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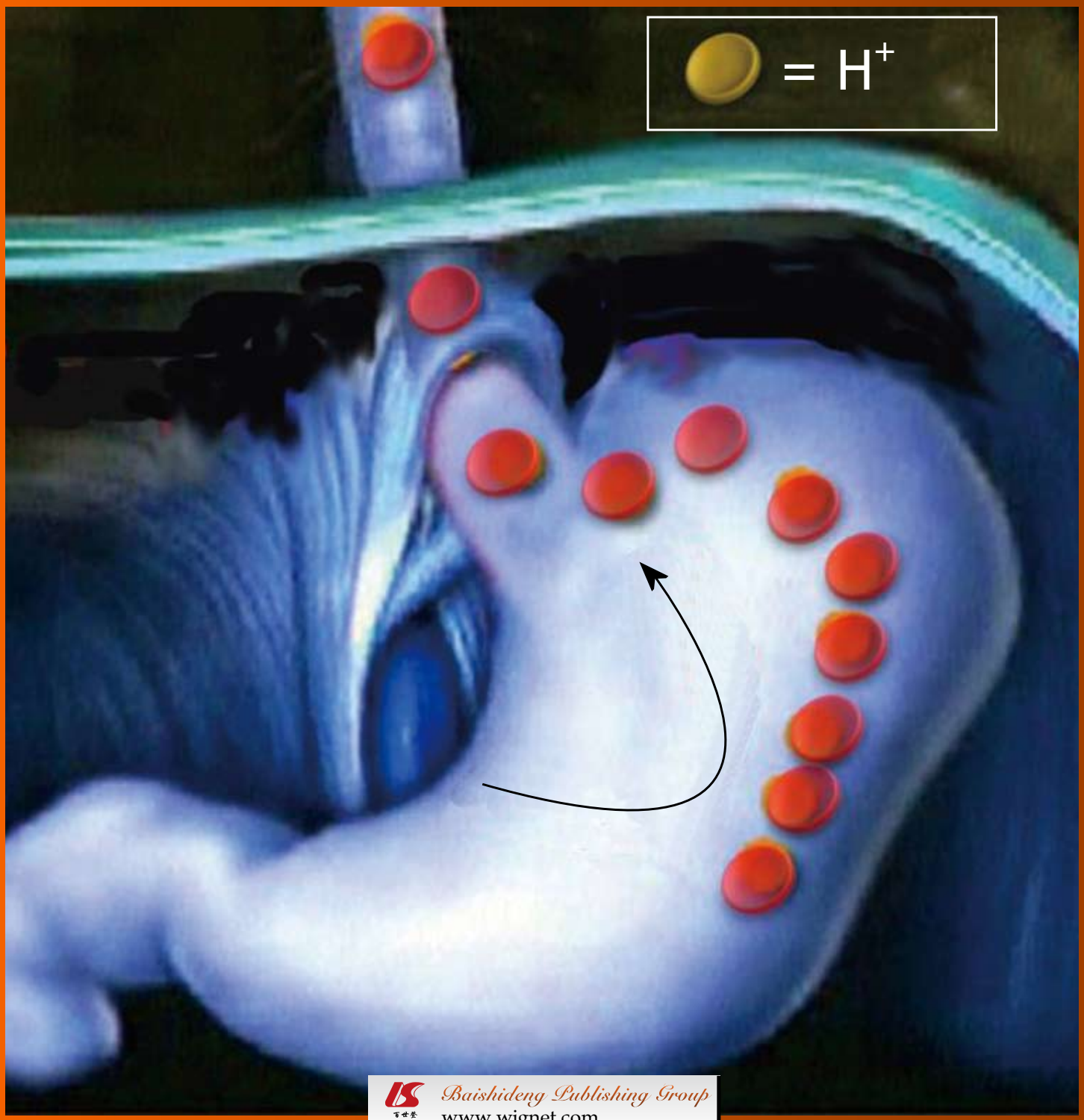

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EDITORIAL OFFICE
Jin-Lei Wang, Director
Xiu-Xia Song, Vice Director
World Journal of Gastroenterology
Room 903, Building D, Ocean International Center,
No. 62 Dongsihuan Zhonglu, Chaoyang District,
Beijing 100025, China
Telephone: +86-10-59080039
Fax: +86-10-85381893
E-mail: wjg@wjgnet.com
http://www.wjgnet.com

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Room 1701, 17/F, Henan Building,
No.90 Jaffe Road, Wanchai, Hong Kong, China
Fax: +852-31158812
Telephone: +852-58042046
E-mail: bpgoffice@wjgnet.com
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EDITORIAL

Helicobacter pylori eradication and reflux disease onset: Did gastric acid get "crazy"?

Angelo Zullo, Cesare Hassan, Alessandro Repici, Vincenzo Bruzzese

Angelo Zullo, Cesare Hassan, Vincenzo Bruzzese, Gastroenterology and Digestive Endoscopy Unit, Nuovo Regina Margherita Hospital, 00153 Rome, Italy
Alessandro Repici, Digestive Endoscopy Unit, IRCCS Istituto Clinico Humanitas, 00100 Milan, Italy
Author contributions: Zullo A and Hassan C designed and edited the review; Repici A reviewed literature; Bruzzese V contributed with constructive criticisms.
Correspondence to: Dr. Angelo Zullo, MD, Gastroenterology and Digestive Endoscopy Unit, Nuovo Regina Margherita Hospital, Via E. Morosini 30, 00153 Roma, Italy. zullo66@yahoo.it
Telephone: +39-6-58446533 Fax: +39-6-58446608
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Abstract

Gastroesophageal reflux disease (GORD) is highly prevalent in the general population. In the last decade, a potential relationship between *Helicobacter pylori* (*H. pylori*) eradication and GORD onset has been claimed. The main putative mechanism is the gastric acid hypersecretion that develops after bacterial cure in those patients with corpus-predominant gastritis. We performed a critical reappraisal of the intricate pathogenesis and clinical data available in this field. Oesophagitis onset after *H. pylori* eradication in duodenal ulcer patients has been ascribed to a gastric acid hypersecretion, which could develop following body gastritis healing. However, the absence of an acid hypersecretive status in these patients is documented by both pathophysiology and clinical studies. Indeed, duodenal ulcer recurrence is virtually abolished following *H. pylori* eradication. In addition, intra-oesophageal pH recording studies failed to demonstrated increased acid reflux following bacterial eradication. Moreover, oesophageal manometric studies suggest that *H. pylori* eradication would reduce - rather than favor - acid reflux into the oesophagus. Finally, data of clinical studies would suggest that *H. pylori* eradication is not significantly associated with either reflux symptoms or erosive oesophagitis onset, some data suggesting also an advantage in curing the infection when oesophagitis is already present. Therefore, the legend of "crazy acid" remains - as all the others - a fascinating, but imaginary tale.

Key words: *Helicobacter pylori*; Oesophageal reflux; Oesophagitis; Eradication; Pathophysiology; Clinical studies

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INTRODUCTION

Although *Helicobacter pylori* (*H. pylori*) infection prevalence is declining in developed countries, such infection remains a worldwide spread disease with a definite morbidity and mortality. Indeed, *H. pylori* may cause non-ulcer dyspepsia, peptic ulcer disease, and gastric tumors, including both low-grade mucosa-associated lymphoid tissue lymphoma and adenocarcinoma[1-3]. In addition, an interaction between *H. pylori* with non-steroidal, anti-inflammatory drugs in damaging the gastroduodenal mucosa has been also recognized[4]. Similarly, the role of *H. pylori* in the pathogenesis of different extra-digestive diseases has also been claimed. However, an association has been consistently proven only between *H. pylori* infection and both idiopathic thrombocytopenic purpura and idiopathic iron deficiency anemia[5,6], whilst conflicting data exist for other diseases[7-9].

Gastroesophageal reflux disease (GORD) is a highly prevalent condition in the general population[10]. It is characterized by the reflux of gastric contents into the

oesophagus, leading to mucosal damage and/or typical and atypical symptoms[11]. Several factors may predispose patients to pathologic reflux, including hiatal hernia, lower esophageal sphincter (LES) hypotension, transient lower oesophageal sphincter relaxation, loss of esophageal peristaltic function, abdominal obesity, gastric hypersecretory states, and delayed gastric emptying[12].

In the last decade, a potential relationship between *H. pylori* infection and GORD has been claimed. In detail, it has been suggested that *H. pylori* eradication may cause both reflux symptoms and erosive oesophagitis. We performed a critical reappraisal of the intricate data available in such a topic.

DOES *H. PYLORI* ERADICATION CAUSE GORD?

The beginning

The first study suggesting a possible association between *H. pylori* and GORD was published by Labenz *et al*[13] on 1997. In this study, 25.8% of patients with duodenal ulcer (DU) cured for *H. pylori* infection developed erosive oesophagitis (Savary-Miller classification) at 3-year follow-up as compared to 12.9% of DU patients with ongoing infection ($P < 0.001$). Identified risk factors were male sex, severity of corpus gastritis, and weight gain. These results deserve some considerations. As shown in Figure 1, the incidence of erosive oesophagitis in *H. pylori* eradicated patients constantly increased at follow-up, so that at 3-year follow-up the curve did not still reach the plateau. This would probably suggest that an even higher oesophagitis incidence is expected at longer follow-up in these patients. However, this contrasts with data of a recent 7-year follow-up study which showed that reflux oesophagitis occurred in only 1.9% of DU patients following *H. pylori* eradication[14]. In addition, it should be noted that the curve of oesophagitis incidence in not eradicated DU patients showed a very similar slope as compared to that of eradicated patients (Figure 1). In detail, following a questionable delay of 1 year, reflux oesophagitis started to increase also in *H. pylori* infected patients, and the increase of incidence parallels with that of eradicated patients. A plausible explanation of such phenomenon could be that several not eradicated DU patients were taking anti-acid therapy, at least for the first year follow-up in this study. Indeed, this patient group included either control cases of *H. pylori* eradication trials (75 cases) or *H. pylori* eradication failure patients (141 cases) in whom DU symptoms most likely persisted[13]. Therefore, the observation of a distorted phenomenon could not be ruled out. On the other hand, it was observed that oesophagitis occurred more frequently in those *H. pylori* cured patients with weight gain > 2 kg [odds ratio (OR): 3.2; 95%CI: 1.2-9.4; $P < 0.05$] within 3-year follow-up. Unfortunately, both mean and range of weigh gain was not provided in this study. However, it is plausible that several patients changed their dietary and voluptuary habits following complete DU healing, with consequent body mass index

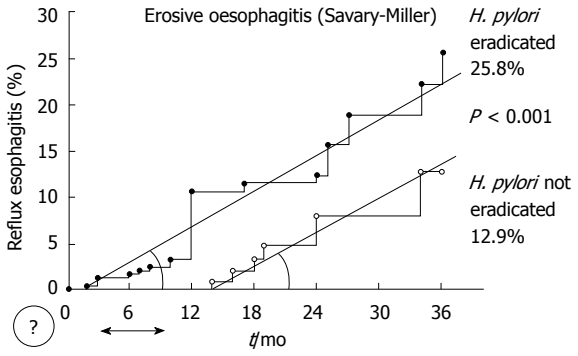

Figure 1 Incidence of erosive oesophagitis in *Helicobacter pylori* eradicated and not eradicated duodenal ulcer patients (Modified from reference 13). The comparable curve slopes would indicate a similar phenomenon. The final difference between patient groups depends on the 1-year delay between curve which remains unexplained. *H. pylori*: *Helicobacter pylori*.

(BMI) increase. The direct relationship between BMI and GORD is widely documented in literature, so that the "guilty" of erosive oesophagitis onset - at least in some patients - probably was the BMI increase rather than the *H. pylori* cure. Unfortunately, the lack of multivariate regression including *H. pylori* eradication as an independent risk factor for GORD does not allow to confirm the significant role observed solely at univariate analysis. Finally, the reason for which the risk of erosive oesophagitis incidence following *H. pylori* eradication was 3.6 (95%CI: 1.1-10.6) higher in males as compared to females remains unclear. This would contrast with the prevalence of GORD in the general population which equally occurs in both sexes[15].

The tale of a "crazy acid"

At least in theory, *H. pylori* eradication could cause GORD by: (1) recovering gastric acid secretion; (2) increasing the amount of reflux in the oesophagus; and (3) reducing the clearance of oesophageal acid exposure.

Regarding the first point, it has been observed that *H. pylori*-associated corpus-predominant gastritis is an independent risk factor (OR: 5.5; 95%CI: 2.8-13.6) for erosive oesophagitis development following *H. pylori* eradication in DU patients[13]. In this study, the body gastritis sum score before *H. pylori* eradication was 4.1 in those who developed oesophagitis as compared to 3.1 of those without oesophagitis onset. Therefore, it has been suggested that the higher degree of recovery leads into a higher acid secretion which, in turn, causes erosive oesophagitis[13]. However, by considering that the sum score (activity plus grade) of gastritis was ranging from 0 (absent) and 8 (severe), it is questionable that acid hypersecretion developed only in those patients in whom corpus gastritis score dropped from 4.1, but not from 3.1, to zero following bacterial cure. At this point, the crucial issue is to understand whether *H. pylori* eradication really causes gastric acid hypersecretion in DU patients. To our knowledge, neither pathophysiology nor clinical studies support such an event. It has been found that infected DU patients have a 3-fold increase of basal acid output (BAO)

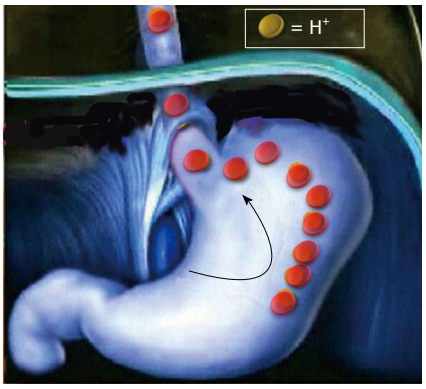

Figure 2 The "tale of a crazy acid". Following *Helicobacter pylori* gastric in duodenal ulcer patients the hypothesized acid hypersecretion would U-turn into the oesophagus instead of in the duodenal bulb.

and a 6-fold increase of stimulated maximal acid output (MAO) as compared to uninfected controls[16]. Of note, both these alterations normalize 1-year following *H. pylori* eradication, suggesting that DU patients cured for the infection completely restore normal acid secretion[16]. The absence of an acid hypersecretive status in these patients is further documented by the clinical observation that DU recurrence is virtually abolished following *H. pylori* eradication[17]. Therefore, it is unclear the reason for which the hypothesized hypersecretion in DU patients after bacterial cure should cause erosive oesophagitis onset, but not DU recurrence. To search for a plausible explication, one could hypothesize the "tale of a crazy acid". According to this theory, following the healing of corpus-predominant gastritis - a typical gastritis pattern of both gastric ulcer and cancer, but infrequent in DU[18] - acid hypersecretion occurs in some DU patients. In these patients the acid should invert its normal route - so failing to cause DU recurrence - and should turn into the oesophagus causing erosions (Figure 2). However, studies on 24-h intraoesophageal pH recording before and after *H. pylori* eradication showed that the percentage time of pH < 4.0 did not significantly change following eradication[19,20]. Other putative mechanisms by which *H. pylori* eradication could favor GORD are alterations of both LES pressure and oesophageal peristalsis. However, two studies found no difference in basal LES pressure between *H. pylori* infected and matched controls[21,22], whilst another study showed an even lower basal LES pressure and higher rate of ineffective oesophageal motility in infected patients[23]. Based on these manometric observations, it could be expected that *H. pylori* eradication would reduce - rather than favor - acid reflux into the oesophagus.

Clinical studies

Several clinical trials assessing the onset of both reflux symptoms and erosive oesophagitis have been performed. A recent meta-analysis evaluated data of 10 trials where data of patients treated for *H. pylori* infection were compared to those receiving placebo[24]. At 8-30 mo follow-up the incidence of reflux symptoms did not significantly differ between the two patient groups (17% *vs* 22.6%), with a trend even favoring bacterial eradication (OR: 0.81; 95%CI: 0.56-1.71). Likewise, erosive oesophagitis equally occurred in both groups (5% *vs* 5.1%; OR: 1.13; 95%CI: 0.72-1.78). Noteworthy, a study on 156 patients with both peptic ulcer and reflux oesophagitis found that the oesophageal lesions improved more frequently in *H. pylori* eradicated patients as compared to those with persistent infection[25]. Overall, these data would suggest that *H. pylori* eradication is not significantly associated with either reflux symptoms or erosive oesophagitis onset, some data suggesting also an advantage in curing the infection when oesophagitis is already present.

CONCLUSION

GORD is highly prevalent in the general population. Several altered mechanisms are involved in its pathogenesis, and different lifestyle and voluptuary habits have been identified as risk factors. In the last decade, *H. pylori* eradication has been charged to cause GORD. The main putative mechanism is that a gastric acid hypersecretion develops following bacterial cure in those patients with corpus-predominant gastritis. However, studies on acid secretion demonstrated that the hypersecretion status of both DU (BAO × 3; MAO × 6) and non-ulcer dyspeptic (MAO × 3) patients observed during *H. pylori* infection normalizes following bacterial eradication[16]. Indeed, DU does not recur following a successful *H. pylori* cure[17]. Therefore, the reported erosive oesophagitis onset after the infection cure in DU patients most likely depends on other factors rather gastric acid hypersecretion (Weight gain? Changes of voluptuary habits?). On the other hand, both oesophageal 24-h pH recording and manometry studies failed to demonstrate a significant role for *H. pylori* in GORD.

Gastric acid secretion may recover with *H. pylori* eradication in those patients with corpus-predominant (or atrophic) gastritis[26]. However, such a gastritis pattern is generally encountered in either gastric ulcer or cancer patients, and it is highly prevalent in Asian countries[18]. However, the reason for which such a hypersecretive status should cause GORD but not DU onset in these patients remains unclear.

The last but not the least, several, placebo-controlled, clinical trials have been recently summarized in a meta-analysis including near 4500 patients[25]. Data found that neither reflux symptoms nor erosive oesophagitis develop following *H. pylori* eradication. Therefore, the legend of "crazy acid" remains - as all the others - a fascinating, but imaginary tale!

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FRONTIER

Current hurdles in the management of eosinophilic oesophagitis: The next steps

Stephen EA Attwood, Michael SJ Wilson

Stephen EA Attwood, Michael SJ Wilson, Department of General Surgery, North Tyneside Hospital, Rake Lane, North Shields, Tyne and Wear NE29 8NH, United Kingdom
Author contributions: Attwood SEA and Wilson MSJ made substantial contributions to the conception,design, drafting and final approval of the final version of the manuscript to be published.
Correspondence to: Stephen EA Attwood, Professor, Department of General Surgery, North Tyneside Hospital, Rake Lane, North Shields, Tyne and Wear NE29 8NH, United Kingdom. stephen.attwood@nhct.nhs.uk
Telephone: +44-191-2934079 Fax: +44-191-2934190
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Abstract

Eosinophilic oesophagitis (EoE) is a chronic, antigen mediated disease of the disease of the oesophagus that may present in both adults and children. It is characterised by intermittent dysphagia, food bolus obstruction and weight loss. The pathogenesis is incompletely understood but is thought to culminate in poor compliance, or reduced distensibility. The condition is being reported and studied in the literature with increasing incidence, although equally it is highly likely that the diagnosis is being missed altogether with alarming frequency. Diagnosis of the condition requires at least one oesophageal biopsy with an eosinophil count greater than 15 per high power field. Endoscopic features include trachealisation, furrows, white exudate, narrowing and in the most severe cases stricture formation although none are pathognomonic of the condition. Therapy is often not required, but in the acute setting may take the form of dietary therapy or topical steroids. Long term maintenance therapy is usually only required in the most severe cases and the most effective treatment is the subject of ongoing research. There are a number of hurdles to be overcome in the management of patients with EoE. These include; improving our understanding of the aetiology of the condition, investigating the individual causes, assessing the true disease severity and planning the best long term maintenance therapy. Distinguishing EoE from EoE gastro-oesophageal reflux disease is also a hurdle because the two conditions, both being common, can co-exist. In order to overcome these hurdles, a multifaceted approach is required. The management of food bolus obstruction requires a management algorithm that is accepted and endorsed by a number of specialties. National and international disease registers should be established in order to facilitate future research but more importantly to address areas where further education or increased diagnostic capabilities may be required. Assessment of disease severity should become a key goal, and the development of specific biomarkers for EoE should also be a priority. Finally, randomised controlled trials of new agents are required to assess the best treatment in both the acute and long term setting.

Key words: Eosinophil oesophagitis; Dysphagia; Food bolus obstruction; Therapy; Gastroscopy

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INTRODUCTION

Eosinophilic oesophagitis (EoE) is an increasingly common cause of dysphagia or food bolus obstruction. EoE is a chronic antigen mediated disease of the oesophagus. It is characterised symptomatically by oesophageal dysfunction and histologically by eosinophil predominant inflammation. The condition was first described almost 30

years ago in a cohort of patients with > 20 intraepithelial eosinophils (IEE) per high power field seen on oesophageal biopsy, with 11 of the 12 demonstrating normal oesophageal acid exposure on 24 pH monitoring[1]. A further cohort of 10 patients with recurrent dysphagia and high concentrations of IEE at endoscopic biopsy was reported in 1994[2].

Until 2007 only 212 cases had been reported in the literature[3]. Since that time, the reporting of cases of EoE has increased dramatically. More than 750 articles incorporating several thousand patients have been published on the subject since being first described with seventy five per cent being published in the last five years.

EoE is sometimes described as "oesophageal asthma" on account of its association with atopy. Up to 50% of patients also have bronchial asthma or allergic rhinitis, and 20% have atopic dermatitis[4]. Males are affected three times more often than females, and patients typically present either in childhood or during the third or fourth decades of life.

The typical presenting features of this condition include intermittent dysphagia, food bolus obstruction and weight loss. In the paediatric population, patients may also present with nausea, vomiting, weight loss and failure to thrive.

Recent consensus guidelines state that histological evidence of at least 15 intraepithelial eosinophils per high power field (eos/hpf) in at least one oesophageal mucosal biopsy stained with haematoxylin and eosin is required for the diagnosis. However, in the correct clinical setting, patients may be considered to have EoE with < 15 eos/hpf. An example would be an atopic male on a proton pump inhibitor (PPI) at the time of diagnostic endoscopy with typical endoscopic findings. Gastro-oesophageal reflux disease (GORD) should be excluded by demonstrating a lack of response to high dose proton pump inhibitor therapy or normal oesophageal pH monitoring[5,6].

Not only is oesophageal biopsy essential in all patients with food bolus obstruction or dysphagia, regardless of the endoscopic appearance, the number of biopsies taken is also important. A minimum of six biopsies should be taken; two each from the upper, mid and lower oesophagus. Using a benchmark of 15 eos/hpf, it has been shown that the diagnostic sensitivity for EoE is 84%, 97% and 100% when 2, 3 and 6 biopsies are taken respectively[7]. A further study demonstrated 100% sensitivity when 5 biopsies were taken[8].

Estimates of the prevalence of EoE in the general population vary in the literature. In Switzerland, the adult prevalence has been estimated to be 0.02%[9], 0.44% in the United States[10] and 1% in Sweden[11]. In more targeted populations, such as those undergoing oesophageal endoscopy the prevalence is significantly greater and in the region of 6%-15%[12,13].

The current hurdles in the management of patients with EoE include; improving our understanding of the aetiology of the condition, investigating the individual causes, assessing the true disease severity and planning the best long term maintenance therapy. Distinguishing EoE from GORD is also a hurdle because the two conditions, both being common, can co-exist.

AETIOLOGY AND PATHOPHYSIOLOGY

The pathophysiology of this condition is incompletely understood and is the subject of ongoing research and debate. Expert consensus has concluded that the condition arises as a result of an antigen mediated immunologic process resulting in oesophageal inflammation. The specific allergen(s) is yet to be identified but a number of theories have been postulated. Skin prick testing has been shown to have a poor predictive value for the identification of specific food allergens in patients with EoE[14]. Given that the condition is being reported with increasing frequency this raises the possibility of an environmental allergen being involved in the pathogenesis. The "hygiene hypothesis" or declining incidence of *Helicobacter pylori* have both been proposed to play a role given that they have coincided with increased incidence of EoE[15,16].

Acid reflux (GORD) is not usually present in patients with EoE. However, it is standard practice to perform diagnostic biopsies while patients are on PPI to exclude the contribution of reflux injury[5]. When symptoms are atypical, or if there is a mixture of reflux symptoms and dysphagic EoE symptoms then 24 h pH monitoring should be employed to guide therapy. There is a subgroup of symptomatically typical EoE patients, with normal pH profiles who seem to respond to PPI, and these PPI responsive patients will benefit from with maintenance therapy[17].

Oesophageal biopsy specimens from patients with EoE compared to normal controls or patients with GORD show increased levels of the T helper 2 (Th2) cytokines, principally interleukin-5 (IL-5) and IL-13[18-20], with increased levels of the eosinophil chemoattractants eotaxin-1 and eotaxin-3 also reported[20-23]. Other studies have also demonstrated increased levels of other cytokines such as tumour necrosis factor-β1 and fibroblast growth factor-9[24,25]. GORD can be distinguished from EoE by its high level of COX-2 activation, while both show high rates of proliferation (Ki-67)[26].

It is thought that long standing oesophageal inflammation results in remodelling of the oesophageal wall in a similar manner to the airway remodelling seen in patients with bronchial asthma. A potential sequela is a fragile mucosa that is likely to tear easily as demonstrated by reported cases of spontaneous or procedure induced oesophageal rupture.

ASSESSING THE TRUE DISEASE SEVERITY

EoE can present at any age, but is more prevalent in childhood or during the third and fourth decades of life[5]. Adult patients typically present with a long history of intermittent, often severe dysphagia. In the most se-

vere cases the dysphagia may be continuous and associated with odynophagia and chest pain. EoE is also prevalent in the paediatric population, and is often associated with other atopic conditions. The clinical presentation varies with age. Infants and toddlers may present with prolonged feeding time or frank denial of food, whereas older children may present with vomiting and odynophagia. Adolescents, like adults tend to present with dysphagia. Less frequently, the child may present with failure to thrive and weight loss[27,28].

EoE is associated with a number of abnormalities of the oesophagus at endoscopy. These include; oesophageal rings (or trachealisation), furrows, white exudate, narrowing and in the most severe cases stricture formation. The pathogenesis of dysphagia in EoE is believed to be poor compliance, or reduced distensibility. If this affects the esophagus diffusely then a narrow bore oesophagus that fails to distend will result. If there is focal fibrosis, a stricture will be evident. The distinction is important when considering interventions such as dilation[29]. However, normal endoscopic findings do not exclude the diagnosis and neither are the above endoscopic findings pathognomonic of the condition. A number of studies have demonstrated histopathological evidence of EoE in patients with normal appearance of the oesophagus at endoscopy. This highlights the importance of oesophageal biopsy for all patients presenting with food bolus obstruction or dysphagia, even in the presence of a normal looking oesophagus[12,30].

Recent research has also shown that up to half of patients who present acutely with food bolus obstruction have underlying EoE. In a prospective series of 43 patients presenting with food bolus obstruction, of whom 29 had biopsies taken from the proximal and distal oesophagus, 14 (50%) fulfilled the histological criteria for EoE[31]. A further study reported that 17 of 31 (55%) patients presenting with food bolus obstruction fulfilled the histological criteria for EoE[32]. It is for these reasons that the most recent consensus guidelines stress the need for oesophageal biopsy of all patients presenting with dysphagia. In patients presenting with acute food bolus obstruction, oesophageal biopsies should also be taken at the time of disimpaction with arrangements made for appropriate clinical follow up[33]. In countries where the management of acute food bolus obstruction is principally managed by non EoE specialists, efforts should be made to raise awareness of the condition in order to avoid missed diagnoses[34].

A disease specific EoE health related quality of life questionnaire has also been developed[35]. This may be useful in identifying those who may benefit from treatment and in the assessment of response to therapy both in the clinical and research setting. There is a need to identify overall disease severity and to establish if symptoms, endoscopy or pathology can predict therapeutic outcomes.

Attention has also turned towards identifying potential biomarkers of the disease. Serum eosinophil derived neurotoxin has recently been highlighted as a potential diagnostic biomarker for EoE that may also be valid in assessing response to therapy and relapse of symptoms[36].

THERAPY

The acute management of EoE has a fairly well established pattern of treatment algorithms. In the paediatric setting, diet and topical steroids are the mainstay of treatment in the acute phase.In adults non obstructive disease is usually treated with topical steroids as the first line therapy, along with avoidance of known food precipitants. For obstructive EoE dilatation is worthwhile. Long term maintenance therapy is believed to be valuable but there is no evidence in controlled studies with long term follow up. Disease severity is both difficult to score and not predictive of long term natural history. The lack of long term therapy does not necessarily lead to the development of long term complications[37].

Dietary therapy in the acute setting can take on a number of forms. The introduction of an elemental diet has been used effectively to induce remission of symptoms and oesophageal inflammation particularly in the paediatric setting[38], with some recent success reported in adult[39]. In adults, the role of dietary therapy is being assessed but is not well established. The major drawback of this approach is the unpalatable nature of the diet. Some diets are intolerable in the long term, and recurrence of symptoms after cessation of the elemental diet is common. An alternative approach has been attempted, again with some success. This involved the introduction of a six-food elimination diet (wheat, milk, eggs, soya, nuts and rice) over a 6-12 wk period and improved patients' symptoms[40,41]. Further work by the same group highlighted the major drawback of dietary therapy in terms of compliance with the avoidance of particular food groups that are so common in western societies[42]. In the paediatric setting there has been some success with single food introduction following the six food elimination diet, with milk being identified as the most common causative food antigen[43].

Topical steroids in the acute setting are usually very effective with 60%-80% symptom resolution[44]. For long term maintenance topical corticosteroids have demonstrated some efficacy[44,45] although there is a risk of candida infection with long term use. Traditionally, topical steroid therapy is in widespread use for the management of bronchial asthma. In EoE the topical steroid is swallowed as opposed to inhaled. The most frequently employed topical steroid is fluticasone propionate at a dose of 300 to 500 micrograms twice daily, but doses of up to 880 micrograms twice daily have also been reported. The aim is to maximise exposure to the oesophagus and therefore spraying the back of the throat and swallowing the agent coats the oesophagus. This should be undertaken twice daily (morning and night time) to maximise the exposure of topical steroid to the oesophagus over a 24-h period. More recently, oral viscous budesonide has

also been shown to be effective[46,47] and in one case report a superior alternative to swallowed fluticasone[48]. Studies of clinical response to topical steroids do show variation in clinical outcome and also poor correlation of histological response with clinical improvement[49]. It is thought that topical corticosteroid therapy acts by reversing the oesophageal remodelling that occurs in EoE[45]. In a paediatric population fluticasone has been associated with improvements in the endoscopic, histologic and immunological parameters of EoE. However, the improvement was less marked in patients with a history of allergy[50].

In some patients reflux type symptoms and occasionally dysphagia is improved by PPI therapy. It is useful to measure the degree of acid reflux in these patients with 24 h pH monitoring to help gauge the need for combinations of PPI and topical steroid therapy.

Monteleukast, a leukotriene receptor antagonist has been proposed as a long term treatment. In an observational study of twelve patients, monteleukast was associated with a good symptomatic response (dysphagia scores and frequency of bolus obstruction) with an inconsistent reduction in the associated concentration of eosinophils[51]. A similar response has been demonstrated in a small cohort of paediatric patients[52]. The use of monteleukast has been criticised in some quarters on account of the lack of a placebo controlled group in the original paper reporting its use, and a further study has shown that monteleukast is inefficient in maintaining steroid reduced remission in EoE[53].

Oesophageal dilatation has been shown to be a safe and effective therapy in EoE, particularly in the presence of strictures[54]. However, mucosal tears are a worrisome occurrence but may be acceptable if an effective dilatation is achieved. Oesophageal perforation is a recognised complication of this procedure, but rare[55]. As a result, this intervention gained an adverse reputation somewhat prematurely. A recent systematic review has estimated a 0.1% risk of oesophageal perforation as a direct result of oesophageal dilatation[56,57], and this is no different to the risk of perforation when dilating a peptic stricture[58].

EoE may cause a stiffening of the oesophageal wall and produce poor compliance. Subsequent attempts to regurgitate or dislodge a food bolus may then cause a tear (usually partial) in the oesophagus[59-61]. The frequency of perforation seems to be greater from the food bolus itself than with dilatation. Management needs careful assessment because if the perforation is only a dissection, with a contained leak and no free fluid in the chest cavity, then non surgical management may be sufficient. The majority of such perforations heal with such a conservative approach[5].

More recently, attention has turned towards potential monoclonal antibody therapies against specific cytokines. Mepolizumab, a monoclonal antibody against IL-5 has been shown in a small randomised controlled trial to reduce the peak oesophageal eosinophil count, but with no improvement in clinical symptoms[62]. Similarly reslizumab (anti IL-5)[63], infliximab (anti-tumor necrosis factor-α)[64] and omalizumab (anti-IgE)[65] have been shown to reduce peak oesophageal eosinophil counts with no improvement in clinical symptoms. Results from larger randomised trials are awaited.

With thoughts towards the future, potential targets for therapy are being evaluated. One candidate currently in phase Ⅱ clinical trials is the chemoattractant receptor expressed on Th2 cells (CRTH2) antagonist[66]. CRTH2 is a receptor expressed on Th2 cells that is known to bind to prostaglandin in asthmatics. By developing a drug with the ability to block this receptor it is hoped that a key component of the inflammatory response in patients with EoE will be disabled with a subsequent clinical improvement for the patient.

FUTURE CHALLENGES

In order to facilitate future clinical research into this condition then the largest centres should collaborate closely. Both national and international disease registers should be established in order to better understand the epidemiology of this novel condition. National and international registers will enable the identification of the largest centres with the highest incidence and prevalence, but more importantly identify those centres with low or zero prevalence. This will enable targeted education to be focused upon these centres as undoubtedly this represents missed or misinterpreted diagnoses.

With recent research highlighting that up to half of patients presenting with acute food bolus obstruction fulfil the diagnostic criteria for EoE, formal protocols should be implemented to ensure that patients undergo oesophageal biopsy at the time of disimpaction to exclude EoE. In a number of countries where the management of acute food bolus obstruction is managed by specialties with little or no knowledge of the existence of this condition this will require collaborative links to be forged between gastroenterology, ENT and surgical colleagues as appropriate. Ensuring these patients undergo oesophageal biopsy is likely to result in a significant increase in the incidence of EoE and sufficient resources should be in place in order to cope. This will require dissemination of the existence of this condition into primary care.

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Hepatocellular carcinoma after ablation: The imaging follow-up scheme

Lin-Na Liu, Hui-Xiong Xu, Yi-Feng Zhang, Jun-Mei Xu

Lin-Na Liu, Hui-Xiong Xu, Yi-Feng Zhang, Jun-Mei Xu, Department of Medical Ultrasound, Shanghai Tenth People's Hospital, Tenth People's Hospital of Tongji University, Shanghai 200072, China

Author contributions: Liu LN collected the materials and wrote the manuscript; Zhang YF and Xu JM discussed the topic; and Xu HX supervised the publication of this commentary.

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Correspondence to: Hui-Xiong Xu, MD, PhD, Professor, Department of Medical Ultrasound, Shanghai Tenth People's Hospital, Tenth People's Hospital of Tongji University, No. 301 Yanchangzhong Road, Shanghai 200072, China. xuhuixiong@hotmail.com
Telephone: +86-21-66301031 Fax: +86-21-66301031

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Abstract

Percutaneous ablation using thermal or chemical methods has been widely used in the treatment of hepatocellular carcinoma (HCC). Nowadays, contrast-enhanced imaging modalities such as computed tomography (CT), magnetic resonance imaging (MRI), and contrast-enhanced ultrasound (CEUS) are widely used to evaluate local treatment response after ablation therapies. CEUS is gaining increasing attention due to its characteristics including real-time scanning, easy performance, lack of radiation, wide availability, and lack of allergy reactions. Several studies have documented that CEUS is comparable to CT or MRI in evaluating local treatment efficacy within 1 mo of treatment. However, little information is available regarding the role of CEUS in the follow-up assessment after first successful ablation treatment. Zheng *et al* found that in comparison with contrast-enhanced computed tomography (CECT), the sensitivity, specificity, positive predictive value, negative predictive value and overall accuracy of CEUS in detecting local tumor progression (LTP) were 67.5%, 97.4%, 81.8%, 94.4% and 92.3%, respectively, and were 77.7%, 92.0%, 92.4%, 76.7% and 84.0%, respectively for the detection of new intrahepatic recurrence. They concluded that the sensitivity of CEUS in detecting LTP and new intrahepatic recurrence after ablation is relatively low in comparison with CECT, and CEUS cannot replace CECT in the follow-up assessment after percutaneous ablation for HCC. These results are meaningful and instructive, and indicated that in the follow-up period, the use of CEUS alone is not sufficient. In this commentary, we discuss the discordance between CT and CEUS, as well as the underlying mechanisms involved. We propose the combined use of CT and CEUS which will reduce false positive and negative results in both modalities. We also discuss future issues, such as an evidence-based ideal imaging follow-up scheme, and a cost-effectiveness analysis of this imaging follow-up scheme.

Key words: Hepatocellular carcinoma; Radiofrequency ablation; Ethanol ablation; Contrast-enhanced ultrasound; Follow-up; Treatment response; Computed tomography

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COMMENTARY ON HOT TOPICS

We read with great interest the recent article by Zheng *et al*[1] evaluating the usefulness of contrast-enhanced ultrasound (CEUS) in the follow-up of patients with he-

patocellular carcinoma (HCC) who had undergone local ablation therapies.

As a minimal invasive and safe treatment method, percutaneous ablation using thermal or chemical methods has been widely used in the treatment of early HCC, recurrent HCC, and even advanced HCC[2-11]. Percutaneous ablation such as radiofrequency ablation (RFA), microwave ablation and ethanol ablation (EA), is regarded as the curative treatment method for early HCC[12,13]. In contrast to surgical resection where the tumor is removed, the tumor is not eradicated from the body but is deactivated by ablation therapy, therefore, it is of paramount importance to evaluate the efficacy of this treatment to determine follow-up treatment and strategy. Currently, the use of contrast-enhanced imaging to detect residual viable tumor or recurrent HCC is widely accepted[14-19]. The underlying mechanism of this imaging method relates to the fact that viable tumor tissue shows arterial hypervascularity (*i.e.*, hyper-enhancement), whereas destroyed tumor shows absence of vascularity (*i.e.*, non-enhancement), thus the distinction between them is achievable. However, this method is not ideal as imaging studies may fail to detect tiny viable tumor tissue, especially when neoangiogenesis is not obvious. Percutaneous biopsy may be another option, however, this technique has significant sample error and it is not ethical and practical to sample all the lesion after ablation. Consequently, contrast-enhanced imaging modalities such as computed tomography (CT), magnetic resonance imaging (MRI), and CEUS are currently widely used to evaluate local treatment response after ablation therapies. Although CT and MRI are accepted as the reference standards, the newly introduced CEUS is also gaining increasing attention due to its characteristics including real-time scanning, easy performance, lack of radiation, wide availability, and lack of allergic reactions[20-38]. Several studies have documented that CEUS is comparable to CT or MRI in evaluating local treatment efficacy within one mo of treatment (Table 1)[18,39-41]. Kim *et al*[42] also reported that they were in favor of CEUS as it had the advantage of being able to detect lesions < 2 cm; therefore, CEUS has been used effectively in diagnostic algorithms for small 1-2 cm newly detected nodules in HCC patients. However, little information is available regarding the role of CEUS in the follow-up assessment after first successful ablation treatment. In the follow-up period, the patient may develop local tumor progression (LTP) or new intrahepatic recurrence, and imaging modalities are used to successfully detect these lesions.

In the study by Zheng *et al*[1], 141 patients with HCCs who underwent percutaneous ablation therapy were assessed by paired follow-up CEUS and contrast-enhanced computed tomography (CECT). Using CECT as the reference standard, the ability of CEUS to detect LTP or new intrahepatic recurrence during follow-up was evaluated. They found that 33 LTP and 131 new intrahepatic recurrent foci were detected on CEUS, whereas 40 and 183 were detected on CECT, respectively (both $P < 0.05$). Compared with CECT, the sensitivity, specificity, positive predictive value (PPV), negative predictive value (NPV) and overall accuracy of CEUS in detecting LTP were 67.5%, 97.4%, 81.8%, 94.4% and 92.3%, respectively, and were 77.7%, 92.0%, 92.4%, 76.7% and 84.0%, respectively for the detection of new intrahepatic recurrence. They concluded that the sensitivity of CEUS in detecting LTP and new intrahepatic recurrence after ablation was relatively low compared with CECT, and that CEUS can not replace CECT in the follow-up assessment after percutaneous ablation for HCC. Their results are interesting and meaningful, and we agree with the authors regarding the role of CEUS in the follow-up of HCC patients after ablation therapy.

The discordance in imaging features between CT and CEUS is well recognized, and is largely due to the difference in pharmacokinetics between CT contrast medium and ultrasound contrast agent[35,43]. The CT contrast medium diffuses into the interstitial space, while the ultrasound contrast agent is a pure blood pool tracer. The CEUS characteristic of real-time scanning is helpful in detecting subtle lesions with transient arterial hyper-enhancement which is hard to visualize on CT. Some lesions may show arterial iso-enhancement on CT and hyper-enhancement on CEUS due to the limited time window in CT scanning[44-49]. On the other hand, CEUS also has its shortcomings as shown by Zheng *et al*[1] who found that image quality was affected by lesions located near the liver dome, and obscuration due to gas from the lung or intestine. The development of new foci may be located in different lobes of the liver, and the arterial hyper-enhancement on CEUS only lasts for several seconds, thus it is difficult to detect all hypervascular lesions in one scanning procedure. Most importantly, in comparison with CT/MRI, there is universal bias in readers' minds with regards to ultrasound images. The quality of the procedure and subsequent results are largely operator-dependent, thus less uniformity is encountered in clinical practice (Table 2).

The data in the above-mentioned article are detailed and reasonable results have been obtained. However, there is still controversy over the role of CEUS *vs* CECT in the diagnosis of HCC after ablation, which is largely dependent on individual opinion and familiarity with the techniques. Frieser *et al*[39] concluded that CEUS is equal to CECT in evaluating treatment response. Gallotti *et al*[46] found that CEUS was excellent in evaluating treatment response after RFA, whereas it was inadequate for evaluating treatment response after EA. It should be noted that despite the vast number of published studies on the subject, a unanimous consensus has not been achieved.

There are a number of unsolved issues regarding the use of CEUS and CECT including the following: (1) The authors should recommend the combined use of CT and CEUS in clinical practice when CEUS is available, which will reduce the number of false positive and negative findings in both modalities[50]. In a study of liver lesion characterization, although no follow-up assessment after

Table 1 Diagnostic values of contrast-enhanced imaging in evaluating treatment response after ablation for liver cancer

Ref.	*n*	Imaging	Accuracy	Sen	Spe	PPV	NPV
[1]Lu *et al*[18]	151 patients	CEUS	96.6%	-	98.20%	-	-
Frieser *et al*[39]	76 patients 118 nodules	CEUS	93.8%	-	-	-	-
		CECT	86.2%	-	-	-	-
		CEUS	100%	-	-	-	-
		CEMRI	88.4%	-	-	-	-
[2]Ricci *et al*[40]	100 patients 100 nodules	CEUS	-	92.3%	100%	100%	97.4%
[2]Salvaggio *et al*[41]	148 nodules	CEUS	97%	83.3%	100%	-	-

[1]In comparison with contrast-enhanced computed tomography (CECT) or contrast-enhanced magnetic resonance imaging (CEMRI); [2]In comparison with CECT. -: Not applicable. Sen: Sensitivity; Spe: Specificity; PPV: Positive predictive value; NPV: Negative predictive value; CEUS: Contrast-enhanced ultrasound.

Table 2 Comparison between contrast-enhanced ultrasound and contrast-enhanced computed tomography in the follow-up scheme after liver cancer ablation

	CEUS	CECT
Pharmacokinetics of the contrast	Early vascular phase; followed by diffusion into the interstitial space	Pure blood pool tracer; without diffusion into the interstitial space
Strong points	Real-time scanning, easy to perform, no radiation, wide availability, and no allergic reactions	High image quality; operator-independent; panoramic imaging; easy to interpret
Weak points	Image quality is apt to be affected by lesions located near the liver dome, and obscuration by gas from the lung or intestine; Inability to imaging multiple lesions in one procedure; operator-dependent	Inferior temporal resolution; allergic reaction to the contrast-medium; unsuitable for patients with kidney function impairment; radiation; inferior availability

CEUS: Contrast-enhanced ultrasound; CECT: Contrast-enhanced computed tomography.

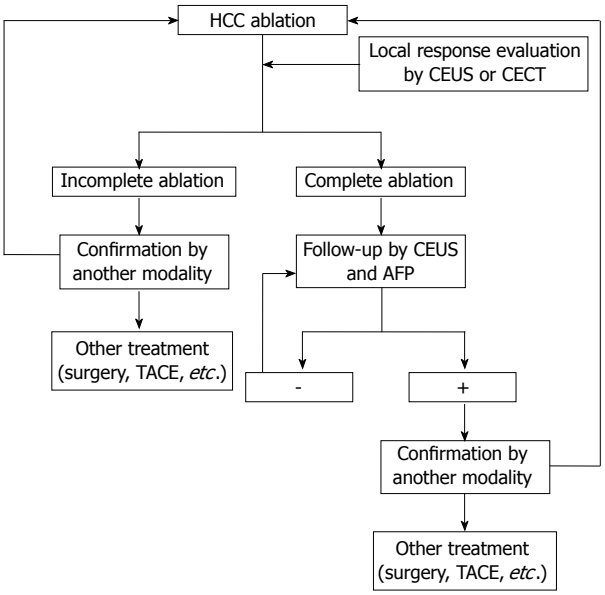

Figure 1 The proposed algorithm combining the strengths of the radiologic techniques of contrast-enhanced ultrasound and contrast-enhanced computed tomography. HCC: Hepatocellular carcinoma; CEUS: Contrast-enhanced ultrasound; CECT: Contrast-enhanced computed tomography; AFP: α fetoprotein; TACE: Transarterial chemoembolization; -: Negative finding; +: Positive finding.

ablation was carried out, the authors found that combined assessment using CEUS/CT provided higher sensitivity (97%, both readers) than separate assessment using CEUS (88% reader 1; 87% reader 2) and CT (74% reader 1; 71% reader 2; $P < 0.05$), while no change in specificity was observed using the combined analysis. The combined assessment of hepatocellular nodule vascularity using CT and CEUS improved diagnostic sensitivity of malignancy in patients with liver cirrhosis[50]. However, the study by Zheng *et al*[1] was retrospective, thus it is difficult to assess the value of the combined diagnostic procedures in clinical practice, which has prompted a future prospective study to evaluate this issue. Fusion imaging may be another solution which combines the virtues of both modalities[51]; (2) The ideal imaging follow-up scheme is not yet available. Future evidence-based studies are necessary to establish this scheme. Every practicing clinician must make a decision on the most accurate, cost-effective radiologic test to be used in the follow-up of liver lesions after local treatment. A reasonable algorithm is proposed and is shown in Figure 1, however, further study is mandatory to evaluate its efficacy; (3) A cost-effectiveness analysis should be performed to select the best imaging follow-up scheme; and (4) Long-term follow-up studies are needed to help guide our approach and therapy.

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REVIEW

What is the role of adiponectin in obesity related non-alcoholic fatty liver disease?

Carmine Finelli, Giovanni Tarantino

Carmine Finelli, Giovanni Tarantino, Center of Obesity and Eating Disorder, Stella Maris Mediterraneum Foundation, 80035 Potenza, Italy
Carmine Finelli, Giovanni Tarantino, Department of Clinical and Experimental Medicine, Federico II University Medical School of Naples, 80131 Naples, Italy
Author contributions: Both authors contributed equally to the design and draft of manuscript.
Correspondence to: Giovanni Tarantino, Professor, Department of Clinical and Experimental Medicine, Federico II University Medical School of Naples, 80131 Naples, Italy. tarantin@unina.it
Telephone: +39-81-7462024 Fax: +39-81-5466152
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Abstract

Non-alcoholic fatty liver disease (NAFLD) is recognized as the most common type of chronic liver disease in Western countries. Insulin resistance is a key factor in the pathogenesis of NAFLD, the latter being considered as the hepatic component of insulin resistance or obesity. Adiponectin is the most abundant adipose-specific adipokine. There is evidence that adiponectin decreases hepatic and systematic insulin resistance, and attenuates liver inflammation and fibrosis. Adiponectin generally predicts steatosis grade and the severity of NAFLD; however, to what extent this is a direct effect or related to the presence of more severe insulin resistance or obesity remains to be addressed. Although there is no proven pharmacotherapy for the treatment of NAFLD, recent therapeutic strategies have focused on the indirect upregulation of adiponectin through the administration of various therapeutic agents and/or lifestyle modifications. In this adiponectin-focused review, the pathogenetic role and the potential therapeutic benefits of adiponectin in NAFLD are analyzed systematically.

Key words: Non-alcoholic fatty liver disease; Obesity; Adiponectin; Visceral fat; Adipokines

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INTRODUCTION

Non-alcoholic fatty liver disease (NAFLD) is the most common type of chronic liver injury in many countries[1,2]. NAFLD includes a spectrum of syndromes, ranging from simple steatosis, non-alcoholic steatohepatitis (NASH) to fibrosis, cirrhosis and hepatocellular carcinoma[3]. The overall prevalence of NAFLD is 15%-40% in Western countries and 9%-40% in the Asian population[4]. NAFLD has dramatically increased over the past 15 years, mainly as a consequence of its close association with two major worldwide epidemics, obesity and type 2 diabetes mellitus (T2DM)[5]. Mortality in patients with NAFLD is significantly higher than in the age and gender-matched general population[6]. Disease progression to NASH and cirrhosis appears to be very slow, and only a few patients develop life-threatening advanced liver disease.

In many cases of NAFLD, the risks of developing metabolic and cardiovascular morbidities are much higher than the risks of developing hepatic diseases[7,8]. In fact, NAFLD is considered as the hepatic manifestation of the metabolic syndrome, which refers to a cluster of cardiovascular risk factors associated with insulin resistance, including central obesity, hypertension, dyslipidemia and T2DM[9]. The association between NAFLD and metabolic syndrome has been established in many cross-sectional and prospective studies[8]. NAFLD significantly increases the risk of diabetes and is a better predictor of the development of metabolic disorders than obesity

itself[10]. Some studies have reported an association of NAFLD with multiple classical and non-classical risk factors for cardiovascular diseases[7]. NAFLD predicts future cardiovascular events independently of other prognostic factors, including the component of metabolic syndrome. In summary, NAFLD is associated with a future high incidence of cardiovascular and metabolic complications and should be considered beyond a liver disease confined to classical boundaries. Understanding the disease and its management is a vital issue in current clinical practice.

PATHOGENESIS OF NAFLD

Although the pathogenesis of NAFLD remains largely unknown, insulin resistance, oxidative stress and inflammation play important roles in the development and progression of NAFLD[11,12]. Fatty liver itself is a status of insulin resistance. Hepatic fat accumulation can lead to hepatic insulin resistance, which may occur before the alterations in peripheral insulin actions and may induce peripheral insulin resistance[13,14]. Insulin regulates the uptake, oxidation and storage of fuel within insulin-sensitive tissues including the liver, skeletal muscle and fat. Peripheral insulin resistance impairs glucose uptake from blood into skeletal muscle and adipose tissue; serum non-esterified fatty acid (NEFA) levels may also be elevated because of the failure of insulin to suppress lipolysis[15,16]. In the liver, insulin resistance is associated with increased cellular contents of fatty acids and their metabolites (fatty acyl-CoAs, diacylglycerides and ceramides)[17-19]. Hyperinsulinemia caused by insulin resistance, in the presence of increased circulating levels of NEFA, enhances the hepatic uptake of fatty acid and promotes lipogenesis[1,20]. In addition, defects in mitochondrial β-oxidation, enhanced fatty acid synthesis and impaired secretion of triacylglyceride-rich very low density lipoproteins also contribute to hepatic steatosis[21-23]. A growing body of evidence from animal models suggests a "two-hit" hypothesis as being responsible for the development of NAFLD[24-26]. According to this theory, the first hit is the occurrence of fatty liver (steatosis), followed by a second event leading to the development of NASH. The potential secondary hits include endotoxin exposure, alcohol consumption and virus infections, which expand hepatic lipid stores, cause hepatocellular injury, and promote oxidative stress and inflammation in the liver. Lipotoxicity, and the release of cytokines and other pro-inflammatory mediators, play important roles during this process. Moreover, inflammation in the development of NASH can further impede insulin signaling[27]. Histologically, NASH is manifested by hepatocyte nuclear ballooning, hepatocyte apoptosis, Mallory's hyaline and inflammation foci[28]. NAFLD patients have a high circulating free fatty acids (FFAs) level that correlates with the severity of liver disease. Overloaded FFAs may exhibit lipotoxicity by inducing the expression of proinflammatory cytokines, such as tumor necrosis factor alpha (TNF-α)[29].

VISCERAL OBESITY, ADIPOKINES AND NAFLD

Obesity, especially visceral obesity, is frequently associated with NAFLD and their coexistence in the same individual increases the likelihood of having more advanced forms of liver disease[30,31]. NAFLD occurs in 60%-95% of people with obesity[32]. Visceral fat is a key mediator of NASH and is strongly associated with alanine aminotransferase (ALT) levels in the nondiabetic obese population[31,33,34]. The importance of visceral fat in the pathogenesis of NAFLD has also been shown in many animal models, including *fa/fa* obese rats. In these animals, surgical resection of intra abdominal fat depots reverses hepatic insulin resistance and steatosis[35].

Recent evidence suggests that visceral adipose tissue is a metabolic and inflammatory organ that signals and modulates the action and metabolism of the brain, liver, muscle and cardiovascular system[36,37]. The imbalanced production of pro- and anti-inflammatory adipokines secreted from fat contributes to the pathogenesis of NAFLD[38]. Modulation of endocrine/immune/inflammatory interactions of adipose tissue may provide novel therapeutic (pharmacological) targets for the treatment of NAFLD. For example, in patients with severe lipodystrophy, injection with leptin reverses nonalcoholic fatty liver diseases[39,40]. However, in cases of NAFLD associated with obesity, serum levels of leptin are increased, and the liver becomes refractory to the "anti-steatotic" effects of leptin[41-43]. Leptin infusion is therefore unlikely to be of therapeutic value for patients with NAFLD. TNF-α, a pro-inflammatory adipokine that interferes with insulin signaling and favors steatosis, may play a casual role in the pathogenesis of NASH[38]. Circulating levels of TNF-α and hepatic expression of its type 1 receptor are increased in NASH, but could not discriminate steatohepatitis from steatosis[44-46]. Neutralization of TNF-α activity improves fatty liver disease in animals[47]. Conversely, nutritional steatohepatitis can still be produced experimentally in both TNF-α and TNF-α type 1 receptor knockout mice, suggesting that this adipokine might not be an essential mediator of NAFLD[48,49]. In contrast to leptin and TNF-α, adiponectin is more closely implicated in the pathogenesis of NAFLD/NASH. Unlike other adipokines, serum levels of adiponectin are decreased in obesity and its associated medical complications[50]. A negative association between serum levels of adiponectin and liver enzyme levels has been shown in healthy subjects[51]. Numerous epidemiological investigations in diverse ethnic groups have identified lower adiponectin level as an independent risk factor for NAFLDs and liver dysfunctions[37]. Compared with healthy controls, adiponectin levels are lower by more than 50% in NASH patients[52]. Adiponectin expression is decreased by 20%-40% during the development of NAFLD, from simple steatosis to NASH[52,53]. Moreover, NASH patients with lower levels of adiponectin show higher grades of inflammation, suggesting that adiponectin deficiency is an important risk factor

for the development of fatty liver, steatohepatitis and other forms of liver injuries[52-55]. In patients with T2DM, plasma adiponectin concentrations are inversely related to hepatic fat content[56]. There is a direct relationship between hypoadiponectinemia and NASH, independent of insulin resistance[52]. Animal-based studies have demonstrated that adiponectin possesses potent protective activities against various forms of liver injury, including those induced by carbon tetrachloride, lipopolysaccharide (LPS)/*D*-galactosamine, pharmacological compounds, bile duct ligations and methionine-deficient diet[57-61]. In animal models of both alcoholic and nonalcoholic steatohepatitis, exogenous adiponectin reduces hepatomegaly, depletes lipid accumulation, quenches hepatic inflammation and decreases hepatic expression and plasma concentrations of TNF-α[62]. Adiponectin knockout mice exhibit an enhanced pattern of hepatic fibrosis induced by carbon tetrachloride[58]. The lack of adiponectin expression could accelerate hepatic tumor formation in a NASH model in mice[63]. Among the known adipokines, adiponectin stands out for its insulin-sensitizing and anti-inflammatory roles, and may be used as a promising drug candidate for the treatment of liver diseases.

HEPATOPROTECTIVE FUNCTIONS OF ADIPONECTIN: STRUCTURAL BASIS AND SIGNALLING MECHANISMS

Four independent groups originally identified adiponectin, also termed Acrp30, AdipoQ, apM1 or GBP28, in both mice and humans[64-67]. This adipokine has attracted much attention because of its multiple beneficial effects on a cluster of obesity-related metabolic and cardiovascular dysfunctions. Hypoadiponectinemia is a key etiological factor contributing to almost all the major pathological conditions associated with obesity[68]. The physiological functions and clinical relevance of adiponectin in obesity-related medical complications have been extensively reviewed elsewhere[50,69-72]. In the following sections, we will discuss recent advances on the structural regulations of adiponectin as well as the molecular evidence supporting the role of adiponectin as a major protective agent against obesity-related NAFLD.

Polymorphism of the multimeric structures of adiponectin

A unique feature of the structure of adiponectin is its ability to assemble into several characteristic oligomeric isoforms, including trimers [low molecular weight (LMW)], hexamers [middle molecular weight (MMW)] and the oligomeric complexes comprising 18 protomers or above [high molecular weight (HMW)][73]. Adiponectin presents predominantly in the circulation as these three oligomeric complexes[74-79]. Trimeric adiponectin is the basic building block of adiponectin. The subunits in the trimer are associated *via* hydrophobic interactions. Two LMW adiponectin molecules linked by disulfide bonds form hexameric adiponectin. The structural properties of the HMW adiponectin remain poorly characterized because of the heterogeneous nature of this isoform. Analysis of adiponectin oligomers by non-denaturing and non-heating gel electrophoresis shows that the human HMW adiponectin composes of a mixture of 18-30 mers, or even larger molecular weight species[73,78,80,81]. Dynamic light scattering and transmission electron microscopy shows that the bovine HMW adiponectin forms a bouquet-like architecture resembling that of complement C1q[82]. Six globular objects can be seen atop a thin stalk, which presumably correspond to the six LMW adiponectins. The stalks bunch together in a manner that is consistent with the requirement for NH2-terminal disulfide bonding. The side views of HMW adiponectin suggest a conical structure of the oligomer with the COOH-terminal portion forming the base. Interestingly, these globular domains are arranged in a tight ring. This circular arrangement might enable polyvalent interactions of the globular domains with a single receptor. Recently, the HMW oligomeric structures formed by multiples of adiponectin trimers have been determined by single-particle analysis of electron micrographs[83]. Pleiomorphic ensembles of collagen-like stretches of the trimers lead to a highly dynamic structure of HMW adiponectin, which can be classified into two major classes: the fan-shaped (Class Ⅰ) and bouquet-shaped (Class Ⅱ). In both of these conformations, the globular domains assume a variety of arrangements, covering an area of up to 4.9 × 105 Å^2 and up to 320 Å apart. The conformational flexibility of the HMW oligomer can allow it to access and cluster disparate target ligands or receptors, which may be necessary to activate cellular signaling leading to the remarkable functional diversity of adiponectin.

HMW adiponectin as a major bioactive form in liver

Obese individuals have different distribution of adiponectin oligomers compared with lean controls. Relatively lower content of HMW adiponectin is closely associated with obesity-related metabolic complications[81]. The increases in the ratio of HMW *vs* total adiponectin, but not total adiponectin level *per se*, correlate well with improved insulin sensitivity during treatment with the insulin-sensitizing drug thiazolidinediones, in both diabetic mice and patients with T2DM. On the other hand, weight reduction by either calorie restriction or gastric bypass surgery results in a selective elevation of the HMW adiponectin, but not the trimeric and hexameric complexes[84-86]. An independent inverse association exists between ALT and HMW adiponectin[87]. Taken together, these epidemiological and genetic data suggest that the beneficial effects of adiponectin in humans might be mediated primarily by its HMW isoform, and the deficiency of this oligomer is an important etiological factor that links obesity with its medical complications.

Evidence from both *in vitro* and animal-based studies also supports the role of the HMW oligomer as the major active form in mediating the multiple actions of adiponectin in liver tissue. Recombinant adiponectin pro-

duced from mammalian cells, which can form the HMW oligomers, potently decreases hyperglycemia in diabetic mice through inhibition of hepatic glucose production[88]. However, bacterially generated full-length adiponectin, which lacks the capacity to form the HMW adiponectin, is almost inactive. Intravenous injection of the HMW adiponectin, but not the hexameric adiponectin, leads to a dose-dependent decrease in serum glucose levels[81]. The formation of the HMW oligomers is obligatory to mediate the insulin sensitizing effects of adiponectin on suppression of hepatic gluconeogenesis in primary rat hepatocytes[80]. Acute injection of recombinant adiponectin enriched with the HMW oligomers results in a marked activation of AMP-activated kinase (AMPK) in the liver, while chronic infusion with this protein leads to prolonged alleviation of hyperglycemia and insulin resistance in *db/db* diabetic mice[89]. This animal-based evidence is consistent with the clinical observations showing that the ratio of HMW/total adiponectin correlates closely with hepatic insulin sensitivity[81]. The role of the HMW oligomer as a predominant active form of adiponectin mediating its hepatic actions is also supported by two recent independent reports demonstrating that the insulin-sensitizing effects of the peroxisome proliferator-activated receptor gamma (PPAR-γ) agonists thiazolidinediones were diminished in *ob/ob* obese mice with the targeted mutation of the adiponectin gene[90,91]. Notably, treatment with thiazolidinediones causes a selective elevation of the HMW oligomeric adiponectin[79,81]. In addition to the hepatic insulin-sensitizing activity, the HMW adiponectin has also been suggested to be the most potent isoform for alleviation of fatty liver disease in high fat diet-induced obese mice[92], and inhibition of apolipoprotein B and E release from human hepatocytes[93]. HMW adiponectin dose-dependently suppressed growth factor-induced hepatic stellate cell proliferation[94]. Taken together, these data suggest that the HMW form predominantly mediates the beneficial effects of adiponectin in hepatic tissue.

Receptors and postreceptor signaling pathways mediating the hepato-protective functions of adiponectin

Two adiponectin receptors (adipoR1 and adipoR2) have been identified and found to be expressed in various tissues[95]. AdipoR1 is abundantly expressed in skeletal muscles, whereas adipoR2 is present predominantly in the liver, suggesting a role of adipoR2 in hepatic adiponectin signaling[68,96]. Recently, several laboratories have investigated the physiological roles of adipoR1 and adipoR2 in *adipoR1/2* knockout mice. Both *adipoR1* and *adipoR2* knockout mice exhibit mild insulin resistance[97]. In *adipoR1/R2* double knockout mice, the binding and actions of adiponectin are abolished, resulting in increased tissue triglyceride content, inflammation and oxidative stress[97]. *AdipoR2* knockout mice reported by Liu *et al*[98] displayed reduced diet-induced insulin resistance, but promoted T2DM. These data support the physiological roles of adipoR1 and adipoR2 as the predominant receptors for adiponectin in the regulation of glucose and lipid metabolism. Despite this information, the detailed roles and expression of adipoRs in NAFLD are not conclusive[38,99-102].

Adiponectin stimulates AMPK in almost all its major target tissues, including skeletal muscle, liver, heart, endothelium, adipocytes and brain[75,89,103-106]. Notably, most biological effects of adiponectin in these target tissues are abrogated by expression of a dominant negative version of AMPK, supporting its obligatory role in mediating adiponectin's multiple actions. The precise mechanisms whereby adiponectin activates AMPK through its receptors remain to be determined. APPL1, an adaptor protein containing a pleckstrin homology domain, a phosphotyrosine binding domain and a leucine zipper motif, appears to be a key signaling molecule that couples adiponectin receptors and its downstream AMPK activation[103,107]. Adiponectin enhances the binding of APPL1 to both adipoR1 and adipoR2, and these interactions are essential for subsequent phosphorylation and activation of AMPK. Studies also indicate the important role of APPL1 in the metabolic syndrome[108,109]. AMPK activation in turn phosphorylates acetyl Coenzyme A carboxylase (ACC) and attenuates ACC activity. Inhibition of ACC reduces lipid synthesis and enhances fatty acid oxidation by blocking the production of malonyl-CoA, an allosteric inhibitor of carnitine palmitoyl transferase 1, the rate-limiting enzyme in fatty acid oxidation. In addition, activation of AMPK downregulates the expression of sterol regulatory element-binding protein 1c (SREBP1c), a transcription factor that regulates cholesterol and lipid synthesis. Reduction of SREBP1c results in downregulation of genes involved in lipogenesis, including ACC, fatty acid synthase, and glycerol-3-phosphate acyltransferase[104,110,111].

PPARα is a transcription factor controlling the transcription of a panel of genes encoding fatty acid oxidation enzymes, such as FATP, acyl-CoA oxidase and long chain acyl-CoA synthetase. Adiponectin stimulates PPARα activity possibly through PPARγ coactivator-1α[112]. These adiponectin-mediated signaling pathways lead to enhanced fat oxidation, reduced lipid synthesis and prevention of hepatic steatosis (Figure 1).

Cellular mechanisms contributing to the anti-inflammatory activities of adiponectin in NAFLD

Inflammatory cytokines are key mediators of hepatic inflammation, cell death, and fibrosis, as well as regeneration after massive or focal liver injury[38,113]. Adiponectin levels are negatively associated with mediators of inflammation, including interleukin-6 (IL-6) and C-reactive protein; but positively related to anti-inflammatory cytokine IL-10[114,115]. It suppresses TNF-α functions by inhibiting its expression and antagonizing its activities[61,62,116,117]. In the liver, cytokines such as IL-6 and TNF-α, are mainly produced from Kupffer cells and hepatic stellate cells (HSC), and partly from inflamed hepatocytes[52,118,119]. Adiponectin ameliorates NASH and liver fibrosis by suppressing the activation of Kupffer

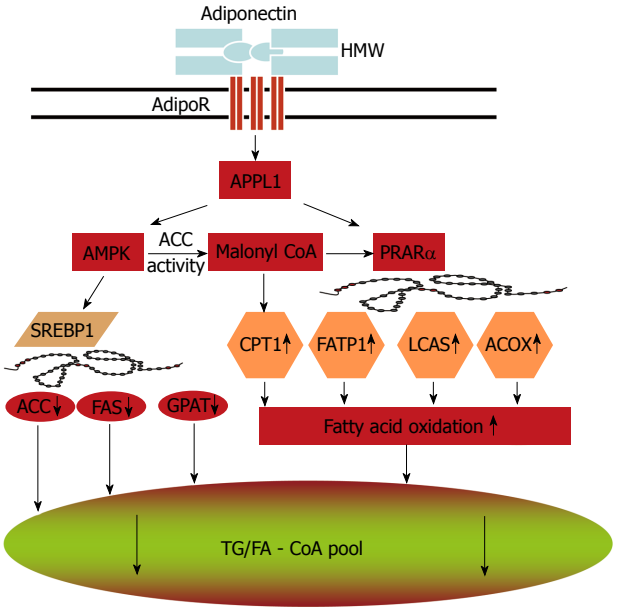

Figure 1 Summary of multiple signaling pathways that mediate the anti-steatotic effects of adiponectin. HMW: High molecular weight; AdipoR: Adiponectin receptor; APPL1: Adaptor protein, phosphotyrosine interaction, PH domain and leucine zipper containing 1; AMPK: AMP-activated kinase; ACC: Acetyl Coenzyme A carboxylase; CPT1: Carnitine palmitoyl transferase 1; SREBP1: Sterol regulatory element-binding protein 1; FAS: Fatty acid synthase; GPAT: Glycerol-3-phosphate acyltransferase; ACOX: Acyl-CoA oxidase; LCAS: Long chain acyl-CoA synthetase; FATP: Fatty acid transport protein; PPARα: Peroxisome proliferator-activated receptor α; TG: Triacylglyceride; FA: Fatty acid.

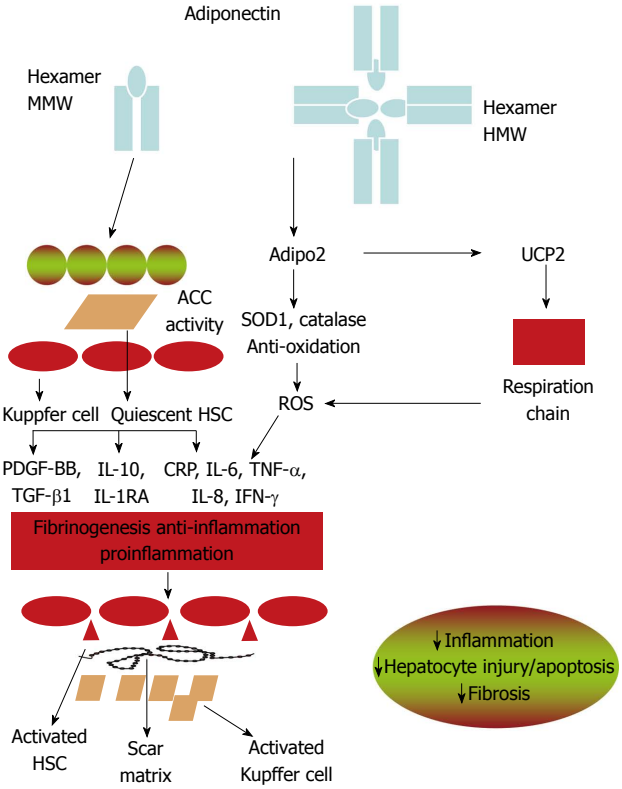

Figure 2 Summary of multiple pathways underlying the protective effects of adiponectin against liver injury. MMW: Middle molecular weight; HMW: High molecular weight; AdipoR: Adiponectin receptor; UCP: Uncoupling protein; SOD1: Superoxide dismutase 1; ROS: Reactive oxygen species; PDGF-BB: Platelet-derived growth factor BB; TGF-β1: Transforming growth factor-β1; CRP: C-reactive protein; IL: Interleukin; IL-1RA: Interleukin-1-receptor antagonist; TNF-α: Tumor necrosis factor-α; HSC: Hepatic stellate cells; IFN-γ: Interferon-γ.

cells and HSC (Figure 2). In porcine blood-derived macrophages, adiponectin suppresses both TNF-α and IL-6 production stimulated by LPS and induces IL10 expression. The attenuation of proinflammatory cytokine production by adiponectin is mediated in part by attenuating the translocation of nuclear factor kappa B (NF-κB) to the nucleus[120]. Adiponectin can also induce the expression of the anti-inflammation cytokine interleukin-1-receptor antagonist[121,122]. The anti-inflammatory effects of adiponectin in macrophages may involve the toll-like receptor-4 (TLR-4) signaling pathway. However, the mechanisms by which adiponectin suppresses TLR-4 mediated responses are not well understood[123].

The transformation of HSC into myofibroblasts is the key step that initiates the fibrotic process during liver injury[124,125]. The activated hepatic stellate cells increase the accumulation of extracellular matrix. Both adiponectin receptors, adipoR1 and adipoR2, are expressed in HSC. Adiponectin treatment maintains HSC quiescence, inhibits platelet-derived growth factor-stimulated proliferation and migration of human HSCs, and reduces the secretion and of monocyte chemoattractant protein-1 through AMPK-dependent mechanisms[94,125,126]. Additionally, adiponectin also regulates hepatic expression of TGFβ1, a pro-fibrotic factor involved in HSC activation[58,127] that plays an important role in neofibrogenesis of NAFLD[128].

Inhibition of adipoR2 expression by short hairpin RNAi-expressing adenovirus can induce TGFβ1 expression, and overexpression of adipoR2 diminishes TGFβ1 mRNA level.

Regulatory role of adiponectin on mitochondria activities

Mitochondrial dysfunction represents a central mechanism linking obesity with associated metabolic complications[129]. In patients with NASH, the hepatic mitochondria exhibit ultrastructural lesions and decreased activity of the respiratory chain complexes[130,131]. In this condition, the decreased activity of the respiratory chain results in accumulation of reactive oxygen species (ROS) that oxidize fat deposits to form lipid peroxidation products, which in turn, cause steatohepatitis, necrosis, inflammation and fibrosis. The increased mitochondrial ROS formation in steatohepatitis could directly damage mitochondria DNA and respiratory chain polypeptides, induce NF-κB activation and the hepatic synthesis of TNFα[132]. Oxidative phosphorylation reactions mediated by mitochondria respiratory chain (MRC) complexes are directly involved in regulating intracellular ROS activities and preventing accumulation of lipids and lipid peroxidation products in the liver.

Mice without adiponectin show an increased lipid

accumulation even under normal chow feeding[117]. This pre-existing hepatic steatotic condition might be the direct consequence of dysregulated mitochondria functions[117]. Adiponectin treatment restores the MRC activities, decreases the levels of mitochondrial lipid peroxidation products through regulating hepatic mitochondrial functions, which might represent a common mechanism underlying the multiple beneficial activities of this hormone in various obesity-related pathologies. Moreover, we have provided evidence supporting an essential role of uncoupling protein 2 (UCP2), a mitochondria inner membrane transporter, in mediating the beneficial effects of adiponectin on MRC activities. The protein and mRNA levels of UCP2 are decreased in the liver tissues of adiponectin knockout mice and can be significantly upregulated by adiponectin treatment. Overexpression of adipoR2 upregulates mRNA levels of UCP2, catalase, and superoxide dismutase 1 in the liver[97]. Furthermore, the effects of adiponectin on MRC activities are dramatically attenuated in *Ucp2*-deficient mice, suggesting that the increased UCP2 expression might be obligatory for adiponectin to elicit its activities on mitochondria functions (Figure 2). UCP2 possesses anti-oxidant activities through inhibition of ROS production from mitochondria[133]. It can also inhibit the production of pro-inflammatory cytokines in both macrophage and Kupffer cells[134]. A growing body of evidence suggests that UCP2 may play a beneficial role in various stages of fatty liver diseases[134,135]. These results suggest the existence of a reciprocal relationship between uncoupling proteins and adiponectin. However, the detailed signaling mechanisms underlying adiponectin-induced UCP2 expression are not clear and warrant further investigation.

ELEVATION OF ADIPONECTIN PRODUCTION AS A THERAPEUTIC STRATEGY FOR TREATMENT OF NAFLD

To date, there have been very few effective drug treatments for NAFLD and NASH. Early diagnosis and management of the underlying condition remains the mainstay of treatment. The present "gold standard" for treatment of NAFLD is weight reduction or a reduction of central obesity[4]. These "life-style adjustment" or anti-obesity measures (including bariatric surgery) impressively reduce liver cell injury, inflammation and hepatic fibrosis, as well as steatosis[136,137]. The potential for correcting steatosis by dietary or pharmacological approaches should provide a sound therapeutic approach for the treatment of steatosis and steatohepatitis. Strategies to block oxidative stress are of great interest, with some evidence that ALT normalization or histological improvement occurs with vitamin E (alone or with vitamin C or pioglitazone) and betaine[138].

Adiponectin and its agonists might represent emerging therapeutic agents for the treatment and/or prevention of liver dysfunctions[139-141]. Adiponectin replacement therapy is not yet available as a treatment option. Pharmacological intervention aimed at elevating adiponectin production might hold promise for the treatment and/or prevention of NAFLD.

CONCLUDING REMARKS

Based on our data, polymorphic UCP1 (AG + GG) obese patients with low adiponectin levels appear to be high-risk subjects for worsening of liver steatosis, an NAFLD, possibly requiring a second-step evaluation by liver biopsy[142].

The role of adiponectin in systemic inflammation and critical illness is not well defined. Early data suggest that plasma levels of adiponectin are decreased in critical illness[143]. Whether this is a result of the disease process itself or whether patients with lower levels of this hormone are more susceptible to developing a critical illness is not known. This observation of lower adiponectin levels then raises the possibility of therapeutic options to increase circulating adiponectin levels[143]. The various options for modulation of serum adiponectin (recombinant adiponectin, thiazolidinediones) are discussed.

Nevertheless, adiponectin-based therapeutics for NAFLD represent a promising area for further investigation.

CONCLUSION

Adiponectin is an abundant adipocyte-derived hormone with well established anti-inflammatory and insulin sensitizing properties. The significance of adiponectin in protecting obesity-related NAFLD has been increasingly recognized. Despite the advances made in recent years, the detailed molecular and cellular mechanisms underlying its hepato-protective functions remain largely uncharacterized.

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REVIEW

Current evidence for histone deacetylase inhibitors in pancreatic cancer

Ioannis Koutsounas, Constantinos Giaginis, Efstratios Patsouris, Stamatios Theocharis

Ioannis Koutsounas, Constantinos Giaginis, Efstratios Patsouris, Stamatios Theocharis, First Department of Pathology, Medical School, National and Kapodistrian University of Athens, GR-11527 Athens, Greece
Constantinos Giaginis, Department of Food Science and Nutrition, University of the Aegean, GR-81400 Lemnos, Greece
Author contributions: Koutsounas I collected and analyzed literature data and wrote the paper; Giaginis C collected and analyzed literature data; Patsouris E collected funds for the study; and Theocharis S designed the study and analyzed literature data.
Correspondence to: Stamatios Theocharis, MD, PhD, Pathologist, Associate Professor of the Department of Pathology, Medical School, National and Kapodistrian University of Athens, GR-11527 Athens, Greece. theocharis@ath.forthnet.gr
Telephone: +30-21-7462413 Fax: +30-21-7716098
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Abstract

Pancreatic cancer is one of the most aggressive human cancers, with more than 200 000 deaths worldwide every year. Despite recent efforts, conventional treatment approaches, such as surgery and classic chemotherapy, have only slightly improved patient outcomes. More effective and well-tolerated therapies are required to reverse the current poor prognosis of this type of neoplasm. Among new agents, histone deacetylase inhibitors (HDACIs) are now being tested. HDACIs have multiple biological effects related to acetylation of histones and many non-histone proteins that are involved in regulation of gene expression, apoptosis, cell cycle progression and angiogenesis. HDACIs induce cell cycle arrest and can activate the extrinsic and intrinsic pathways of apoptosis in different cancer cell lines. In the present review, the main mechanisms by which HDACIs act in pancreatic cancer cells *in vitro*, as well as their antiproliferative effects in animal models are presented. HDACIs constitute a promising treatment for pancreatic cancer with encouraging anti-tumor effects, at well-tolerated doses.

Key words: Pancreatic cancer; Histone deacetylases; Histone deacetylase inhibitors; Experimental studies

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INTRODUCTION

Pancreatic cancer is one of the most lethal human cancers and continues to be a major unsolved health problem at the beginning of the 21st century. Worldwide, over 200 000 people die annually of pancreatic cancer, with the highest incidence and mortality rates found in developed countries. Pancreatic cancer is the 4th and 6th leading cause of cancer death in United States and Europe, respectively. Pancreatic cancer incidence and mortality rates are almost equal because of the high fatality rate. A lack of illness indicators and screening tests mean that pancreatic cancer is usually diagnosed at the late stages of the natural history of the disease[1,2].

In Western communities, the 1- and 5-year survival rates for pancreatic cancer are less than 25% and 5%, respectively, and the mortality rates are essentially identical. Although survival rates are highest (16.6%) when the tumor is localized at diagnosis, less than 10% of tumors are detected at that time. On the other hand, the survival rates have been only slightly improved over the past decade because of a lack of significant medical advances in early detection and the poor outcome of treatment approaches. Pancreatic cancer is rare in the first three decades of life. The majority of pancreatic cancers occur

in the exocrine pancreas and the vast majority (> 90%) have ductal differentiation. Men consistently have higher incidence and mortality rates than women, worldwide[3].

Treatment of advanced pancreatic cancer

Traditionally, 5-fluorouracil (5-FU)-based chemotherapy and/or radiotherapy have been used in the treatment of locally advanced pancreatic cancer; however, the value of radiotherapy remains unclear[4]. Today, *gemcitabine*-based therapy is the acceptable treatment approach for both unresectable locally advanced and metastatic pancreatic cancer. Several phase Ⅲ trials were undertaken with gemcitabine in combination with a range of chemotherapy agents. However, combining gemcitabine with 5-FU[5], as well as *irinotecan*, *oxaliplatin*, *pemetrexed*, *exatecan* and *cisplatin*[6-10], all failed to show superiority over gemcitabine monotherapy. In a recent phase Ⅲ trial, the combination of *capecitabine* with gemcitabine significantly improved the objective response rate and progression-free survival, but did not show superiority in overall survival in patients with advanced pancreatic cancer[11]. Additionally, in a randomized phase Ⅱ trial of *folfirinox* (5-FU/leucovorin, irinotecan, and oxaliplatin) versus gemcitabine, the median overall survival was 11.1 mo in the folfirinox group compared with 6.8 mo in the gemcitabine group, indicating that folfirinox is an option for the treatment of patients with metastatic pancreatic cancer[12].

Targeted therapies have also been investigated for advanced pancreatic cancer. Erlotinib is a small-molecule tyrosine kinase inhibitor of the human epidermal growth factor receptor (EGFR). A multicenter, randomized, double-blind, placebo-controlled phase Ⅲ clinical trial of erlotinib in combination with gemcitabine, in patients with locally advanced or metastatic pancreatic adenocarcinoma met its primary endpoint, with the combination regimen being the first gemcitabine combination to demonstrate a statistically significant survival advantage over gemcitabine monotherapy and the regimen was consequently approved for metastatic disease[13].

Many molecular-targeted agents that interact with crucial pathways for cell survival in pancreatic cancer are currently being explored. These include agents that target *poly ADP*-ribose polymerase, histone deacetylase (*HDAC*), *Src/Abl kinases,* and mammalian targets of rapamycin[14].

Histone acetyltransferases and deacetylases

The principal structure of eukaryotic chromatin is the nucleosome. Each nucleosome consists of approximately 146 bp of DNA wrapped around a core of eight basic proteins called histones, two each of H2A, H2B, H3 and H4. Nucleosomal structure not only facilitates packing DNA into a relatively small nucleus, but also exerts important regulatory functions. The N- and C- terminal tails of the nucleosomal core undergo post-translational modifications, participating in chromatin assembly regulation and/or DNA accessibility. Nucleosomes containing highly charged hypoacetylated histones bind tightly to the phosphate backbone of DNA, inhibiting transcription, preventing transcription factors, regulatory complexes and RNA polymerase to access the DNA. Acetylation neutralizes the charge of the histones generating a more open DNA conformation. Transcription factors may then access the DNA, promoting the expression of the corresponding genes. Therefore, histone acetylation is generally associated with transcriptional activation. Histone acetylation is carried out by a group of proteins called histone acetyl transferases (HATs), and the acetyl groups can be removed by HDACs. These molecules play a pivotal role in cellular functions, such as chromosome remodelling, gene transcription and cell proliferation[15,16].

Eighteen different human HDAC isoforms have been described and are classified into four classes. Class Ⅰ HDACs (HDACs 1, 2, 3 and 8) are associated with RPD3 deacetylase, and are primarily located in the nucleus. Class Ⅱ HDACs are divided into two subclasses, class Ⅱa (HDACs 4, 5, 7 and 9) and class Ⅱb (HDACs 6 and 10) and are homologous to the yeast Hda1 deacetylase. Class Ⅲ HDACs consists of seven HDACs (SIRT1 to SIRT7) that share homologies with the yeast silent information regulator 2 (Sir2) family. The class Ⅳ family of HDACs has only one member, HDAC11. Classes Ⅰ, Ⅱ and Ⅳ require Zn^{2+} for activity, while class Ⅲ has a unique catalytic mechanism that requires the co-factor NAD^{+}. To achieve the acetylation of histones, an acetyl group of acetyl coenzyme A is linked to the ε-amino group of lysine by HATs, which can be removed by HDACs. When HDACs remove the acetyl group from a histone lysine, a positive charge on the lysine residue condensing the structure of nucleosomes is restored. The active site of HDACs consists of a cylindrical pocket in which the lysine residue fits when deacetylation takes place. Zn^{2+} is located near the bottom of the cylindrical pocket[17].

In addition to deacetylating histones, HDACs have also been reported to interact with non-histone proteins. Such protein targets of HDACs include transcription factors and regulators, signal transduction mediators, DNA repair enzymes, nuclear import regulators, chaperone proteins, structural proteins, inflammation mediators and viral proteins. These HDAC substrates are involved in numerous important cell pathways, including control of gene expression, regulation of cell proliferation, differentiation, migration and death. Altered expression of HDACs has been reported in several types of human neoplasms[18,19].

HDAC inhibitors

HDAC inhibitors (HDACIs) have three common structural characteristics: a Zn binding moiety, an opposite capping group, and a straight chain alkyl, vinyl or aryl linker connecting the two. The majority of HDACIs are designed to interfere with the catalytic domain of HDACs, blocking substrate recognition and inducing gene expression. Aberrant expression of different HDAC isoforms has been associated with different malignancies; thus, HDACIs represent a potent and specific strategy for cancer treatment. Most of the described HDACIs only

affect the Zn-dependent classes I and Ⅱ HDACs. The HDACIs described so far vary in structure and origin, being divided into different classes based on their chemical properties. The *hydroxamic acids* include trichostatin A (TSA), SAHA (vorinostat), LBH589 (panobinostat) and PXD101 (belinostat). The *short-chain fatty acids* comprise another class, including sodium butyrate (NaBu), 4-phenylbutyrate and valproic acid. A third class includes the *cyclic tetrapeptides*, such as FK228/depsipeptide (romidepsin). A fourth class of HDACIs is the *benzamides*, including MS-275 (entinostat), CI-994 and MGCD0103[20,21].

The mechanisms of action of HDACIs are complex and not completely understood. HDACIs have multiple biological effects related to acetylation of histones and many non-histone proteins, such as those involved in regulation of gene expression, apoptosis, cell cycle progression, DNA repair, cell migration and angiogenesis. HDACIs induce cell cycle growth arrest in both normal and transformed cells, and can activate the extrinsic and intrinsic pathways of apoptosis. Both *in vitro* and *in vivo* data and ongoing clinical trials have indicated that HDACIs could be used against different solid tumors and hematological malignancies; thus, comprising one of the most promising classes of new anticancer agents[22,23]. In the present review, the latest knowledge on the effect of HDACIs on pancreatic cancer is discussed.

EXPERIMENTAL *IN VITRO* STUDIES

The data available so far regarding the different classes of HDACIs used in pancreatic cancer cell lines are presented in the following section. Additionally, the targets modulated by different HDACI compounds are listed in Table 1.

Hydroxamic acids

Suberoylanilide hydroxamic acid (SAHA, *N*-hydroxy-*N*'-phenyl-octanediamide, vorinostat) is a synthetic hydroxamic acid that is structurally related to the natural product, trichostatin A {TSA, 7-[4-(dimethylamino)phenyl]-*N*-hydroxyl-4,6-dimethyl-7-oxo-(2*E*,4*E*,6*R*)-2,4-heptadienamide}, which is produced by selected strains of *Streptomyces platensis*, *Streptomyces hygroscopicus* Y-50 or *Streptomyces sioyaensis*. Hydroxamic acids have a high affinity to biometals, including Fe^{3+}, Ni^{2+} and Zn^{2+}. The synthesis of SAHA and its potency to induce differentiation of murine erythroleukemia (MEL) cells was first reported in 1996. SAHA and TSA contain a hydroxamic acid-based metal-binding domain that coordinates the catalytic Zn^{2+} in the HDAC active site, a 5 (TSA) or 6 (SAHA)-membered carbon-based linker that mimics the Cα functional group of lysine, and a hydrophobic motif that interacts with the periphery of the HDAC binding pocket[24].

TSA: TSA strongly inhibited the cellular growth of nine pancreatic adenocarcinoma cell lines (MiaPaCa-2, PANC1, PSN1, PT45P1, CFPAC1, HPAF-Ⅱ, T3M4, PaCa44 and PC), although a marked difference in sensitivity to the drug was noted. TSA-induced cell cycle arrest was associated with a block in the G2 phase and apoptotic death. TSA treatment in T3M4 and PaCa44 cell lines resulted in p21WAF1/CIP1 induction, an increase in caspase-3 activity and the downregulation of p27 and cyclin A2 mRNA expression[25].

Global gene expression profiles were also examined in several pancreatic cancer cell lines (CFPAC1, HPAF, MiaPaCa-2, Panc1, PC, PSN1, PT-45P1 and PaCa44) post-TSA treatment. Three point four percents of genes involved in a wide variety of cellular processes, such as cell proliferation, signaling, regulation of transcription, and apoptosis, were altered after TSA treatment. The cyclin-dependent kinase (cdk) inhibitors p21, p19 and p57 were all upregulated, while cyclin A and cdk10 were downregulated. Additionally, BIM, a proapoptotic BCL-2 family member, was significantly induced, while the expression of the antiapoptotic genes *BCL-XL* and *BCL-W* was repressed by TSA treatment[26].

Different pancreatic cancer cell lines co-express high-level TNF-related apoptosis-inducing ligand receptor (TRAIL-R), Fas and TNF-R1 but are strongly resistant to apoptosis triggered by the death receptors. The drug combinations geldanamycin/PS-341, TSA/PS-341 and TSA/geldanamycin with low-dose TRAIL were tested and all were found to be effective in initiating apoptosis in four pancreatic cancer cell lines (AsPC-1, BxPC-3, MiaPaCa-2 and Panc-1) compared with single drug-based treatments. This killing effect was enhanced when Bcl-XL was depleted. When Bcl-XL-depleted cells and control counterparts were exposed to TSA/PS-341, TRAIL induced cell death in Bcl-XL knockdown cells. However, under the same experimental conditions fewer control cells were killed, indicating that Bcl-XL depletion significantly increased TSA/PS-341 killing effects on pancreatic cancer cells in the presence of TRAIL[27].

TSA and SAHA induced apoptosis in pancreatic cancer cell lines IMIM-PC-1, IMIM-PC-2 and RWP-1, independently of their intrinsic resistance to conventional antineoplastic agents. Caspase-3 activity was slightly increased in IMIM-PC-1 and RWP-1 cells, but significantly increased in IMIM-PC-2 cells after TSA treatment. On the other hand, caspase-8 and -9 activities were not altered. In addition, PARP-1 was only partially cleaved after TSA treatment. An inhibitor of the human serine protease Omi/HtrA2, called ucf-101, was able to block the cell death induced by TSA in the three cell lines through a caspase-independent mechanism. In the same experimental setting, Bax protein levels were dramatically increased, but those of Bcl-2 and p21 were not significantly modified[28].

TSA and SK-7041, a novel hybrid synthetic HDACI, both induced apoptosis and G2-M cell cycle arrest in the pancreatic cancer cell lines Panc-1 and ASPC-1. They caused increased H4 histone acetylation, and also suppressed the expression of the antiapoptotic proteins Mcl-1 and Bcl-XL, but did not affect either Bcl-2 or the proapoptotic Bax and Bak proteins. TSA and SK-7041 also enhanced the expression of p21 and of cyclin D2

Table 1 Histone deacetylase inhibitors and targets modulated in different pancreatic cancer cell lines

HDACI compound		Targets modulated		Ref.
		Upregulated	Downregulated	
Hydroxamic acids	TSA	p21, p19, p57, Bim, Bax, caspase-3, -7, cyclin D2, TGF-β, OC18, MGMT, TFAR19, maspin, CDKN1C, MUC2, MUC5B, miR-107	p27, cyclin A2 , -B1, cdk6, cdk10, Bcl-XL, Mcl-1, PARP-1, NF-κB, K-Ras, MEK1/2, phosph. MEK, ERK1/2, NPM, TCTP	[25-30,37-52]
	SAHA	p21, p27, p57, Bax, RARa, E-cadherin, C/EBPa, caspase-3, -7, HHIP, RELN, DAB1	Bcl-2, cyclin D1, -B1, c-myc, Ptc-1, survivin, EGFR, NF-κB, RelA/p65	[53-60,62,63,66]
Cyclic peptides	FK228	p21, p16, caspase-3, DR5, GATA4	Survivin	[68-70]
Short chain fatty acids	VPA	NEP/CD10, RELN, DAB1		[62,73]
	4-PB	p21, p16, miR-127, caspase-8, Bid, JNK	Bcl-6, PARP	[78-80]
	NaBu	ALP, K23, NEP/CD10, caspase-3, -7, -9, GnT-Ⅳa, 5-hydroxytryptamine, CEA, DU-PAN-2, CA19-9, IRT, Lcu7, synaptophysin, p21, p27, Bax	Bcl-XL, TGF-α, PKC, b4 and b7 integrin, EGFR, ezrin, cyclin D1, mut-p53, Bcl-2, survivin	[73,82-99]
Benzamides	MS-275	p21, p27, gelsolin, caspase-3	pRb, Bcl-2, cyclin D1	[101,102]

HDACI: Histone deacetylase inhibitor; TSA: Trichostatin A; SAHA: Suberoylanilide hydroxamic acid; VPA: Valproic acid; NF-κB: Nuclear factor kappa B; TGF: Transforming growth factor; EGFR: Epidermal growth factor receptor; CEA: Carcinoembryonic antigen.

and reduced that of cyclin B1[29].

TSA and the selective 26S proteasome inhibitor PS-341, synergistically induced apoptosis in eight pancreatic adenocarcinoma cell lines (AsPC-1, BxPC-3, CFPAC-1, Capan-2, Mia PaCa-2, Panc-1, SU86, and SW1990). Combining TSA with PS-341 induced apoptosis by increasing caspase-3 and -7 activities and enhanced PARP cleavage. Their combination also effectively blocked nuclear factor kappa B (NF-κB) signaling pathway and downregulated the NF-κB dependent anti-apoptotic factor Bcl-XL. Moreover, they inactivated the Ras-MAP kinase pathway by depleting several key components of MAP kinase cascades, including K-Ras, MEK1/2, phosphorylated MEK and ERK1/2[30].

TSA strongly inhibited proliferation of pancreatic endocrine carcinoma cell lines (CM, metastatic insulinoma; BON, metastatic carcinoid; and QGP-1, somatostatinoma) by causing cell cycle G2/M arrest and apoptosis. TSA-induced apoptosis of CM cells was shown to be a retarded event with respect to that observed in BON and QGP-1 cells. Such effect was ascribed to modifications in the expression of proteins related to cell proliferation, gene expression, signal transduction, cytoskeleton organization, chromatin organization, as also RNA splicing and protein folding[31]. Another study examined the effect of TSA or 5-Aza-C, a DNA methyltransferase inhibitor, treatment on the proliferation of the pancreatic endocrine cancer cell lines QGP-1, CM and BON. TSA treatment resulted in cell cycle arrest at G1 (QGP-1) or G2 (BON and CM) phase, whereas 5-Aza-C blocked the cell cycle in G2 phase only in BON cells. The combined treatment did not significantly increase the cytostatic effect obtained with TSA alone, suggesting that the synergistic cell growth inhibition by the two drugs may be not caused by cell cycle arrest[32].

TSA and gemcitabine synergistically inhibited the proliferation of several human pancreatic adenocarcinoma cell lines (T3M4, PANC1, PC, CFPAC1, YAPC, DANG and Panc-89). In the cell lines tested, TSA enhanced apoptosis, but not the cell cycle arrest induced by gemcitabine[33,34]. TSA significantly inhibited the viability of BxPC-3 cells in a time- and dose-dependent manner by inhibition of cell proliferation and induction of apoptosis. Cell cycle analysis showed an increase of cells in the G0/G1 phase post-TSA treatment, indicating cell cycle arrest. Additionally, TSA induced the apoptosis of BxPC-3 cells and led to alterations in the expression levels of miRNAs. Although some variation at the gene transcription level was observed among Panc-1, BxPC-3, SOJ-6 and MiaPaCa-2 cell lines, the amount of HDAC proteins produced seemed to be comparable[35]. The effects of known inhibitors of class Ⅲ HDACs, such as Nicotinamide and Sirtinol, on the growth of pancreatic cancer cells, in addition to those of TSA, were also examined. Treatment of pancreatic cells with different drugs concentrations resulted in a dose-dependent inhibition of cell growth, with TSA being the most effective compound. Sirtinol induced G1 arrest in SOJ-6; however, TSA induced G1 arrest in BxPC-3 cells. Treatment of cells with HDACIs resulted in elevated cell numbers in the sub-G1 peak region, suggesting induction of cell DNA degradation by Sirtinol and TSA. Sirtinol and TSA treatment also involved the mitochondrial pathway of apoptosis induction[36].

Transforming growth factor beta (TGF-β) plays a significant role in the growth inhibition of most normal epithelial, and some cancer, cells. TGF-β mediates its biological affects through cell surface receptors known as type Ⅰ (RⅠ) and Ⅱ (RⅡ) receptors. TGF-β resistance caused by loss of receptors expression has been linked to tumor formation and progression. The TGF-β RⅡ promoter contains two consensus Sp1 sites. The Sp gene family consists of four members, whose protein products are referred to as Sp1-Sp4. Sp1, Sp2 and Sp4 are activators of gene transcription, whereas Sp3 can be an activator or a repressor. In this aspect, TSA treatment of MiaPaCa-2 cell line induced accumulation of acetylated histones in chromatin associated with the TGF-β RⅡ gene. MiaPaCa-2 pancreatic cancer cells acquired resistance to growth inhibition by TGF-β associated with

reduced transcription of TGF-β RⅡ. Accumulation of TGF-β RⅡ with highly acetylated histones H3 and H4 was noted in TSA-treated compared to untreated MiaPaCa-2 cells[37]. Furthermore, TSA activated TGF-β RⅡ promoter activity in a panel of five pancreatic cancer cell lines (BxPC-3, PANC-1, CFPAC-1, MiaPaCa-2 and UK Pan-1), by mechanisms involving induction of Sp1 acetylation and changes in a multiprotein complex containing p300, PCAF, Sp1 and NF-Y[38,39].

Treating the pancreatic carcinoma cell line MIA PaCa-2 with TSA, increased the O(6)-Methylguanine-DNA methyltransferase (MGMT) mRNA and protein levels by 2-3-fold, caused by increased histone acetylation in the endogenous MGMT promoter region, which was also associated with CBP/p300. MGMT is a suicide enzyme that repairs pre-mutagenic, pre-carcinogenic and pre-toxic DNA damage O(6)-methylguanine. MGMT also likely protects against therapy-related tumor formation caused by highly mutagenic drugs. The MGMT expression level provides important information on cancer susceptibility and the success of therapy[40]. TSA treatment also resulted in a marked (5-fold) induction of 2.3% of genes in AsPC1, 1.9% in Hs766T, 1.1% in MiaPaCa2, and 2.5% in Panc1. A large panel of novel targets for silencing by histone deacetylation were identified, including several known tumor suppressor or cell cycle-regulatory genes, such as *ING1*, *p57KIP2*, *CHES1*, *CHFR* and *GADD45B*. One of the novel findings of this study was that TSA alone induced the expression of four of the 11 genes whose CpG islands were identified as aberrantly methylated in pancreatic cancer[41]. Among the proteins with altered expression post-TSA treatment in Paca44 and T3M4 pancreatic cancer cell lines, of particular interest are the two downregulated proteins nucleophosmin and translationally controlled tumor protein, which are involved in oncogenesis and tumor reversion, respectively. Additionally, several other proteins were found to be upregulated, including programmed cell death protein 5 (TFAR19), which is involved in the regulation of cell apoptosis, and stathmin (OC18), which promotes microtubule depolymerization during interphase and late mitosis. TSA could inhibit cell proliferation of the pancreatic adenocarcinoma cell line Paca44 by cell growth arrest at the G2 phase and apoptosis[42,43].

Maspin is a unique member of the serpin family of protease inhibitors with tumor suppressive activity in different cancer types. Interestingly, the maspin gene is located on chromosome 18q21.3 and its promoter region contains the binding sites of several transcription factors that positively regulate its expression, including Ets, AP1, HER and p53. When PANC-1 cells were exposed to 5-Aza-C, maspin mRNA expression was restored in a dose dependent manner. TSA also led to re-expression of maspin[44]. Five pancreatic cancer cell lines (AsPC1, CFPAC1, Hs766T, MiaPaCa-2, and Panc1) were screened for genes that displayed expression patterns associated with hypomethylation. This analysis identified 1485 transcripts that were likely to be variably expressed in pancreatic cancer cell lines. Among the 1485 transcripts identified, 392 were found to be elevated by 3-fold or greater in any of the pancreatic cancer cell lines after combined treatment with 5-Aza-C and TSA. This list included several genes that have been reported to be overexpressed in pancreatic cancer, such as those encoding cysteine-rich protein 1 (CRIP1), decay accelerating factor for complement (CD55), maspin/SERPINB5, S100 calcium-binding protein P (S100P), and tissue-type plasminogen activator (PLAT). Treatment of MiaPaCa-2 cells with 5-Aza-C restored the expression of maspin mRNA, whereas treatment with TSA alone did not, but combined treatment with 5-Aza-C and TSA strongly induced the maspin expression in a synergistic manner[45,46]. CDKN1C is a potent inhibitor of several G1 cyclin complexes, and is a negative regulator of cell proliferation. Treatment of the pancreatic cancer cell lines AsPC1 and BxPC3, where the CDKN1C gene was silenced, with 5-Aza-C or TSA, or their combination, resulted in restoration of CDKN1C expression, more potently with TSA and the combined treatment[47].

MUC2, *MUC5AC*, *MUC5B* and *MUC6* mucin genes encode large secreted O-glycoproteins that participate in mucus formation and play an important role as a physiological barrier against various attacks on the underlying epithelia. Among the four 11p15 mucin genes, *MUC2* and *MUC5B* were highly susceptible to DNA methylation and histone modifications, whereas *MUC5AC* was rarely influenced by epigenetic regulation and *MUC6* was not. In this context, pancreatic cell lines CAPAN-1 and PANC-1 were treated with 5-Aza-C or TSA. In PANC-1 cells TSA treatment induced MUC2 expression, while in CAPAN-1 cells 5-Aza-C treatment induced an increase of *MUC5AC* mRNA and *MUC5B* expression. Histone deacetylation was also involved in *MUC5B* repression, as TSA treatment induced its expression[48]. Treatment of the human pancreatic cancer cell lines PANC1 (MUC2-negative) and BxPC3 (MUC2-positive) with both 5-Aza-C and TSA, resulted in a definite increase of the expression level of *MUC2* mRNA[49]. In pancreatic cancer, *MUC4* overexpression is associated with a bad prognosis and has become an important molecular target. In this aspect, the pancreatic cancer cell lines PANC-1, CAPAN-1 and CAPAN-2 were tested. In *MUC4*-nonexpressing pancreatic PANC-1 cells, treatment with 5-Aza-C and TSA induced *MUC4* expression at the mRNA level, and at protein level in a small number of cells. In *MUC4*-high expressing pancreatic CAPAN-1, 5-Aza-C treatment did not affect MUC4 mRNA, whereas TSA treatment induced a strong inhibition of *MUC4* mRNA levels, correlated to a strong decrease of the apomucin level in the cells[50].

Two pancreatic cancer cell lines, MiaPACA-2 and PANC-1, were treated with 5-Aza-C or TSA, and their combination. Fourteen miRNAs were upregulated by ⩾ 2-fold in each of the cell lines following exposure to both agents. Enforced expression of miR-107 in MiaPACA-2 and PANC-1 cells downregulated *in vitro* growth that was associated with repression of the putative miR-107 target,

cdk6, thereby providing a functional basis for the epigenetic inactivation of this miRNA in pancreatic cancer[51].

Evaluating the *in vitro* growth inhibition of several pancreatic cancer cell lines established from primary tumors, as well as that of others established from metastatic tumors, to gemcitabine and 5-FU, newer generation cytotoxic agents (oxaliplatin, irinotecan), targeted therapy (gefitinib) and TSA, demonstrated that the combination of TSA and irinotecan increased growth inhibition on the highest percentage of cell lines (80%). TSA proved to be the best partner for all drugs, with the exception of 5-FU. Notably, PSN1, the most sensitive cell line to single-drug treatments, became the most resistant cell line to all combined treatments. In addition, all pairwise combinations were less effective in PaCa3, which contains a functional p53 gene, supporting the hypothesis that the p53 gene status may by not relevant for cell sensitization by TSA[52].

SAHA: Both SAHA and its novel compounds 17a and 9, inhibited PANC-1 and PT-45 cells' proliferation in a dose dependent manner, with the novel compounds having a more potent antiproliferative activity. Although p21 gene expression of PANC-1 cells was significantly increased after treatment with SAHA and 17a, none of the HDACIs tested affected the expression of the p27 gene[53].

In another study, SAHA inhibited the growth of BxPC-3 and COLO-357 cell lines, by 42% and 50%, respectively, but not that of PANC-1 cells. SAHA induced a G1 cell cycle arrest and upregulation of p21 in BxPC-3 and COLO-357. On the other hand, PANC-1 cells remained unaffected. According to this study, p21 upregulation was necessary for SAHA-induced cell cycle arrest in COLO-357, but not in BxPC-3 cells. PANC-1 cells, which were resistant to gemcitabine alone, exhibited a marked increase in sensitivity when treated with both gemcitabine and SAHA[54].

Additive and time-dependent reduction of cell proliferation and induction of apoptosis by SAHA and the novel DNA methyltransferase inhibitor Zebularine was reported in pancreatic cancer cell lines YAP C, DAN G and Panc-89. In fact, the apoptosis induction was associated with downregulation of Bcl-2 and upregulation of Bax[55]. SAHA inhibited the cell growth in six pancreatic cancer cell lines in a dose-dependent manner. G2/M cell cycle arrest was also induced by SAHA in most cell lines. Remarkably, SAHA and 5-Aza-C treated cells, presented higher levels of acetylated-H3 than those noted in cells treated with SAHA alone. Treatment with SAHA markedly enhanced histone H3 acetylation in the promoter region of the p21 gene. In PANC-1 cells, p21 protein expression increased to the same levels after exposure to either 5-Aza-C, SAHA or both, while p27, Bax and Bcl-2 levels remained unaltered. Levels of p57, E-cadherin and RARa increased in the presence of SAHA, either alone or with 5-Aza-C. Additionally, SAHA decreased expression of cyclin D1, B1 and c-myc independently of the β-catenin pathway and increased C/EBPa[56].

Treatment of PANC1, MiaPaca2 and ASPC-1 cells with concentrations of SAHA and sorafenib that are sustainable in patient serum resulted in a greater than additive increase in tumor cell killing, as assessed by short-term death assays, and a synergistic increase in killing assessed by colony formation assays. Suppression of caspase 8 function, as well as expression of dominant negative caspase 9 or Bcl-XL also blunted sorafenib-SAHA lethality. Sorafenib-SAHA, but not treatment with the individual drugs, activated CD95 and caused formation of a death-inducing signal complex containing caspase 8, FADD, ATG5 and Grp78/BiP. Additionally, a clinically relevant and sustainable concentration of sodium valproate, another HDACI, enhanced sorafenib lethality in a synergistic fashion in pancreatic tumor cells derived from either humans or rodents. Finally, it was speculated that small-molecule antagonists of Bcl-2 family proteins (HA14-1, GX15-070) enhanced sorafenib-HDACI lethality *via* autophagy and partial activation of the intrinsic apoptosis pathway, independently of death receptor functionality[57-59].

A combination of SAHA and the Smoothened antagonist, SANT-1, was evaluated for their ability to suppress growth of the gemcitabine-resistant pancreatic adenocarcinoma cell lines Panc-1 and BxPC-3. The combination of SAHA and SANT-1 supra-additively suppressed cellular proliferation and colony formation *via* induction of apoptotic cell death, cell cycle arrest in G0/G1 phase and ductal epithelial differentiation. Cell death was associated with nuclear localization of survivin, increased Bax expression and activation of caspases-3 and -7. Consistent with the cell cycle arrest and cytodifferentiation, the CdkIs p21 and p27 were upregulated and cyclin D1 was downregulated. The potentiated anti-proliferative effect by the combination of SAHA and SANT-1 was attributed to cooperative suppression of the Hedgehog pathway activity, as shown by the upregulation of hedgehog interacting protein by SAHA, and enhanced repression of Ptc-1 mRNA expression[60].

SAHA induced apoptosis in IMIM-PC-1, IMIM-PC-2 and RWP-1 cell lines with a serine protease-dependent and caspase-independent mechanism. SAHA induced a decrease in the number of cells in S phase in all three cell lines, while an increase in the sub-G1 peak was noted, suggesting that the three cell lines underwent apoptosis[28].

SAHA and the proteasome inhibitor bortezomib (PS-341) were tested in a panel of pancreatic cancer cell lines. Both SAHA and TSA blocked bortezomib-induced aggresome formation in pancreatic cells, which is a cytoprotective mechanism, and dramatically sensitized aggresome-positive cells to bortezomib-induced apoptosis[61].

RELN, a key regulator of neuronal migration, is frequently silenced in pancreatic cancers. RELN is a secreted extracellular protein that plays an essential role in brain development and function. Underexpression of RELN and its components (ApoER2, VLDLR and DAB1) is related to cell motility, invasiveness, and colony-forming ability in cancer. Treatment of pancreatic adenocarcinoma cell lines Panc1 and AsPC1 with SAHA and valproic acid, restored

the expression of RELN and DAB1 in a dose-dependent manner, and also inhibited cell migration[62]. SAHA inhibited proliferation of MiaPaCa-2 and AsPC-1 PDAC cells in a dose-dependent manner and further enhanced the radiation-induced apoptosis (additive but not synergistic). Radiosensitization of these cells was ascribed to inhibition of DNA repair and suppression of radiation-induced EGFR and NF-κB prosurvival signaling pathways[63]. In another study, using 3-D agarose colonies of MiaPaCA cells, synergy occurred when SAHA was combined with carboplatin at short exposure times, providing evidence that SAHA may allow a reduction in the standard dose of carboplatin, with improvement in the overall therapeutic index[64]. Thirty genes were identified as constitutively silenced in PANC-1 cells, and were upregulated post-SAHA and/or 5-Aza-C treatment. Interestingly, among them, 10 genes were known cancer antigens, suggesting that many of these antigens may be silenced by acetylation and/or methylation in PANC-1 cells[65]. The Rel/NF-κB family consists of various members of transcription factors, such as RelA/p65, which are responsible for the regulation of cytokines, their receptors and cell adhesion molecules. Overexpression or dysregulation of certain regulatory proteins of the NF-κB pathway, have been associated with poor prognosis in different cancer types. Treatment of the pancreatic cancer cell line PANC-1 with SAHA resulted in a time dependent reduction of RelA/p65 activity of up to 50%, while valproic acid (VPA) treatment decreased RelA/p65 activity by approximately 25%, affecting also its subcellular localization. Neither SAHA nor VPA affected the protein levels of IκBα, but inhibited its phosphorylation. According to this study, strong antineoplastic effects of SAHA could be partly based on an alteration of the NF-κB signaling pathway[66].

Cyclic peptides

FK228 (FR901228, depsipeptide, romidepsin): Depsipeptide (1S,4S,7Z,10S,16E,21R)-7-ethylidene -4,21-bis (1-methyletheyl)-2-oxa-12,13-dithia-5,8,20,23-tetraazabicyclo [8.7.6] tricos-16-ene-3,6,9,19,22-pentone, is a bicyclic peptide isolated from *Chromobacterium Violaceum* and has demonstrated potent *in vitro* cytotoxic activity against human tumor cell lines and *in vivo* efficacy against human tumor xenografts. Upon entering cells, FK228 is reduced to an active compound, capable of preferentially interacting with the zinc in the active site of the HDAC class I enzymes; however, it is still generally classified as a broad-spectrum inhibitor as it also inhibits class Ⅱ enzymes. It was approved by the United States Food and Drug Administration for the treatment of cutaneous T-cell lymphoma[67].

FK228 markedly inhibited the proliferation of five pancreatic cancer cell lines, with the greatest effect on MIAPaCa-2 cells. FK228 treatment induced cell cycle arrest at the G1 or G2/M phase and subsequent apoptosis. The induced hyperacetylation of histone H3 was accompanied by p21 overexpression and caspase-3 activation, leading to cleavage of p21, and by dramatic downregulation of survivin[68]. Treatment with FK228, bortezomib or both, sensitized two out of three pancreatic cancer cell lines tested, to NK cells. Tumors sensitized to NK cell cytotoxicity showed a significant increase in surface expression of DR5, which induces the TRAIL pathway of apoptosis. Expressions of MHC class I , MIC-A/B, DR4 and Fas did not alter in different cell lines[69]. Interestingly, in another study, depsipeptide could induce demethylation of both the p16 and GATA4 promoters in PANC1 cells, among other cancer cell lines, assayed with bisulfite sequencing (region D of the p16 promoter and region B of the GATA4 promoter). Additionally, depsipeptide could induce a significant inhibition of cell proliferation in PANC1 cells. In this study, a novel mechanism of HDACI-mediated DNA demethylation *via* suppression of histone methyltransferases was suggested[70].

Short chain fatty acids

VPA: VPA is now an established antiepileptic drug through its effect on the function of the neurotransmitter GABA. The finding that VPA was an effective inhibitor of HDACs came from the observations that VPA was able to relieve transcriptional repression of a peroxisomal proliferation and activation of a glucocorticoid receptor (GR)-peroxisome-proliferation-associated receptor (PPAR)ε hybrid receptor and a RAR-dependent reporter gene expression system, suggesting that it acts on a common factor in gene regulation, such as corepressor-associated HDACs, rather than on individual transcription factors or receptors. Consistent with this finding, it was shown that VPA causes hyperacetylation of the N-terminal tails of histones H3 and H4 *in vitro* and *in vivo* and was found to inhibit HDAC enzymatic activity at a concentration of 0.5 mmol/L[71]. VPA has shown potent antitumor effects in a variety of *in vitro* and *in vivo* systems, by modulating multiple pathways, including cell cycle arrest, apoptosis, angiogenesis, metastasis, differentiation and senescence. Most of preclinical and clinical data on the anticancer effects of VPA has been generated for malignant hematological diseases[72].

Neutral endopeptidase (NEP/CD10) is a cell surface Zn metalloprotease that inactivates multiple physiologically active peptides. Loss of, or decrease in, NEP/CD10 expression has been reported in many types of malignancy. VPA treatment resulted in increase of NEP/CD10 protein expression accompanied with a significantly reduced growth in PATU-8988T and HUP-T4 cells. The VPA effect on the proliferation in HUP-T4, as well as in HUP-T3, cells was much lower than in the high NEP/CD10-expressing PATU-8988T cells[73]. VPA treatment of DanG cells resulted in a significant reduction of cell proliferation, an effect that was depended on the drug exposure time. VPA evoked a significant blockade of both DanG tumor cell adhesion to HUVECs, particularly when the compound was applied for 5 days. Additionally, VPA treatment modified the integrin surface profile on pancreatic cancer DanG cells[74]. MiaPaCa2 and Panc1 cell lines were also treated with the topoisomerase Ⅱ inhibitor etoposide and VPA. VPA-mediated depletion of HDAC2

was observed in MiaPaCa2 and Panc1 cells. The apoptotic fraction of VPA/etoposide co-treated MiaPaCa2 and Panc1 cells was significantly increased compared to etoposide-treated pancreatic cancer cells. Sensitization by VPA to etoposide (DNA-damage induced apoptosis) was noted in both cell lines. This effect was not observed when VPA was used in combination with other drugs, such as gemcitabine, 5-FU or oxaliplatin. Such data suggested that HDAC2 inhibition might result in specific sensitization towards DNA damage-induced apoptosis[75]. Treatment of Panc1 cells with VPA and SAHA, at different concentrations, induced the mRNA expression of RELN and DAB1 in a dose-dependent manner. VPA was also shown to prevent the epigenetic downregulation of RELN, leading to the inhibition of migration of Panc-1 cells[62].

4-phenylbutyrate: 4-phenylbutyrate (4-PB) is a short-chain fatty acid that reversibly inhibits class I and Ⅱ HDACs. It is considered as an HDAC inhibitor of the first generation, as the HDAC inhibitory effect is not specific. Working concentrations are rather high, in the millimolar range, and the effects are pleiotropic. 4-PB exerts multiple effects in the cell, including the modulation of protein isoprenylation, which regulates the ras proto-oncoprotein, and activation of the nuclear steroid PPAR[76]. 4-PB exerts a potent antitumor effect *in vitro* and causes growth inhibition and differentiation in various human cancer cell lines[77].

A set of normal fibroblast and cancer cell lines, among them the pancreatic cancer CFPAC-1, were treated with 5-Aza-C and 4-PB. Upregulation of the cell cycle inhibitors p16 and p21 was induced post-5-Aza-C and 4-PB treatment. Interestingly, both normal and cancer cells presented very similar induction levels after combination treatment[78]. Expression profiling of different cancer cell lines revealed upregulation of several miRNAs by simultaneous treatment with 5-Aza-C and 4-PB. One of these, miR-127, is embedded in a CpG island and is highly induced from its own promoter after treatment. miR-127 is usually expressed as part of an miRNA cluster in normal but not in cancer cells, suggesting that it is subject to epigenetic silencing. Additionally, the proto-oncogene *BCL6,* a potential target of miR-127, was translationally downregulated after treatment of several lines with the combination of drugs[79]. 4-PB inhibited HDAC activity by 60%-70% in the cancer cell lines T3M-4 and BxPc3. Treatment with 4-PB inhibited growth and induced apoptosis of Panc1, T3M-4, COLO 357 and BxPc3 pancreatic adenocarcinoma cell lines in a dose- and time-dependent manner. Concentration-dependent cell cycle arrest was noted in T3M-4 and COLO 357 cells, which was not verified in Panc 1 and BxPc3 cells. In addition, 4-PB increased gap junction communication between T3M-4 cells, allowing exchange of apoptotic signals between neighboring cells. Furthermore, 4-PB inhibited cellular export mechanisms. 4-PB increased gemcitabine-mediated apoptosis of two resistant cell lines T3M-4 and BxPc3. Activation of caspase-8 was enhanced, as well as that of Bid and PARP-cleavage. No differences in the expression levels of caspase -2 and -3 and IAPs were noted. Finally, no influence of MEK or p38 on gemcitabine-mediated cell death in these cells was found. In contrast, inhibition of JNK completely abolished the sensitizing effect of 4-PB[80].

Sodium butyrate (NaBu): Sodium butyrate has multiple effects on cultured mammalian cells, including inhibition of proliferation, induction of differentiation and induction or repression of gene expression. NaBu inhibits most HDACs, except class Ⅲ HDAC and class Ⅱ HDAC6 and -10. Promoters of butyrate-responsive genes have butyrate response elements, and the action of butyrate is often mediated through Sp1/Sp3 binding sites[81].

NaBu induced a dramatic decrease in cell proliferation and an increase in ALP activity in PANC-1 cells. NaBu also induced a number of morphologic alterations in these cells, including increase in the cytoplasmic secretory elements and enhancement of differentiation[82]. Both NaBu and the natural butyrate prodrug tributyrin, inhibited growth and induced apoptosis in MiaPaca-2 and Capan-l cells and stimulated differentiation in Capan-l cells, as indicated by alterations of ALP levels[83]. NaBu also induced differentiation and apoptosis in the human pancreatic cancer cell line AsPC-1, as well as increased K23 mRNA levels[84]. NaBu treatment resulted in a significant reduced cell growth of PATU-8988T cells and an increase of NEP/CD10 protein levels[73]. Additionally, NaBu treatment induced cell growth inhibition and apoptosis in four lines (ASPC-1, PANC-1, PT45 and PACA44) with different susceptibility. Bcl-xL expression was strongly downregulated by NaBu in a time-dependent manner, whereas Bax expression was not affected. NaBu enhanced the intrinsic pathway of apoptosis, including mitochondrial membrane depolarization, cytochrome c translocation to the cytosol, caspase-3 and -9 activation, although it had no effect on caspase-8. NaBu also enhanced the extrinsic pathway of apoptosis, sensitizing pancreatic cancer cell lines to Fas-mediated signals[85]. Moreover, NaBu inhibited the ability of several pancreatic cell lines to form colonies in soft agar. Cellular ALP levels were markedly increased post-treatment[86,87]. Treatment of pancreatic cancer cell line CAPAN-1 with 1 mmol/L NaBu reduced the rate of cellular growth and inhibited colony forming ability in soft agar, but did not suppress cell growth. These effects were completely reversible on removal of NaBu and were therefore not causing a terminal differentiation step or a loss of cell viability. Significant changes in proteins and glycoproteins of CAPAN-1 occurred with NaBu treatment[88]. Treatment with NaBu strongly inhibited growth of pancreatic carcinoid BON cell line. It was found that NaBu increased levels of 5-hydroxytryptamine in the cells, as a differential effect[89]. TGF-α but not TGF-β mRNA levels were decreased after NaBu treatment in CAPAN-1 cells, while the membrane-bound protein kinase C activity was also reduced[90].

Oligosaccharide antigens are commonly used as tumor markers. Such antigens control tumor cell adhesion,

motility and invasiveness, being synthesized by a series of glycosyltransferases. In MiaPaCa-2, PSN-1 and PK59 cell lines, the expression of GnT-IVa (N-acetylglucosaminyltransferase-IVa) was increased after NaBu treatment[91]. Carcinoembryonic antigen (CEA) expression of human pancreatic adenocarcinoma cells and differentiation features were studied and compared in the well differentiated and CEA-producing CAPAN-1 and the poorly differentiated PANC-1 cell line post-NaBu treatment. NaBu reduced colony formation in both cell lines by approximately 50%. Significant ultrastructural alterations were noted only in the PANC-1 cells, including increased intercellular desmosomes, tonofilaments and lipid droplets. NaBu increased CEA expression in CAPAN-1 cells, but had no effect on CEA expression in PANC-1 cells. Thus, CEA expression and state of differentiation were independently affected[92]. NaBu inhibited the growth of pancreatic cancer cell lines PC-1 and PC-1.0 (hamster) and HPAF, CD11, CD18 and PANC-1 (human), and induced cell enlargement, an increase in secretory material, microfilaments and pseudopodia. NaBu increased the expression of blood group A, DU-PAN-2 and CA 19-9 tumor associated antigens[93]. Treatment with NaBu, slightly increased immunoreactive trypsin 1 (IRT) levels in both human pancreatic adenocarcinoma cell lines CF-PAC-1 (established from a patient with cystic fibrosis) and CAPAN-1, while growth inhibition was significant. Consequently, IRT levels or differentiation state did not correlate with cellular growth[94].

NaBu treatment inhibited the cell growth of four pancreatic cancer lines (PT45, PaTu-Ⅱ, Panc-1 and A818-1) more potently than all-trans retinoic acid (ATRA). Additionally, neuroendocrine markers synaptophysin and Lcu7 in Panc-1 cells were highly expressed[95]. The expression of b4 and b7 integrin chains correlates with tumor invasiveness. In this aspect, it was documented that NaBu inhibited b4 integrin expression in AsPC-1 cells, inhibiting pancreatic tumor invasion. NaBu also reduced the expression of the b7 integrin chain, which was expressed only in the more aggressive pancreatic cancer cell lines[96]. The cellular morphological characteristics of the PANC-1 cell line treated with NaBu appeared more differentiated, in a dose-dependent manner. The EGFR expression of NaBu-treated PANC-1 cells was decreased in a dose-dependent manner. Ezrin is a cytosolic molecule that cross-links the plasma membrane to actin filaments and has functions related to cell motility, signal transduction, cell-cell and cell-matrix recognition, invasion and metastasis. Both membranous ezrin expression of PANC-1 cells and mRNA expression of ezrin were decreased[97].

A novel bioconjugate (HA-But) obtained by the esterification of butyric acid (BA) with hyaluronic acid (HA), the main constituent of the ECM, which selectively recognizes transmembrane receptor CD44, was developed. All HA-But treated cell lines, including the pancreatic cancer cell line MiaPaCa, were responsive to the antiproliferative effect of HA-But in a dose-dependent manner with cell growth inhibition higher than that observed in the presence of BA alone. Like BA, HA-But induced hyperacetylation of histone H4, a dose-dependent overexpression of some G1/S transition-related proteins, including the CdkIs p27 and p21, and the block of cell growth in the G0/G1 phase of the cell cycle[98]. In another study, HA-But induced a dose-dependent inhibitory effect with an almost complete suppression of MIA PaCa-2 cell growth at the highest concentration used. A decrease in the number of cells in S phase and a concomitant increase of those in G0/G1 or G2/M phase were noted. HA-But decreased cyclin D1 and mut-p53 and increased p27 and p21 protein levels. Additionally, HA-But slightly increased the level of Bax and caspase-7, slightly decreased Bcl-2, but strongly decreased survivin protein levels, providing to be active on both Bcl-2 and survivin-mediated apoptosis pathways[99].

Benzamides

MS-275: This synthetic benzamide derivative 3-pyridylmethyl-N-{4-[(2-aminophenyl)carbamoyl]benzyl}carbamate inhibits HDACs, and has anti-tumor activity in many preclinical models. The first clinical trial with this agent in 2005 included patients with advanced solid tumors or lymphoma. At high concentrations of MS-275, there is a marked induction of reactive oxygen species, mitochondrial damage, caspase activation and apoptosis. Treatment of sensitive tumor cell lines with MS-275 induces gelsolin, a maturation marker, and produces a change in the cell cycle distribution with a decrease in S phase and an accumulation of cells G_1. The *in vivo* therapeutic efficacy of MS-275 has been demonstrated in a variety of human tumor xenograft models[100].

The addition of MS-27-275 to cell cultures resulted in the accumulation of hyperacetylated H4 molecules. MS-27-275 transcriptionally induced p21 and gelsolin (tumor suppressor) through acetylation of histones affecting cell cycle progression. In addition, pRb molecules were reduced. The response of Capan-1 cell line, presenting a p53 mutation, to MS-27-275 treatment was moderate, although expression of gelsolin was induced. The gelsolin induction by MS-27-275 seemed to have no correlation with the sensitivity of the cells to the treatment. Additionally, p21 induction was considered crucial for the action of MS-27-275[101].

TSA, NaBu and MS-275 inhibited the growth of the NET cell lines CM and BON in a dose-dependent manner. In both cell lines, HDAC inhibition resulted in a dose-dependent increase of caspase-3 enzyme activity without affecting cell membrane integrity or exerting immediate necrotic effects. Treatment with HDACIs resulted in cell cycle arrest of the NET cells at G1, thereby decreasing those in the S phase. MS-275 treatment of CM and BON cells resulted in a dose-dependent decrease of Bcl-2, whereas Bax remained unaffected in both cell lines. Cyclin D1 was also downregulated in CM and BON cells by MS-275 treatment, while p21 and p27 were markedly increased[102].

MGCD0103: MGCD0103 is an isotype-specific aminophenylbenzamide that inhibits HDAC classes I and Ⅳ,

Table 2 Studies of histone deacetylase inhibitors and different pancreatic cancer cell lines in xenograft models

HDAC inhibitor	Pancreatic cancer cell line	Time schedule	Dosage schedule	Results	Ref.
TSA (+ gemcitabine)	T3M4	q28 d	0.25 mg/kg *ip*, biweekly	50% tumor weight reduction 3-fold H4 increase	[33]
SAHA (+ bortezomib)	L3.6pl	q21 d	50 mg/kg *ip*, daily	About 70% tumor weight reduction aggresome disruption	[61]
SAHA (+ Zebularine)	Panc-89, YAP C	(1) q7 d or q14 d (2) q7 d	(1) 50 mg/kg *ip*, daily (2) 50 mg/kg *ip*, daily	Tumor growth inhibition Upregulation of CK7, CK20 Downregulation of CK8, Vimentin, chromogranin-A	[55]
FK228	CAPAN-1	q14 d-q21 d	1.5 mg/kg *ip*, biweekly	50% tumor growth inhibition	[108]
MS-275	CAPAN-1	q28 d	(1) 12.3 mg/kg per os (2) 24.5 mg/kg per os (3) 49 mg/kg per os, 5 × weekly	Moderate growth inhibitory effect	[101]
	CAPAN-1, MiaPaca, Panc-1, Panc-15	Not defined	per os once daily (dosage not defined)	Mixed response: moderate growth inhibitory effect/ resistant to inhibition	[109]
NVP-LBH589 (+ gemcitabine)	HPAF-2, L3.6pl	q28 d	25 mg/kg *ip*, 5 × weekly	63% tumor weight reduction (HPAF-2) About 80% tumor weight reduction (L3.6pl) MIB-1 slight reduction TUNEL slight induction (HPAF-2)	[113]

HDAC: Histone deacetylase; TSA: Trichostatin A; HPAF: Suberoylanilide hydroxamic acid; TUNEL: Transferase dUTP nick end labeling.

with almost no class II effect. MGCD0103 is well-tolerated and exhibits favorable pharmacokinetic and pharmacodynamic profiles, demonstrating target inhibition and clinical responses. It induces cell death and autophagy, synergizes with proteasomal inhibitors and affects non-histone targets, such as microtubules[103].

A comparative study in order to estimate the pharmacological properties of second generation HDACIs with the hydroxamate and benzamide head group, namely SAHA, LAQ824/LBH589, CI-994, MS-275 and MGCD0103 was carried out. SAHA and LAQ824/LBH589 seemed to behave as quite unselective HDACIs, while the benzamides CI-994, MS275 and MGCD0103 were more selective HDAC1 and HDAC3 inhibitors. All the compounds induced histone H3 hyperacetylation, as well as cell differentiation and apoptosis and inhibited proliferation. A broad cytotoxicity was seen across different tumor cell lines, among them the pancreatic lines AsPC-1, BxPc3 and Panc-1, with LAQ824/LBH589 being the most potent agents[104,105]. The inhibitory activities of MGCD0103, MS-275 and SAHA were also compared using a panel of cancer cell lines, among them Panc-1. Although the measured IC50 values varied between cell lines, MGCD0103 was always more potent than the comparator molecules in all cases examined, being at least 7-fold more potent than SAHA in PANC-1 pancreatic cancer cells[106].

IN VIVO EXPERIMENTAL STUDIES

The data available so far regarding the different classes of HDACIs used in *in vivo* animal studies of pancreatic cancer are presented in the following section and are listed in Table 2.

Hydroxamic acids

TSA: *In vivo* studies on xenografts of pancreatic cancer line T3M4 in nude mice, showed that the combination of TSA (0.25 mg/kg) and gemcitabine (2.5 mg/kg), given biweekly for 4 wk, led to a reduction in the mean tumor weight by about 50% compared to control or single drug treatments. Additionally, TSA treatment resulted in only a 3-fold increase of H4 acetylation levels *in vivo* compared to the 18-fold increase noted *in vitro*. None of the treatments produced any toxicity, as indicated by lack of change in body weight, and none of the animals developed ulcerating tumors[33]. Finally, TSA exerted an inhibitory effect on the DMBA-induced carcinogenesis model and the growth of pancreatic ductal adenocarcinoma in rats, by upregulating *KiSS-1*, a metastasis suppressor gene located on 1q32, which is considered to exert an important role in inhibiting the invasion and metastasis of pancreatic cancer[107].

SAHA: Effects of bortezomib and SAHA in orthotopic human pancreatic tumors (cell line L3.6pl) were investigated. Tumors were treated biweekly with 1 mg/kg bortezomib, daily with 50 mg/kg SAHA, or a combination of the two agents for 21 d. *In vivo* data showed aggresome disruption by the combination and reduction of pancreatic tumor weight, with minimal toxicity noted, what there was being related to bortezomib[61]. Another experiment in mice presented tumor suppression when treated with SAHA and the DNA methyltransferase inhibitor Zebularine. The first experimental setting included a single intraperitoneal (*ip*) bolus injection with SAHA (50 mg/kg), Zebularine (1 g/kg) or the combination of both agents, and the animals were sacrificed after 7 or 14 d. For daily treatment, animals received the same doses of drugs over a time-course of 1 wk. Gemcitabine as a control therapy was administered *ip* every fourth day. The hypermethylated cell line Panc-89 was more susceptible to SAHA than YAP C. No adverse effects were noted and all animals

survived. Furthermore, the expression of CK7, which is a marker associated with pancreatic cancer cell differentiation, was higher in Zebularine and combined Zebularine/SAHA-treated xenografts, as well as that of the glandular differentiation marker CK20. The expression of CK8, Vimentin and chromogranin-A was lower or constant in Zebularine and Zebularine/SAHA-treated animals[55].

Cyclic peptides

FK228 (FR901228, depsipeptide): More than 90% of human pancreatic cancers are associated with oncogenic mutations of RAS, in particular K-RAS at codon 12. The Tyr-kinase inhibitors, PP1 and AG, block the RAS mutation-induced activation of PAK1, which is the Rac/CDC42-dependent Ser/Thr kinase. PAK1 is essential for RAS-transformation. Based on these data, the therapeutic potential of either FK228, the combination of these two Tyr-kinase inhibitors or GL-2003, a water-soluble derivative of AG 879, on human pancreatic cancer (Capan-1) xenograft in mice, was examined. Capan-1 cells were injected sub-cutaneously (*sc*) into several groups of nude mice. Each group was treated *ip* with either FK228 (1.5 mg/kg), GL-2003 (20 mg/kg), a combination of PP1 and AG 879 (20 mg/kg of each drug), a combination of PP1 and GL-2003 (20 mg/kg of each drug), or vehicle alone (0.1 mL of 1% DMSO in PBS) as the control, twice a week for 2-3 wk. No adverse effects were detected. The most effective combination was that of GL-2003/PP1 that suppressed cell growth by around 80%, while FK228 alone showed only 50% inhibition. The synergy between GL-2003 and PP1 in blocking the RAS-induced PAK1 activation was not observed *in vitro*[108].

Benzamides

MS-275: MS-275 administered orally, once daily, 5 d per week for 4 wk, strongly inhibited the growth in 7 out of 8 tumor cell lines implanted into nude mice, although most of these did not respond to 5-FU. Tumors were passaged several times before starting *in vivo* antitumor testing, a tumor lump (2-3 mm in diameter) was transplanted *sc* into the flank of a nude mouse, and the therapeutic efficacy of MS-27-275 was examined. MS-27-275 at 49 mg/kg showed a moderate effect against the only pancreatic Capan-1 tumor. The drug at 24.5 mg/kg and 12.3 mg/kg also showed significant effects against these tumors. As the dose of 49 mg/kg was the maximum tolerated one in this administration schedule, and apparent signs of toxicity, such as weight loss and poor appearance, were reported. The maximum dose of the drug was lowered to 24.5 mg/kg, at which no gross weight loss was observed[101]. Additionally, the pancreatic carcinoma cell lines Capan1, MiaPaCa, Panc1 and Panc15 were grown as xenografts in nude mice and afterwards, treated per os once daily with MS-275. For most cell lines used, antitumor activity and dose-dependent response of MS-275 *in vivo* were observed. While various tumor cell lines from other malignancies showed an almost complete response, in pancreatic cell lines a mixed response was achieved, with half of the models tested to present either a moderate growth inhibitory effect or resistance to treatment[109].

CI-994: CI-994 or N-acetyldinaline[4-(acetylamino)-N-(2-amino-phenyl) benzamide] is a novel oral compound with a wide spectrum of antitumor activity in preclinical models. The mechanism of action may involve inhibition of histone deacetylation and cell cycle arrest. CI-994 is currently undergoing clinical trials. Although several changes in cellular metabolism induced by the drug have been characterized, the primary molecular mechanism of its antitumor activity remains unknown[110].

CI-994 was previously identified as having cytotoxic and cytostatic activity against several murine and human xenograft tumor models. CI-994 had activity against 8/8 solid tumors tested among them pancreatic adenocarcinoma 02 and 03[111]. Notably, CI-994 was active against a Pan-02 pancreatic tumor of C57BL/6 mouse origin[112].

Other HDACIs

Pancreatic tumors were induced in nude mice by *sc* injection of HPAF-2 and L3.6pl cells. Animal groups received either NVP-LBH589 (25 mg/kg, 5 × weekly) or gemcitabine (5 mg/kg, 1 × weekly) or a combination of both (NVP-LBH589 at 25 mg/kg, 5 × weekly plus gemcitabine at 5 mg/kg, 1 × weekly) *ip*, whereas the control group received placebo only, for 28 consecutive days. Three days after commencement of NVP-LBH589 or combination treatment, HPAF-2 cell tumors showed a significantly reduced volume compared with the control. Combination therapy was significantly more efficient than gemcitabine treatment alone and significantly more efficient than NVP-LBH589 therapy alone, in both cell lines. Regarding side effects, weight loss was 6% and 25% for the combination treatment in HPAF-2 and L3.6pl cell tumor bearing mice, respectively. Treatment with NVP-LBH589 and the combination slightly reduced proliferation (Ki-67 index) and slightly induced apoptosis markers in HPAF-2 cell bearing mice, whereas proliferation was not decreased and apoptosis only slightly increased in L3.6pl cell bearing mice[113].

CONCLUSION

In this review, the recent advances in the understanding and clinical development of HDACIs were discussed. The exact mechanism and the molecular basis for the antitumor effects of these new drugs are complex and not completely understood. HDACIs regulate the acetylation of histones and many non-histone proteins that are involved in gene expression, cell proliferation, migration and death. HDACIs have been shown to induce differentiation and cell cycle arrest, activate the extrinsic or intrinsic pathways of apoptosis and to inhibit invasion, migration and angiogenesis in different cancer cell lines. Normal cells are relatively more resistant to HDACIs-induced cell death. Although not completely elucidated, the main mechanisms by which HDACIs act in pancre-

atic cancer are common. The cdk inhibitor p21 is one of the most commonly induced genes in various pancreatic cancer cell lines by all HDACIs tested. Transcriptional induction of p21 is associated with G1 cell cycle arrest and growth inhibition. Cell cycle arrest and growth inhibition is also correlated with transcriptional activation of other cell cycle regulatory genes such as p16, p27, cyclin E and gelsolin, while inhibition of cyclins A, B1, D1 and D2 were also noted in many cancer cell lines. The pro-apoptotic proteins Bax, Bad and Bim were upregulated, among others, whereas anti-apoptotic proteins, such as Bcl-2, Bcl-XL and survivin, were downregulated. HDACIs reduced the expression of angiogenetic factors, such as VEGF receptors -1 and -2, and affected the expression of a panel of metastasis promoting genes (Table 1).

Additionally, these drugs exhibited antiproliferative effects in cancer animal models. Various pancreatic carcinoma cell lines were grown as xenografts in nude mice and treated with HDACIs (Table 2). For most lines used, anti-tumor activity and dose-dependent response were observed, with reduction of cell proliferation (Ki-67 index) and induction of apoptosis (transferase dUTP nick end labeling test). The reduction of pancreatic tumor weight was achieved with minimal toxicity.

These results support the efficiency that HDACIs presented *in vitro;* however, more studies and well-controlled experiments are required to obtain stronger *in vivo* data. Furthermore, phase Ⅱ/Ⅲ trials including patients with pancreatic cancer are needed to determine the clinical efficacy of these new drugs.

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REVIEW

Diagnosis and management of insulinoma

Takehiro Okabayashi, Yasuo Shima, Tatsuaki Sumiyoshi, Akihito Kozuki, Satoshi Ito, Yasuhiro Ogawa, Michiya Kobayashi, Kazuhiro Hanazaki

Takehiro Okabayashi, Yasuo Shima, Tatsuaki Sumiyoshi, Akihito Kozuki, Department of Surgery, Kochi Health Sciences Center, Kochi 781-8555, Japan
Satoshi Ito, Yasuhiro Ogawa, Department of Radiology, Kochi Medical School, Nankoku 783-8505, Japan
Michiya Kobayashi, Kazuhiro Hanazaki, Department of Surgery, Kochi Medical School, Nankoku 783-8505, Japan
Author contributions: Okabayashi T and Hanazaki K designed research; Sumiyoshi T, Kozuki A and Ito S contributed analytic tools; Shima Y, Ogawa Y and Kobayashi M supervised the paper; and Okabayashi T wrote the paper.
Supported by Kochi Organization for Medical Reformation and Renewal Fund
Correspondence to: Dr. Takehiro Okabayashi, MD, PhD, Department of Surgery, Kochi Health Sciences Center, 2125-1 Ike, Kochi 781-8555, Japan. takehiro_okabayashi@khsc.or.jp
Telephone: +81-88-8373000 Fax: +81-88-8376766
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Abstract

Insulinomas, the most common cause of hypoglycemia related to endogenous hyperinsulinism, occur in 1-4 people per million of the general population. Common autonomic symptoms of insulinoma include diaphoresis, tremor, and palpitations, whereas neuroglycopenic symptoms include confusion, behavioural changes, personality changes, visual disturbances, seizure, and coma. Diagnosis of suspected cases is based on standard endocrine tests, especially the prolonged fasting test. Non-invasive imaging procedures, such as computed tomography and magnetic resonance imaging, are used when a diagnosis of insulinoma has been made to localize the source of pathological insulin secretion. Invasive modalities, such as endoscopic ultrasonography and arterial stimulation venous sampling, are highly accurate in the preoperative localization of insulinomas and have frequently been shown to be superior to non-invasive localization techniques. The range of techniques available for the localization of insulinomas means that blind resection can be avoided. Intraoperative manual palpation of the pancreas by an experienced surgeon and intraoperative ultrasonography are both sensitive methods with which to finalize the location of insulinomas. A high proportion of patients with insulinomas can be cured with surgery. In patients with malignant insulinomas, an aggressive medical approach, including extended pancreatic resection, liver resection, liver transplantation, chemoembolization, or radiofrequency ablation, is recommended to improve both survival and quality of life. In patients with unresectable or uncontrollable insulinomas, such as malignant insulinoma of the pancreas, several techniques should be considered, including administration of ocreotide and/or continuous glucose monitoring, to prevent hypoglycemic episodes and to improve quality of life.

Key words: Pancreas; Insulinoma; Neuroendocrine pancreatic tumor; Diagnosis; Management; Continuous blood glucose monitoring

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INTRODUCTION

Insulinomas are the most common functioning endocrine neoplasm of the pancreas[1-4]. They are insulin-secreting tumors of pancreatic origin that cause hypoglycemia[5-7]. Insulinomas occur in 1-4 people per million in the general population and represent 1%-2% of all pancreatic neoplasms[8-10]. Insulinomas can occur at any age and have an equal gender distribution. As many as 90% of insulinomas have been reported to be benign, 90% are solitary, > 90% occur at intrapancreatic sites, and 90% are < 2 cm in

diameter[10-13]. Insulinomas are evenly distributed over the entire pancreas. Most insulinomas are located in the pancreas or are attached directly to the pancreas. Extrapancreatic insulinomas causing hypoglycemia are extremely rare (incidence < 2%); extrapancreatic insulinomas are most commonly found in the duodenal wall[8]. The etiology and pathogenesis of insulinomas are not known.

Following biological and biochemical confirmation of an insulinoma, preoperative localization is sought using computed tomography (CT)[14-16], magnetic resonance imaging (MRI)[16-19], endoscopic ultrasonography (EUS)[20-22], intra-arterial calcium stimulation test with hepatic venous sampling[23], and/or angiography and arterial stimulation venous sampling (ASVS)[24-28]. Surgical resection is the primary treatment modality for insulinomas, and so accurate localization of the tumor before or during surgery is important. Intraoperative manual palpation of the pancreas by an experienced surgeon and intraoperative ultrasonography are both sensitive methods with which to localize insulinomas, supporting the argument by some surgeons that preoperative localization of the tumors is not necessary[29-33]. The present review describes some of the latest findings regarding the clinical diagnosis and medical management of insulinomas in the adult population that are not associated with either multiple endocrine neoplasms or von Hippel-Lindau disease.

CLINICAL SIGNS

Insulinomas are the most common cause of hypoglycemia related to endogenous hyperinsulinism. The episodic nature of the hypoglycemic attack is due to the intermittent secretion of insulin by the tumor[8]. Common autonomic symptoms of an insulinoma include diaphroresis, tremor, and palpitations, whereas neuroglycopenenic symptoms include confusion, behavioral changes, personality changes, visual disturbances, seizures and coma[34,35].

Diagnosis of insulinomas can be challenging. Although it was originally considered that symptoms only became evident in the fasting state or following exercise, it is now known that patients with an insulinoma can also present with postprandial symptoms[36,37]. The classical diagnosis of insulinoma depends on satisfying the criteria of Whipple's triad, which remains the cornerstone of the screening process: (1) hypoglycemia (plasma glucose < 50 mg/dL); (2) neuroglycopenic symptoms; and (3) prompt relief of symptoms following the administration of glucose (Table 1)[38]. In adults with symptoms of neuroglycopenia or documented low blood glucose levels, the gold standard for biochemical diagnosis remains measurement of plasma glucose, insulin, C-peptide, and proinsulin during a 72-h fast (Table 1). This prolonged fasting test can detect up to 99% of insulinomas[39]. Endogenous hypoglycemia due to insulinomas was previously based on findings of abnormal serum levels of insulin, C-peptide, and, more recently, proinsulin at the time of fasting hypoglycemia. To date, there is some general agreement regarding the diagnostic thresholds that must be reached for insulin, C-peptide, and proinsulin levels to be considered abnormal. Several years ago, ratios calculated from insulin and blood glucose levels were used, with the insulin/C-peptide ratio in patients diagnosed with insulinoma reported to be < 1.0[40,41]. It is of note that a normal insulin level does not exclude the disease, because the absolute insulin level is not elevated in all patients with insulinoma. In addition, because the proportion of proinsulin secreted by insulinoma cells is generally higher than that secreted by normal β-cells, high proinsulin levels have been suggested as being diagnostic of insulinoma, regardless of concomitant blood glucose levels[42]. The availability of proinsulin assays has led to the use of serum proinsulin thresholds as a diagnostic tool: it has been recommended that a cut-off level of 20 pmol/L proinsulin at the time of hypoglycemia < 45 mg/dL is indicative of the presence of an insulinoma (Table 1)[42-44].

Table 1 Diagnosis of insulinoma

Classical diagnosis
Hypoglycemia (plasma glucose < 50 mg/dL)
Neuroglycopenic symptoms
Prompt relief of symptoms following the administration of glucose
Present consensus
At the time of hypoglycemia during a 72-h fasting test:
5 mIU/L (36 pmol/L) insulin threshold
0.6 ng/mL (0.2 nmol/L) C-peptide threshold
Insulin/C-peptide ratio < 1.0
20 pmol/L proinsulin cut-off level
Absence of sulfonylurea (metabolites) in the plasma or urine

Delays in the diagnosis of insulinoma are common because the symptoms usually precede detection of a tumor and there may be misattribution of the symptoms to psychiatric, cardiac, or neurological disorders[45]. Once a diagnosis of insulinoma is considered, it is important that patients are managed in a timely and safe manner. As a general rule, patients with insulinoma can be cured by surgical resection of the tumor. The need for preoperative localization of insulinomas and the methods used remain contentious. In the past, confirmation of the presence of the Whipple triad (symptoms known or likely to be caused by hypoglycemia; a low plasma glucose measured at the time of the symptoms; relief of symptoms when the glucose is raised to normal) usually meant that a patient was led directly to surgery[46,47]. However, at present, most agree that knowledge of the site of the tumor before surgery is helpful in that it allows one to determine not only whether enucleation (the surgical removal of a mass) of the neoplasm or pancreatic resection is likely to be required, but also whether the tumor is amenable to removal *via* a laparoscopic approach. Preoperative localization should also mean that the operation itself can be performed more quickly, thereby reducing associated morbidity and mortality[27]. It is important to remember that most tumors are intrapancreatic, 90% are solitary, 90% are < 2 cm in diameter, and the tumors are distributed equally within the head, body and tail of the pancreas.

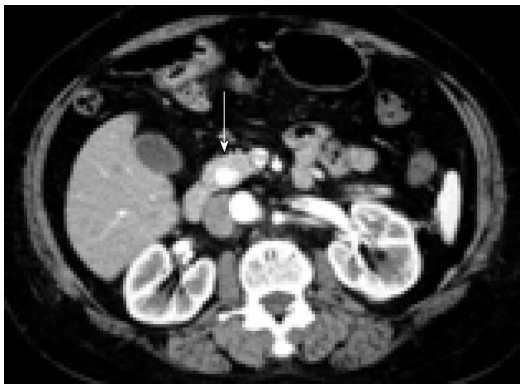

Figure 1 Computed tomography of insulinoma of the pancreas. Typically, insulinomas (arrow) are hypervascular and, as a result, demonstrate a greater degree of enhancement than normal pancreatic parenchyma during the arterial and capillary phases of contrast bolus.

NON-INVASIVE IMAGING

A number of non-invasive techniques are available for the localization of a suspected insulinoma, including transabdominal ultrasonography, CT and/or MRI. The sensitivity of transabdominal ultrasonography in the localization of insulinomas is poor (ranging from 9% to 64%)[48]. However, insulinomas demonstrate characteristic features when imaged with both CT and MRI and the sensitivity of these techniques has been reported to be 33%-64% and 40%-90%, respectively[10,49]. The sensitivity and specificity of MRI is generally superior to that of CT, as is the detection of extrapancreatic extensions[16].

CT is a safe and simple procedure to perform that is operator independent. CT visualizes the exact location of an insulinoma, its relationship to vital structures, and the presence of metastases[49]. Typically, insulinomas are hypervascular and, as a result, demonstrate a greater degree of enhancement than normal pancreatic parenchyma during the arterial and capillary phases of contrast bolus (Figure 1)[16]. An atypical CT appearance of insulinomas is occasionally encountered and can include hypovascular and hypodense lesions post-contrast, hyperdense lesions precontrast, cystic masses, and calcified masses[16]. Calcification, when it occurs, tends to be discrete and nodular, and is more common in malignant than benign tumors[50,51]. Technical advances have improved the quality of CT, with a recent study reporting that using a multidetector CT enabled visualization of 94.4% of insulinomas[52]. CT is currently accepted as the first-line investigation for the visualization of insulinomas.

Currently, there is strong evidence emerging for the use of MRI in the imaging of insulinomas, and investigators have shown a high sensitivity for MRI in the detection of insulinomas[16,49]. Like CT, MRI is safe, non-invasive, rapid, and facilitates the detection of metastases. Insulinomas generally demonstrate low signal intensity on T_1-weighted images and high signal intensity on T_2-weighted images (Figure 2)[50]. However, limitations in the use of MRI in the detection of insulinomas include the standard contraindications for MRI. The modern MRI system allows rapid triphasic, breath-held T_1 rapid gadolium-enhanced sequences, and/or diffusion-weighted imaging[19,49]. These sequences significantly reduce motion artifacts and enable accurate assessment of the pancreas in both the arterial and venous phase[49]. MRI has all the advantages of CT and recent evidence suggests that it may be the more sensitive tool. In current practice, MRI is a second-line investigation for the localization of insulinomas, but it could potentially take over from CT in the future as it becomes more widely available and expertise improves.

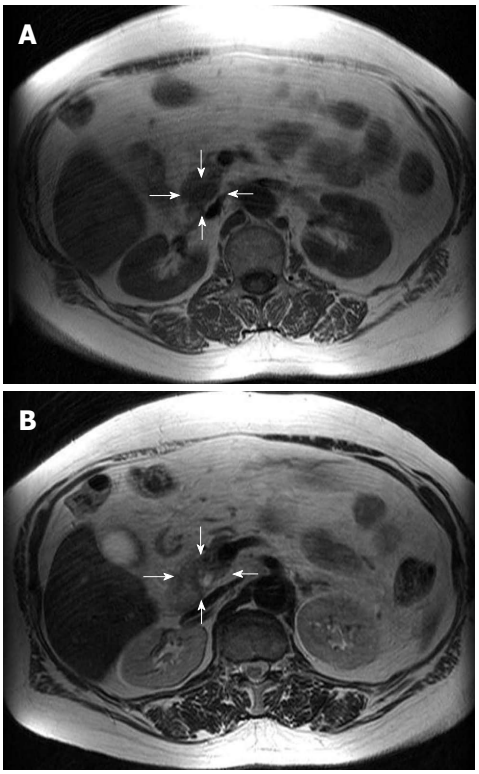

Figure 2 Magnetic resonance imaging of insulinoma of the pancreas. Insulinomas (arrows) generally demonstrate low signal intensity on T1-weighted images (A) and high signal intensity on T2-weighted images (B).

INVASIVE DIAGNOSTIC MODALITIES

The diagnostic procedure in cases of suspected insulinoma is based on standard endocrine examinations, especially the prolonged fasting test. Non-invasive imaging procedures are used to localize the source of pathological insulin secretion after a diagnosis of insulinoma has been established[20]. Invasive modalities, such as EUS and ASVS, have been shown to be highly accurate in the preoperative localization of insulinomas, and have frequently been shown to be superior to non-invasive localization techniques.

EUS is currently the test of choice in most Western centers, with reported detection rates of 86.6%-92.3%[10,46]. The appearance of insulinomas on EUS is quite characteristic, with most tumors homogeneously hypoechoic, rounded in shape, and with distinct margins (Figure 3).

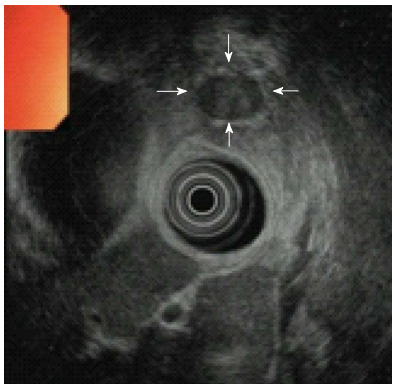

Figure 3 Endoscopic ultrasound features of insulinoma of the pancreas. The appearance of insulinomas (arrows) on endoscopic ultrasonography is quite characteristic, with most tumors homogeneously hypoechoic, rounded in shape, and with distinct margins.

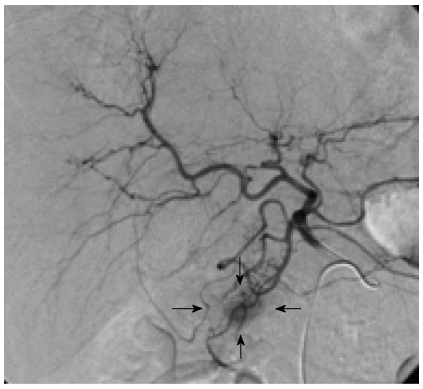

Figure 4 Angiography and arterial stimulation venous sampling. Using arterial stimulation venous sampling, insulinomas (arrows) are seen as well-defined, round or oval vascular blushes that are of increased vascularity compared with the surrounding normal pancreatic parenchyma.

Although EUS is a highly reliable procedure for the preoperative localization of insulinomas, there are several problems associated with the detection of these tumors using EUS. First, EUS may yield both false-positive and false-negative results, with the quality of the EUS findings largely dependent on the examiner's experience[22]. Second, some insulinomas are missed by preoperative EUS because they are completely isoechoic. A low body mass index, female gender, and young age may be risk factors for negative imaging[20]. Third, the sensitivity of EUS for insulinomas depends on the location and size of the tumor; sensitivity is greatest for tumors in the head of the pancreas and lowest for those in the tail of the pancreas or those that are extrapancreatic[10]. Once the site of the tumor has been determined, fine-needle aspiration (FNA) of the pancreas allows for a preoperative diagnosis of insulinoma. Advances in EUS have made EUS-guided FNA particularly useful in the diagnosis of insulinomas, because most functioning tumors are small. EUS-guided FNA is becoming increasingly popular, and it seems likely that it will eventually become the standard for the diagnosis and staging of pancreatic tumors[9].

There can be little doubt that angiography combined with ASVS should not precede non-invasive investigations, such as CT and MRI, but it remains a highly sensitive technique for the precise localization of insulinomas and will usually provide more information than EUS[27]. Morphological imaging modalities do not reflect hormonal function; however, the addition of ASVS helps regionalize a tumor by verifying hormonal function[24]. The use of ASVS allows for a more accurate surgical approach and can minimize the likelihood of re-operation[24]. For atypical insulinomas, preoperative localization of insulinomas by ASVS is particularly important. The accuracy of ASVS in localizing insulinomas has been reported to range from 94% to 100%[23,28]. Using ASVS, insulinomas are seen as well-defined, round or oval vascular blushes that are of increased vascularity compared with the surrounding normal pancreatic parenchyma (Figure 4). Insulinomas are visualized during the early arterial phase and persist for a variable length of time into the venous phase of the run. The localization of an insulinoma by ASVS relies on the fact that a hyperosmolar concentration of calcium in the vessels supplying the tumor will cause degranulation of cells within the neoplasm, releasing insulin into the portal venous system, which results in a detectable rise in insulin in venous samples obtained from the hepatic vein[27]. The splenic, gastroduodenal, superior mesenteric, and proper hepatic arteries are the vessels most commonly studied during ASVS; an increase in insulin concentrations in the hepatic vein will localize the insulinoma to the body/tail of the pancreas, an anterosuperior site of the pancreatic head, and postero-inferior site of the pancreatic head, respectively. An increase in insulin concentrations after injection of calcium into the proper hepatic artery suggests that hepatic metastases may be present. Changes in serum insulin levels plotted as a function of time after calcium injection indicate that insulin concentrations are markedly elevated only in the feeding arteries of the insulinoma (Figure 5).

The range of imaging modalities now available means that blind resection for insulinoma can be avoided because of accurate preoperative and intraoperative localization of the tumor[53]. Manual palpation of the pancreas by an experienced surgeon and ultrasonography are both sensitive methods for the intraoperative detection of the site of insulinomas[29-31]. The sensitivity of these two methods is clinically acceptable and has been reported as 75%-95% and 80%-100%, respectively[29,32,54].

MEDICAL MANAGEMENT OF BENIGN INSULINOMAS

Most patients with benign insulinomas can be cured with surgery, although other techniques for the management of insulinomas, including injection of octreotide, EUS-guided alcohol ablation, radiofrequency ablation (RFA), or embolization of an insulinoma of the pancreas, have been described[55-61].

After identification of an insulinoma, surgery is indicated for all localized tumors. The choice of procedure will depend on the features of the tumor mass, such as

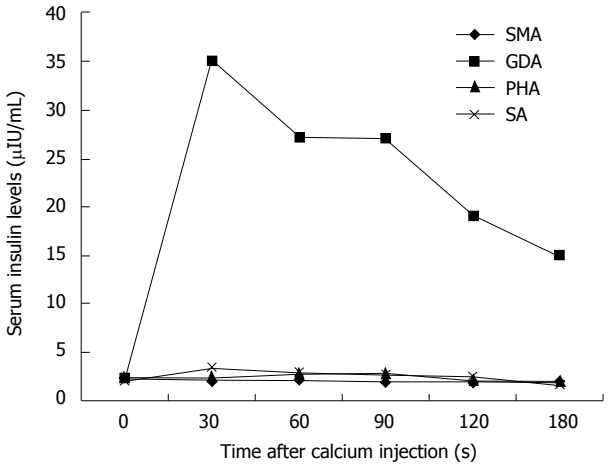

Figure 5 Changes in serum insulin levels. Changes in serum insulin levels plotted as a function of time after calcium injection indicate that insulin concentrations are markedly elevated only in the feeding arteries of the insulinoma. SMA: Superior mesenteric artery; GDA: Gastroduodenal artery; PHA: Proper hepatic artery; SA: Splenic artery.

type, size, and localization. Atypical resection, including enucleation, partial pancreatectomy, or middle pancreatectomy, has the advantage of preserving the pancreatic parenchyma as much as possible, thereby reducing the risk of late exocrine/endocrine insufficiency[62]. To date, laparoscopic resection has often been performed for insulinomas that are benign, small, and/or located in the body or tail of the pancreas[63]. Radical resection should be considered for patients in whom the lesion is not single, not well-capsulated, > 4 cm in diameter, and involves or is near the main pancreatic duct. Lymphadenectomy is not usually performed. Although the cure rate after resection for insulinoma is very high, it is necessary to be aware of the potential for postoperative complications after pancreatic surgery, especially postoperative pancreatic fistula[64-66].

However, there is a considerable risk of morbidity and mortality associated with the surgical management of insulinomas, which precludes surgery in high-risk patients. Alcohol ablation and RFA have been established as minimally invasive procedures in the treatment of primary liver tumors and hepatic metastases. Recently, successful EUS-guided alcohol ablation and CT-guided RFA of pancreatic insulinomas have been reported in humans[56,57]. These two patients were in poor general condition and were experiencing recurrent symptomatic episodes of hypoglycemia. Because it was considered that surgical management for benign insulinoma of the pancreas was impossible in both cases, ablation of the solitary mass was performed. Both patients were discharged without any complications and reported no further hypoglycemic episodes. Embolization of an insulinoma of the pancreas is another non-surgical alternative[55,67,68]. Because angiographically the insulinoma is demonstrated in the arterial phase as a hypervasculated mass, embolization could be performed using flow to direct particles exclusively into the tumor. Although it remains contentious as to whether these procedures are a viable treatment for patients with an insulinoma, they may be offered as an alternative for certain patients, such as those who refuse surgery, those who are of advanced age, those with a poor general condition, those who have already undergone multiple abdominal surgeries, or those with an increased risk of postoperative complications due to other reasons.

Insulinomas are rare endocrine tumors, most of which can be cured by surgery. Medical treatment to normalize blood glucose is useful during the preoperative period, as well as for patients who cannot be cured by surgery, such as those with diffuse β-cell disease, multiple insulinomas, unresectable malignant insulinoma, those in whom surgery is contraindicated, or patients who refuse surgery[59]. Octreotide is a somatostatin analog that inhibits insulin secretion and the peripheral action of many gastrointestinal hormones, primarily *via* activation of somatostatin sst_2 receptors. Octreotide has been used for the treatment of insulinoma, with successful control of blood glucose levels[60,61]. In addition, ocreotide may have an antiproliferative effect, as well as a moderate antitumoral action, on pancreatic endocrine tumors[69]. Therapy may be initiated with short-acting ocreotide two to four times daily, or 20-30 mg long-acting ocreotide every 4 wk[70]. Initiation of therapy with short-acting ocreotide can be used to assess systemic tolerability, particularly any gastrointestinal side-effects[71]. Thus, pharmacotherapy with somatostatin to control hypoglycemia represents a feasible option for the non-surgical management of insulinomas.

MEDICAL MANAGEMENT OF MALIGNANT INSULINOMAS

To be considered malignant, insulinomas must show evidence of local invasion into the surrounding soft tissue or there must be verification of lymph node or liver metastasis[72]. The reported incidence of malignant insulinomas ranges between 7% and 10%[72-74], and the 10-year survival has been reported to be 29%[2]. The major sites of metastasis or recurrence are the liver and regional lymph nodes. Aggressive surgical resection is recommended because these tumors are much less virulent than their malignant ductal exocrine counterparts, in which there are severe hormonal symptoms that cannot be controlled by medical treatment. RFA can be used to reduce the tumor mass in the liver, thereby reducing hormonal symptoms[58,75]. Selective embolization alone or in combination with intra-arterial chemotherapy is an established procedure to reduce both hormonal symptoms and liver metastases. Although experience is limited, liver transplantation for multiple liver metastases of malignant insulinomas may be considered in patients with no extrahepatic metastases[76,77]. Aggressive sequential multimodal therapy (chemoembolization, RFA, liver resection, liver transplantation) can prolong the survival of patients with sporadic malignant insulinoma, even in the presence of liver metastases.

Malignant insulinomas remain extremely rare tumors. In many patients with malignant insulinomas, the tumors are unresectable and medical treatment therapy is limited

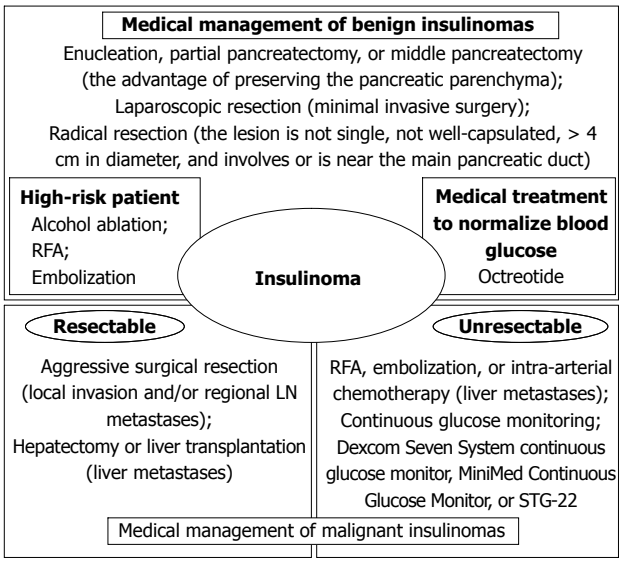

Figure 6 In patients who have unresectable or uncontrollable malignant insulinomas of the pancreas, several strategies need to be considered to both control hypoglycemic episodes and improve quality of life, including administration of ocreotide and continuous glucose monitoring. RFA: Radiofrequency ablation; LN: Lymph node.

in its ability to prevent hypoglycemic episodes[78,79]. Continuous glucose monitoring in patients with insulinomas can detect hypoglycemia, monitor responses to medical therapy, and confirm a cure postoperatively. In the literature, continuous glucose monitoring has been reported using a Dexcom Seven System continuous glucose monitor (Dexcom, San Diego, CA, United States) or the MiniMed Continuous Glucose Monitor (CGM Medtronic MiniMed, Northridge, CA, United States)[78,79]. These studies reported that continuous glucose monitoring is a useful addition to the armamentarium for the prevention of hypoglycemia. These techniques are considered an effective adjunct to therapy to reduce hypoglycemic episodes by alerting patients to low glucose concentrations before they develop neuroglycopenic symptoms; however, patients should respond promptly to oral glucose intake after hypoglycemia has been detected by these machines. In patients with a poorer general condition, malignant insulinomas that are unresectable, and uncontrolled hypoglycemia, it is proposed that blood glucose concentrations are monitored using the STG-22 (Nikkiso Co., Tokyo, Japan). The STG-22 is a reliable and accurate device for the measurement of blood glucose concentrations compared with the ABL 800FLEX machine (Radiometer Medical ApS, Brønshøj, Denmark) that is recommended by the National Committee for Clinical Laboratory Standards[80,81]. The STG-22 closed-loop glycemic control system is composed of a glucose sensor for the detection and/or monitoring of glucose and pumps for infusing an appropriate amount of insulin or glucose. The insulin and glucose pumps are computer regulated based on a target blood glucose value that is defined prior to initiation of the system[82,83]. It is has been proven clinically that the STG-22 device is safe and beneficial for maintaining glycemic control without hypoglycemic episodes in surgical patients[84-86] (Figure 6).

CONCLUSION

Insulinomas are the most common neuroendocrine tumors of the pancreas and cause hypoglycemia related to endogenous hyperinsulinism. More than 90% of insulinomas are benign and usually small, well-encapsulated, solitary tumors. Surgical resection is the treatment of choice for insulinomas and offers the only chance for cure. Most insulinomas can be identified intraoperatively by an experienced surgeon. However, what should be done in an operating theater when an insulinoma cannot be identified? Based on information presented in this review, we recommended the use of various non-invasive and invasive imaging modalities when the tumor cannot be detected using conventional diagnostic procedures. Blind surgical resection of the pancreas should not be undertaken in any patient who suffers from hypoglycemic episodes due to an insulinoma. Identifying the location of the insulinoma enables the surgeon to proceed with surgery uninterrupted, minimizing time in the operating theater, reducing the likelihood for re-operation, limiting perioperative complications, and ensuring, in most cases, a successful outcome[38].

In patients with malignant insulinomas, aggressive surgical resection, including extended pancreatic resection, liver resection, and/or liver transplantation, should be attempted when possible to improve patient survival[58,75-77]. Furthermore, aggressive secondary treatments may be indicated, such as chemoembolization or RFA for liver metastases from malignant insulinomas of the pancreas to control hypoglycemia. In patients who have unresectable or uncontrollable malignant insulinomas of the pancreas, several strategies need to be considered to both control hypoglycemic episodes and improve quality of life, including administration of ocreotide and continuous glucose monitoring. Insulinomas of the pancreas remain rare and may occur simply by chance. To refine the diagnosis and management of these tumors, epidemiologic and pathologic data should continue to be collected.

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Hepatitis B vaccine in celiac disease: Yesterday, today and tomorrow

Giovanna Vitaliti, Andrea Domenico Praticò, Carla Cimino, Giovanna Di Dio, Elena Lionetti, Mario La Rosa, Salvatore Leonardi

Giovanna Vitaliti, Andrea Domenico Praticò, Carla Cimino, Giovanna Di Dio, Elena Lionetti, Mario La Rosa, Salvatore Leonardi, Department of Pediatrics, University of Catania, 95100 Catania, Italy

Author contributions: All authors equally contributed to the building up of the paper, the literature bibliography research and to the writing of the paper.

Correspondence to: Salvatore Leonardi, MD, Department of Pediatrics, University of Catania, 95100 Catania, Italy. leonardi@unict.it

Telephone: +39-953-782764 Fax: +39-953-782385

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Abstract

Some studies showed that in celiac patients the immunological response to vaccination is similar to that one found in general population except for vaccine against hepatitis B virus (HBV). The non-responsiveness to HBV vaccine has also been described in healthy people, nevertheless the number of non-responders has been demonstrated to be higher in celiac disease (CD) patients than in healthy controls. Several hypothesis explaining this higher rate of unresponsiveness to HBV vaccine in CD patients have been described, such as the genetic hypothesis, according with CD patients carrying the disease-specific haplotype HLA-B8, DR3, and DQ2, show a lower response to HBV vaccine both in clinical expressed CD patients and in healthy people carrying the same haplotype. On the other hand, it has been demonstrated that the gluten intake during the vaccination seems to influence the response to the same vaccine. Moreover, it has been demonstrated a possible genetic predisposition to hepatitis B vaccine non-responsiveness likely due to the presence of specific human leukocyte antigen haplotypes and specific single nucleotide polymorphism in genes of cytokine/cytokine receptors and toll like receptors, but the pathogenic mechanism responsible for this low responsiveness still remains unclear. The aim of this review is to focus on the possible pathogenic causes of unresponsiveness to HBV vaccine in CD patients and to propose an alternative vaccination schedule in order to improve the responsiveness to HBV vaccine in this at-risk patients.

Key words: Celiac disease; Non responders; Hepatitis B vaccine; Vaccination schedules

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INTRODUCTION

It is estimated that more than one third of the world's population has been infected with the hepatitis B virus (HBV), causing acute and chronic liver diseases, ranging from fulminant hepatitis to cirrhosis and eventually hepatocellular carcinoma, with an incidence of 620 000 death per year[1-3].

On this regard, in order to prevent this serious health problem, since 1982 a safe and effective hepatitis B vaccine has been available. The first vaccines were plasma-derived, which contained purified hepatitis B surface antigen (HBsAg) obtained from the plasma of people with chronic HBV infection. In the following years, recombinant DNA hepatitis B vaccine has been developed, containing purified HBsAg obtained by culturing genetically engineered yeast or mammalian cells carrying the *HBsAg* gene. Currently recombinant DNA hepatitis B vaccines are predominant-

ly being used, while plasma-derived hepatitis B vaccines are still being used in several low-income countries[4].

At the beginning, the hepatitis B vaccine was considered for use in high risk individuals for acquiring HBV infection. Actually, it has become more widely used and recommendations for hepatitis B vaccination have been extended to all infants in an attempt to achieve protection against HBV infection[5]. Despite these recommendations, 8 countries in Northern Europe (Denmark, Finland, Iceland, Ireland, Netherlands, Norway, Sweden, and the United Kingdom) have yet to implement such a policy, currently adopting an "at-risk" strategy. In 2007, Zuckerman *et al*[6] recommend a reassessment in these country of their hepatitis B prevention strategies considering the difficulty in identifying all at-risk individuals and the lack of effectiveness of the vaccination on at-risk subjects in reducing the overall incidence of hepatitis B.

Italy was one of the first countries to implement an universal strategy of hepatitis B vaccination[7]. Since 1984 HBV vaccination has been recommended and offered free of charge by the National Health Service to high-risk groups-*e.g.*, family members of HBsAg carriers, healthcare workers and in 1991, vaccination became mandatory for all new born and for twelve year-old adolescents[8].

Consequent to this global strategy, a decrease in prevalence of chronic HBV infection among vaccinated children and adolescents has been documented, including people living in high risk area such as China, Hong Kong, Taiwan, the Gambia, Senegal, Alaska and Italy[9].

Usually, a single course of three doses of hepatitis B vaccine administered in a variety of schedules, such as at birth, 1 and 6 mo schedule, the 6, 10 and 14 wk doses and the 2, 4 and 6 mo schedule induces protective levels of antibody to HBsAg in nearly 95% of healthy infants and children[10].

The antibody response to hepatitis B vaccine has been found occurring in more than 90% of the healthy vaccinated subjects[11-13]. However some studies showed that serum anti-HBs levels decreases with the time following vaccination[14,15]. Several factors, such as age, body mass index and smoking at the time of vaccination, have been found to be associated with a lower rate of antibody response to hepatitis B vaccine[16,17], and the decline of HBs antibody titer seemed mainly to be proportional to the antibody titer originally obtained[18].

Long-term follow-up studies of newborn vaccination showed that antibodies become negative in 15%-50% among the vaccine responders within 5 to 10 years[19-21].

The clinical significance of the disappearance of specific antibodies in immunocompetent patients responders to previous vaccination remains object of discussion.

Successfully vaccinated people who have lost antibodies after primary vaccination, usually show a rapid anamnestic response when boosted. This means that the immunological memory for HBsAg can outlast antibody detection, providing long-term protection against the disease. Hence, there is consensus that there is no need to administer booster doses of vaccine to ensure long-term protection in immunocompetent patients[22-25].

On the other hands some authors suggest specific strategies for the vaccination of certain groups of people at high risk such as haemodialysis, celiac and thalassemia patients[26-31].

Vaccinated subjects with an anti-HBs titer less than 10 mIU/mL after completion of primary vaccine series are called "no responders".

Several factors have been associated with a non response to the HBV vaccine. These include inappropriate vaccine storage conditions, vaccination in buttocks, obesity, smoking, drug abuse, infections[32]. There are also chronic conditions, such as chronic alcoholism, chronic kidney disease, human immunodeficiency virus infection, immune-suppression, type I Diabetes Mellitus and Celiac disease that are characterized by a lower response to HBV vaccination[33-45]. Recently, it has been demonstrated a possible genetic predisposition to hepatitis B vaccine non-responsiveness likely due to the presence of specific human leukocyte antigen (HLA) haplotypes and specific single nucleotide polymorphism (SNP) in genes of cytokine/cytokine receptors and toll like receptors (TLR)[42,46], but the pathogenic mechanism responsible for this low responsiveness still remains unclear.

CELIAC DISEASE AND HBV VACCINE: THE HISTORY

Celiac disease (CD) is an autoimmune disorder characterized by a permanent intolerance to ingested gluten, which results in immunologically mediated inflammatory damage to intestinal mucosa[47]. Genetic, environmental and immunological factors seem to be responsible for the disease.

CD is usually characterized by various gastrointestinal (GI) symptoms (*e.g.*, diarrhea, malabsorption, weight loss) associated with consumption of grains containing gluten (wheat, barley, rye). Although some CD patients may have primarily GI symptoms, CD may be detected due to associated extra intestinal disorders, even without GI symptoms, or due to screening for CD based on a positive family history. CD has a strong association with HLA-DQ2 and HLA-DQ8[48]. HLA-DQ2 is present in 90% to 95% of patients with CD[49].

The link between HBV infection and celiac disease seems to be controversial. Relatively little data exist on the relationship between HBV and CD, although one third of the world's population (around 2 billion people) have been infected with HBV. It has been reported that the response rate to HBV vaccination in CD-infected individuals is lower (30%-50%) than in the general population (4%-10%)[42,43] (Table 1). Recently, it has been hypothesized that non intestinal inflammatory diseases may trigger immunologic gluten intolerance in suscepti-

ble individuals, and HBV as far as hepatitis C virus (HCV) were thought to be suitable candidates. However this assumption is still matter of debate. Relatively few data exist on the relationship between HBV and CD. It could be speculated that HBV as well as HCV infections may trigger immunologic gluten intolerance in genetically susceptible people and that chronic HBV segregates a higher percentage of CD patients[50,51]. However this assumption is still a matter of debate.

Possible activation of CD due to the treatment of hepatitis infection is another controversial point. There have been some reports indicating that autoimmune disorders such as insulin-dependent diabetes mellitus and celiac disease can develop during treatment with interferon (IFN)-α for viral hepatitis because of its immune modulatory properties[52,53]. CD activation during interferon α or interferon α plus ribavirin therapy has recently been observed in HCV-positive patients[54], confirming that IFN-α therapy could trigger CD in susceptible subjects during treatment. In the study of Leonardi *et al*[30], although evidence suggested that IFN-α can activate CD4T cells in the lamina propria and cause intestinal tissue damage[55], no patient treated in childhood showed any serological marker of CD at the time of the present study. Due to the small sample size authors could not claim that there was no association between CD and HBV, although a sample size more representative of the prevalence of CD in Italy should help to better examine the relationship between CD and HBV.

Some studies showed that in celiac patients the immunological response to vaccination is similar to that one found in general population except for vaccine against HBV[42,56]. On this regard, it is well known that unresponsiveness to hepatitis B vaccine in healthy people has been attributed to failure of class II major histocompatibility complex molecules in the interaction with processed protein antigen, in the stimulation of T-helper cells, or in both[57,58].

Analysis of previous studies suggested a very high incidence of a particular extended HLA haplotype in no responders to hepatitis B vaccine. In fact, homozygotes for HLA-B8, DR3, and DQ2 were found to have a significantly higher incidence of hepatitis B vaccine non-response[58]. In 1989, Alper *et al*[59] prospectively vaccinated 5 homozygotes and 9 heterozygotes for extended MHC aplotype (HLA-B8, SC01, DR3). Four of the 5 homozygotes produced low levels of HBsAb two months after their third HBV vaccination, whereas all 9 heterozygotes had significantly higher titers of HBsAb. In 1995, Livenson *et al*[60] studied 153 patients with end-stage renal disease immunized with a recombinant HBV vaccine. Homozygotes for HLA-A1, HLA-B8, HLA-DR3 and HLA-DQ2 were found almost exclusively in the non-responder group and a significant higher number of heterozygotes for these alleles was found in the non-responder group compared to the responders[59]. In the same year, Martinetti *et al*[61] performed an HLA study in 9 absolute non-responder (serum titer of anti-HBsAg < 2 mIU/mL) and 8 low-responder (serum antibody level between 2 and 9.9 mIU/mL) infants who underwent, in neonatal period, HBV vaccination. The investigation pointed out that many of these subjects carry HLA haplotypes classically involved in autoimmune diseases: HLA DR7, DQ2, DR4, DQ8 and DR3. Further Godkin *et al*[58] investigated the binding affinities of envelope and core peptides of HBV to particular HLA glycoproteins, and their data supported the direct involvement of HLA-DR3 in HBV vaccine no responsiveness.

Table 1 People who have anti-HBs titre after completion of primary vaccine series less than 10 mIU/mL

	Celiac patients (no responders, %)	Control subjects (no responders, %)
Leonardi *et al*[30]	30/60 (50)	7/60 (11.7)
Ahishali *et al*[42]	8/25 (32)	0/20 (0)
Zingone *et al*[43]	31/51 (60.7)	13/48 (27.08)
Ertekin *et al*[44]	20/52 (38.5)	2/20 (10)
Noh *et al*[56]	13/19 (68.4)	-

Since the HLA-DQ2 haplotype is over-represented in celiac population, it has been postulated that this genetic profile may play a crucial role in predisposing celiac patients to a lower grade of immunization to hepatitis B vaccine[62].

On the other hand some studies argue that in celiac individuals gluten intake at the time of vaccination may influence the vaccine-induced immune response[41,44].

Nevertheless all these hypotheses are still argument of debate and no responsiveness of celiac patients to the HBV vaccine remains a significant public health problem, since the worldwide spread of the disease although there is no convincing evidence that patients with viral hepatitis B carry an increased risk of celiac disease[63].

In 2003, Noh *et al*[62] studied 19 celiac patients and found that 13 did not respond to vaccination. HLA typing was performed on 15 of these subjects (13 no responders and two responders). All tested subjects were apparently either homozygous or heterozygous for DQ2. The authors postulated that the non responsiveness to the hepatitis vaccine in celiac patients is due to the suppression of the Th2 response and to the B cell differentiation due to the high protection performed by the IFN-gamma.

In 2009, Leonardi *et al*[30] retrospectively analysed the response to HBV vaccine in 60 celiac patients and they found that 30 of 60 CD patients (50%) and 7 of 60 control subjects (11.6%) were non-responders to HBV vaccination ($P < 0.0001$).

It is well known that in celiac disease the intestinal damage is caused by interaction between specific deaminated glutamine residues of gliadin and HLA-DQ2 or DQ8 molecules[63].

Both HBsAg protein fragments and gliadin peptides bind to HLA-DQ2 molecules, and their competition

may result in a defective antibody response against the recombinant HBsAg vaccine in active CD[49].

In 2008, Nemes *et al*[41] studied 128 children and adolescent with celiac disease and 113 age-matched control subjects. For all celiac patients diet compliance and CD activity were monitored by measurement of antibodies against transglutaminase and endomysium. The authors demonstrated that the rate of primary non-response to the standard regimen of recombinant HBV vaccination was surprisingly high (74.1%) in undiagnosed and untreated celiac adolescents.

In 2010, Ertem *et al*[64] evaluated anti-HBs titer in celiac patients and healthy children. They found that anti-HBs negativity was significantly higher in celiac patients than in healthy controls. They also demonstrated that response to HBV vaccine in children with CD who were compliant with the gluten free diet (GFD) was not different from that found in the healthy population.

In 2010, even Zingone *et al*[44] hypothesized the possible role of dietary gluten in induction of a suboptimal immune response in celiac patients. They studied 51 celiac patients who were on a GFD and were negative for anti-transglutaminase-IgA antibody at the time of testing. Eleven years after primary vaccination the proportion of vaccinated subjects with anti-HBs antibody $\geqslant$ 10 mIU/mL was significantly lower in celiac than in controls (68.6% *vs* 91.7%; $P < 0.01$). Only three (5.9%) celiac patients were on GFD at the time of primary vaccination. Fourteen out of 16 (87.5%) celiac patients and all controls with anti-HBs < 10 mIU/mL received a booster dose of vaccine. Two weeks after the booster dose, 4 (28.6%) celiac patients and 3 (75%) controls showed an anamnestic response. Three out of 10 celiac patients who did not respond to the booster dose agreed to complete a new vaccine cycle. One month after vaccination completion, all showed anti HBs between 10 and 100 mIU/mL. Thus, the authors concluded that the gluten intake at the time of vaccination could decrease the efficacy of the same vaccination.

To confirm the role of gluten in the unresponsiveness to HBV vaccine in celiac patients, Ertekin *et al*[45] observed 52 children with CD and 20 age healthy children who received HBV vaccination according to the standard immunization schedule. They found that anti-HBs positivity was significantly higher in celiac patients who were compliant to GFD than in those who were noncompliant.

Even in the study leaded by Leonardi *et al*[30], the authors found a significantly higher number of responders in patients younger than 18 mo at diagnosis and a significantly lower number of responders in adolescent patients older than 14 years at diagnosis. This data confirms that in celiac patients a complete cycle of revaccination during a well controlled GFD might be more effective than the primary vaccination performed on a gluten-containing diet.

FUTURE DIRECTION

We have underlined how celiac disease is characterized by a low responsiveness to vaccinations such as HBV vaccine, and both for the widespread of celiac disease, that is high in prevalence of morbidity, and for the wild range of not response to HBV vaccine. The problem of unresponsiveness could represent a matter of world health, because the group of non-responders patients could be considered as a large reservoir of HBV-susceptible people that will persist as healthy carriers, leading to a diffusion of the diseases even in healthy subjects.

This problem drew the literature research towards possible reassessments of immunization strategies to protect this population and to achieve the goal of universal protection.

It would be important, therefore, to assess the possible vaccination strategy in order to reduce this "healthy-reservoir" of infection. On this regard, new vaccination strategies for celiac patients were proposed in literature: the first one the use of booster doses of HBV vaccine by intramuscular route (IM), the second one is the performance of booster doses of HBV vaccine by intradermal route (ID).

In their scientific paper, Zingone *et al*[44] gave an IM dose of HBV vaccine to celiac patients with anti HBs titre < 10 mIU/mL and found that, one month after vaccination completion, all showed anti-HBs titer between 10 and 100 mIU/mL. They concluded that celiac patients may require higher doses of vaccine and/or more injection to achieve full protection. Supporting this hypothesis, in 2008, Ashaili *et al*[43] studied 25 celiac patients and control subjects who were negative for anti-HBs. HBV vaccine was administered to all celiac patients and control subjects in three doses, at month 0, 1, 6 by intramuscular injection into the deltoid muscle. Four weeks after the last dose, anti-HBs was measured quantitatively. Seventeen celiac patients (68%) and all control subjects (100%) were found to respond to HBV vaccination[44].

On the other hands, supporting the hypothesis of a possible role of gluten in the unresponsiveness to HBV vaccine in celiac patients and in severe liver disease[40], Nemes *et al*[41] recommended a revaccination by IM route for celiac patients after treatment with gluten-free diet. They administered a dose of IM vaccine to anti-HBs-negative patients with CD during a controlled gluten-free diet and found that 97.3% of them seroconverted after revaccination. They concluded that the no responder status to primary HBV vaccination is not permanent in CD and may improve after gluten exclusion.

The second strategy addresses on the use of ID booster of HBV vaccine in non-responders patients. The rational of this is that in contrast to IM, which relies on a T-cell mediated response, vaccines introduced directly into the skin activate a dendritic-cell-mediated immune response through a lower doses of antigen[65].

According to these results, in 2010, Leonardi *et al*[66]

revaccinated celiac patients who were non-responders to HBV vaccination with a 2 mg dose of recombinant hepatitis B vaccine administered intradermally. After the first ID dose we found that 40% of patients achieved anti-HBs titer ⩾ 1000 mIU/mL, 20% between 100 and 1000 mIU/mL, and 15% between 10 and 99 mIU/mL.

In order to compare the efficacy and the safety of the two HBV vaccination strategy (IM *vs* ID) in a non-responder population, in 2011, Leonardi *et al*[67] re-vaccinated non-responder celiac patients with ID or IM vaccine. The Authors found a higher percentage of "responders" after the first booster dose (ID = 76.7% *vs* IM = 78.6%) and a deeper increase after the third dose (ID = 90% *vs* IM = 96.4%) of vaccine in both groups. These data seem to suggest that both ID and IM route are effective options to administer a booster dose of HBV vaccine in celiac patients. However the ID route seems to be a better vaccination strategy, as demonstrated by the higher percentage of patients with an anti-HBs title > 1000 IU/L found in ID than in IM group.

In literature there are no other studies comparing the efficacy of HBV vaccination administered by IM or ID route in celiac patients. The two different methods are extensively compared in other pathologic conditions in which we observed a low responsiveness to HBV vaccination. For example in a meta-analysis about the HBV vaccination response in patients with chronic kidney disease, the authors found that pooling of study results demonstrated a decreased risk of failure to respond to HBV vaccine among patients who were vaccinated by intradermal *vs* intramuscular route[27].

There is another topic that should be considered. Actually it is still not clear if non-responder patients are characterized by an immunological anergy since birth, or they lose their immune competence during the time. With this regard, there is general consensus in literature that successfully vaccinated people who have lost antibodies years after primary vaccination usually show a rapid anamnestic response when boosted[68]. This means that the immunological memory for HBsAg can outlast antibody detection, providing long-term protection against the disease and the development of the carrier state. Hence, there should be no need to administer booster doses of vaccine to ensure long-term protection in subjects initially responding to vaccination[22-25]. Thus, it is still not clear if celiac non-responders show an immunity anergy since birth and do not respond since the first dose of vaccination or they loose their antibody protection with the flow of time, as physiologically happens in normal people, even if in a shorter period of time, remaining, thus, protected by an intra-cellular immunity.

For this reason it would be important an early identification of potential "pure" non-responsive patients through the identification of specific markers of unresponsiveness. Recent acquisitions show a possible role of toll-like receptors, cytokines and cytokine receptors polymorphisms associated with no response to hepatitis B vaccine in healthy population. As a matter of fact, Chen *et al*[46] hypothesized that the variations in these structures may act individually or cooperatively in the influence of the duration and intensity of immune response elicited by the hepatitis B vaccine, finding that 4 specific SNPs in the *IL-4*, *IL-4RA*, *IL-13* and *TLR2* genes were closely associated with the serum anti HBsAg response to HBV vaccine. These cytokines and TLR2 seemed to be associated with a status of hepatitis B vaccine-induced protective humoral immune response. If an early identification of responsiveness to HBV vaccine could be speculated in general population by the analysis of specific SNPs, it could be postulated the use of the same analysis in celiac patients, in whom the rate of non-responsiveness is higher, representing a specific marker of the "pure" non-responders. Nevertheless, further studies should clarify the role of these polymorphisms and their possible use as markers of un-responsiveness to HBV vaccine.

In conclusion, we suggest that all patients with CD should be revaccinated to achieve the goal of universal protection. In consideration of the possible relationship between anti-HBs titers and compliance with GFD, they should be revaccinated after the decrease of specific celiac antibodies, that usually occurs after about 1 year of a strict GFD. We also suggest the use of the intradermal route for the revaccination of these patients. The increase of anti-HBs antibodies is in fact satisfactory after ID injection in all patients, a lower dose of the vaccine can be used for immunization and the cellular immune responsiveness to HBsAg can be easily assessed by the development of a skin reaction at the injection site[31]. Thanks to the appearance of this reaction there is no need to test the serum anti-HBs concentration after the booster dose to value the vaccine efficacy, and this could represent a less expensive strategy for the Health Organization to perform HBV booster doses[69]. A recent retrospective cost-benefit analysis of ID hepatitis B vaccination reported a cost reduction exceeding 50% compared with a standard IM vaccine regimen[63]. Moreover the ID route would allow a lower performance of venous withdraws in all patients, reducing the costs linked to serial follow-up withdraws. In literature it is also demonstrated that strong immunological memory persists more than 10 years after immunisation of infants and adolescents with a primary course of vaccination[70]. Furthermore, we suggest to administer a booster dose of vaccine every 10 years to all celiac patients, independently their status of "pure" unresponsiveness, and as they are genetically predisposed to loose their anticorpal memory, these boosters would favour a better immunologic strategy in order to protect celiac non-responders from a possible HBV infection.

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ORIGINAL ARTICLE

Accommodation and peristalsis are functional responses to obstruction in rat hypertrophic ileum

Simona Bertoni, Francesca Saccani, Rita Gatti, Alberto Rapalli, Lisa Flammini, Vigilio Ballabeni, Elisabetta Barocelli

Simona Bertoni, Francesca Saccani, Alberto Rapalli, Lisa Flammini, Vigilio Ballabeni, Elisabetta Barocelli, Department of Pharmacological, Biological and Applied Chemical Sciences, University of Parma, 43124 Parma, Italy
Rita Gatti, Department of Experimental Medicine, University of Parma, 43125 Parma, Italy
Author contributions: Bertoni S performed the research and wrote the paper; Saccani F and Gatti R performed the immunohistochemistry studies; Rapalli A and Flammini L performed the functional studies; Ballabeni V analyzed the data; Barocelli E designed the research study and wrote the paper.
Correspondence to: Dr. Simona Bertoni, Department of Pharmacological, Biological and Applied Chemical Sciences, University of Parma, Parco Area delle Scienze 27/a, 43124 Parma, Italy. simona.bertoni@unipr.it
Telephone: +39-521-905090 Fax: +39-521-905091
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Abstract

AIM: To investigate the effects of chronic obstruction on enteric reflexes evoked by electrical stimulation (EFS) or intraluminal distension of the rat hypertrophic ileum.

METHODS: Motor responses to EFS and to intraluminal distension were studied in the absence and in the presence of various inhibitors of enteric mediators. Ileum segments from operated (chronic ileal obstruction), sham-operated (control) and normal rats were horizontally mounted, connected to a pressure transducer and intraluminally perfused. The effects of selective serotonin receptor (5-HTR) blockers were investigated on distension-induced responses. The cellular localization of 5-HT3Rs was also examined in control and hypertrophic tissues through confocal microscopy.

RESULTS: In non-obstructed segments, EFS elicited tetrodotoxin (TTX)-sensitive responses with high amplitude contraction followed by weak relaxation. In hypertrophic tissues, EFS lowered the baseline pressure and evoked TTX-sensitive contractions significantly larger than normal ($P < 0.01$) or control ($P < 0.05$), and devoid of any relaxation phase ($P < 0.01$ *vs* normal). Incubation with atropine and guanethidine [non-adrenergic non-cholinergic (NANC) conditions] did not modify intestinal tone in normal and control preparations, but reversed the accommodation produced by EFS in hypertrophic tissues, and depressed the amplitude of contractions in all types of tissues. L-NAME and α-chymotrypsin blocked residual NANC motility in all tissues and augmented intraluminal pressure in hypertrophic segments ($P < 0.05$ *vs* NANC conditions). Intraluminal distension of the intestinal wall evoked non-propulsive cycles of contractions and relaxations in non-obstructed tissues. In all hypertrophic segments, strong propulsive strokes, markedly wider ($P < 0.001$), and larger than normal ($P < 0.001$) or control ($P < 0.05$) were elicited. Both motor patterns were blocked under NANC conditions and with simultaneous incubation with L-NAME and α-chymotrypsin. In all types of tissues, incubation with ketanserin or GR125487 did not modify distension-induced motility. In contrast, blockade of 5-HT3Rs by ondansetron concentration-dependently inhibited motor responses in normal and control tissues, but only slightly impaired enteric reflexes in the hypertrophic preparations. Finally, confocal microscopy did not reveal a different cellular distribution of 5-HT3Rs in control and hypertrophic ileum.

CONCLUSION: Accommodation and distension-induced peristalsis of rat hypertrophic ileum are controlled by cholinergic and peptidergic transmission and are negligibly affected by 5-HT3Rs, which modulate distension-induced motility in non-obstructed tissues.

Key words: 5-HT3 receptor; Intestinal obstruction; Peristalsis; Rat intestinal motility; Serotonin

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INTRODUCTION

Small bowel obstruction is a common clinical problem resulting from a variety of causes: intraluminal (food bolus, gallstones), intramural (neoplasms, strictures) or extramural (adhesions, hernias) factors. It can be partial or complete and, if not properly diagnosed and treated, it may rapidly lead to death[1]. Experimentally, striking morphological and neurochemical changes, affecting all the layers of the gut wall, have been reported to occur orally to the site of a mechanical long-standing partial obstruction. Massive thickening of the muscle coats, neuronal hypertrophy, neoangiogenesis and neoformation of collagen[2], a loss of interstitial cells of Cajal[3,4] and an altered expression of enteric neuropeptides[3,5] are the most prominent alterations detected in the obstructed intestine. Functionally, along with pronounced changes in biomechanical and motility patterns, detected both *in vitro*[6,7] and *in vivo*[8], preservation of the peristaltic reflex in the isolated guinea pig ileum has also been described[9]. Indeed, peristaltic reflex, responsible for the mixing and aboral propulsion of luminal contents *in vivo*, is the result of a highly sophisticated and localized integration between neural and myogenic components; *in vitro*, it can be initiated by local mechanical stimulation of the mucosa or by muscle stretch, as elegantly shown by Trendelenburg's pioneering work[10]. Hypertrophic growth, induced by mechanical obstruction of the rat ileum, affects the functional responses of both smooth muscle layers and of intrinsic innervation[7,11]. Therefore, the present work focused on the consequences of a partial long-standing stenosis on enteric reflexes evoked by electrical field stimulation (EFS) or by intraluminal distension of rat isolated terminal ileum. To this end, the changes in intraluminal pressure elicited by neurogenic stimulation of rat obstructed intestine were investigated in the absence and in the presence of different inhibitors of enteric neurotransmitters which control gut motility, and compared with those triggered in preparations obtained from normal or sham-operated (control) rats. Secondly, given the pivotal role played by endogenous serotonin (5-HT) in the initiation and propagation of enteric reflexes, such as the peristaltic reflex[12,13], special attention was directed at evaluation of its role in specific distension-induced motor patterns exhibited by non-hypertrophic and hypertrophic tissues by investigating the effects of different selective 5-HT receptor (5-HTR) antagonists. The cellular localization of 5-HT_3R was also investigated through confocal microscopy.

MATERIALS AND METHODS

Surgical procedure

Adult female Wistar rats (180-200 g), bred from a local colony, were housed in single cages with a 12 h/12 h light/dark cycle and received food and water *ad libitum*. The rats were randomly assigned to normal group (age-matched animals that did not undergo any type of surgery), a control group (age-matched sham-operated animals subjected to the same intestinal manipulation except for ileal obstruction) and an operated group (25 animals per group). Under general anesthesia (sodium pentobarbital 33 mg/kg *ip*), the rats were operated on according to Gabella's method[14], as previously described[7]. Briefly, a polyethylene ring of diameter 1-2 mm larger than that of the intestine, was applied around the ileum, proximal to the ileo-cecal junction, keeping it initially free for turning around. Postoperatively, the animals were monitored daily with regard to weight, general well-being and distension of the abdomen. Fourteen days after ileal obstruction, when previous experiments had shown that intestinal hypertrophy is fully and homogeneously developed over 10 cm aboral to the stenosis[7], the rats were killed, and functional or immunohistochemical studies were performed on ileal tissue. The experimental protocol complied with the requirements of animal care and was approved by Ministero della Salute, Italy (DL 116/92).

Functional studies

Rats were killed by CO_2 asphyxiation. Full-thickness 4-cm long segments of terminal ileum, immediately proximal to the site of occlusion excised from operated rats, and loops of the same part of the gut excised from normal or control rats, were cleared of their contents by flushing with Krebs-Henseleit solution (composition: NaCl 118.9 mmol/L, KCl 4.6 mmol/L, $CaCl_2$ 2.5 mmol/L, KH_2PO_4 1.2 mmol/L, $NaHCO_3$ 25 mmol/L, $MgSO_4$ 1.2 mmol/L, glucose 11 mmol/L). From each animal, only one preparation was obtained either immediately proximal to the point of obstruction, where hypertrophy was maximal and uniform, or from the corresponding portion of terminal ileum of normal and control rats. According to the method described by Costall *et al*[15], the segments were cannulated at the oral and anal ends, and were mounted in a Mayflower horizontal 20-mL tissue bath (Hugo Sachs Elektronik-Harvard Apparatus GmbH D-79232 March Hugstetten, Germany) filled with Krebs-Henseleit solution gassed with a mixture of 5%CO_2/95%O_2 at 37 ℃, and stretched at their initial length of 4 cm. The inlet cannula was connected *via* tubing to a reservoir filled with nutritive solution, maintained at 37 ℃ and continuously oxygenated, and to a pressure transducer (TSD104A Biopac Systems, 2Biological Instruments, Besozzo, VA, Italy) connected to a MacLab digital data acquisition system to record changes in the intraluminal pressure. The outflow tube at the aboral end had two outlets: one at the level of the bath (A) while the other (B) could be raised above the level of the tissue. A continuous flow of fresh

oxygenated solution was delivered intraluminally at a flow rate of 0.5 mL/min and extraluminally at a speed of 2 mL/min by means of the same peristaltic pump (Gilson Minipuls-3, Gilson Italia SRL, MI, Italy) to remove perfusate and extraluminal solution from the bath. The extraluminal flow was temporarily suspended during the period of incubation of drugs.

Electrical field stimulation: After a period of equilibration of 30 min, during which the fluid level in the outflow tube was equal to that in the bath (drain A open), the intraluminal pressure was augmented by closing drain A and by raising drain B 5 cm above the level of the tissue in the bath (5 cm hydrostatic resistance or low distension, corresponding to a pressure of 3.68 mmHg); 30 V square pulses of 1 ms duration were then delivered to the tissues at 3 Hz frequency through platinum wire electrodes positioned at the two opposite sides of ileum and connected to a generator that provided trains lasting 20 s at 120 s intervals. EFS-evoked changes in intraluminal pressure were registered in the absence (basal conditions) and in the presence of the muscarinic receptor antagonist atropine 1 μmol/L and sympatholytic agent guanethidine 4 μmol/L [non-adrenergic non-cholinergic (NANC) conditions]. The effects produced by the application of nitric oxide synthase (NOS) inhibitor N^G-nitro-*L*-arginine methyl ester (L-NAME) (300 μmol/L) and by the protease α-chymotrypsin (10 IU/mL) on NANC responses evoked by EFS were investigated. Drugs were added to the extraluminal solution.

Distension-induced motility: In a second series of experiments, after a period of equilibration of 30 min, drain A was closed and drain B was elevated 8 cm above the level of the tissue in the bath (8 cm hydrostatic resistance or high distension, corresponding to a pressure of 5.88 mmHg). After another 30 min, once regular motility had been evoked by the increase in the intraluminal pressure, the effects of simultaneous application of atropine 1 μmol/L and guanethidine 4 μmol/L or of single concentrations of the 5-HT_{2A}R antagonist ketanserin (1 μmol/L), the 5-HT_3R antagonist ondansetron (1-10 μmol/L) and the 5-HT_4R antagonist GR125487 (1 μmol/L) were studied over the next 30 min. Each preparation was challenged with a single application of each drug in order to minimize time-dependent variability. About 25% of non-obstructed tissues, which failed to develop a regular motility following distension, were discarded from further investigation in the experiment.

Immunohistochemistry

Tissue preparation and processing. Full-thickness 2-cm long segments of terminal ileum, excised as described above from hypertrophic and control animals, were cleared of their contents by flushing with phosphate buffered saline (PBS) 100 mmol/L at pH 7.4, opened along the mesenteric border, cleaned and pinned flat onto slabs of Sylgard 184 (Dow Corning), with the serosal side exposed. After 2 h fixation with 4% formaldehyde in 100 mmol/L PBS, whole-mount preparations containing the longitudinal muscle layer and the adherent myenteric ganglia were peeled off from the mucosa and the circular muscle under a dissection microscope using fine forceps, and were processed by the immunofluorescence method for double labeling using a free floating technique. After washing in 100 mmol/L PBS, whole mounts were incubated with a mixture of rabbit-anti-rat immunoglobulin G (IgG) primary antibody directed against 5-HT_3R 1:500 (No. 95247, a generous gift from CURE/UCLA Digestive Disease Research Center, Los Angeles, CA, United States) and mouse-anti-rat clathrin IgG 1:100 (Becton Dickinson Italia SpA) for 48 h at 4 ℃. This was followed by a 2-h incubation period at room temperature with a mixture of Alexafluor 488 donkey-anti-rabbit IgG 1:100 (Invitrogen Ltd, United Kingdom) and DyLight 549-conjugated AffiniPure donkey-anti-mouse IgG 1:200 (Jackson ImmunoResearch). Tissues were finally washed with 100 mmol/L PBS, mounted on slides and cover-slipped with ProLong Gold Antifade Reagent (Invitrogen Ltd, United Kingdom). Specificity controls were obtained by omitting primary or secondary antibodies from the incubation solution. Primary and secondary antibodies were diluted in Triton X-100 (0.5% in 100 mmol/L PBS, Sigma). Normal donkey serum (Jackson ImmunoResearch) was added to a final concentration of 10% to reduce the unspecific background staining.

Confocal microscopy specimens were analyzed with a confocal system LSM 510 Meta scan head integrated with the Axiovert 200 mol/L inverted microscope (Carl Zeiss, Jena, Germany). Specimens were observed through a 63 × 1.4 NA oil objective. Alexafluor and DyLight were excited with 488 nm argon and 543 nm He-Ne laser lines, respectively. Image acquisition was carried out in a multitrack mode, with the relevant beamsplitters; barrier filters were 505-530 band pass and 585 long pass for the above signals, respectively. A series of x-y sections was acquired with a z-step of 0.5 μm, to cover the whole height of the samples.

Presentation of results

Data were expressed as mean ± SE of 5 distinct experiments. The following parameters were measured: changes in intestinal tone (baseline intraluminal pressure), index of accommodation capacity; amplitude (area under the pressure-time curve or area under curve for each wave of contraction/relaxation), height and frequency of electrically- or distension-induced contractions/relaxations over a 15 min period immediately prior to and after 15 min incubation with each drug tested. When peristalsis, identifiable by propagating and propulsive waves of contraction (visually confirmed as rings of contraction within 1 cm from the oral end of the loop moving towards the distal end), was induced by distension, a preparatory and an emptying phase could be detected. During the preparatory phase, the intraluminal pressure increased slowly until the threshold pressure was reached and the emptying phase, marked by an abrupt increase in the intraluminal

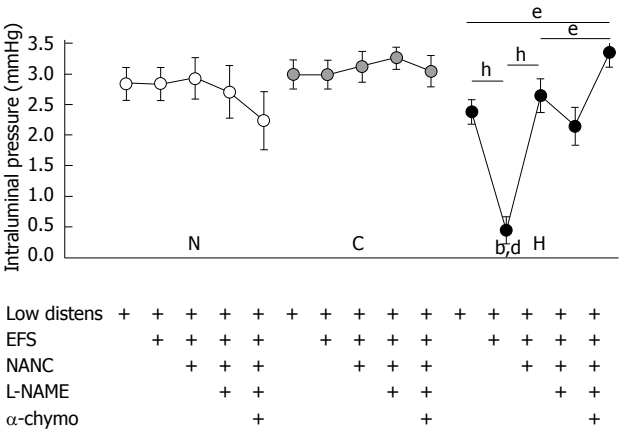

Figure 1 Basal intestinal tone. Changes in basal intestinal tone of normal (N), control (C) and hypertrophic (H) ileal segments, expressed as values of intraluminal pressure (mmHg), exposed to different subsequent conditions: hydrostatic pressure of 5 cm H_2O (Low distens), electrical field stimulation (EFS), NANC conditions (NANC), incubation with N^G-nitro-*L*-arginine methyl ester (L-NAME) 300 μmol/L (L-NAME), incubation with α-chymotrypsin 10 IU/mL (α-chymo). Each value is the mean ± SE of 5 distinct experiments per group. $^bP < 0.001$ *vs* corresponding normal values; $^dP < 0.001$ *vs* corresponding control values; $^eP < 0.05$; $^hP < 0.001$; analysis of variance test followed by Bonferroni's post-test.

pressure, was triggered (peristaltic stroke). The average compliance of the intestinal wall during the preparatory phase (volume infused in the preparatory phase/change in pressure in the preparatory phase) and the average power generated by the intestine during the peristaltic stroke (average pressure in emptying phase × volume expelled/duration of emptying phase) were measured according to Waterman *et al*[16] for 5-8 complete waves of contraction immediately prior to and after 15 min incubation with the drug. In order to calculate the average power, the volume of fluid expelled from the aboral end of the intestine by each peristaltic wave was measured with a measuring cylinder.

Statistical analysis

Data acquisition and analysis were carried out using MacLab digital data acquisition system and applying PowerLab Chart v4.1.1 software (PowerLab/4SP ADI Instruments, Ugo Basile, Comerio, VA, Italy). Statistical analysis was performed by two-way analysis of variance test followed by Bonferroni's post-test unless otherwise indicated. A P value < 0.05 was considered significant, a P value < 0.01 highly significant and a P value < 0.001 extremely significant.

Drugs

The following drugs were used: tetrodotoxin citrate (TTX), atropine sulfate, guanethidine sulfate, L-NAME, α-chymotrypsin, ketanserin tartrate, ondansetron hydrochloride dihydrate, purchased from Sigma Aldrich (St Louis, MO, United States), and GR125487 sulfamate, bought from Tocris Bioscience (Bristol, United Kingdom). All other reagents were of the highest grade commercially available. The stock solutions were prepared by dissolving all drugs in distilled water. The working solutions were prepared fresh on the day of the experiment by diluting the stock solutions with distilled water or nutritive solution. The volume of the drugs added to the organ bath was 1% of the final volume of the bath solution.

RESULTS

Motor responses to electrical field stimulation

During the equilibration period, normal, control and hypertrophic tissues exhibited no or only sporadic spontaneous motility. The raising of the outflow block to 5 cm above the level of the tissue in the bath increased the intraluminal pressure by about 2.5-3.0 mmHg in all the tissues tested (Figure 1), but it did not evoke any wave of contraction or relaxation. In normal and sham-operated segments, EFS did not further modify the intestinal tone but elicited biphasic TTX-sensitive responses comprising a transient high amplitude contraction followed by a weak relaxation (Figure 2A, B and D). In hypertrophic tissues, EFS lowered the intraluminal pressure almost to the same level as in the equilibration phase ($P < 0.001$ *vs* low distension), in contrast to that observed in normal and control tissues ($P < 0.001$) (Figure 1). EFS also evoked TTX-sensitive phasic contractions of significantly larger amplitude in hypertrophic tissue (95.5 ± 17.1 mmHg.s) compared with that in normal tissue (40.3 ± 8.9 mmHg.s, $P < 0.01$) or control tissue (56.0 ± 8.7 mmHg.s, $P < 0.05$), and which were devoid of any relaxation phase ($P < 0.01$ *vs* normal, Figure 2C and D). Incubation with atropine 1 mmol/L and guanethidine 4 mmol/L (NANC conditions) did not modify the intestinal tone in normal and sham-operated preparations but reversed the accommodation produced by EFS in hypertrophic tissues, with intraluminal pressure slowly increasing to the same level reached after raising the outflow block (Figures 1 and 2A-C). Furthermore, the amplitude of contractions in all types of tissues was markedly depressed (Figure 2D). Subsequent exposure to the NOS inhibitor L-NAME 300 mmol/L moderately, although not significantly, increased the contractile responses compared with NANC conditions, while simultaneous incubation with the protease α-chymotrypsin 10 IU/mL blocked the residual NANC motor activity in all the tissues (Figure 2D) and significantly augmented intraluminal pressure in hypertrophic segments ($P < 0.05$ *vs* low distension and *vs* NANC conditions) (Figure 1).

Distension-induced motility

Distension of the intestinal wall, produced by elevating the outflow block to 8 cm above the level of the tissue in the bath, evoked distinct motor patterns in normal and control preparations compared with hypertrophic tissue. In normal and control tissues, intraluminal pressure increased steeply by about 6.5-7.0 mmHg, and non-propulsive, local cycles of contractions and relaxations were exhibited by 75% of the preparations (Figure 3A and B). In contrast, in all hypertrophic segments, intestinal tone first gradually and slowly increased up to a threshold pressure. At this point, rhythmic, anally propagating, strong propulsive strokes, markedly wider than normal and control tissues ($P < 0.001$) (Figure 3D) and markedly

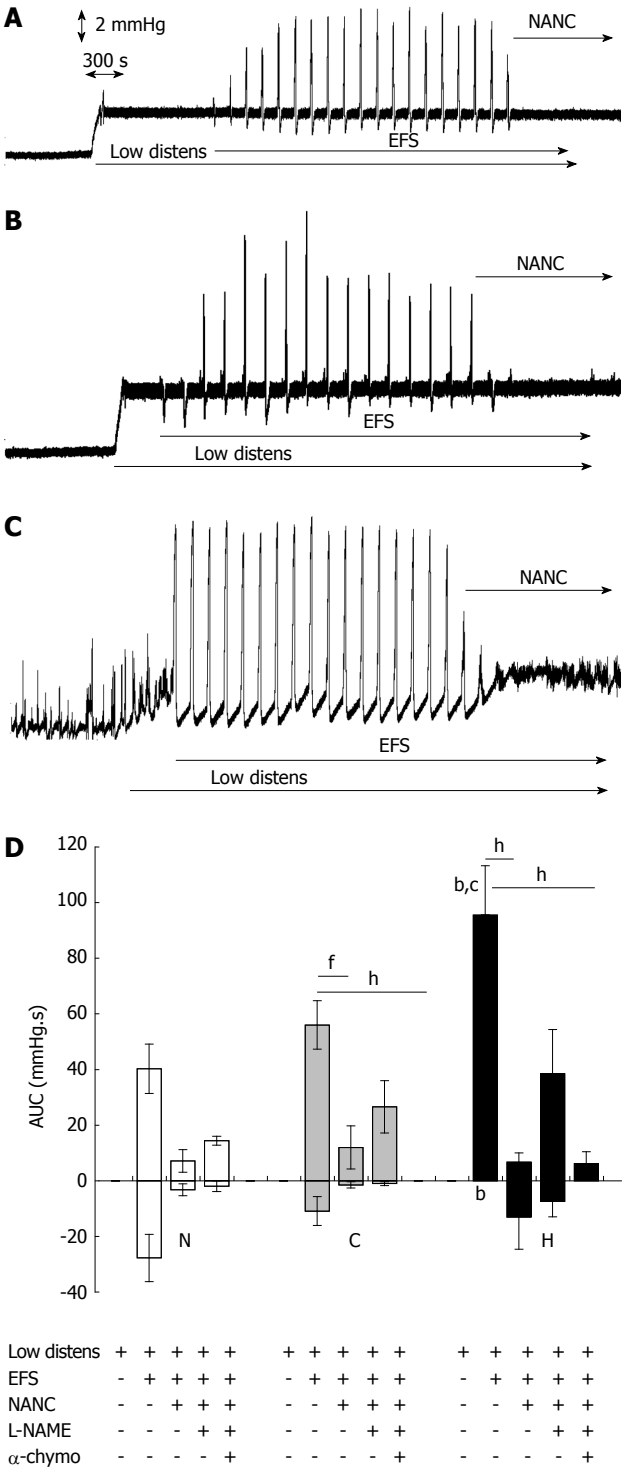

Figure 2 Motor responses to electrical field stimulation. Original tracings representing changes of intraluminal pressure of ileal segments in basal conditions and following subsequent application of a hydrostatic pressure of 5 cm H_2O (Low distens), EFS (30 V, 1 ms, 3 Hz, trains lasting 20 s every 120 s) and NANC conditions (NANC). A: Normal tissues; B: Control tissues; C: Hypertrophic tissues; D: Area under the pressure-time curve of motor responses in normal (N), control (C) and hypertrophic (H) ileal segments following subsequent application of a hydrostatic pressure of 5 cm H_2O (Low distens), EFS (30 V, 1 ms, 3 Hz, trains lasting 20 s every 120 s), NANC conditions (NANC), incubation with L-NAME 300 μmol/L (L-NAME) and incubation with α-chymotrypsin 10 IU/mL (α-chymo). Each value is the mean ± SE of 5 distinct experiments per group. $^{b}P < 0.01$ *vs* corresponding normal values; $^{c}P < 0.05$ *vs* corresponding control values; $^{f}P < 0.01$; $^{h}P < 0.001$; analysis of variance test followed by Bonferroni's post-test.

higher than normal ($P < 0.001$) or control tissue ($P < 0.05$) (Figure 3E) were elicited; simultaneously, basal intraluminal pressure progressively decreased to equilibration values (Figure 3C). Both motor patterns were blocked under NANC conditions and with simultaneous incubation with L-NAME 300 μmol/L and α-chymotrypsin 10 IU/mL (data not shown). In all types of tissues, incubation with the 5-HT_{2A}R antagonist ketanserin 1 μmol/L did not modify any parameter of the distension-induced motility (Figure 3D-F). In contrast, blockade of 5-HT_3R by ondansetron concentration-dependently reduced the amplitude, height and frequency of contractions and relaxations in normal and control tissues; in hypertrophic preparations, only the highest concentration of ondansetron tested (10 μmol/L) had an effect, with a decreased height of the peristaltic strokes without modification of their area or frequency (Figure 3D-F). Regarding antagonism of 5HT_4R by GR125487 1 μmol/L, no significant changes were produced in the distension-induced motility of any tissue (Figure 3D-F).

When the propulsive ability of hypertrophic tissues was taken into consideration, none of the 5-HTR antagonists tested modified the average compliance of the intestinal wall during the preparatory phase (Figure 4), or the efficacy of the propulsion of luminal fluid during the emptying phase, expressed by the average power (data not shown).

Immunohistochemistry

In both sham-operated and hypertrophic specimens, 5-HT_3R immunoreactivity was detected in many neurons and fibers of the ileal myenteric plexus, appearing concentrated primarily near the neuronal plasma membrane; only slight staining was observed also in the cytoplasm (Figure 5A and D). Double-labeling with immunoreactivity for 5-HT_3R and clathrin, a marker for early endosomes, whose distribution seemed mainly cytosolic (Figure 5B and E), did not show any clear significant co-localization, suggesting that in control and hypertrophic tissues the internalization of this ligand-gated ionotropic receptor appeared as an occasional event under basal conditions (Figure 5C and F).

DISCUSSION

The results of the present study indicate that hypertrophy of the intestinal wall induced by a partial chronic obstruction located at the terminal portion of rat ileum is associated with striking changes in the shape and nature of motor patterns elicited by EFS and by intraluminal distension. In particular, an increased accommodation capacity and little involvement of serotonergic neurotransmission in the control of enteric reflexes in hypertrophic tissues compared with normal and control reflexes can be hypothesized. First, when the motor responses evoked by electrical stimulation of intrinsic innervation are taken into consideration, it is worth noting that the typical biphasic profile, comprising a transient contraction fol-

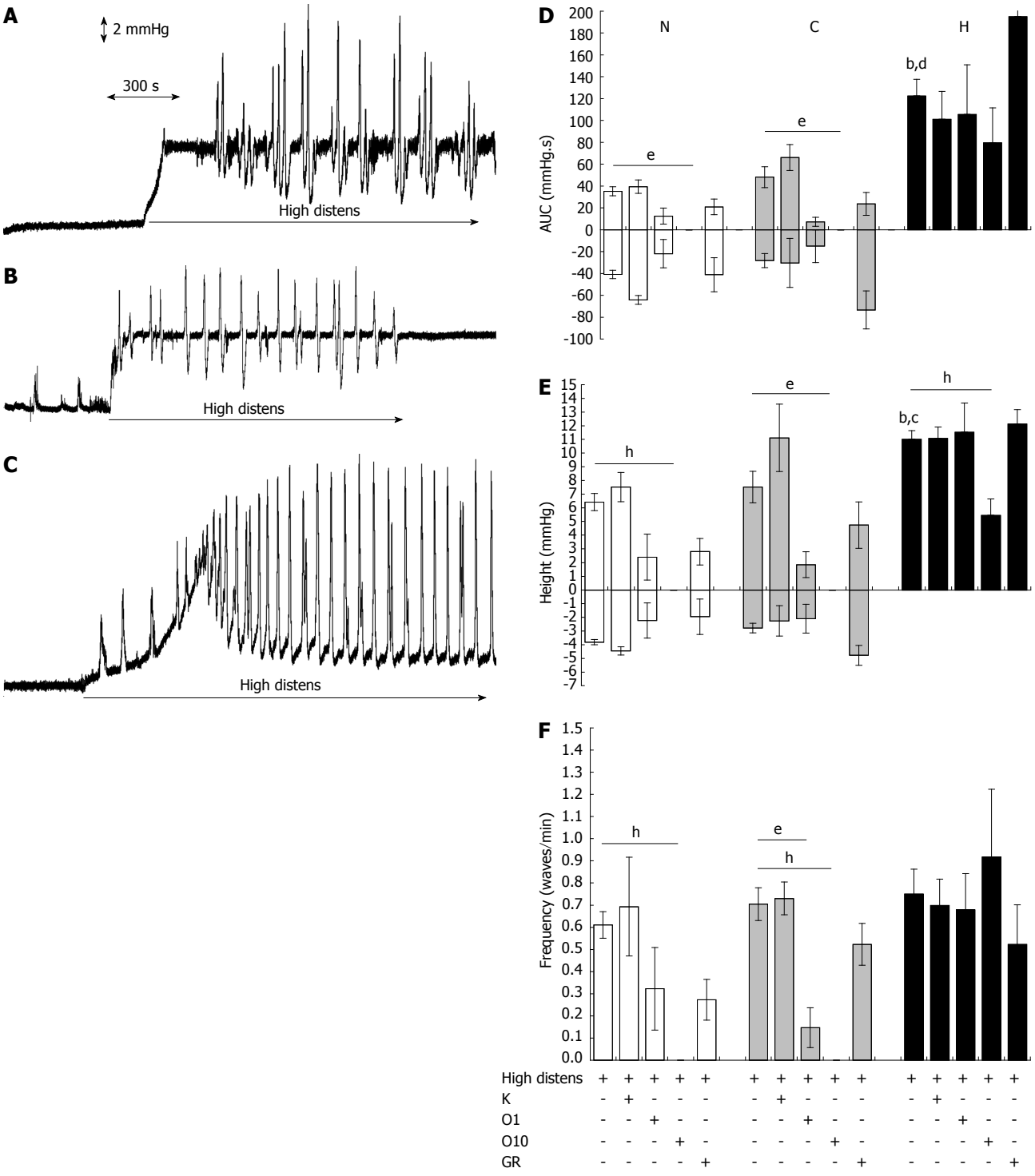

Figure 3 Distension-induced motility. Original tracings representing changes of intraluminal pressure of ileal segments in basal conditions and following application of a hydrostatic pressure of 8 cm H_2O (high distens). A: Normal tissues; B: Control tissues; C: Hypertrophic tissues; D-F: Motor responses in normal (N), control (C) and hypertrophic (H) ileal segments following application of a hydrostatic pressure of 8 cm H_2O (high distens) and incubation with ketanserin 1 μmol/L (K), ondansetron 1 (O1) or 10 (O10) μmol/L or GR125487 1 μmol/L (GR). D: Amplitude (area under the pressure-time curve or AUC); E: Height; F: Frequency. Each value is the mean ± SE of 5 distinct experiments per group. $^{b}P < 0.001$ *vs* corresponding normal values; $^{c}P < 0.05$, $^{d}P < 0.001$ *vs* corresponding control values; $^{e}P < 0.05$; $^{h}P < 0.01$. Analysis of variance test followed by Bonferroni's post-test.

lowed by a weak relaxation in normal and control tissues, was converted into a high-amplitude monophasic contraction in hypertrophic tissue. This modification, similar to that already detected in electrically stimulated hypertrophic ileum when longitudinally mounted[7], can be accounted for by two different factors: a greater force of contraction due to increased muscle mass developed by hypertrophic growth; an increased accommodation capacity as shown by the quick drop in basal intraluminal pressure at the onset of EFS. The subsequent pharmacological investiga-

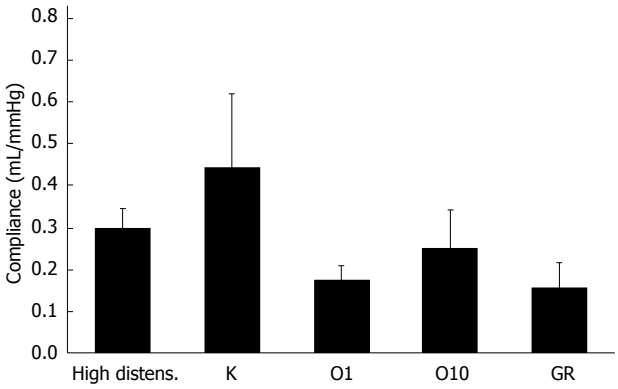

Figure 4 Average compliance of hypertrophic ileum. Changes of the average compliance of hypertrophic intestinal wall following application of a hydrostatic pressure of 8 cm H_2O (high distens.) and incubation with ketanserin 1 μmol/L (K), ondansetron 1 (O1) or 10 (O10) μmol/L or GR125487 1 μmol/L (GR). Each value is the mean ± SE of 5 distinct experiments per group.

tion confirmed the pivotal role played by acetylcholine not only as an excitatory neurotransmitter in normal, control and especially hypertrophic tissues[7], but also as one of the primary transmitters of descending interneurons, corroborating the findings collected in guinea pig small intestine[17]. Indeed, incubation with atropine and guanethidine markedly reduced the contractile phase in all preparations and nearly abolished it in hypertrophic tissues. Furthermore, in obstructed segments, atropine and guanethidine impaired the accommodation reflex elicited by electrical stimulation, by increasing the intraluminal pressure to pre-stimulation values.

Along with acetylcholine, other mediators were involved in the regulation of intestinal motility both in physiological conditions and after chronic obstruction: only simultaneous incubation with the NOS inhibitor L-NAME and the protease α-chymotrypsin completely blocked neurogenic motor responses in all the preparations and further impaired the accommodation capacity in the obstructed tissue. It is worth noting that an increased sensitivity to relaxing mediators, including nitric oxide and vasoactive intestinal peptide (VIP), was exhibited by hypertrophic smooth muscle layers compared with non-obstructed ones[11], possibly concurring with the higher accommodation capacity shown by hypertrophic ileum in these conditions.

In the second part of the research, the increase in hydrostatic pressure on the intestinal wall and the resulting distension triggered atropine- and α-chymotrypsin-sensitive typical motor patterns in normal, control and hypertrophic tissues. In particular, in chronically obstructed segments, distension evoked wide, rhythmic and propulsive peristaltic waves propagating aborally, with shape similar to those elicited by electrical stimulation in the same type of tissues and totally different from the stationary, non-propulsive clusters of cycles arising in normal and control preparations in these experimental conditions. The precise mechanisms underlying this particular motor pattern are not known and the possible contribution given by each morphological and neurochemical alteration, already thoroughly described in the hypertrophic intestine[2-5], requires further investigation. The augmented total collagen content, possibly acting as a force transducer among the components of the contractile system[18], or the higher density of inhibitory neurons expressing VIP or pituitary adenylate cyclase-activating polypeptide[3], may be only two of the reasons for this motor behavior.

The collected results seem however to be consistent with the enhanced responsiveness to contractile agents and relaxing mediators which is seen in isolated hypertrophic smooth muscle layers[11], both aspects likely playing a critical role in the powerful peristaltic reflex. According to the original idea attributed to Bulbring *et al*[13], intraluminal distension initiates the peristaltic reflex through stimulation of intrinsic sensory primary afferent neurons by 5-HT released into the wall of the gut from enterochromaffin cells. Starting from this premise it seemed interesting to investigate the role played by endogenous 5-HT in the modulation of enteric motor reflexes through the application of selective $5HT_{2A}R$, $5\text{-}HT_3R$ or $5\text{-}HT_4R$ antagonists both in non-obstructed and in hypertrophic distended ileum. The findings provided by the present work suggest that in normal and control tissues, distension-induced motility is independent of the activation of smooth muscle $5\text{-}HT_{2A}R$, mediating rat ileum contraction[19], and only slightly affected by blockade of $5HT_4R$. These receptors are chiefly located on terminals of submucosal intrinsic primary afferent neurons, where, by promoting the release of acetylcholine and calcitonin gene-related peptide, they are reported to play a critical role in facilitating the peristaltic reflex along with the putative $5\text{-}HT_{1P}R$[20], towards which no selective antagonist is currently available. Also $5\text{-}HT_3R$ are involved in peristalsis: they are mainly localized on extrinsic sensory nerves and on myenteric neurons, where they initiate giant migrating contractions[20], and may participate in the triggering and propagation of the peristaltic reflex[21,22]. The application of $5\text{-}HT_3R$ antagonist concentration-dependently inhibited distension-induced motor responses in normal and control tissues, while, only slightly at the highest tested concentration did it impair the motor reflexes activated by wall distension in hypertrophic segments, which were refractory to the other 5-HTR antagonists. With regard to this behavior, it is tempting to speculate, and worth further investigation, that the mucosa, hypertrophied following chronic obstruction[23], could contain massive levels of 5-HT, stored in enterochromaffin cells. The mediator, once released by distension of the gut wall, may strongly stimulate $5\text{-}HT_3R$, possibly leading to two effects: the initiation of the peristaltic reflex and the internalization of the ligand-gated ion channel. Indeed, a long-term increase in 5-HT content in the intestinal mucosa has been demonstrated by Freeman *et al*[24] to result in a pronounced $5\text{-}HT_3R$ internalization in myenteric neurons of rat ileum. The results obtained through confocal microscopy in the present study do not seem to support the hypothesis of a different cellular localization of $5\text{-}HT_3$ receptors in control and hypertrophic tissues: on the contrary, in the adopted experimental conditions, internalization of this receptor subtype appears equally unlikely in both kinds of preparations. This

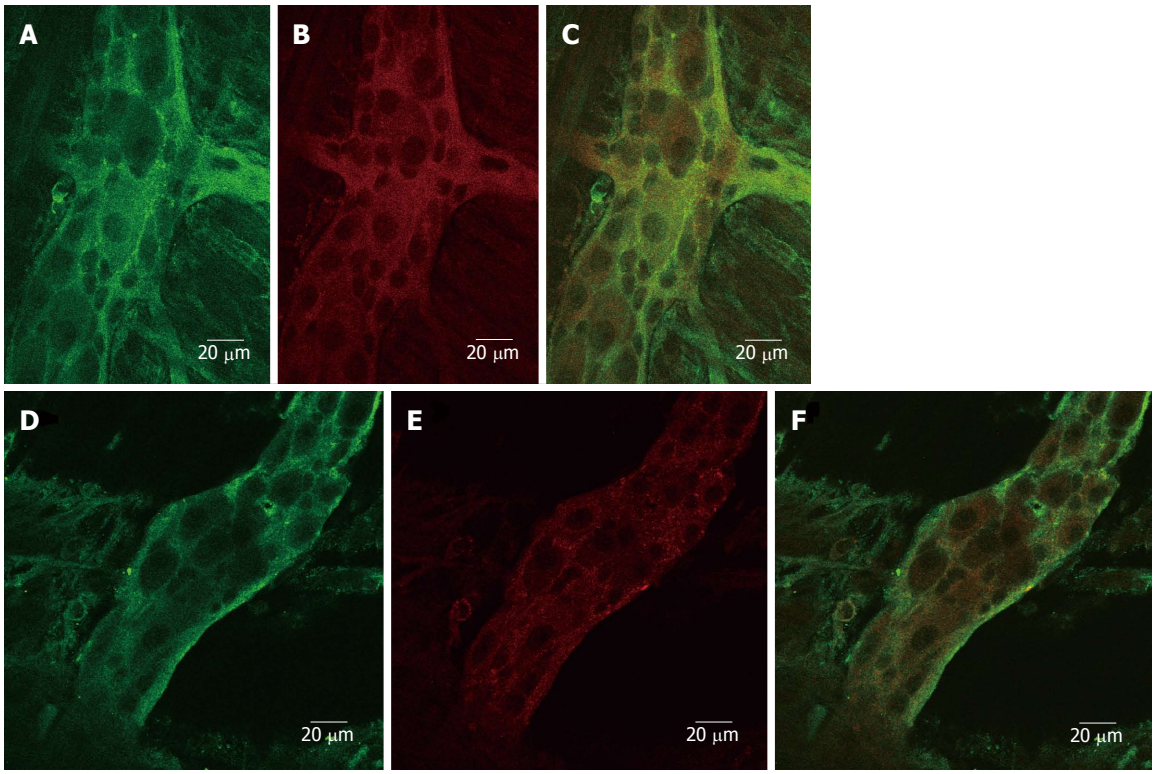

Figure 5 Cellular localization of 5-HT3 receptors in control and hypertrophic ileum. Confocal images (0.5 μm sections; scale bar 20 μm) of myenteric plexus neurons isolated from control and hypertrophic ileal tissues. A: Image showing 5-HT3 receptor immunoreactivity visualized using donkey-anti-rabbit immunoglobulin G (IgG) conjugated with AlexaFluor 488 (green); B: Image showing clathrin immunoreactivity visualized using a donkey-anti-mouse IgG conjugated with Dylight 549 (red); C: Merged images from A and B; D: Image showing 5-HT3 receptor immunoreactivity visualized using donkey-anti-rabbit IgG conjugated with AlexaFluor 488 (green); E: Image showing clathrin immunoreactivity visualized using a donkey-anti-mouse IgG conjugated with Dylight 549 (red); F: Merged images from D and E.

finding, together with the inability of ondansetron even at high concentrations to affect the peristaltic response, casts doubts over the actual involvement of 5-HT$_3$R in the motor reflexes stimulated by mechanical distension in hypertrophic ileum. Given the complexity of factors possibly altering 5-HT availability and metabolism and, therefore, also affecting the pharmacological response to 5-HT$_3$R antagonists, future studies will help to elucidate the role exerted by 5-HT in this experimental model and in intestinal secreto-motor disorders in general, where refractoriness to blockade of 5HT$_3$R has sometimes emerged[25].

In conclusion, on the basis of the findings of this study, it is rational to hypothesize that the ability of hypertrophic intestine to elicit a powerful peristaltic reflex *in vitro* is an expression of the functional plasticity of neural and muscular intestinal tissue, a property aimed at preserving its physiological role in more demanding conditions, and allowing it to maintain chyme propulsion even in the presence of a point of abnormal resistance. This distinctive motor pattern appears to be primarily controlled by cholinergic and peptidergic mediators and only slightly affected by serotonergic transmission, which is critically involved, *via* 5-HT$_3$R, in the modulation of non-propulsive motility triggered by mechanical distension in non-obstructed tissues.

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COMMENTS

Background

Intestinal obstruction is a frequently encountered clinical problem, resulting from congenital malformations (as in Hirschsprung's disease) or by an acquired obstruction, such as a surgical-induced stenosis or a neoplasm, possibly leading to death if not properly diagnosed and treated. Morphological, biomechanical and neurochemical changes have been thoroughly described orally to the site of an experimental chronic obstruction; however, functional investigations, which would help to gain a deeper insight into the regulatory mechanisms of motility in this pathological condition, are still limited.

Research frontiers

Disruption of digestive motor activity, changes in slow wave activity but also preservation of the peristaltic reflex have been functionally described in different experimental models of intestinal stenosis. In rat ileum, hypertrophic growth, induced by mechanical obstruction, has been demonstrated to affect the responses of both smooth muscle layers and of intrinsic innervation, but no information is available on its effects on a highly sophisticated and integrated process between neural and myogenic components such as intestinal peristalsis.

Innovations and breakthroughs

To the best of knowledge, this is the first report showing that, following obstruction, rat isolated terminal ileum develops a propulsive activity triggered simply by intraluminal distension. Furthermore, the peristaltic activity exhibited by rat hypertrophic ileum appears only slightly affected by serotoninergic transmission, a property quite unexpected on the basis of the pivotal role played, in

general, by endogenous serotonin in the initiation and propagation of enteric reflexes and, in particular, in the non-propulsive motility triggered by mechanical distension in non-obstructed tissues.

Applications

The results of this research improve the understanding of the changes in motility patterns and enteric reflexes triggered by intestinal obstruction, through a pharmacological investigation. Moreover, the observed refractoriness to 5-HT$_3$ antagonists makes this system an interesting model to study conditions of variable sensitivity to blockade of 5-HT$_3$ receptors, such as that described for diarrhea-predominant irritable bowel syndrome in female patients treated with alosetron.

Terminology

Peristalsis: A coordinated motor behavior, which allows the intestine to propel its contents in an anal direction; Accommodation: Indicates the ability of the intestine to adapt itself to the distension pressure, and, conversely, is a reflection of the resistance of the intestinal wall to the infused fluid; 5-HT$_3$ receptor: Ligand-gated ion channel receptor activated by serotonin or 5-HT, localized on extrinsic sensory nerves and on myenteric neurons, where they initiate giant migrating contractions and may participate in the triggering and propagation of the peristaltic reflex.

Peer review

This is a nice study, the findings are interesting and potentially important for clinical medicine. The authors showed that accommodation capacity and distension-induced peristalsis of rat hypertrophic ileum are primarily controlled by cholinergic and peptidergic transmission and negligibly by 5-HT$_3$ receptors.

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ORIGINAL ARTICLE

Role of contrast-enhanced ultrasound in follow-up assessment after ablation for hepatocellular carcinoma

Shu-Guang Zheng, Hui-Xiong Xu, Ming-De Lu, Xiao-Yan Xie, Zuo-Feng Xu, Guang-Jian Liu, Lin-Na Liu

Shu-Guang Zheng, Hui-Xiong Xu, Lin-Na Liu, Department of Medical Ultrasound, Tenth People's Hospital of Tongji University, Shanghai 200072, China
Shu-Guang Zheng, Hui-Xiong Xu, Ming-De Lu, Xiao-Yan Xie, Zuo-Feng Xu, Guang-Jian Liu, Lin-Na Liu, Department of Medical Ultrasonics, the First Affiliated Hospital, Sun Yat-Sen University, Guangzhou 510080, Guangdong Province, China
Author contributions: Zheng SG and Xu HX contributed equally to this work; Xie XY, Xu ZF and Liu GJ performed the majority of experiments; Zheng SG, Xu HX and Lu MD provided analytical tools and were also involved in revising the manuscript; Xu HX and Lu MD collected all the human materials and provided financial support for this work; Zheng SG, Xu HX, Lu MD and Liu LN designed the study and wrote the manuscript.
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Correspondence to: Ming-De Lu, MD, DMSc, Department of Medical Ultrasonics, the First Affiliated Hospital, Sun Yat-Sen University, No. 58, Zhongshan Road 2, Guangzhou 510080, Guangdong Province, China. lumd@live.com
Telephone: +86-20-87765183 Fax: +86-20-87765183
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Abstract

AIM: To assess the usefulness of contrast-enhanced ultrasound (CEUS) during follow-up after percutaneous ablation therapy for hepatocellular carcinoma (HCC).

METHODS: A total of 141 patients with HCCs who received percutaneous ablation therapy were assessed by paired follow-up CEUS and contrast-enhanced computed tomography (CECT). The follow-up scheme was designed prospectively and the intervals between CEUS and CECT examinations were less than 14 d. Both images of follow-up CEUS and CECT were reviewed by radiologists. The ablated lesions were evaluated and classified as local tumor progression (LTP) and LTP-free. LTP was defined as regrowth of tumor inside or adjacent to the successfully treated nodule. The detected new intrahepatic recurrences were also evaluated and defined as presence of intrahepatic new foci. On CEUS and CECT, LTP and new intrahepatic recurrence both were displayed as typical enhancement pattern of HCC (*i.e.*, hyper-enhancing during the arterial phase and washout in the late phase). With CECT as the reference standard, the ability of CEUS in detecting LTP or new intrahepatic recurrence during follow-up was evaluated.

RESULTS: During a follow-up period of 1-31 mo (median, 4 mo), 169 paired CEUS and CECT examinations were carried out for the 141 patients. For a total of 221 ablated lesions, 266 comparisons between CEUS and CECT findings were performed. Thirty-three LTPs were detected on CEUS whereas 40 LTPs were detected on CECT, there was significant difference ($P < 0.001$). In comparison with CECT, the numbers of false positive and false negative LTPs detected on CEUS were 6 and 13, respectively; the sensitivity, specificity, positive predictive value (PPV), negative predictive value (NPV) and overall accuracy of CEUS in detecting LTPs were 67.5%, 97.4%, 81.8%, 94.4% and 92.3%, respectively. Meanwhile, 131 new intrahepatic recurrent foci were detected on CEUS whereas 183 were detected on CECT, there was also significant difference ($P < 0.05$). In comparison with CECT, the numbers of false positive and false negative intrahepatic recurrences detected on CEUS were 13 and 65, respectively; the sensitivity, specificity, PPV, NPV and overall accuracy of CEUS in detecting new intrahepatic recurrent foci were 77.7%, 92.0%, 92.4%, 76.7% and 84.0%, respectively.

CONCLUSION: The sensitivity of CEUS in detecting LTP and new intrahepatic recurrence after percutaneous ablation therapy is relatively low in comparison

with CECT.

Key words: Contrast-enhanced ultrasound; Contrast-enhanced computed tomography; Hepatocellular carcinoma; Radiofrequency ablation; Microwave ablation

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INTRODUCTION

Hepatocellular carcinoma (HCC) is the sixth most common cancer worldwide, the incidence of which is continuously increasing in both western and eastern countries[1]. Percutaneous ablation therapy, such as radiofrequency ablation (RFA) and microwave ablation (MWA), as a minimally invasive and effective treatment modality, has been accepted in the management of hepatic malignance and regarded as one of the best treatment options for patients with early stage HCC who are not suitable for resection or transplantation[2-5]. Several randomized clinical trials have also confirmed that for small HCC, treatment efficacy of thermal ablation is comparable to that of surgical resection[2,6,7].

The evaluation of treatment efficacy after percutaneous ablation therapy for HCC is essential for the determination of subsequent treatment and follow-up strategy[8], which is usually performed by means of imaging modalities such as contrast-enhanced computed tomography (CT) and magnetic resonance imaging (MRI)[2,9,10]. Both of them have been regarded as reliable and accurate imaging tools for post-treatment efficacy evaluation and follow-up[2]. However, contrast-enhanced CT (CECT) and contrast-enhanced MRI (CEMRI) are relatively expensive. In addition, CECT is unsuitable for patients with renal function impairment and allergic reaction to contrast agent. Finally, the radiation exposure in CT examination is always a concern for both clinicians and patients.

The technique of contrast-enhanced ultrasound (CEUS) has the potential to be a substitute for CECT or CEMRI. By using ultrasound contrast agents (UCAs) and contrast specific imaging techniques, CEUS is able to depict the micro- and macro-circulation in the liver and the treated lesion, thus allowing assessment of the treatment efficacy for HCC after percutaneous ablation therapy in a similar fashion with CECT or CEMRI[2,8,11-14]. Besides, UCAs are very safe and the incidence of severe hypersensitivity or allergic reaction is lower than that of current X-ray contrast agents and comparable to MR contrast agents[13,15]. CEUS has been confirmed to be comparable to CECT for characterization of HCC[16-19]. A prospective multicenter study also proved that CEUS is equal to CECT or CEMRI for the assessment of the local treatment response after ablation therapy[11,12]. After local treatment response assessment, the patient is always enrolled into a long-term follow-up scheme for surveillance of local tumor progression (LTP) or new intrahepatic foci[11,12]. In theory, CEUS may be inferior to CECT or CEMRI since the development of new foci may be multiple and may be located in different lobes of the liver, and the arterial enhancement on CEUS only lasts several seconds, thus it is hard to detect all the hypervascular lesions. In addition, the new foci in the blind areas such as liver dome may be invisible on CEUS. To our knowledge, there has been no study evaluating the role of CEUS in the follow-up assessment after percutaneous ablation therapy for HCC[2,12,19,20]. To confirm the hypothesis that CEUS might not be competent to CECT in follow-up assessment after local ablation for HCC, the study was carried out to assess the usefulness of CEUS in detecting LTP and new intrahepatic recurrence in the follow-up after ablation therapy for HCC, with CECT as the reference standard.

MATERIALS AND METHODS

Patients

Between May 2007 and March 2011, 466 consecutive patients with HCCs were referred to the institution for ultrasound (US)-guided percutaneous ablation therapy. These patients met the following enrollment criteria: (1) single HCC not greater than 6 cm in diameter; (2) multiple HCCs up to 5 in number with each tumor measuring 3 cm or smaller; (3) absence of portal venous thrombosis or extrahepatic metastases; (4) liver cirrhosis classified as Child-Pugh class A or B; and (5) prothrombin time ratio greater than 50% and platelet count greater than 60 000/mm^3 (60 × 10^9 /L). Among them, 141 patients (132 men, 9 women; mean age, 53.4 ± 12.1 years; age range, 27-81 years) were enrolled to this follow-up study after ablation. The inclusion criteria were as follows: (1) the patients had no allergic reaction to iodinated contrast agent; (2) CECT confirmed complete ablation of the tumors within 1 mo after ablation therapy; and (3) paired CEUS and CECT were performed during the follow-up and the time interval between CEUS and CECT was less than 14 d. All the data of the 141 patients, including baseline data, clinical data, and imaging data, were collected prospectively and stored in a dedicated database for further analysis. The study was approved by the Institutional Review Board, and written informed consent was obtained from all patients.

Among the 141 patients, 60 patients had recurrent HCCs after partial hepatectomy for primary HCCs and the remaining 81 patients with primary HCCs were treated by US-guided percutaneous ablation therapy as the first therapy. The diagnoses of HCC were confirmed by histopathological examination with specimens obtained from US-guided percutaneous biopsy (n = 35) or clinical data (n = 106). The clinical diagnostic criteria for HCC mainly followed the AASLD and EFSUMBS

practice guideline: the presence of typical CECT and CEUS features (*i.e.*, hyper-enhancement in arterial phase and washout in portal-venous or late phase)[2,15]. Among the clinically confirmed 106 patients, 46 were diagnosed by characteristic imaging findings on CECT and serum α-fetoprotein ≥ 200 ng/mL; 60 patients were diagnosed by history of partial hepatectomy for HCC and typical appearance of HCC recurrence on CECT. The US-guided percutaneous ablation therapies for them included radiofrequency ablation (RFA) (n = 83), percutaneous ethanol ablation (EA) (n = 29), RFA in combination with EA (n = 26), and microwave ablation (MWA) (n = 3).

Ablation techniques

Percutaneous ablation therapy for HCC was performed with local anesthesia and conscious sedation. RFA was carried out with a cooled-tip RFA ablation system (Cool-tip, Radionics, MA, United States), which is a 480 kHz alternative current generator that can produce a maximum power of 200W through a 17 Gmonopolar, cooled-tip needle electrode. The radiofrequency electrode temperature was maintained at less than 18 ℃ by the application of a circulating chilled saline solution to the cannula sheath. A single 3 cm exposed tip RFA electrode was applied[21,22]. MWA was carried out with a microwave delivery system (FORSEA; Qinghai Microwave Electronic Institute, Nanjing, China), which consisted of an MTC-3 microwave generator (FORSEA) with a frequency of 2450 MHz, a power output of 10-150 W, a flexible low-loss cable, and a 14-gauge cooled-shaft antenna. The RFA electrode or MWA antenna was firstly placed at the bottom of the tumor and withdrawn 1.5-2 cm each time to ablate the more superficial portion for large tumors. Multiple insertions were applied to treat tumors larger than 1.5 cm for RFA and 3.0 cm for MWA[21,22]. EA was performed using a Quadra-Fuse™ multi-pronged needle (Rex Medical, Radnor, PA, United States). In general, no greater than 30 mL of 95% ethanol was injected until the hyperechoic cloud covered the whole tumor. For patients with tumor adjacent to critical structures such as hilum or great vessels, RFA was performed in combination with EA. In general, EA was carried out in advance of RFA and RFA was performed 5 min after EA, the aim of which was to increase the coagulation volume whereas limit the damage to adjacent critical structures[23-25]. To prevent possible bleeding or tumor seeding, the needle track was cauterized when the RFA electrode or the MWA antenna was withdrawn. The aim of the procedure was to completely ablate the tumor along with an ablative margin of 0.5-1.0 cm[26,27].

Contrast-enhanced US examination

All the US examinations were performed by one of three skillful radiologists who had more than 7 years experience in CEUS and were unaware of clinical and other imaging information of the patients. Two US machines were used in this study. One was an Acuson Sequoia 512 machine (Siemens Medical Solutions, Mountain View, CA, United States) and the other was an Aplio XV machine (Toshiba Medical Systems, Tokyo, Japan). A 4V1 vector transducer with a frequency range of 1.0-4.0 MHz was applied for Sequoia 512 and a 375 BT convex transducer with a frequency range of 1.9-6.0 MHz was applied for Aplio XV. The installed contrast-specific imaging modes were contrast pulse sequencing (CPS) for Sequoia 512 and contrast harmonic imaging (CHI) for Aplio XV. Both modes work under low acoustic power, and the corresponding mechanical index (MI) ranges were 0.15-0.21 for CPS in Sequoia 512 and 0.05-0.08 for CHI in Aplio XV.

Baseline US (BUS) investigation in B-mode was firstly applied to scan the whole liver, including Doppler technique. Once the treated lesion was found, the lesion size, echogenicity, and location were recorded, and the images that showed best the above-mentioned features were stored digitally in the US machine. Then the transducer was moved to scan other liver to detect if there were suspected new recurrence foci and the above-mentioned features were also recorded if new foci were present. Afterward, the imaging mode was shifted to CEUS mode and the imaging settings were optimized to ensure sufficient tissue cancellation with the maintenance of adequate depth penetration, with the diaphragm remaining barely visible.

The US contrast agent used was SonoVue (Bracco, Milan, Italy), a sulfur hexafluoride-filled microbubble contrast agent. A total of 2.4 mL contrast agent was given intravenously as a 2.4 mL bolus within 2-3 s through the antecubital vein, followed by 5 mL saline flush. Upon start of the SonoVue injection, the stop clock was started and digital cine was recorded simultaneously. During early period of CEUS procedure, the transducer was firstly kept in a stable position to observe the enhancement pattern of the treated lesion and then switched to scan other liver parenchyma. The first 2 min was continuously observed and subsequent intermittent scanning was performed until the disappearance of contrast agent in liver parenchyma. According to the previous studies, the CEUS process was divided into arterial (*i.e.*, 8-30 s from the beginning of contrast agent administration), portal (31-120 s), and late (121-360 s) phases[11,15]. A second or third injection of SonoVue was performed when suspicious new foci were documented on BUS or hypoenhancing new foci were detected in the late phase on CEUS. No patient received more than 3 injections.

Contrast-enhanced CT examination

For the CT examination, the Aquilion 64-slice helical CT machine (Tokyo, Japan) was used. The intervals between CEUS and CECT examinations were less than 14 d and no additional treatment was carried out during this period. The imaging protocol for CT examinations was as follows: 0.5 mm × 64 mm collimation, 120 kV, 150-200 mAs for 64-slice helical CT examination. The standard triphasic scan procedure was used. An unenhanced helical sequence scan through the liver was performed first; thereafter nonionic iodinated contrast material (Ultravist,

Schering, Berlin, Germany) (1.5 mL/kg) was administered via antecubital vein with power injection at a rate of 4 mL/s for 64-slice helical CT. The arterial phase sequence was obtained 25-32 s after contrast material administration, followed by a portal venous phase sequence 70 s after contrast agent administration.

Image interpretation

Two of the three skillful radiologists, who had more than 7-year experience in liver CEUS, evaluated the CEUS images and two experienced radiologists, who had more than 15-year experience in liver CECT, evaluated the treatment response using the CT images. The reviewers were not involved in the US or CT scanning, and were unaware of clinical and other imaging information of the patients. The findings of the treated lesions and new intrahepatic recurrence were observed and the treatment response was evaluated. Complete ablation was defined as nonenhancement in the ablated area; otherwise, ablation was considered incomplete. During the follow-up period, local tumor progression (LTP) was defined as regrowth of tumor inside or adjacent to the successfully treated nodule, which appeared as a hyper-enhancing area during the arterial phase and wash-out during portal-late phases inside the treated lesion on CEUS or CECT[3]. Non-enhancement in the treated area was defined as LTP-free. New intrahepatic recurrence was defined as presence of intrahepatic new foci with typical enhancement pattern of HCC on CEUS or CECT (*i.e.*, hyper-enhancing during the arterial phase and wash-out in the late phase). Development of portal venous tumor thrombosis was also defined as new intrahepatic recurrence. Non-recurrence was defined as no additional HCC lesions found.

Follow-up assessment

In the study design, the local effectiveness of ablation was assessed by CEUS or CECT within one month after ablation. Only the patients with complete ablation were enrolled into the prospectively designed follow-up scheme, and those with incomplete ablation were referred to further treatment, *e.g.*, additional ablation, transcatheter arterial chemoembolization (TACE), Sorafenib, *etc.*, according to the liver function status and tumor staging.

In the follow-up scheme, all patients were evaluated simultaneously with CECT and CEUS every 3 mo for the first 6 mo. If no positive findings were present and the ablation area shrank or disappeared, follow-up at an interval of 6-12 mo was made. At the same time, all patients were also monitored monthly with abdominal color Doppler US, serum AFP, chest radiography and liver function tests for the first 6 mo, and thereafter every 3 to 6 mo. When there were suspicious findings on US (*i.e.*, enlargement of the treated area, changes in US pattern, presence of intralesional Doppler signal, and appearance of new lesion) or elevated AFP, then paired CEUS and CECT were performed to confirm the diagnosis. Once the LTP or new intrahepatic recurrences were detected, the follow-up was over and the patients were referred to further treatment.

Statistical analysis

Continuous data were expressed as mean ± SD. The χ^2 test was used to compare the differences in detecting LTP and new intrahepatic recurrence between CECT and CEUS. Two-tailed $P < 0.05$ was considered statistically significant. With CECT as the reference standard, the sensitivity, specificity, positive predictive value (PPV), negative predictive value (NPV), and overall accuracy of CEUS in detecting LTP were computed on the basis of the assessment results of the ablation lesions on each follow-up examination, and those of CEUS in detecting new intrahepatic recurrence were computed on the basis of the patients' screening results on each follow-up examination. Statistical analysis were performed using the SPSS 13.0 software package (SPSS Inc., Chicago, IL, United States).

RESULTS

After percutaneous ablation therapy, the 141 patients with 221 HCCs (the maximum diameter ranged from 0.6 cm to 5.7 cm; mean ± SD, 2.4 ± 1.0 cm) were followed up for 1-31 mo (median, 4 mo; mean ± SD, 6.7 ± 6.4 mo) after complete ablation was confirmed by CECT 1 mo after ablation. The interval between every paired CEUS and CECT examination ranged from 0 to 14 d (median, 1 d; mean ± SD, 3.1 ± 4.3 d).

During the follow-up period, the 141 patients received 169 (once, $n = 118$; twice, $n = 18$; three times, $n = 5$) paired CEUS and CECT examinations. For the 221 ablated lesions (the diameter ranged from 1.2 cm to 7.4 cm; mean ± SD, 4.1 ± 1.1 cm), 266 comparisons between CEUS and CECT findings (185 ablated lesions were examined once; 27 ablated lesions were examined twice; 9 ablated lesions were examined three times) were performed.

During the follow-up, 40 LTPs (the diameter ranged from 0.4 cm to 5.5 cm; mean ± SD, 2.0 ± 1.1 cm) and 183 new intrahepatic recurrences (the diameter ranged from 0.3 cm to 3.8 cm; mean ± SD, 1.5 ± 0.8 cm) were detected on CECT, whereas only 33 and 131 were detected on CEUS. The locations of the LTPs and the new intrahepatic recurrences are summarized in Table 1.

In the 266 comparisons between paired CECT and CEUS for all ablation lesions, CEUS determined that 233 lesions were LTP-free and all showed non-enhancement in the arterial, portal, and late phases (Figure 1A and B). The remaining 33 lesions were determined to be LTP on CEUS and all showed hyper-enhancement in the arterial phase and wash-out ($n = 28$) (Figure 2A and B) or iso-enhancement ($n = 5$) in the portal-late phases. However, on CECT, 226 lesions were determined to be LTP-free and all showed non-enhancement in the arterial, portal, and late phases (Figure 1C and D); and the remaining 40 lesions were determined to be LTP and all showed hyper-enhancement in the arterial phase and wash-out in the portal-late phase (Figure 2C and D).

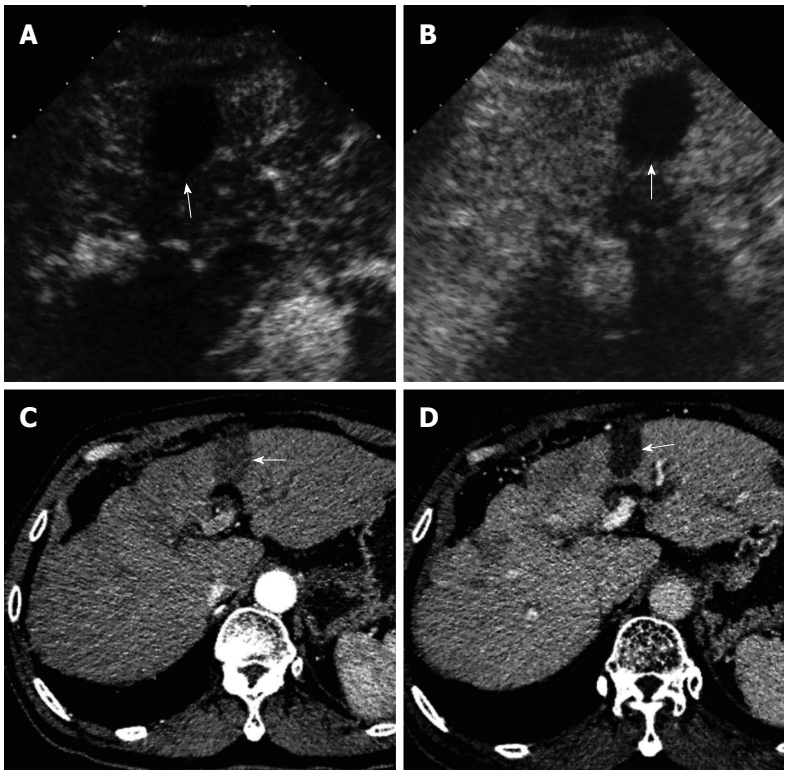

Figure 1 A 57-year-old male patient with hepatocellular carcinoma. Two months after radiofrequency ablation for hepatocellular carcinoma in segment 3 of the liver. On both contrast-enhanced computed tomography (A, B) and contrast-enhanced ultrasound (C, D), the treated lesion (arrow) showed complete necrosis without any enhancement in all vascular phases.

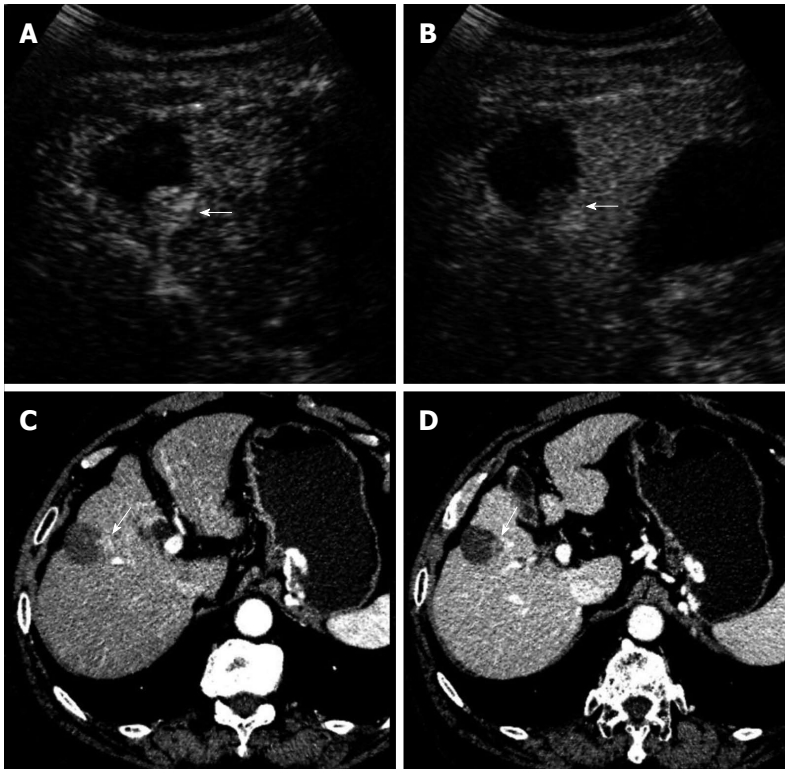

Figure 2 A 70-year-old male patient with hepatocellular carcinoma. Local tumor progression (arrow) was detected 6 mos after radiofrequency ablation in combination with ethanol ablation for hepatocellular carcinoma in segment 5 of the liver. Local tumor progression showed hyper-enhancement in the arterial phase and iso-enhancement in the portal-late phase on contrast-enhanced ultrasound (A, B), whereas hyper-enhancement in the arterial phase and wash-out in the portal-venous phase on contrast-enhanced computed tomography (C, D).

By comparing the number of the ablated lesions, there was significant difference between CECT and CEUS in detecting LTP ($P < 0.001$, Table 2). Differences between CECT and CEUS were also found in the subgroups (< 3 cm *vs* ≥ 3 cm in diameter; single *vs* multiple ablated lesions), (both $P < 0.001$) (Table 2). With CECT as the reference standard, the sensitivity, specificity, PPV, NPV and overall accuracy of CEUS in detecting LTP after percutaneous ablation were 67.5% (27/40), 97.3% (220/226), 81.8% (27/33), 94.4% (220/233), 92.9% (247/266), respectively.

A total of 183 new intrahepatic recurrences were detected on CECT and all showed hyper-enhancement in the arterial phase and wash-out in the portal-venous

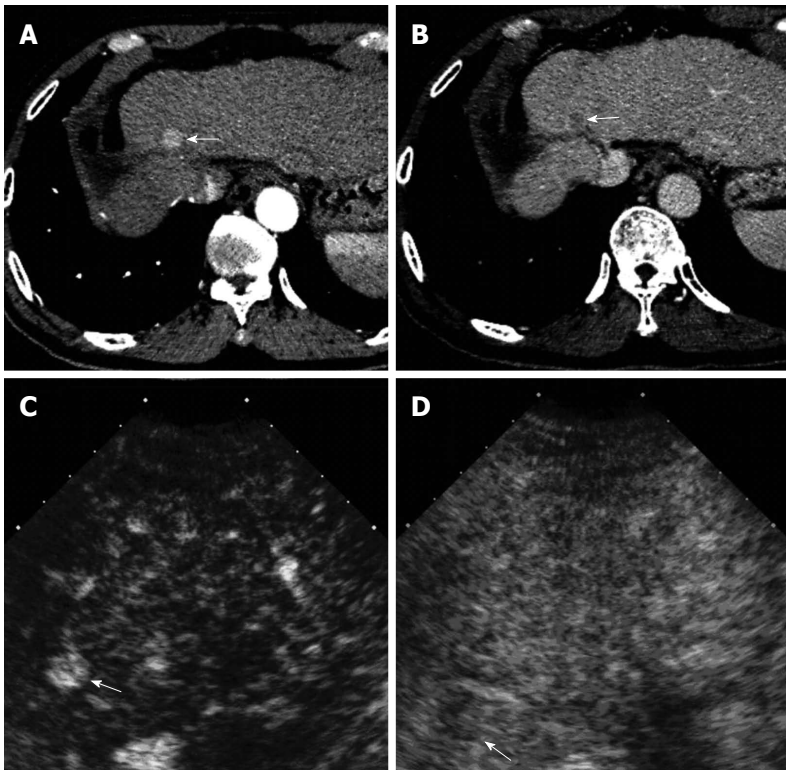

Figure 3 The same patient as shown in Figure 1. A, B: Two months after radiofrequency ablation for hepatocellular carcinoma. A new intrahepatic recurrence (arrow) was detected in segment 4 of the liver, which showed hyper-enhancement in the arterial phase and wash-out in the portal-venous phase on contrast-enhanced computed tomography; C, D: Similar findings were found in the arterial phase and the portal-late phases with contrast-enhanced ultrasound.

Table 1 Location of local tumor progression and new intrahepatic recurrence on contrast-enhanced computed tomography and contrast-enhanced ultrasound

Location	Local tumor progression		New intrahepatic recurrence	
	CEUS	CECT	CEUS	CECT
S1	0	0	1	2
S2	1	2	14	22
S3	2	2	7	13
S4	8	10	27	38
S5	5	7	17	18
S6	7	5	14	23
S7	3	4	17	18
S8	7	10	25	36
PV	0	0	9	11
HV	0	0	0	2
Total	33	40	131	183

Data show the number of the detected lesions. S: Liver segment; PV: Portal vein; HV: Hepatic vein; CEUS: Contrast-enhanced ultrasound; CECT: Contrast-enhanced computed tomography.

phases (Figure 3A and B). Conversely, only 131 recurrent lesions were detected on CEUS (Figure 3C and D). Among them, 107 lesions were in the arterial phase with hyper-enhancement (n = 104) or iso-enhancement (n = 3) and the remaining 24 lesions were missed during the arterial phase. And 124 lesions showed wash-out in the portal-late phases on CEUS and the remaining 7 lesions showed iso-enhancement.

There was significant difference between the follow-up CECT and CEUS in detecting new intrahepatic recurrence when comparing the number of the detected lesions (P = 0.02, Table 3) or the number of the patients with detected lesions (P < 0.01, Table 3). With CECT as the reference standard, the sensitivity, specificity, PPV, NPV and overall accuracy of CEUS in detecting recurrence were 77.7% (73/94), 92.0% (69/75), 92.4% (73/79), 76.7% (69/90), 84.0% (142/169), respectively. The numbers of new intrahepatic recurrence in each patient detected by each follow-up CEUS and CECT are shown in Table 4.

The numbers of false positive and false negative LTPs detected on CEUS were 6 and 13, respectively. Compared with CECT, among the 6 false positive LTPs, 5 misinterpreted hepatic blood vessels (Figure 4) and 1 new intrahepatic recurrence were misdiagnosed as LTPs. The main reasons for false negative LTPs (Figure 5) on CEUS were as follows: near liver dome and obscuration by lung gas (n = 5); deep location (n = 1; depth > 10 cm); obscuration by gastrointestinal tract gas (n = 2); obscuration by enhanced portal vein (n = 1); small lesion (n = 3, all < 0.7 cm in diameter); misdiagnosis as new intrahepatic recurrence (n = 1).

Compared with CECT, the numbers of false positive and false negative intrahepatic recurrences detected on CEUS were 13 and 65, respectively. The causes of false positive recurrences were as follows: 3 regenerative nodules, 9 misinterpreted hepatic blood vessels and 1 LTP were misdiagnosed as new intrahepatic recurrence. Among the 65 false negative recurrences, 4 regenerative nodules and 1 LTP were misdiagnosed as recurrence, and the remaining 60 new intrahepatic recurrences were missed on CEUS. Compared with CECT, the reasons for the missed new intrahepatic recurrences were as follows: multiple lesions (n = 7, > 2 in number), obscuration by gastrointestinal tract gas (n = 10); deep location (n = 4, > 10 cm in depth), near liver dome and obscuration by lung gas (n = 19), small lesion (n = 5, < 1 cm in diameter) and

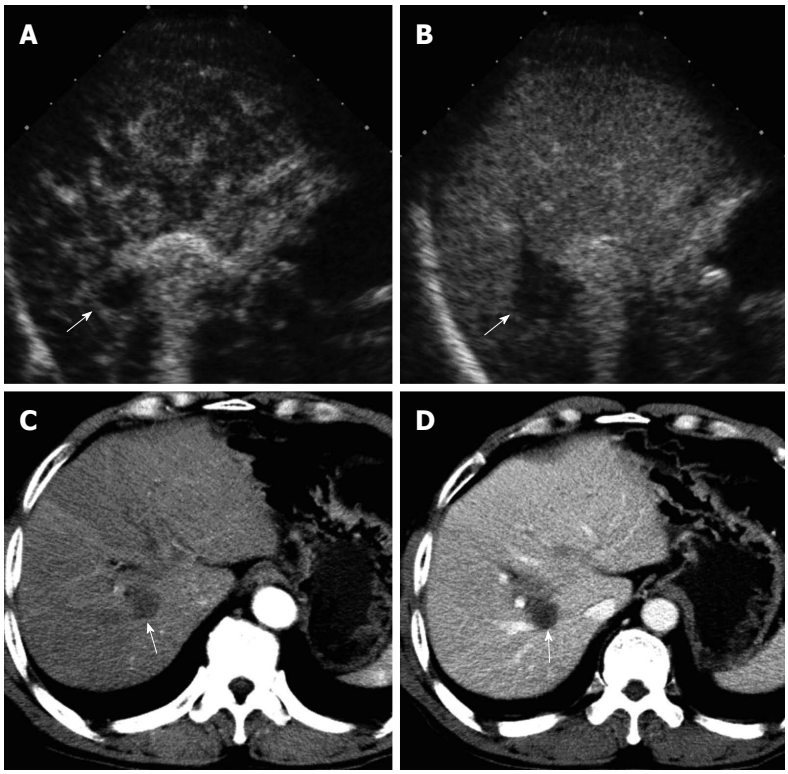

Figure 4 A 62-year-old male patient with hepatocellular carcinoma. Two months after percutaneous ethanol ablation for hepatocellular carcinoma in segment 8 of the liver. A, B: The false positive local tumor progression (arrow) was detected. It showed rim-like hyperenhancement in the arterial phase, wash-out in the portal-late phase on contrast-enhanced ultrasound; C, D: On contrast-enhanced computed tomography, the treated area (arrow) showed complete necrosis without any enhancement in all the vascular phases, but several enhanced hepatic vessels around the treated area.

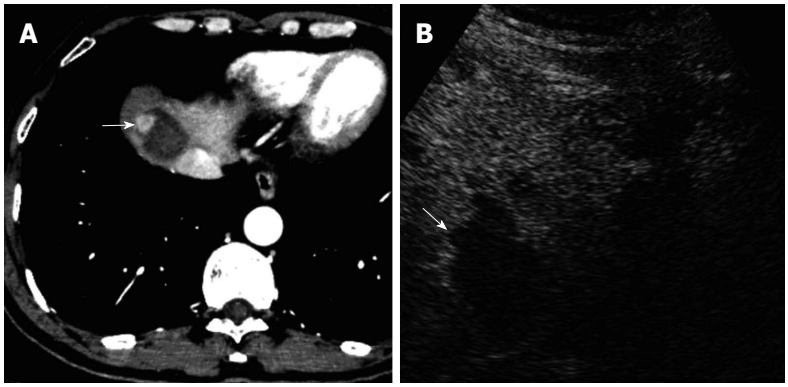

Figure 5 A 54-year-old male patient with hepatocellular carcinoma. Three months after radiofrequency ablation in combination with ethanol ablation for hepatocellular carcinoma in segment 8 of the liver. A: Contrast-enhanced computed tomography showed local tumor progression (arrow) at the periphery of the treated area; B: On contrast-enhanced ultrasound, local tumor progression (arrow) could not be detected, and the treated area was not clearly observed due to unfavorable location near the liver dome.

Table 2 Comparison between contrast-enhanced computed tomography and contrast-enhanced ultrasound in detecting local tumor progression after percutaneous ablation therapy for hepatocellular carcinoma during follow-up

CEUS	CECT														
	All			< 3 cm			≥ 3 cm			Single			Multiple		
	LTP	LTP-free	Total	LTP	LTP-free	Total	LTP	LTP-free	Total	LTP	LTP-free	Total	LTP	LTP-free	Total
LTP	27	6	33	18	4	22	9	2	11	15	5	20	12	1	13
LTP-free	13	220	233	10	165	175	3	55	58	3	74	76	10	146	156
Total	40	226	266	28	169	197	12	57	69	18	79	97	22	147	169
χ^2		125.6			86.7			32.66			48.51			70.79	
P value		< 0.001			< 0.001			< 0.001			< 0.001			< 0.001	

Data show the numbers of the ablated lesions. LTP: Local tumor progression; CEUS: Contrast-enhanced ultrasound; CECT: Contrast-enhanced computed tomography.

unknown causes (n = 15).

DISCUSSION

The treatment efficacy assessment after percutaneous ablation therapy for HCC mainly involves short-term local treatment response evaluation and long-term follow-up assessment. The short-term local treatment response evaluation is difficult as microscopic residual viable HCC is hardly detected by current imaging techniques. There-

Table 3 Comparison in number of detected new intrahepatic recurrence and lesion between contrast-enhanced computed tomography and contrast-enhanced ultrasound

CEUS	CECT		
	Yes	No	Total
New intrahepatic recurrence			
Yes	118	13	131
No	65	0	65
Total	183	13	196
Lesion			
Yes	73	6	79
No	21	69	90
Total	94	75	169

Data show the numbers of detected new intrahepatic recurrence and lesion on CEUS and CECT. The χ^2 test with Yates's correction indicated significant difference in detecting intrahepatic recurrence ($\chi^2 = 5.40$, $P = 0.02$) and lesion ($\chi^2 = 8.33$, $P < 0.01$) between CEUS and CECT. CEUS: Contrast-enhanced ultrasound; CECT: Contrast-enhanced computed tomography.

fore, follow-up scheme after ablation therapies is important. Early detection of LTP or new recurrence during follow-up after percutaneous ablation for HCC is critical and will facilitate retreatment at an early stage[3]. The short-term local treatment response evaluation is usually carried out within 1 mo after ablation therapy[2,8,28]. Contrast-enhanced imaging studies are the most widely accepted modalities to assess the local treatment response[10,12,28]. In contrast to the follow-up assessment, local treatment response focused on the specified known lesion, whereas not the whole liver. Many studies have shown that in local treatment response evaluation, CEUS is comparable with CECT or CEMRI[11,13,20].

However, up till now, no studies have been performed to evaluate the efficacy of CEUS in the follow-up assessment. This issue is very important since some centers may only use CEUS for follow-up because of the convenience of CEUS and the unwareness of the limitations of CEUS. In this study, the efficacy of CEUS in follow-up was firstly evaluated, in comparison with the widely accepted modality of CECT. The long-term follow-up assessment (up to 31 mo; mean ± SD, 6.7 ± 6.4 mo) provided a sufficient surveillance for HCC progression after ablation and the short interval (3.1 ± 4.3 d) between the paired CEUS and CECT examination was able to guarantee that the lesions were observed under the same status of vascularity for comparison between CEUS and CECT.

Many studies have confirmed the accuracy of CEUS in local treatment response evaluation, with the CECT or CEMRI as the reference standard. Among these studies, a prospective multicenter study showed that the sensitivity and the accuracy were as high as 97.0% and 94.2%, respectively[11,20]. The accuracy (92.9%) of follow-up CEUS in detecting LTP in this study was comparable to that in local treatment response evaluation, so were the specificity (97.3%), PPV (81.8%) and NPV (94.4%). However, the relatively low sensitivity (67.5%) showed that CEUS was not comparable to CECT ($P < 0.001$). The low sensitivity was partly due to short arterial phase duration of CEUS and the intrinsic shortcomings of US technique such as inability to detect the lesions in the dome or small lesions, and obscuration by gas from gastrointestinal tract or lung.

It is unknown whether CEUS is competent for the detection of new intrahepatic recurrence after HCC ablation as compared with CECT. According to the published literatures, although CEUS is comparable with CECT/MRI in the detection of liver metastasis, CEUS is incompetent to CECT for HCC surveillance, owing to the insufficient access to the lesion near the liver dome, short duration in arterial phase and the variable appearances in late phase[15,29-31]. Correas *et al*[32] found that CEUS had a sensitivity of 78% and an accuracy of 70% for detection of liver metastases by scanning entire liver parenchyma, similar to the 77.7% and 84% for detection of new intrahepatic recurrences in our study. In this study, CECT was significantly superior to CEUS for the detection of new intrahepatic recurrent foci and CEUS was unable to detect 65 (35.5%) of 183 lesions. The possible reasons might be as follows: small lesion, unfavorable location (i.e., deep location, near liver dome, near gastrointestinal tract or large hepatic blood vessel), atypical enhancement pattern, and background of fatty or cirrhotic liver[19,28,33,34].

During the routine CEUS procedure, the hepatic arterial phase starts from 10-20 s after injection of UCAs, and lasts approximately 10-15 s. Furthermore, the arterial phase presents the optimal contrast enhancement for detecting LTPs and recurrence[15]. However, the short duration of the arterial phase makes it hard to scan the whole liver and screen all suspected lesions, while CECT can scan the entire liver in a few seconds[35]. In this study, a total of 24 (18.3%, 24/131) new intrahepatic recurrences were missed in the arterial phase.

In the portal-late phases, though CEUS can guarantee sufficient duration for whole liver scan, some LTPs and new intrahepatic recurrences usually show iso-enhancement [5 (15.2%, 5/33) and 7 (5.3%, 7/131) in this study, respectively], making them indistinguishable from the surrounding liver parenchyma[28,33,36].

Besides the above-mentioned limitations of CEUS, there were some factors related to the false negative results on CEUS, *e.g.*, near liver dome and obscuration by lung gas (5 LTPs and 19 recurrences) and obscuration by gastrointestinal tract gas (2 LTPs and 10 recurrences), which usually were displayed on CECT whereas not on CEUS. On the other hand, deep location (depth > 10 cm; 1 LTP and 4 recurrences) and small size (< 1.0 cm in diameter; 1 LTP and 5 recurrences) were not easy to be detected on CEUS due to acoustic attenuation and inconspicuous enhancement. Additionally, in this study 15 recurrences were missed on CEUS and it was unable to figure out definite reason in comparison with CECT.

It was notable that the misinterpreted abnormal hepatic blood vessels due to the presence of hepatic blood vessel enhancement in the vicinity of suspicious lesion (Figure 4) was the main reason of the false positive results

Table 4 Number of new intrahepatic recurrence detected by follow-up contrast-enhanced ultrasound and contrast-enhanced computed tomography

CEUS	CECT						
N	0	1	2	3	4	5	Total
0	69 (0/0)	13 (0/13)	7 (0/14)	1 (0/3)	0 (0/0)	0 (0/0)	90 (0/30)
1	4 (4/0)	25 (25/25)	9 (9/18)	8 (8/24)	1 (1/4)	0 (0/0)	47 (47/71)
2	2 (4/0)	5 (10/5)	8 (16/16)	3 (6/9)	0 (0/0)	0 (0/0)	18 (36/30)
3	0 (0/0)	0 (0/0)	0 (0/0)	7 (21/21)	1 (3/4)	1 (3/5)	9 (27/30)
4	0 (0/0)	0 (0/0)	0 (0/0)	0 (0/0)	3 (12/12)	1 (4/5)	4 (16/17)
5	0 (0/0)	0 (0/0)	0 (0/0)	0 (0/0)	0 (0/0)	1 (5/5)	1 (5/5)
Total	75 (8/0)	43 (35/43)	24 (25/48)	19 (35/57)	5 (16/20)	3 (12/15)	169 (131/183)

N represents the number of the detected recurrence in each patient. Data are the numbers of follow-up examinations and data in parenthesis are the numbers of new intrahepatic recurrences (detected on CEUS/CECT). CEUS: Contrast-enhanced ultrasound; CECT: Contrast-enhanced computed tomography.

on CEUS, which mainly involved arteriovenous shunting or marginally enhanced artifact of large vessel and may be caused by the ablation or severe liver cirrhosis. The misinterpreted hepatic blood vessels usually showed hyperenhancement in the arterial phase and iso-enhancement in the portal-late phase on CEUS[13,34]. In this study, a total of 14 misinterpreted hepatic blood vessels were misdiagnosed as false positive LTPs (83.3%, 5/6) or new intrahepatic recurrence (69.2%, 9/13) by CEUS.

There are some limitations in this study: firstly, although the study was designed prospectively, not all the HCC patients who underwent ablation therapy received follow-up assessment by this paired CEUS and CECT examinations due to the low compliance of the patients, which may lead to bias of patient selection. Secondly, CECT was used as the reference standard in this study and not all the detected LTPs and new intrahepatic recurrences were confirmed by pathology. However, it was hard to obtain pathological results for all the lesions found in the follow-up due to the ethical concern and under current situation CECT is still acceptable to be used as the standard. Thirdly, we did not evaluate the role of CEUS using different ablation techniques. There might be some differences of the incidence of LTP or new intrahepatic recurrence between different ablation techniques such as RFA and EA, *etc.*, because they have different efficacies in treating HCC. However, the role of the study was not to evaluate the treatment efficacy of different ablation therapies, but to evaluate the ability of CEUS in treatment response assessment. In theory, the viable tumor tissue will show arterial hypervascularity on CEUS, whether it is residual tumor tissue or the recurrent tumor, and whether it is after RFA or after EA. In addition, the number of patients undergoing EA was small. Finally, further prospective study with a large number of cases is necessary to confirm the results of the present study and to evaluate the real value of CEUS in the follow-up.

In conclusion, the sensitivity of CEUS in detecting LTP and new intrahepatic recurrence after percutaneous ablation therapy is relatively low in comparison with CECT. CEUS cannot replace CECT in the follow-up assessment after percutaneous ablation for HCC.

COMMENTS

Background

For the patients with hepatocellular carcinoma (HCC) after percutaneous ablation therapy, the regular follow-up after ablation can detect local tumor progression (LTP) and new recurrence as early as possible, so as to facilitate further treatment in time, and therefore can benefit the survival. Thus the follow-up assessment, similar to surveillance and diagnosis, plays a key role in the management of HCC. However, at present, only contrast-enhanced computed tomography (CECT) and contrast-enhanced magnetic resonance imaging (CEMRI), are recommended as accurate and reliable imaging tools and applied to the follow-up assessment. Unfortunately, some factors, such as high cost, radiation and side effect of agents, limit its application.

Research frontiers

Contrast-enhanced ultrasound (CEUS) is a new imaging technique developed in recent decade. A lot of previous studies have demonstrated that CEUS is comparable to CECT and CEMRI in the area of characterization and treatment response assessment of HCC. Regarding the role of CEUS in follow-up assessment for HCC after ablation, most studies just focus on targeted lesion assessment and seldom investigate its capability of detecting LTP and new intrahepatic recurrence by scanning whole liver. Whether CEUS can be competent to this follow-up assessment is still controversial.

Innovations and breakthroughs

In most of the previous studies, CEUS has a good performance for treatment response assessment after HCC ablation, while just for the specific lesions. In this study, the authors aimed to investigate the ability of CEUS by scanning whole liver for detecting the LTPs and new intrahepatic recurrences, most of which are unknown in number, size and location, *etc*. It is concluded that CEUS is inferior to CECT in the follow-up assessment of HCC after ablation, which is mainly due to the innate defect of CEUS, such as limited acoustic penetration, display scope and relatively short duration of artery phase. Additionally, the incompetence of CEUS in the follow-up assessment might result from some traits of LTPs and new intrahepatic recurrences after HCC ablation, such as its small size, deep location, and atypical enhancement patterns.

Applications

The study results suggest that in follow-up assessment after HCC ablation CEUS can not take place of CECT and CEMRI for whole liver scanning, but can act as an adjuvant imaging tool for assessing the specific lesions.

Terminology

Treatment response assessment is performed in a month after HCC ablation by using CECT or CEMRI to assess the efficacy of ablation. Follow-up assessment: after complete ablation of HCC is confirmed by treatment response assessment, the patients will be follow-up regularly for monitoring the progression and recurrence.

Peer review

In this prospective study, the authors investigated extensively the role of CEUS in the follow-up of HCC patients undergoing radiofrequency ablations with CECT as the reference standard. The conclusion drawn by the authors is that the ability of CEUS in detecting LTP and new intrahepatic recurrence after percutaneous abla-

tion therapy is inferior to CECT. This is the first study to evaluate the role of CEUS in the follow-up assessment after percutaneous ablation therapy for HCC and the results are relevant and objective. The conclusions are very important, which indicate that people should not overestimate the role of CEUS in the follow-up even though it is meaningful in local treatment evaluation.

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ORIGINAL ARTICLE

Interference of suppressor of cytokine signaling 3 promotes epithelial-mesenchymal transition in MHCC97H cells

Yuan-Yuan Ji, Zhi-Dong Wang, Zong-Fang Li, Ke Li

Yuan-Yuan Ji, Ke Li, Scientific Research Center, the Second Affiliated Hospital, Xi'an Jiaotong University School of Medicine, Xi'an 710004, Shaanxi Province, China
Zhi-Dong Wang, Zong-Fang Li, Department of General Surgery and Engineering Research Center of Biotherapy and Translation Medicine, Shaanxi Province, the Second Affiliated Hospital, Xi'an Jiaotong University School of Medicine, Xi'an 710004, Shaanxi Province, China
Author contributions: Ji YY and Wang ZD designed the research and contributed equally to this work; Ji YY and Wang ZD performed the research; Ji YY, Wang ZD and Li K contributed to materials and methods; Ji YY and Li K analyzed the data; Ji YY, Wang ZD and Li ZF wrote the manuscript.
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Correspondence to: Zhi-Dong Wang, MD, PhD, Department of General Surgery and Engineering Research Center of Biotherapy and Translation Medicine, Shaanxi Province, the Second Affiliated Hospital, Xi'an Jiaotong University School of Medicine, No. 157, West 5th Road, Xi'an 710004, Shaanxi Province, China. xawzd@163.com
Telephone: +86-29-87679386 Fax: +86-29-87679386
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Abstract

AIM: To investigate the role of suppressor of cytokine signaling 3 (SOCS3) silencing in epithelial-mesenchymal transition (EMT) involved in a human hepatocellular carcinoma MHCC97H cell line.

METHODS: MHCC97H cells were transiently transfected with SOCS3 small-interfering RNA (siRNA). Morphological changes of the transfected cells were observed under microscope. Expressions of E-cadherin, Vimentin and α-smooth muscle actin (α-SMA) were identified with immunofluorescence. Furthermore, protein expressions and mRNA levels of characteristic markers of EMT (E-cadherin, Vimentin, α-SMA and Snail) were detected by Western blotting, quantitative real-time polymerase chain reaction. Transforming growth factor-β1 (TGF-β1) levels in the supernatant were measured with enzyme-linked immunosorbent assay.

RESULTS: The transfected cells with SOCS3 siRNA showed a morphological alteration from a typical cobblestone morphology to mesenchymal spindle-shaped and fusiform features. SOCS3 siRNA lessened immunofluorescent expression of E-cadherin, but elicited immunofluorescent expressions of Vimentin and α-SMA in MHCC97H cells. More importantly, compared with the negative control, depletion of SOCS3 resulted in the decrease of the epithelial marker E-cadherin ($P < 0.05$), and the increase of the mesenchymal markers Vimentin and α-SMA and the transcription factor Snail in MHCC97H cells ($P < 0.05$). Moreover, compared with the negative control, SOCS3 siRNA evidently enhanced TGF-β1 secretion in MHCC97H cells (200.20 ± 29.02 pg/mL *vs* 490.20 ± 92.43 pg/mL, $P < 0.05$).

CONCLUSION: SOCS3 silencing is able to promote EMT in MHCC97H cells *via* changing the phenotypic characteristics and modulating the characteristic markers.

Key words: Hepatocellular carcinoma; Epithelial-mesenchymal transition; Suppressor of cytokine signaling; E-cadherin; Snail

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INTRODUCTION

Hepatocellular carcinoma (HCC), the primary malignancy of the liver, ranks the sixth in incidence and the third in cancer-related deaths worldwide[1]. Poor prognosis of HCC is associated with a high potential of vascular invasion, metastasis, and recurrence even after curative surgical resection[2]. The main cause of death in HCC patients is intrahepatic metastasis, but the underlying mechanism is still not fully understood. Epithelial-mesenchymal transition (EMT) is the morphological and molecular change that occurs when epithelial cells lose their characteristics, gain mesenchymal properties and become motile, which is a key event in tumor invasion and metastasis[3-5]. The most common character of EMT is that the cells turn to spindle-like morphology from compact and well-arranged epithelial structure[6]. The epithelial marker E-cadherin plays a central role in cell-cell adhesion junctions in maintenance of cell polarity[7-9]. Loss of E-cadherin expression is commonly related to tumor invasiveness, metastasis and poor prognosis, including HCC[8]. Furthermore, the expression of mesenchymal markers such as vimentin and α-smooth muscle actin (α-SMA), along with the expression of transcription factors such as Snail is an essential molecular marker of EMT[10]. Snail is able to repress E-cadherin and overexpression of Snail has been reported to be correlated with HCC metastasis through the induction of EMT[11,12]. Moreover, the levels of transforming growth factor-β1 (TGF-β1) are very high in many cancer cells, which are related to EMT and a high incidence of metastasis[4]. In particular, TGF-β1 induced-EMT has been found to be associated with HCC invasion and metastasis[13]. EMT is being increasingly recognized as a crucial step that promotes tumor invasiveness and metastasis, thus understanding the influential factor of EMT could allow the development of novel therapies targeting at HCC invasion and metastasis.

Suppressor of cytokine signaling (SOCS) family proteins have been implicated in the negative regulation of various cytokines[14,15]. Recently, emerging evidence suggests that SOCS may be tumor suppressors. It has been postulated that SOCS can decelerate or inhibit the progression of cirrhosis and HCC[16,17]. Intriguingly, SOCS3 silencing is a significant predictor or poor survival indicating that SOCS3 might play a special role in limiting late-stage HCC progression[18]. Although SOCS3 may be involved in the suppression of tumor growth and metastasis of HCC[19,20], its role in hepatoma carcinoma cells has not been completely established. Thus, it will be of interest to learn more about whether SOCS3 depletion is able to promote EMT of HCC. The objectives of the present study were to primarily investigate the role of SOCS3 silencing in EMT involved in MHCC97H cells.

MATERIALS AND METHODS

Reagents

Dulbecco's modified Eagle's medium (DMEM) and fetal bovine serum (FBS) were purchased from Gibco BRL (Carlsbad, CA, United States). Polyclonal anti-human E-cadherin, Vimentin, Snail, SOCS3, anti-α-SMA and anti-β-SMA antibodies were products of Santa Cruz Biotechnology (Santa Cruz, CA, United States). Human TGF-β1 enzyme-linked immunosorbent assay (ELISA) kit was obtained from Invitrogen (Carlsbad, CA, United States). siRNA specific for SOCS3 (siGENOME SMARTpool, M-004299-08-0005) and negative control siRNA (siGENOME Non-Targeting siRNA Pool, D-001206-13-05), siGLO Green (6-FAM) Transfection Indicator (D-001630-01-05), DharmaFECT 4 transfection reagent (T-2002-04), and siGLO Green (6-FAM) Transfection Indicator (D-001630-01-05) were purchased from Dharmacon (Lafayette, CO, United States)

Cell culture

Human MHCC97H cell, a typical HCC cell line with a high metastatic potential[21], was obtained from Liver Cancer Institute of Fudan University (Shanghai, China). MHCC97H cells were cultured with DMEM supplemented with 10% FBS in a humidified incubator at 5% CO_2 and 37 ℃.

Small-interfering RNA transfection

MHCC97H cells (1×10^5) were seeded into 6-well plates and were grown until 60%-80% confluent. The cells were transiently transfected with 25 nmol/L of SOCS3 small-interfering RNA (siRNA) or negative control siRNA (NC siRNA) using Dharma FECT 4 transfection reagents according to the manufacturer's instructions. After 24 h, fluorescent images of transfected cells were observed under fluorescence microscope (Nikon, Japan). After 48 h, protein expression and mRNA levels of SOCS3 were detected by Western blotting, quantitative real-time polymerase chain reaction (PCR) and reverse transcription (RT)-PCR. Transfection rates of 60%-70% of the cells were accepted for all the experiments.

Examination of morphological changes

After application of NC siRNA or SOCS3 siRNA for 48 h, morphological changes of the transfected cells were observed under an inverted phase-contrast microscope (Nikon, Japan). The photographs were taken at 100× and 200× magnifications by a digital camera.

Immunofluorescence

MHCC97H cells were plated on cover slips and grown to confluence, and were transiently transfected with 25 nmol/L of SOCS3 siRNA or NC siRNA for 48 h. After the treatment, the cells were fixed with 4% formaldehyde-PBS for 15 min. The cell membranes were fenestrated with 0.3% Triton-100-PBS, and nonspecific binding sites were blocked with 10% goat serum. The cells were incubated with anti-human antibody including E-cadherin (1:100) or Vimentin (1:200) or α-SMA (1:100) and then incubated with the secondary antibody conjugated to fluorescein isothiocyanate. The immunolabeled cells were observed under fluorescence microscope (Nikon, Japan).

Table 1 Primers used for real-time polymerase chain reaction analysis

Gene	Primer sequence	Accession number	Expected size (bp)
E-cadherin	5′-CCCGGGACAACGTTTATTAC-3′ 5′-GCTGGCTCAAGTCAAAGTCC-3′	NM_004360.3	190
Vimentin	5′-AAAGTGTGGCTGCCAAGAAC-3′ 5′-AGCCTCAGAGAGGTCAGCAA-3′	NM_003380.2	200
α-SMA	5′-GCGCAAATACTCGGTGTGGA-3′ 5′-CCCCCCCATTGAGAAGATTC-3′	NM_001141945.1	170
Snail	5′-TGGTTGCTTCAAGGACACAT-3′ 5′-GTTGCAGTGAGGGCAAGAA-3′	NM_003068.3	141
SOCS3	5′-CAGGAATGTAGCAGCGATGGAA-3′ 5′-CCTGTCCAGCCCAATACCTGA-3′	NM_003955.3	125
β-actin	5′-ATCGTGCGTGACATTAAGGAGAAG-3′ 5′-AGGAAGGAAGGCTGGAAGAGTG -3′	NM_001101	179

α-SMA: α-smooth muscle actin; SOCS3: Suppressor of cytokine signaling 3.

ELISA

After application of NC siRNA or SOCS3 siRNA for 48 h, concentration of TGF-β1 in the supernatant of the cells was measured by ELISA kits according to the manufacturer's instructions.

Western blotting analysis

As described previously[22], protein samples (25 μg) were separated on sodium dodecyl sulfate polyacrylamide gel electrophoresis gels and transferred onto a polyvinylidene difluoride membrane (Bio-Rad Laboratories, Hercules, CA, United States). The membranes were blocked with 5% nonfat dry milk in Tris-buffered saline containing 0.1% Tween 20, and incubated with specific antibodies against E-cadherin (1:200), Vimentin (1:200), Snail (1:100), SOCS3 (1:200), α-SMA (1:400) and β-actin (1:400). The expression of β-actin was used as a loading control. Reagents (Pierce Corp., Rockford, IL, United States) for the enhanced chemiluminescence were applied to the blots, and the light signals were detected by X-ray film. Optical densities of the bands were scanned and quantified with the Syngene Gene Tools (Syngene Corp., Cambridge, United Kingdom). Three independent experiments were carried out to study protein expression.

Quantitative real-time PCR and RT-PCR

mRNA levels were determined by our previous method[22]. Total RNA was isolated using a TRIzol Kit (Invitrogen Corp., Carlsbad, CA, United States). cDNA was synthesized from 1 μg samples of total RNA using Revert Aid™ First Strand cDNA Synthesis Kit (Fermentas, St. Leon-Rot, Germany) following the manufacturer's instructions. Real-time PCR was performed with the SYBR Premix Ex Taq™ Ⅱ Perfect Real Time kit (Takara, Japan) on an ABI QPCR System (Applied Biosystems, CA, United States) following the manufacturer's instructions. The samples were run in triplicate. Primers for human E-cadherin, Vimentin, Snail, SOCS3, α-actin and β-actin were designed with Beacon designer v 4.0 (Premier Biosoft, United States) (Table 1). Traditional PCR was performed according to the manufacturer's instructions. The RT-PCR products were analyzed by electrophoresis through 2% agarose gels containing ethidium bromide. A melting point dissociation curve generated by the instrument was used to confirm that only a single product was present. Quantization of relative gene expression was calculated by the comparative Ct method ($2^{-\Delta\Delta CT}$) as described by the manufacturer. Data were normalized to human β-actin mRNA levels. Three independent experiments were carried out to study mRNA levels.

Statistical analysis

All data were expressed as the mean ± SD from three different experiments. Statistical analysis were carried out with Student's *t* test for independent samples. In all cases, a value of $P < 0.05$ was considered statistically significant.

RESULTS

Fluorescent detection of transfected cells

After MHCC97H cells were transiently transfected with SOCS3 siRNA (25 nmol/L) and siGLO Green Transfection Indicator (50 nmol/L) for 24 h, fluorescent expression was observed under fluorescence microscope. Compared with the control, the transfected cells with SOCS3 siRNA showed a green fluorescence (Figure 1). The results illustrated that SOCS3 siRNA had been successfully transfected into MHCC97H cells.

Effect of SOCS3 siRNA on morphological changes of MHCC97H cells

To investigate the role of SOCS3 in morphological changes of MHCC97H cells, the cells were transiently transfected with NC siRNA or SOCS3 siRNA for 48 h. As shown in Figure 2, knockdown of SOCS3 resulted in a significant change in cell morphology, as demonstrated by phase-contrast microscopy, with transition from a typical cobblestone morphology to mesenchymal spindle-shaped and fusiform features. The acquisition of a fibroblastic morphology suggested that MHCC97H cells could undergo the mesenchymal change after treated with SOCS3 siRNA.

Effect of SOCS3 siRNA on immunofluorescent expressions of E-cadherin, Vimentin and α-SMA in MHCC97H cells

To examine the effect of SOCS3 on immunofluorescent expressions of E-cadherin, Vimentin and α-SMA in MHCC97H cells, the cells were transiently transfected with NC siRNA or SOCS3 siRNA for 48 h, and expressions of E-cadherin, Vimentin and α-SMA were identified with immunocytofluorescence. The results showed that SOCS3 siRNA might lessen immunofluorescent expression of E-cadherin, but elicit immunofluorescent expressions of Vimentin and α-SMA in MHCC97H cells (Figure 3).

Effect of SOCS3 siRNA on characteristic markers of EMT in MHCC97H cells

To further explore the impact of SOCS3 on characteristic markers of EMT in MHCC97H cells, the cells were

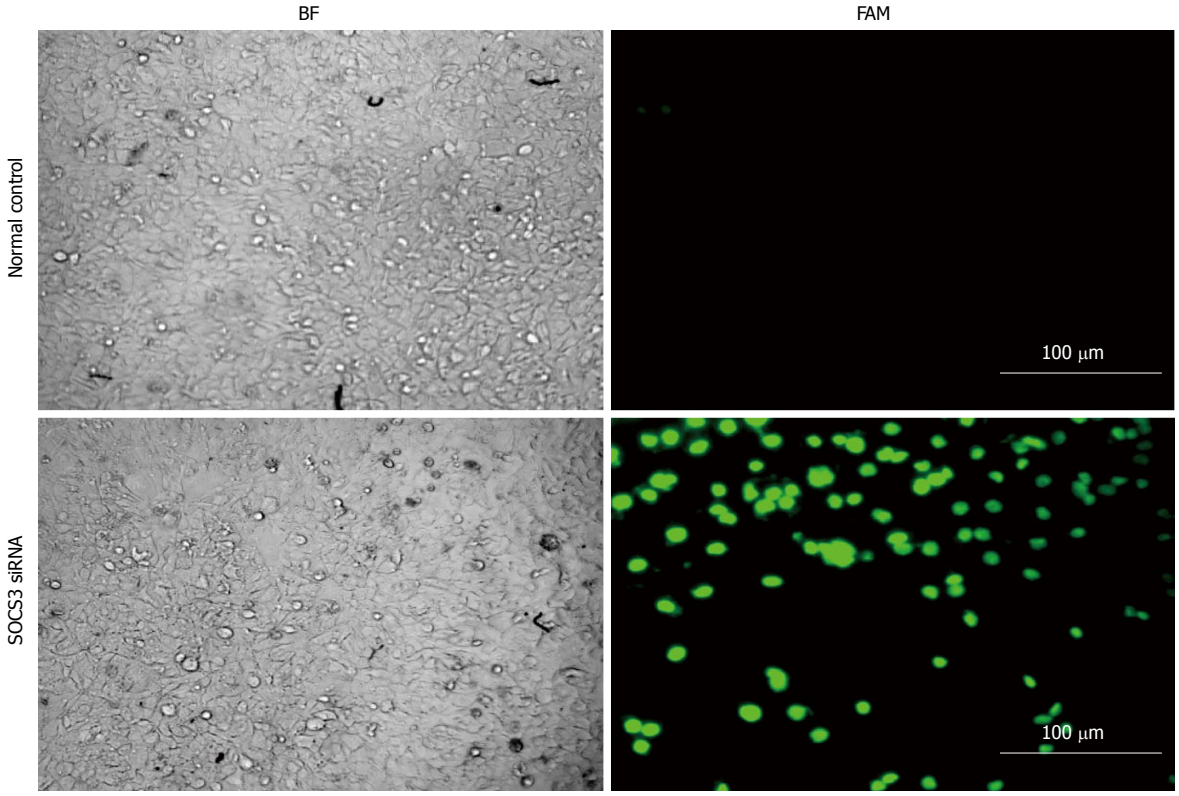

Figure 1 Fluorescent detection of transfected cells. MHCC97H cells were transiently transfected with suppressor of cytokine signaling 3 (SOCS3) small-interfering RNA (siRNA) for 24 h, green fluorescence was observed under fluorescence microscope. BF: Bright field; FAM: Fluorescence from fluorescein-labeled scramble-siRNA.

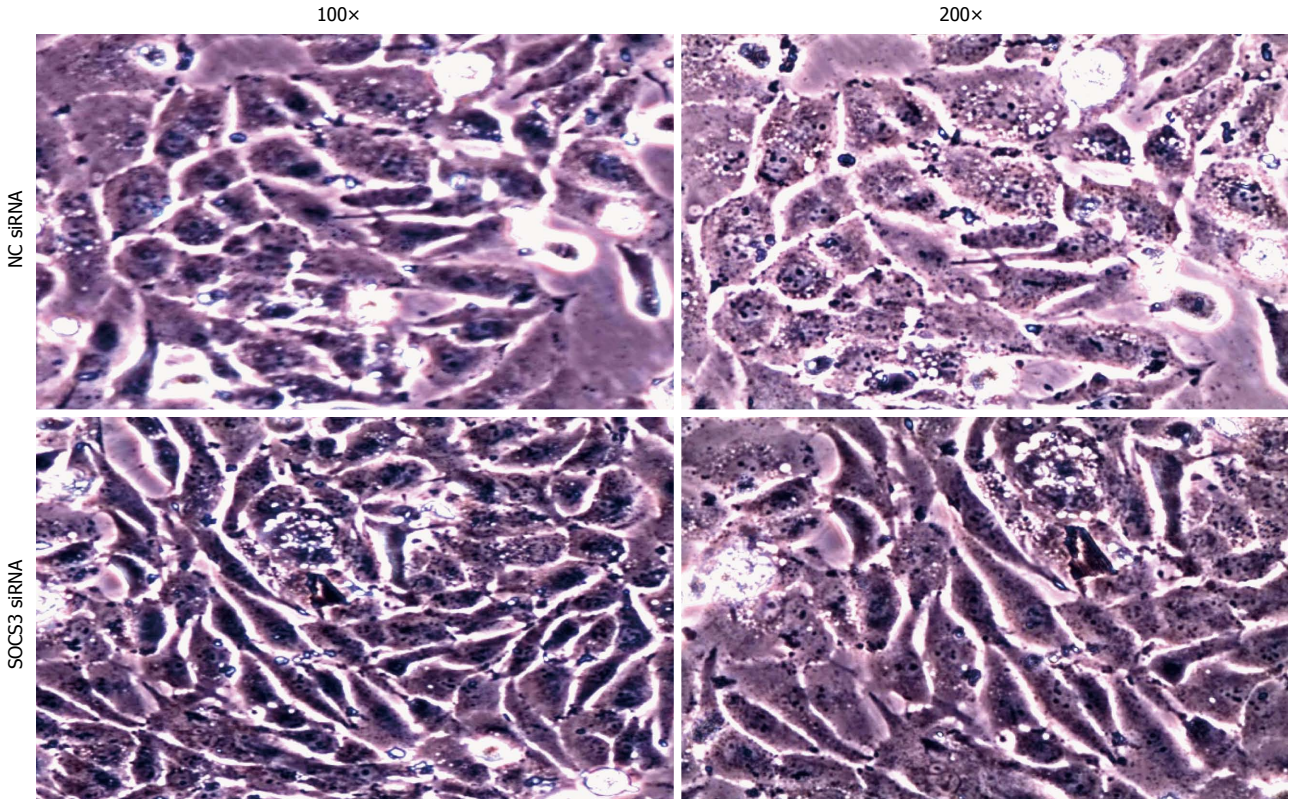

Figure 2 Effect of suppressor of cytokine signaling 3 small-interfering RNA on morphological changes of MHCC97H cells. After application of negative control (NC) small-interfering RNA (siRNA) or suppressor of cytokine signaling 3 (SOCS3) siRNA for 48 h, morphological changes of cells were recorded with an inverted phase-contrast microscope.

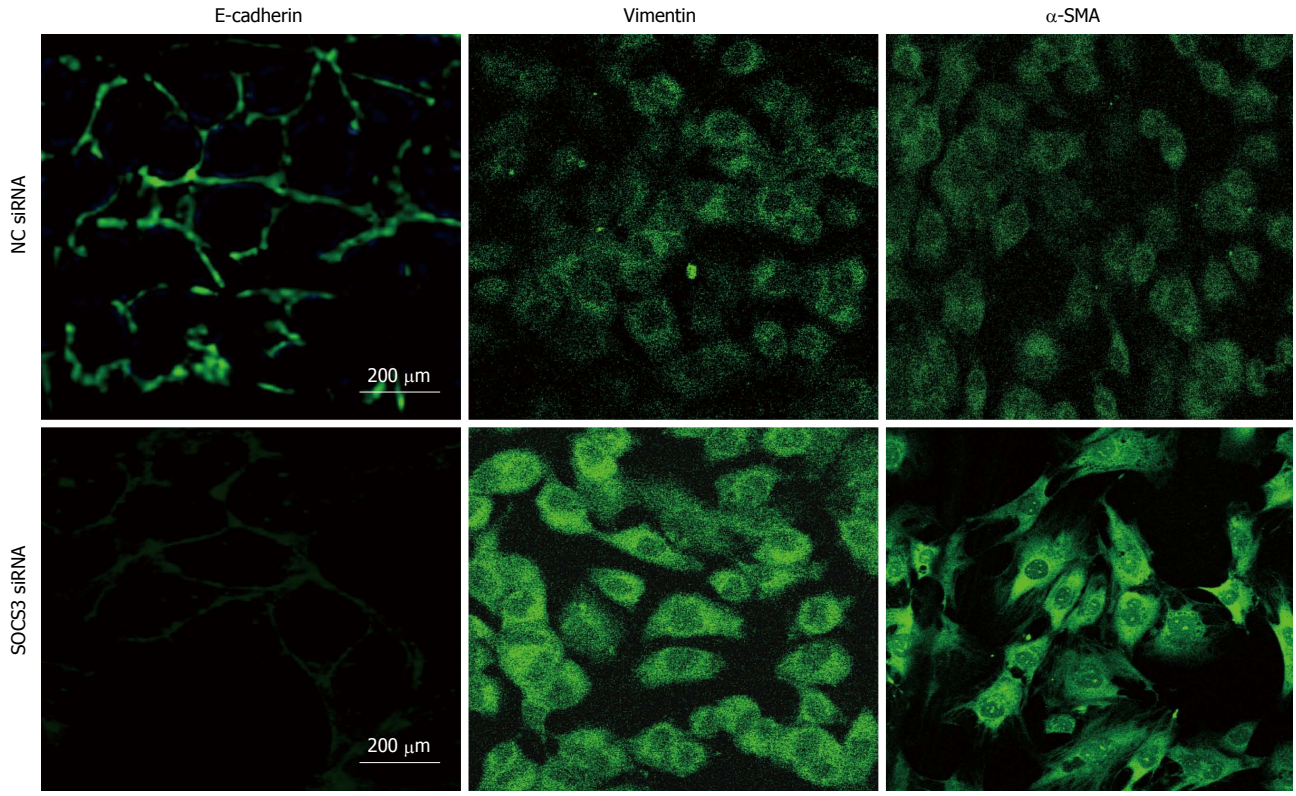

Figure 3 Effect of suppressor of cytokine signaling 3 small-interfering RNA on immunofluorescent expressions of E-cadherin, Vimentin and α-smooth muscle actin in MHCC97H cells. The cells were transiently transfected with negative control (NC) small-interfering RNA (siRNA) or suppressor of cytokine signaling 3 (SOCS3) siRNA for 48 h, expressions of E-cadherin, Vimentin and α-smooth muscle actin (SMA) were identified with immunofluorescence.

transiently transfected with NC siRNA or SOCS3 siRNA for 48 h, and protein expression and mRNA levels of SOCS3 were detected. Knock down efficiency of SOCS3 was 69.2% or 63.5% as determined by Western blot or quantitative real-time PCR ($P < 0.05$) (Figure 4A and B). These results showed that SOCS3 siRNA could efficiently reduce protein expression and mRNA levels of SOCS3. Meanwhile, depletion of SOCS3 resulted in the decrease of the epithelial marker E-cadherin, and the increase of the mesenchymal markers Vimentin, α-SMA and Snail in MHCC97H cells ($P < 0.05$) (Figure 4C and D).

Effect of SOCS3 siRNA on TGF-β1 levels in MHCC97H cells

The above characteristic markers of EMT (E-cadherin, Vimentin, α-SMA and Snail) modulate the process of EMT, and TGF-β1 is believed to play a major role in this process. We then observed TGF-β1 levels in MHCC97H cells after SOCS3 silencing. As described in Figure 5, compared with the negative control, depletion of SOCS3 remarkably aggravated TGF-β1 secretion in MHCC97H cells (200.20 ± 29.02 pg/mL *vs* 490.20 ± 92.43 pg/mL, $P < 0.05$), suggesting that SOCS3 is able to decrease TGF-β1 generation of MHCC97H cells.

DISCUSSION

The present study demonstrated that the transfected cells with SOCS3 siRNA showed a morphological alteration from a typical cobblestone morphology to mesenchymal spindle-shaped and fusiform features, suggesting that MHCC97H cells might adopt the mesenchymal cell phenotype. More importantly, we found that lack of SOCS3 resulted in the decrease of the epithelial marker E-cadherin, and the increase of the mesenchymal markers Vimentin and α-SMA and the expression of transcription factor Snail in MHCC97H cells. In addition, SOCS3 siRNA evidently enhanced TGF-β1 secretion in MHCC97H cells. These results reveal that interference of SOCS3 is able to facilitate EMT involved in MHCC97H cells.

SOCS3 functions as a negative regulator of the Jak/Stat signal transduction pathway and has been described as a tumor suppressor in human cancers[23,24]. It has also been reported that SOCS3 silencing plays a role in human HCC and SOCS3 has a tumor suppressor role in mouse models of HCC[25]. So far, the relationship between SOCS3 and EMT in HCC is still elusive. As a result, our present study has focused on the effect of SOCS3 silencing on EMT in MHCC97H cells. We found for the first time that SOCS silencing was associated with EMT process in MHCC97H cells because of changes of the phenotypic characteristics.

EMT is a key and dynamic process that facilitates invasion of tumor cells. In addition to changes of the phenotypic characteristics, tumor cells are reported to gain the molecular characteristics of EMT including down-regulation of epithelial marker (E-cadherin) and up-regulation of mesenchymal markers (Vimentin and α-SMA)[26].

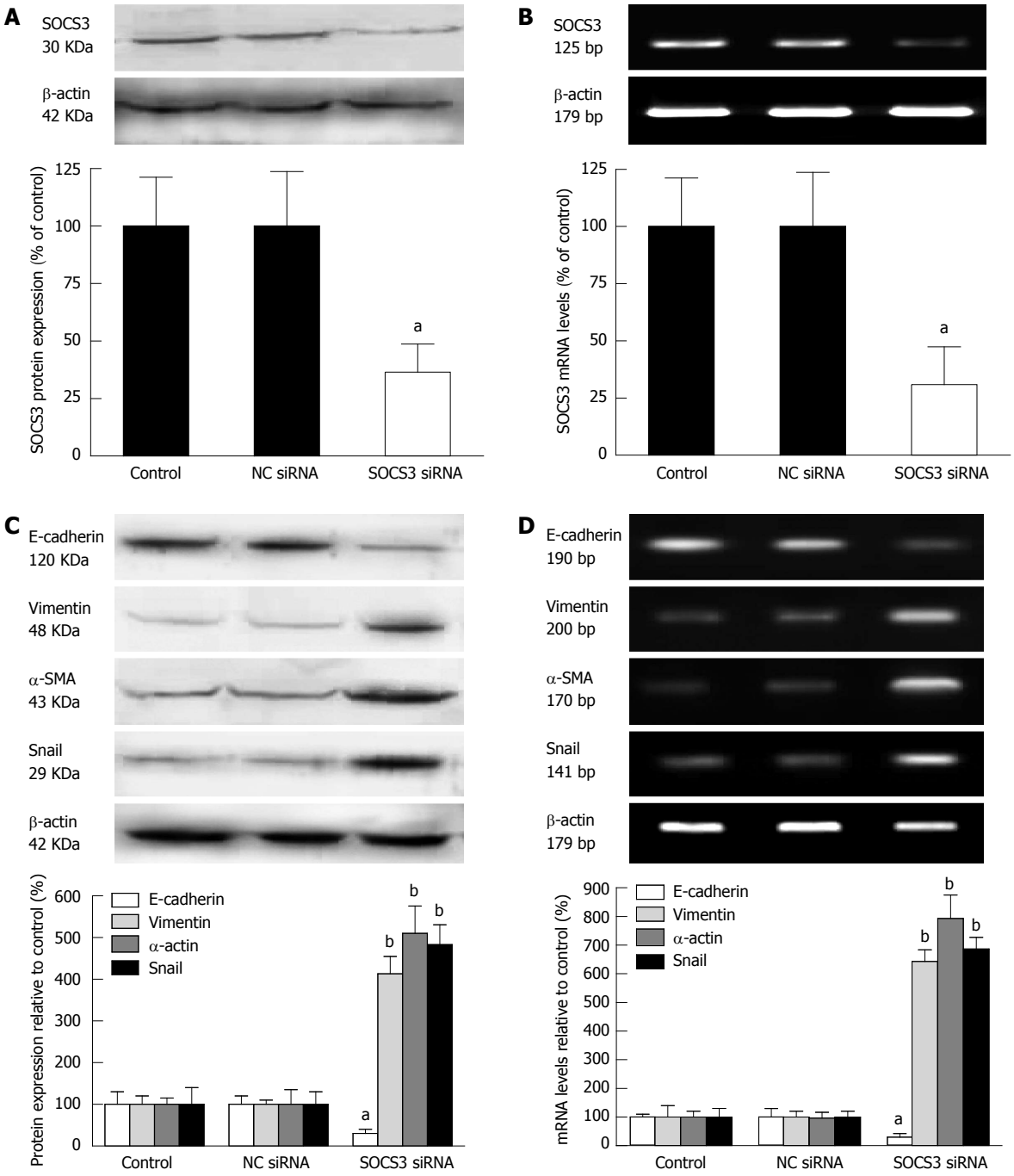

Figure 4 Effect of suppressor of cytokine signaling 3 small-interfering RNA on expression of epithelial-mesenchymal transition-associated molecules in MHCC97H cells. The cells were transiently transfected with negative control (NC) small-interfering RNA (siRNA) or suppressor of cytokine signaling 3 (SOCS3) siRNA for 48 h. A: Protein expression and mRNA levels of SOCS3 in MHCC97H cells were detected by Western blotting; B: Reverse transcription-polymerase chain reaction (RT-PCR) and quantitative real-time PCR; C: Protein expression and mRNA levels of epithelial-mesenchymal transition-associated molecules in MHCC97H cells were analyzed by Western blotting; D: RT-PCR and quantitative real-time PCR after normalization to β-actin mRNA. Data are presented as the mean ± SD from three independent experiments. $^{a}P < 0.05$, $^{b}P < 0.01$ *vs* NC siRNA.

Loss of E-cadherin and gain of Vimentin or α-SMA are the most important consequence of EMT that facilitate invasion and metastasis of tumor cells[27-29]. In the present study, transfection of MHCC97H cells with SOCS3 siRNA resulted in down-regulation of epithelial marker E-cadherin and up-regulation of mesenchymal markers Vimentin and α-SMA, further confirming the promotion of EMT by SOCS3 silencing in MHCC97H cells.

Snail has been shown to be a strong repressor of transcription of the E-cadherin gene and evokes tumorigenic and invasive properties in epithelial cells upon overexpression[30]. It has been reported that the high expression of Snail leads to EMT and enhances motility and invasiveness of tumor cells[31]. Inverse correlation of Snail and E-cadherin was detected in HCC-derived cell lines[32]. In the present study, examination of Snail expression in

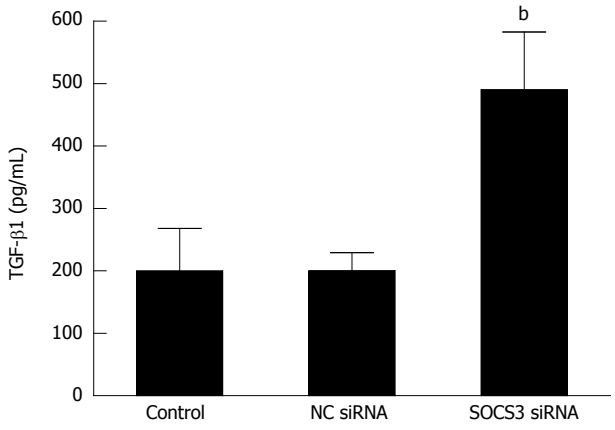

Figure 5 Effect of suppressor of cytokine signaling 3 small-interfering RNA on transforming growth factor-β1 levels in MHCC97H cells. After application of negative control (NC) small-interfering RNA (siRNA) or suppressor of cytokine signaling 3 (SOCS3) siRNA for 48 h, concentration of transforming growth factor-β1 in the supernatant of the cells was measured by enzyme-linked immunosorbent assay. Data are presented as the mean ± SD from three independent experiments. $^{b}P < 0.01$ *vs* NC siRNA. TGF: Transforming growth factor.

MHCC97H cells with SOCS3 siRNA transfection indicated a significant increase in protein expression and mRNA levels of Snail, possibly leading to loss of E-cadherin resulting in the induction of EMT. These results are consistent with the mechanism of induction of EMT[11,12].

TGF-β1 is known to be the most potent inducer of EMT, and it initiates morphological transition of the cells from an epithelial to a fibroblastic appearance, accompanied by loss of epithelial cell marker such as E-cadherin and a gain of mesenchymal cell marker such as Vimentin[33,34]. During the late stages of HCC tumorgenesis, TGF-β1 stimulates cellular invasion through the EMT program[35]. A previous study also demonstrates that TGF-β1 expression is enhanced by SOCS3 gene deletion in HCC[25]. In this work, we further detected TGF-β1 levels in MHCC97H cells transfected with SOCS3 siRNA. The results displayed that depletion of SOCS3 remarkably aggravated TGF-β1 secretion in MHCC97H cells, further illustrating the induction of EMT by SOCS3 silencing.

In conclusion, the present study provides direct evidence that SOCS3 silencing is able to promote EMT in MHCC97H cells *via* changing the phenotypic characteristics and modulating the characteristic markers, suggesting that SOCS3 could play a potential role in EMT in MHCC97H cells. We also identified a novel relationship between SOCS3 silencing and EMT in MHCC97H cells, however, further *in vitro* and *in vivo* studies are necessary to find out the exact molecular mechanism associated with the effect of SOCS3 in EMT of HCC.

COMMENTS

Background

The main cause of death of patients with hepatocellular carcinoma (HCC) is intrahepatic metastasis, but the underlying mechanism is still not fully understood. Epithelial-mesenchymal transition (EMT) is being increasingly recognized as a crucial step that promotes HCC invasiveness and metastasis. However, the role of suppressor of cytokine signaling (SOCS) in EMT involved in HCC is not well documented.

Research frontiers

This study demonstrated that depletion of SOCS3 resulted in the decrease of the epithelial marker E-cadherin, and the increase of the mesenchymal markers Vimentin and α-smooth muscle actin and the expression of transcription factor Snail in MHCC97H cells. In addition, SOCS3 small-interfering RNA evidently enhanced transforming growth factor-β1 secretion in MHCC97H cells. These results reveal that interference of SOCS3 is able to facilitate EMT involved in MHCC97H cells.

Innovations and breakthroughs

The present study provides direct evidence that SOCS3 silencing has the ability to promote EMT in MHCC97H cells *via* changing the phenotypic characteristics and modulating the characteristic markers, suggesting that SOCS3 could play a potential role in EMT in MHCC97H cells.

Applications

The authors found a novel relationship between SOCS3 silencing and EMT in HCC, thus understanding the influential factor of EMT could allow the development of novel therapies targeting at HCC invasion and metastasis.

Terminology

EMT is the morphological and molecular changes that occur when epithelial cells lose their characteristics, gain mesenchymal properties and become motile, which is a key event in tumor invasion and metastasis; The SOCS proteins are a family of negative regulatory proteins related to wide cytokine and growth correlation factor, especially by the signal molecule in the pathway of Janus kinase/signal transducer and transcription activator.

Peer review

This study presented results suggesting that SOCS3 silencing has the ability to promote EMT in MHCC97H cells. It will help to define the role of SOCS3 as a tumor suppressor in HCC. The manuscript is well presented and of interest and it can contribute to increase the knowledge of SOCS3 silencing on EMT involved in MHCC97H cell line.

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BRIEF ARTICLE

Intraductal ultrasound substantiates diagnostics of bile duct strictures of uncertain etiology

Tobias Meister, Hauke S Heinzow, Carina Woestmeyer, Philipp Lenz, Josef Menzel, Torsten Kucharzik, Wolfram Domschke, Dirk Domagk

Tobias Meister, Hauke S Heinzow, Carina Woestmeyer, Philipp Lenz, Josef Menzel, Torsten Kucharzik, Wolfram Domschke, Dirk Domagk, Department of Medicine B, University of Münster, D-48149 Münster, Germany
Tobias Meister, Department of Medicine Ⅱ, HELIOS Albert-Schweitzer University Teaching Hospital, D-37154 Northeim, Germany
Josef Menzel, Department of Medicine Ⅱ, Klinikum Ingolstadt, D-85049 Ingolstadt, Germany
Torsten Kucharzik, Department of Medicine, University Teaching Hospital Lüneburg, D-21339 Lüneburg, Germany
Author contributions: Meister T and Heinzow HS contributed equally to this work; Meister T contributed to design of the study, analysis and interpretation of data, drafting of the manuscript; Heinzow HS contributed to analysis and interpretation of data, drafting of the manuscript, technical and material support; Woestmeyer C contributed to acquisition of data; Lenz P contributed to critical revision of the manuscript for important intellectual content, technical support; Menzel J contributed to performance of the intraductal ultrasound (IDUS) procedures, revision of the manuscript for important intellectual content; Kucharzik T contributed to performance of the IDUS procedures, critical revision of the manuscript for important intellectual content, study concept, analysis and interpretation of data; Domschke W contributed to critical revision of the manuscript for important intellectual content; Domagk D contributed to senior author, study concept and design, performance of the IDUS procedures, analysis and interpretation of data, drafting of the manuscript, statistical analysis, study supervision and final approval of the version to be published.
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Correspondence to: Tobias Meister, MD, Department of Medicine Ⅱ, HELIOS Albert-Schweitzer Teaching Hospital, Sturmbäume 8-10, D-37154 Northeim, Germany. tobiasmeister@gmx.de
Telephone: +49-5551-971240 Fax: +49-5551-971420
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Abstract

AIM: To report the largest patient cohort study investigating the diagnostic yield of intraductal ultrasound (IDUS) in indeterminate strictures of the common bile duct.

METHODS: A patient cohort with bile duct strictures of unknown etiology was examined by IDUS. Sensitivity, specificity and accuracy rates of IDUS were calculated relating to the definite diagnoses proved by histopathology or long-term follow-up in those patients who did not undergo surgery. Analysis of the endosonographic report allowed drawing conclusions with respect to the T and N staging in 147 patients. IDUS staging was compared to the postoperative histopathological staging data allowing calculation of sensitivity, specificity and accuracy rates for T and N stages. The endoscopic retrograde cholangio-pancreatography and IDUS procedures were performed under fluoroscopic guidance using a side-viewing duodenoscope (Olympus TJF 160, Olympus, Ltd., Tokyo, Japan). All procedures were performed under conscious sedation (propofol combined with pethidine) according to the German guidelines. For IDUS, a 6 F or 8 F ultrasound miniprobe was employed with a radial scanner of 15-20 MHz at the tip of the probe (Aloka Co., Tokyo, Japan).

RESULTS: A total of 397 patients (210 males, 187 females, mean age 61.43 ± 13 years) with indeterminate bile duct strictures were included. Two hundred and sixty-four patients were referred to the department of surgery for operative exploration, thus surgical histopathological correlation was available for those patients. Out of 264 patients, 174 had malignant disease proven by surgery, in 90 patients benign disease was found. In these patients decision for surgical exploration was made due to suspicion for malignant disease in multimodal diagnostics (computed tomography scan, endoscopic ultrasound or magnetic resonance imaging). Twenty benign bile duct strictures were misclassified by IDUS as malignant while 14 patients with malignant strictures were initially misdiagnosed by IDUS as benign resulting in sensitivity, specificity and accuracy rates

of 93.2%, 89.5% and 91.4%, respectively. In the subgroup analysis of malignancy prediction, IDUS showed best performance in cholangiocellular carcinoma as underlying disease (sensitivity rate, 97.6%) followed by pancreatic carcinoma (93.8%), gallbladder cancer (88.9%) and ampullary cancer (80.8%). A total of 133 patients were not surgically explored. 32 patients had palliative therapy due to extended tumor disease in IDUS and other imaging modalities. Ninety-five patients had benign diagnosis by IDUS, forceps biopsy and radiographic imaging and were followed by a surveillance protocol with a follow-up of at least 12 mo; the mean follow-up was 39.7 mo. Tumor localization within the common bile duct did not have a significant influence on prediction of malignancy by IDUS. The accuracy rate for discriminating early T stage tumors (T1) was 84% while for T2 and T3 malignancies the accuracy rates were 73% and 71%, respectively. Relating to N0 and N1 staging, IDUS procedure achieved accuracy rates of 69% for N0 and N1, respectively. Limitations: Pretest likelihood of 52% may not rule out bias and overinterpretation due to the clinical scenario or other prior performed imaging tests.

CONCLUSION: IDUS shows good results for accurate diagnostics of bile duct strictures of uncertain etiology thus allowing for adequate further clinical management.

Key words: Intraductal ultrasound; Bile duct strictures; Accuracy

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INTRODUCTION

There is still ongoing debate about adequate diagnostics in bile duct strictures of unknown etiology. The application of endoscopic retrograde cholangio-pancreatography (ERCP) is considered to be an essential tool in bile duct strictures in which additional need for intervention is given[1]. The main advantage of ERCP over other imaging modalities is the ability to achieve biliary decompression and to take transpapillary specimens for histological or cytological analysis in the very same session. A large trial on biliary brush cytology, however, described poor results thus indicating the need for further studies in the analysis of appropriate tissue sampling[2]. The use of intraductal ultrasound (IDUS) performed during ERCP enables the investigator to obtain additional information concerning the bile duct wall and the periductal tissue[3]. Specifically, IDUS gives clinically important data by visualizing the wall layers in biliary strictures and estimating the extent of potentially cancerous infiltration, also enabling the investigator to perform targeted biopsies. Thus, IDUS might be instrumental in choosing the appropriate therapeutic approach and may improve our potential to differentiate benign and malignant strictures. In this matter, however, previous studies, although partly of a prospective design, have only investigated limited numbers of patients[4-6]. On the other hand, cholangioscopy is at present time a promising diagnostic technique in diagnosing bile duct strictures of unknown origin. However, so far only limited data evaluating the diagnostic impact of cholangioscopy is available. In a prospective study with 35 patients included, the sensitivity of SpyGlass-directed biopsy of intraductal lesions was 71%[7,8].

In the present large cohort of patients, we aimed to evaluate the diagnostic yield of IDUS in patients scheduled for ERCP due to indeterminate strictures or filling defects of the common bile duct.

MATERIALS AND METHODS

Data collection

At the tertiary referral center of Münster University Hospital, we retrospectively analyzed the data of our patient cohort undergoing ERCP in combination with IDUS for diagnostics of indeterminate strictures of the bile duct during 2002-2009. All patients with bile duct stenosis who had undergone ERCP and IDUS during the study period could be identified by looking for codes K83.1 and procedures 3.055 and 1440.6 according to the International Classification of Diseases. A total of 397 patients were found in our analysis who were referred to the Department of Medicine B at Münster University Hospital. Clinical records of patients were collected and carefully analyzed. Baseline characteristics, diagnostic techniques employed and histopathology were retrieved as shown in Table 1.

Exclusions

Patients not having been histopathologically controlled by surgery, forceps biopsy or with an endoscopic follow-up < 1 year in suspected benign biliary stenosis were excluded from this study.

Procedures

All 397 patients enrolled in the present study underwent ERCP with the additional application of intraductal ultrasound. In our cohort, there was no case in which a positive tissue diagnosis was already known at the time of IDUS investigation. The individual procedure was performed after written informed consent had been obtained from the patients or related persons for the endoscopic procedures. All endoscopic maneuvers were executed by highly experienced investigators according to the generally accepted guidelines with an ERCP case volume above 200/year[9]. The ERCP and IDUS procedures were performed under fluoroscopic guidance using a side-viewing duodenoscope (Olympus TJF 160, Olympus, Ltd., Tokyo, Japan). All procedures were performed

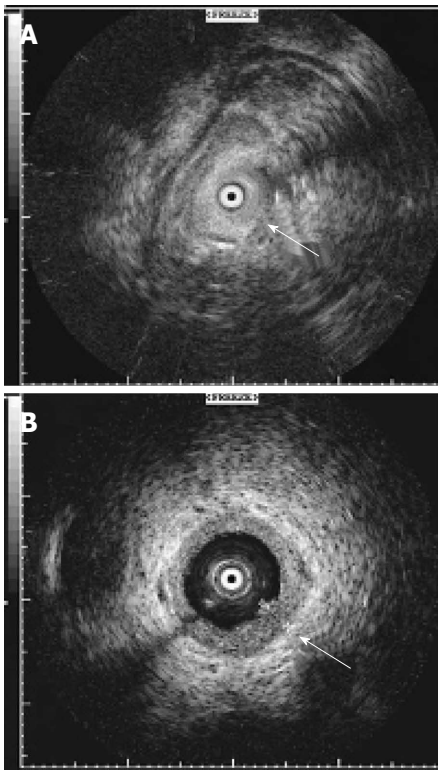

Figure 1 An exemplary benign and malignant bile duct stenosis. A: Benign bile duct stricture showing a homogeneous hyperechoic lesion with smooth margins (arrow); B: Malignant stricture showing bile duct wall thickening and irregular margins (arrow).

under conscious sedation (propofol combined with pethidine) according to the German guidelines[10]. For IDUS, a 6 F or 8 F ultrasound miniprobe was employed with a radial scanner of 15-20 MHz at the tip of the probe (Aloka Co., Tokyo, Japan). Thus a radial real-time image of 360° view was possible for optimum investigation of the area surrounding the probe. The visible depth was about 20 mm with a resolution of up to 0.1 mm (Figure 1). Three hundred and twelve IDUS procedures included additional forceps biopsies. Endoscopic transpapillary biopsies (n = 4-8 specimens) were taken out of the biliary strictures by straight or angled endoscopic forceps (MTW Endoscopy, Wesel, Germany). If insertion of the forceps into the stricture was not feasible, biopsies were retrieved from the distal margins of the bile duct stenosis[11]. Patients with eligibility for surgery were transferred to the Department of General Surgery, Münster University Hospital.

IDUS T and N staging

Two hundred and sixty-four patients were surgically explored (66% of patients). In 174 cases malignancy was proven, of those 14 patients were initially misdiagnosed by IDUS as benign, thus in these cases uT stages were not available. Thirteen of the 174 patients had surgical exploration but due to extended disease with inoperability T and N stage assessment was not done. Thus, retrospective analysis of the endosonographic report allowed drawing conclusions with respect to the T and N staging in 147 patients. IDUS staging was based on the latest TNM classification system[12]. IDUS classification of N stages was as follows: N0-no regional lymph node metastasis; N1-regional lymph node metastasis. Lymph nodes were considered positive, if at least one of the following criteria could be assessed: lymph node larger than 10 mm, delineated borders, hypoechoic structure resembling the primary tumor, roundish shape.

IDUS staging was compared to the postoperative histopathological staging data allowing calculation of sensitivity, specificity and accuracy rates for T and N stages. Due to the limited penetration depth of IDUS, M staging was not performed.

Follow-up

All patients with suspected benign strictures had routine follow-up the day following intervention as well as every three months the first year, every 6 mo the second year and annually up to the third year after intervention at our department. The follow-up procedure was performed according to a surveillance protocol and included laboratory testing and abdominal ultrasound. In cases of biliary plastic stent insertion, the follow-up procedures included ERCP and where appropriate biliary plastic stent changing.

Statistical analysis

Data were analyzed using SPSS 17.0 (Chicago, IL, United States). Results were expressed as medians and ranges. For each of the diagnostic measures, sensitivity, specificity and accuracy rates were calculated for each individual T and N stage. In all cases, gold standard was the histopathologic staging of specimens. Comparison of accuracy rates between groups (localization of stricture) was performed by using the Mann-Whitney U test and the χ^2 test as appropriate. Differences were considered statistically significant if $P < 0.05$. For statistical analysis, sensitivity, specificity, pre-test likelihood and accuracy rates were calculated as follows: sensitivity = true positives/(true positives + false negatives); specificity = true negatives/(true negatives + false positives); pre-test likelihood = truly malignant cases/total cases; accuracy = (true positives + true negatives)/total cases.

RESULTS

Patient characteristics

The study cohort included 397 patients (210 men and 187 women; median age 61.4 ± 13 years). Two hundred and sixty-four patients were referred to the department of surgery for operative exploration, thus surgical histopathological correlation was available for those patients. Out of 264 patients, 174 had malignant disease proven by surgery, in 90 patients benign disease was found. In these patients decision for surgical exploration was made due to suspicion for malignant disease in multimodal diagnostics [computed tomography (CT) scan, endoscopic ultrasound (EUS) or magnetic resonance imaging (MRI)]. A total of 133 patients were not surgically explored.

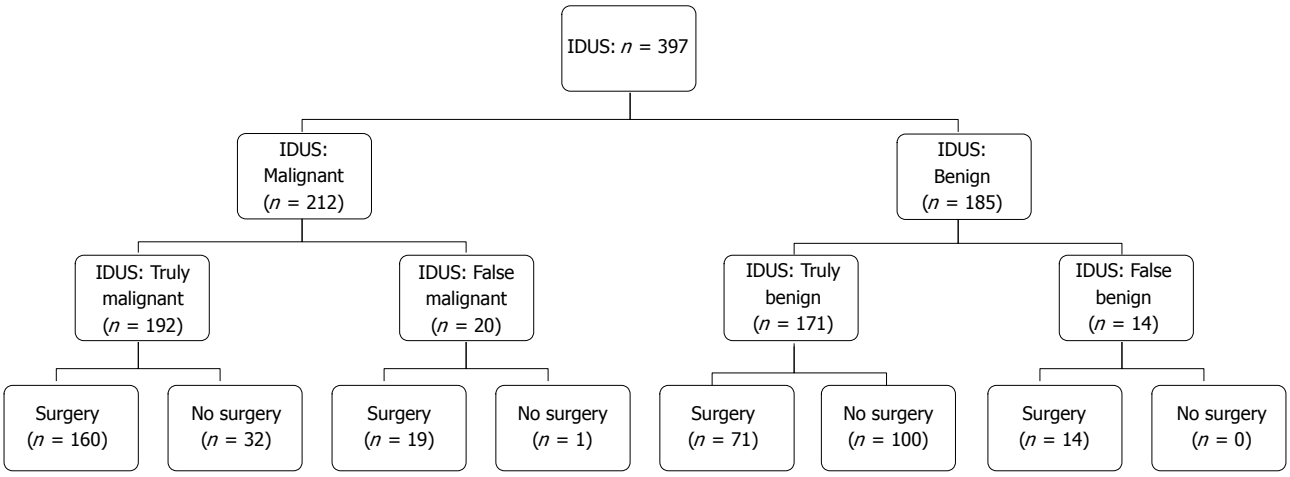

Figure 2 Flow chart showing the enrollment of study patients and distribution based on intraductal ultrasound diagnosis. IDUS: Intraductal ultrasound.

Table 1 Baseline characteristics of the patient cohort

Baseline characteristics	
Patients (*n*)	397
Age (yr), mean ± SE	61.43 ± 13
Sex (M/F)	210/187
IDUS performed (*n*)	397
Follow-up (mo), mean ± SE	39.7 ± 23.1
Follow-up range (mo)	12-100
Procedures (*n*)	
Clinical follow-up	95
Surgery	264
Palliative therapy	32
Calculus extraction	6
Localization of stricture (CBD)	
Proximal third	59
Middle third	46
Distal third	292
Final diagnosis (*n*)	
Normal bile duct	25
Benign disease	
Papillitis	30
Ampullary adenoma	18
Cholangitis	17
PSC	8
Mirizzi syndrome	7
Choledocholithiasis	14
Pancreatitis	59
Pseudocyst	4
Portal vein thrombosis	1
Papilloma of pancreatic duct	3
Choledochal cyst	1
Postoperative stenosis	1
Pancreatic cystadenoma	2
Caroli's syndrome	1
Malignant disease	
Cholangiocarcinoma	85
Pancreatic carcinoma	80
Ampullary carcinoma	26
Gallbladder carcinoma	9
Hepatocellular carcinoma	6

CBD: Common bile duct; PSC: Primary sclerosing cholangitis; M: Man; F: Female; IDUS: Intraductal ultrasound.

Thirty-two patients had palliative therapy due to extended tumor disease in IDUS and other imaging modalities. In 6 patients calculus extraction was performed due to choledocholithiasis. Ninety-five patients had benign diagnosis by IDUS, forceps biopsy and radiographic imaging and were followed by a surveillance protocol with a follow-up of at least 12 mo; the mean follow-up was 39.7 mo (Table 1). A flow chart showing the enrollment of the study patients with distribution based on IDUS diagnosis is presented in Figure 2.

IDUS

IDUS was performed in all patients enrolled. Pre-test likelihood for malignant stenosis in our patient cohort of 52% (95%CI: 47%-57%) was calculated. Twenty benign bile duct strictures were initially misclassified by IDUS as malignant while 14 malignant bile duct strictures were initially interpreted by IDUS as benign resulting in sensitivity, specificity and accuracy rates of 93.2%, 89.5% and 91.4%, respectively (Tables 2 and 3). In the subgroup analysis of malignancy prediction, IDUS showed best performance in cholangiocellular carcinoma as underlying disease (sensitivity rate, 97.6%) followed by pancreatic carcinoma (93.8%), gallbladder cancer (88.9%) and ampullary cancer (80.8%) (Tables 2 and 3).

Accuracy rates of IDUS for malignant stenoses in the proximal, middle or distal third of the common bile duct did not differ statistically in terms of localization (Table 4).

Accuracy of IDUS in T and N staging

Overall T and N staging results with the intraductal ultrasound miniprobe are given in Tables 5 and 6. The accuracy rate for discriminating early T stage tumors (T1) was 84% while for T2 and T3 malignancies the accuracy rates were 73% and 71%, respectively (Table 5). Relating to N0 and N1 staging, IDUS achieved accuracy rates of 69% each (Table 6).

DISCUSSION

Endoscopic ultrasound has proved to be an accurate imaging device in the diagnostics and staging of malig-

Table 2 Staging results of intraductal ultrasound (*n* = 397)

Method, classification	Final diagnosis according to histopathology or long-term follow-up						
	Benign lesion	Carcinoma	CCC	Pancreatic CA	Ampullar CA	GB CA	HCC
IDUS, benign	171	14	2	5	5	1	1
IDUS, malignant	20	192	83	75	21	8	5

CA: Carcinoma; GB CA: Gallbladder cancer; CCC: Cholangiocellular carcinoma; HCC: Hepatocellular carcinoma; IDUS: Intraductal ultrasound.

Table 3 Sensitivity, specificity and accuracy rates of intraductal ultrasound (*n* = 397)

Tumor	Sensitivity (95%CI)	Specificity (95%CI)	Accuracy (95%CI)
All tumors	0.93 (0.90-0.97)	0.89 (0.85-0.94)	0.91 (0.89-0.94)
CCC	0.98 (0.94-1.0)	0.98 (0.94-1.0)	0.92 (0.89-0.95)
Pancreatic CA	0.94 (0.88-0.99)	0.90 (0.85-0.94)	0.91 (0.87-0.94)
Ampullary CA	0.81 (0.66-0.96)	0.90 (0.85-0.94)	0.89 (0.84-0.93)
GB CA	0.89 (0.68-1.0)	0.90 (0.85-0.94)	0.90 (0.85-0.94)
HCC	0.83 (0.54-1.0)	0.90 (0.85-0.94)	0.89 (0.85-0.94)

CA: Carcinoma; GB CA: Gallbladder cancer; CCC: Cholangiocellular carcinoma; HCC: Hepatocellular carcinoma; IDUS: Intraductal ultrasound.

Table 4 Statistical analysis of intraductal ultrasound accuracy rates relating to localization of bile duct strictures (*n* = 397)

Localization of stenosis	*N*	Accuracy (95%CI)	Test for significance	*P* value
Proximal third	55/59	0.93 (0.87-1.0)	Proximal *vs* middle third	0.958
Middle third	43/46	0.93 (0.87-1.0)	Proximal *vs* distal third	0.280
Distal third	260/292	0.89 (0.85-0.93)	Middle *vs* distal third	0.308

Table 5 Histopathological *vs* intraductal ultrasound T staging

IDUS	Histopathology			
	pT1	pT2	pT3/4	∑
uT1	12	9	13	34
uT2	1	26	24	51
uT3	1	5	56	62
∑	14	40	93	147
Sensitivity (95%CI)	0.86 (0.67-1.0)	0.65 (0.50-0.80)	0.60 (0.50-0.70)	
Specificity (95%CI)	0.83 (0.77-0.90)	0.77 (0.69-0.85)	0.89 (0.81-0.97)	
Accuracy (95%CI)	0.84 (0.78-0.89)	0.73 (0.66-0.81)	0.71 (0.63-0.78)	

IDUS: Intraductal ultrasound.

Table 6 N staging accuracy-histopathology *vs* intraductal ultrasound

IDUS	Histopathology		
	pN0	pN1	∑
uN0	44	23	67
uN1	23	57	80
∑	67	80	147
Sensitivity (95%CI)	0.66 (0.61-0.81)	0.61 (0.57-0.79)	
Specificity (95%CI)	0.61 (0.54-0.77)	0.66 (0.54-0.77)	
Accuracy (95%CI)	0.69 (0.61-0.76)	0.69 (0.61-0.76)	

IDUS: Intraductal ultrasound.

nant bile duct strictures[13]. However, in previous studies, although partly of a prospective design, only limited numbers of patients were investigated[4-6]. Therefore, in the present to our knowledge largest patient cohort, we aimed to substantiate the diagnostic yield of IDUS.

IDUS has also proved superior to other imaging modalities such as CT and MRI[14-16]. Its limitation, however, is the minor ultrasonic penetration depth. Consequently, intraductal ultrasonography tends to understage tumors of the pancreaticobiliary tract. Therefore, IDUS does not seem useful in extensive lymph node staging[17].

It is consistently accepted that in the bilio-pancreatic tract depiction of tumors by means of ultrasound and CT is difficult, but these tumors can appropriately be represented through endoscopic ultrasound[18] or IDUS[19,20]. Although IDUS is superior to EUS regarding diagnostics of tumor extension in the pancreatic and biliary duct, the use of IDUS has not been taken up widely yet.

A promising diagnostic tool in the differentiation of bile duct strictures is single- operator peroral cholangioscopy (SOC) as it provides direct visualization of the bile duct and facilitates diagnostic procedures and therapeutic intervention. The diagnostic utility of SOC for indeterminate biliary lesions has been the topic of a few recently published studies[21,22].

However, only limited data with smaller patient cohorts exist. Promising results with an accuracy of SOC for diagnosing malignant lesions has been reported to range between 64% and 89%. SOC-guided biopsies have been shown to be adequate in 72% to 82%[21-23]. Therefore, its therapeutic and diagnostic yield in patients with bile duct strictures remains uncertain. A prospective randomized trial comparing various endoscopic imaging and radiographic imaging modalities with each other would be desirable, especially in a multi-center study design.

In our patient cohort of 397 patients with bile duct strictures of initially unknown etiology, using IDUS we observed sensitivity, specificity and accuracy rates of 93%, 89% and 91%, respectively, for discriminating malignant from benign lesions. In previous studies with a limited number of patients, IDUS accuracy rates between 84% and 95% are described as presented in Table 7. IDUS also showed excellent results in regard to localization of biliary stenoses with an accuracy rate of 93% (proximal part of bile duct), 93% (middle) and 89% (distal), respectively (*P* values not significant) (Table 4). In our study, endoscopic transpapillary biopsies (ETP) were additionally obtained in 312 cases. Out of 226 bile duct strictures initially diagnosed to be benign by ETP, 101 cases eventually turned out as malignancies. There were no false-positive results in forceps biopsy histopathology leading to sensitivity, specificity and accuracy rates of 46%, 100% and 67.6%, respectively[11]. Thus the authors conclude that ETPs are of limited value for detecting additional malignant bile

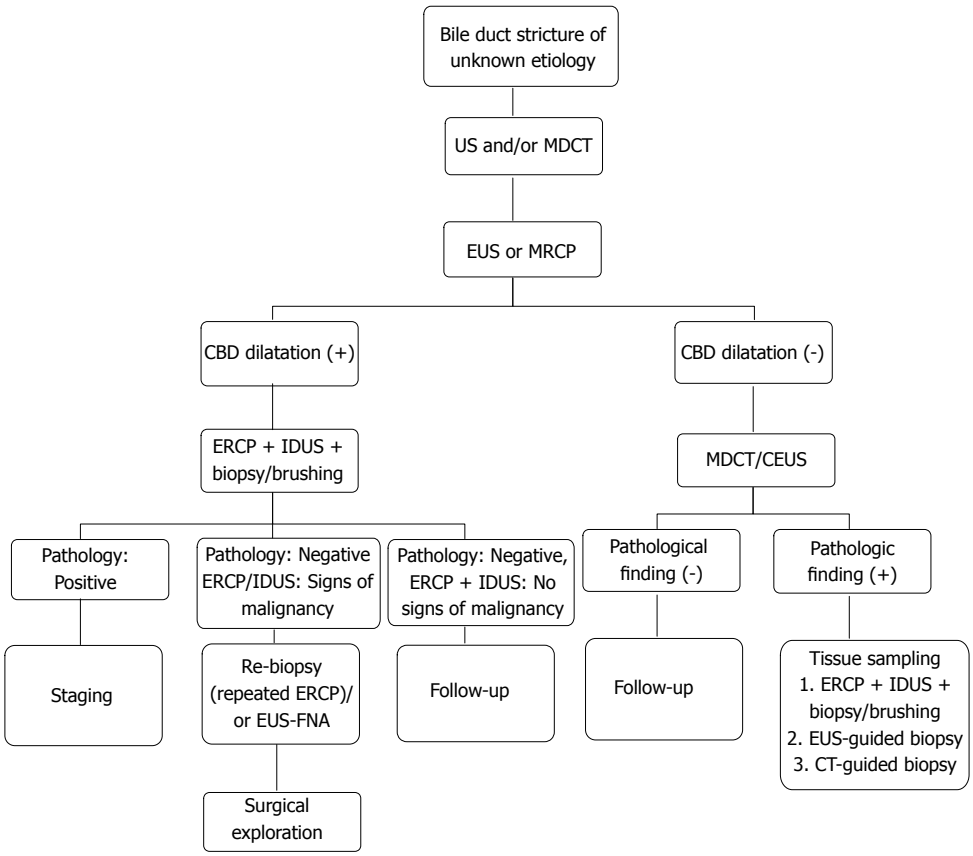

Figure 3 Suggested algorithm for the evaluation of bile duct strictures of uncertain etiology. US: Ultrasound; MDCT: Multidetector computed tomography; EUS: Endoscopic ultrasound; MRCP: Magnetic resonance cholangio-pancreatography; CBD: Common bile duct; IDUS: Intraductal ultrasound; CEUS: Contrast enhanced ultrasound; EUS-FNA: Endoscopic ultrasound with fine needle aspiration; CT: Computed tomography.

Table 7 Meta-analysis of intraductal ultrasound accuracy in detecting bile duct malignancy

Study	No. of patients	Accuracy of IDUS
Menzel *et al*[15]	57	89%
Tamada *et al*[30]	19	84%
Domagk *et al*[14]	60	88%
Domagk *et al*[31]	33	88%
Domagk *et al*[29]	30	95%

IDUS: Intraductal ultrasound.

duct strictures.

In matters of tumor entity, IDUS demonstrated best results in cholangiocellular carcinoma (CCC) with accuracy and sensitivity rates of 92% and 98%, respectively. As far as T staging is concerned, upon IDUS the normal bile duct wall appears as either two or three layers. However, in some patients differentiation of the fibromuscular layer from the perimuscular connective tissue can be difficult thus limiting the ability to distinguish CCC stages 1 and 2, although this distinction is usually not clinically relevant with respect to treatment options[24]. According to a study conducted earlier by our group[25], the various layers of the extrahepatic bile duct wall as described by the Union for International Cancer Control 7th tumor-node-metastasis (TNM) classification[12] are not consistently demonstrable histomorphologically, immunohistochemically or by endosonographic imaging. Domagk *et al*[25] suggested that difficulties in distinguishing tumor invasion encroaching beyond the bile duct (T2) and into the pancreas (T3) pose problems for the present TNM-classification. A better metric may be to combine T2- and T3-staged tumors into one single class.

T staging of other tumor entities such as pancreatic, ampullary or gallbladder cancer may be limited in mini-probe intraductal ultrasound due to its maximum penetration depth of 20 mm. The T stage, *e.g.*, of pancreatic tumors among other factors relies on tumor size. Further, the assessment of vascular tumor infiltration is restricted due to the technical limitation of penetration depth and absence of potential duplex sonography. In a study by Tamada *et al*[26,27], vascular infiltration of CCC was assessed: Depiction of the right hepatic artery was possible in 100% of the cases while in less than 20% of the cases depiction of the common hepatic artery or the left hepatic artery succeeded. On that account, the assessment of tumor infiltration into the above mentioned vessels was inaccurate.

In summary, IDUS tends to understage tumors of the pancreaticobiliary tract. Specific analysis of our patient cohort revealed overall T staging accuracy rates as follows: T1 84%, T2 73% and T3/T4 71% as displayed in Table 5. N staging accuracy was calculated as 69% (Table 6).

In recent years, other imaging techniques like magnetic resonance cholangio-pancreatography (MRCP) and multi-detector computed tomography have also been evaluated for their diagnostic sensitivity and specificity in biliary duct tumors. In a first prospective comparison of the diagnostic accuracy of ERCP, MRCP, CT and EUS in biliary duct strictures by Rösch *et al*[28], 50 patients were

analyzed. In their patient cohort, relating to diagnosis of malignancy sensitivity and specificity rates of 85%/75% for ERCP/PTC, 85%/71% for MRCP, 77%/63% for CT, and 79%/62% for EUS were observed. Those authors concluded that although MRCP provides the same imaging information as direct cholangiography, it has only limited specificity for the diagnosis of malignant strictures[28]. A prospective study by our group confirmed superiority of ERCP supplemented by IDUS in differentiating benign from malignant strictures as compared with MRI[29]. Classification of T stages by endolumenal ultrasound was also the topic of an investigation conducted by Menzel *et al*[15] demonstrating similar accuracy rates (77.7%) for T staging as shown in the present study.

Admittedly, as limitations of the present study the retrospective design and certain bias have to be mentioned. In our cohort, there was no case in which a positive tissue diagnosis was already known at the time of IDUS investigation but our pre-test likelihood of 52% may not rule out bias and over-interpretation due to the clinical scenario or other prior performed imaging tests. Our patient cohort is certainly a highly selected one making the probability of malignant bile duct stricture more obvious. On the one hand, the prevalence of malignancy in our patient cohort was 52% as expressed by the pre-test likelihood. On the other hand, the accuracy of IDUS for detecting malignant bile duct strictures exceeds 91%. Therefore, our analysis of a very large patient cohort shows good results of IDUS with regard to accurate diagnostics of bile duct strictures of uncertain etiology and, thus, allows for adequate further clinical management. In particular, IDUS is suitable for early T stage prediction.

Summing up, we suggest the following algorithm for the evaluation of bile duct strictures of uncertain etiology as displayed in Figure 3.

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COMMENTS

Background

Adequate diagnostics in bile duct strictures of unknown etiology is still a challenging task. Many different imaging techniques compete with each other. Intraductal ultrasound (IDUS) might be instrumental in choosing the appropriate therapeutic approach and may improve our potential to differentiate benign and malignant strictures.

Research frontiers

The authors undertook the largest European retrospective study to evaluate the diagnostic yield of IDUS in patients scheduled for endoscopic retrograde cholangio-pancreatography due to indeterminate strictures of the common bile duct.

Innovations and breakthroughs

IDUS shows good results for accurate diagnostics of bile duct strictures of uncertain etiology thus allowing for adequate further clinical management. In particular, IDUS is suitable for early T stage prediction.

Applications

By analyzing the accuracy of IDUS in the diagnostics of bile duct strictures of uncertain etiology, the authors contribute to a better diagnostic strategy of this difficult issue. This may lead to an improved patient care with an optimal diagnostic approach to patients with bile duct strictures of uncertain etiology.

Terminology

IDUS enables the investigation of the common bile duct wall and the periductal tissue with high resolution.

Peer review

Authors evaluated the role of IDUS in the differential diagnosis of indeterminate biliary strictures. This retrospective study involved such a large number of patients with surgical standards. It is a well-written paper, in which the authors have studied an important issue in hepatobiliary medicine.

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BRIEF ARTICLE

Chronic intestinal ischemia and splanchnic blood-flow: Reference values and correlation with body-composition

Helle Damgaard Zacho, Jens Henrik Henriksen, Jan Abrahamsen

Helle Damgaard Zacho, Jan Abrahamsen, Department of Clinical Physiology, Viborg Regional Hospital, 8800 Viborg, Denmark
Jens Henrik Henriksen, Department of Clinical Physiology, Hvidovre Hospital, University of Copenhagen, 2650 Hvidovre, Denmark
Author contributions: Zacho HD designed and performed the experiments, analysed data, wrote the manuscript and has approved of the final draft submitted; Henriksen JH analyzed data, edited the manuscript, and has approved of the final draft submitted; Abrahamsen J designed and performed the experiments, supervised the analysis of data, edited the manuscript, and has approved of the final draft submitted; all authors discussed the results and implications and commented on the manuscript at all stages.
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Correspondence to: Dr. Helle Damgaard Zacho, Department of Clinical Physiology, Viborg Regional Hospital, Heibergs Allé 4, 8800 Viborg, Denmark. helle.damgaard@viborg.rm.dk
Telephone: +45-78-443306 Fax: +45-78-443399
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Abstract

AIM: To determine the splanchnic blood flow and oxygen uptake in healthy-subjects and patients and to relate the findings to body-composition.

METHODS: The total splanchnic blood flow (SBF) and oxygen uptake (SO_2U) were measured in 20 healthy volunteers (10 women) and 29 patients with suspected chronic intestinal ischemia (15 women), age 40-85 years, prior to and after a standard meal. The method is based on the Fick principle using the continuous infusion of an indicator (99mTechnetium-labelled mebrofenin) and catheterization of an artery and the hepatic vein. An angiography of the intestinal arteries was performed during the same investigation. A whole-body dual-energy x-ray absorptiometry scan was performed in healthy volunteers to determine body composition.

RESULTS: Angiography revealed no atherosclerotic lesions in the intestinal arteries. The mean baseline SBF was 1087 mL/min (731-1390), and this value increased significantly to 1787 mL/min after the meal in healthy volunteers ($P < 0.001$). The baseline SBF in patients was 1080 mL/min, which increased to 1718 mL/min postprandially ($P < 0.001$). The baseline SBF was independent of age, sex, lean body mass and percentage of body fat. The mean meal-induced increase in SBF was equal to 282 mL/min + 5.4 mL/min × bodyweight, ($P = 0.025$). The SO_2U in healthy volunteers and patients was 50.7 mL/min and 48.0 mL/min, respectively, and these values increased to 77.5 mL/min and 75 mL/min postprandially, respectively. Both baseline and postprandial SO_2U were directly related to lean body mass. Age and sex exerted no impact on SO_2U.

CONCLUSION: A direct correlation between body weight and the postprandial increase in SBF was observed. The effect of body weight should be considered in the diagnosis of chronic intestinal ischemia.

Key words: Splanchnic circulation; Postprandial period; Body composition; Mesenteric vascular occlusion; Middle aged

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INTRODUCTION

Hepatic and total splanchnic blood flow (SBF) can be

quantified using the Fick principle with the continuous infusion of an indicator, the catheterization of the liver vein and subsequent blood sampling. This method has been thoroughly described in the literature[1,2], and it has been validated[3]. SBF is used for the investigation of splanchnic oxygen uptake (SO_2U) or liver metabolism[4] and the examination of the liver clearance of endogen substances and pharmaceuticals[5]. This method is used in Scandinavian countries as a diagnostic tool for chronic intestinal ischemia (CII)[6,7]. SBF is measured at baseline and after a meal to quantify the meal-induced SBF response.

The diagnosis of CII is based on the inability to enhance SBF after a standard meal, and an increase less than 250 mL/min is abnormal[6-8]. Reference values for SBF are based on few and often young individuals without consideration of their body composition or the morphology of their intestinal arteries[6,8,9]. The lack of this information is deplorable because atherosclerotic lesions in the intestinal arteries are commonly observed in a population of otherwise healthy middle-aged individuals[10,11]. Furthermore, patients suffering from CII are frequently severely underweight[7,12], and the large variation in the anthropometrics of individuals may influence SBF values. Therefore, investigations are required to explore the association between SBF, SO_2U, age, and body composition.

The present study determined the SBF and SO_2U prior to and after a standard meal in a group of middle-aged healthy volunteers with angiography-proven normal intestinal arteries. This study also related SBF and SO_2U values to anthropometric measures of the body in healthy volunteers and in a cohort of patients with suspected chronic intestinal ischemia due to weight loss and abdominal pain but with angiography-proven normal intestinal arteries.

MATERIALS AND METHODS

Ethical approval

The protocol was performed under a license from the Ethical Committee, Central Denmark Region, and the Danish Data Protection Agency. Individually signed informed consent forms were obtained according to the Helsinki II declaration. No complications or side effects were encountered in the study. Twenty healthy volunteers aged 40-70 year (10 women) participated in the present study. None of the volunteers exhibited any signs of cardiovascular disease, abdominal complaints or weight loss one year prior to the investigation. Apart from appendectomy $n = 2$, no former abdominal surgery was performed in any of the volunteers. A total of 180 patients with suspected CII were routinely referred for SBF measurements due to weight loss and abdominal pain from June 2002 to October 2011. Patients with a digital subtraction angiography that revealed three normal intestinal arteries were included in the present study for comparison only. Therefore, 32 patients participated in the study; 31 (97%) patients suffered from postprandial abdominal pain, and 25/32 had experienced unintentional weight loss (mean 10.4 kg). Three patients were excluded: two patients due to ischemic colitis as verified on biopsy and one patient due to pulmonary cancer that had metastasized to the ventricle and colon. Table 1 presents the anthropometric data of the healthy volunteers and patients.

Table 1 Demographic data from 20 healthy volunteers and 29 patients suspected of chronic intestinal ischemia but normal intestinal arteries

	Healthy volunteers		Patients suspected of CII, normal angiography		
	Female $n = 10$	Male $n = 10$	Female $n = 15$	Male $n = 14$	*P* value
Age (yr)	53.8 (40-64)	54.5 (43-69)	63 (44-85)	60.4 (45-76)	0.01
Weight (kg)	72 (55.0-92.0)	83 (65.0-94.8)	57.9 (34.0-114)	74.9 (52.0-98.0)	0.02
Height (cm)	165 (160-170)	177 (170-188)	165 (153-172)	176 (170-186)	0.88
BMI (kg/m^2)	26.5 (21.2-34.5)	26.6 (21.7-30.6)	21.4 (12.5-41.9)	24 (15.0-31.6)	0.02
Body fat (%)	34.4 (28.0-48.2)	21.9 (13.1-29.4)	NA	NA	
LBM (kg)	47.4 (39.6-54.6)	65.5 (55.8-70.6)	NA	NA	
BSA (m^2)	1.81 (1.56-2.07)	2.01 (1.77-2.16)	1.61 (1.25-2.29)	1.89 (1.64-2.19)	0.02

All results are given as mean and range. The *P* value is the difference between healthy volunteers and patients. CII: Chronic intestinal ischemia; LBM: Lean body mass; BSA: Body surface area; BMI: Body mass index; NA: Not available.

All of the healthy volunteers and patients underwent the following protocol.

Catheterization

Subjects underwent catheterization of the femoral artery and vein in the morning following an overnight fast using the Seldinger technique under local analgesia. A 5F sheath was used for the artery, and a 7F sheath was used for the vein. The venous catheter (Schwan-Ganz, Edwards, CA, United States) was placed in a central hepatic vein using fluoroscopy, and the arterial catheter (pigtail catheter, Cordis, NJ, United States) was positioned in the abdominal aorta.

SBF and SO_2U

The SBF equals the hepatic blood flow in normal subjects, and it was measured using a standardized protocol of the indirect Fick principle with 99mTechnetium labeled Bridatec (Mebrofenin®, GE Healthcare, Suluggia, Italy) [99mTc-Mebrofenin (MBF)] as the indicator. This method was originally introduced by Bradley[1], and it was used with corrections for unsteady state and small urinary excretion of 99mTc-MBF, as recommend by Henriksen *et al*[2].

Briefly, a bolus injection of 99mTc-MBF was administered followed by a constant infusion at 2.02 mL/min (range: 1.96-2.17 mL/min), which equaled an infusion rate of 0.323 MBq/min. Less than 80 MBq was used in total. An equilibration period of 20 min was interposed

before blood samples were collected to obtain steady state measurements. Splanchnic plasma flow (SPF) was calculated as SPF = E/(Ca-Cv), where E is the hepatobiliary excretion rate of 99mTc-MBF, which equals the corrected infusion rate[2]. Ca and Cv are the concentrations of 99mTc-MBF in the abdominal aorta and the hepatic vein, respectively. The level of 99mTc-MBF in plasma samples was determined using a Cobra Ⅱ Auto-Gamma counter (Packard Bioscience Company, Frankfurt, Germany). At least 10 000 counts were obtained and corrected for decay, background and dead time.

SBF was calculated as SPF/(1-hematocrit fraction). The splanchnic oxygen uptake (SO_2U) was calculated as hemoglobin × (arterial oxygen saturation-hepatic venous oxygen saturation) × SBF × 1.34 mL O_2/g of hemoglobin. The blood samples were analyzed using an ABL 700 series (Radiometer Medical A/S, Brønshøj, Denmark), which was operated according to the manufacturer's instructions.

Blood samples were taken from a central hepatic vein and the abdominal aorta simultaneously every ten minutes during the first hour. A mean baseline value was calculated. The participants ingested a 4000 kJ/400 mL standard liquid meal that consisted of 33% protein, 33% carbohydrates and 33% fat after one hour. Blood samples were collected every ten minutes for an additional hour, and blood glucose levels were measured at each sampling time as a control of gastric emptying and intestinal absorption. The extraction fraction (EF) of 99mTc-MBF was calculated as (Ca-Cv)/Ca.

The wedged and free hepatic vein pressures were measured using a capacitance transducer at the end of the session to exclude the presence of portal hypertension and resulting porto-systemic shunting. The mean gradient wedged-to-free hepatic vein pressure was 2.5 mmHg (range 1-5 mmHg).

Angiography

An angiography was performed during the same session *via* a 4F pigtail catheter in the abdominal aorta as a control for the morphology of the intestinal arterial blood supply. The angiography included an antero-posterior and lateral horizontal abdominal projection to visualize the individual origin of the mesenteric artery branches. Iomeprol (Iomeron® 200 mg iodine/mL, Bracco, Milan, Italy; 15 mL for each image) was used as a contrast agent, and a total of 45 mL was administered on average.

Each artery was classified as normal (0%-9% lumen reduction) or exhibiting slight stenosis (10%-49% lumen reduction), moderate stenosis (50%-69% lumen reduction), significant stenosis (70%-99% lumen reduction) or occlusion. The investigator who evaluated the angiographies was blinded to the results of the SBF, and both positive and negative controls were included.

Dual-energy x-ray absorptiometry-scanning

Only healthy volunteers were subjected to a dual-energy x-ray absorptiometry (DEXA) scan. Body composition, which was evaluated as the percentage of body fat and fat-free mass, was measured using whole-body scanning in a Delfi Discovery A S/N 70879 and software version 12.6 (Hologic, MA, United Stastes) for quantification. Each participant was immobilized in supine position during evaluation with the arms and legs away from the body. The equipment was calibrated daily according to the manufacturer's instructions.

Statistical analysis

Statistical analysis was performed using STATA®11 (StataCorp LP, Station, Texas, United States). All results are presented as mean values, SD and ranges. Paired and unpaired Student's *t* tests were used for intergroup comparisons. Relationships between variables were analyzed using linear or multiple linear regression analysis and repeated measurements when appropriate. A P value < 0.05 was considered statistically significant.

RESULTS

The average values and ranges of SBF and SO_2U during fasting and after the meal are presented in Table 2. Significant differences ($P < 0.01$) between these values were observed at baseline and after the standard meal. The mean body weight among patients (65.4 kg) was significantly ($P = 0.016$) smaller than the group of healthy volunteers (77.5 kg). Eleven of the 29 patients were underweight [defined as body mass index (BMI) < 20]. None of the healthy volunteers were underweight. However, no differences in the mean baseline SBF were observed between patients (1080 mL/min) and healthy volunteers (1087 mL/min) ($P = 0.95$) or the mean postprandial SBFs, which were 1718 mL/min in patients and 1787 mL/min in healthy volunteers. Figure 1 presents the mean SBF and standard deviation as a function of time in healthy volunteers and the patient group.

Healthy volunteers

Visceral arteriography revealed no significant stenotic vessels or occlusions in the group of healthy volunteers. A slight stenosis (10%-49% lumen reduction) of the celiac artery was present in five cases. The presence of these minor stenoses did not impact the SBF at baseline (1001 mL/min, $P = 0.31$) or alter the postprandial increase (648 mL/min, $P = 0.49$). Baseline SBF and SO_2U values did not change over time (1045 mL/min to 1068 mL/min, $P = 0.89$ and 48 mL O_2/min to 50 mL O_2/min, $P = 0.66$, respectively). Variations in SBF were significantly larger between individuals than within individuals ($P = 0.002$), and the mean baseline SBF was 1087 mL/min (range 731-1390 mL/min), which increased to 1787 mL/min (range 1387-2343 mL/min) postprandially ($P < 0.001$). The corresponding SO_2U increased from 50.7 mL O_2/min (range 32.1-84.5 mL O_2/min) to 77.5 mL O_2/min (range 49.1-118.9 mL O_2/min) ($P < 0.01$).

The baseline values and the increase in SBF after meals were independent of age, sex, body weight, body surface area, and lean body mass in the group of healthy volunteers. However, SO_2U differed significantly between gen-

Table 2 Results of glucose, splanchnic blood flow and oxygen consumption from 20 healthy volunteers and 29 patients suspected of chronic intestinal ischemia but normal intestinal arteries

	Healthy volunteers		Patients suspected of CII, normal angiography		*P* value
	Female *n* = 10	Male *n* = 10	Female *n* = 15	Male *n* = 14	
Fasting plasma glucose (mmol/L)	5.7 (5.1-6.9)	5.8 (5.2-6.9)	5.7 (4.5-11.6)	5.6 (4.7-7.5)	0.59
Postprandial plasma glucose (mmol/L)	6.8[b] (5.9-7.8)	7.2[b] (6.3-8.8)	7.0[b] (5.4-12.1)	7.2[b] (5.9-9.9)	0.27
Mean fasting SBF (mL/min)	1044 (731-1319)	1129 (800-1390)	1040 (619-2781)	1123 (429-2740)	0.95
Mean postprandial SBF (mL/min)	1731[b] (1386-1987)	1844[b] (1485-2343)	1581[b] (906-2340)	1863[b] (1332-3163)	0.54
Postprandial rise in SBF (mL/min)	686 (515-915)	714 (314-1145)	542 (-441-1014)	707 (293-1325)	0.47
Mean fasting oxygen consumption (mL/min)	43.4 (32.1-53.8)	58.1 (40.5-84.5)	44.5 (34.6-76.1)	51.8 (15.2-78.2)	0.48
Mean postprandial oxygen consumption (mL/min)	65.5[b] (49.1-84.0)	89.6[b] (60.1-118.9)	68.3[b] (46.2-104.7)	82.2[b] (45.0-118.2)	0.63

All results are given as mean and range. The *P* value is the comparison difference between healthy volunteers and patients. [b]$P < 0.001$ *vs* fasting values. CII: Chronic intestinal ischemia; SBF: Splanchnic blood flow.

ders ($P = 0.001$). This difference disappeared when gender was corrected for lean body mass ($P = 0.99$). Therefore, the gender-related differences in baseline SO_2U and the postprandial increase were solely attributed to the gender-related difference in lean body mass ($P = 0.001$). Lean body mass did not influence SBF. Therefore, the SO_2U increase was accommodated by a larger oxygen extraction from the blood, which lowered hepatic venous blood saturation (data not shown).

The percentage of body fat influenced baseline SO_2U, but it did not influence the postprandial increase ($P = 0.003$). The baseline SO_2U decreased by 4.4 mL/min for a five percent increase in body fat percentage.

Figure 2 illustrates the EF as a function of time in each healthy volunteer. The arrow indicates the time of the standard meal. The mean EF of 99mTc-MBF decreased significantly ($P < 0.01$) from 0.46 to 0.39 during the baseline period. A steep drop in EF from 0.39 to 0.24 ($P < 0.01$) and an increase in SBF were observed 20 min after the meal. The EF did not change thereafter.

Patients

Figure 3A presents the mean baseline SBF for individual patients and healthy volunteers as a function of weight. Two of the patients exhibited high baseline SBF values (2740 and 2781 mL/min). Figure 3B presents the postprandial increase in SBF in individuals as a function of weight. One of the outliers with hyperperfusion at baseline experienced a decreased SBF after the meal; this individual had the negative increase in Figure 3B.

The increase in baseline and postprandial SBF did not depend on weight ($P = 0.47$ and $P = 0.12$, respectively). However, weight did exert a significant ($P = 0.025$) impact on the postprandial increase but not the baseline SBF in all individuals ($n = 49$) independent of health status. Therefore, an average person weighing 60 kg would demonstrate a 605 mL/min (95%CI: 510-701 mL/min) postprandial increase in SBF. Each additional kg in bodyweight would augment the postprandial increase by 5.4 mL/min. No significant difference between genders or any relationship to age was observed. Baseline and postprandial SO_2U values were 48.0 mL O_2/min and 75.0 mL O_2/min, respectively, and these values in patients did not differ significantly from the healthy volunteers ($P = 0.48$ and $P = 0.68$). Patients also exhibited a significant difference between genders in postprandial SO_2U ($P = 0.03$).

DISCUSSION

Despite the redundancy in the visceral circulation, with its numerous interconnections between the three abdominal arteries, the celiac trunk, and the superior and inferior mesenteric arteries, symptoms of CII ultimately arise when the genuine and collateral arteries can no longer accommodate the postprandial oxygen demand. Therefore, investigations of the morphology of the intestinal arteries cannot stand alone; functional tests that evaluate the physiological consequences are required[12]. An understanding of splanchnic hemodynamics and SO_2U in healthy volunteers is crucial. None of the healthy volunteers with stable weight exhibited a BMI < 20. Therefore, a group of patients who underwent SBF measurements due to a suspicion of CII with weights of 34 to 114 kg and angiography-proven normal intestinal arteries served as a control group. The mean baseline SBF of 1087 mL/min and the increase to 1787 mL/min after the ingestion of a standard meal that was observed in this study are consistent with Madsen *et al*[8], who performed a study in a comparable population of healthy volunteers but without knowledge of arterial morphology.

Previous investigations of SBF have been performed in volunteers who were 20 to 30 years of age. This age group is not representative of the population of interest for CII. These studies reported a total splanchnic

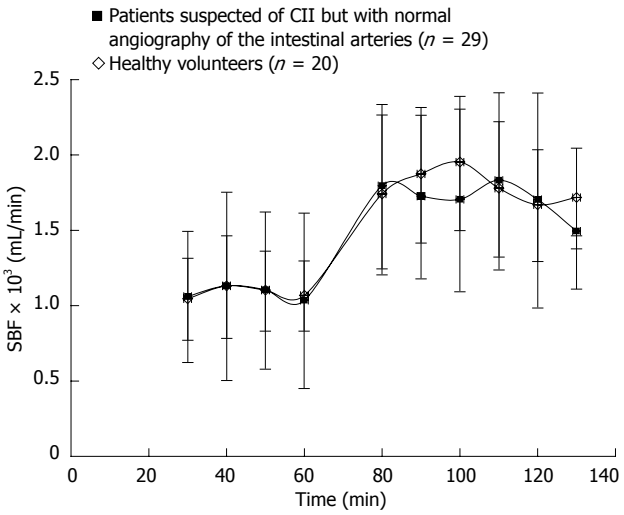

Figure 1 Splanchnic blood flow ± SD at each sampling time. The standard meal of 4000 kJ was served after 60 min. CII: Chronic intestinal ischemia; SBF: Splanchnic blood flow.

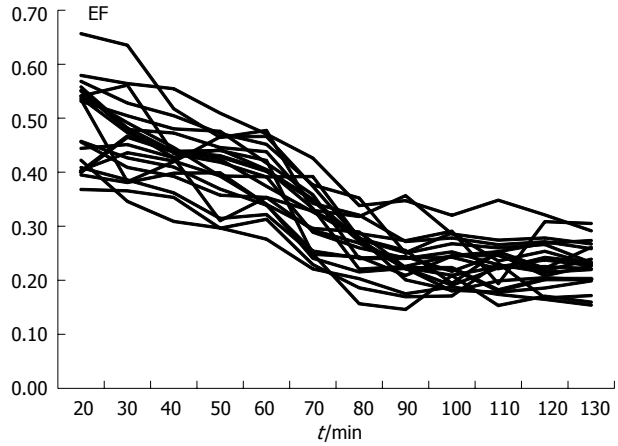

Figure 2 Extraction fraction as a function of time for each of the healthy individuals. The standard meal is served after 60 min as marked by the line, which causes a sharper decrease in extraction fraction (EF) during the first 20 min after the meal.

plasma flow of 900 mL/min during fasting[4,9,13], which corresponds to an SBF of 1500 mL/min given a normal hematocrit. These flow values are 35% higher than the results in the present study. Although we did not observe an age-related effect in participants aged 40 to 85 years, age may be a factor in comparisons of young adults to middle-aged and elderly individuals.

The frequent presence of atherosclerotic changes in asymptomatic 60- to 70-year-old adults renders the knowledge of arterial morphology in a study population essential. However, this knowledge has been neglected in previous studies. The present study demonstrated no atherosclerosis in any mesenteric arteries in healthy volunteers, and only patients with normal intestinal arteries were included.

Healthy volunteers demonstrated no correlation between SBF and body composition, which was described as body weight, body surface area and lean body mass. This result contrasts a previous study[8] in which both baseline SBF and SO_2U were directly related to the body surface area. One reason for this discrepancy may be that the ranges of body surface area or lean body mass in the healthy volunteers in the present study were too small to detect a correlation. This limitation was encountered because of the use of a group of patients with a weight range from 34 to 114 kg. The inclusion of weight in the analysis further augmented the postprandial increase by 5.4 mL/min for each kg bodyweight. Even patients who weighed 34 kg, which represented the lightest patient at our clinic, should have experienced a mean postprandial SBF increase of 465 mL/min (95%CI: 275-656) using this calculation. The exclusion of two patients with hyperperfusion at baseline slightly changed these values (the mean postprandial SBF increased 4.9 mL/min for each kg of bodyweight). These results support previous studies[6,8], which have reported that all individuals should demonstrate a postprandial response larger than 250 mL/min independent of body weight. However, the present study recommends the consideration of body weight when postprandial SBF is used to diagnose CII.

Interestingly, weight did not influence baseline SBF. A minor tendency toward an increase in baseline SBF of 2.2 mL/min (95%CI: -1.6 to 6.1, $P = 0.25$) for each kg increase in body weight was observed. However, a significant correlation between weight and baseline SBF must be rejected.

A significant difference in SO_2U between genders was observed in both groups. These differences between genders were uniquely correlated to the differences in lean body mass in the healthy volunteers who underwent a DEXA scan. This difference may also apply to the patient group, but this group did not undergo DEXA scans. Therefore, the correlation of gender differences in SO_2U to differences in body mass remains unknown in this group. The whole-body DEXA scan can reveal the body composition in each region of the body. A large total lean body mass is closely associated with a large truncal lean body mass, which may explain the increased demand for oxygen in the splanchnic territory that was observed in the present study.

The observed decrease in the EF of 99mTc-MBF in healthy volunteers was most pronounced in the 20 min following the meal. This decrease is an inherent consequence of the Fick principle in which the EF decreases with an increase in flow according to the following formula: SPF = E/(Ca-Cv). However, the decrease in EF throughout the baseline period, which was characterized by a constant SBF, remains unexplained. The decrease in EF during continuous infusion has been observed previously in pigs[3] and patients with fatty liver and cirrhosis[2]. This observation may be due to the limited capacity of hepatocytes to process 99mTc-MBF from the blood to the bile.

International guidelines[14,15] on CII suggest the exclusive use of investigations that describe the morphology of the intestinal arteries for the diagnosis of CII. The diagnostic criteria include a combination of relevant symptoms, such as postprandial abdominal pain and

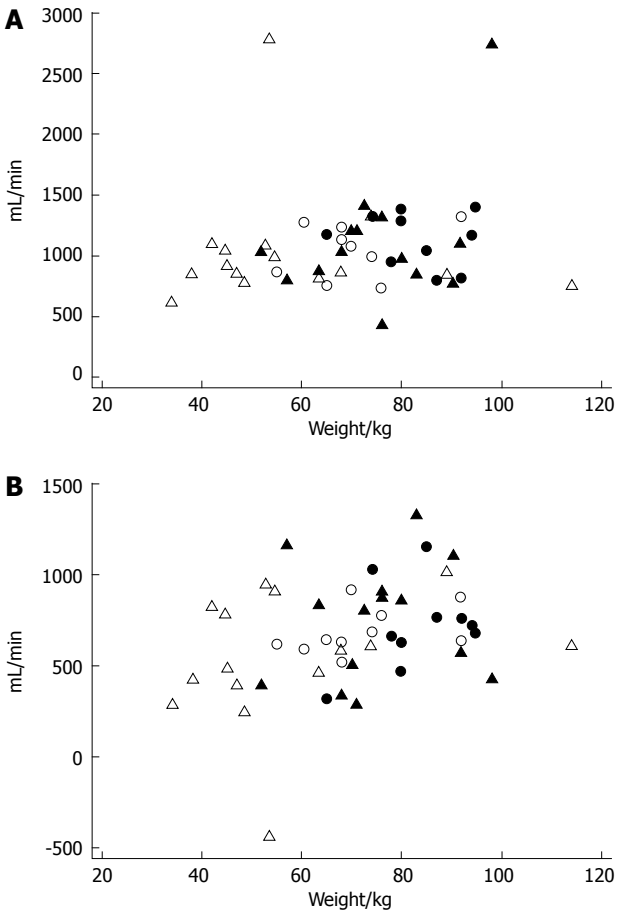

Figure 3 Postprandial increase in splanchnic blood flow for each individual as a function of bodyweight. A: Baseline splanchnic blood flow (SBF): the linear correlation between bodyweight and baseline SBF is given by the equation. SBF = 858 mL/min + 3.2 mL/min × bodyweight, the correlation is however not significant (P = 0.35); B: Postprandial increase in SBF: the linear correlation between bodyweight and the postprandial increase in SBF can be described by the equation: SBF (increase) = 282 mL/min + bodyweight × 5.4 mL/min (P = 0.025). △: Female patients with normal angiography; ▲: Male patients with normal angiography; ○: Healthy female volunteers; ●: Healthy male volunteers.

weight loss, and at least two significant stenoses/occlusions in the intestinal arteries. This approach ignores the well-documented knowledge of the frequent presence of inconsequential arterial stenosis[11,16,17]. Furthermore, patients with single-vessel disease or non-occlusive mesenteric ischemia are neglected.

The major drawbacks of physiological investigations, such as SBF measurements or tonometry, are that these techniques are often time consuming[18,19] and invasive. Therefore, the use of non-invasive investigations, such as duplex ultrasound (DUS) and MRI, are widely used. The wide availability and non-invasive nature of DUS make it an attractive screening tool. The overall accuracy, sensitivity and specificity for the identification of stenotic or occluded vessels for both the CA and the SMA are above 90% compared to DSA[20]. The meal-induced differences in blood flow velocity responses and the resistivity index in the celiac and superior mesenteric arteries can be assessed using DUS[21] as a surrogate marker for blood flow in healthy young volunteers. However, the calculation of blood flow volume is dependent on an accurate cross-sectional area of the vessel, which is difficult to obtain using DUS, especially in the intestinal arteries, and this area is almost impossible to obtain in patients with disseminated atherosclerosis. Therefore, DUS exhibits a disadvantage because only the morphology of the vessels is examined. Another major disadvantage of DUS is the requirement of an experienced examiner because obesity and overlying bowel gas may compromise the results and the lack of the visualization of the inferior mesenteric artery.

MRI has demonstrated promising results for the quantification of blood flow in the portal and superior mesenteric veins[22,23], and MR angiography has a short acquisition time (17 s)[24]. Another promising study reported the possible quantification of small bowel perfusion for the differentiation of normal individuals from patients with CII[25]. However, the capability of a functional assessment of CII using MRI has not been applied in a clinical setting despite the promise of previous reports. This fact assigns the measurement of SBF as one of a few clinical applicable investigations for the functional evaluation of CII.

In conclusion, the present study recommends the consideration of body weight in the diagnosis of CII using the postprandial increase in SBF.

COMMENTS

Background

Chronic intestinal ischemia is characterized by postprandial abdominal pain and weight loss. Atherosclerosis of the intestinal arteries is the most common cause of chronic intestinal ischemia. However, the rich collateral blood supply in the intestines may further develop in the presence of arterial stenosis to provide an adequate blood flow despite the significant stenosis of the intestinal arteries. Therefore, the application of physiological testing is required. The measurement of the splanchnic blood flow prior to and after a meal allows for an assessment of the physiological consequences of arterial stenosis in the intestines.

Research frontiers

Reference values for the total splanchnic blood flow and splanchnic oxygen uptake are lacking. The effect of body size and body composition should be considered because the majority of patients are underweight.

Innovations and breakthroughs

Previous investigations of the splanchnic blood flow have not considered the morphology of the intestinal arteries or the possible effect of body composition. An intestinal angiography and a whole-body dual-energy X-ray absorptiometry scan were performed in each of the healthy volunteers in the present study. A group of patients was included as a second group to achieve a weight range that represented individuals with very low bodyweight. All patients exhibited a completely normal angiography of the intestinal arteries. The splanchnic blood flow during fasting was independent of age, sex, body weight and body composition, but the meal-induced increase was directly related to body weight.

Applications

The study suggests that the meal-induced increase in splanchnic blood flow is directly related to body weight. This relationship should be considered in the diagnosis of chronic intestinal ischemia based on splanchnic blood flow measurements.

Terminology

Chronic intestinal ischemia is a clinical entity that is characterized by abdominal pain after eating. The pain causes the patient to eat smaller meals at longer intervals, which often leads to weight loss. The total splanchnic blood flow equals the blood flow through the liver, which can be calculated using the Fick principle.

Peer review

The paper makes sense to search for and refine new diagnostic tools, espe-

cially with regard to the functional aspects of the chronic mesenteric ischemia. The study design is valid and the data is sufficient.

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BRIEF ARTICLE

Mucosal healing effect of mesalazine granules in naproxen-induced small bowel enteropathy

István Rácz, Milán Szalai, Valéria Kovács, Henriett Regőczi, Gyöngyi Kiss, Zoltán Horváth

István Rácz, Milán Szalai, Valéria Kovács, Henriett Regőczi, Gyöngyi Kiss, Department of Gastroenterology, Petz Aladár County and Teaching Hospital, 9024 Győr, Hungary
Zoltán Horváth, Department of Mathematics and Computational Sciences, Széchenyi István University, 9026 Győr, Hungary
Author contributions: Rácz I designed the research; Szalai M, Kovács V, Regőczi H and Kiss Gy performed the research; Horváth Z analyzed the data; and Rácz I wrote the paper.
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Correspondence to: István Rácz, MD, PhD, Department of Gastroenterology, Petz Aladár County and Teaching Hospital, 9024 Győr, Hungary. raczi@petz.gyor.hu
Telephone: +36-96-418244 Fax: +36-96-418486
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Abstract

AIM: To investigate the effect of mesalazine granules on small intestinal injury induced by naproxen using capsule endoscopy (CE).

METHODS: This was a single center, non-randomized, open-label, uncontrolled pilot study, using the PillCam SB CE system with RAPID 5 software. The Lewis Index Score (LIS) for small bowel injury was investigated to evaluate the severity of mucosal injury. Arthropathy patients with at least one month history of daily naproxen use of 1000 mg and proton pump inhibitor co-therapy were screened. Patients with a minimum LIS of 135 were eligible to enter the 4-wk treatment phase of the study. During this treatment period, 3 × 1000 mg/d mesalazine granules were added to ongoing therapies of 1000 mg/d naproxen and 20 mg/d omeprazole. At the end of the 4-wk combined treatment period, a second small bowel CE was performed to re-evaluate the enteropathy according to the LIS results. The primary objective of this study was to assess the mucosal changes after 4 wk of mesalazine treatment.

RESULTS: A total of 18 patients (16 females), ranging in age from 46 to 78 years (mean age 60.3 years) were screened, all had been taking 1000 mg/d naproxen for at least one month. Eight patients were excluded from the mesalazine therapeutic phase of the study for the following reasons: the screening CE showed normal small bowel mucosa or only insignificant damages (LIS < 135) in five patients, the screening esophagogastroduodenoscopy revealed gastric ulcer in one patient, capsule technical failure and incomplete CE due to poor small bowel cleanliness in two patients. Ten patients (9 female, mean age 56.2 years) whose initial LIS reached mild and moderate-to-severe enteropathy grades (between 135 and 790 and ⩾ 790) entered the 4-wk therapeutic phase and a repeat CE was performed. When comparing the change in LIS from baseline to end of treatment in all patients, a marked decrease was seen (mean LIS: 1236.4 ± 821.9 *vs* 925.2 ± 543.4, $P = 0.271$). Moreover, a significant difference between pre- and post-treatment mean total LIS was detected in 7 patients who had moderate-to-severe enteropathy gradings at the inclusion CE (mean LIS: 1615 ± 672 *vs* 1064 ± 424, $P = 0.033$).

CONCLUSION: According to the small bowel CE evaluation mesalazine granules significantly attenuated mucosal injuries in patients with moderate-to-severe enteropathies induced by naproxen.

Key words: Nonsteroidal anti-inflammatory drug; Small bowel enteropathy; Mesalazine granules; Mucosal healing; Capsule endoscopy

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INTRODUCTION

Non-steroidal anti-inflammatory drugs (NSAIDs) are among the most commonly prescribed medications worldwide[1,2]. The side effects of NSAIDs on the upper gastrointestinal tract are well-known, however, toxicity to the small bowel mucosa is an increasingly recognized problem and is emerging as a more prevalent site of injury than the stomach[3-7].

Advances in small bowel imaging have led to direct observation at enteroscopy and during the last decade by video capsule endoscopy (CE) allowing a full investigation of the entire small intestine[8-15].

Capsule endoscopy is capable of demonstrating NSAIDs-induced pathology in the small bowel. CE studies comparing NSAIDs users and healthy volunteers found a higher rate of lesions in the former group (55%-76% *vs* 7%-11%)[16-19]. The most common NSAID-induced lesions in the small bowel are mucosal breaks, reddened folds, petechial or red spots, and blood in the lumen without a visualized source of bleeding. The conclusion from these initial studies was that CE is the optimal diagnostic tool to obtain direct evidence of macroscopic injury of the small intestine, resulting from short-term (2-4 wk) administration of conventional NSAIDs.

Studies in long-term NSAID users suggest that 60%-70% have an asymptomatic enteropathy characterized by increased intestinal permeability and mild mucosal inflammation[18,20]. Occult intestinal bleeding resulting from long-term NSAID usage is an increasing clinical challenge, especially in elderly patients[21,22].

Management options for NSAID enteropathy are limited. Co-administration of proton pump inhibitors failed to prevent NSAID-induced small intestinal damage[7,8]. Cessation of NSAIDs is not tolerable for most arthropathy patients. Cyclooxygenase (COX)-2 inhibitors have fewer toxic effects in the small bowel[23-25]. However, due to increased cardiovascular event risk many physicians prefer to prescribe conventional NSAIDs[17]. Although a recently published short-term study showed promising results indicating that prostaglandin (PG) reduced the incidence of small-intestinal lesions induced by diclofenac sodium[19], there are no convincing long-term clinical data on the usefulness of PG co-therapy with NSAIDs. Sulfasalazine significantly reduced NSAID-provoked intestinal permeability in an experimental study[26].

Mesalazine has not yet been studied in the treatment of NSAID-induced enteropathy. However, according to the release patterns of the oral preparations of mesalazine some therapeutic efficacy was noted in Crohn's disease, *e.g.*, with Salofalk® tablets from the duodenum to the ileum[27-29]. Pharmacokinetic data indicate a release pattern of 5-aminosalicylate from Salofalk® granules preparation starting in the same intestinal region as that for Salofalk® tablets[30].

Thus, it would be valuable in a clinical setting to determine the therapeutic effect of mesalazine granules on NSAID-induced small intestine injury. Therefore, we investigated the effect of mesalazine granules on small intestinal injury induced by NSAIDs in an open label, uncontrolled pilot study. We assessed naproxen-induced mucosal changes using CE before and after 4 wk of treatment with 3 g mesalazine granules.

MATERIALS AND METHODS

This was a single center, non-randomized, open label, uncontrolled pilot study. Entry criteria included men and women, 18-75 years of age, with at least one month history of daily naproxen use of 1000 mg for osteoarthrosis, rheumatoid arthritis, or nonspecific arthritis. To avoid gastroduodenal mucosal injury, the patients were treated with a standard dose of proton pump inhibitor. A complete small bowel CE examination within 7 d of inclusion was performed to diagnose small bowel NSAID-induced enteropathy. Patients with a Lewis CE scoring index of at least 135 were eligible to enter the 4-wk treatment phase of the study. During this treatment period 3 × 1000 mg/d mesalazine granules (Salofalk® Granu-Stix®) medication was added to ongoing 1000 mg/d naproxen and 20 mg/d omeprazole therapy. At the end of the 4-wk combined therapy period, a second small bowel CE examination was performed to re-evaluate the CE enteropathy scoring index.

Patients with evidence of small bowel strictures as previously proven by barium small bowel follow through, enteroclysis or patency test capsule, or with swallowing disorders, gastroduodenal ulcer in the patient history or proven by upper endoscopy were excluded. Further exclusion criteria were a history of IBD and colon diverticulosis, mesalazine treatment within 12 wk and corticosteroid treatment within 30 d prior to baseline endoscopy. Known previous or concurrent malignancy was also an exclusion criterion.

This study was approved by the central ethics committee of the National Health Scientific Board, and all patients provided written informed consent before their enrollment in this study.

Capsule endoscopy

We used the PillCam SB capsule endoscopy system with RAPID 5 software (Given Imaging Ltd, Yokneam, Israel). The patients had a liquid diet on the day before the CE examination. For preparation of the small bowel, 2 L of polyethylene glycol (PEG) solution on the day before and 1 L of PEG on the morning of the CE examination were administered. The CE procedure and methodology for review of images were conducted as previously described[5,7]. Briefly, the patients were equipped with a sensor array and recorder-battery belt pack, and then swallowed a capsule, which continuously transmitted video images at 2 frames per second for 8 h, at which point, the apparatus was disconnected and the images were processed. All video images were independently analyzed by 2 reviewers per video for pathology detection. All images were saved for final comprehensive analysis.

Evaluation of small bowel injury

We investigated the Lewis Index Score (LIS) for small

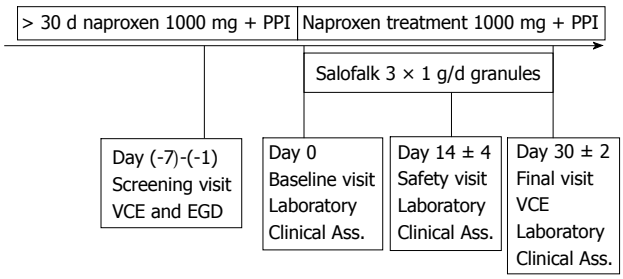

Figure 1 Study design. PPI: Proton pump inhibitor; VCE: Video capsule endoscopy; EGD: Esophagogastroduodenoscopy; ASS.: Assessment.

bowel enteropathy alterations to strengthen the validity of the results[31,32]. The LIS is incorporated as an integral part of Given's RAPID 5 version. The scoring index is based on three capsule endoscopy variables: villous appearance, ulceration, and stenosis. The changes in villous appearance and ulceration were assessed by tertiles, dividing the small bowel transit time into three equal time allotments. Villous appearance was defined as edema where villous width was equal or greater than villous height. Ulcerations were defined as mucosal breaks with white or yellow bases surrounded by red or pink collars. Ulcer size was based on the entire lesion including its surrounding collar and was measured according to the percentage of the capsule image occupied by the ulcerated lesion. The evaluation of stenosis was carried out for one entire study. The total score was the sum of the highest tertile score plus the stenosis score. The results were classified into the three categories by the final numerical score: normal or clinically insignificant change (< 135), mild change (between 135 and 790), and moderate-to-severe change (≥ 790).

The rate of successful arrival of the capsule in the cecum was assessed on the capsule endoscopic images. Gastric transit time was defined as the time taken from the first gastric image to the first duodenal image. Small bowel transit time was defined as the time from the first duodenal image to the first cecal image.

Small bowel cleanliness was graded using a 4-point scale (excellent[1] or good[2], fair[3] or poor[4]) for each tertile. Each small bowel tertile was scored separately, with the final cleansing score being the average of the sum of the three tertile cleansing scores[31].

Study protocol

At the screening visit, patients taking 1000 mg/d naproxen and 20 mg/d omeprazole first underwent esophagogastroduodenoscopy (EGD) to exclude active peptic ulcer. Those without active ulcer underwent the baseline CE examination. Eligible patients with LSI higher than 135 were administered 1000 mg mesalazine granules three times daily and 500 mg naproxen two times daily, and 20 mg omeprazole once daily immediately after meals for a period of 4 wk. Post-treatment CE was performed within 24 h after completion of the drug regimen (Figure 1). Patients who developed symptoms that warranted withdrawal from the study or with incomplete CE were excluded from the study and analysis.

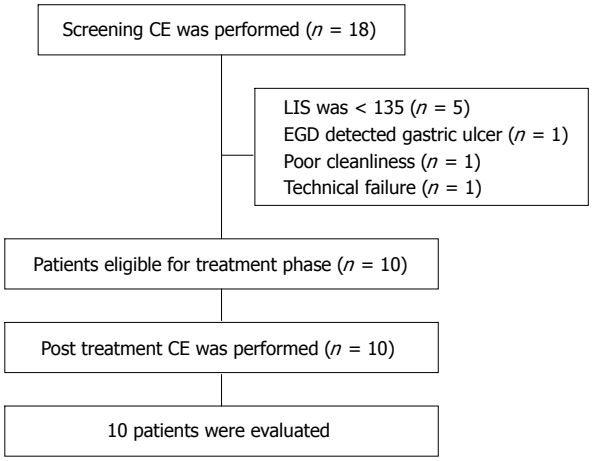

Figure 2 Flow chart of the study patients. LIS: Lewis Index Score; EGD: Esophagogastroduodenoscopy; CE: Capsule endoscopy.

Statistical analysis

The Lewis Index Score results, gastric transit time, small bowel transit time, cleanliness scores, hemoglobin results, results of baseline CE and the 4 wk control were compared and tested using the Wilcoxon signed rank test. In addition, paired *t* tests were also performed. Data were expressed as mean ± SD. *P* values < 0.05 were considered statistically significant. The effect of treatment on the presence of ulcers with a diameter of at least 3 mm was tested using the binomial sign test. McNemar tests were also performed but due to the small sample size, the binomial test was more relevant.

RESULTS

A total of 18 patients (16 females), ranging in age from 46 to 78 years (mean age 60.3 years) were screened, all had been taking 1000 mg/d naproxen for at least one month. Eight patients were excluded from the mesalazine therapeutic phase of the study for the following reasons: the screening CE showed normal small bowel mucosa or only insignificant damage (LIS < 135) in five patients, the screening EGD revealed gastric ulcer in one patient, and capsule technical failure and incomplete CE due to poor small bowel cleanliness in two patients (Figure 2). Ten patients whose initial LIS reached the mild and moderate-to-severe enteropathy grades (between 135 and 790, and ≥ 790, respectively) entered the therapeutic study phase and a second CE was carried out 4 wk later (Table 1). The CE enteropathy scores for small bowel are presented in Table 2.

At the screening CE, seven out of the ten enteropathy patients showed moderate-to-severe abnormalities according to the LIS grades (≥ 790).

In six of these seven moderate-to-severe small bowel enteropathy cases, the characteristic alterations were already seen in the first tertile, and in only three patients in the third tertile.

Table 1 Baseline characteristics of the intent-to-treat patient population (n = 10)

Characteristic	
Sex (female)	9
Age, yr (mean ± SD)	56.2 ± 9.7
Reason for NSAID therapy	
Rheumatoid arthritis	2
Osteoarthritis	8
Medication use	
Anti-hypertensive agents	3
Anti-diabetic agents	2
Low dose aspirin	NA
Statin	2

NA: Not available; NSAID: Non-steroidal anti-inflammatory drugs.

Table 2 Capsule endoscopy results according to Lewis Index Score evaluation of the small bowel mucosa at inclusion and 4 wk after mesalazine granules treatment

Subject No.	First tertile	Second tertile	Third tertile	Total LIS score
NSAID				
1	429	3040	1404	3040
2	225	450	0	450
3	1630	225	225	1630
4	1462	204	1462	1462
5	247	247	0	247
6	1104	654	562	1104
7	1312	712	135	1312
8	361	0	0	361
9	1068	1068	225	1068
10	1690	393	135	1690
NSAID + mesalazine				
1	900	1208	1237	1208
2	1462	165	0	1462
3	247	0	247	247
4	1068	339	1012	1068
5	112	0	0	112
6	1068	900	225	1068
7	1068	1068	233	1068
8	225	225	135	225
9	1104	0	0	1104
10	1690	1554	1104	1690

NSAID: Non-steroidal anti-inflammatory drugs; LIS: Lewis Index Score.

The average small bowel cleanliness grades were 1.3 ± 0.67 at the screening CE, and 1.4 ± 0.69 at the control CE examinations, respectively. The mean hemoglobin levels at screening and at the end of therapy were 131.1 ± 5.8 g/L and 127.1 ± 7.8 g/L, respectively (Table 3).

The most frequently observed NSAID-induced morphological alterations were villous edema which was present in all 10 patients at inclusion, and 19 ulcers were detected at baseline CE examinations (Figure 3). No stenosis was seen in any of the patients, either at inclusion or at the control CE examinations.

No dyspepsia or other significant abdominal symptoms were reported by the patients at any of the study visits. When comparing the LIS results assessed at inclusion and at the control CE after treatment with mesalazine granules, a marked decrease was seen (mean LIS: 1236.4 ± 821.9 *vs* 925.2 ± 543.4), however, the difference was not significant (P = 0.271).

Table 3 Capsule endoscopy results of the patients with naproxen and naproxen + mesalazine granules therapy (n = 10)

	Inclusion	After mesalazine gran therapy	P value
Total Lewis Index Score	1236.4 ± 821.9	925.2 ± 543.4	0.271
1st tertile	952.8 ± 584.9	894.4 ± 534.3	0.569
2nd tertile	699.3 ± 877.9	545.9 ± 580.9	0.649
3rd tertile	415.3 ± 561.5	319.3 ± 403.3	0.247
Ulcers with a diameter of > 3 mm, n			
1st tertile	8	7	1.000
2nd tertile	8	6	0.625
3rd tertile	3	4	1.000
Gastric transit time (min)	43.8 ± 32	49.8 ± 35.6	0.470
Small bowel transit time (min)	261.9 ± 108	250.9 ± 93.9	0.470
Cleanliness score	1.3 ± 0.67	1.4 ± 0.69	0.345
Hemoglobin	131.1 ± 5.8	127.1 ± 7.8	0.103

In contrast, a significant difference in pre- and post-treatment LIS was detected in the seven patients who had moderate-to-severe enteropathy gradings at the inclusion CE visit (mean LIS: 1615 ± 672 *vs* 1064 ± 424, P = 0.033). Further analysis showed that two patients (subject number 9 and 10) in the group with moderate-to-severe NSAID-induced enteropathy at inclusion, the total LIS score was unchanged or worsened after mesalazine treatment. However, even in these two patients a decrease in LIS scores was detected either in the first or second small bowel tertile which was also seen in the other 5 cases (Figure 4).

DISCUSSION

In the present pilot study we found that mesalazine granules reduced the severity of small intestine lesions induced by the administration of naproxen, a non-selective NSAID. Patients, who received naproxen therapy with mesalazine granules showed markedly lower enteropathy scores compared with the period when naproxen was taken alone. Importantly, the co-administration of mesalazine granules significantly attenuated the mucosal damage caused by naproxen in patients with moderate-to-severe enteropathy.

NSAIDs are known to increase intestinal permeability; the severity of the mucosal injury is dose-dependently related to the potency of NSAIDs to inhibit COX-1. The increase in intestinal permeability is the most important factor which provokes NSAID-induced inflammation and injury in the small intestine[2,3].

Previous studies evaluating NSAID-associated mucosal injury have shown various ways to interpret and compare CE data. Goldstein *et al*[17], who compared the effects of naproxen versus celecoxib on the small bowel, simply counted the number of mucosal breaks per tertile to measure adverse drug effects. Graham *et al*[18] assessed small bowel mucosal injury in chronic NSAIDs users. In their study, CE lesions were scored as normal, red spots, small erosions, large erosions or ulcers. Maiden *et al*[16]

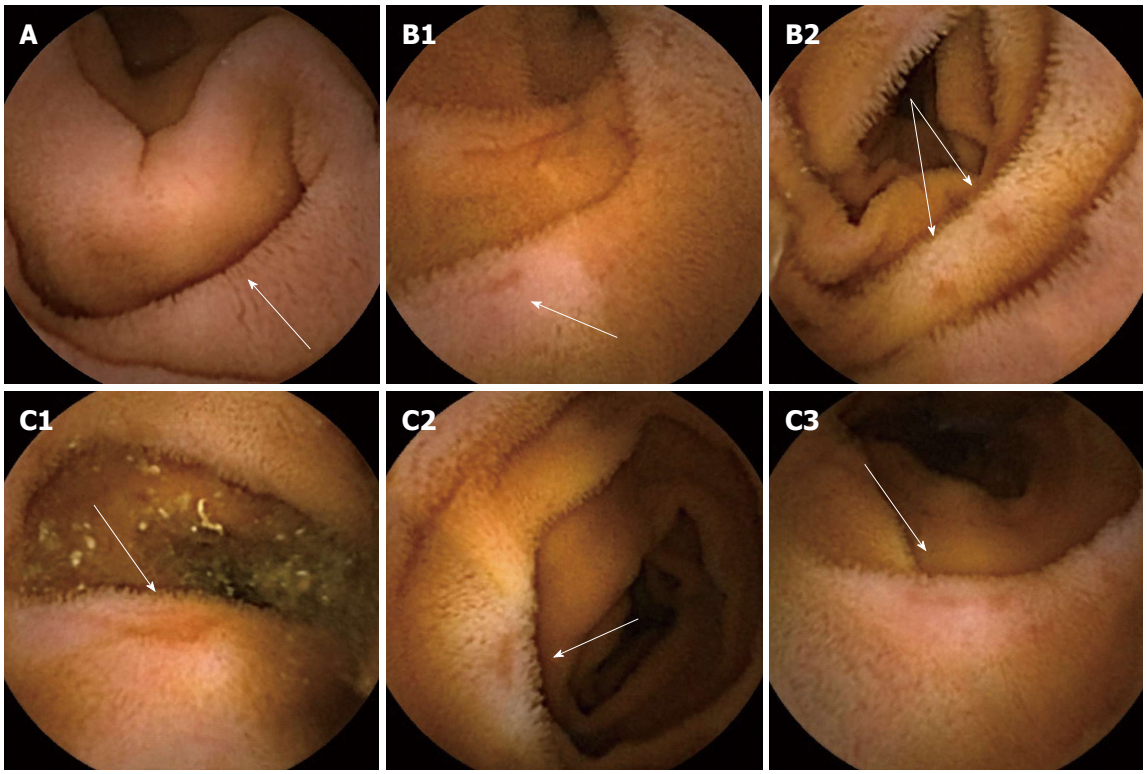

Figure 3 Examples of capsule endoscopy images in non-steroidal anti-inflammatory drug-induced enteropathy patients. A: Villous edema (arrow); B: Erosions (arrows); C: Non-steroidal anti-inflammatory drug- induced ulcers (arrows).

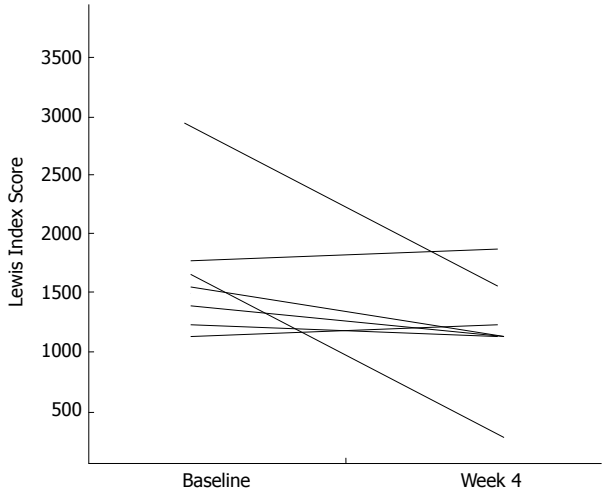

Figure 4 Lewis Index Score results before and after mesalazine granules treatment in patients with moderate-to-severe naproxen-induced enteropathy (*n* = 7).

graded diclofenac-induced lesions as category 1 (reddened folds), category 2 (denuded area), category 3 (petechia/red spot), category 4 (mucosal break), or category 5 (presence of blood without a visualized lesion).

A new score index has been developed by Gralnek *et al*[32] to assess mucosal changes in the small bowel detected by CE. The LIS offers objective scoring of small bowel injuries and has the potential to measure and document mucosal healing in response to therapy[33-36]. Therefore, in our study we used the LIS to assess the mucosal effect of naproxen as well as the effects of mesalazine granules therapy.

In the present pilot study we found mild and moderate-to-severe changes in the LIS in 66% of patients (10/15) who received NSAID and proton pump inhibitor therapy. These results are in a good correlation with the data from other CE studies in which NSAID-induced intestinal injury was directly evaluated. Maiden *et al*[16] found intestinal lesions using CE in 68% of healthy volunteers; Goldstein *et al*[17] reported that 55% of subjects on naproxen developed small-bowel injuries, while Fujimori *et al*[19] found small bowel mucosal breaks in 53% of subjects taking diclofenac. In these three studies, CE examinations were performed in healthy volunteers after two wk of NSAID medication. Graham *et al*[18] performed small bowel CE in chronic NSAID users with at least 3 mo history of daily NSAIDs for rheumatologic diseases. Small bowel injury was seen in 71% of these patients. Similar to this study we also recruited patients taking NSAIDs chronically which better reflects the real life situation in the small bowel compared to short-term NSAID exposure.

We analyzed the LIS results for NSAID-induced mucosal changes according to the small bowel tertiles. This analysis revealed that mucosal injuries tended to decrease in severity in the distal part of the small bowel. This result is inconsistent with a previous report in which chronic low dose aspirin-associated ulcers were observed mainly in the distal part of the small bowel[36]. However, there are no other available data regarding the topical dis-

tribution of NSAID-induced small bowel changes.

Management of NSAID-induced enteropathy is an unsolved problem. Moreover, the clinical significance of small bowel lesions is also a challenge. Proton-pump-inhibitors have been proved to be ineffective for the prevention of NSAID-induced injury[16-19]. Celecoxib, a selective COX-2 inhibitor effectively decreased the number of mucosal breaks and the percentage of subjects with at least one mucosal break[17]. Nevertheless, due to the risk of cardiovascular events, the use of COX-2 inhibitors is limited. In a recent pilot study co-administration of misoprostol was effective in preventing small bowel injury induced by NSAID medication in healthy volunteers[19]. Although the results of this short-term trial are promising, the clinical outcome of misoprostol therapy in typical chronic NSAIDs users is still an open question.

The role of sulfasalazine has been evaluated in 40 patients with rheumatoid arthritis on NSAIDs[26]. After 3-9 mo treatment, sulfasalazine (1.5-3.5 g/d) significantly reduced intestinal permeability, as measured by ^{111}In-labelled leucocytes.

Mesalazine has not yet been studied for the prevention or treatment of NSAID-induced enteropathy. Based on the hypothesis that mesalazine granules may have some therapeutic effect against NSAID-induced small bowel injury we designed a short-term therapeutic trial instead of a preventive trial. The primary objective of our pilot study was to assess the mucosal changes according to the LIS results detected by CE after 4 wk of treatment with mesalazine granules in patients receiving naproxen therapy. To avoid to inclusion of patients with clinically insignificant small bowel injuries, only patients with a LIS > 135 were recruited into the mesalazine granules therapeutic phase. When comparing the pre- and post-therapeutic LIS results in all 10 patients, a marked but not significant improvement in NSAID-induced enteropathy was observed. However, in the analysis of the 7 patients with moderate-to-severe initial enteropathy a significant improvement in the LIS was seen after treatment with mesalazine granules. These results demonstrate that patients whose initial enteropathy was more advanced benefited most from mesalazine treatment. Importantly, the improvement in LIS results was detected mainly in the first and second small bowel tertiles indicating that the mesalazine granules were effective even in the upper part of the small intestine. Although this is in contrast to the normal release profile of mesalazine, this may be due to the concomitant therapy with omeprazole.

According to the patient diaries and despite continuous NSAID treatment, our patients did not have dyspeptic symptoms or any other adverse effects. Significant anemia was not detected, either at inclusion or at the controls during the treatment phase. Ulcer-like clinical symptoms and dyspepsia are driven most probably by the gastroduodenal effect of NSAIDs which can be abolished or prevented by omeprazole co-therapy.

This is the first study to demonstrate using CE that mesalazine granules can reduce NSAID-induced macroscopic damage throughout the small intestine. This was also the first study to use the LIS system to evaluate the therapeutic effect of mesalazine granules on NSAID-induced small bowel injury. However, the trial has some inherent limitations. First, our study included only a small number of patients. Second, there was no parallel control group included to test the mucosal effect of the NSAID without mesalazine treatment. Third, this study was an open-label trial, which has a potential bias against neutrality.

Consequently, further studies are necessary to confirm the beneficial effect of mesalazine granules detected in the present pilot study.

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COMMENTS

Background

The side effects of non-steroidal anti-inflammatory drugs (NSAIDs) on the upper gastrointestinal tract are well-known, however, toxicity to the small bowel mucosa is an increasingly recognized problem and is emerging as a more prevalent site of injury than the stomach. Capsule endoscopy (CE) is capable of demonstrating NSAID-induced pathology in the small bowel. CE is the optimal diagnostic tool to obtain direct evidence of macroscopic injury of the small intestine, resulting from short-term (2-4 wk) administration of conventional NSAIDs. Studies in long-term NSAID users suggest that 60%-70% have an asymptomatic enteropathy characterized by increased intestinal permeability and mild mucosal inflammation. Management options for NSAID-induced enteropathy are limited. Mesalazine has not yet been studied in the treatment of NSAID-induced enteropathy. In this article the authors investigated the effect of mesalazine granules on small intestinal injury induced by naproxen in an open label, uncontrolled pilot study. The authors assessed naproxen-induced mucosal changes using CE before and after 4 wk of treatment with 3 g mesalazine granules.

Research frontiers

Entry criteria included men and women, 18-75 years of age, with at least one month history of daily naproxen use of 1000 mg for osteoarthrosis, rheumatoid arthritis, or nonspecific arthritis. To avoid gastroduodenal mucosal injury, the patients were treated with a standard dose of proton pump inhibitor. A complete small bowel CE examination within 7 d of inclusion was performed to diagnose small bowel NSAID-induced enteropathy. Patients with a Lewis capsule endoscopy scoring index of at least 135 were eligible to enter the 4-wk treatment phase of the study. During this treatment period, 3 × 1000 mg/d mesalazine granules (Salofalk® Granu-Stix®) medication was added to ongoing 1000 mg/d naproxen and 20 mg/d omeprazole therapy. At the end of the 4-wk combined therapy period, a second small bowel CE examination was performed to re-evaluate the capsule endoscopy enteropathy scoring index.

Innovations and breakthroughs

The authors found that mesalazine granules reduced the severity of small intestine lesions induced by the administration of naproxen, a non-selective NSAID. Patients, who received naproxen therapy with mesalazine granules showed markedly lower enteropathy scores compared with the period when naproxen was administered alone. Importantly, the co-administration of mesalazine granules significantly attenuated the mucosal damage caused by naproxen in patients with moderate-to-severe enteropathy.

Applications

This study offers a better understanding of the NSAID-induced enteropathy changes evaluated by the Lewis Index Score (LIS). The results demonstrated that patients whose initial naproxen-induced enteropathy was the most advanced benefited most from treatment with mesalazine granules.

Terminology

The LIS was used to investigate small bowel enteropathy alterations to

strengthen the validity of the results. The LIS is incorporated as an integral part of Given's RAPID 5 version. The scoring index is based on three capsule endoscopy variables: villous appearance, ulceration, and stenosis. The changes in villous appearance and ulceration were assessed by tertiles, dividing the small bowel transit time into three equal time allotments. The results were classified into the three categories by the final numerical score: normal or clinically insignificant change (< 135), mild change (between 135 and 790), and moderate-to-severe change (⩾ 790).

Peer review

Management of NSAID enteropathy is an unsolved problem. The authors present a pilot study in a group of patients with NSAID macroscopic lesions found in small intestine. The authors evaluate the effect of treatment by mesalazine in this group of patients using CE and an objective score (LIS). The result has limited statistical power due to the scarce number of patients included in the study. Nevertheless, the figures are promising and, as said by the authors, open an investigational field on NSAID side effects prevention/treatment, needing further investigation.

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BRIEF ARTICLE

Effect of *Gongronema latifolium* on gastric emptying in healthy dogs

Sylvester O Ogbu, Kenneth K Agwu, Isaac U Asuzu

Sylvester O Ogbu, Kenneth K Agwu, Department of Medical Radiography and Radiological Sciences, Faculty of Health Sciences and Technology, College of Medicine, University of Nigeria Enugu Campus, Enugu State 400001, Nigeria
Isaac U Asuzu, Department of Veterinary Physiology and Pharmacology, Faculty of Veterinary Medicine, University of Nigeria Nsukka Campus, Enugu State 400001, Nigeria
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Correspondence to: Dr. Sylvester O Ogbu, Department of Medical Radiography and Radiological Sciences, Faculty of Health Sciences and Technology, College of Medicine, University of Nigeria Enugu Campus, Enugu State 400001, Nigeria. sylvester.ogbu@unn.edu.ng
Telephone: +234-80-33760550 Fax: +234-42-771977
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Abstract

AIM: To investigate sonographically the effect of *Gonogronema latifolium* (*G. latifolium*) on gastric emptying of semi-solid meals in healthy dogs.

METHODS: In a randomized, placebo-controlled experiment, twenty-five clinically healthy dogs were randomly allotted into five groups of five dogs in each group. The placebo group served as the control, and the low, moderate and high dose groups ingested the methanolic leaf extract of *G. latifolium* in capsules at 100 mg/kg, 250 mg/kg and 500 mg/kg, respectively, while the prokinetic group ingested 0.5 mg/kg capsules of metoclopramide. After a 12-h fast, each group ingested its treatment capsules 30 min before the administration of a test meal. Measurements of gastric emptying and blood glucose levels were obtained 30 min before and immediately after the ingestion of the test meal and thereafter every 15 min for 4 h. This was followed by further measurements every 30 min for another 2 h.

RESULTS: The gastric emptying times of the placebo, low dose, moderate dose, high dose and prokinetic dose groups were 127.0 ± 8.2 min, 135.5 ± 3.7 min, 155.5 ± 3.9 min, 198.0 ± 5.3 min and 59.0 ± 2.5 min, respectively. Gastric emptying times of the moderate and high dose groups were significantly slower than in the placebo control group (155.5 ± 3.9 min, 198.0 ± 5.3 min *vs* 127.0 ± 8.2 min, $P = 0.000$). No significant difference in gastric emptying between the low dose and placebo control groups was noted (135.5 ± 3.7 min *vs* 127.0 ± 8.2 min, $P = 0.072$). Gastric emptying of the prokinetic group was significantly faster than that of the control group (59.0 ± 2.5 min *vs* 127.0 ± 8.2 min, $P = 0.000$). The hypoglycaemic effect of *G. latifolium* and gastric emptying were inversely related ($r = -0.95$, $P = 0.000$).

CONCLUSION: *G. latifolium* delays gastric emptying and lowers postprandial blood glucose in healthy dogs. It reduces the postprandial blood glucose by delaying gastric emptying.

Key words: *Gonogronema latifolium*; Gastric emptying; Sonography; Postprandial blood glucose; Semi-solid meals

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INTRODUCTION

Gastric emptying (GE) is the rate with which substances

leave the stomach after ingestion[1]. The process involves the storage of food, mixing with gastric secretions, grinding the solid food into particles of 1-2 mm in diameter, and subsequent delivery of the chyme into the small intestine at a rate designed to optimize digestion and absorption[2]. GE is one of the factors that affect the rate and completeness of intestinal nutrient absorption[3] and is a major determinant of postprandial glycaemic excursions not only in healthy subjects but in type 1 and type 2 diabetic patients[2].

The GE process can be influenced by a variety of physiological, pathological, pharmacological and dietary factors[4]. The effect on GE of *Gongronema latifolium* (*G. latifolium*) (Asclepiadaceae), which is used as a dietary and pharmacological agent for the control of postprandial blood glucose excursions[5,6], has, as far as we know, not been investigated previously. None of the relatively scanty information available on the mechanism(s) of action of *G. latifolium* relates to its effect on GE. We hypothesized that ingestion of a hypoglycaemic agent like *G. latifolium* might accelerate gastric emptying in healthy individuals just as insulin does[7].

In this study we investigated with the use of ultrasonography the effect of the methanolic leaf extract of *G. latifolium* on GE in health, as well as the relationship between its hypoglycaemic effect and GE, using the dog as an animal model. The study further investigated whether the effect of *G. latifolium* on GE in healthy individuals is dose dependent. This study is important as new therapies that aim to change blood glucose by modulating GE are being actively explored and evaluated.

MATERIALS AND METHODS

Plant preparation

Fresh *Gongronema latifolium* leaves were supplied, identified and authenticated by Mr. Ozioko AO, a taxonomist with the Bioresources Development and Conservation Programme Center, Nsukka, South Eastern Nigeria, an International Centre for Enthomedicine and Drug Development (INTERCEDD). A voucher specimen (INTERCEDD/170) was deposited for reference at the centre. The fresh *G. latifolium* leaves were air-dried, pulverized and the extract was prepared according to the previously described method by Ugochukwu and Babady[5]. The 2.2 kg dried powder of *G. latifolium* was extracted with 80% Romil-SA Methanol (MRS Scientific Ltd Essex United Kingdom) and dried in a hot air oven (Gallenkamp, England) at 40 ℃. The filtrates were concentrated at 40 ℃ using a vacuum rotary evaporator and freeze-dried to yield about 146.6 g of green coarse powder. The powder was further pulverized and encapsulated in doses of 100 mg, 250 mg and 500 mg for use in the study.

Animals

Clinically healthy mongrel dogs with no clinical and laboratory evidence of gastrointestinal disease, diabetes, gastroparesis, cardiovascular, pulmonary, renal, and hepatic diseases as ascertained by a veterinary doctor were used in this study. Pregnant female dogs confirmed by palpation and ultrasound were excluded. The dogs were dewormed with 5 mg/kg Levamizole® (Levamisole hydrochloride, Eagle chemical Co. Ltd, N. Korea) one week prior to the GE study. Food was withheld from the dogs for 12 h while water was withheld for 2 h before the study. The age, weight and fasting blood glucose concentration of the dogs did not differ between the control and the treatment groups ($P = 0.7$; $P = 0.2$; $P = 0.7$) (Table 1).

Table 1 Demographic and baseline clinical characteristics of the healthy groups (mean ± SD)

Characteristics	Placebo control	Low dose	Moderate dose	High dose	Prokinetic dose
Age (mo)	6.6 ± 0.7	6.5 ± 1.1	5.9 ± 0.8	6.4 ± 1.1	6.1 ± 0.3
Weight (kg)	6.2 ± 0.7	5.3 ± 0.3	5.6 ± 0.4	5.8 ± 0.7	5.7 ± 0.5
FBG (mmol/L)	4.0 ± 0.6	3.9 ± 0.1	4.0 ± 0.5	3.9 ± 0.2	4.2 ± 0.1

FBG: Fasting blood glucose.

Test meal

The test meal used consisted of 100 g proprietary canned Nestle Cerelac (Maize and Milk infant cereal, Nestle Nigeria plc) food and 150 mL of water. The calories and nutritional components in the 100 g food and 150 mL water are: Calories 1730 KJ (414 kcal); Protein 15 g; Fat 9 g; Carbohydrates 68.2 g; Dietary fibre 2 g; Minerals (ash) 3.3 g; Moisture 2.5 g.

Study design

The study was approved by the University of Nigeria Ethical Committee UNTH Enugu. The guidelines of the National Institutes of Health (NIH) *Principles of Laboratory Animal Care* (NIH Publication No. 86-23, revised 1985) were followed. This clinic-based experimental study was carried out in the University of Nigeria Veterinary Teaching Hospital, Nsukka. A randomized, placebo-controlled experimental design was adopted in this study and the dog was used as an animal model because of its established performance in the assessment of gastrointestinal motility[3] and in many physiological and pharmacological studies[8]. The dogs were randomly allotted into five groups of five dogs in each group. The placebo group served as the control; the low, moderate and high dose groups ingested the *G. latifolium* leaf extract capsules at 100 mg/kg, 250 mg/kg, 500 mg/kg, respectively, while the prokinetic dose group ingested 0.5 mg/kg capsules of metoclopramide (Mederax® 10 mg, Jiangsu Peng YAO Pharmaceutical Inc China). The prokinetic dose group served as prokinetic control. The prokinetic effect of metoclopramide is comparable to the insulin effect on gastrointestinal motility[7].

After a 12-h fast, each group ingested its treatment capsules 30 min before the administration of the test meal. Measurements of GE and blood glucose levels were obtained 30 min before and immediately after the ingestion of the test meal and then every 15 min for 4 h for each dog. Further measurements were made every 30 min for another 2 h. The three doses of *G. latifolium* were

Table 2 Gastric emptying and incremental postprandial blood glucose concentration of the healthy groups (mean ± SD)

Group	GE (min)	AUC of *i*PPBG (mmol/L × min)
Placebo control	127.0 ± 8.2	1028.6 ± 204.2
Low dose	135.5 ± 3.7	938.1 ± 40.0
Moderate dose	155.5 ± 3.9[b]	559.1 ± 101.8[b]
High dose	198.0 ± 5.3[b]	223.5 ± 52.2[b]
Prokinetic dose	59.0 ± 2.5[b]	1426 ± 108.2[b]

[b]$P < 0.0001$ *vs* control group. *i*PPBG: Incremental postprandial blood glucose concentrations; GE: Gastric emptying; AUC: Area under the curve.

introduced to assess its dose-dependent effect. The minimum dose of 100 mg/kg was based on the dose used in mice and rats[5,9]. All the treatment capsules ingested by the dogs were visually identical. The dogs ingested the test meal under natural free-feeding circumstances.

Measurement of gastric emptying

Gastric emptying was measured using an ultrasound technique as described by Chalmers *et al*[10] and McLellan *et al*[11]. The examinations were performed using a veterinary ultrasound machine with a 6-8 MHz microconvex transducer (Medison SA-600 v; 2006; Medison Co., Ltd., South Korea). Each dog was gently restrained while erect and the transducer was placed in a longitudinal orientation on the ventral midline, caudal to the xiphoid. The ultrasound beam was maintained in the sagittal plane and directed cranially until the liver was located and the stomach identified immediately caudal to it. The stomach was observed using real-time imaging, allowing the image to be frozen between peristaltic contractions when it was at a constant, maximal distension. Electronic callipers were used to measure the craniocaudal and ventrodorsal diameters of the antrum between the serosal margins. The antral area was calculated by using the software incorporated in the ultrasound machine to predict the area inside the elliptical shape defined by the craniocaudal and ventrodorsal diameters of the stomach. Three measurements of antral area were taken at each time, and their mean was used for further calculations. Baseline values were subtracted from the measurements made at each subsequent time point and the values expressed as a percentage of maximal antral area. The percentages of the maximal antral areas measured during each test were plotted against time. The gastric half-emptying time with ultrasonography (T50) that correlated significantly with $t_{1/2}$ of carbon 13-labelled octanoic acid breath test in dogs[11] was used to describe the rate of GE. The T50 was defined as the time at which the antral area decreased to 50 percent of its maximal area. T50 was calculated by linear interpolation between two points in the curve.

Measurement of blood glucose

Five millilitres of blood was drawn from each dog's ear at specific time intervals indicated in the study design. Blood glucose was determined by using a portable Accu-chek® Advantage glucometer (Roche Diagnostics GmbH Mannheim Germany). The incremental blood glucose concentrations were computed and plotted against time, and the blood glucose area under the curve (AUC) was calculated from the blood glucose curve.

Statistical analysis

All the data were expressed as mean ± SD. Dunnett's test was used for parametric multiple comparisons between the control and the treatment groups. Analysis of variance linear trend test was used to assess the dose trend. Pearson correlation was used to assess the linear association between the values of two variables. The values were considered to be significant when the P value was less than 0.05. Graphpad prism version 5.03 for windows (Graphpad Software San Diego California United States) and SPSS 15.0 for Windows Evaluation Version (United States) were used for statistical analysis.

RESULTS

Effect of G. latifolium leaf extract on GE

The GE times of the moderate and high dose groups were significantly ($P = 0.000$) slower than in the placebo control, while the GE of the prokinetic group was significantly ($P = 0.000$) faster than in the placebo control group (Table 2). No significant ($P = 0.072$) difference in GE was observed between the low dose and control groups. The effect of *G. latifolium* on GE was also significantly ($P = 0.000$) dose dependent (Figure 1A).

Effect of G. latifolium leaf extract on postprandial blood glucose concentration

The AUC values of incremental postprandial blood glucose concentrations (*i*PPBG) of the moderate and high dose groups were significantly ($P = 0.000$) smaller than in the placebo control, while the AUC of *i*PPBG of the prokinetic group was significantly ($P = 0.000$) larger than that of the placebo control (Table 2). No significant ($P = 0.442$) difference in AUC of *i*PPBG was observed between the low dose and control groups. The effect of *G. latifolium* on AUC of *i*PPBG of healthy dogs was significantly ($P = 0.000$) dose-dependent (Figure 1B).

The hypoglycaemic effect of G. latifolium and GE

The hypoglycaemic effect of *G. latifolium* and GE were inversely related ($r^2 = 0.95$) (Figure 2).

DISCUSSION

The gastric emptying process can be influenced by a variety of physiological, pathological, pharmacological and dietary factors[4] but the effect on GE of *G. latifolium*, which is used as a dietary and pharmacological agent for the control of postprandial blood glucose excursions[5,6], has, as far as we know, not been investigated in animals/humans previously. In this study, we demonstrate that the ingestion of the methanolic extract of *G. latifolium* delayed the gastric emptying of a semi-solid meal dose-dependently.

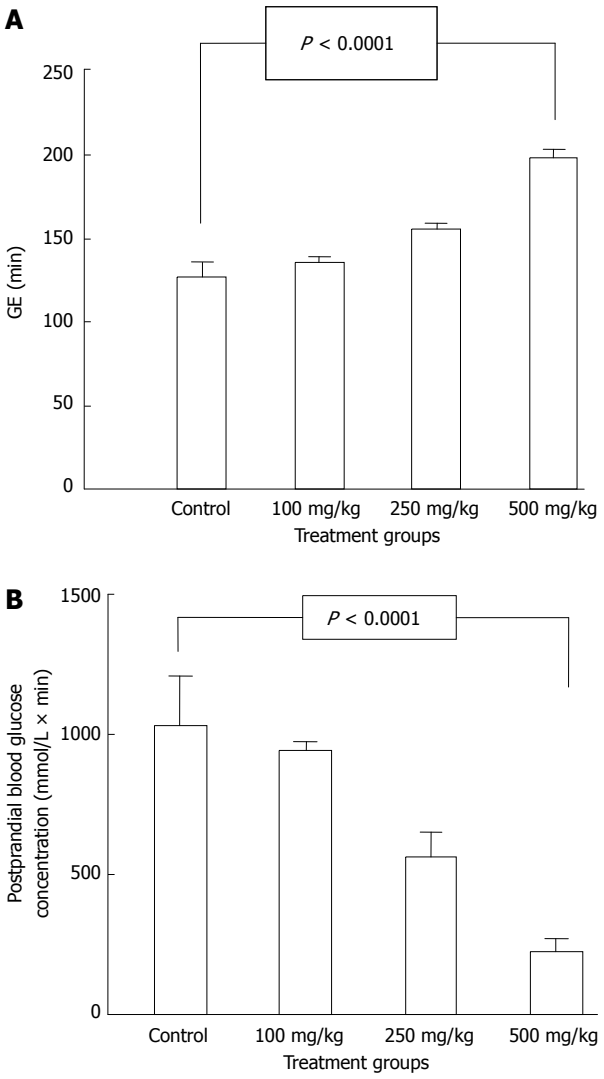

Figure 1 Dose-dependent effect of *Gongronema latifolium* on gastric emptying and postprandial incremental blood glucose concentrations. A: Dose-dependent effect of *Gongronema latifolium* (*G. latifolium*) on gastric emptying; B: Dose-dependent effect of *G. latifolium* on postprandial incremental blood glucose concentrations. GE: Gastric emptying.

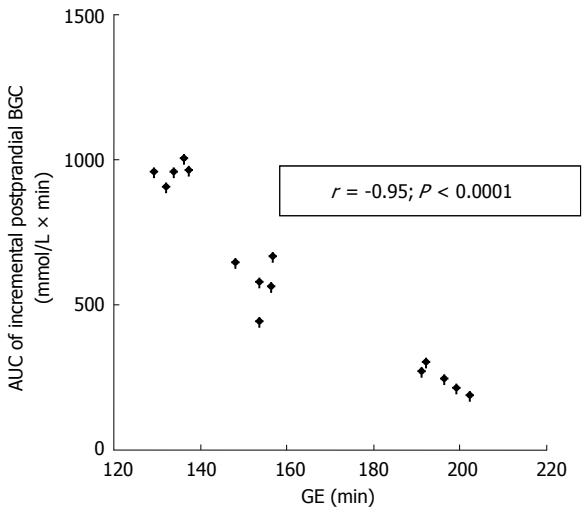

Figure 2 Relationship between the hypoglycaemic and gastric emptying effects of *Gongronema latifolium*. GE: Gastric emptying; AUC: Area under the curve; BGC: Blood glucose concentration.

The effect is similar to that of *Trigonella foenum-graecum*[12] and that of *Gymnema sylvestre*[13,14] which belong to the same Asclepiadaceae plant family as *G. latifolium*[15,16], but opposite to that of metoclopramide as noted in this study.

The presence of saponins in *G. latifolium*[17] and *G. sylvestre*[13,14] may be responsible for the similarity in their GE effects. Saponins significantly and dose-dependently delay gastric emptying[12-14,18]. GE is linearly associated with changes in the antral area[11,19,20]; therefore, our data suggest that *G. latifolium* causes less rapid reductions in antral areas and an increase in antral areas. This agrees with works which associated substances that slow GE with a relative increase in the content of the distal stomach[21,22] and inhibition of antral motility[23]. The more rapid reductions in antral areas and a decrease in antral areas observed with metoclopramide in this study are because it improves GE by increasing amplitude and frequency of antral contractions[4]. Therefore, the observed difference between the GE effect of metoclopramide and that of *G. latifolium* in this study may be due to their different mechanisms of action.

The mechanisms underlying the delayed effects of *G. latifolium* on GE were not evaluated in this study, but we speculate that the effect may be mediated by vagal mechanisms[21,24-26] and/or the release of gastrointestinal hormones[27]. *G. latifolium* may have elicited a gastrointestinal motor and/or sensory function that caused antral distension and subsequently suppressed antral contractions to result in a slower rate of antral delivery of the ingested semi-solid meals. The demonstration of the potential of *G. latifolium* to slow the rate of antral delivery of the ingested semi-solid meals from the stomach into the small intestine by this study is very important as new therapies that aim to change blood glucose by modulating GE are being actively explored and evaluated. The effect has hitherto not been demonstrated in animals or humans.

This study also demonstrates that there is an inverse relationship between the GE effect of *G. latifolium* and blood glucose concentration. It agrees with the fact that the pharmacologic acceleration of GE results in higher postprandial glucose concentrations, while delaying GE results in lower postprandial glucose concentrations after a physiologic meal[28]. Although the demonstration of a correlation does not establish causation, this finding suggests that *G. latifolium* when ingested with a meal may, through the mechanism of delayed GE, slow digestion and prolong the postprandial absorption of food, with a resultant improvement or reduction in postprandial blood glucose concentrations after a semi-solid meal. The rates of meal-derived glucose appearance in the systemic circulation are determined mainly by GE[29,30]. A previous work indicates that saponins with hypoglycaemic activity also inhibited GE while other saponins that have no hypoglycaemic activity did not affect GE[31]. Thus, saponins in *G. latifolium* may be responsible for the correlation between GE and its hypoglycaemic effects in healthy dogs. Some authors have proposed that saponin compounds act as hypogly-

caemic agents by delaying the transfer of glucose from the stomach to the small intestine, the main site of glucose absorption, and by inhibiting the glucose transport at the site of intestinal brush border membranes[32,33].

In this study, 100 mg/kg of *G. latifolium* did not significantly slow GE, probably due to low dose-response effect. The 100 mg/kg dose of *G. latifolium* might be below the threshold required to cause a significant delay on GE. Our findings that *G. latifolium* in a dose-dependent manner affects both the rate and extent of carbohydrate absorption by slowing the transfer of food from the stomach into the small intestine and thereby reducing or delaying exposure of nutrient to small bowel mucosa are clinically relevant with regard to improving postprandial blood glucose and triglycerides and consequently lowering the risk of chronic disease. Distension of the stomach is one factor that promotes the feeling of satiety[34]. Therefore, *G. latifolium* may, through the mechanism of delayed GE, play an important role in the regulation of appetite and energy intake. Finally, since interventions directed at modulating upper gastrointestinal motor and absorptive functions have a major effect on postprandial blood glucose excursion and are likely soon to enter the mainstream of therapy for diabetes[2,35], *G. latifolium* may be relevant in the current treatment and management of diabetes. Alternatively hyperglycaemia can cause a decrease in GE rate[36,37]. This factor is unlikely to have influenced the result of this study much since there was no statistically significant difference between the preprandial blood glucose concentrations in the subgroups when compared with the placebo control.

The neural, humoral and cellular mechanisms by which *G. latifolium* affects GE were not investigated, therefore further studies are required to elucidate them. Although the dog is an animal model for the study of human GE in many physiological and pharmacological studies[8] and an established model for the assessment of gastrointestinal motility[3], the pattern of findings may not be exactly the same in humans as demonstrated in this study.

Gongronema latifolium delays GE and lowers postprandial blood glucose in healthy dogs. It reduces the postprandial blood glucose by delaying GE. The effect has also been noted to be dose-dependent. Therefore it can play an important role in the current dietary and pharmacological approaches in the prevention, treatment and management of metabolic diseases like diabetes.

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COMMENTS

Background

Gastric emptying is a major determinant of postprandial glycaemic excursions not only in healthy subjects but in diabetic patients. Interventions directed at modulating gastric emptying have a major effect on postprandial blood glucose excursion. *Gongronema latifolium* (*G. latifolium*) (Asclepiadaceae) is currently used as a dietary and pharmacological agent for the control of postprandial blood glucose excursions, but its effect on gastric emptying has, as far as we know, not been reported.

Research frontiers

Previous studies have demonstrated the hypoglycaemic effect of *G. latifolium*, but none of the relatively scanty information available on its mechanism(s) of action relates to its effect on gastric emptying. In this study, the authors demonstrated that the gastric emptying delaying effect of *G. latifolium* could be one of the pathways for reducing the postprandial blood glucose level.

Innovations and breakthroughs

New therapies that aim to change blood glucose by modulating gastric emptying are being actively explored and evaluated. This is believed to be the first study to explore the effect of *G. latifolium* on gastric emptying in health as well as the relationship between its hypoglycaemic effect and gastric emptying.

Applications

The findings that *G. latifolium* in a dose-dependent manner slows the rate of antral delivery of the ingested semi-solid meals from the stomach into the small intestine may be clinically relevant in the management approach of postprandial blood glucose and triglycerides and consequently in diabetics.

Terminology

Gastric emptying is the rate with which substances leave the stomach after ingestion. The process involves the storage of food, mixing with gastric secretions, grinding the solid food into particles of 1-2 mm in diameter, and subsequent delivery of the chyme into the small intestine at a rate designed to optimize digestion and absorption. *G. latifolium* (Asclepiadaceae) is an edible tropical rainforest plant that is widely used in folk medicine.

Peer review

The authors present data on the effects of various doses of the methanolic leaf extract of *G. latiifolium* on gastric emptying and postprandial plasma glucose levels in a dog animal model. Gastric emptying was measured by ultrasonography. The data demonstrate dose related effects on inhibiting gastric emptying and plasma glucose.

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BRIEF ARTICLE

Ampulla dilation with different sized balloons to remove common bile duct stones

Neng-Ping Li, Jiang-Qi Liu, Zhi-Qiang Zhou, Tao-Ying Ji, Xiao-Yan Cai, Qing-Yun Zhu

Neng-Ping Li, Jiang-Qi Liu, Zhi-Qiang Zhou, Tao-Ying Ji, Xiao-Yan Cai, Qing-Yun Zhu, Department of General Surgery, Shanghai Gongli Hospital, Shanghai 200135, China
Author contributions: Li NP designed and performed the study, analyzed data and wrote the manuscript; Liu JQ instructed the study and revised the manuscript; Zhou ZQ performed the study and collected and analyzed data; Ji TY followed up the patients and collected and analyzed data; Cai XY collected data and supplemented references; and Zhu QY revised the manuscript.
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Correspondence to: Neng-Ping Li, MS, Chief Doctor, Department of General Surgery, Shanghai Gongli Hospital, No. 219, Miaopu Road, Pudong New Area, Shanghai 200135, China. linengp@163.com
Telephone: +86-21-58858730 Fax: +86-21-38821635
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Abstract

AIM: To assess the outcomes of ampulla dilation with different sized balloons to remove common bile duct (CBD) stones.

METHODS: Patients (n = 208) were divided into five groups based on the largest CBD stone size of < 5, 6-8, 8-12, 12-14, and > 14 mm. Patients underwent limited endoscopic sphincterotomy (EST) alone or limited EST followed by endoscopic papillary balloon dilation with 8, 10, 12 and 14 mm balloons, such that the size of each balloon did not exceed the size of the CBD. Short- and long-term outcomes, such as post-endoscopic retrograde cholangiopancreatography (ERCP) pancreatitis, perforation, bleeding, and pneumobilia were compared among the five groups.

RESULTS: The overall rate of successful stone removal in all groups was 100%, and all patients were cured. Eight (3.85%) patients had post-ERCP pancreatitis, none had perforations, and 6 (2.9%) had bleeding requiring transfusion. There were no significant differences in early complication rates among the five groups. We observed significant correlations between increased balloon size and the short- and long-term rates of post-ERCP pneumobilia. Post-ERCP pancreatitis and bleeding correlated significantly with age, with post-ERCP pancreatitis occurring more frequently in patients aged < 60 years, and bleeding occurring more frequently in patients aged > 70 years. We observed a significant correlation between patient age and the diameter of the largest CBD stone, with stones > 12 mm occurring more frequently in patients > 60 years old.

CONCLUSION: Choosing a balloon size based on the largest stone diameter is safe and effective for removing CBD stones. Balloon size should not exceed 15 mm.

Key words: Endoscopic papillary balloon dilation; Endoscopic sphincterotomy; Common bile duct stone; Endoscopic retrograde cholangiopancreatography; Pancreatitis

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INTRODUCTION

Endoscopic sphincterotomy (EST) and endoscopic papillary balloon dilation (EPBD) with balloons < 12 mm in diameter are the methods of choice for the removal of stones from the common bile duct (CBD)[1-4]. Both methods, however, have distinct advantages and disadvantages. EST is associated with serious short-term complications, such as hemorrhage, perforation, and pancreatitis,

and long-term complications such as permanent loss of sphincter of Oddi (SO) function and recurrent bile duct infection[5-7]. Although complications of bleeding and perforation seldom occur during EPBD[8-10] and SO function can be preserved[11,12], it is difficult to remove large CBD stones using EPBD because the biliary opening is not as enlarged as it is with EST, and EPBD is associated with a higher rate of post-endoscopic retrograde cholangiopancreatographic (ERCP) pancreatitis.

More recently, EPBD with large sized balloons (12-20 mm) has been used to remove large CBD stones following limited EST[13-16]. It is unclear, however, whether the increase in balloon size is associated with increased rates of short-term complications, such as perforation and hemorrhage, or with preservation of SO function. Combining the advantages of EST and EPBD, by selecting the correct sized balloon and EST incision length to achieve a high rate of stone extraction, while minimizing complications of both procedures, would be of great benefit to the patients. We therefore prospectively investigated the short- and long-term outcomes of different sized balloons, chosen according to each patient's maximum CBD stone size, to dilate the papilla following limited EST.

MATERIALS AND METHODS

Patients

Patients with possible CBD stones, as diagnosed by biliary symptoms and abnormality of biliary enzymes, or whose presence was suspected through imaging modalities, such as ultrasound (US), computed tomography (CT) and/or magnetic resonance imaging (MRI) were screened. Patients were excluded if they had (1) severe acute pancreatitis (APACHE Ⅱ ≥ 8, or Balthazar CT score ≥ 4); (2) severe cholangitis with disturbance of consciousness and shock; (3) coagulopathies; (4) malignant diseases; (5) a history of previous EPBD or EST; (6) age > 85 years; (7) a CBD filled with stones; and (8) had undergone a Billroth Ⅱ gastrectomy. Patients who met these criteria and lived in Shanghai, enabling follow-up, were fully informed about the methods and possible complications of the procedure, and were asked to provide written informed consent before ERCP. The study was approved by the Ethics Committee of Shanghai Gongli Hospital, and was supported by the Shanghai Municipal Health Bureau.

Patients were enrolled if selective deep cannulation to the CBD was successful, CBD stones were diagnosed by ERC, and an incision was made to the mid-portion of the papilla with a pull-type sphincterotomy. The diameter of the largest stone was determined by comparing it with the size of the endoscope tip. Patients were divided into five groups based on the largest CBD stone size, of (1) < 5 mm, (2) 6-8 mm, (3) 8-12 mm, (4) 12-14 mm, and (5) > 14 mm. These groups underwent limited EST alone without EPBD, and EPBD with balloons of 8, 10, 12 and 14 mm in diameter, respectively, such that the size of each balloon did not exceed the size of the CBD.

Methods

Pharyngeal anesthesia and premedication before the procedure, including the intravenous administration of diazepam, meperidine hydrochloride, and scopolamine, were performed in the same manner as for general endoscopy. ERCP was performed with a side-viewing endoscope (JF240; JF260V; Olympus, Tokyo, Japan).

Limited EST was performed according to the standard methods using a pull-type sphincterotomy. The incision was made up to the mid-portion of the papilla.

Endoscopic papillary balloon dilation

A balloon dilation catheter of 8, 10, 12 or 14 mm in diameter (Wilson-Cook Medical Inc., NC, United States), was inserted and inflated slowly with diluted contrast fluid until the waistline was obliterated under fluoroscopic monitoring and maintained for one min at 6 atm or 8 atm as required. After the balloon was deflated, the stones were extracted using a retrieval basket (Wilson-Cook Medical Inc., NC, United States) and/or a retrieval balloon (Extracter XL; Boston Scientific Corporation, MA, United States).

When the stone diameter was > 16 mm, as shown by diagnostic ERCP, a mechanical lithotripter (ML; BML-4Q; Olympus Corporation, Tokyo, Japan) was used to break the stones into fragments.

Follow-up

All patients were seen at the outpatient clinic six months to one year after discharge and every year thereafter. At each visit, blood and liver function tests, abdominal US and CT were performed. Other relevant examinations were performed when deemed necessary. If stone recurrence was suspected from symptoms, laboratory data, and/or images, ERCP was performed, and the recurrent stone was removed.

Outcome measures

Short-term outcomes included the rates of post-ERCP pancreatitis, bleeding requiring transfusion, perforation, pneumobilia and mortality. Long-term (2-5 years) outcomes included the rates of reflux cholangitis, pneumobilia, and recurrence of CBD stones.

Statistical analysis

Statistical analyses were performed using statistical software (SPSS 12.0 for Windows; SPSS Inc., Chicago, IL, United States). Quantitative data were presented as the mean ± SD. The χ^2 test or Fisher's exact test was used to compare sex distribution, and rates of mechanical lithotripter (ML) use, gallbladder *in situ*, concomitant gallbladder stones, and early and later complications in the 5 groups. ANOVA was used to compare age, number of stones, diameter of largest stone, and relationships between age and post-ERCP pancreatitis and bleeding in the 5 groups. A P value < 0.05 was considered statistically significant.

Table 1 Demographic data and baseline characteristics of the 5 patient groups

	Balloon diameter					
	EST alone (n = 42)	8 mm (n = 35)	10 mm (n = 87)	12 mm (n = 29)	14 mm (n = 15)	P value
Sex (F/M)	20/22	19/16	50/37	15/14	9/6	0.832
Age (yr)	55.6 ± 13.1	59.5 ± 14.2	66.8 ± 15.5	72.8 ± 11.8	74 ± 5.3	0.003
No. of stones	2.0 ± 1.0	2.2 ± 1.0	2.1 ± 1.4	2.1 ± 1.0	2.3 ± 1.0	0.994
Diameter of largest stone (mm)	5.5 ± 1.5	7.0 ± 2.0	9.3 ± 2.1	10.3 ± 2.7	14.7 ± 1.2	0.000
Use of mechanical lithotripter	0	0	0	0	4	0.000
Gallbladder *in situ*	33	29	74	21	10	0.358
Concomitant gallbladder stones	26	22	61	18	7	0.795
Sessions required for complete stone removal						
Single session	42	35	87	28	13	
Two sessions	0	0	0	1	2	

F: Female; M: Male; EST: Endoscopic sphincterotomy.

RESULTS

Patient characteristics and early complications

We enrolled 208 consecutive patients (95 males and 113 females), all of whom were diagnosed with CBD stones by ERC and underwent successful selective deep cannulation to the CBD at our institution between January 1, 2006, and January 1, 2008. Mean patient age was 62.4 ± 15.0 years. Stones were successfully removed from all the patients in all 5 groups. The demographic data and baseline characteristics of the 5 groups are shown in Table 1.

We observed a significant correlation between patient age and the diameter of the largest stone, with stones > 12 mm occurring more frequently in patients > 60 years old. The ML was used more often in patients with larger CBD stones, especially for stones > 16 mm in diameter. Although the overall success rate of stone removal was 100% in all groups, two patients in the 14 mm balloon group and one in the 12 mm group each required 2 sessions for stone removal due to patient intolerance of a long operation time to remove large stones.

Early complications

All patients were cured, none died, and none had a perforation. We found that 8 (3.85%) patients had post-ERCP pancreatitis, including 1 in the 10 mm balloon dilation group who had severe pancreatitis. All patients were cured by conservative treatments. Six (2.9%) patients experienced upper gastrointestinal bleeding requiring transfusions, including 2 patients with bleeding in the stomach and 4 with bleeding in the duodenal papilla. Two patients were cured by angiographic embolization and 1 by laparotomy to ligate the bleeding vessel after 2 attempts of endoscopic clamping and 1 of angiographic embolization all failed. The other 3 patients were cured conservatively. There were no significant differences in early complication rates among the five groups.

Pneumobilia occurred in 55 (26.4%) patients at a mean of 4.9 ± 0.7 d (range, 3-7 d) after ERCP. We observed a significant correlation between increased balloon size and the incidence of pneumobilia, suggesting that dilation with a large balloon may cause more damage to SO function. Details of early complications are described in Table 2.

Interestingly, post-ERCP pancreatitis and bleeding correlated significantly with age, with post-ERCP pancreatitis occurring more frequently in patients aged < 60 years, and bleeding occurring more frequently in patients aged > 70 years. The 8 patients with post-ERCP pancreatitis were significantly younger than the 200 who did not develop post-ERCP pancreatitis (51.1 ± 8.3 years *vs* 63.5 ± 15.1 years, P = 0.026). Conversely, the 6 patients with bleeding were significantly older than the 202 who did not develop bleeding (75.7 ± 7.1 years *vs* 61.4 ± 15.0 years, P = 0.024).

Later complications

Of the 208 patients, 192 (92.3%) were followed up for at least 2 years, with a mean follow-up time of 3.2 ± 1.1 years (range, 2-5 years). There were no significant differences in the rates of later complications, including reflux cholangitis and recurrence of CBD stones, among the 5 patient groups.

The incidence of pneumobilia one year after ERCP was significantly lower than shortly after ERCP, suggesting that SO function had recovered, at least partially, in these patients. We observed a significant correlation between the size of the dilation balloon and the 1-year incidence of pneumobilia, suggesting that larger balloons may cause more damage to SO function. The details of later complications in each group are shown in Table 2.

DISCUSSION

As no standard endoscopic procedure has been developed to date to maximize the effects and minimize the complications of EST and EPBD[17,18], we prospectively assessed a method combining EPBD with limited EST. CBD stone sizes vary, from 3-5 mm in diameter to 15-30 mm in diameter, or even larger, suggesting that an endoscopic treatment method should be based on stone size. We therefore utilized limited EST alone for CBD stones < 5 mm in diameter, and limited EST followed by EPBD with balloons of 8, 10, 12 and 14 mm for CBD stones 6-8, 8-12, 12-14 and > 14 mm, respectively. We found that tailoring balloon size to stone size was safe and effective,

Table 2 Early and later complications in the 5 patient groups

	Balloon size					
	EST alone (n = 42)	8 mm (n = 35)	10 mm (n = 87)	12 mm (n = 29)	14 mm (n = 15)	P value
Early complications						
Post-ERCP pancreatitis	1	2	3	1	1	0.918
Perforation	0	0	0	0	0	
Bleeding	1	1	3	1	0	0.961
Incidence of pneumobilia	7	5	25	11	7	0.039
Later complications						
Long term outcome						
Incidence of pneumobilia	2	1	10	5	4	0.029
Reflux cholangitis	0	0	2	2	1	0.235
Recurrence of CBD stones	0	0	2	1	0	0.624

ERCP: Endoscopic retrograde cholangiopancreatography; EST: Endoscopic sphincterotomy; CBD: Common bile duct.

with low rates of short- and long-term complications.

Limited EST was sufficient to remove CBD stones < 5 mm in diameter, as the biliary opening was large enough to remove these stones. EBPD was not required as balloons larger than CBD stones can cause more damage to SO function. We found that limited EST did not cause any perforations, an often fatal complication and even more serious than pancreatitis and bleeding, and preserved SO function.

We also found that limited EST followed by EPBD with balloons < 12 mm in size could partially preserve SO function. Although limited EST plus EPBD with balloons 12-14 mm in size did not cause any perforations, it was associated with higher rates of pneumobilia, both shortly after ERCP and ⩾ 2 years later, compared with limited EST alone or followed by EPBD with smaller balloons, suggesting that large balloons result in greater damage to SO function.

Limited EST followed by EPBD has several benefits, including a lower incidence of post-ERCP pancreatitis. After EST, the openings of the pancreatic duct and common bile duct separate, decreasing the pressure on the pancreatic duct caused by EPBD and papillary edema[19]. Using limited EST in all our patients with CBD stones, we found that the overall incidence of post-ERCP pancreatitis was 3.85%, lower than previously reported[5-7,10]. Limited EST combined with EPBD can also make cannulation easier and reduce the procedure and fluoroscopy times[20] by shortening the cannulation length, and is safer than full EST or EPBD alone, because full EST may lead to perforation, while EPBD alone may lead to post-ERCP pancreatitis. Furthermore, limited EST is easier to perform than full EST. We have successfully utilized this method to remove large CBD stones since 1999[21], and have found that it is a good choice for different sized CBD stones.

Large balloon dilation of the papilla may make the removal of CBD stones easier, reducing the need for an ML, and shortening cannulation and stone removal times, thus decreasing the incidence of post-ERCP pancreatitis. However, we did not use a balloon > 15 mm. A recent study in animals showed that EPBD with balloons < 15 mm was safe, with no perforations, whereas balloons > 15 mm was associated with a significantly higher rate of perforation[22]. In contrast, the use of 8 mm balloons in animals showed that EPBD was not associated with fibrosis or altered papillary architecture[23] and many clinical reports have shown that EPBD with large balloons > 15 mm was effective and safe[13-16]. However, the risk of perforation is higher with large balloons, prolonging hospitalization and increasing costs. We also found that increased dilation size was significantly associated with an increased incidence of pneumobilia, indicating that dilation with large balloons may cause more damage to SO function. Although we found no significant differences in later complications, such as reflux cholangitis and recurrence of CBD stones among the 5 patient groups, follow-up time may not have been sufficiently long. SO function is important in preventing biliary diseases, such as acute cholecystitis, cholangitis and recurrence of CBD stones[24], suggesting that preserving SO physiological function may be advantageous, especially in younger patients. Our findings also indicate that 14 mm balloons were large enough to remove CBD stones > 15 mm, assisted by an ML. Taken together, our findings indicate that limited EST, followed by EPBD with balloons < 15 mm is safe.

We found that the rate of post-ERCP pancreatitis and bleeding correlated with patient age, with patients < 60 years more frequently having post-ERCP pancreatitis and patients > 70 years more prone to bleeding. The progressive decline in pancreatic exocrine function with age may protect older patients from pancreatic injury[25,26]. In contrast, the Oddi muscle may be stronger in younger than in older patients, resulting in more difficult dilation in the former and a higher rate of pancreatitis. Although bleeding has seldom been reported after EBPD, we found that 6 (2.9%) of our patients had upper gastrointestinal bleeding requiring transfusions. All 6 were > 65 years old, with a mean age of 76 years. Older patients may be more prone to bleeding due to the relative inelasticity of their blood vessels. We also observed a correlation between CBD stone size and patient age, with stones > 12 mm occurring more frequently in patients > 60 years old.

The main limitation of this study was that we evaluated SO function by pneumobilia incidence, and not by endoscopic manometry. Manometry requires cannulation to the CBD, making it painful for patients and unacceptable during follow-up. Other limitations include the performance of this study at a single center, the relatively small number of patients, and the relatively short follow-up period.

In conclusion, limited EST, alone or followed by EPBD with balloons 8-14 mm, is safe and effective for the removal of different sized CBD stones. Choosing balloon size based on CBD stone size can maximize outcomes and minimize the complications of both EST and EPBD. Balloons > 15 mm in size are not necessary.

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COMMENTS

Background

Common bile duct (CBD) stones are very common, and patients are traditionally treated by open CBD exploration. With the advent of invasive endoscopic techniques, more patients are being treated endoscopically. However, endoscopists are often faced with difficult treatment decisions: endoscopic sphincterotomy (EST) or endoscopic papillary balloon dilation (EPBD)? Both methods have distinct advantages and disadvantages. The authors of this study prospectively investigated the short-and long-term outcomes of different sized balloons to dilate the papilla following limited EST.

Research frontiers

Both EST and EPBD are used for patients with CBD stones. Limited EST combined with EPBD has become popular for the removal of CBD stones in recent years, as it is thought to maximize outcomes and minimize the complications of both EST and EPBD.

Innovations and breakthroughs

This study investigated the short- and long-term outcomes of different sized balloons, chosen according to each patient's maximum CBD stone size, to dilate the papilla following limited EST. The authors found that choosing a balloon size no more than 15 mm based on the diameter of each patient's largest CBD stone is a good choice for removing CBD stones.

Applications

EPBD using a balloon size no more than 15 mm based on the diameter of patient's largest CBD stone following limited EST was proven to be a safe and effective treatment for CBD stones, and should be recommended for patients with CBD stones.

Terminology

ERCP: endoscopic retrograde cholangiopancreatography, a technique that combines the use of endoscopy and fluoroscopy to diagnose and treat certain problems of the biliary or pancreatic ductal systems; EST: endoscopic sphincterotomy, a minimally invasive surgery that was developed on the basis of ERCP to treat biliary or pancreatic ductal disease, the incision is made up to the full-portion of the papilla; Limited EST: like EST, the incision is made up to the mid-portion of the papilla; EPBD: endoscopic papillary balloon dilation, the papilla is dilated with a balloon to facilitate the removal of CBD stones.

Peer review

This is a good study in which authors evaluate the short- and long-term outcomes of different sized balloons to dilate the papilla following limited EST. The results are interesting and suggest that choosing balloon size no more than 15 mm based on the diameter of each patient's largest CBD stone is a good choice for removing CBD stones.

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BRIEF ARTICLE

Novel *CDH1* germline mutations identified in Chinese gastric cancer patients

Qin-Hua Chen, Wei Deng, Xiao-Wei Li, Xiu-Fang Liu, Jing-Mei Wang, Li-Feng Wang, Nong Xiao, Qiong He, Ya-Ping Wang, Yi-Mei Fan

Qin-Hua Chen, Wei Deng, Xiao-Wei Li, Xiu-Fang Liu, Ya-Ping Wang, Yi-Mei Fan, Department of Medical Genetics, Medical School, Nanjing University, Nanjing 210093, Jiangsu Province, China
Qin-Hua Chen, Wei Deng, Xiao-Wei Li, Xiu-Fang Liu, Ya-Ping Wang, Yi-Mei Fan, Jiangsu Key Laboratory of Molecular Medicine, Nanjing 210093, Jiangsu Province, China
Jing-Mei Wang, Department of Pathology, Drum Tower Hospital Affiliated to Medical School, Nanjing University, Nanjing 210093, Jiangsu Province, China
Li-Feng Wang, Department of Oncology, Drum Tower Hospital Affiliated to Medical School, Nanjing University, Nanjing 210093, Jiangsu Province, China
Nong Xiao, Qiong He, Lujiang Hospital, Lujiang 231500, Anhui Province, China
Author contributions: Chen QH and Deng W contributed equally to this work; Fan YM conceived and designed the study, and drafted the manuscript; Deng W also helped to draft the manuscript; Chen QH and Deng W carried out mutation screening, case-control and bioinformatics analysis; Chen QH and Liu XF performed promoter luciferase activity assay; Li XW carried out RNA splicing analysis; Wang JM, Wang LF, Xiao N and He Q contributed to the collection of samples and clinical data; Wang YP reviewed and modified the paper; and all of the authors have read and approved the final manuscript.
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Correspondence to: Dr. Yi-Mei Fan, Associate Professor, Department of Medical Genetics, Medical School, Nanjing University, Hankou Road 22, Nanjing 210093, Jiangsu Province, China. ymfan@nju.edu.cn
Telephone: +86-25-83593374 Fax: +86-25-83686559
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Abstract

AIM: To give a comprehensive report of E-cadherin gene (*CDH1*) variations in a population at a high risk for gastric cancer (GC).

METHODS: The samples consisted of 178 men and 58 women with a mean age of 62.3 ± 9.4 years and an age range of 30-84 years. A total of 240 cancer-free controls were recruited (mean age of 61.8 ± 10.1 years, age range of 26-82 years). Samples were screened for *CDH1* germline mutations by high-resolution melting analysis or directly sequencing. Luciferase reporter assay, RNA splicing assay and bioinformatic analysis were used to evaluate the effect of mutations.

RESULTS: Four novel *CDH1* sequence alterations were identified in GC patients including a G>T transition 49 bp before the start codon; a three-nucleotide deletion, c.44_46del TGC; one missense mutation, c.604G>A (V202I); and one variation in the intron, c.1320+7A>G. In addition, polymorphism frequencies were observed for *CDH1*-164delT, -161C>A, -73A>C, c.48+6C>T, c.48+62_48+63delinsCGTGCCCCAGCCC, c.894C>T (A298A), c.1224G>A (A408A), c.1888C>G (L630V), c.2076T>C (A692A), and c.2253C>T (N751N) which is similar to the data reported in http://www.ncbi.nlm.nih.gov/projects/SNP/. RNA splicing analysis suggested that the c.1320+7A>G and c.1224G>A variations did not affect exon splicing ability. Luciferase reporter assay demonstrated that the c.-49T variation might be helpful for *E-cadherin* transcription, though the increase in transcription activity is limited (only 33%). SIFT score and PolyPhen analysis both demonstrated that the L630V missense mutation probably damages protein function, while the V202I variant does not.

CONCLUSION: This study reveals novel mutations in sporadic GC patients which had been poorly investigated for susceptibility genes.

Key words: Gastric cancer; Germline mutation; *CDH1*; Luciferase reporter assay; RNA splicing analysis

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INTRODUCTION

Gastric cancer (GC) is one of the most common malignancies worldwide and the leading cancer in East Asian countries[1]. There are two histopathological types of gastric cancer, differentiated and undifferentiated[2], or intestinal and diffuse, respectively[3]. Genetic factors are important for the etiology of GC. The E-cadherin gene (*CDH1*), a calcium-dependent transmembrane glycoprotein, is critical for epithelial architecture, intercellular adhesion, and cell invasion[4]. E-cadherin consists of a large extracellular domain composed of five repeat domains and smaller transmembrane and cytoplasmic domains[5]. Mutations in the *CDH1* gene and perturbation of E-cadherin expression are the most frequent genetic alterations in hereditary diffuse gastric cancer (HDGC)[6,7]. The *CDH1* germline mutation spectrum is heterogeneous and includes point mutations, small deletions, and insertions distributed along the entire coding sequence[8-10]. In *CDH1* germline variation carriers, the lifetime penetrance is estimated to be approximately 70%[11]. The identification of *CDH1* mutations offers the opportunity for the development of cancer risk-reduction strategies for unaffected at-risk individuals. About 90% of gastric carcinoma presents a sporadic setting and only 10% shows a familial cluster; among this group, about 15% are considered as hereditary syndromes, such as the HDGC. For sporadic GC, germline *CDH1* mutations are seldom reported.

In this study, we carried out a comprehensive screen of *CDH1* germline mutations in 236 Chinese GC patients (175 sporadic cases and 61 cases with hereditary predisposition) (Table 1) and identified four novel germline *CDH1* mutations in sporadic GC patients. In addition, the *CDH1* polymorphism frequencies in Chinese GC patients and controls were determined. Furthermore, functional assays were carried out to evaluate the impact of the novel mutations identified.

MATERIALS AND METHODS

Subjects

Gastric cancer patients from the East District of China having disease onset between January 1 and December 31, 2008, in whom tumors had been confirmed using histology, were investigated. The samples consisted of 178 men and 58 women with a mean age of 62.3 ± 9.4 years and an age range of 30-84 years. A total of 240 cancer-free controls were recruited (mean age of 61.8 ± 10.1 years, age range of 26-82 years) (Table 1). Details regarding the following information are summarized in Table 1: gastric cancer family history, age of onset and histological and tumor-node-metastasis (TNM) staging classifications. Informed consent, according to the Ethics Committee of the Medical School of Nanjing University, was obtained from all subjects who underwent genetic testing.

Table 1 Frequency distributions of variables in gastric cancer cases and controls *n* (%)

Variables	Cases	Controls	*P* value
Number	236	240	
Age (yr)			0.997
≤ 49	19 (8.1)	19 (7.9)	
50-59	65 (27.5)	65 (27.1)	
60-69	94 (39.8)	98 (40.8)	
≥ 70	58 (24.6)	58 (24.2)	
Gender			0.915
Male	178 (75.4)	180 (75.0)	
Female	58 (24.6)	60 (25.0)	
Family history			
Familial recurrence for gastric cancer[1]	6 (2.5)		
Low familial recurrence for gastric cancer[2]	39 (16.5)		
Young age (< 50 yr) of sporadic disease	16 (6.8)		
Old age (≥ 50 yr) of sporadic disease	175 (74.2)		
Histologic grade[3]			
Poorly differentiated adenocarcinoma	64 (39.5)		
Moderately differentiated adenocarcinoma	69 (42.6)		
Well differentiated adenocarcinoma	29 (17.9)		
Depth invasion (pT)[3,4]			
pT1	12 (7.4)		
pT2	32 (19.8)		
pT3	109 (67.3)		
pT4	9 (5.5)		
Lymph node involvement (pN)[3,4]			
pN0	35 (21.6)		
pN1	3 (1.9)		
pN2	65 (40.1)		
pN3	59 (36.4)		
Distant metastasis (M)[3,4]			
M0	161 (99.4)		
M1	1 (0.6)		
TNM stage[3,4]			
Stage Ⅰ	8 (4.9)		
Stage Ⅱ	39 (24.1)		
Stage Ⅲ	115 (71.0)		

[1]Individuals with gastric cancer and two or more first-degree relatives with gastric cancer or related cancers; [2]Individuals with gastric cancer and one first-degree relative with gastric cancer or related cancers; [3]Available for 162 cases; [4]According to the National Comprehensive Cancer Network guidelines on tumor-node-metastasis (TNM) Staging Classification for Carcinoma of the Stomach (7th ed., 2010) by the American Joint Committee on cancer.

Genotyping analysis

Genomic DNA was extracted from peripheral blood leukocytes using the QIAamp DNA Blood Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Mutation screening of *CDH1* exons 2-16 and neighboring intronic sequences was performed using

polymerase chain reaction (PCR) and high-resolution melting analysis using a LightScanner system (Idaho technology, Salt Lake City, UT, United States). The samples that presented abnormal profiles were sequenced on an ABI 3130-Avant automated sequencer (Applied Biosystems, Foster City, CA, United States). The region around the transcription start site (TSS) of the *CDH1* gene (From promoter region to intron 1 of *CDH1* gene) was genotyped using PCR and directly sequenced on the ABI 3130-Avant automated sequencer. Primer sequences and PCR conditions are available upon request.

Promoter luciferase activity assay

A dual-luciferase reporter assay system (http://www.promega.com) was used to examine the effects of novel sequence variation in the promoter region on the transcriptional activity of *CDH1*. Briefly, DNA fragments around the TSS (-345 to 271 bp) were amplified by PCR using genomic DNA containing either the particular variant sequence or *CDH1* wild type as a template. The amplified fragments were designed to contain the region possessing basal promoter activity. The following primer sequences were used: 5'-ATGCCTCGAGCCATCTCCAAAACGAACAAAC-3' (forward) and 5'-ATGCAAGCTTGAAGGGAAGCGGTGACGAC-3' (reverse), which include the restriction sites (underlined) for Xho I and Hind Ⅲ, respectively. The PCR products were digested with Xho I and Hind Ⅲ and subsequently cloned into the pGL3-basic vector carrying the firefly luciferase gene (Promega). The nucleotide sequence of the fragment inserted into each plasmid was confirmed by DNA sequencing. Plasmids were then transiently transfected into Hela cells using the Lipofect transfection reagent (Tiangen Biotech, Beijing, Co., Ltd., China). All plasmids were co-transfected with the renilla luciferase gene containing the pRL-CMV plasmid (Promega) as an internal standard. Cell extracts were prepared, and luciferase activity was measured by a luminometer instrument (Promega) using the dual-luciferase reporter assay system (Promega). The transcriptional activity in each cell extract was determined from the level of firefly luciferase after normalization to renilla luciferase activity. Four independent experiments were performed using DNA from plasmid preparations.

RNA splicing analysis on clinical samples

Because it has been recognized that DNA sequence variants localized in exon-intron boundaries could be pathogenic by affecting exon definition and the splicing of pre-mRNA[12,13], we used RNA splicing assay to evaluate the variant located at the 5' terminal of intron 9. Total RNA from frozen tumor tissue and paired normal tissue was extracted using RNAiso Plus (TaKaRa Biotechnology [(Dalian) Co., Ltd.]. Reverse transcription (RT)-PCR was performed in 2 steps. First strand cDNA synthesis was performed using PrimerScript RT reagent Kit (TaKaRa) with random DNA hexamers and oligo-dT primer according to the manufacturer's protocol. cDNA was amplified in the region of exons 7-10. Primer sequences were 5'-GGACCGAGAGAGTTTCCCTACG-3' (sense) and 5'-GTTATTTTCTGTTCCATAAATG-3' (antisense). PCR conditions were as follows: 35 cycles at 94 ℃ for 30 s, 58 ℃ for 30 s and 72 ℃ for 30 s, followed by a final extension at 72 ℃ for 5 min. Agarose gel electrophoresis was carried out using 2% gels run at 100 V for 40 min. The purified amplification products were sequenced on the ABI 3130-Avant automated sequencer.

Bioinformatics analysis of CDH1 variants

The impact of amino acid allelic variants on protein structure/function can be predicted via analysis of multiple sequence alignments and protein 3D-structures. The sorting intolerant from tolerant (SIFT) algorithm and *Poly*morphism *Phen*otyping (PolyPhen) were adopted.

SIFT is a program that predicts the effect of amino acid substitutions on protein function based on sequence conservation during evolution and the nature of the amino acids substituted in a gene of interest[14]. The SIFT score was calculated online (http://sift.jcvi.org/). If the value is less than 0.05, the amino acid substitution is predicted as intolerant, while those with a value greater than or equal to 0.05 are classified as tolerated.

PolyPhen is an automatic tool for prediction of the possible impact of an amino acid substitution on the structure and function of a human protein based on straightforward physical and comparative considerations[15] (http://genetics.bwh.harvard.edu/pph/). Each of the two amino acid residues [the original residue and the single-nucleotide polymorphism (SNP)] was entered and the difference between the position-specific independent counts (PSIC) scores of the two residues was computed. The higher a PSIC score difference is, the higher the functional impact a particular amino acid substitution is likely to have. A PSIC score difference of 1.5 and above is considered to be damaging.

Statistical analysis

χ^2 tests or Fisher's exact tests were used to compare the distribution of variables between cases and controls. Luciferase activities were compared using Student's unpaired *t* test. All statistical tests were two-sided, with a *P* value of 0.05 considered to be significant, using SPSS software (version 16).

RESULTS

Characteristics of the study population

The study was comprised of 236 gastric cancer cases and 240 cancer-free controls. There were no significant differences in the distributions of age or gender between the cases and controls ($P = 0.997$ and 0.915, respectively) (Table 1). The majority of studied cases were sporadic; approximately 20% had a family history of cancer. The tumor type was assessed in 162 cases, and more than 80% of the cases had poorly differentiated or moderately differentiated adenocarcinoma; more than 70% of the cases were in TNM Stage Ⅲ (Table 1).

Table 2 Novel *CDH1* mutations and polymorphism identified in Chinese gastric cancer patients *n* (%)

Gene location	Sequence variant	Consequence	Genotype		
			Gastric cancer patients (*n* = 236)	Control (*n* = 240)	*P* value
5′UTR	c.-49 G>T	Substitution at the 5′UTR	4 (1.7)	3 (1.3)	0.723
Exon 1	c.44_46delTGC	Loss of the 15th code (Leu)	2 (0.85)	0 (0.0)	0.245
Exon 5	c.604G>A	Missense V202I	1 (0.4)	0 (0.0)	0.496
Intron 9	c.1320+7 A>G	Substitution of invariant A	1 (0.4)	0 (0.0)	0.496

Two-sided χ^2 test or Fisher's exact test for genotype distribution

Table 3 Polymorphisms identified in *CDH1* in Chinese gastric cancer patients and controls

Gene location	Sequence variant	Condon	MAF in 236 GC patients	MAF in 240 controls	Reported MAF[1]
Promoter	-164del T(g.4837delT)	-	0.004del T	0.000del T	rs5030658: NA
Promoter	-161C>A(g.4840C>A)	-	0.242A	0.227A	rs16260: 0.227A
Promoter	-73A>C (g.4928A>C)	-	0.129C	0.146C	rs28372783: 0.040C
Intron 1	c.48+6C>T	-	0.280C	0.271C	rs3743674: 0.214C
Intron1	c.48+62_48+63delinsCGTGCCCCAGCCC	-	0.280del[2]	0.271del[2]	rs3833051: NA
Exon 7	c.894C>T	A298A	0.002T	0.000T	rs139110184: NA
Exon 9	c.1224G>A	A408A	0.002A	0.000A	rs200161607: 0.001A
Exon 12	c.1888C>G	L630V	0.004G	0.004G	rs2276331: 0.002G
Exon 13	c.2076T>C	A692A	0.377T	0.404T	rs1801552: 0.307T
Exon 14	c.2253C>T	N751N	0.089T	0.102T	rs33964119: 0.058T

[1]From http://www.ncbi.nlm.nih.gov/projects/SNP/; [2]Coincident with MAF of c.48+6C>T variant. GC: Gastric cancer; MAF: Minor allele count; NA: Not available.

Table 4 Available clinical-pathologic characteristics of the gastric cancer cases with novel *CDH1* mutations

ID code	Age onset (yr)	Gender	Family history	Histologic grade	Depth invasion (pT)	Lymph node involvement (pN)	Distant metastasis (M)	CDH1 variants
G45	66	Female	Sporadic	Poorly differentiated adenocarcinoma	pT3	pN3	M0	c.44_46delTGC
G68	51	Male	Sporadic	NA	NA	NA	NA	c.44_46delTGC
G150[1]	58	Male	Sporadic	Moderately differentiated adenocarcinoma	pT2	pN3	M0	c.604G>A (V202I)
G26	56	Male	Sporadic	Moderately differentiated adenocarcinoma	pT3	pN2	M0	c.1320+7 A>G

[1]Also harbors the MLH1 c.2101C>A (Q701K) mutation[16]. NA: Not available.

CDH1 genetic screening revealed four novel germline sequence variants

Four novel *CDH1* germline variations were identified in gastric cancer patients. One of the variants was located in the *CDH1* 5'UTR (c.-49 G>T) and is seemed to be a polymorphism since it is found in both the cases and controls. The other three were only detected in the GC cases and not seen in the controls. One was a missense mutation in the coding region [c.604G>A (V202I)], one was a three-nucleotide deletion in exon 1 (c.44_46del TGC) and the other was an intronic variation (c.1320+7A>G) (Figure 1 and Table 2). In addition, ten *CDH1* polymorphisms (and their frequencies) were observed. The polymorphisms frequencies are similar to the data available at the SNP website (http://www.ncbi.nlm.nih.gov/projects/SNP/) (Table 3).

Clinical-pathologic characteristics of the GC cases with novel CDH1 mutations

The cases carrying novel *CDH1* mutations were all sporadic GC patients, with poorly differentiated or moderately differentiated adenocarcinoma (Table 4). The case with *CDH1* c.604G>A (V202I) mutation harbors the *MLH1* c.2101C>A (Q701K) mutation as well[16].

Functional characterization of the novel CDH1 variants

c.-49T variation contribute a slightly higher promoter activity of the *CDH1* gene than the wild type: The c.-49T alteration was near the TSS of the *CDH1* gene (49 bp before the start codon, and +76 bp relative to the TSS). To examine the potential effect of the c.-49T variation on E-cadherin gene transcription, a 616-bp promoter of the E-cadherin gene (-345 to 271) carrying either the G or T allele was inserted upstream of the luciferase gene in the pGL3 promoterless enhancer plasmid vector. The activity of the E-cadherin G/T promoter-luciferase reporter gene constructs was assessed using transient transfection assays in Hela cells. As shown in Figure 2, slightly higher luciferase activities were observed for the pGL-T construct compared with the pGL-G (wild type)

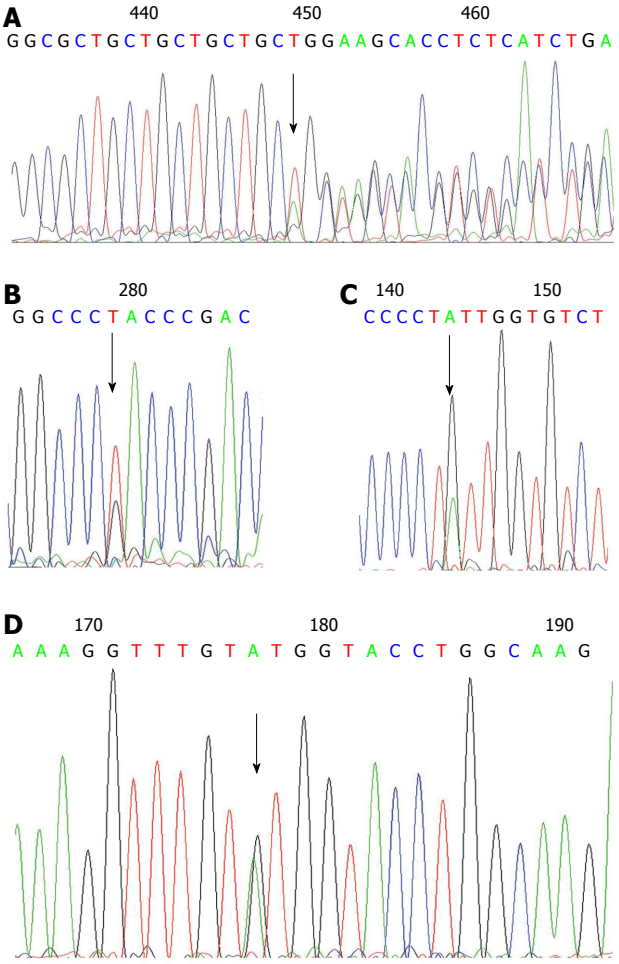

Figure 1 Novel variations detected in this study. A: c.44_46del TGC; B: c.-49G>T; C: c.604G>A (V202I); D: c.1320+7A>G.

construct. The average activity of the promoter having the c.-49T variation was 133.7% (P = 0.002) relative to the *CDH1* wild type promoter.

c.44_46del TGC variant causes the loss of one leucine in the signal peptide region of the E-cadherin protein: Codon sequence analysis demonstrated that the three-nucleotide deletion c.44_46del TGC causes the loss of a single amino acid [the 15th codon (Leucine)] in exon 1 of CDH1, which is in the signal peptide region of the E-cadherin protein.

Intron variation (c.1320+7A>G) and the silent mutation [c.1224G>A (A408A)] do not induce CDH1 splicing defects: To evaluate functional consequence of those novel mutations detected on pre-mRNA splicing, we analyzed cDNA produced *in vivo* from tissues retrieved from the patients harboring the novel mutations. The PCR fragment generated using primers flanking the silent mutation [c.1224G>A (A408A)] and the one intron variation (c.1320+7A>G) indicated normal-sized mRNA. This result demonstrated that a splicing defect was not likely to occur as a consequence of these mutations (Figure 3).

Table 5 *CDH1* missense mutations analyzed by sorting intolerant from tolerant and PolyPhen

Sequence variant	Structural alteration	SIFT scores	PSIC score difference	Prediction
c.604G>A	V202I	0.20	0.381	Benign
c.1888C>G	L630V	0.02	1.748	Probably damaging

SIFT: Sorting intolerant from tolerant; PSIC: Position-specific independent counts.

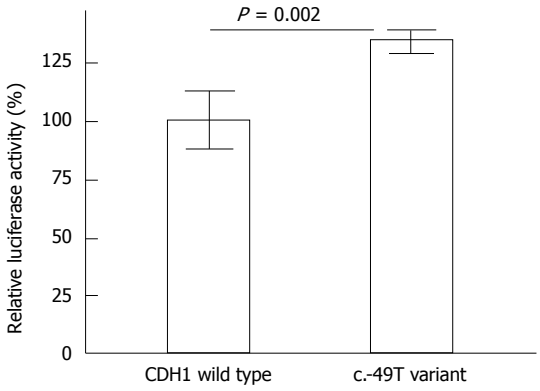

Figure 2 Luciferase reporter assay for the *CDH1* c.-49 G>T variant. The average relative luciferase activity (with standard deviation) is shown. The activity of *CDH1* wild type was defined as 100%.

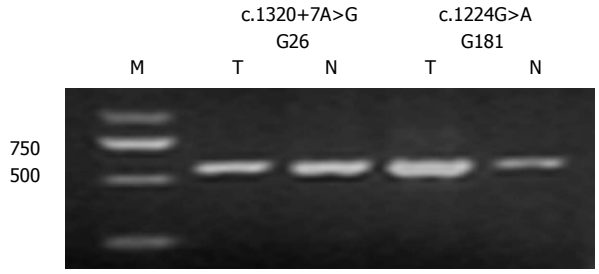

Figure 3 Results of reverse transcription polymerase chain reaction for *CDH1* in patient G26 (c.1320+7A>G, intron 9) and patient G181 [c.1224G>A(A408A), exon 9] using primers in exons 7 and 10. Both patients had an expected band of 567 bp. M: Molecular size markers; T: Tumor tissue; N: Paired normal tissue.

c.1888C>G (L630V) missense mutation might impair E-cadherin protein function while the V202I mutation does not: Two *CDH1* missense mutations were detected. Both the SIFT score and the PolyPhen analysis demonstrated that the L630V variant was sorted as being intolerant, suggesting that this amino acid substitution is predicted to damage protein function. The other variant, V202I, of CDH1 was sorted as tolerant (Table 5).

DISCUSSION

The discovery of genetic variants responsible for the pathogenesis of gastric cancer is important in understanding this disease. Although screening of *CDH1* germline mutations in hereditary GC has been fairly well established, the report of *CDH1* germline mutations in

sporadic GC is limited. Bacani *et al*[17] identified a germline deletion (nt41delT) in a 30-year-old sporadic GC patient and suggested that 2%-3% of cases of early-onset gastric cancer in North America may be owing to high-risk genetic mutations. Garziera *et al*[18] reported a germline missense mutation in *CDH1* exon 6, c. 820 G>A (G274S) in one sporadic Italian gastric cancer patient. Here, we report a population-based study of GC to determine the role of germline mutations in a population at a high risk for GC. The majority of studied cases were sporadic. We have studied all of the coding and promoter core regions of the most important gene implicated in GC and have identified four novel *CDH1* sequence variants distributed along the entire coding sequence and the non-coding regions in the *CDH1* gene. This is consistent with previously published reports[19], suggesting that *CDH1* mutations have arisen without mutational hotspots.

The c.-49 G>T transition was detected in 4/236 (1.7%) of GC patients and 3/240 (1.3%) of cancer-free controls; however, these differences did not achieve significance (P = 0.723, Table 2). One patient with this variant had low familial recurrence for gastric cancer, but IHC showed normal CDH1 protein expression. Previous studies have demonstrated that a fragment spanning -399 to +31 bp relative to the TSS of the *CDH1* gene possesses basal promoter activity[20]. As this variant is around the TSS of the *CDH1* gene, a luciferase reporter assay was carried out. This *in vitro* assay showed that the c.-49T promoter had 33% higher activity than the promoter containing the c.-49G (Figure 2). So this polymorphism might be helpful for E-cadherin transcription, though the increase in transcription activity is limited (only 33%).

The c.44_46del TGC variant was detected in two sporadic GC patients of 51 years and 66 years and was not detected in the 240 controls. One patient's pathological data was available, which showed a poorly differentiated adenocarcinoma (Table 4). To the best of our knowledge, this variant has never been reported in the open access mutation database and literatures. As the parents of the probands are not available for mutation analyzing, we are not certain whether it's a *de novo* mutation. This variant seems to be a rare variant with an allele frequency in GC patients of 0.85% and can even be considered as a kind of mutation hotspot, as it has been detected in two patients with no relationship. Recently rare variants have been reported in several diseases, include cancer. The identified rare variants often have functional effects on protein-protein interactions. Further, rare variants might confer a stronger increase in disease risk than common variants and may make a substantial contribution to the multifactorial inheritance of common chronic diseases[21-24]. Codon sequence analysis demonstrated that the c.44_46del TGC variant causes the loss of a single amino acid [the 15th codon (Leucine)] in exon 1 of CDH1 which is in the signal peptide region of the E-cadherin protein. This amino acid loss might have effect on E-cadherin protein, and further functional analysis should be carried out to investigate associations of the variant with phenotype.

Growing evidence has shown that rare single base substitutions localized in exon-intron boundaries can disrupt one of the cis-transcriptional elements known as exonic splicing enhancers and affect normal pre-mRNA splicing[12,13]. Therefore, it appears reasonable to verify the effect of variants at the mRNA level. The sequence alteration c.1320+7A>G, located in an exon-intron boundary, was detected in a 56-year-old GC patient and not in the 240 controls (Table 2). RNA splicing assay demonstrated that this variation did not affect exon splicing ability (Figure 3) and might be rare polymorphism.

The CDH1 molecule consists of five tandemly repeated extracellular domains (EC1-EC5, containing exons 4-13), each about 110 amino acids in length. This large extracellular domain is responsible for Ca^{2+} binding and is important for cell-cell adhesion. The NH2-terminal EC1 domain is required for lateral E-cadherin dimerization contributing to the intercellular junction[25-27]. A novel missense mutation, V202I, is located in the middle of EC1. While EC1 shows remarkably high conservation between various species[28], SIFT and PolyPhen analyses both showed that V202I might be a tolerant variation (Table 5). The GC patient with this variation carried the *MLH1* c.2101C>A (Q701K) mutation as well. IHC analysis in the index patient demonstrated a loss of MLH1 protein and normal expression of MSH2 and E-cadherin[16]; therefore, we suggest that the *MLH1* c.2101 C>A (Q701K) mutation, and not the *CDH1* c.604G>A (V202I) variation, might be the cause of GC in this patient.

In addition, frequencies of *CDH1* polymorphisms in Chinese GC patients and controls were reported which were similar to those reported at http://www.ncbi.nlm.nih.gov/projects/SNP/ (Table 3). It needs to say something about the rare polymorphism, c.1888C>G (L630V). The SIFT score of the CDH1 variants and the PolyPhen analysis both showed that the L630V variant probably damages protein function (Table 5). However, data from case-control analysis did not support an effect of this L630V variant (Table 3). Accordingly, the pathogenic role of this polymorphism remains elusive and *in vitro* approaches should be performed to elucidate its function.

In conclusion, this study reveals novel mutations in sporadic GC patients in China, a high-incidence country for GC. Though the pathogenic role of the variants remains still uncertain, our findings display the necessity to scan germline *CDH1* variants in sporadic gastric cancer population.

COMMENTS

Background

Although E-cadherin gene (*CDH1*) germline mutations are well implicated in hereditary diffuse gastric cancer, the report of *CDH1* germline mutations in sporadic gastric cancer (GC) is limited.

Research frontiers

Here, the authors report a population-based study of GC to determine the role

of *CDH1* germline mutations in a population at a high risk for GC. The majority of studied cases were sporadic.

Innovations and breakthroughs

The authors have studied all of the coding and promoter core regions of *CDH1* and identified four novel *CDH1* sequence variants, including one transition near the transcription start site, one three-nucleotide deletion in code region, one missense mutation, and one variation in exon-intron boundary. Three of the four variants were detected only in sporadic GC patients and not in the 240 cancer-free controls. Though the functional significance of the variants remains still uncertain, this study reveals novel mutations in sporadic GC patients which had been poorly investigated for susceptibility genes

Applications

The findings display the necessity to scan germline *CDH1* variants in sporadic gastric cancer population.

Terminology

High-resolution melting analysis, is a high-throughput single-nucleotide polymorphism genotyping technology based on the analysis of the melting profile of polymerase chain reaction products.

Peer review

In this study, the authors identified 4 novel *CDH1* germline mutations in different patients harbouring GC with a sporadic setting. The identification of mutations represents an important discovery, to assess the cancer risk for the novel generations.

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BRIEF ARTICLE

Effects of early enteral nutrition on immune function of severe acute pancreatitis patients

Jia-Kui Sun, Xin-Wei Mu, Wei-Qin Li, Zhi-Hui Tong, Jing Li, Shu-Yun Zheng

Jia-Kui Sun, Xin-Wei Mu, Jing Li, Shu-Yun Zheng, Department of Intensive Care Unit, Nanjing First Hospital, Nanjing Medical University, Nanjing 210006, Jiangsu Province, China
Jia-Kui Sun, Wei-Qin Li, Zhi-Hui Tong, Department of General Surgery, Jinling Hospital, Nanjing University School of Medicine, Nanjing 210002, Jiangsu Province, China
Author contributions: Sun JK, Li WQ and Tong ZH designed the research; Sun JK, Mu XW, Li J and Zheng SY performed the research; Sun JK and Li WQ analyzed the data; and Sun JK wrote the paper.
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Correspondence to: Wei-Qin Li, Professor, Department of General Surgery, Jinling Hospital, Nanjing University School of Medicine, No. 305, Zhongshan East Road, Nanjing 210002, Jiangsu Province, China. sjk0935119@163.com
Telephone: +86-25-80860066 Fax: +86-25-84803956
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Abstract

AIM: To investigate the effects of early enteral nutrition (EEN) on the immune function and clinical outcome of patients with severe acute pancreatitis (SAP).

METHODS: Patients were randomly allocated to receive EEN or delayed enteral nutrition (DEN). Enteral nutrition was started within 48 h after admission in EEN group, whereas from the 8th day in DEN group. All the immunologic parameters and C-reactive protein (CRP) levels were collected on days 1, 3, 7 and 14 after admission. The clinical outcome variables were also recorded.

RESULTS: Sixty SAP patients were enrolled to this study. The CD4+ T-lymphocyte percentage, CD4+/CD8+ ratio, and the CRP levels in EEN group became significantly lower than in DEN group from the 7th day after admission. In contrast, the immunoglobulin G (IgG) levels and human leukocyte antigen-DR expression in EEN group became significantly higher than in DEN group from the 7th day after admission. No difference of CD8+ T-lymphocyte percentage, IgM and IgA levels was found between the two groups. The incidences of multiple organ dysfunction syndrome, systemic inflammatory response syndrome, and pancreatic infection as well as the duration of intensive care unit stay were significantly lower in EEN group than in DEN group. However, there was no difference of hospital mortality between the two groups.

CONCLUSION: EEN moderates the excessive immune response during the early stage of SAP without leading to subsequent immunosuppression. EEN can improve the clinical outcome, but not decrease the hospital mortality of SAP patients.

Key words: Early enteral nutrition; Immune; Severe acute pancreatitis

Sun JK, Mu XW, Li WQ, Tong ZH, Li J, Zheng SY. Effects of early enteral nutrition on immune function of severe acute pancreatitis patients. *World J Gastroenterol* 2013; 19(6): 917-922 Available from: URL: http://www.wjgnet.com/1007-9327/full/v19/i6/917.htm DOI: http://dx.doi.org/10.3748/wjg.v19.i6.917

INTRODUCTION

Severe acute pancreatitis (SAP) is a systemic disease characterized by a high mortality because of multiple organ dysfunction syndrome (MODS) and infectious complications[1,2]. During recent years, numerous studies concluded that immune dysregulation might play an important role in the development of MODS and infectious complications in SAP patients[3-5]. In the early stage of SAP, immune imbalance mainly appears as excessive immune

response, which is associated with systemic inflammatory response syndrome (SIRS) and MODS[3-6]. Therefore, some clinicians advocate using immunomodulatory therapy in the acute phase of SAP[4,7,8]. However, no consensus on the efficacy of immunotherapy has been reached due to the conflicting results of relevant experiments.

As an essential therapeutic modality for SAP, early enteral nutrition (EEN) could increase antioxidant activity, modulate inflammatory response, and decrease the incidence of SIRS and subsequent MODS[9]. Hence, we infer that EEN might exert some underlying influence on the immune function of SAP patients. However, there have been few trials investigating the effects of EEN on the immune function of the patients during the initial stage of SAP. Zou *et al*[10] reported that enteral immunonutrition enhanced the immune function in pigs with SAP. Belabed *et al*[11] found that the immune enhancing diets was efficient in preserving lymphocyte function in head-injured rats. Nevertheless, the impact of standard enteral nutrition on the immunologic function of SAP patients remains unclear.

Therefore, this study aimed to investigate the influence of EEN on the immune function and clinical outcome of early-stage SAP patients.

MATERIALS AND METHODS

Study design

This is a single-center, prospective, and randomized controlled clinical trial. Patients were randomly allocated to receive either EEN or delayed enteral nutrition (DEN) on admission. The study protocol was approved by the Ethics Committee of the hospital, and informed consent was obtained from each patient or his/her first-degree relatives. This study was also registered at Clinical Trials.gov (Identifier: NCT01507766).

Patients

All adult SAP patients (aged 18-70 years) admitted within 3 d of symptom onset to the Surgical Intensive Care Unit (SICU), the Department of General Surgery, Jinling Hospital from September 2010 to September 2011 were enrolled into this study. SAP was defined as presence of one or more local complications (*e.g.*, pseudocyst, necrosis or abscess) or/and organ failure and acute physiology and chronic health evaluation Ⅱ (APACHE Ⅱ) score > 8 according to the widely used Atlanta criteria formulated in 1992[12]. Patients with chronic organs dysfunction or immunodeficiency or malnutrition, patients who had received artificial nutrition (either enteral nutrition or parenteral nutrition) before admission, and patients with ileus or pancreatitis in pregnancy were all excluded. All the SAP patients received specialized medical therapy for SAP[2,13] such as intensive monitoring, oxygen administration, fluid resuscitation, stopping oral feeding, exocrine pancreatic suppression, and antibiotic prophylaxis.

Patients in EEN group received enteral nutrition within 48 h after admission, whereas patients in the DEN group received enteral nutrition beginning from the 8th day after admission.

Nutrition protocols

A nasojejunal tube (size 10F, Flocare, Nutricia Ltd, Wuxi, China) was placed beyond the Treitz' ligament endoscopically or radiologically, and the position of tip was confirmed by fluoroscopy. The tube was placed within 24 h after admission in EEN group, and enteral nutrition was started from the next 24 h. Patients in DEN group received feeding on the 8th day after admission, and a nasojejunal tube was placed on the 7th day. Peptide-based formula (Peptisorb, Nutricia Ltd) was used in the first 24-48 h of feeding, and if patients were tolerant, whole protein formula (Nutrison Fibre, Nutricia Ltd) would be established subsequently. The goal intake of enteral nutrition was calculated as 20-25 kcal/kg per day and protein need was calculated as 1.5 g/kg per day[14,15]. The feeding rate was initiated at 15-20 mL/h and increased gradually by 15-20 mL every 6-8 h, using a pump. If the patients were intolerant due to abdominal distension and so on, we would slow down the feeding rate, dilute the feedings concentration, and use prokinetic agents to improve the intestinal motility.

Total parenteral nutrition was given during the first week of admission in DEN group. The caloric intake of parenteral nutrition was determined as 20-25 kcal/kg per day and the calorie: nitrogen ratio was calculated as 120-150:1[16,17]. Fifty to seventy percentages of total energy need were supplied by glucose, and the use of lipids was on the basis of serum triglyceride levels[16]. Furthermore, sufficient vitamins, electrolytes, trace elements, and insulin were also added into the intravenous solution.

Data collection

The baseline characteristics of patients including age, sex, body mass index, and etiology were recorded on admission. The APACHE Ⅱ score, sequential organ failure assessment score, and C-reactive protein (CRP) levels were also collected. The CRP levels and the immunologic parameters including CD4+, CD8+ T-lymphocyte percentage, CD4+/CD8+ ratio, human leukocyte antigen-DR (HLA-DR), immunoglobulin A (IgA), IgG and IgM in peripheral blood were collected on days 1, 3, 7 and 14 after admission[4,6,18]. T-lymphocyte subset percentage and HLA-DR expression were detected by flow cytometry (BD FACS Aria, United States). IgA, IgG and IgM were measured by enzyme-linked immunosorbent assay. The incidences of SIRS and MODS during the first 2 wk after admission, the incidence of surgical operation, the development of pancreatic infection, the duration of ICU stay, and the hospital mortality were also recorded.

Statistical analysis

All the data were presented as median (interquartile range), if not stated otherwise. Categorical variables were expressed as absolute numbers or in percentages, and were analyzed using χ^2 test. Continuous variables were com-

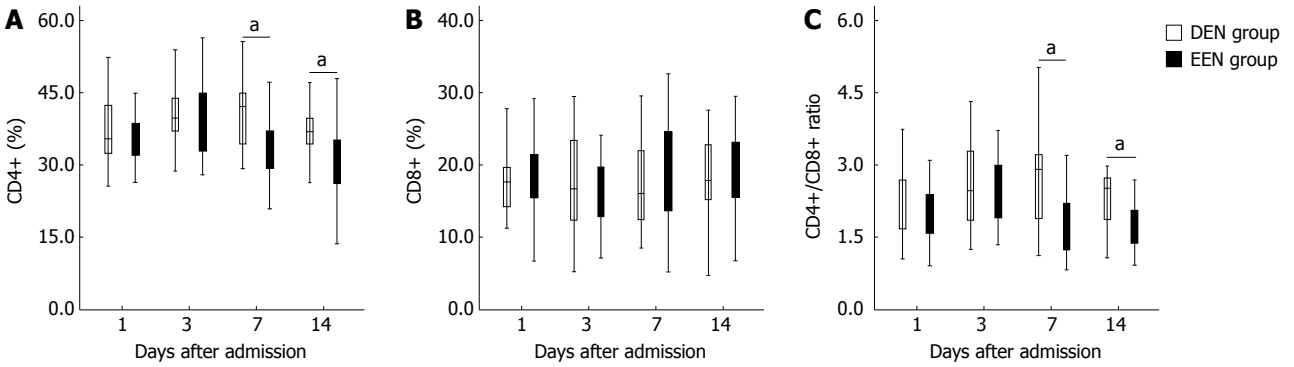

Figure 1 Comparisons of T-lymphocyte subsets percentage between early enteral nutrition and delayed enteral nutrition groups. Results are expressed as median (interquartile range). A: Comparison of CD4+ T-lymphocyte percentage between early enteral nutrition (EEN) and delayed enteral nutrition (DEN) groups; B: Comparison of CD8+ T-lymphocyte percentage between EEN and DEN groups; C: Comparison of CD4+/CD8+ ratio between EEN and DEN groups. $^{a}P < 0.05$, EEN groups *vs* DEN groups.

Table 1 Demographic data and clinical parameters on admission

	DEN group (n = 30)	EEN group (n = 30)	P value
Age (yr)	43 (34.5-51)	45 (35-52)	0.594
Sex (male:female)	18:12	20:10	0.287
Etiology n (%)			
Biliary origin	17 (57)	19 (63)	0.278
Hyperlipidemia	8 (27)	6 (20)	0.373
Alcohol abuse	3 (10)	4 (13)	1.000
Idiopathic	2 (7)	1 (3)	1.000
BMI	24.6 (23.5-26.8)	25.8 (23.9-28.8)	0.158
APACHE Ⅱ score	9.5 (8.5-11)	10 (8-11.5)	0.994
SOFA score	4.5 (3.5-5.5)	4 (3-5)	0.880
CRP (mg/L)	203.5 (188-253)	195 (161-247.5)	0.214

DEN: Delayed enteral nutrition; EEN; Early enteral nutrition; BMI: Body mass index; APACHE Ⅱ: Acute physiology and chronic health evaluation Ⅱ; SOFA: Sequential organ failure assessment; CRP: C-reactive protein.

pared by the Mann-Whitney *U* test or Wilcoxon signed-rank test as appropriate. Statistical Package for the Social Sciences (SPSS, version 17.0, Chicago, IL, United States) software was used for statistical analysis. $P < 0.05$ was considered statistically significant.

RESULTS

A total of 60 eligible patients with SAP (30 in each group) were enrolled into this clinical trial during the study period. The principal etiological factors of SAP were biliary origin (60%, 36/60) and hyperlipidemia (23%, 14/60). The demographic data and clinical parameters of the patients on admission are presented in Table 1. Three patients (5%, 3/60) died of MODS and septic shock during hospital stay.

Comparison of immune parameters and CRP levels between two groups

Figure 1 shows the differences of T-lymphocyte subsets percentage between DEN and EEN groups. As shown in Figure 1A, patients in EEN group had significantly lower CD4+ T-lymphocyte percentage from the 7th day ($P < 0.05$) after admission. Similar results were detected in the CD4+/CD8+ ratio (Figure 1C). However, there was no difference of CD8+ T-lymphocyte percentage between the two groups during the two weeks after admission (Figure 1B).

Figure 2 presents the disparities of immunoglobulin subtypes between the two groups. As shown in Figure 2A, patients in EEN group had significantly higher IgG levels from the 7th day ($P < 0.05$) after admission. Nevertheless, no difference of IgM and IgA between the two groups was found during the two weeks after admission (Figure 2B and C).

As shown in Figure 3A, patients in EEN group had significantly higher HLA-DR expression from the 7th day ($P < 0.05$) after admission. In contrast, the CRP levels of patients in EEN group were significantly lower than in patients of the DEN group from the 7th day ($P < 0.05$) after admission (Figure 3B).

Table 2 Clinical outcome variables n (%)

	DEN group (n = 30)	EEN group (n = 30)	P value
Hospital mortality	1 (3)	2 (7)	1.000
ICU stay (d)	12 (8-21)	9 (5-14)	0.033
Pancreatic infection	10 (33)	3 (10)	0.028
MODS	13 (43)	5 (17)	0.024
SIRS	22 (73)	12 (40)	0.009
Surgical operation	4 (13)	2 (7)	0.671

DEN: Delayed enteral nutrition; EEN: Early enteral nutrition; ICU: Intensive care unit; MODS: Multiple organ dysfunction syndrome; SIRS: Systemic inflammatory response syndrome.

Comparison of outcome variables between two groups

As presented in Table 2, patients in EEN group had significantly lower incidences of MODS, SIRS and pancreatic infection as well as shorter ICU stay during hospital stay. However, no difference of hospital mortality and operation incidence was found between the two groups.

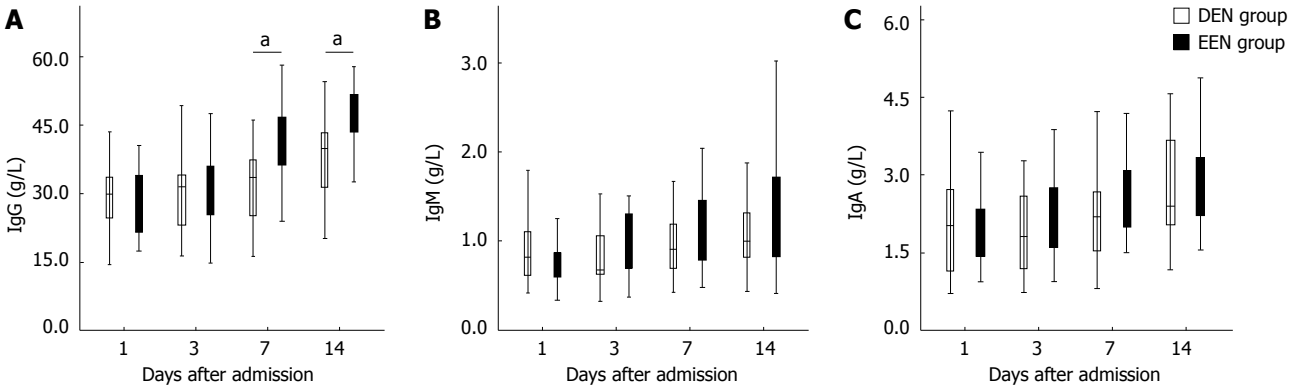

Figure 2 Comparisons of immunoglobulin subtypes between early enteral nutrition and delayed enteral nutrition groups. Results are expressed as median (interquartile range). A: Comparison of immunoglobulin G (IgG) levels between early enteral nutrition (EEN) and delayed enteral nutrition (DEN) groups; B: Comparison of IgM levels between EEN and DEN groups; C: Comparison of IgA levels between EEN and DEN groups. $^{a}P < 0.05$, EEN groups *vs* DEN groups.

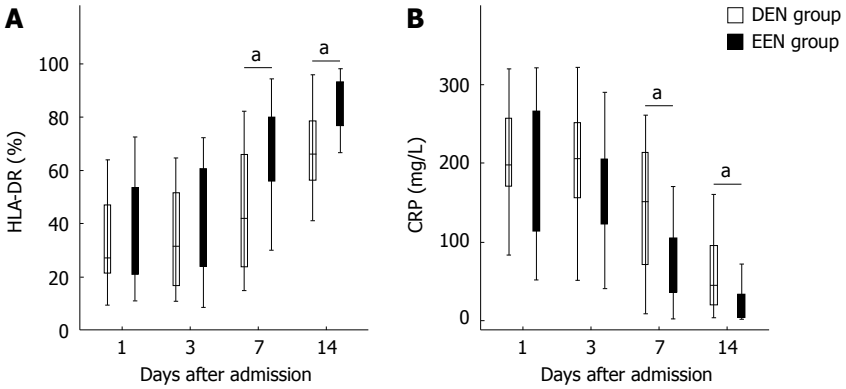

Figure 3 Comparisons of human leukocyte antigen-DR expression and C-reactive protein levels between early enteral nutrition and delayed enteral nutrition groups. Results are expressed as median (interquartile range). A: Comparison of human leukocyte antigen-DR (HLA-DR) expression between early enteral nutrition (EEN) and delayed enteral nutrition (DEN) groups; B: Comparison of C-reactive protein (CRP) levels between EEN and DEN groups. $^{a}P < 0.05$, EEN groups *vs* DEN groups.

DISCUSSION

This clinical study investigated the effects of EEN on the immune function as well as on the clinical outcome of SAP patients. We found that EEN could moderate the excessive immune response during the early stage of SAP without leading to subsequent immunosuppression. Moreover, EEN administration could improve the clinical outcome, but not decrease the hospital mortality of SAP patients.

Immune dysregulation is one of the main causes responsible for a high mortality of SAP. Recent studies have shown that excessive immune response might play an important role in the development of SIRS and MODS in the early stage of SAP[3-6,19]. The imbalance of Th1/Th2 cells (differentiated from the Th0 cells which were derived from CD4+ T-lymphocytes) is the principal mechanism of excessive immune response[4,19]. Th1 cells mainly produce some anti-inflammatory factors, such as interleukin 10 (IL-10) and IL-4, whereas Th2 cells mainly release the pro-inflammatory factors, such as IL-6, and tumor necrosis factor-α (TNF-α)[3,4,19]. Pietruczuk *et al*[19] found that Th1 cells were suppressed more strongly than Th2 cells in the acute phase of SAP, thus the pro-inflammatory factors produced by Th2 cells were over-activated and then triggered the strong inflammatory reaction. For this reason, some researchers considered using immunosuppressive therapy for the patients with early-stage SAP. Up till now, a number of immunosuppressive agents (*e.g.*, dexamethasone, IL-10, and anti-tumor necrosis factor monoclonal antibodies) have been studied in SAP animal experiments[4,8]. Unfortunately, the findings were inconsistent or even conflicting, and very few of these studies involved SAP patients. Moreover, inappropriate immunosuppression might be associated with subsequent infectious complications during the late stage of SAP[3,4,6]. Therefore, the effects of immunomodulatory therapy in SAP were still unclear, and further clinical trails are extremely needed.

Gut is the primary immune organ providing an initial barrier against extraneous antigens and microbes[20,21]. The previous studies reported that gut immune response was highly associated with enteral nutrition, and deficiency of enteral stimulation might induce immune suppression[20,21]. In addition, enteral nutrition (especially EEN) had been confirmed to modulate inflammatory response, maintain gut integrity, and release immunomodulating agents[9,22]. Based on the above findings, we postulated that EEN might have some underlying impacts on the immune function of SAP patients, however, few clinical trials about it have been conducted. Therefore, the principal purpose of this clinical study was to investigate the influence of EEN on the immune function of early-stage SAP patients.

CD4+, CD8+ T-lymphocyte percentage and CD4+/CD8+ ratio were closely related to the cellular immune

function of SAP[23,24]. Uehara *et al*[5] observed that both CD4+ percentage and CD4+/CD8+ ratio were significantly reduced in patients with acute pancreatitis, whereas Liu *et al*[6] reported that the CD4+ percentage was increased significantly on the 7th day in SAP patients after admission. In the present study, we found that EEN could reduce CD4+ T-lymphocyte percentage as well as CD4+/CD8+ ratio from the 7th day after admission. And patients in EEN group also had significantly lower CRP levels from the same day. Furthermore, the initial incidences of SIRS and MODS in EEN group were significantly lower than in DEN group. These results indicated that EEN might be able to suppress the excessive immune response during the early phase of SAP. It has been demonstrated that EEN can prevent the release of pro-inflammatory mediators (*e.g.*, IL-6 and TNF-α) and promote the release of anti-inflammatory factors (*e.g.*, IL-10)[8,9]. In other words, EEN might suppress the overactivation of Th2 cells and promote the production of Th1 cells, and then moderate the imbalance of Th1/Th2 cells. This may be the underlying mechanisms of our conclusion.

In addition, since excessive or prolonged immunosuppressive therapy might induce the development of infectious complications, whether EEN could lead to subsequent immunosuppression still need to be further investigated. Immunoglobulin concentrations and HLA-DR expression levels were also closely related to the immune function of SAP patients. Zou *et al*[10] reported that the serum IgG levels of SAP pigs were significantly decreased on day 2 after induction of SAP, and then were increased significantly on day 8. Yu *et al*[25] found that continuous HLA-DR suppression was highly associated with infectious complications and poor outcome in SAP patients, and the HLA-DR suppression was inversely correlated with CRP levels. In our study as shown in Figures 2A and 3A, patients in EEN group had significantly higher IgG levels and HLA-DR expression from the 7th day after admission. And the IgM and IgA levels of patients in EEN group were also not lower than that of patients in DEN group. Furthermore, the incidence of pancreatic infection in EEN group was significantly lower than in DEN group, and this result is also consistent with the findings of previous studies[9,22]. These phenomena suggested that EEN did not lead to immunosuppression during the late stage of SAP.

There are several limitations in this study. Due to the small sample size and the single-center design, our results might be uncertain for a definite conclusion, and the accuracy should be tested by further large-sized studies. Moreover, since this study did not base on a pathophysiological model, the precise mechanisms of EEN in SAP patients should be verified by more basic experiments.

In conclusion, EEN could moderate the excessive immune response during the early stage of SAP without leading to subsequent immunosuppression. Moreover, EEN could improve the clinical outcome, but not decrease the hospital mortality of SAP patients. However, the precise mechanisms of EEN for SAP are still not clear, and further studies are required to verify our conclusions.

COMMENTS

Background

The immune dysregulation might play an important role in the development of multiple organ dysfunction syndrome (MODS) and infectious complications in severe acute pancreatitis (SAP) patients. In the early stage of SAP, immune imbalance mainly appears as excessive immune response, therefore, some clinicians advocate using immunomodulatory therapy for SAP. However, no consensus on the efficacy of immunotherapy has been reached due to the conflicting results of relevant experiments.

Research frontiers

Early enteral nutrition (EEN) could increase antioxidant activity, modulate inflammatory response, and decrease the incidence of systemic inflammatory response syndrome (SIRS) and subsequent MODS. Hence, the authors infer that EEN might have some underlying influence on the immune function of SAP patients. However, there have been few trials investigating the effects of EEN on the immune function of the patients with SAP at the initial stage.

Innovations and breakthroughs

The study found that EEN could moderate the excessive immune response during the early stage of SAP without leading to subsequent immunosuppression; and EEN could improve the clinical outcome, but not decrease the hospital mortality of SAP patients.

Applications

The results of this study indicate that enteral feedings should be provided as early as possible to correct the immune dysregulation of SAP patients.

Terminology

MODS, is a progressive condition usually characterized by combined dysfunction of multiple organs or systems due to various etiological factors in critically ill patients; SIRS, is diagnosed when two or more of the following criteria are met: body temperature < 36 ℃ or > 38 ℃; heart rate > 90 beats/min; tachypnea > 20 breaths/min, or an arterial partial pressure of carbon dioxide < 32 mmHg; white blood cell count less than 4 × 10^9/L or greater than 12 × 10^9/L, or the presence of > 10% immature neutrophils (band forms).

Peer review

Overall, presented paper is of a very good quality. Study design is appropriate and clear, helps to answer a clinical relevant question. Data analysis is sufficient. Manuscript is well structured, language is of sufficient quality.

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BRIEF ARTICLE

Testing for hepatitis B infection in prospective chemotherapy patients: A retrospective study

Ying Wang, Xin-Mei Luo, Dan Yang, Jie Zhang, Hong-Yu Zhuo, Jian Zhang, Yu Jiang

Ying Wang, Xin-Mei Luo, Dan Yang, Jie Zhang, Hong-Yu Zhuo, Jian Zhang, Yu Jiang, Department of Medical Oncology, Cancer Center, State Key Laboratory of Biotherapy, West China Hospital, Sichuan University, Chengdu 610041, Sichuan Province, China

Author contributions: Wang Y and Luo XM performed the majority of this study and contributed equally to this work; Yang D, Zhang J, Zhuo HY and Zhang J collected vital clinical data and were involved in revising the manuscript; Wang Y, Luo XM and Jiang Y wrote the manuscript; and Jiang Y designed the study and provided financial support for this work.

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Correspondence to: Yu Jiang, MD, Department of Medical Oncology, Cancer Center, State Key Laboratory of Biotherapy, West China Hospital, Sichuan University, Chengdu 610041, Sichuan Province, China. jiangyu1973@hotmail.com
Telephone: +86-28-85422683 Fax: +86-28-85423278

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Abstract

AIM: To estimate hepatitis B virus (HBV) infection testing rate in cancer patients before chemotherapy with a focus on HBV reactivation.

METHODS: A retrospective study was conducted from January 1, 2009 to June 30, 2010. Inclusion required that patients be naïve to cancer chemotherapy but have indications for it. Patients who did not receive chemotherapy for any reason were excluded. Important clinical information, such as the levels of HBV DNA and serological markers were collected. HBV reactivation was defined as an increase in serum HBV DNA to > 1 log higher than that of the pre-exacerbation baseline, or serum HBV DNA conversion from negative to positive. HBV DNA levels > 1000 copies/mL were defined as HBV DNA positive. The χ^2 or Fisher's exact test was used for analysis of categorized data. Multiple logistic regression analysis was used to estimate the odd ratio and 95%CI of the HBV screening rate.

RESULTS: Of 6646 patients, 5616 (84.5%) received chemotherapy. Only 17.1% of the cancer patients received pre-chemotherapy HBV testing (43.2% for hematological malignancies and 14.9% for solid tumors). Patients who had received rituximab therapy, had elevated aminotransferase levels, or had hematological malignancies were more likely to receive HBV testing. The prevalence of hepatitis B surface antigen (HBsAg) positivity was 13.4%. HBV reactivation (appearance of HBV DNA or an increase in HBV DNA levels by 1 $\log_{10}$) was observed in 33.1% (53/160) of the patients after chemotherapy. Among patients without prophylactic antiviral therapy, the reactivation rate was 43.9% (43/98) in the solid tumor group. Two reactivation cases occurred in patients who were HBsAg negative, but positive for hepatitis B core antibody. HBV reactivation was more likely to occur in patients with lymphoma, high levels of HBV DNA, or hepatitis B e antigen, and in men.

CONCLUSION: Less than 20% of patients received HBV testing before chemotherapy. HBV reactivation would have occurred in about 50% of infected patients with solid tumors without antiviral prophylaxis.

Key words: Chemotherapy; Hematologic malignancy; Hepatitis B virus; Hepatitis B virus reactivation; Solid tumor

Wang Y, Luo XM, Yang D, Zhang J, Zhuo HY, Zhang J, Jiang Y. Testing for hepatitis B infection in prospective chemotherapy patients: A retrospective study. *World J Gastroenterol* 2013; 19(6): 923-930 Available from: URL: http://www.wjgnet.com/1007-9327/full/v19/i6/923.htm DOI: http://dx.doi.org/10.3748/wjg.v19.i6.923

INTRODUCTION

Hepatitis B virus (HBV) infection is a global health problem. According to estimates of the World Health Organization, nearly 2 billion people worldwide have been infected with HBV, and > 350 million have chronic infection[1]. Hepatitis B is endemic in China, and the weighted prevalence of hepatitis B surface antigen (HBsAg) for the Chinese population is 7.2%[2]. Cancer patients receiving intensive chemotherapy may be at risk for HBV reactivation[3-8]. A previous study has reported that the reactivation rate of HBV infection after chemotherapy was as high as 73%[8]. It has been observed in a meta-analysis that the average reactivation rate of HBV infection was nearly 34% (162/475 patients), and the mortality rate was about 7% (27/394 patients)[9]. HBV reactivation might lead to a delay in chemotherapy, therefore, antiviral therapy should be administered immediately[3]. However, this might affect the efficacy of chemotherapy.

Corticosteroids, monoclonal antibodies (such as rituximab and alemtuzumab), and nearly all types of chemotherapeutic agents have been involved in HBV reactivation[5]. A diagnosis of lymphoma is another risk factor for HBV reactivation[4]. A multivariate analysis has revealed that patients with lymphoma or breast cancer are more likely to develop HBV reactivation after chemotherapy[10].

Recently, more attention has been paid to HBV testing before chemotherapy. Many clinical practice guidelines, such as the associated guidelines of the European Association for the Study of Liver Disease (EASL) and the American Association for the Study of Liver Diseases, advise HBV testing before starting chemotherapy[11,12]. The United States Centers for Disease Control and Prevention (CDC) also recommend testing for chronic HBV infection in cancer patients receiving chemotherapy[13]. It has been reported in recent years that the HBV infection testing rates before chemotherapy in Canada and the United States were 14%[14] and 16.7%[15], respectively. However, to the best of our knowledge, few studies have focused on this issue in a high endemic region of HBV infection such as China.

The aim of this study was to assess the rate of HBV serological testing prior to initiation of chemotherapy and the rate of HBV reactivation after chemotherapy in cancer patients in China.

MATERIALS AND METHODS

Patients

From January 1, 2009 to June 30, 2010, all cancer patients who were seen in the Cancer Center of West China Hospital were analyzed. Inclusion in this study required that patients be naïve to cancer chemotherapy but have indications for it. Patients who did not receive chemotherapy for any reason were excluded. This study was approved by the Institutional Review Board of West China Hospital and carried out according to the provisions of the Helsinki Declaration.

Methods and definitions

A retrospective study of the chart information on cancer patients was undertaken. HBV reactivation was defined as an increase in serum HBV DNA to > 1 log higher than that of the pre-exacerbation baseline, or serum HBV DNA conversion from negative to positive[16]. In our center, HBV DNA levels > 1000 copies/mL were defined as HBV DNA positive. Important clinical information, such as the levels of HBV DNA and serological markers [including HBsAg, hepatitis B surface antibody (anti-HBs), hepatitis B e antigen (HBeAg), HBeAg antibody and hepatitis B core antibody (anti-HBc)], were collected and measured by a microparticle enzyme-linked immunosorbent assay (Santa Cruz Biotechnology, CA, United States). All hepatitis B serological marker results were reported as positive or negative. EASL guidelines recommend that all patients requiring chemotherapy be tested for HBsAg and anti-HBc prior to initiation of treatment[11], therefore, the presence of either HBsAg-positive or isolated anti-HBc-positive results was defined as positivity for an HBV serological marker (HBV-sm). HBV DNA levels were tested using a real-time polymerase chain reaction detection system (Applied Biosystems, Foster City, CA, United States). Individuals with chronically elevated alanine aminotransferase (ALT) or aspartate aminotransferase (AST) should be routinely tested for HBV[12], and ALT and AST levels were analyzed using an automatic biochemistry analyzer (Olympus AU5400, Olympus Corporation, Tokyo, Japan). Values for ALT or AST above the upper limit of normal (58 IU/L in our hospital) were defined as elevated.

Statistical analysis

Frequencies and percentages were used for statistical descriptions. The χ^2 or Fisher's exact test was used for analysis of categorized data. A nonparametric Mann-Whitney/Wilcoxon test or *t* test was used for analysis of quantitative data. Multiple logistic regression analysis was used to estimate the odd ratio (OR) and 95%CI of the HBV screening rate. All statistical analyses were carried out using SPSS version 18.0 statistical software, and statistical significance was defined as $P < 0.05$.

RESULTS

Patient characteristics

From the beginning of January 2009 to the end of June 2010, a total of 6646 cancer patients were reviewed. Among these patients, 5616 (84.5%) received chemotherapy (Figure 1). Combined with other chemotherapeutic agents, rituximab was given to 49 patients (17.4%) and/or steroids were given to 203 (72.2%) patients with hematological malignancies. Patient characteristics are shown in Table 1.

Testing for HBV infection

As shown in Figure 1, 49.3% (2770/5616) patients were screened for HBV before initiation of chemotherapy.

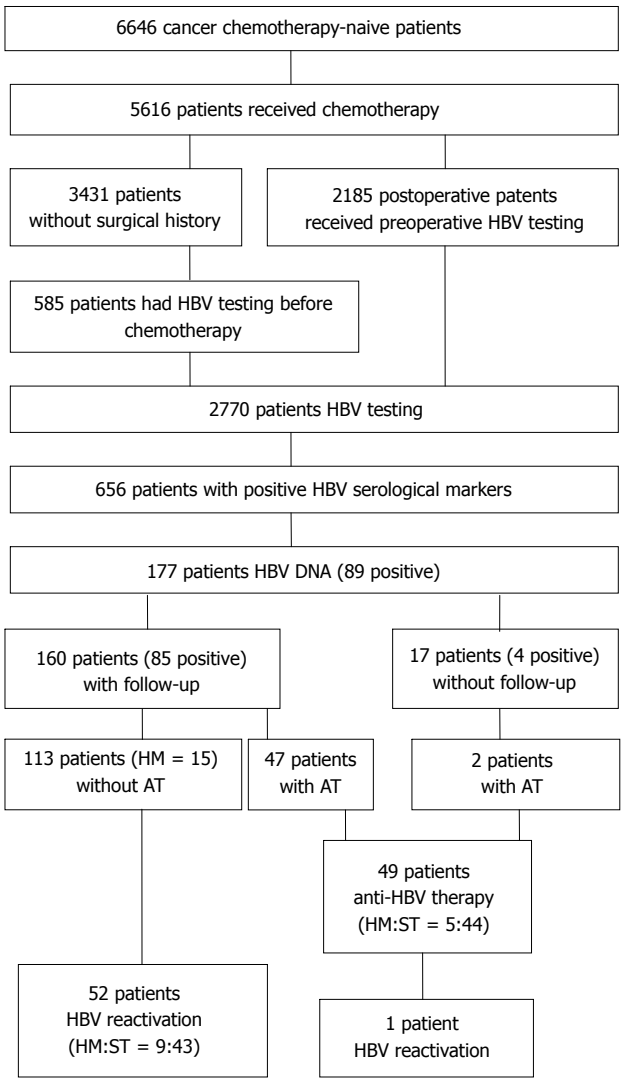

Figure 1 Study flow diagram. HM: Hematological malignancy; ST: Solid tumor; AT: Anti-HBV therapy; HBV: Hepatitis B virus.

The testing rate for patients with hematologic malignancies and solid tumors was 47.7% (134/281) and 49.4% (2636/5335), respectively. However, the reason for HBV serological testing in 2185 patients was not in preparation for chemotherapy, but as a routine check before surgery. Only 17.1% (585/3431) of the cancer patients received pre-chemotherapy HBV testing. The exact pre-chemotherapy testing rate for hematological malignancies and solid tumors was 43.2% (112/259) and 14.9% (473/3172), respectively. As shown in Table 2, in univariate analysis, the characteristics of age > 50 years, male sex, history of HBV infection, rituximab use, elevated aminotransferases, and hematological malignancies were associated with HBV testing. In multiple logistic regression analysis, patients with rituximab therapy (OR: 1.96, 95%CI: 1.04-3.67, $P < 0.05$), elevated aminotransferase levels (OR: 10.88, 95%CI: 8.01-14.78, $P < 0.001$), and hematological malignancies (OR: 4.17, 95%CI: 3.10-5.61, $P < 0.001$) were more likely to have received pre-chemotherapy HBV serological testing.

Table 1 Characteristics of 5616 cancer patients who received chemotherapy *n* (%)

Characteristics	Hematological malignancy	Solid tumor		Total
	(*n* = 281)	HCC (*n* = 237)	Others (*n* = 5098)	(*n* = 5616)
Age (yr), mean (range)	50.5 (18-83)	53.1 (39-67)	54.4 (41-67)	54.1 (29-79)
Sex				
Men	176 (62.6)	201 (84.8)	2567 (50.4)	2944 (52.4)
Women	105 (37.4)	36 (17.9)	2531 (49.6)	2672 (47.6)
Surgical history	22 (7.8)	6 (2.5)	2157 (42.3)	2185 (38.9)
History of HBV infection[1]	27 (9.6)	126 (53.2)	179 (3.5)	332 (5.9)
Aminotransferase				
Normal (< 58 IU/mL)	251 (89.3)	144 (60.8)	4816 (94.5)	5211 (92.8)
Elevated	30 (10.7)	93 (39.2)	282 (5.5)	405 (7.2)

[1]History of hepatitis B surface antigen positivity. HCC: Hepatocellular carcinoma; HBV: Hepatitis B virus.

Table 2 Univariate logistic regression analysis of factors associated with testing for hepatitis B virus serological markers prior to chemotherapy, but excluding those tested preoperatively (*n* = 3431)

	No.	Screening *n* (%)	*P* value
Age (yr)			< 0.01
≤ 50	1215	240 (19.8)	
> 50	2216	345 (15.6)	
Sex			< 0.05
Men	2031	374 (18.4)	
Women	1400	211 (15.1)	
Rituximab therapy[a]			< 0.01
Yes	49	25 (51.0)	
No	3382	560 (16.6)	
History of HBV infection			< 0.01
Yes	141	45 (31.9)	
No	3290	540 (16.4)	
ALT and/or AST[b]			< 0.01
Abnormal	205	128 (62.4)	
Normal	3226	457 (14.2)	
Types of tumor[c]			< 0.01
Hematological	259	112 (43.2)	
Solid	3172	473 (14.9)	< 0.05
Liver cancer	78	18 (23.1)	
Others	3094	455 (14.7)	

[a]$P = 0.038$ (OR: 1.96, 95%CI: 1.04-3.67); [b]$P < 0.001$ (OR: 10.88, 95%CI: 8.01-14.78); [c]$P < 0.001$ (OR: 4.17, 95%CI: 3.10-5.61). OR: Odds ratio; HBV: Hepatitis B virus; ALT: Alanine aminotransferase; AST: Aspartate aminotransferase.

HBV serological markers

The prevalence of HBsAg positivity was 13.4%. The prevalence of HBV-sm positivity was 23.7%. The prevalence of HBsAg positivity ($P < 0.001$) and HBsAg and/or anti-HBc positivity ($P < 0.001$) in the male patients were significantly higher than in the female patients. In patients < 20 years old, the rate of HBV-sm positivity was significantly lower than in patients in the older age group ($P < 0.001$) (Table 3).

Table 3 Prevalence of hepatitis B virus serum markers and DNA in 2770 patients *n* (%)

	No.	HBsAg+	HBsAb+	HBeAg+	HBeAb+	Isolated HBcAb+	HBV-sm positive[1]	Screening HBV DNA[2]
Total	2770	372 (13.4)	1367 (49.4)	43 (1.6)	600 (21.7)	284 (10.3)	656 (23.7)	177 (27.0)
Age (yr)								
≤ 20	27	2 (7.4)	14 (51.9)	1 (3.7)	2 (7.4)	1 (3.7)	3 (11.1)	1 (33.3)
>20	2743	370 (13.5)	1353 (49.3)	42 (1.5)	598 (21.8)	283 (10.3)	653 (23.8)	176 (27.0)
Sex								
Women	1495	144 (9.6)	780 (52.2)	15 (1.0)	280 (18.7)	146 (9.8)	290 (19.4)	52 (17.9)
Men	1275	228 (17.9)	587 (46.7)	28 (2.2)	320 (25.5)	138 (10.8)	366 (28.7)	125 (34.2)
Types of tumor								
Hematological	134	21 (15.7)	51 (38.1)	3 (2.2)	26 (19.4)	7 (5.2)	28 (20.9)	23 (82.1)
Solid	2636	351 (13.3)	1316 (49.9)	40 (1.5)	574 (21.8)	277 (10.5)	628 (23.8)	154 (24.5)
Liver cancer	177	114 (64.4)	45 (25.4)	17 (9.6)	114 (64.4)	27 (15.3)	141 (79.7)	68 (48.2)
Others	2459	207 (8.4)	1271 (41.7)	23 (0.9)	114 (64.4)	250 (10.2)	487 (19.8)	84 (3.5)

[1]HBV-sm positive: HBV serological marker positive (HBsAg positive and/or isolated HBcAb positive); [2]Percentage equals the screening number of HBV DNA/number of HBV-sm positive. HBV: Hepatitis B virus; HBsAg: Hepatitis B surface antigen; HBsAb: Hepatitis B surface antibody; HBeAg: Hepatitis B e antigen; HBeAb: Hepatitis B e antibody; HBcAb: Hepatitis B core antibody.

Table 4 Characteristics of 160 hepatitis B surface antigen-seropositive cancer patients undergoing cytotoxic chemotherapy *n* (%)

	Patients developed HBV reactivation (*n* = 53)	Patients who did not develop HBV reactivation (*n* = 107)	*P* value
Sex[a]			< 0.05
Men	43 (38.4)	69 (61.6)	
Women	10 (20.8)	38 (79.2)	
Age (yr), median (range)	51.5 (24.8-78.2)	52.9 (27.8-77.9)	
Tumor type[b]			< 0.05
Lymphomas	10 (55.6)	8 (44.4)	
Solid tumor	43 (30.3)	99 (69.7)	
Liver cancer	25 (35.7)	45 (64.3)	
Others	18 (25.0)	54 (75.0)	
ALT levels, median (range) (normal < 58 IU/mL)	69.1 (21.9-116.3)	55.7 (18.2-93.2)	
HBeAg status[c]			< 0.01
Positive	17 (73.9)	6 (26.1)	
Negative	36 (26.3)	101 (73.7)	
HBV DNA status[d]			< 0.01
Positive	47 (45.2)	57 (54.8)	
Negative	6 (10.7)	50 (89.3)	
Use of rituximab			< 0.05
Yes	6 (60)	4 (40)	
No	47 (31.3)	103 (68.7)	
Use of corticosteroids			< 0.05
Yes	8 (61.5)	5 (38.5)	
No	45 (30.6)	102 (69.4)	
Receiving antiviral therapy			< 0.01
Yes	1 (2.1)	46 (97.9)	
No	52 (46.0)	61 (54.0)	

[a]*P* = 0.040 (OR: 2.634, 95%CI: 1.046-6.63); [b]*P* = 0.002 (OR: 5.700, 95%CI: 1.869-17.380); [c]*P* = 0.000 (OR: 6.064, 95%CI: 2.213-16.621); [d]*P* = 0.006 (OR: 5.982, 95%CI: 1.689-21.194). OR: Odds ratio; HBV: Hepatitis B virus; ALT: Alanine aminotransferase; HBeAg: Hepatitis B e antigen.

HBV DNA and antiviral therapy

Among the 656 patients who were positive for HBV serological markers, 177 (27.0%) received HBV DNA testing and 160 (24.4%) underwent re-examination. The HBV DNA testing rate was significantly higher in the hematological cancer group ($P < 0.001$). Antiviral therapy (lamivudine) was given to 55.1% (49/89) of the patients who were positive for HBV DNA. All five HBV-DNA-positive patients with hematological malignancies received antiviral therapy, but only 52.4% (44/84) of the HBV-DNA-positive patients with solid tumors were given antiviral therapy (Figure 1 and Table 3).

HBV reactivation

Two cases of reactivation occurred in HBsAg-negative but anti-HBc-positive patients. One patient had diffuse large B-cell lymphoma while the other had breast cancer. As shown in Figure 1, HBV reactivation was observed in 33.1% (53/160) of patients after chemotherapy. Within the virus-untreated cohort ($n = 113$), the reactivation rate was 46.0% (52/113 patients). Among the 47 patients who received antiviral therapy, only one case of reactivation (2.1%) occurred. However, HBV reactivation was observed in 60.0% (9/15 patients) in the hematological malignancy group and 43.9% (43/98) in the solid tumor group who had not received any antiviral therapy. The median onset time of HBV reactivation was 11 wk (range: 3-25 wk) after initiation of chemotherapy. HBV reactivation developed earlier ($P < 0.001$) in patients with lymphoma (9 wk, range: 4-14 wk) than in those with other cancers (15 wk, range: 9-21 wk). HBV reactivation also developed earlier ($P < 0.001$) in patients receiving CHOP (cyclophosphamide, doxorubicin, vincristine and prednisolone)-like regimens (8 wk, range: 3-15 wk) compared to non-CHOP protocols. Baseline HBV status (positive or negative) was not significantly associated with the time interval between initiation of chemotherapy and onset of HBV reactivation. In multivariate analysis (Table 4), HBV reactivation was more likely to occur in patients with lymphoma, high levels of HBV DNA, HBeAg positivity, and male patients. It was also found that antiviral prophylaxis for patients who were positive for HBV DNA significantly reduced the incidence of chemotherapy-induced HBV reactivation (OR: 0.009,

95%CI: 0.001-0.073, $P < 0.001$).

Chemotherapy drugs and HBV reactivation

Of the 160 patients who were re-tested for HBV, 13 had received corticosteroids (all patients had lymphoma) and 61.5% (8/13) developed HBV reactivation. In univariate analysis, HBV reactivation was more likely to occur in patients who had received corticosteroids ($P = 0.023$) and botanical chemotherapeutic drugs ($P = 0.048$). However, the components of the chemotherapy regimens (including corticosteroids) seemed not to be associated with HBV reactivation in multivariate analysis.

Hepatitis C virus co-infection

Of 5616 patients, 2679 (47.7%) were tested for hepatitis C virus (HCV). In this group, 305 and 12 patients were HBsAg positive and HCV RNA positive, respectively. Only four out of 305 patients (1.3%) were both HBsAg and HCV-RNA positive, and none of these patients developed HBV reactivation.

DISCUSSION

In recent years, many organizations have advocated that all chemotherapy candidates should receive HBV serological testing before starting treatment[11-13]. In the present study, it was observed that 49.3% of patients had received HBV serological testing prior to chemotherapy, which was much higher than reports from Canada (14%)[14] and the United States (16.7%)[15]. However, most of the patients were not for HBV infection because of impending chemotherapy, but as part of a preoperative routine (Figure 1). Therefore, only 17.1% of the cancer patients received pre-chemotherapy HBV testing, which is close to the testing rates in other published studies[14,15].

Patients with elevated aminotransferase levels prior to chemotherapy were more likely to be screened for HBV infection (OR: 10.88, $P < 0.001$). This is reasonable because elevated aminotransferase levels usually indicate liver inflammation, which may lead physicians to screen for HBV as a possible etiology. Guidelines have recommended that individuals with chronically elevated ALT or AST should be routinely screened for HBV infection[12]. However, not all patients with elevated aminotransferases receive HBV testing. It has been reported that only 30%-70% of oncologists screen for HBV infection before chemotherapy in patients with abnormal liver-associated enzymes[17,18]. The testing rate of only 62.4% in the current study is consistent with the data from other studies. The lack of HBV testing might be because some patients without a history of HBV infection had only slightly elevated aminotransferase levels that were insufficiently high to be a contraindication for chemotherapy.

In the current study, a relationship was found between the type of tumor and the rate of HBV serological testing. Patients with hematological malignancies (OR: 4.17, $P < 0.001$) were more likely to receive HBV testing before chemotherapy. The same phenomenon was also observed in an American study[19], which might be partly because lymphoma is a known risk factor for HBV reactivation[4,10]. Rituximab is a standard treatment for diffuse large B-cell lymphoma. Rituximab treatment was found to be an important indicator associated with HBV testing prior to chemotherapy (OR: 1.96, $P = 0.038$) in the present study. Testing for HBV infection before anticancer therapy is likely because of the known risk of HBV reactivation after rituximab administration[20].

In the current study, the HBV reactivation rate was found to be 33.1%, which was very close to the average reactivation rate (34%) observed in a prior meta-analysis[9].

The median onset time of HBV reactivation after initiation of chemotherapy was 11 wk (range: 3-25 wk), which was similar to that reported previously (16 wk, range: 4-36 wk)[21]. HBV reactivation was observed to occur earlier in patients with lymphoma and those receiving CHOP-like regimens in the current study. Previous studies have identified many risk factors related to HBV reactivation during anticancer therapy, such as male sex, younger age, absence of anti-HBs, HBeAg seropositivity, diagnosis of lymphoma or breast cancer, high pre-chemotherapy HBV DNA levels, and the use of steroids or rituximab[4,10,20,22]. In addition, it has also been reported that HBV DNA contains a glucocorticoid responsive element that stimulates HBV replication[23,24]. Multivariate analysis in the present study identified high baseline levels of HBV DNA, male patients, HBeAg positivity, and lymphoma to be predictive of HBV reactivation (Table 4). Further study is needed to determine the relationship between HBV reactivation and steroids (including their dose).

HBV reactivation rates were significantly higher in the non-lamivudine group compared with the lamivudine group (46.0% *vs* 2.1%). Moreover, multivariate analysis identified that antiviral prophylaxis significantly reduced the incidence of chemotherapy-induced HBV reactivation, which has also been reported previously[25-28]. Therefore, for patients with high baseline levels of HBV DNA, prophylactic lamivudine might be considered before chemotherapy. This is already recommended by EASL and CDC[11,13]. However, high baseline HBV viral loads are associated with the development of resistance to lamivudine (an antiviral agent with a low genetic barrier)[29]. After prophylactic administration of lamivudine, the rate of HBV reactivation was 5%-13.3% in previous studies[25,27,28]. It was observed in some small studies that none of the HBsAg-positive patients receiving chemotherapy or immunosuppressive therapy developed HBV reactivation after prophylactic entecavir[30,31]. Therefore, prophylactic administration of other antiviral agents with high genetic barriers to resistance, such as entecavir or tenofovir, might be considered suitable substitutes for lamivudine for HBV prophylaxis prior to chemotherapy.

In the current study, it was observed that 372 patients (13.4%) were HBsAg seropositive (Table 3). According to an epidemiological serological survey of hepatitis B[2], the

weighted prevalence of HBsAg for the Chinese population aged 1-59 years was 7.2%. Therefore, the prevalence of HBsAg in the current study seems to be higher. This might be because most of the patients in our study were > 20 years old (Table 3), and therefore, born before the introduction of the universal vaccination program[32]. Another reason might be that our patients with a history of HBV or elevated aminotransferases were more likely to be tested for HBV infection (Table 2).

Previous studies have reported that 0.72%-3.3% of patients who were negative for HBsAg developed *de novo* HBV-related hepatitis after chemotherapy[33-35]. Therefore, it is not sufficient to test for the presence of HBsAg alone as a marker of HBV infection. This is consistent with the EASL recommendations that cancer patients should be tested for both HBsAg and anti-HBc before chemotherapy[11]. In the current study, 284 patients (10.5%) were HBsAg negative but anti-HBc positive (Table 3), and two of these had HBV reactivation; one with diffuse large-B lymphoma and the other with breast cancer.

As mentioned before, high baseline HBV DNA levels are an independent risk factor for HBV reactivation[10,22]. EASL has recommended that cancer patients who are HBsAg or anti-HBc positive should have their HBV DNA levels measured[11]. In the current study, of the 656 patients who were HBsAg positive and/or anti-HBc positive, only 177 patients (27.0%) had been tested for HBV DNA (Table 3). Oncology physicians should pay more attention to this problem.

A high prevalence of occult HBV infection has been observed in HCV-infected patients[36,37]. The HCV co-infection rate among HBsAg carriers ranged from 3% to 18%[38,39], depending on the geographic location and selection of patients. In the current study, 4 patients (1.3%) were positive for both HBsAg and HCV RNA, and none developed HBV reactivation. This might have been because HCV replication is suspected to suppress HBV replication strongly[40].

The limitations of this study include the fact that it was a single-institution, retrospective study. Some important information was not recorded, such as the reasons why physicians made decisions regarding testing for HBV infection. This limitation may be resolved by a prospective study in the future.

In conclusion, only 17.1% of cancer patients received complete HBV testing prior to chemotherapy. Patients who were to receive rituximab therapy, had elevated aminotransferase levels, or had hematological malignancies were more likely to receive HBV testing. Only 27% of the patients who were positive for HBsAg and/or anti-HBc received testing for HBV DNA. HBV reactivation was observed in 33.1% of the patients, and was more likely to occur in patients with lymphoma, high levels of HBV DNA, HBeAg positivity, and male patients. Prophylactic antiviral therapy for patients with positive HBV DNA can significantly reduce the incidence of chemotherapy-induced HBV reactivation.

COMMENTS

Background

Cancer patients receiving intensive chemotherapy may be at risk for hepatitis B virus (HBV) reactivation. Recently, more attention has been paid to HBV testing before chemotherapy. Many clinical practice guidelines advise HBV testing before starting chemotherapy. However, few studies have focused on this issue in regions of high HBV endemicity, such as China.

Research frontiers

Authors demonstrated that only 17.1% of cancer patients received pre-chemotherapy HBV testing (43.2% for hematological malignancies and 14.9% for solid tumors). Patients who had received rituximab therapy, had elevated aminotransferase levels, or had hematological malignancies were more likely to receive HBV testing. HBV reactivation was more likely to occur in patients with lymphoma, high levels of HBV DNA, hepatitis B e antigen positivity, and male patients.

Innovations and breakthroughs

In recent years, more organizations have advocated that all chemotherapy candidates should receive HBV serological testing before starting treatment. This is believed to be the first study to report the exact pre-chemotherapy HBV testing rate in China; a region of high endemicity for HBV infection. The rate of HBV reactivation after chemotherapy and the median onset time are also reported.

Applications

Many clinical practice guidelines advise HBV testing before starting chemotherapy. However, the results in this study showed that only 17.1% of cancer patients received pre-chemotherapy HBV testing.

Terminology

HBV reactivation was defined as an increase in serum HBV DNA to > 1 log higher than that of the pre-exacerbation baseline, or serum HBV DNA conversion from negative to positive.

Peer review

In this retrospective analysis, the authors assessed the rate of HBV serological testing prior to the initiation of chemotherapy and the rate of HBV reactivation after chemotherapy in Chinese patients with cancer. The authors concluded that < 20% of patients received HBV testing before chemotherapy, and HBV reactivation would have occurred after chemotherapy in nearly half of these infected patients who did not receive pre-emptive antiviral therapy.

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BRIEF ARTICLE

Quadruple therapy for eradication of *Helicobacter pylori*

Hai-Jun Ma, Jin-Liang Wang

Hai-Jun Ma, Jin-Liang Wang, Department of Gastroenterology, the First Affiliated Hospital of Henan University of Science and Technology, Luoyang 471003, Henan Province, China
Author contributions: Ma HJ collected data on the subject and prepare the manuscript; Wang JL conceived and designed the topic, reviewed and analyzed the literature.
Correspondence to: Jin-Liang Wang, Professor, Department of Gastroenterology, the First Affiliated Hospital of Henan University of Science and Technology, No. 24 Jinghua Road, Luoyang 471003, Henan Province, China. wangjinliang3008@163.com
Telephone: +86-379-64830750 Fax: +86-379-64907159
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Abstract

AIM: To investigate quadruple therapy with rabeprazole, amoxicillin, levofloxacin and furazolidone for the eradication of *Helicobacter pylori* (*H. pylori*) infection.

METHODS: A total of 147 patients were divided into the experimental treatment group (n = 78) and the standard triple treatment group (n = 69). The experimental treatment group received rabeprazole 20 mg, amoxicillin 1.0 g, levofloxacin 0.2 g and furazolidone 0.1 g, twice daily. The standard triple treatment group received omeprazole 20 mg, amoxicillin 1.0 g and clarithromycin 0.5 g, twice daily.

RESULTS: One month after treatment, the ^{13}C urea breath test was carried out to detect *H. pylori*. The eradication rate using per-protocol analysis was 94.3% in the experimental treatment group and 73% in the standard triple treatment group (P < 0.05), and using intention to test analysis, these figures were 86% and 67% in the two groups, respectively. Side effects were observed in 34 patients, and included mild dizziness, nausea, diarrhea and increased bowel movement. Eleven of the 34 patients needed no treatment for their side effects.

CONCLUSION: Rabeprazole, amoxicillin, levofloxacin and furazolidone quadruple therapy is a safe method for the eradication of *H. pylori* with high efficacy and good tolerability.

Key words: Rabeprazole; Amoxicillin; Levofloxacin; Furazolidone; *Helicobacter pylori*

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INTRODUCTION

Helicobacter pylori (*H. pylori*) is closely related to a number of gastrointestinal diseases. It is the main pathogenic factor in chronic gastritis and peptic ulcer disease, and is also the initiating factor in gastric cancer and gastric mucosa-associated lymphoid tissue lymphoma[1]. The ability to reliably eradicate this pathogen is important in the management of these diseases[2-5].

The eradication of *H. pylori* has been a research focus for the effective treatment of the above diseases. Currently, the worldwide standard triple therapy consists of a proton pump inhibitor (PPI) or bismuth, amoxicillin and metronidazole or clarithromycin. However, with the extensive use of antibiotics and the irregularity of treatment, there is increasingly high *H. pylori* resistance, especially resistance to metronidazole and clarithromycin. The effectiveness of "legacy triple therapy", which was recommended by the Maastricht Ⅲ Consensus Report, has demonstrated disappointingly low treatment success (*i.e.*, below 80%) worldwide[6]. The difficulty of *H. pylori* eradication is increasing. The consensus of the Third National Lushan Conference held at Lushan Mountain in Jiangxi in August 2007 states that there is high *H. pylori* resistance to metronidazole and clarithromycin, but low resistance to furazolidone, tetracycline and quinolones,

therefore, the effects of these antibiotics are relatively high, and may be used as the initial treatment choice. In our study, quadruple therapy with rabeprazole, amoxicillin, levofloxacin and furazolidone achieved good results.

MATERIALS AND METHODS

General information

All 147 patients underwent the treatment with either rabeprazole 20 mg, amoxicillin 1.0 g, levofloxacin 0.2 g and furazolidone 0.1 g, twice daily or omeprazole 20 mg, amoxicillin 1.0 g and clarithromycin 0.5 g, twice daily at the First Affiliated Hospital of Henan University of Science and Technology from January 2009 to December 2011 and were included in this study as sample cases. However, 78 patients who chose treatment with rabeprazole 20 mg, amoxicillin 1.0 g, levofloxacin 0.2 g and furazolidone 0.1 g, twice daily were included in the experimental treatment group, and 69 patients who chose treatment with omeprazole 20 mg, amoxicillin 1.0 g and clarithromycin 0.5 g, twice daily were included in the standard triple treatment group. All patients, who did not receive treatment before enrolling in this study, were diagnosed with chronic erosive gastritis or peptic ulcer disease by endoscopy. In addition, all patients had a positive rapid urease test and antrum mucosal tissue was obtained to detect the presence of *H. pylori*.

The patients by their own volition, were divided into the experimental treatment group (n = 78) and the standard triple treatment group (n = 69). The experimental treatment group consisted of 41 males and 37 females, aged 20 to 65 years, with an average age of 44.5 years; 34 patients had chronic erosive gastritis, 28 had a gastric ulcer and 16 had a duodenal ulcer. The standard triple treatment group consisted of 38 males and 31 females, aged 22 to 67 years, with an average age of 43.5 years; 31 patients had chronic erosive gastritis, 17 had a gastric ulcer and 21 had a duodenal ulcer. There were no statistically significant differences between the two groups regarding age and gender (Table 1).

Exclusion criteria

Patients with a history of gastrointestinal surgery, gastroesophageal reflux disease, non-steroidal anti-inflammatory drug-related stomach problems or ulcers, those taking PPIs or H_2 blockers, bismuth, antibiotics or other drugs which might affect gastric physiology two weeks before enrolling in the study and those who were allergic to the medicine tested were excluded.

Methods

The experimental treatment group received rabeprazole (Jiangsu Stockhausen Pharmaceuticals, Nanjing, China) 20 mg, amoxicillin (Shiyao Pharmaceutical Group, Shijiazhuang, China) 1.0 g, levofloxacin (Jiangsu Yabang Epson Pharmaceutical, Nanjng, China) 0.2 g and furazolidone (Wuhan Huawei Medicine, Wuhan, China) 0.1 g; the standard triple treatment group received omeprazole (Beijing Taiyang Pharmaceutical, Beijing, China) 20 mg, amoxicillin (Shiyao Pharmaceutical Group, Shijiazhuang, China) 1.0 g and clarithromycin (Guangzhou Baiyunshan Pharmaceutical Group, Guangzhou, China) 0.5 g. The drugs were given half an hour before meals in the morning and evening for 7 d.

Table 1 Baseline characteristics of the patients

Group	No. of patients	Age (yr)	Male[1]	Female[1]	Chronic erosive gastritis[2]	Gastric ulcer[2]	Duodenal ulcer[2]
Experimental treatment	78	44.5	41	37	34	28	16
Standard treatment	69	43.5	38	31	31	17	21

[1]χ^2 = 0.093, P > 0.05; [2]χ^2 = 2.96, P > 0.05.

Observed indicators

Patients were followed up four weeks after treatment, and the ^{13}C urea breath test (^{13}C-UBT) was carried out to detect the presence of *H. pylori*.

Ethics

This was a clinical study carried out in humans, and the protocol for this work was approved by the Institutional Ethics Committee of the First Affiliated Hospital of Henan University of Science and Technology. Before the study began, the patients signed an informed consent form which included name, age, gender, chief complaint, drug history and past medical history.

Statistical analysis

The *H. pylori* eradication rate was assessed based on intention-to-treat (ITT) and per-protocol (PP) analysis. The 95%CI were also calculated for both the ITT and PP analyses and the eradication rate. The patients who were lost to follow-up or could not complete the treatment course due to severe adverse reactions, were considered treatment failures and were excluded in the PP analysis. The χ^2 test and Fisher's exact test were used to compare the differences between the two groups in terms of baseline data, eradication rate and adverse reactions. $P < 0.05$ was considered significant.

RESULTS

Treatment results

In the experimental treatment group, 7 of the 78 patients were lost to follow up (9%), and of the 71 remaining patients, 67 were negative by the ^{13}C-UBT. In the standard triple treatment group, 6 of the 69 patients were lost to follow up (8.6%), and of the remaining 63 patients, 46 were negative by the ^{13}C-UBT.

The eradication rate following the PP analysis in the experimental treatment group was 94.3% (67/71), and was 73% (46/63) in the standard triple treatment group. The eradication rate following the ITT analysis was 86%

Table 2 *Helicobacter pylori* eradication rate in the two groups

Group	Eradication	No eradication	Follow-up lost	Eradication rate[1] (PP) (95%CI)	Eradication rate[2] (ITT) (95%CI)
Experimental treatment group	67	4	7	94% (88%-99%)	86% (78%-94%)
Standard triple treatment	46	17	6	74% (64%-84%)	67% (57%-77%)

[1]χ^2 = 12.10, P < 0.05; [2]χ^2 = 7.615, P < 0.05. PP: Per-protocol; ITT: Intention-to-treat.

Table 3 *Helicobacter pylori* eradication rates in relation to disease *n* (%)

Group	Chronic erosive gastritis	Gastric ulcer	Duodenal ulcer
Experimental treatment	29 (34)	24 (28)	14 (16)
Standard treatment	21 (31)	11 (17)	14 (21)

χ^2 = 2.29, P > 0.05

and 67% in the two groups, respectively. The eradication rates in the two groups were statistically significant (P < 0.05) (Table 2). The eradication rate in patients with different diseases in the two groups was not significantly different (Table 3).

Side effects

Major adverse reactions were abdominal pain, dry mouth, dizziness, nausea, vomiting and bloating symptoms, however, these reactions were mild and well tolerated. All patients were able to complete the treatment regimen (Table 4).

DISCUSSION

Warren and Marshall reported the isolation of Campylobacter unidentified (unidentified curved bacilli) from the stomach in 1983, which was officially named *H. pylori* in 1989. *H. pylori* is considered to be the etiologic cause of gastritis, peptic ulcer disease and is associated with the development of gastric cancer[7-9]. It is a spiral or curved gram-negative microaerophilic flagellated bacillus. The prevalence of *H. pylori* infection varies worldwide, and depends on socioeconomic status and sanitation conditions[10]. The diagnosis of *H. pylori* can be performed using invasive and noninvasive methods. Invasive methods include the urease test, which has a sensitivity ranging from 79.7% to 97.5% and a specificity from 97.2% to 100%[11-14], it is performed in the endoscopy unit and is a suitable rapid indirect test to confirm the presence of *H. pylori* in biopsy samples. The urea breath test is considered the gold standard for the diagnosis of *H. pylori* infection, and has a sensitivity and a specificity ranging from 90% to 100%[15-18]. Therefore, we suggest using the ^{13}C-UBT, or esophagogastroduodenoscopy for the assessment of *H. pylori* status.

With increased research on bacteria and drug treatment trials for *H. pylori* infection, it is now clear that antibiotics, antacids, metal preparations and other drugs are effective. Antibiotics used in the treatment of *H. pylori* infection include amoxicillin, tetracycline, clarithromycin, quinolones, furazolidone, metronidazoles and other drugs which have either bactericidal or bacteriostatic effects. Antacids include PPIs and histamine2-receptor antagonists. PPI-clarithromycin-amoxicillin or metronidazole treatment for 7 to 14 d is the first treatment choice for *H. pylori* infection[19]. However, several large clinical trials and meta-analyses have shown that the eradication rate with standard triple therapy has generally declined to unacceptable levels (*i.e.*, 80% or less) recently[20,21].

With increasing clinical use of antibiotics, *H. pylori* resistance appears to have significantly increased. Abuse and irrational drug use as well as repeated anti-*H. pylori* treatment failure are important causes of drug resistance[22].

According to the *H. pylori* Study Group of Digestive Diseases Division of the Chinese Medical Association, which investigated multiple regions of the country in 2007, the resistance rates of *H. pylori* to metronidazole, clarithromycin and amoxicillin were 75.6%, 27.6% and 2.7%, respectively[23]. *H. pylori* resistance to antibiotics is the most important factor in treatment failure. Therefore, the choice of antibiotics is the key to successful eradication of *H. pylori*, in the absence of drug susceptibility testing, and it is safe to use the less frequently used antibiotics which are less likely to induce antibiotic resistance.

The mechanism of action of PPIs involve: (1) direct inhibition of the growth of *H. pylori*; (2) inhibition of urease activity; and (3) increased intragastric pH and enhanced antibiotic activity[24]. Hepatic oxidation is mediated by the cytochrome P450 2C19 (CYP2C19) gene which is polymorphic and produces fast metabolizers and low metabolizers. This is another important factor in the failure of *H. pylori* eradication therapy, and one study suggested that this is the second leading cause of bacterial resistance[25]. The elimination of omeprazole, lansoprazole and pantoprazole involves hepatic oxidation mediated by cytochrome CYP2C19 and CYP3A4, while that of rabeprazole involves its reduction *via* a non-enzymatic pathway to rabeprazole-thioether and partial metabolism by CYP2C19 and CYP3A4[21]. Thus, rabeprazole is less susceptible to the influence of genetic polymorphisms of either CYP2C19 or CYP3A4, and was found to depend only on its pharmacokinetic and pharmacodynamic characteristics. The pKa of rabeprazole is 5.0 and those of other PPIs, such as omeprazole and lansoprazole, are around 4.0, rabeprazole accumulates in an acidic space up to a concentration ten-fold greater than do other PPIs. In addition, rabeprazole is known to transform into the acid-activated form much faster than other PPIs, which further contributes to the inhibition of proton pumps immediately after the arrival of rabeprazole at the target site[26].

Levofloxacin is a third-generation fluoroquinolone, its antibacterial mechanism involves inhibition of bacterial type Ⅱ topoisomerase. Topoisomerase controls DNA to-

Table 4 Side effect profile of patients

Group	No adverse reactions	Abdominal pain	Nausea	Vomiting	Dry throat	Bloating	Dizziness	Total
Experimental treatment	58	8	10	6	4	5	7	40
Standard triple	55	7	6	5	3	2	1	24
Total episodes	113	15	16	11	7	7	8	64

$\chi^2 = 2.13$, $P > 0.05$.

pology in DNA replication and repair, it is a key enzyme in transcription. Levofloxacin, blocks DNA replication by inhibiting bacterial DNA gyrase A subunit and inhibits topoisomerase Ⅳ activity, thus has a bactericidal effect[27]. In addition, levofloxacin has no cross-resistance to *B*-lactam and macrolide antibiotics.

The clinical use of furazolidone is not very extensive, and it is not a commonly used drug in the standard regimen. However, it has a significant effect on *H. pylori* and does not tend to produce drug resistance. The reported furazolidone sensitivity rate is generally 100%[28], and it is inexpensive, therefore, we chose this medication for our *H. pylori* infected patients, and the results showed that inclusion of this drug in the quadruple regimen for *H. pylori* infection resulted in a high eradication rate, thus, this agent has good efficacy. The most common side effects of furazolidone are nausea, vomiting and other gastrointestinal reactions. In this study, gastrointestinal reactions were seen in patients taking furazolidone, however, multiple neuritis was not noted, and the drug was well tolerated.

In summary, these experimental results demonstrated that rabeprazole - amoxicillin - levofloxacin - furazolidone quadruple combination therapy is a safe, highly effective and well tolerated regimen for eradication of *H. pylori*.

COMMENTS

Background

Helicobacter pylori (*H. pylori*) eradication rates with standard triple therapy are declining worldwide. The optimal management of *H. pylori* is evolving and new treatment combinations for antibiotic resistant *H. pylori* strains are required.

Research frontiers

Standard triple therapy represents the accepted standard therapy for *H. pylori* as the organism is known to be susceptible to clarithromycin, and local antimicrobial resistance rates are below 20%, while newer treatment regimens (sequential, quadruple, concomitant and hybrid therapies) and various combinations of new and old antibiotics aimed at eradicating the organism more effectively are increasing in popularity.

Innovations and breakthroughs

In this study, the authors provide further evidence of the efficacy and tolerability of quadruple eradication therapy for *H. pylori* infection.

Applications

Empirical therapy can be employed to treat *H. pylori* infection if antimicrobial sensitivity data are unavailable.

Terminology

A 7-d quadruple therapy consisting of rabeprazole (20 mg *bid*) amoxicillin (1.0 g, *bid*) levofloxacin (0.2 g, *bid*) and furazolidone (0.1 g, *bid*), has high efficacy in the treatment of *H. pylori* infection with an eradication rate of 86% by intention-to-treat analysis and 94% by per-protocol analysis.

Peer review

This is a nice study, particularly for populations who have a relative high prevalence of CYP2C19 polymorphism. The article represents an important contribution to the treatment of *H. pylori*.

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META-ANALYSIS

Pre-existing diabetes mellitus increases the risk of gastric cancer: A meta-analysis

Jae Moon Yoon, Ki Young Son, Chun Sick Eom, Daniel Durrance, Sang Min Park

Jae Moon Yoon, Sang Min Park, Department of Family Medicine, Seoul National University Hospital, Seoul National University College of Medicine, Seoul 110-744, South Korea
Ki Young Son, Center for Health Promotion, Seoul National University Hospital, Seoul 110-744, South Korea
Chun Sick Eom, Department of Family Medicine, Hallym University, Chuncheon Sacred Heart Hospital, ChunCheon 200-704, South Korea
Daniel Durrance, Family Medicine, Graduate School of Korea University College of Medicine, Seoul 110-799, South Korea
Author contributions: Yoon JM and Son KY have contributed equally to this article; Yoon JM and Son KY designed study, analyzed data and wrote manuscript; Eom CS contributed discussion and wrote manuscript; Durrance D edited manuscript; Park SM designed study, contributed discussion and edited manuscript.
Supported by Grant from the Seoul National University Hospital Research Fund, No. 04-2011-0540
Correspondence to: Dr. Sang Min Park, Department of Family Medicine, Seoul National University Hospital, Seoul National University College of Medicine, 101 Daehak-ro, Jongno-gu, Seoul 110-744, South Korea. sangmin.park.snuh@gmail.com
Telephone: +82-2-20723331 Fax: +82-2-7663276
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Abstract

AIM: To systematically assess the association between diabetes and incidence of gastric cancer.

METHODS: We searched MedLine (PubMed), EMBASE, and the Cochrane Library without any limitations with respect to publication date or language, we also searched the references of qualifying articles. Case-control studies and cohort studies comparing the risk of gastric cancer between diabetic patients and control subjects were included. We excluded studies reporting only standardized incidence ratios without control groups and those that investigated only mortality but not incidence. Seventeen studies met our criteria, and the qualities of these studies were assessed using the Newcastle-Ottawa Quality Assessment Scale. We performed a meta-analysis of pre-existing diabetes and gastric cancer incidence using the DerSimonian-Laird method for random-effects. For subgroup analyses, we separated the studies by study type, region, sex and method to determine confounding factors and reliability. We also conducted subgroup analyses to examine the effects of smoking, *Helicobacter pylori* (*H. pylori*) infection, and cancer site. Publication bias was evaluated using Begg's test.

RESULTS: A random-effects model meta-analysis showed an increased gastric cancer risk in diabetic patients [relative risk (RR) = 1.19; 95%CI: 1.08-1.31]. Subgroup analyses indicated that this result persisted in cohort studies (RR = 1.20; 95%CI: 1.08-1.34), in studies on populations of both Western (RR = 1.18; 95%CI: 1.03-1.36) and Eastern countries (RR = 1.19; 95%CI: 1.02-1.38), in a female subgroup (RR=1.24; 95%CI: 1.01-1.52), and in highly qualified studies (RR = 1.17; 95%CI: 1.05-1.31). Moreover, these results persisted when the analysis was confined to studies adjusted for well-known gastric cancer risk factors such as smoking (RR = 1.17; 95%CI: 1.01-1.34) and *H. pylori* infection (RR = 2.35; 95%CI: 1.24-4.46).

CONCLUSION: Pre-existing diabetes mellitus may increase the risk of gastric cancer by approximately 19%. This effect seems to be unrelated to geographical region.

Key words: Disease association; Diabetes mellitus; Gastric cancer; Incidence; Risk; Meta-analysis

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INTRODUCTION

Diabetes mellitus is currently an epidemic. Approximately 171 million individuals worldwide had diabetes mellitus in 2000, and it is estimated that 366 million people will have diabetes by 2030, which corresponds to nearly 4.5% of the global population[1]. The estimated prevalence of diabetes among adults in the United States currently ranges from 5.9% to 12.4% (median 8.3%)[2], and some cancers, such as colorectal, endometrial, breast, liver, and pancreatic cancer, are reportedly more prevalent in diabetes patients[3-7]. Diabetes mellitus has also been associated with an increased risk of long-term and all-cause mortality in cancer patients[8].

Although the incidence and associated mortality of gastric cancer is decreasing, it remains the fourth most common cancer and the second leading cause of cancer-related mortality worldwide[9]. Despite the success in gastric cancer control, the persistence of the public health burden of gastric cancer indicates the need to expand efforts to identify and address the individual and social determinants of the disease. The public health significance of a potential causal link between diabetes mellitus and gastric cancer highlights the need for a systematic assessment of the association between these 2 diseases.

Therefore, several systematic reviews have recently been performed to examine the above mentioned associations. One review suggested that diabetic individuals have an increased risk of gastric cancer[10]. However, this review had some limitations as it included studies without control groups[11-14] and excluded appropriate studies[15,16]. Two other reviews showing inconclusive results[17,18] did not include any recent studies[19,20], and did not differentiate between "incidence" and "mortality" in terms of describing the risk[17]. Such methodological weaknesses of the previous meta-analysis made it difficult to understand the association between preexisting diabetes mellitus and the risk of gastric cancer.

In the present study, we aimed to clarify the association between diabetes and gastric cancer through an extensive search of the literature, which we reviewed using strict criteria.

MATERIALS AND METHODS

Methods

The procedures performed in this meta-analysis are in accordance with recent guidelines for the reporting of meta-analysis (PRISMA guidelines).

Data sources and searches

We conducted a systematic search of electronic databases and the bibliographies of all eligible studies to identify all relevant studies. We initiated the search on February 7, 2012 without any limitations with respect to publication date or language. The electronic databases searched included MedLine (PubMed), EMBASE, and the Cochrane Library. The search strategy included terms for diabetes (glucose, diabetes, or hyperglycemia), gastric cancer (stomach cancer, gastric cancer, stomach malignant neoplasm, or gastric malignant neoplasm), and risk (incidence, prevalence, or risk). We also searched the references of included articles.

Study selection

Case-control studies, cohort studies, and randomized controlled trials comparing the risk of gastric cancer between diabetic patients and control subjects were eligible for inclusion. We included studies evaluating self-reported diabetes, registered diabetes, and high HbA1c and blood glucose levels. To be included in our meta-analysis, articles had to contain both of the following: (1) a risk estimate (hazard ratio, relative risk, or odds ratio relating preexisting diabetes to subsequent occurrences of gastric cancer); and (2) an estimate of precision (standard error or 95%CI). We also included articles that failed to report precision directly, but from which we could reconstruct a precision estimate using the data described[21,22]. We excluded studies reporting only standardized incidence ratios without control groups. We also excluded studies that investigated only mortality without incidence and studies in which the classification of the subjects' diabetes status (diabetic or non-diabetic) was not possible due to insufficient ranges in blood glucose levels. Studies were excluded if either the abstract[23] or the full text[24] was unavailable after author contact was made.

Data extraction and quality assessment

The titles, abstracts, and full articles were reviewed independently by two authors (Yoon JM and Son KY). Yoon JM performed a full abstraction of the data, and Son KY verified the accuracy. Disagreements were resolved by discussion, consensus, and arbitration by a third author (Park SM). Abstracted data included type of study, study population characteristics, criteria for diabetes mellitus or hyperglycemia, duration of follow-up, incidence of cancer, adjustment variables, and study quality. Quality was assessed using the Newcastle-Ottawa Quality Assessment Scale (NOS) for case-control or cohort studies.

Statistical analysis

To avoid overlapping patient populations, we compared data sources and geographic locations. If a patient population was found to overlap, we included the article with the most comprehensive population or the most adjusted risk estimate associated with preexisting diabetes. If an article reported estimates separately by sex[15,19,25-29] or by different cohorts[16], we regarded the study as 2 independent studies. If an article did not report the risk ratio and confidence interval, we estimated the risk using Fisher's exact test without adjustment[20-22], and the estimates were rounded off to the nearest hundredth. In case-control studies, odds ratios were regarded as relative risks because of the low prevalence of gastric cancer[9,30,31].

For the meta-analysis, we calculated pooled estimates for all the studies. For the subgroup analyses, we separated studies by study type, region, sex, and method to determine the confounding factors and reliability. We also

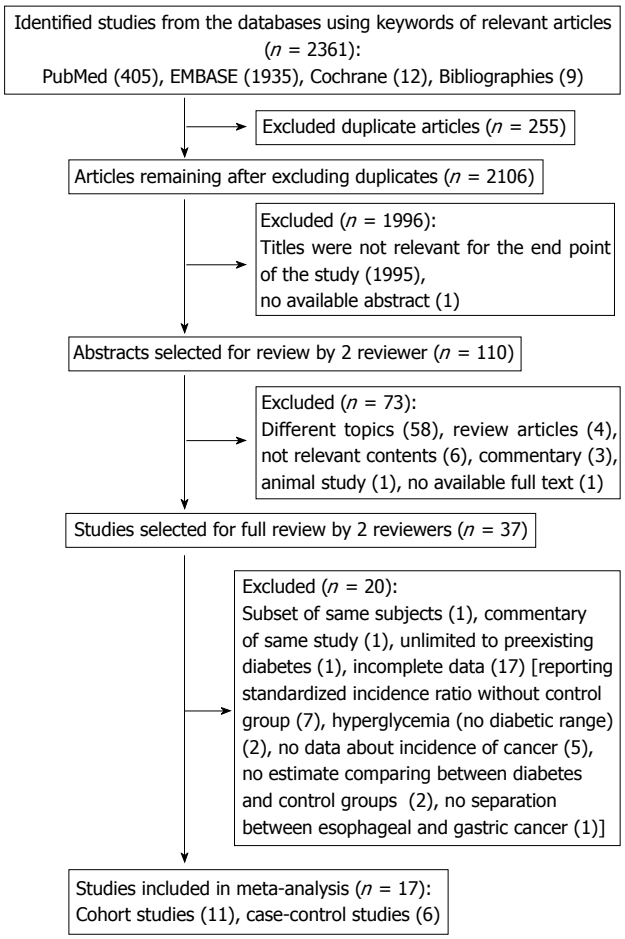

Figure 1 Flow diagram of studies identified and selected.

conducted subgroup analyses to examine the effects of smoking, *Helicobacter pylori* (*H. pylori*) infection, and cancer site, which are major factors influencing the risk of gastric cancer[32-34]. We calculated all the pooled estimates using the DerSimonian-Laird method for random effects. We also reported I^2 values for heterogeneity assessment. Publication bias was evaluated using Begg's test. All analyses were conducted using Stata software(version12.1, StataCorp, United States).

RESULTS

The systematic literature search identified 2361 relevant references (Figure 1). After screening the titles, we excluded 255 duplicated and 1996 non-relevant studies. By reviewing the abstracts, we further excluded 73 articles. The full texts of the 37 remaining articles were retrieved for formal review. After independent review, 20 studies were excluded; 2 studies[35,36] used the same populations as another trial[37], and 1 did not limit the exposure group to individuals with preexisting diabetes[38].

Seventeen studies reported only limited data (uncertain diabetes diagnosis[39,40], usage of a standardized incidence ratio[11,13,14,41-44], lack of an acceptable control group[45,46], reporting of only mortality data[47-51], and uncertain gastric cancer diagnosis[52]). Table 1 provides the details of the 17 studies that met our predefined inclusion and exclusion criteria. In total, 11 cohort[15,16,19,22,26-28,37,53-55] and 6 case-control[20,21,25,29,56,57] studies were used. We were unable to find any suitable randomized control trials.

Description of studies

The 17 articles included in our analysis were heterogeneous in many respects. Geographically, 6 studies were conducted in East Asia, 1 in West Asia, 5 in North America, and 5 in Europe. According to the cohort studies, the prevalence of diabetes varied from 3.0% to 13.2%. Four studies used measurement criteria to define diabetes mellitus, and 2 studies differentiated between gastric cardia and non-cardia cancer. The reporting of age and follow-up time varied widely. In 13 studies, a time interval of more than 1 year between the diagnoses of diabetes and cancer was required in order to minimize reverse causality, although 2 studies showed only the estimates without time intervals[16,54]. Quality varied from 4 to 9 stars according to the NOS, but 3 studies were downgraded because we used recalculated estimates without adjusting for pooled analysis[20-22]. After downgrading, the mean NOS score was 7.3. We therefore conducted subgroup analysis according to methodological quality (with "high-quality" defined as a score of $\geqslant 8$).

Fifteen studies included both sexes and 10 reported data separated by sex. In one of the latter, fewer than 5 female patients were diagnosed with both diabetes mellitus and subsequent gastric cancer[15]; the estimate was therefore not reported. In total, we found 28 independent groups separated by sex and cohort, and we yielded 27 eligible estimates. Only 2 studies described the specific risk ratios of cardia and non-cardia gastric cancers[20,22].

Overall

As shown in Figure 2, diabetes mellitus was associated with a significantly increased risk of gastric cancer in all included studies using random effects model analysis [relative risk (RR) = 1.19; 95%CI: 1.08-1.31; I^2 = 70.7%; 95%CI: 56.8%-80.2%].

Subgroup meta-analysis

We examined methodological differences when determining the quality of the studies. According to Figure 2, subgroup meta-analysis by study design revealed significant associations in cohort studies (RR = 1.20; 95%CI: 1.08-1.34), while the results of case-control studies were not statistically significant. Positive associations were observed in more reliable subgroup studies using the registration record or laboratory measurements as diabetes criteria and studies that were highly graded by NOS. A subgroup meta-analysis of studies designed to minimize reverse causality, including the shortest cancer-free interval, indicated that diabetes seemed to increase subsequent gastric cancer (Table 2).

Regional and gender-based differences are presented in Table 2. East Asian (RR = 1.19; 95%CI: 1.02-1.38) and Western studies (RR = 1.18; 95%CI: 1.03-1.36) showed

Table 1 Characteristics of 15 studies included in the meta-analysis of the gastric cancer risk of preexisting diabetes

Study	Study type	Country	DM criteria	Age (yr)	Follow up (yr)	Least interval[1] (yr)	Sex	DM prevalence	RR or OR (95%CI)	Adjustment variables/matched variable of selection	NOS
Atchison *et al*[53]	CS	United States (veterans)	Registration	18-100	27	1	Male	13.2%	0.95 (0.89-1.02)	Age, time, latency, race, number of visits, diagnoses of alcohol-related conditions, obesity and COPD	8
Carstensen *et al*[19]	CS	Denmark	Registration	No limit	15	Not mentioned	Male Female		1.28 (1.15-1.43) 1.34 (1.14-1.58)	Age, calendar time, date of birth	8
Jiang *et al*[20]	CC	United States	Self reported	30-74		1	Both		1.51 (1.10-2.07)[2]	Age, sex, race, education, birth place, smoking and BMI	7 (5)[5]
Lin *et al*[22]	CS	United States	Self reported	50-71	10	2	Male Female	9.9% 7.2%	1.52 (1.21-1.92)[2] 1.62 (0.98-2.68)[2]	Age, calories, alcohol consumption, smoking, fruit consumption, vegetable consumption, ethnicity, education, and physical activity	8 (6)[5]
Wotton *et al*[16] ORLS1 ORLS2	CS	England	Registration	⩾ 30	35 9	0	Both	5.5% 4.0%	1.11 (0.89-1.37) 2.05 (1.30-3.10)	Sex, age in 5-year bands, time and district of residence	8
Chodick *et al*[54]	CS	Israel	Registration	⩾ 21	8	0	Male Female		1.44 (0.98-2.11) 0.99 (0.55-1.80)	Age, region, SES level, use of healthcare, BMI, and history of CVD/ matched for age, sex	8
Ikeda *et al*[37]	CS	Japan	HbA1c (5.0%-5.9% *vs* 7.0%)	⩾ 40	14	Not mentioned	Both	3.9%	2.69 (1.24-5.85)	Age, sex, *H. pylori*, history of peptic ulcer, BMI, TC, alcohol intake, smoking, dietary factor[4]	8
Ogunleye *et al*[55]	CS	Scotland	Registration (only type 2 DM)	No limit	11	1	Both		0.77 (0.36-1.66)	Deprivation deciles of Carstairs/matched for age, sex and doctor practice	8
Kuriki *et al*[29]	CC	Japan	Self reported	40-80		Not mentioned	Male Female		1.16 (0.93-1.44) 1.70 (1.16-2.48)	Age, BMI, drinking, smoking, exercise, bowel movement, FHx of cancer and DM, dietary restriction, vegetable intake, greasy food, snack	6
Inoue *et al*[27]	CS	Japan	Self reported	40-69	13	5	Male Female	6.7% 3.0%	1.09 (0.79-1.5) 1.92 (1.06-3.47)	Age, study area, history of CVD and IHD, smoking, BMI, physical activity, alcohol, vegetable, coffee	9
Jun *et al*[57]	NCC	Korea	FBS (< 99 mg/dL *vs* ⩾ 126 mg/dL)	⩾ 35	Case 2.4/ control 6.5 h	2	Both		1.77 (0.57-5.45)	*H. pylori*, smoking, drinking, and education level/matched on age, sex, year and area of enrollment, and follow-up duration	9
Khan *et al*[28]	CS	Japan	Self reported	40-79	19	2	Male Female	7.5% 4.6%	0.72 (0.40-1.09) 0.26 (0.08-0.82)	Age, BMI, smoking, drinking	8
Rapp *et al*[15]	CS	Austria	FBS (4.2-5.2 mmol/ L *vs* 7.0 mmol/L)	> 19	14	1	Male Female	3.9% 3.0%	0.84 (0.38-1.87) -[3]	Age, smoking, occupation, BMI	8
Rousseau *et al*[56]	CC	Canada	Self reported	35-70		2	Male		1.0 (0.5-1.8)	Age, income, education, ethnicity, proxy status, BMI, smoking, beta-carotene, alcohol	7
Jee *et al*[26]	CS	South Korea	FBS (< 90 mg/dL *vs* ⩾ 126 mg/dL) or DM medication	30-95	10	1	Male Female	5.1% 4.5%	1.11 (1.04-1.2) 1.15 (0.99-1.34)	Age, age squared, smoking, alcohol	8
La Vecchia *et al*[25]	CC	Italy	Self reported	⩽ 75		Not mentioned	Male Female		0.6 (0.4-1.0) 0.7 (0.4-1.2)	Age	6
O'Mara *et al*[21]	CC	USA (only white)	Self reported	30-89		1	Male Female		0.91 (0.28-2.27)[2] 1.57 (0.48-3.94)	Age	4 (3)[5]

[1]Interval between diabetes mellitus (DM) and gastric cancer to be selected as population; [2]Calculated from table without adjustment by Fisher's exact test; [3]No estimate was reported due to less than 5 cases in DM patients; [4]Energy, fat, salt, vitamin, dietary fibers; [5]The first numbers are Newcastle-Ottawa Quality Assessment Scale (NOS) of original studies. The second numbers in brackets are downgraded NOS because we calculated new estimates without adjustment. Subgroup analysis were done according to the second numbers. CS: Cohort study; CC: Case control study; NCC: Nested case-control study; BMI: Body mass index; FBS: Fasting blood glucose; FHx: Family history; TC: Total cholesterol; CVD: Cardiovascular disease; IHD: Ischemic heart disease; ORLS: Oxford Record Linkage Study; *H. pylori*: *Helicobacter pylori*; COPD: Chronic obstructive pulmonary disease.

similar increased risks. In the sexual subgroup, only women exhibited statistical significance (RR = 1.24; 95%CI: 1.01-1.52).

Pooled estimates adjusted for well-known gastric cancer risk factors (cigarette smoking and *H. pylori* infection) were also significant. The pooled estimate adjusted for ciga-

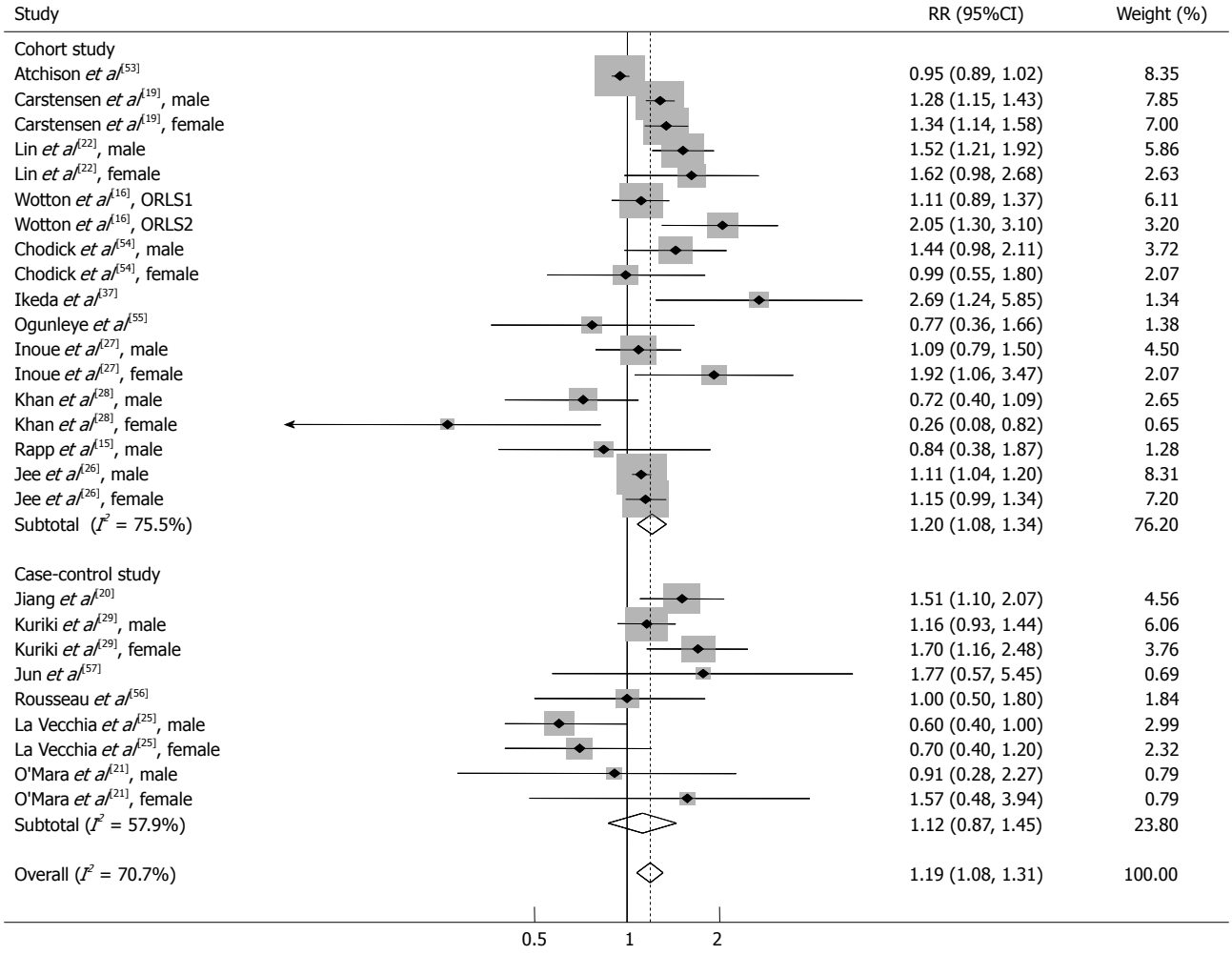

Figure 2 Meta-analysis and pooled relative risk of gastric cancer comparing non-cancer population with and without preexisting diabetes mellitus by random effect model. ORLS: Oxford Record Linkage Study.

rette smoking status was similar to the overall results (RR = 1.17; 95%CI: 1.01-1.34). The pooled estimate adjusted for *H. pylori* infection was greater than the overall estimate (RR = 2.35; 95%CI: 1.24-4.46). Nevertheless, subgroup analyses on either cardia or noncardia cancer did not produce significant results.

Publication bias

Begg's test indicated no publication bias in our analysis (Figure 3).

DISCUSSION

Summary of results

We found a significant association between preexisting diabetes and gastric cancer incidence. In diabetic patients, we found that the risk of subsequent gastric cancer incidence was increased by approximately 19%, and this effect was significant in women. The subgroups of cohort, Western and Eastern studies, showed similar results. These results persisted in studies using self-reported criteria and those adjusted for known risk factors such as cigarette smoking and *H. pylori* infection.

Explanations

We observed some discrepancy between our analysis and previous meta-analysis. Three previous meta-analysis have explored the association between diabetes and gastric cancer incidence. One of these, a meta-analysis of cohort studies[18], produced a misleading result due to the exclusion of appropriate cohort studies[16,19,54] and the inclusion of a study using a glucose level that was not clearly defined and an unrepresentative range of diabetes mellitus[40]. The study also presented an incomprehensible method for summarizing 2 estimates of cardia and noncardia cancer from the same control group. A different study[17] used the term "risk" without a distinction between incidence and mortality. This methodological weakness made the result heterogeneous and biologically implausible. Another meta-analysis[10] also excluded several recent studies[15,16,19,20] and included studies reporting a standardized incidence ratio without quality assessment.

To supplement the previous insufficient meta-analyses, we restricted inclusion in our analysis to studies reporting a risk ratio compared to a control group, and we assessed the quality of these studies using various criteria. As a result, we found that diabetes and the risk of gastric

Table 2 Preexisting diabetes and risk of gastric cancer by subgroup meta-analysis using random effect model

	Studies (*n*)	RR (95%CI)	I^2 % (95%CI)
Overall	27	1.19 (1.08-1.31)	70.7 (56.8-80.2)
Countries			
East Asian countries[1]	10	1.19 (1.02-1.38)	60.1 (20.0-80.1)
Western countries[2]	17	1.18 (1.03-1.36)	75.6 (61.0-84.8)
Study type			
Cohort study	18	1.20 (1.08-1.34)	75.5 (61.2-84.5)
Case control study	9	1.12 (0.87-1.45)	57.9 (11.8-79.9)
Sex			
Male	12	1.10 (0.97-1.24)	74.9 (55.7-85.8)
Female	9	1.24 (1.01-1.52)	58.9 (14.1-80.3)
DM criteria			
Registration	8	1.21 (1.02-1.44)	83.0 (67.8-91.0)
Measured[3]	5	1.15 (1.01-1.32)	35.5 (0.0-75.8)
Self report	14	1.13 (0.93-1.39)	65.2 (38.6-80.3)
Interval[4]			
One and more years	15	1.15 (1.02-1.31)	67.5 (44.3-81.0)
Quality (NOS)			
High quality (8 and more)	17	1.17 (1.05-1.31)	73.4 (57.0-83.6)
Low quality (7 and less)	10	1.20 (0.97-1.49)	61.2 (22.6-80.5)
Adjustment for risk factor			
Smoking[5]	12	1.17 (1.01-1.34)	52.6 (8.7-75.4)
H. pylori[5]	2	2.35 (1.24-4.46)	0.0 (UC)
Cancer site			
Cardia cancer	2	1.39 (0.72-2.69)	82.3 (UC)
Noncardia cancer	2	1.19 (0.80-1.77)	59.6 (UC)

[1]Japan, South Korea; [2]United States, Canada, Europe and Israel; [3]Using fasting blood glucose or HbA1c as criteria; [4]The least cancer-free time of subject according to study design; [5]The subgroup of studies designed to include the risk factor as adjustment variables. RR: Relative risk; UC: Unable to calculate due to low degree of freedom; NOS: Newcastle-Ottawa Quality Assessment Scale; *H. pylori*: *Helicobacter pylori*.

cancer were positively associated with each other. This positive association may be reliable based on consistent results from well-designed studies including cohort studies, studies of high quality based on assessment with the NOS, studies examining the time interval between onset of diabetes and subsequent gastric cancer, and studies using objective diabetic criteria (diabetes registry or laboratory measurements) that were adjusted based on important factors (smoking or *H. pylori* infection). Although a time interval of 1 year between diabetes and gastric cancer may be insufficient evidence of a causal relationship, we were able to minimize the reverse causality.

Regarding biological plausibility, the results of this study are consistent with previous studies in which preexisting diabetes was associated with an increased risk of a variety of cancers. The oncogenic properties of diabetes have been well documented. The increased insulin concentration in early diabetes can stimulate cell proliferation through activation of the insulin receptor or insulin-like growth factor-I receptor (IGF-IR) and the inhibition of IGF binding proteins, which may result in increased free and bioavailable IGF-I[58]. Additionally, a high level of serum IGF-I has been demonstrated to increase the risk for development of several carcinomas including gastrointestinal carcinomas[59]. Exogenous IGFs stimulate the proliferation of gastric cancer cells, while the blocking of

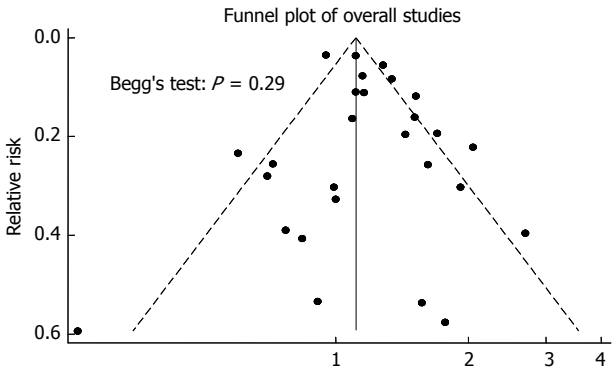

Figure 3 Funnel plots for publication bias.

IGF-IR inhibits tumor development[60,61].

Vascular endothelial growth factor (VEGF), the levels of which are increased in the blood of diabetic patients[62], is another cytokine that is related to the oncogenic properties of diabetes. VEGF is correlated with tumor vascularity and the frequency of hepatic metastasis, and it induces the proliferation and dilation of lymphatics culminating in node metastasis[63-65]. Moreover, the IGF/IGF-IR axis has also been shown to interact with the VEGF/VEGFR system in various tumors including gastrointestinal malignancies[66,67].

DNA damage in diabetic patients is another potential mechanism of oncogenesis. The increased production of reactive oxygen species could result in greater oxidative damage to DNA[68]. Furthermore, in an experimental study, high blood glucose levels were shown to directly induce DNA damage[69].

While we found that the association between diabetes and gastric cancer persisted irrespective of *H. pylori* infection, it nevertheless seems probable that diabetes and *H. pylori* infection, which is a known risk factor for gastric cancer, work synergistically to increase the risk of gastric cancer. Indeed, a previous cohort study in Japan demonstrated that a high HbA1c level and a concomitant *H. pylori* infection increased the gastric cancer risk synergistically[37]. It is possible that reactive oxygen-dependent DNA damage enhances the modifying effect of *H. pylori* on epithelial cell proliferation.

To confirm this synergistic effect, we also performed subgroup analyses by cancer site. Although noncardia gastric cancers were known to be strongly associated with *H. pylori* infection[70,71], we failed to show a similar relationship between diabetes mellitus and specific sites of gastric cancer. As further studies dealing with site-specific data are performed, a reassessment will be required.

Potential confounders

A positive association between diabetes and gastric cancer incidence was observed among women but not among men. These results must be interpreted carefully as there were only a few studies differentiating men and women. Sex hormones could represent one possible reason for this difference. In breast cancer, it is well established that

estrogen and IGF-1 interactively affect cell proliferation[72]. Gastrointestinal tissues, whether normal or cancerous, contain estrogen receptor β, and estrogen can bind to them[73,74]. Through an undefined mechanism, estrogen levels in diabetic women could influence or stimulate the proliferation of gastric cancer cells.

This gender-based difference could also be due to disparate methods of diabetes intervention. Metformin[46,75], aspirin[76,77], and statins[78] are known to protect against a number of cancers, while insulin is reported to increase the risk of several cancers[75]. Although gender differences in this matter were not explored in many previous studies, clues were obtained from the following studies: One study suggested that gender differences in adherence to diabetes management were minimal[79]. However, an Italian study reported that diabetic women were more likely to use insulin than men[80]. According to an American study, men use statins more frequently in type 2 diabetes mellitus[81]. These factors, however small, might contribute to the above-mentioned gender differences.

Limitations

Our study has several limitations. First, the studies we examined were highly heterogeneous. In particular, diabetes mellitus was defined by various methods and cut-off values. Diabetic prevalence and subject age also differed. Second, dietary food factors such as salt, nitrite[82-84], and fresh vegetable[85] intake could not be considered in the analysis despite being potential confounders. Finally, the selected studies contained no details regarding diabetes interventions that were sufficient to adjust for the effects of diabetes treatments.

In conclusion, despite the limitations, our meta-analysis suggests that preexisting diabetes mellitus may increase the risk of gastric cancer. Further prospective studies assessing confounders such as diabetes interventions are needed to specifically test the effects of diabetes mellitus on gastric cancer risk.

COMMENTS

Background

The burden of diabetes mellitus is increasing globally. Gastric cancer is the fourth most common cancer and the second leading cause of cancer-related mortality worldwide. Although the association between diabetes mellitus and a variety of cancers is well established, the association between diabetes mellitus and gastric cancer is currently unclear.

Research frontiers

Over the past 3 decades, many studies have been performed to understand the associations between preexisting diabetes mellitus and subsequent gastric cancer incidence. Moreover, several systematic reviews were recently performed to investigate these associations. However, these reviews were methodologically insufficient and thus could not achieve a comprehensive conclusion.

Innovations and breakthroughs

Based on this meta-analysis, preexisting diabetes mellitus may increase the risk of gastric cancer by approximately 19%. Similar associations were indicated in subgroup analyses of East Asian, Western, cohort, and high-quality studies. These findings were not presented clearly in previous systematic reviews.

Applications

Diabetes mellitus appears to be either directly or indirectly associated with the risk of gastric cancer. An exploration of the mechanism for this association may help them to reduce the gastric cancer risk.

Terminology

Insulin-like growth factors (IGFs) are proteins similar to insulin. IGF is a part of the cellular communication system. Recently, IGFs were proposed to play a role in aging. Vascular endothelial growth factor (VEGF) is a protein related to angiogenesis. In diabetes mellitus, the level of VEGF in the blood is high.

Peer review

This is a well-performed meta-analysis of currently available studies on the potential role of diabetes mellitus in the development of gastric cancer. The authors found that pre-existing diabetes mellitus may increase the risk of gastric cancer by approximately 19%. This finding was found to be unrelated to geographical region. This is a good meta-analysis and the authors have included many relevant issues missed by other research groups.

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CASE REPORT

Dubin-Johnson syndrome coinciding with colon cancer and atherosclerosis

Eva Sticova, Milan Elleder, Helena Hulkova, Ondrej Luksan, Martin Sauer, Irena Wunschova-Moudra, Jan Novotny, Milan Jirsa

Eva Sticova, Ondrej Luksan, Milan Jirsa, Center for Experimental Medicine, Institute for Clinical and Experimental Medicine, 14021 Prague 4, Czech Republic
Eva Sticova, Third Faculty of Medicine, Charles University, 10000 Prague 10, Czech Republic
Milan Elleder, Helena Hulkova, Institute of Inherited Metabolic Disorders, First Faculty of Medicine, Charles University and VFN, 12808 Prague 2, Czech Republic
Martin Sauer, Department of Surgery IPVZ, Masaryk Hospital, 40113 Usti nad Labem, Czech Republic
Irena Wunschova-Moudra, Department of Thoracic Surgery, Masaryk Hospital, 40113 Usti nad Labem, Czech Republic
Jan Novotny, Gastroenterology Department, Masaryk Hospital, 40113 Usti nad Labem, Czech Republic
Author contributions: Sticova E and Jirsa M analyzed the mutations, the pathological data and were involved in writing and editing the manuscript; Elleder M and Hulkova H performed histochemical, immunohistological and ultrastructural analysis; Luksan O participated in mutation analysis; Sauer M, Wunschova-Moudra I and Novotny J were involved in collecting the clinical and laboratory data.
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Correspondence to: Eva Sticova, MD, Center for Experimental Medicine, Institute for Clinical and Experimental Medicine, Videnska 1958/9, 14021 Prague 4,
Czech Republic. eva.sticova@ikem.cz
Telephone: +420-236-055229 Fax: +420-241-721666
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Abstract

Hyperbilirubinemia has been presumed to prevent the process of atherogenesis and cancerogenesis mainly by decreasing oxidative stress. Dubin-Johnson syndrome is a rare, autosomal recessive, inherited disorder characterized by biphasic, predominantly conjugated hyperbilirubinemia with no progression to end-stage liver disease. The molecular basis in Dubin-Johnson syndrome is absence or deficiency of human canalicular multispecific organic anion transporter MRP2/cMOAT caused by homozygous or compound heterozygous mutation(s) in *ABCC2* located on chromosome 10q24. Clinical onset of the syndrome is most often seen in the late teens or early adulthood. In this report, we describe a case of previously unrecognized Dubin-Johnson syndrome caused by two novel pathogenic mutations (c.2360_2366delCCCTGTC and c.3258+1G>A), coinciding with cholestatic liver disease in an 82-year-old male patient. The patient, suffering from advanced atherosclerosis with serious involvement of coronary arteries, developed colorectal cancer with nodal metastases. The subsequent findings do not support the protective role of Dubin-Johnson type hyperbilirubinemia.

Key words: Dubin-Johnson syndrome; *ABCC2*; Hyperbilirubinemia; Oxidative stress; Atherosclerosis; Cancer

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INTRODUCTION

Recent data have indicated the potent antioxidant properties of mild or moderately elevated serum bilirubin levels with substantial positive clinical consequences and especially their protective effect on atherogenesis and cancerogenesis[1]. A direct link between low serum bilirubin levels and peripheral vascular disease and the protective

effect of mild or moderate unconjugated hyperbilirubinemia on atherosclerosis were confirmed in numerous clinical trials[2-5]. Antiproliferative, cytostatic and pro-apoptotic effects of bilirubin are well-known and have been demonstrated in both *in vitro* and *in vivo* studies[6-10]. However, conflicting data on the possible protective effect of elevated bilirubin levels against sporadic colorectal carcinoma have been reported in the literature[11-13].

Dubin-Johnson syndrome (DJS, OMIM 237500) is a rare, autosomal recessive disorder characterized by non-hemolytic hyperbilirubinemia with no progression to end-stage liver disease[14]. Both the conjugated and unconjugated form of bilirubin can be elevated in DJS subjects, with the former ranging from 17% to 88% of the total bilirubin with a mean value of 60%[14,15]. In addition to the fluctuating jaundice caused by biphasic hyperbilirubinemia due to absence or deficiency of human canalicular multispecific organic anion transporter MRP2/cMOAT[16-18], DJS subjects may suffer from non-specific symptoms such as weakness and abdominal discomfort. Urinary coproporphyrin output is normal; however, 80% of the coproporphyrin fraction is represented by coproporhyrin I (normally 25%)[19]. The biliary excretion of anionic dyes including bromosulfophthalein, indocyanine green and cholescintigraphy radiotracers is delayed. Liver histology in DJS shows an accumulation of distinctive melanin-like lysosomal pigment in an otherwise normal liver, which gives the organ a characteristic dark pink or even black colour[14,20].

In this report, we describe the case of unintentionally detected DJS complicating liver injury in an 82-year-old patient who was being followed for previously diagnosed colorectal cancer and ischemic heart disease.

CASE REPORT

An 82-year-old Caucasian male patient, a normotensive non-smoker with a history of right-sided hemicolectomy due to colorectal adenocarcinoma with nodal metastases five years ago and ischemic heart disease, was referred to the hospital due to clinical jaundice with elevated serum bilirubin (total bilirubin 220 μmol/L, direct bilirubin 171 μmol/L), gamma-glutamyltransferase (γGT) activity (4.7 μkat/L) and fluctuating alkaline phosphatase (ALP) activity (1.3-3.6 μkat/L) to exclude bile duct obstruction or metastatic liver disease. The patient complained of slight upper abdominal discomfort without pruritus or nausea. Complete blood count and serum electrolyte levels were normal, as was his serum lipid profile (serum cholesterol 3.5 mmol/L, high-density lipoprotein cholesterol 0.9 mmol/L, low-density lipoprotein cholesterol 2.5 mmol/L, triglycerides 1.3 mmol/L). Abdominal ultrasonography, endoscopic retrograde cholangiopancreatography and magnetic resonance cholangiopancreatography did not detect any obstacle or dilation of the biliary tree, and only slightly imperfect filling of the ductus choledochus was observed. Serological examinations for viral hepatitis (hepatitis A virus, hepatitis B virus and hepatitis C virus) and autoantibodies were completely negative. During the patient's hospitalization, the activity of γGT and ALP gradually decreased but, due to persistent marked hyperbilirubinemia, a percutaneous liver biopsy was performed.

The liver tissue obtained at biopsy was standardly processed. Sections cut at 4-6 μm were stained with hematoxylin and eosin, periodic acid Schiff, Schmorl's and van Gieson's method. Analysis of the liver biopsy specimen revealed preserved architecture of liver parenchyma with perivenular and perisinusoidal fibrosis, subtle steatosis and only discrete intracellular cholestasis in centrilobular hepatocytes. The most striking feature, however, was an intense parenchymal pigmentation, with centrilobular and midzonal accentuation, consisting of coarse brown black pigment (Figure 1, Panel A-C). Special stainings (Gömöri, silver ammonium complex-Masson's and Perls Prussian blue method) for pigment characterization were added. The pigment was negative in the Perls reaction and displayed rudimentary autofluorescence with gradually increasing intensity, especially in Shandon mounting medium and simultaneously reduced Masson's solution. A retrospective analysis of the patient's medical records back to 1994 revealed long-term, persistent, mixed, predominantly conjugated hyperbilirubinemia (total bilirubin fluctuating within the range 23.0-215.6 μmol/L, conjugated bilirubin 17.7-170 μmol/L). The conjugated-to-total bilirubin ratio was within the range of 40% (total bilirubin 202 μmol/L and conjugated bilirubin 80.6 μmol/L in 2009) to 89% (total bilirubin 41.8 μmol/L and conjugated bilirubin 37.2 μmol/L in 1994). Taking into account the normal activity of aminotransferases, γGT and ALP, DJS was suspected.

For immunohistochemical analysis, 4 to 6 μm-thick sections were incubated with the anti-MRP2 mouse monoclonal antibody (clone M2Ⅲ-6, Kamiya, Seattle, WA). The EnVision Peroxidase Kit (Dako, Glostrup, Denmark) was used for visualization and counterstaining with Harris's hematoxylin was performed. As a positive control, sections of an adult liver without cholestasis were stained, and liver sections incubated without primary antibody were used as negative controls. Immunohistochemical analysis for MRP2 protein was completely negative (Figure 1D).

Ultrastructural analysis was performed on the liver sample fixed with 4% buffered paraformaldehyde, osmicated, dehydrated in ascending ethanol solutions and embedded into Epon-Araldite mixture. Ultrathin sections were double-stained with uranyl acetate and lead nitrate and then examined under a JEM 1200 EX electron microscope. Intralysosomal localization of the pigment was demonstrated.

A mutation analysis of the *ABCC2* gene was indicated to confirm the diagnosis of DJS at the molecular level. Written informed consent was obtained from the patient before the genetic investigation. *ABCC2* was analyzed by direct sequencing of genomic DNA extracted from peripheral leucocytes. All 32 exons, with the adjacent parts of the intronic sequences, were amplified by polymerase chain reaction (PCR) using the intronic oligonucleotide primers listed in Table 1. Amplified fragments were gel-purified, extracted from the gel with QIA quick spin col-

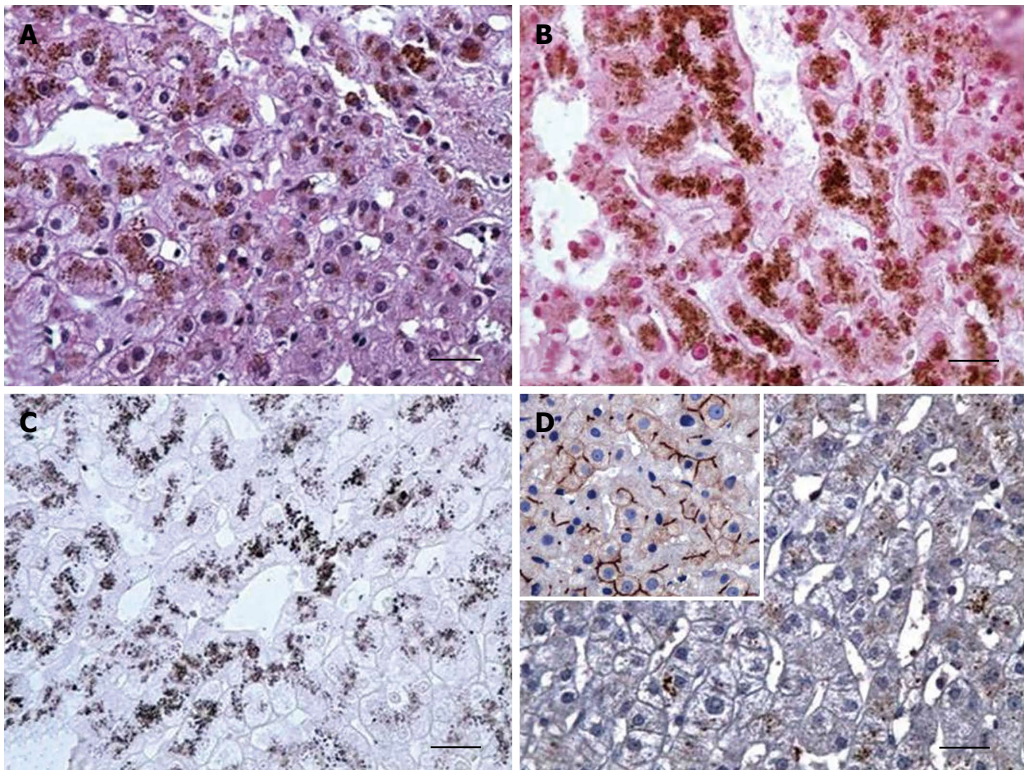

Figure 1 Histochemistry and immunohistology of the liver in Dubin-Johnson syndrome patient. A: Accumulation of distinctive dark brown pigment in hepatocytes was detected in hematoxylin and eosin staining; B: The pigment was negative in Perls reaction; C: The pigment reduced Masson's solution; D: Immunohistochemical analysis for ABCC2/MRP2 protein was negative compared to the positive control (inset). Original magnification ×400 (bars correspond to 100 μm).

Table 1 Primer pairs used for amplification of *ABCC2/MRP2* gene exons and promoters

Pair No.	Sequences of polymerase chain reaction primer pairs used to amplify the *ABCC2/MRP2* gene from genomic DNA		Exon No.
	Forward primer	Reverse primer	
1	5=-TTTACAATGCCTGGCAAAGG-3=	5=-CAGCATGATTCCTGGACTGC-3=	Promoter
2	5=-TCCCACATTCTGGATTTTGAC-3=	5=-ATAAAATAGCTTATCCAGTGTGAGC-3=	Promoter
3	5=-GGTCAGACCAATTTACATTTCCATC-3=	5=-CATAACCACCCATGCAGTATCC-3=	Promoter
4	5=-TACTTTGGGAACTGGTGAGTCT-3=	5=-AGAAGGCAATTTTGCGACTA-3=	1
5	5=-CACAAATAGGAAAATACGGATA-3=	5=-CCTGGGACAGCTGCTTA-3=	2
6	5=-CTGAATCACTGCATACCGCTTTT-3=	5=-CCAACCAAGCTTTGCCTCAC-3=	3
7	5=-CTGAATCACTGCATACCGCTTTT-3=	5=-AATTCGATCCTGGAGCTCAAC-3=	3_4
8	5=-TCATAGTAAATGGCATCAAGT-3=	5=-GGTGGAACATGAGCTTGAGT-3=	5_6
9	5=-AGTGGTGGAGATAGCCTC-3=	5=-GCTATAAAAATGTAAGGACA-3=	7
10	5=-GCCAGGGAGAGATGATCAAA-3=	5=-GGCCAGTCAACATTAAGTG-3=	8_9
11	5=-TGGAGCACATCCTTCCATTG-3=	5=-TTGCCCAAACTCCCATTAAG-3=	10
12	5=-TCACTGGGCACCTCAAGTTC-3=	5=-AGCAGGAATCCATCACCTCT-3=	11
13	5=-ATTTTGGGGACTATATCT-3=	5=-GATGTGATAGCCAGTCATTC-3=	12_13
14	5=-GTTCGGTGGAGATTAGGAG-3=	5=-TCTTATGCAAGCATAGGCTC-3=	14
15	5=-TTCACCTCCTGTTAGCGTA-3=	5=-ACCGAAGACATGCACATAGC-3=	15
16	5=-TTCACCTCCTGTTAGCGTA-3=	5=-CAAGACCTCACCTACTAGCC-3=	15_16
17	5=-ACAAGCACGTGAATACATATCAG-3=	5=-ACCCCTGTGTAGTTCTT-3=	17
18	5=-GTAAGATTTTTAACCCCTTG-3=	5=-GCCCAGGCATAGAGTTTC-3=	18_19
19	5=-GTATGGAGTATTTATGGAGT-3=	5=-TGTAAGTATGCGTTCAAT-3=	20_21
20	5=-GTGGTTGGCATTCTAGGT-3=	5=-CATAATAATTCCTCCCTATCA-3=	22_23
21	5=-CTGGGAACACACAGAATCCAAC-3=	5=-GGCTCCTGGGTATGTCAACA-3=	24
22	5=-GGCTTTTGTCTTGTTCAGACG-3=	5=-CTTGGTAAACGGCAGA-3=	25
23	5=-CCCGATCAAGTCAAAC-3=	5=-GGCATTCATGTCTACTTAGGA-3=	26
24	5=-GGAGGCAAGGATTGTC-3=	5=-TCTGCATACTGTGGACCTTAT-3=	27_28
25	5=-ACAGCTGCCAAGAGAGTCCAT-3=	5=-GCTCAAGTATCCCGAGTAGA-3=	29
26	5=-CCTTGCGGAAGCTCAACC-3=	5=-TGCCAGGCATCACCTAACACG-3=	30
27	5=-GTTTTGAAAGTCTGATCTG-3=	5=-AGGAAGTACGATCGAGGTA-3=	31_32

umns (Qiagene, Hilden, Germany), and sequenced on an ABI 3130 Genetic Analyzer (Applied Biosystems, Foster

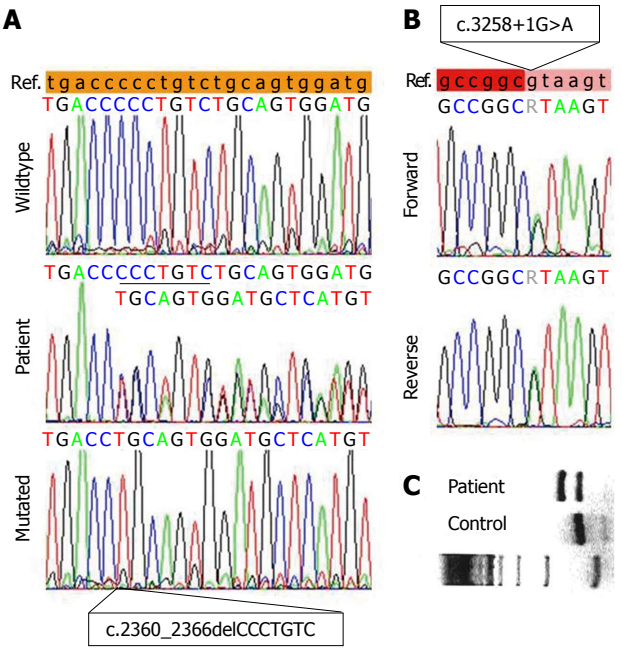

Figure 2 Mutation analysis results. A: Presence of a heterozygous deletion c.2360_2366delCCCTGTC in exon 18 of *ABCC2* (panel A, middle lane) was confirmed by sequence analysis of individual alleles separated by cloning (wildtype allele - panel A, upper lane; mutated allele - panel A, lower lane); B: In addition, a heterozygous splice site mutation c.3258+1G>A was detected in intron 23; C: Presence of the latter mutation was confirmed by polymerase chain reaction-Bsh1236I restriction fragment length polymorphism analysis.

City, CA). The obtained sequence was compared with the reference sequences GenBank NM_000392 (mRNA) and NT_030059 (genomic DNA). An exon with suspected deletion was cloned into a pCR4.1-TOPO plasmid vector (Invitrogen, Carlsbad, CA), and the wild-type and mutated alleles were sequenced separately. Presence of the second mutation was confirmed by PCR-restriction fragment length polymorphism analysis (PCR-Bsh1236I RFLP).

Analysis of the *ABCC2*/*MRP2* gene disclosed two novel mutations: a heterozygous deletion c.2360_2366delCCCTGTC in protein coding exon 18 (Figure 2A), and a heterozygous mutation c.3258+1G>A in intron 23 (Figure 2B and C). The former mutation in exon 18 was predicted to cause a reading frame shift and premature termination of DNA translation at position 803 with protein alteration p.Pro787LeufsX7. The latter mutation affecting the donor splice site of intron 23 was predicted to cause abnormal splicing of mRNA. Therefore, both mutations were considered as pathogenic. Histochemistry and mutation analysis accordingly established the diagnosis of DJS.

The patient suddenly died several months after being released from the hospital. The patient's autopsy revealed no liver metastases and confirmed the diagnosis of advanced atherosclerosis with serious involvement of coronary arteries with signs of chronic myocardial ischemia.

DISCUSSION

The diagnosis of DJS is indicated by the presence of fluctuating predominantly conjugated hyperbilirubinemia, an increased ratio of urinary coproporphyrin I to coproporphyrin Ⅲ, and a unique cholescintigram showing delayed visualization of the liver and biliary tract. Neither a percutaneous liver biopsy with evidence of melanin-like pigment and the absence of MRP2 protein nor mutation analysis are necessary for the diagnosis. Nonetheless, both methods may be helpful in complicated cases to establish the correct diagnosis and rule out more serious liver pathology.

In our patient, neither an examination of urinary coproporphyrin levels nor cholescintigraphy was performed. The diagnosis was based on biochemical evidence of fluctuating biphasic hyperbilirubinemia and characteristic findings in the liver biopsy, and further supported by disclosure of two pathogenic mutations by subsequent mutational analysis. Considering the clinical picture and the results of laboratory tests, the two mutations are supposed to be in transposition. Unfortunately, no living relatives of the patient were found to confirm our presumption.

As the clinical onset of DJS is most often seen during the teenage years or in early adulthood, the first recognition of the disease in old age is highly unusual. To the best of our knowledge, our patient is the oldest reported newly diagnosed DJS patient confirmed by mutation analysis.

The clinical presentation and laboratory findings of our case indicated the coincidence of DJS with mild cholestatic liver damage, the etiology of which remained obscure. The simultaneous occurrence of the syndrome with another hepatobiliary disease is well-known and the coincidence with another disease or pathologic stimulus can modify the clinical picture and results of laboratory tests, including histomorphology[21-24].

A significant fact in our case is the presence of colorectal adenocarcinoma with nodal metastases in a patient with chronic mixed hyperbilirubinemia. Numerous clinical trials have demonstrated the protective effects of hyperbilirubinemia against the development of sporadic colorectal cancer and an association of low bilirubin levels with an increased risk of colon cancer morbidity and mortality[10-12].

In addition, there are reports describing the protective effect of bilirubin on the development of atherosclerosis emphasizing the antioxidative and anti-inflammatory properties of bilirubin[2-5]. Interestingly, our patient, a lifelong normotensive non-smoker with normal lipidogram, suffered from ischemic heart disease and advanced atherosclerosis affecting both large arteries and peripheral circulation, especially coronary arteries.

In conclusion, our case demonstrates that DJS should be included in the differential diagnosis of liver diseases even in atypical age categories. The fact that our patient with DJS developed colorectal adenocarcinoma and clinically significant atherosclerosis indicates that Dubin-Johnson hyperbilirubinemia may not be sufficient to protect from atherogenesis and cancer development, even in the absence of established risk factors.

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CASE REPORT

Successful balloon-occluded retrograde transvenous obliteration for bleeding duodenal varices using cyanoacrylate

Rintaro Hashimoto, Keitaro Sofue, Yoshito Takeuchi, Kentaro Shibamoto, Yasuaki Arai

Rintaro Hashimoto, Keitaro Sofue, Yoshito Takeuchi, Kentaro Shibamoto, Yasuaki Arai, Department of Diagnostic Radiology, National Cancer Center Central Hospital, Tokyo 104-0045, Japan
Author contributions: Hashimoto R wrote the manuscript and performed additional data analysis; Sofue K wrote the manuscript and revised it critically for important intellectual content; Takeuchi Y and Shibamoto K performed procedures; and Arai Y helped by final approval of the version to be published.
Correspondence to: Keitaro Sofue, MD, Department of Diagnostic Radiology, National Cancer Center Hospital, 5-1-1, Tsukiji, Chuo-ku, Tokyo 104-0045, Japan. keitarosofue@yahoo.co.jp
Telephone: +81-3-35422511 Fax: +81-3-35475989
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Abstract

A 76-year-old woman with hepatitis C cirrhosis presented with tarry stools and hematemesis. An endoscopy demonstrated bleeding duodenal varices in the second portion of the duodenum. Contrast-enhanced computed tomography revealed markedly tortuous varices around the wall in the duodenum. Several afferent veins appeared to have developed, and the right ovarian vein draining into the inferior vena cava was detected as an efferent vein. Balloon-occluded retrograde transvenous obliteration (BRTO) of the varices using cyanoacrylate was successfully performed in combination with the temporary occlusion of the portal vein. Although no previous publications have used cyanoacrylate as an embolic agent for BRTO to control bleeding duodenal varices, this strategy can be considered as an alternative procedure to conventional BRTO using ethanolamine oleate when numerous afferent vessels that cannot be embolized are present.

Key words: Bleeding duodenal varices; Balloon-occluded retrograde transvenous obliteration; Cyanoacrylate; Combination therapy; Temporary portal vein occlusion

Hashimoto R, Sofue K, Takeuchi Y, Shibamoto K, Arai Y. Successful balloon-occluded retrograde transvenous obliteration for bleeding duodenal varices using cyanoacrylate. *World J Gastroenterol* 2013; 19(6): 951-954 Available from: URL: http://www.wjgnet.com/1007-9327/full/v19/i6/951.htm DOI: http://dx.doi.org/10.3748/wjg.v19.i6.951

INTRODUCTION

Bleeding duodenal varices is a rare complication in patients with portal hypertension, occurring in only 0.4% of these patients, and is often life-threatening because of the difficulty in diagnosis and treatment[1]. Treatment options include a surgical procedure, endoscopic treatment[2], and endovascular treatment, including transjugular intrahepatic portosystemic shunts (TIPS)[3,4] and balloon-occluded retrograde transvenous obliteration (BRTO)[5-10]. Although several studies have reported successful results using BRTO alone[5-8], some difficult cases with large varices or numerous collaterals requiring a combined approach have been reported[7,9], and no previous publications have used cyanoacrylate as an embolic agent for BRTO to control bleeding duodenal varices. We herein report a case with bleeding duodenal varices that were successfully embolized using cyanoacrylate and BRTO in combination with temporary occlusion of the portal vein.

CASE REPORT

A 76-year-old woman with liver cirrhosis secondary to hepatitis C presented with tarry stools and hematemesis. An urgent endoscopy demonstrated bleeding varices in the second portion of the duodenum (Figure 1A). She had no esophageal or gastric varices. Although banding

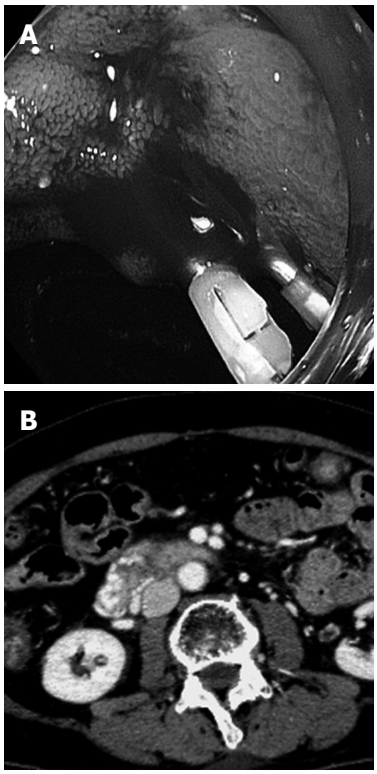

Figure 1 Endoscopy and computed tomography of the duodenum. A: Endoscopy demonstrates bleeding varices in the second portion of the duodenum; B: Contrast-enhanced computed tomography reveals markedly tortuous varices around the wall in the second and third portion of the duodenum.

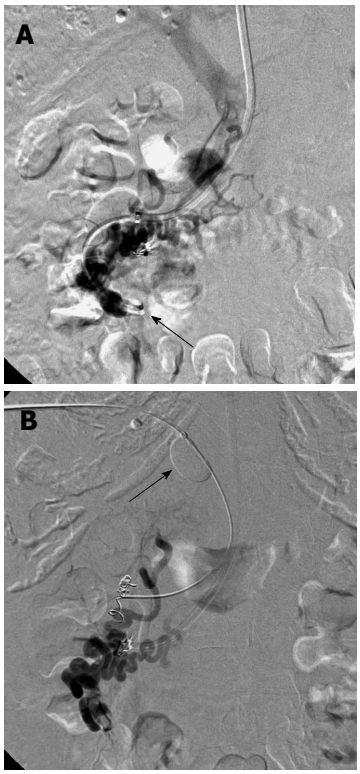

Figure 2 Balloon-occluded retrograde venography. A: Balloon-occluded retrograde venography (BRTV) shows the dilated efferent vein and the duodenal varices, but the contrast material quickly disappears through several afferent veins. Note that the balloon was inflated in the right ovarian vein (arrow); B: BRTV with occlusion of the main portal trunk (arrow) after embolization of one of the afferent veins reveals the complete opacification of the duodenal varices.

and clipping for the varices was attempted, the bleeding continued and frequent blood transfusions were required. Laboratory findings were as follows: red blood cell, 232 $\times 10^4/\mu L$; hemoglobin, 6.8 g/dL; hematocrit, 20.4%; platelets, 96 000/mL; total bilirubin, 1.36 mg/dL; serum albumin, 3.3 g/dL; and prothrombin time, 15.7 s (reference, 11.3 s). Neither ascites nor encephalopathy was observed. Child-Pugh's classification was graded as B. Contrast-enhanced computed tomography (CT) revealed markedly tortuous varices around the wall in the second and third portion of the duodenum (Figure 1B). Several afferent veins of the varices appeared to have developed, and the right ovarian vein draining into the inferior vena cava was detected as an efferent vein. We planned BRTO to embolize the duodenal varices after obtaining informed consent from the patient.

An 8-French guiding sheath introducer was inserted into the inferior vena cava *via* the right internal jugular vein. A 5.2-French, 9-mm cobra-shaped balloon catheter was inserted into the efferent vein through the right ovarian vein, and the balloon was inflated to occlude the efferent vein. Balloon-occluded retrograde venography (BRTV) showed that the dilated efferent vein and the duodenal varices were filled with contrast material, but the contrast material quickly disappeared through several afferent veins (Figure 2A). Because BRTO alone may have failed to achieve adequate sclerosant accumulation because of the leakage into the portal vein, antegrade transhepatic embolization of the afferent veins was attempted.

A 5-French sheath introducer was inserted through the left lateral portal branch, and one of the afferent veins was embolized using two microcoils (MicroNester coil; Cook, Inc, Bloomington, Indiana, United States). However, several remaining afferent veins could not be embolized, and the contrast material also disappeared through the afferent veins. We then placed a balloon catheter into the main portal trunk to control the hepatopetal flow of the afferent veins. BRTV with occlusion of the main portal trunk revealed the disappearance of the hepatopetal portal flow and complete opacification of the duodenal varices (Figure 2B). A microcatheter was coaxially advanced to the duodenal varices through the retrograde route, and a total of 4 mL of 20% cyanoacrylate with ethiodized oil was injected into the duodenal varices (Figure 3).

The following day, a contrast-enhanced CT examination confirmed the complete accumulation of the ethiodized oil replacement in the duodenal varices (Figure 4) and the patency of either portal vein or systemic circulation. Liver function was preserved after the procedure. Four days after the procedure, an endoscopy showed that hemostasis of the bleeding duodenal varices had been achieved. No evidence of bleeding of the duodenal varices was found on follow-up CT and endoscopy examinations performed four months after the procedure.

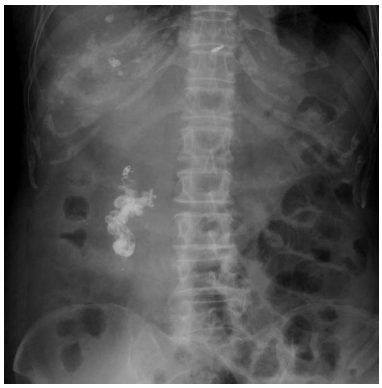

Figure 3 Radiograph after embolization of the duodenal varices demonstrates complete and adequate accumulation of the ethiodized oil in the varices.

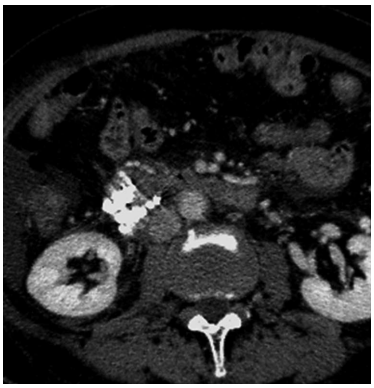

Figure 4 Contrast-enhanced computed tomography after embolization of the duodenal varices shows the complete accumulation of ethiodized oil in the varices.

DISCUSSION

BRTO is an established endovascular treatment for gastric varices[11] but has only been described for the treatment of bleeding duodenal varices in a few reports with limited numbers of clinical patients[5-10]. The advantages of BRTO over TIPS for duodenal varices are that it can completely embolize targeted varices and that it does not reduce portal flow, avoiding further exacerbation of hepatic function and encephalopathy without a significant mortality rate[4,8]. However unlike gastric varices, successful treatment with BRTO alone for duodenal varices is not always feasible and often require combined therapies with an endoscopic or anterograde transhepatic approach, as significant communications or complex hemodynamics between the efferent and afferent veins often complicate treatment and necessitate combined therapy[7,9].

In the present case, some of the afferent veins may have enabled collateral hepatopetal flow during balloon occlusion of the afferent vein, and pressure among the duodenal varices varied, resulting in insufficient filling with the contrast material. At first, coil embolization was attempted *via* a transhepatic portal venous approach, as reported by previous investigators[5,7-9], but not all the afferent veins could be embolized because of the difficulty in catheterizing the tortuous vessels. Second, we performed temporary balloon occlusion of the portal vein. This method was effective because a change in the hemodynamics of the duodenal varices occurred. Temporary occlusion of the main portal trunk may increase the pressure of hepatopetal flow, and the direction of flow in the afferent veins changes from hepatopetal to hepatofugal. This mechanism is similar to that of temporary balloon occlusion of the splenic artery during BRTO for gastric varices to control the portal pressure gradient[12].

In our case, we used cyanoacrylate, not ethanolamine oleate, as a sclerosant. Every investigator has used ethanolamine oleate as the most suitable sclerosant during BRTO for duodenal varices[5-10]. However, ethanolamine oleate was not suitable in our case, because it requires several hours to achieve full effort and may increase the risk of portal venous thrombosis under temporary portal venous balloon occlusion. On the other hand, cyanoacrylate rapidly solidifies with fast polymerization upon exposure to an ionic solution[13], and we believe that this was the best way of minimizing the duration of portal venous occlusion. The potential shortcomings of cyanoacrylate are adhesion to the balloon catheter system or inadvertent embolization upon balloon removal. This should be kept in mind as a note of caution whenever attempting to use cyanoacrylate. To prevent this complication, it would be advantageous to ensure that a microcatheter is advanced to the targeted duodenal varices and only duodenal varices are embolized, with minimal volume of cyanoacrylate.

Endoscopic injection sclerotherapy using cyanoacrylate has been performed as an effective measure[2], but it has the drawback of perforation, tissue injury, and unclear visualization because of massive hemorrhage. Moreover, endoscopic injection of cyanoacrylate also carries a risk of embolism of either portal vein or systemic circulation[14]. Endovascular injection of cyanoacrylate can prevent untargeted embolization such as portal vein or pulmonary artery, confirming the hemodynamics of the duodenal varices using contrast material.

Bleeding duodenal varices is a rare condition that is difficult to diagnose and is potentially life-threatening. BRTO using cyanoacrylate was successfully performed for control of bleeding duodenal varices in the present case. This is an alternative procedure to conventional BRTO using ethanolamine oleate when insufficient filling of the varices with sclerosant occurs and several afferent vessels cannot be adequately embolized.

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CASE REPORT

Rescue endoscopic band ligation of iatrogenic gastric perforations following failed endoclip closure

Joung-Ho Han, Tae Hoon Lee, Yunho Jung, Suck-Ho Lee, Hyun Kim, Hye-Suk Han, Heebok Chae, Seon Mee Park, Seijin Youn

Joung-Ho Han, Hyun Kim, Hye-Suk Han, Heebok Chae, Seon Mee Park, Seijin Youn, Division of Gastroenterology, Department of Internal medicine, Chungbuk National University College of Medicine, Cheongju 361-711, South Korea
Tae Hoon Lee, Yunho Jung, Suck-Ho Lee, Division of Gastroenterology, Department of Internal Medicine, Soon Chun Hyang University College of Medicine, Cheonan Hospital, Cheonan 330-721, South Korea
Author contributions: Han JH and Lee TH contributed equally to this work; Jung Y, Park SM, Kim H, Han HS, Chae H, Lee SH and Youn S provided clinical advice and analyzed the data; Han JH and Lee SH performed the procedures; Han JH and Lee TH designed case report; and Lee TH and Han JH wrote the paper.
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Correspondence to: Tae Hoon Lee, MD, PhD, Division of Gastroenterology, Department of Internal Medicine, Soon Chun Hyang University College of Medicine, Cheonan Hospital, 23-20 Bongmyung-dong, Cheonan-si, Cheonan 330-721, South Korea. thlee9@lycos.co.kr
Telephone: +82-41-5703662 Fax: +82-41-5745762
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Abstract

Iatrogenic gastric perforation is one of the most serious complications during therapeutic endoscopy, despite significant advances in endoscopic techniques and devices. This case study evaluated the clinical efficacy and safety of the rescue endoscopic band ligation (EBL) technique in iatrogenic gastric wall perforation following the failure of primary endoclip closure. Five patients were enrolled in this study. These patients underwent emergency endoscopy following the onset of acute gastric wall perforation during endoscopic procedures. The outcome measurements were primary technical success and immediate or delayed procedure-related complications. Successful endoscopic closure using band ligation was reported in all patients, with no complication occurring. We conclude that EBL may be a feasible and safe alternate technique for the management of acute gastric perforation, especially in cases where closure is difficult with endoclips.

Key words: Gastric perforation; Endoscopy; Band ligation

Han JH, Lee TH, Jung Y, Lee SH, Kim H, Han HS, Chae H, Park SM, Youn S. Rescue endoscopic band ligation of iatrogenic gastric perforations following failed endoclip closure. *World J Gastroenterol* 2013; 19(6): 955-959 Available from: URL: http://www.wjgnet.com/1007-9327/full/v19/i6/955.htm DOI: http://dx.doi.org/10.3748/wjg.v19.i6.955

INTRODUCTION

The management of gut perforation, including successful primary endoscopic repair, has been increasingly reported in endoscopic trials. Primary endoscopic therapy is now often recommended as an alternative to surgical intervention in select cases[1-3]. However, the commonly used endoclip technique for endoscopic closure has some limitations, depending on the perforation size and anatomic site. Endoscopic clip closure may be difficult for a large perforation, one with a tangential angle, and/or on a necrotic or ulcerated surface. Even when clipping is successful, dehiscence might occur due to strong wall tension. Newly developed devices have been introduced and show promise, but many of these devices are expensive, require additional equipment, and are not readily available in many countries[2,3].

Endoscopic band ligation (EBL) is commonly performed in the management of varix or Dieulafoy's bleed-

ing[4-6]. EBL is a safe and feasible method that can reduce procedure time and be useful in the primary repair of colonic or duodenal perforations[7-9]. Our group previously reported the use of EBL to successfully close two colonic perforations in which endoclip closure initially failed due to the presence of a large perforation and a severe tangential angle in endoscopic procedures assisted with a transparent cap[9].

In this case study, we evaluated the clinical efficacy and safety of the rescue EBL technique in iatrogenic gastric wall perforations in which primary endoclip closure failed.

CASE REPORT

Patients with acute gastric wall perforations were enrolled in this study between September 2011 and August 2012. Written informed consent was obtained from all patients and the study was approved by the institutional ethics committee.

All patients underwent emergency endoscopy following the onset of acute gastric wall perforation during endoscopic procedures. Primary endoscopic closure using an endoclip (Endoclip HX-600-090L; Olympus Optical Co., Ltd., Tokyo, Japan) was attempted initially, immediately upon recognition of the perforation. Patients in whom primary endoclip closure failed or was technically difficult subsequently underwent rescue EBL to achieve closure. After endoscopic confirmation of the perforation site, the endoscope was withdrawn and reinserted after attachment of a pneumoactive, single-band ligator (MD-48709; Akita Sumitomo Bakelite Co, Ltd, Tokyo, Japan). The hood of the ligation device was then placed over the target lesion site. Following successful ligation of the approximate targeted edge of the perforation, additional bands or clips were used to completely close the site (Figure 1). After endoscopic closure, patients' recovery was managed with vital sign monitoring, no oral intake, and intravenous fluid therapy with broad-spectrum intravenous antibiotics and acid suppression. Early oral intake was allowed when clinical symptoms such as abdominal pain or fever resolved, appetite and bowel function returned, and laboratory test values were normalized. Surgical intervention was planned if the patient's clinical condition deteriorated.

The outcome measurements were primary technical success and immediate or delayed procedure-related complications. All endoscopic procedures, including EBL, were performed by experienced faculty endoscopists in tertiary referral centers.

Table 1 presents the clinical characteristics of five patients who underwent rescue closure by EBL. Gut perforation rates following therapeutic endoscopic procedures were ranging from 0.9% to 1.8% in two participated units. Three patients underwent endoscopic mucosal resection with ligation (EMR-L) due to gastric adenoma (n = 2) or neuroendocrine tumor (n = 1); one patient received endoscopic submucosal dissection due to adenocarcinoma; and one patient underwent endoscopic biopsy due to a chronic gastric ulcer. The mean perforation size was 8.6 (range 5-11) mm. The primary causes of endoclip failure were difficulty in approximating the location of adjacent gastric mucosa due to wall tension and a tangential angle. In the case of ulcer base perforation occurred by biopsy (iatrogenic perforation following biopsy; Figure 2), the fibrotic ulcer base made endoscopic clipping difficult.

Table 1 Baseline patient characteristics

No.	Age (yr)/sex	Diagnosis	Cause	Perforation location	Size (mm)	Causes of clip failure
1	65/M	Neuroendocrine tumor	EMR-L	Fundus	9	Tangential angle and wall tension
2	52/M	Gastric adenoma	EMR-L	Angle	11	Severe belching
3	68/M	Gastric adenoma	EMR-L	Upper body, great curvature	8	Tangential angle
4	91/F	Gastric ulcer	Biopsy	Angle	5	Fibrotic tissue and tension
5	73/M	Adenocarcinoma	ESD	Antrum, great curvature	10	Friable mucosa

EMR-L: Endoscopic mucosal resection with ligation; ESD: Endoscopic submucosal dissection; M: Male; F: Female.

Table 2 Procedure outcomes of rescue endoscopic band ligation

No.	Procedure time for endoclips (s)	Procedure time for EBL (s)	No. of bands	No. of clips before/after EBL	Success	Days until diet resumption/discharge
1	198	66	1	3/3	Yes	3/7
2	102	42	2	2/1	Yes	4/5
3	138	78	1	0/4	Yes	2/4
4	894	334	2	6/0	Yes	7/14
5	353	39	1	1/2	Yes	4/7

EBL: Endoscopic band ligation.

The rescue EBL technique was performed successfully in all patients (Table 2). The mean procedure time for complete band ligation was 111.8 (range 39-334) s. Two band ligations were performed in the cases of large and ulcer base perforations. Additional endoclips were applied in four cases to achieve complete closure. No procedure-related complication or delayed dehiscence occurred following successful EBL with or without clipping. Patients resumed their normally scheduled diet an average of 4 (range 2-7) d after the procedure, and were discharged an average of 7.4 (range 4-14) d after the procedure.

DISCUSSION

Iatrogenic gut perforations occurring during endoscopic procedures are generally managed surgically. Although surgical operation remains the gold standard treatment for intestinal perforation, the clinician's familiarity with endoclips and their immediate availability and proper use

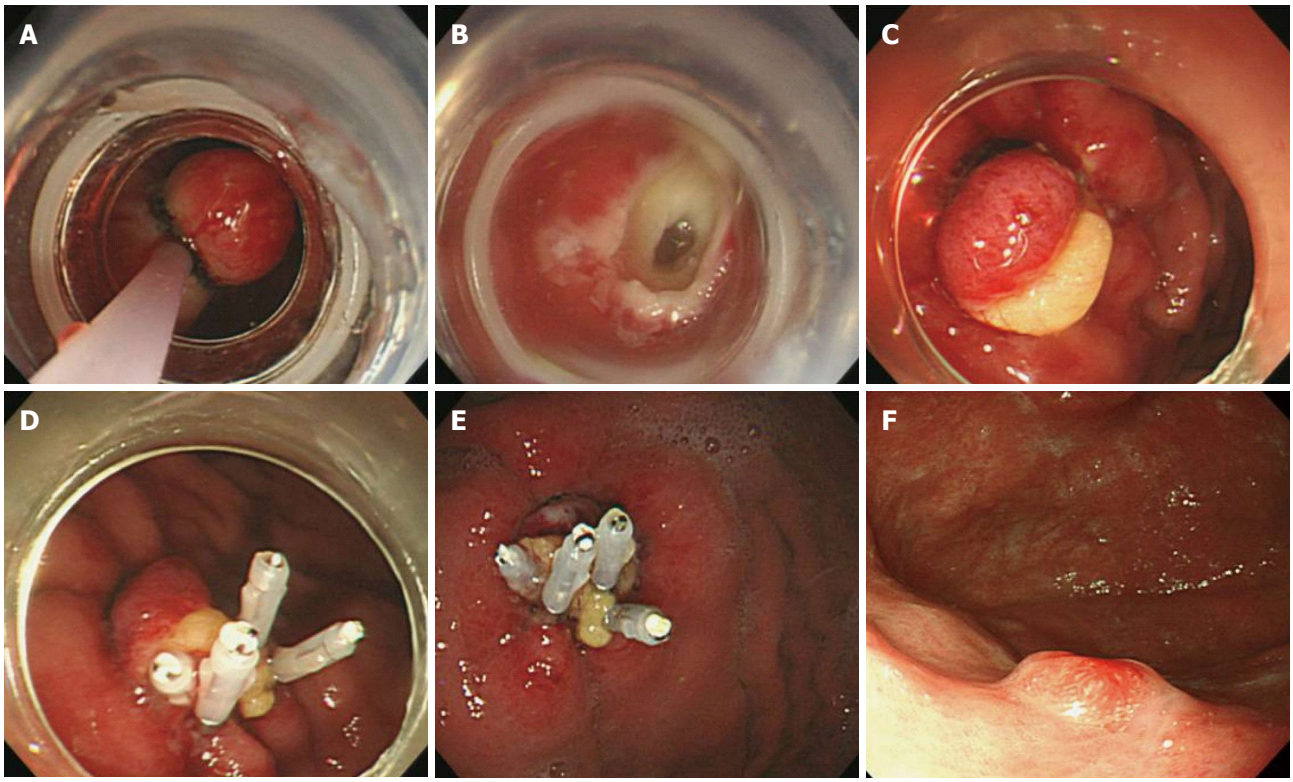

Figure 1 Endoscopic band ligation in iatrogenic gastric wall perforation. A: Endoscopic view of endoscopic mucosal resection with ligation (EMR-L) due to gastric adenoma on the greater curvature of the upper gastric body; B: Iatrogenic gastric wall perforation following EMR-L; C: Primary endoscopic band ligation (EBL) was successful following technical difficulty with endoclip closure; D: Additional clips were applied around the band and surrounding mucosa; E: Follow-up endoscopy 1 d later shows band and multiple clips, with no complication; F: Endoscopic view 1 mo after EBL, showing the absence of the band and clips.

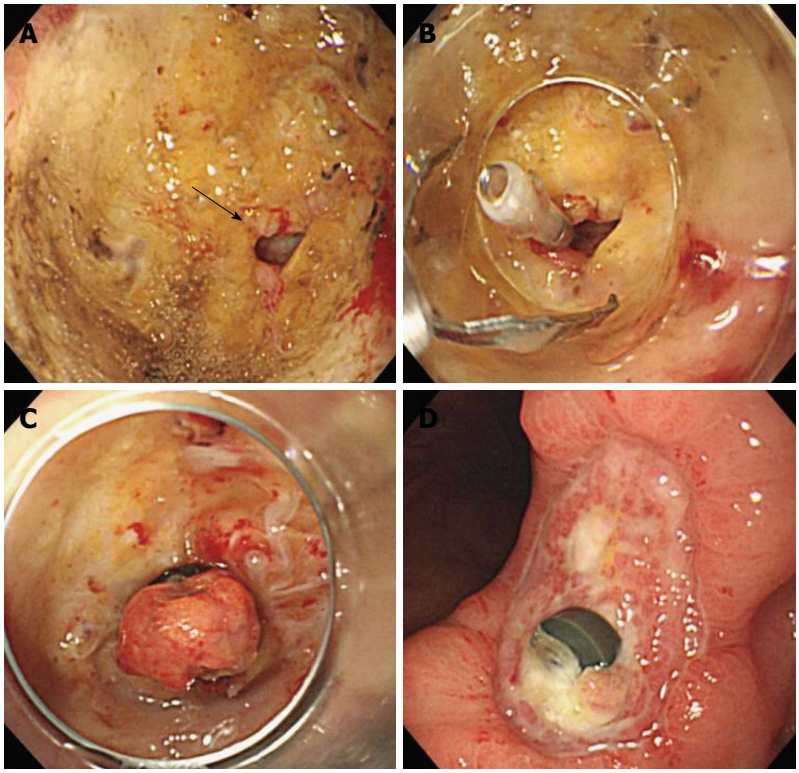

Figure 2 Endoscopic band ligation in iatrogenic ulcer base perforation following endoscopic biopsy. A: Iatrogenic gastric ulcer base perforation following biopsy (arrow); B: The fibrotic ulcer base made endoscopic clipping difficult; C: Successful endoscopic band ligation following technical difficulty with endoclip closure; D: Endoscopic view 2 wk later, showing the healed base of ulcer with remaining bands.

may replace surgery for a selected group of patients with a high surgical risk. The mainstays of treatment of early perforations without systemic upset are nasogastric suction, antibiotics, bowel rest, and parenteral nutrition. This type of conservative management may be undertaken in patients with asymptomatic perforations or localized

peritonitis that is expected to improve clinically without complication[1,10-14]. In presented cases, perforation was recognized immediately during procedure, and treated by endoscopic band and clipping successfully. So that, did not use nasogastic suction. However, in patients whose condition deteriorates despite conservative management or alternative endoscopic management, surgical treatment should be considered immediately.

Recent studies have reported high technical success rates for primary closure of an acute iatrogenic perforation with endoclips, newly developed devices, or a band[1,10-12]. Endoscopic repair using endoclips can be limited in large perforations or in those with tangential angles. A wide perforation is difficult to close because of slippage of the perforation edge from the clip while the clip is maneuvered across the defect to grasp the opposite edge of the perforation. Everted perforation edges also make it impossible to grasp the tissue with endoclips. Newly developed devices have recently been introduced and successfully used to close perforations[15]. These devices include through-the-scope (TTS) clips, such as the QuickClip 2 (Olympus Inc., Center Valley, PA, United States), the Resolution clip (Boston Scientific Inc., Natick, MA, United States), and the Tri-Clip and Instinct clip (Cook Medical, Winston-Salem, NC, United States); the over-the-scope clip (OTSC) system (Ovesco Endoscopy AG, Tubingen, Germany); and endoscopic suturing devices such as T-tags (Ethicon Endo-Surgery, Cincinnati, OH, United States) and the flexible Endo Stitch (Covidien, Mansfield, MA, United States). Closure of luminal perforation > 20 mm in size may be difficult. For larger gastric defects, TTS clips can be placed around the circumference of the perforation and lassoed together with a detachable plastic snare (Endo-loop; Olympus)[16]. Among the newly developed devices, OTSC was approved for the closure of perforation < 20 mm in size, and ex vivo studies shown that colon defects 10 to 30 mm in size can be closed with a single OTSC[2]. However, although some techniques have been developed to correct deficits in clip placement, they are not commonly practiced. Some of these devices may prove suitable for the closure of defects throughout the intestinal tract, but their use is limited by the endoscopist's experience, device availability, and cost[2]. Currently, no particular technique has demonstrated proven efficacy or greater reliability than other closure modalities.

EBL was first used in 1988 to treat bleeding from esophageal varices[15]. The simplicity of the technique and low complication rates compared with sclerotherapy have contributed to its growing popularity[4]. Technically, EBL is a simple procedure, even if the targeted site must be approached tangentially or is located in the posterior wall of the proximal body. EBL has been widely used in the management of non-variceal hemorrhage from Dieulafoy's ulcer, gastric angiodysplasia, and polypectomy-induced bleeding[4-6]. In addition, several reports have described the use of EBL in rectal and duodenal perforations during EMR[7-9]. Our group previously reported the use of EBL to successfully close two colonic perforations when endoscopic closure with endoclips initially failed[9]. Primary endoscopic repair can be difficult in some cases because the clips may not hold the tissue of large perforations together successfully, the tissue may slip in perforations with everted edges, or a fibrotic ulcer base may hinder successful manipulation. In contrast, acute perforations with no hardening can be readily closed with suction and band ligation.

Theoretically, EBL can readily approximate both edges of the perforation. Thus, complete suture to the remaining wall by additional bands or endoclips may be simple, even with a large perforation. EBL can also reduce procedure time in comparison with clipping. Immediate closure could prevent the need for surgery or the development of serious peritonitis caused by gastric content leakage. Additionally, in the case of ulcer base perforation, clipping of the fibrotic membrane is difficult due to strong wall tension, which may result in tearing or dehiscence after closure. EBL with suction could reduce such damage throughout the lesion. No dehiscence occurred after EBL in our cases. Finally, the use of additional clips to suture the perforation after EBL might not be necessary. Follow-up endoscopy has detected clip deterioration, with only the band holding the perforated mucosa tightly. Prudent banding may thus be important for successful closure, and one or more band ligations may be sufficient. However, due to the limited number of cases included in our study, we could not confirm that additional clipping is unnecessary, and we generally recommend additional clipping to maintain tight closure of the primary band.

Several factors can be identified as limitations in our study. Our case study was not comparative and included a limited number of patients, preventing us from drawing concrete conclusions. Also, this result cannot be generalized due to an experimental trial. Additionally, we did not evaluate closure dehiscence or ischemic necrosis following band ligation. Although ischemic necrosis induced by band ligation is limited to the mucosa or submucosa, further prospective studies should be conducted to examine whether the perforation risk may be lower with conventional sclerosant injection therapy, heater probe therapy, or electrocoagulation[4,17]. Secondary risks may also develop due to suction of the adjacent mesentery. None of these complications was observed in our study. In summary, EBL can be used as a rescue method to repair full-thickness perforations and may facilitate complete repair by enabling the approximation of the perforation when initial band ligation is not sufficient to achieve complete closure. EBL may be a feasible and safe alternate technique for the management of acute gastric perforation, especially in cases where closure with endoclips is difficult due to tangential angles, severe belching, or narrow space availability. To evaluate the suitability of EBL for wide clinical use, comparative controlled studies should be conducted.

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CASE REPORT

Misdiagnosis of left supraclavicular lymph node metastasis of hepatocellular carcinoma: A case report

Tao Liu, Jun-Fang Gao, Yong-Xiang Yi, Hai Ding, Wei Liu

Tao Liu, Yong-Xiang Yi, Hai Ding, Department of Hepatobiliary Surgery, the Second Affiliated Hospital of Southeast University, Nanjing 210003, Jiangsu Province, China
Jun-Fang Gao, Department of Hepatic Surgery and Liver Transplantation Centre, the First Affiliated Hospital of Nanjing Medical University, Nanjing 210029, Jiangsu Province, China
Wei Liu, Department of Pathology, the Second Affiliated Hospital of Southeast University, Nanjing 210003, Jiangsu Province, China
Author contributions: Liu T conceptualized the case report and wrote the paper as a major contributor; Gao JF, Yi YX and Ding H were involved in drafting the article and revising it for intellectual content; Liu W carried out the pathological analysis; and all authors read and approved the final manuscript.
Correspondence to: Tao Liu, MD, Department of Hepatobiliary Surgery, the Second Affiliated Hospital of Southeast University, Nanjing 210003, Jiangsu Province, China. lt9821@163.com
Telephone: +86-25-83626393 Fax: +86-25-83626227
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Abstract

Left supraclavicular lymph node metastasis is a rare presentation of hepatocellular carcinoma (HCC). This phenomenon is easily neglected in the clinic. A 56-year-old man presented with HCC. On examination, a 1cm long left supraclavicular lymph node was palpated. Auxiliary examination indicated a lesion located in the right lobe of the liver. Fine needle aspiration cytology (FNAC) of the enlarged lymph node was performed; however, only necrosis was found. Hepatectomy was performed and HCC was confirmed by Hematoxylin-Eosin staining. However, 14 d after surgery, significantly enlarged left supraclavicular lymph nodes, a new intrahepatic lesion, and pulmonary and mediastinal metastasis appeared. An excisional biopsy of the left supraclavicular lymph node was performed, and its findings confirmed metastatic HCC. The patient's HCC rapidly progressed and he died one month later. It is possible for HCC to metastasize to the left supraclavicular lymph node. Surgeons should always consider an overall physical examination. When left supraclavicular lymphadenopathy of unknown origin is encountered, FNAC should be performed initially. If the results are negative, an excisional biopsy and subsequent Positron emission tomography - computed tomography scanning should be performed. These are very important for making the correct diagnosis and for selecting reasonable therapies.

Key words: Left supraclavicular lymph node; Metastasis; Hepatocellular carcinoma; Fine needle aspiration cytology; Misdiagnosis

Liu T, Gao JF, Yi YX, Ding H, Liu W. Misdiagnosis of left supraclavicular lymph node metastasis of hepatocellular carcinoma: A case report. *World J Gastroenterol* 2013; 19(6): 960-963 Available from: URL: http://www.wjgnet.com/1007-9327/full/v19/i6/960.htm DOI: http://dx.doi.org/10.3748/wjg.v19.i6.960

INTRODUCTION

Hepatocellular carcinoma (HCC) is a common malignancy of the digestive tract. The main route of spread is hematogenous, that is, by invasion of the portal venous system or hepatic venous system[1]. Lymphatic metastases are uncommon. For patients with HCC larger than 5 cm, tumor-related factors predict outcomes and survival[2]. Left supraclavicular lymphadenopathy may be the sign of a metastatic tumor, mostly from lung cancer, gastric cancer, nasopharyngeal cancer and breast cancer[3]. However, HCC rarely metastasizes to the left supraclavicular lymph node and few relevant reports are available in the literature. Here, we present a case of HCC that manifested as left supraclavicular lymphadenopathy and analyze the reasons for the initial misdiagnosis.

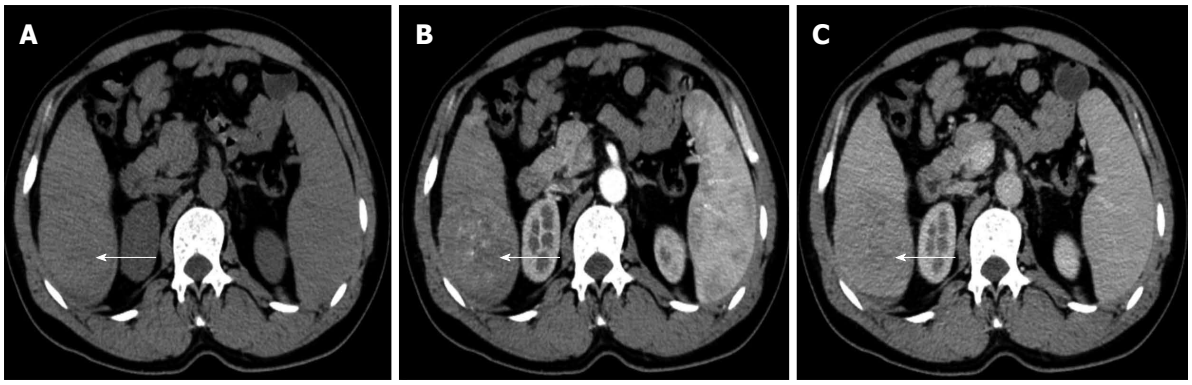

Figure 1 Abdominal computed tomographic images before surgery. A: Non-contrast-enhanced computed tomography scan showing the lesion (arrow) located in the right lobe of the liver; B: The lesion (arrow) showed enhancement during the hepatic arterial phase; C: The lesion (arrow) showed no enhancement during the portal phase.

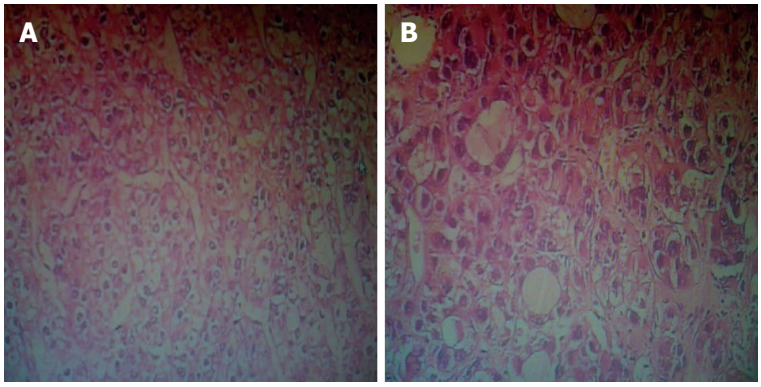

Figure 2 Histopathology of the primary tumor and enlarged left supraclavicular lymph node. A: Cancer cells having oval and single nuclei, with less mitosis and less heteromorphosis in the primary lesion; hematoxylin and eosin (HE) stain, ×400; B: The same changes in cell morphology in left supraclavicular lymph node; HE stain, ×400.

CASE REPORT

A 56-year-old man suffering from HCC was referred to our hospital. His chief complaint was abdominal pain. He had hypertension that was successfully treated with oral medication. On examination, a 1 cm long left supraclavicular lymph node was palpated and it was slightly tender. Examination of the chest did not reveal any signs of bronchospasm. Varicose veins on the thoracic and abdominal wall were not observed. Neurological examination did not demonstrate any deficit. Laboratory examination revealed a platelet count of 89×10^9/L. His alpha-fetoprotein (AFP) level was 695.4 ng/mL (normal < 10.9 ng/mL). Serum hepatitis B surface antigen was positive. HBV DNA quantification was within normal limits. Renal and liver function tests, coagulation function, electronic laryngoscopy and gastroscopy were also normal. Abdominal computed tomography indicated an 8 cm lesion located in the right lobe of the liver (VI segment) (Figure 1A), which was showed significant enhancement during the hepatic arterial phase (Figure 1B), but no enhancement during the portal phase (Figure 1C). The vascular system was normal, and no enlarged lymph nodes were found. No new lesions were found by contrast-enhanced ultrasound. A chest X-ray revealed no lung metastasis. Fine needle aspiration cytology (FNAC) of the enlarged left supraclavicular lymph node showed only necrosis. A hepatectomy was performed without hepatic hilar occlusion. Doppler ultrasound was used to define the tumor margin during the operation. The dissection line was 1 cm outside the tumor margin. Diagnosis was confirmed as HCC with moderate differentiation by Hematoxylin-Eosin staining (Figure 2A), and the resection margin was negative. Initially, the patient showed a good recovery. However, 14 d after surgery, enlarged left supraclavicular lymph nodes were observed to be firm and fixed. To exclude the possibility of malignant lymphoma, an excisional biopsy of the left supraclavicular lymph node was performed, and its findings confirmed metastatic HCC (Figure 2B). Thoracic computed tomography showed significantly enlarged left supraclavicular lymph nodes (Figure 3A), part of which were fused into masses (Figure 3B). Their diameters ranged from 2 cm to 6 cm. Enlarged lymph nodes appeared in mediastinum (Figure 3C) and round nodules were distributed in both lungs (Figure 3D and E). A new lesion was detected in the liver *via* Doppler ultrasound (Figure 3F). Laboratory examination revealed an AFP level > 1050 ng/mL. The patient rapidly declined and died one month later.

DISCUSSION

Distant metastasis of HCC occurs in three main ways: Hematogenous dissemination, lymphatic metastasis and implantation metastasis. Hematogenous dissemination is common, whereas lymphatic metastasis is uncommon. Lymphatic metastasis has been documented in 25.5% of patients at different stages of HCC. Hilar lymph nodes

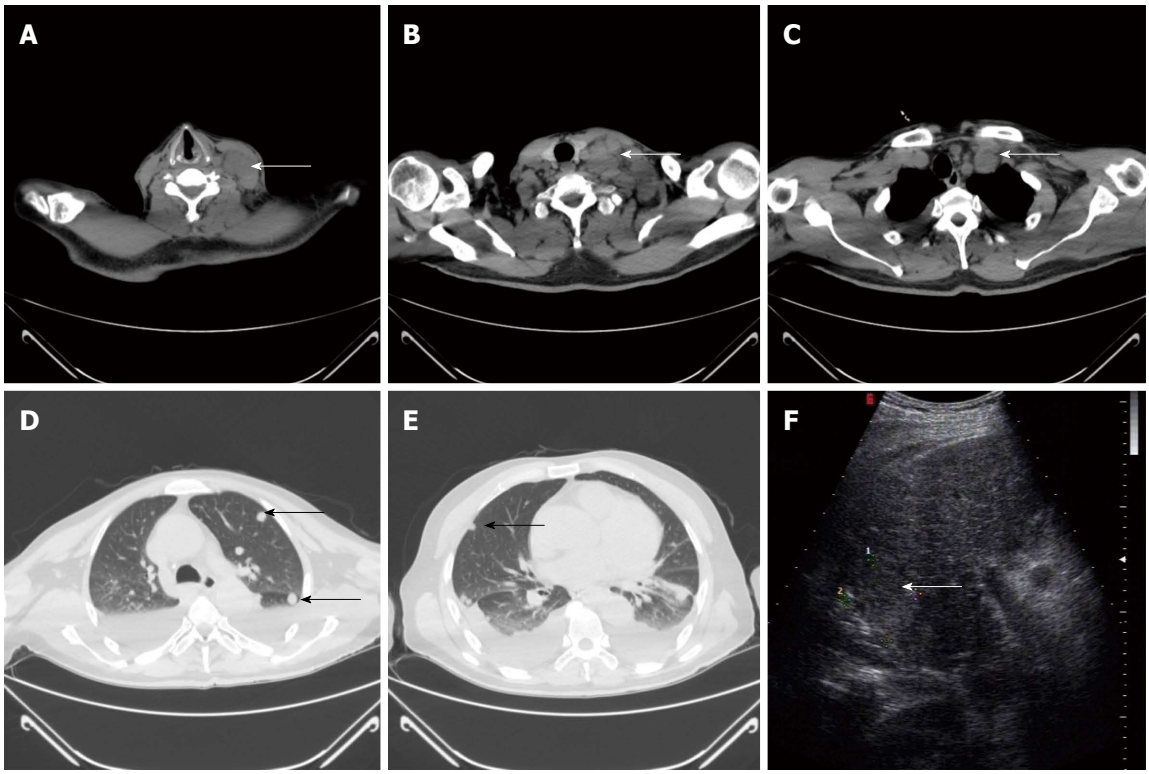

Figure 3 Thoracic computed tomographic and Doppler images after surgery. A: Non-contrast-enhanced computed tomography scan showing enlarged left supraclavicular lymph nodes (arrow), which were easily observed; B: Part of enlarged left supraclavicular lymph nodes (arrow) fused into the mass; C: Enlarged lymph nodes in the mediastinum (arrow) was observed; D and E: Round nodules (arrows) distributed in both lungs; F: A new intrahepatic lesion (arrow) was detected in the non-operating region *via* Doppler ultrasound.

and para-aortic nodes are the major metastatic sites[4]. Nowadays, a hepatectomy has become one of the most popular treatments for HCC, and understanding lymph node metastasis in HCC is indispensable at surgery for improving the patient's prognosis. A previous study revealed that the incidence of lymph node metastasis in operable HCC patients was low, but patients with lymph node metastasis had a poorer prognosis[5].

HCC metastasis to left supraclavicular lymph nodes, although uncommon, does occur, probably *via* the hepatic node and then through the thoracic duct[6]. Early discovery of distant metastasis is important for tumor staging, prognosis judgment and therapy determination in those patients with HCC. However, left supraclavicular lymphadenopathy is not the initial presentation in most conditions; therefore, overall physical examination, especially of the supraclavicular fossa, should not be overlooked. When the left supraclavicular lymph node is involved in the tumor, patients already have advanced disease. In this situation, it is more appropriate for patients to adopt non-operative treatment, including hepatic arterial chemoembolization, molecular targeted therapy, chemotherapy and radiotherapy, instead of surgery. For palpable left supraclavicular lymph nodes, FNAC is not only useful for diagnosing various lesions, but also can help in deciding on appropriate management[7]. However, the diagnosis may sometimes be difficult, with the potential for clinically important diagnostic errors. Therefore, FNAC can also show false negative results for positive lesions[8]. It is presumed that multiple factors can result in misdiagnosis[9], including inadequate specimens, poor smears, inaccurate puncture location, and lack of ancillary studies (cell blocks, immunocytochemistry and electron microscopy)[10].

For a patient with left supraclavicular lymphadenopathy who is highly suspected to have a metastatic tumor, FNAC can be used as a first line diagnostic modality in the evaluation of enlarged lymph nodes. If negative findings are obtained, an excisional biopsy is necessary, which is the gold standard for diagnosis, and Positron emission tomography - computed tomography (PET-CT) scanning should be performed, which allows identification of the primary site and metastatic lesions, including bone and soft tissue metastases, in a single examination.

In our case, an enlarged left supraclavicular lymph node was noticed preoperatively. The negative findings of FNAC and no further examination resulted in incorrect tumor staging. The disease progressed rapidly after surgery. Metastatic HCC was not confirmed until an excisional biopsy was performed.

In summary, we have presented an uncommon case of advanced HCC with left supraclavicular lymph node metastasis. When similar cases are encountered, FNAC should be performed initially. When the results of FNAC, are negative, further tests, such as an excisional biopsy and PET-CT scanning will help to achieve a definitive diagnosis. Such diagnoses will help to prioritize the patient's management protocol.

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CASE REPORT

Severe hepatic necrosis of unknown causes following ABO-incompatible liver transplantation

Hao Lu, Chuan-Yong Zhang, Wei Ding, Yun-Jie Lu, Guo-Qiang Li, Feng Zhang, Ling Lu

Hao Lu, Chuan-Yong Zhang, Wei Ding, Yun-Jie Lu, Guo-Qiang Li, Feng Zhang, Ling Lu, Liver Transplantation Center, First Affiliated Hospital of Nanjing Medical University, Nanjing 210029, Jiangsu Province, China

Author contributions: Lu H, Zhang CY and Ding W contributed equally to this work; Zhang F and Lu L designed the research; Ding W and Lu YJ analyzed the data; Zhang CY, Li GQ and Zhang F performed the operation; and Lu H and Ding W wrote the paper.

Correspondence to: Ling Lu, MD, PhD, Liver Transplantation Center, First Affiliated Hospital of Nanjing Medical University, Nanjing 210029, Jiangsu Province, China. lvling@njmu.edu.cn
Telephone: +86-25-83672106 Fax: +86-25-83672106
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Abstract

Emergency ABO-incompatible (ABO-I) liver transplantations (LTx) have been performed increasingly to treat severe liver failure. Herein, we report a case of severe hepatic necrosis after ABO-I LTx. A 53-year-old man with blood group O was diagnosed as having severe hepatitis B and acute-on-chronic liver failure, and underwent an emergency liver transplantation implanting a blood-group-B liver from a cardiac-death donor. A routine anti-rejection, anti-infection and anti-virus therapy was given after operation. On post-operative day (POD) 16, the recipient had fever and erythra. Laboratory and radiographic examinations suggested a severe hepatic necrosis of unknown causes. The patient was managed with a 10-d methylprednisolone pulse therapy. He was discharged on POD 35 with stable condition, and no recurrent disease was found during the follow-up.

Key words: Liver transplantations; ABO-incompatible; Hepatic necrosis; Graft rejection; Pulse therapy

Lu H, Zhang CY, Ding W, Lu YJ, Li GQ, Zhang F, Lu L. Severe hepatic necrosis of unknown causes following ABO-incompatible liver transplantation. *World J Gastroenterol* 2013; 19(6): 964-967 Available from: URL: http://www.wjgnet.com/1007-9327/full/v19/i6/964.htm DOI: http://dx.doi.org/10.3748/wjg.v19.i6.964

INTRODUCTION

Acute-on-chronic liver failure (ACLF) has a poor prognosis and a high mortality. It may result in multiple syndromes, including jaundice, coagulopathy, ascites, and even multiple organ dysfunction syndrome induced by precipitating factors (*e.g.*, hepatotropic virus reactivation, infection/sepsis, and hepatotoxic drugs), and secondary compensated liver diseases (*e.g.*, chronic hepatitis and stable liver cirrhosis). ACLF is characterized by acute deterioration of hepatic function, which develops aggressively[1,2]. Given the long-term survival without liver transplantation (LTx) or self-recovery in ACLF subjects is very low, emergency LTx has been introduced as a first-line therapy to significantly improve the short- and long-term survival of the patients[3], in spite of the recently emerging debate on the benefit of artificial liver support to improve the short-term prognosis[4-7].

ABO blood group is considered as a barrier to LTx. Immune rejection, especially antibody-mediated rejection (AMR), is the main cause of graft loss in ABO-incompatible (ABO-I) LTx. Due to the presence of A/B antigen on the surface of vascular endothelium, bile duct epithelium and liver sinusoidal endothelial cells, an ABO-I graft might be attacked by the existing hemagglutinin, causing damage to the blood vessel and bile duct of the graft, characterized by liver parenchymal necrosis and biliary strictures. Other causes of graft loss include vascular or biliary complications, and recurrence of viral hepatitis.

Therapeutic advancement has improved the outcomes of ABO-I LTx and made a long-term survival possible[8-10]. In this report, we present a case of severe hepatic necrosis of unknown reasons after ABO-I LTx. However,

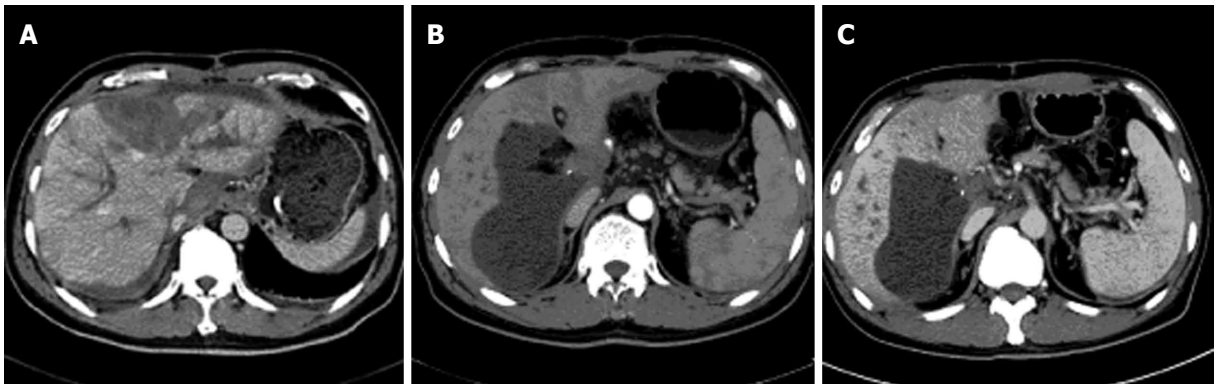

Figure 1 Computed tomography scan and computed tomography angiography images. A: Intrahepatic bile duct expansion and left lobe liver necrosis on post-operative day (POD) 19; B: A low-density cyst (6.1 cm × 13 cm) and a filling defect (2.1 cm × 1.9 cm) at portal area on POD 48; C: Computed tomography (CT) scan and CT angiography on POD 118 showed a smaller low-density cyst (5 cm × 10 cm) and a same-sized filling defect (2.1 cm × 1.9 cm) at portal area on POD 118 compared with earlier findings.

the patient was cured and discharged.

CASE REPORT

A 53-year-old male patient was transferred from the internal medical ward to the liver transplant unit after he was diagnosed as having severe hepatitis B and ACLF, and he then underwent an emergency ABO-I LTx. The liver graft was obtained from a cardiac-death donor with blood group B, which mismatched the recipient's blood group O. Cold ischemia time of the graft was 5 h. The modified piggy orthotopic liver transplantation was performed with a 60-min anhepatic phase and without splenectomy. Massive ascites (about 4000 mL), omentum and gallbladder edema, enlarged tough spleen and severe atrophic and cirrhotic liver were visualized after a right upper incision, confirming the diagnosis of ACLF. The operation was successful. Basiliximab 20 mg and methylprednisolone 1000 mg were administered for immune-induction and hepatitis B immunoglobulin (HB-Ig) 1000 U for prevention of hepatitis B virus (HBV) recurrence before anhepatic phase. A quadruple therapy was given to prevent rejection: (1) Rituximab 100 mg on post-operative day (POD) 1 and Basiliximab 20 mg on POD 4 for induction of immune tolerance; (2) Tacrolimus (Tac) at a dose adjusted according to a blood level of 10-15 ng/mL; (3) Methylprednisolone (MP) at a dose gradually reduced from 1000 mg *qd* to a maintenance of 10 mg *bid*; and (4) Mycophenolatemofetil, 0.5 g, *bid*. Other treatments included administration of antibiotics, antiviral therapy with lamivudine, proton pump inhibitor and liver-protective medication. Primary monitoring examinations included blood routine, liver function, coagulation function, HBV serum virology, serum drug level of Tac, B-ultrasound (B-US) and computed tomography (CT) scan. Pathological examination, lymphocyte subsets analysis and ImmuKnow Assay (T cell function test) results showed no rejection. On POD 16, the patient had fever and erythra. B-US displayed a sub-hepatic, pelvic and thoracic fluid sonolucent area, while CT scan showed abnormal left liver lobe infusion and slight intrahepatic bile duct dilatation (Figure 1). Both alanine aminotransferase (ALT) and aspartate aminotransferase (AST) were elevated (ALT 54.3 U/L and AST 26.5 U/L *vs* ALT 1204.2 U/L and AST 1555.3 U/L at POD 12 and 16, respectively). All these results indicated a severe hepatic necrosis. After a 10-d MP pulse therapy, the patient's symptoms were relieved and aminotransferase was reduced. The patient was discharged on POD 35, and was followed up once a month. The patient is doing well without any recurrent disease.

DISCUSSION

The initial clinical impression of severe hepatic necrosis on POD 16 was rejection. AMR is triggered by alloantibody binding and compliment activation. In the case of ABO-I solid organ transplantation, A, B or AB blood group antigens expressed on graft could be detected by specific natural antibodies. Incompatible blood group antigens activated B cells directly with the help of T cells and resulted in transcription of cytokines and antibodies[11]. AMR can be caused by performed antibodies, anamnestic B-cell responses, early *de novo* and late *de novo* B-cell responses. The strength of isohemagglutinins and natural antibodies in ABO-I recipients can be detected and quantified by multiple methods. Antibody titer is reported to correlate with the survival of recipients. ABO-I living donor liver transplantation with high preoperative and postoperative immunoglobulin (Ig) G and IgM levels sustained bile duct complication and hepatic necrosis, respectively[12], while the safe value of isohemagglutinin titer is $\leqslant$ 1:16 for kidney transplantation[13,14] and $\leqslant$ 1:4 for pediatric heart transplantation[15,16]. In this case, on POD 16, the recipient run a fever, and had poor liver function, and CT scan showed intrahepatic bile duct dilatation and liver necrosis in the left lobe (Figure 1A). But lymphocyte subset analysis indicated a slight increase of T cells and a drop of B cells (CD3+ 77.9%, CD3+CD4+ 49.7%, CD3+CD8+ 23.9%, CD3-CD16+56 14.4% and CD19+ 1%), which was contradictory to AMR with an enhanced B cell function. Meanwhile, ImmuKnow Assay (T cell function tests) before LTx and on POD 34 and

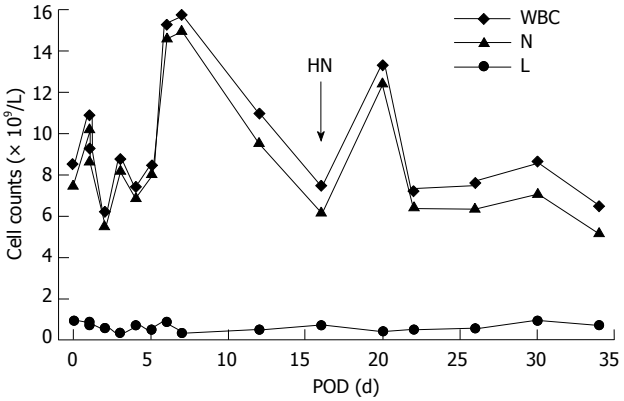

Figure 2 Blood routine test results after ABO-incompatibleliver transplantation. Due to treatment with tacrolimus and methylprednisolone, the number of lymphocyte stayed stable before and after the occurrence of severe hepatic necrosis, while the total number of white blood cell counts was mainly affected by neutrophil changes, which might be induced by non-specific reasons, *e.g.*, phagocytose and absorption of necrotic tissues. LTx: Liver transplantation; POD: Post-operative day; HN: Hepatic necrosis; WBC: White blood cell; L: Lymphocyte; N: Neutrophil.

90 were 156.7 ng/mL, 101.7 ng/mL and 70.3 ng/mL, respectively, suggesting a depression of T cell function after LTx resulting from immunosuppressive therapy[17,18]. The total number of white blood cell was mainly affected by neutrophils (Figure 2). Levels of IgG, IgA, IgM, C3 and C4 were normal or almost normal before LTx and on POD 118 (18.1 g/L, 3.45 g/L, 0.94 g/L, 0.20 g/L and 0.0159 g/L *vs* 7.1 g/L, 1.38 g/L, 0.51 g/L, 1.07 g/L and 0.305 g/L, respectively). Therefore, rejection was not considered as the cause of hepatic necrosis initially.

Another factor leading to severe hepatic necrosis after LTx was recurrent diseases. To prevent recurrent hepatitis B, HB-Ig and/or lamivudine was administered. A 10-year follow-up study in 24 individuals with chronic hepatitis B demonstrated the effectiveness of HB-Ig and/or lamivudine[19]. Nineteen patients were alive after the follow-up duration with well-functioning graft. None of them developed recurrent hepatitis B. Among them, 12 patients received HB-Ig and lamivudine, 3 received lamivudine only, 1 received adefovir, and 3 received no antivirals. HbsAg and HbcAb were both positive in the serum virology test in this patient before LTx. Antiviral treatments included HB-Ig 1000 U injection during LTx and lamivudine *p.o.* after operation. Post-LTx HBV examination results were all negative. Pathological examination of the explanted liver showed classic necrotic changes, without hepatocellular carcinoma. Taken together, there was no recurrent hepatitis or carcinoma till the last follow-up in this case, although CT scan showed dilation of bile duct and inflammation of left liver lobe (Figure 1A), which could result from recurrent viral hepatitis[20].

Blood supply disorders and biliary complications could also lead to hepatic necrosis after LTx. CT scan and CT angiography (CTA) also suggested normal hemodynamics on POD 3, but a low-density cystic echo (6.1 cm × 13 cm) and a filling defect (2.1 cm × 1.9 cm) at the portal area (Figure 1B). However, as shown in Figure 3A, total bilirubin and creatinine levels turned normal on

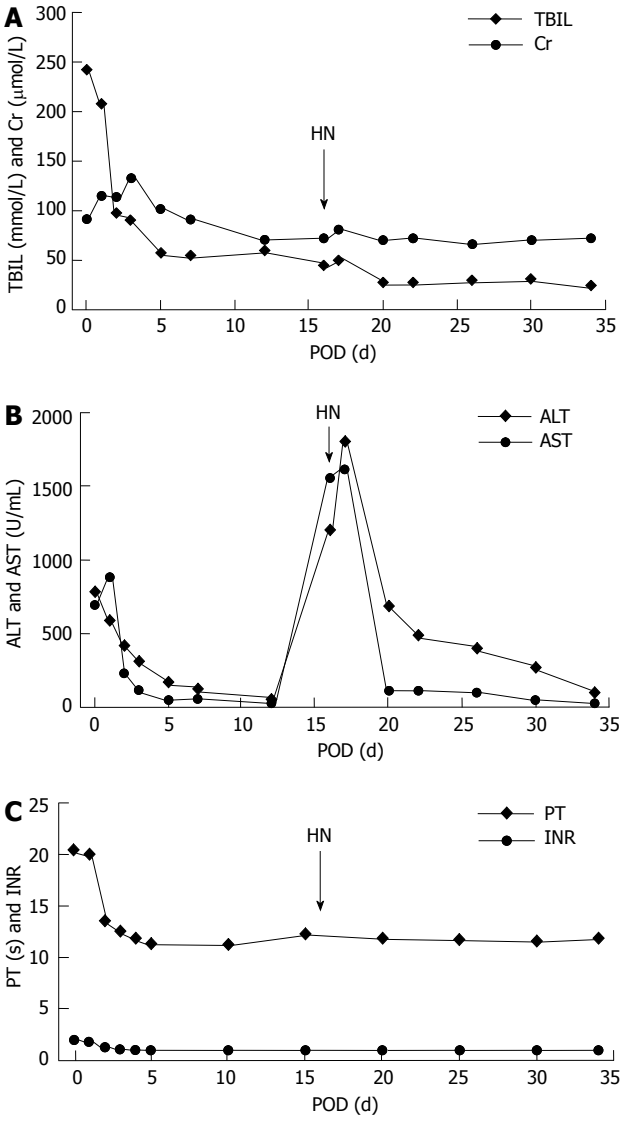

Figure 3 Total bilirubin and creatine, liver function, prothrombin time and international normalized ratio changes after liver transplantation. A: Total bilirubin (TBIL) and creatine (Cr) levels elevated after graft implantation and then decreased in 2 wk. Severe hepatic necrosis resulted in a slight rise, which was reversed by methylprednisolone(MP) pulse therapy; B:Serum alanine aminotransferase (ALT) and aspartate aminotransferase (AST) dropped significantly within 5 d after liver transplantation (LTx), and then remained in a low level till post-operative day (POD) 16. Severe hepatic necrosis led to a sharp increase of both ALT and AST which were under control after 4-d intravenous drip of 500 mg MP. Dosage of MP was reduced gradually to 40 mg on POD 25; C: Prothrombin time (PT) and international normalized ratio (INR) changes after LTx. Coagulation function became normal within 5 d. HN: Hepatic necrosis.

POD 7 according to Li *et al*[21], and remained stable during hepatic necrosis after LTx. Besides, ALT and AST levels started to fall on POD 20, *i.e.*, 4 d after an MP pulse therapy for severe hepatic necrosis, indicating that liver function had been improved (Figure 3B). As demonstrated in Figure 3C, PT and INR became normal on POD 5[21], and B-US on POD 16 showed no sign of intrahepatic thrombosis and stricture. To prevent postoperative bleeding, hemocoagulase was injected which would not result in prothrombin increase and thrombosis. So we excluded vascular or biliary complications. Low molecular weight

heparin calcium injection was also given till POD 30 for 14 d. However, CT scan and CTA on POD 118 showed a smaller low-density cyst (5 cm × 10 cm) and a same-sized filling defect (2.1 cm × 1.9 cm) at portal area compared with the earlier findings (Figure 1C), which indicated no deterioration of blood supply.

In conclusion, coagulatory, immunologic and hepatic functions after LTx were complicated, and we have not encountered such case before at our center. This patient had severe hepatic necrosis on POD 16, which might be attributed to the rejection with atypical manifestation due to immune suppressive therapy. However, the patient recovered in liver function after treatment with MP, and discharged on POD 34. Till the last follow-up 10 mo after LTx, there were no symptoms or signs indicating bad prognosis. Although the mechanism for hepatic necrosis and recovery of similar cases needs further studies, our experience has provided evidence that emergency ABO-I LTx is an effective treatment option for ACLF, and secondary liver necrosis of unknown causes might be treated by MP.

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Editorial office
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Xiu-Xia Song, Vice Director
World Journal of Gastroenterology
Room 903, Building D, Ocean International Center,
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Fax: +86-10-85381893

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Format

Journals

English journal article (list all authors and include the PMID where applicable)

1 **Jung EM**, Clevert DA, Schreyer AG, Schmitt S, Rennert J, Kubale R, Feuerbach S, Jung F. Evaluation of quantitative contrast harmonic imaging to assess malignancy of liver tumors: A prospective controlled two-center study. *World J Gastroenterol* 2007; **13**: 6356-6364 [PMID: 18081224 DOI: 10.3748/wjg.13.6356]

Chinese journal article (list all authors and include the PMID where applicable)

2 **Lin GZ**, Wang XZ, Wang P, Lin J, Yang FD. Immunologic effect of Jianpi Yishen decoction in treatment of Pixu-diarrhoea. *Shijie Huaren Xiaohua Zazhi* 1999; **7**: 285-287

In press

3 **Tian D**, Araki H, Stahl E, Bergelson J, Kreitman M. Signature of balancing selection in Arabidopsis. *Proc Natl Acad Sci USA* 2006; In press

Organization as author

4 **Diabetes Prevention Program Research Group**. Hypertension, insulin, and proinsulin in participants with impaired glucose tolerance. *Hypertension* 2002; **40**: 679-686 [PMID: 12411462 PMCID:2516377 DOI:10.1161/01.HYP.00000 35706.28494.09]

Both personal authors and an organization as author

5 **Vallancien G**, Emberton M, Harving N, van Moorselaar RJ; Alf-One Study Group. Sexual dysfunction in 1, 274 European men suffering from lower urinary tract symptoms. *J Urol* 2003; **169**: 2257-2261 [PMID: 12771764 DOI:10.1097/01.ju.0000067940.76090.73]

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6 21st century heart solution may have a sting in the tail. *BMJ* 2002; **325**: 184 [PMID: 12142303 DOI:10.1136/bmj.325.7357.184]

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7 **Geraud G**, Spierings EL, Keywood C. Tolerability and safety of frovatriptan with short- and long-term use for treatment of migraine and in comparison with sumatriptan. *Headache* 2002; **42** Suppl 2: S93-99 [PMID: 12028325 DOI:10.1046/j.1526-4610.42.s2.7.x]

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9 Outreach: Bringing HIV-positive individuals into care. *HRSA Careaction* 2002; 1-6 [PMID: 12154804]

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Personal author(s)

10 **Sherlock S**, Dooley J. Diseases of the liver and billiary system. 9th ed. Oxford: Blackwell Sci Pub, 1993: 258-296

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11 **Lam SK**. Academic investigator's perspectives of medical treatment for peptic ulcer. In: Swabb EA, Azabo S. Ulcer disease: investigation and basis for therapy. New York: Marcel Dekker, 1991: 431-450

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12 **Breedlove GK**, Schorfheide AM. Adolescent pregnancy. 2nd ed. Wieczorek RR, editor. White Plains (NY): March of Dimes Education Services, 2001: 20-34

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13 **Harnden P**, Joffe JK, Jones WG, editors. Germ cell tumours V. Proceedings of the 5th Germ cell tumours Conference; 2001 Sep 13-15; Leeds, UK. New York: Springer, 2002: 30-56

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14 **Christensen S**, Oppacher F. An analysis of Koza's computational effort statistic for genetic programming. In: Foster JA, Lutton E, Miller J, Ryan C, Tettamanzi AG, editors. Genetic programming. EuroGP 2002: Proceedings of the 5th European Conference on Genetic Programming; 2002 Apr 3-5; Kinsdale, Ireland. Berlin: Springer, 2002: 182-191

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15 Morse SS. Factors in the emergence of infectious diseases. Emerg Infect Dis serial online, 1995-01-03, cited 1996-06-05; 1(1): 24 screens. Available from: URL: http://www.cdc.gov/ncidod/eid/index.htm

Patent (list all authors)

16 **Pagedas AC**, inventor; Ancel Surgical R&D Inc., assignee. Flexible endoscopic grasping and cutting device and positioning tool assembly. United States patent US 20020103498. 2002 Aug 1

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Write as mean ± SD or mean ± SE.

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