Chemotherapy with cladribine.

Related reports

Aggressive systemic mastocytosis without skin involvement is uncommon.

Given the hematological picture, the correct diagnosis was established based on ancillary tests for mast cells using bone marrow aspirates and biopsy. The extensive involvement of the liver and gastrointestinal tract was histologically documented.

Experiences and lessons

Infiltrative diseases, particularly systemic mastocytosis, should be considered as a differential diagnosis in a clinical case of portal hypertension of unknown etiology.

Peer-review

This paper shows a rare case of systemic mastocytosis with non-cirrhotic portal hypertension. It illustrates the challenge in establishing a diagnosis of aggressive systemic mastocytosis especially when the clinical picture is atypical and without skin involvement.

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