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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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Chronic mesenteric ischemia: Time to remember open revascularization

Michael Keese, Thomas Schmitz-Rixen, Thomas Schmandra

Michael Keese, Thomas Schmitz-Rixen, Thomas Schmandra, Clinic for Vascular and Endovascular Surgery, Johann Wolfgang Goethe University Hospital, 60590 Frankfurt, Germany
Author contributions: Keese M and Schmandra T wrote the paper; Schmitz-Rixen T provided technical discussions and overall responsibility.

Correspondence to: Dr. Michael Keese, Clinic for Vascular and Endovascular Surgery, Johann Wolfgang Goethe University Hospital, 60590 Frankfurt, Germany. michael.keese@kgu.de
Telephone: +49-69-63015349 Fax: +49-69-63015336

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Abstract

Chronic mesenteric ischemia is caused by stenosis or occlusion of one or more visceral arteries. It represents a therapeutic challenge and diagnosis and treatment require close interdisciplinary cooperation between gastroenterologist, vascular surgeon and radiologist. Although endovascular treatment modalities have been developed, the number of restenoses ultimately resulting in treatment failure is high. In patients fit for open surgery, the visceral arteries should be revascularized conventionally. These patients will then experience long term relief from the symptoms, a better quality of life and a better overall survival.

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Key words: Chronic mesenteric ischemia; Stent; Vascular surgery; Restenosis; Prognosis

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VASCULAR ANATOMY

In embryogenesis segmental arteries regress until three major visceral vessels remain: the celiac artery, the superior mesenteric artery (SMA) and the inferior mesenteric artery (IMA). Hereby, frequent variants with complex anatomy may develop. In health, the visceral vascular supply is highly collateralized to ensure sufficient visceral blood supply. Retroduodenal and retropancreatic collaterals shunt blood from the coeliac artery to the SMA and vice versa^[1]. The SMA supplies the small bowel and parts of the colon. It originates only 1.5 to 2 cm below the coeliac artery, crosses the horizontal part of the duodenum and then enters the mesenteric root. At the splenic colon flexure the marginal artery of Drummond represents an important collateral which anastomoses the middle colic artery (MCA) with the left colic artery. The riolan anastomosis is another vascular arcade present in the colonic mesentery that connects the proximal MCA artery with the proximal IMA. The IMA supplies the descending colon, the sigmoid colon and the upper rectum. Here multiple collaterals are preformed with the hypogastric artery *via* the medial rectal artery^[2].

These interconnections between the celiac artery, the SMA and the IMA may easily compensate if stenotic lesions in one major visceral vessel develops. Therefore chronic mesenteric ischemia (CMI) with intestinal malperfusion is rarely seen in clinical practice but represents a serious and complex vascular disorder.

EPIDEMIOLOGY

In a population-based study, 553 healthy elderly persons underwent duplex sonography of the visceral arteries. Relevant stenosis was detected in 17.5% of the study cohort. The majority had isolated celiac disease. SMA stenosis and celiac artery occlusion demonstrated a significant and independent association with weight loss and concurrent renal artery disease^[3]. Among the patients

with relevant stenosis of visceral arteries only few develop symptoms. In a natural history study of 980 patients who received consecutive aortograms to identify significant mesenteric stenoses, eighty-two patients (8%) were found to have 50% stenosis of at least one mesenteric artery. Sixty of these patients (6%) had significant occlusions involving one, two, or all three mesenteric arteries. Mesenteric ischemia developed in only four patients (0.4%). Each of these four patients had significant three-vessel disease^[4].

CLINICAL PRESENTATION

The majority (over 70%) of CMI-patients are woman. Classically the symptoms include abdominal angina which presents as postprandial pain resulting in significant weight loss. Patients may develop a fear to eat, therefore among the differential diagnosis malignant diseases and functional disorders have to be considered. Atherosclerosis is by the most common etiology of CMI. Other etiologies causing this clinical entity include fibromuscular dysplasia, Buerger disease, and aortic dissection. In these patients atherosclerosis will often have manifested in other vascular beds by a history of myocardial infarction, stroke, or intermittent claudication. Atypical symptoms include diarrhea, constipation, vomiting and lower gastrointestinal bleeding which is associated with ischemic colitis or ischemic gastropathy. Endoscopic findings are unspecific and therefore subjective to inter observer variance. Previous research studies have suggested a possible role for tonometry, laser doppler, magnetic resonance (MR) oximetry, and MR measurement of mesenteric venous blood flow and spectroscopic oxymetric devices in the assessment of intestinal ischemia^[5]. All of these are still under investigation and their clinical usefulness has not been adequately established. In parallel to peripheral artery disease which may progress from claudication to tissue loss CMI may also progress from post-prandial pain to mesenteric infarction^[6]. Evidence of significant occlusion of two or more of these vessels is often found when classic symptoms and endoscopy suggest bowel ischemia. Single-vessel disease, usually of the SMA, has also been described as a cause of symptomatic CMI, particularly if collateral connections have been disrupted^[7].

DIAGNOSIS

Established non-invasive diagnostic means are duplex ultrasound or computed tomography (CT)- and MR-angiography. Especially duplex ultrasound is fast and readily available. Diagnosis is based on morphology and flow velocities. The largest series correlated angiograms and duplex sonography of 153 patients^[8]. Peak systolic velocity (PSV), end diastolic velocity, and SMA or colic artery/aortic PSV ratio were used to detect $\geq 50\%$ and $\geq 70\%$ stenosis. PSV threshold value for detecting $\geq 50\%$ SMA stenosis was ≥ 295 cm/s (sensitivity 87%, specificity 89% and OA 88%); and for detecting $\geq 70\%$

SMA ≥ 400 cm/s. PSV and ratio measurements were less reliable. Duplex ultrasound of the mesenteric artery requires a high level of technical expertise. It also depends on the fasting status of the patients. In ideal patients even higher sensitivity and specificity have been reported^[9]. There is also the tendency to measure higher flow velocities in stented arteries as compared to non-stented vessels. Therefore, the degree of in-stent stenosis may be overestimated^[10]. Native CT-scans without the application of a contrast agent already provide valuable information on the calcification status of the aorta. This is necessary to plan a revascularisation. In multiplanar reconstruction the complete aorta with all visceral branches can be visualized. Hereby vascular diameters, collateral formation and vascular variants can be imaged. Therefore, therapy should be planned on the basis of a 3-D reconstructed CT and on an invasive digital subtraction angiography^[11]. Magnetic resonance angiography plays no role in the diagnosis of chronic mesenteric ischemia. The digital subtraction angiography should show both lateral and frontal projections of the aorta and all major vessels. Furthermore the individual major branches should be imaged to gain information on collateralisation (*i.e.*, the patency of the Drummond artery or the arc of Riolan).

TREATMENT

With stenoses exceeding 70% in one or more visceral arteries patients may develop symptoms of mesenteric insufficiency. In these cases revascularisation is strongly recommended. While analgesia and parenteral nutrition may be used for optimal preparation of the patient for open surgery or endovascular intervention, conservative treatment will not slow disease progression. Therefore the clinical decision has to be made between endovascular treatment or conventional vascular revascularisation.

ENDOVASCULAR TREATMENT

In the majority of patients endovascular treatment of a mesenteric stenosis is feasible. The choice of vascular access depends on the patient's vascular anatomy. If the right femoral or iliac artery is heavily calcified, occluded or severely kinked the left femoral artery or the brachial artery may be used. Brachial access is also advisable in patients that show a steep angle between the SMA and the aorta. The regular transfemoral vascular access is achieved by puncture or cut down. A 7 F sheath normally allows the introduction of appropriately sized stents. *Via* sidewinder catheter the SMA is catheterized and a stiff wire with a long intervention sheath is placed into the SMA. Any wire manipulation has to be confirmed by angiography to avoid vessel injury. In the case of ostial calcifications balloon expandable stents should be used which are placed a few millimetres into the lumen of the aorta. Flexible nitinol stents may be used in the distal SMA. All patients should be heparinised to lower the risk



Figure 1 Conventional angiography of a 68 year-old patient who presented with weight loss and abdominal angina. The angiography shows an ostial stenosis of the celiac artery and a 1 cm stenosis of the superior mesenteric artery.



Figure 2 3-D reconstruction of a postoperative computed tomography. It shows the successful revascularisation of the common hepatic artery and the superior mesenteric artery by a ninfra-diaphragmatic bifurcation prosthesis.

of thromboembolism and/or stroke. Retrograde cannulation and stent placement of the SMA during laparotomy is another alternative approach which combines endovascular and open revascularisation of the occluded vessels.

CONVENTIONAL REVASCUARISATION

Open surgery requires a medial laparotomy since both the supracolic and the infracolic segments of the aorta have to be dissected. The celiac artery and the SMA are most frequently implicated in the disease process, and their involvement may result in chronic ischemia of the small intestine (Figure 1). Stenosis or occlusion of the IMA may lead to ischemic colitis. If both the celiac artery and the mesenteric arteries are involved, all arteries should be reconstructed to ensure long term prognosis^[12]. After mobilisation of the left hepatic lobe and transection of the right crus of the diaphragm the aorta may be tangentially clamped and longitudinal aortio-coeliac or aortomesenteric bypasses may be constructed using vein or prosthetic material (> 7 mm) (Figure 2). Bypass conduction to the mesenteric artery should follow a retro-pancreatic route to enter the mesenteric root. Here, a tunnel is formed left of the aorta. For a better hemodynamic approach to the SMA, bypasses are conducted curved around the left renal vein. Bypasses should be created tension free and kink-resistant. If longer clamping times are necessary, perfusion catheters may lengthen ischemic tolerance. For short ostial stenosis either transaortic endarterectomy with patch plasty or orthotopic short vein bypasses may be used.

EVIDENCE

So far no level 1 evidence governs the decision whether patients should receive endovascular or open revascularisation in chronic mesenteric ischemia. Early series showed a similar survival at 2 years between patients who received open revascularisation and patients who received a stent pta. No difference in the incidence of symptomatic or radiographic recurrence was found. However,

primary and assisted patency was significantly lower in the percutaneous transluminal angioplasty (PTA)/stent groups compared to OR^[13,14]. At the Cleveland clinics (United States) angioplasty and stenting was performed in 28 patients with chronic mesenteric ischemia. This cohort was compared to 85 patients who received open revascularisation^[15]. While no significant differences were found in terms of mortality, morbidity and restenosis rates, long term symptom recurrence was significantly higher in the endovascular group. In a large retrospective study major complications occurred in 7% of patients who underwent mesenteric artery stenting and resulted in higher mortality, morbidity, and longer hospital length of stay. The use of antiplatelet therapy reduced the risk of distal embolization or vessel thrombosis^[16].

A retrospective comparison at the Mayo clinic which involved 229 patients found a higher morbidity and longer hospital stay in patients after open surgery. Mortality was not different between the groups. Restenosis was five times more common in the endovascular group and symptom recurrence seven times more common than after open surgery^[17]. Restenosis within the stents occurs in nearly 40% of patients after stent placement in the mesenteric artery within the first 29 mo. Half of these patients require reintervention because of symptom recurrence or progression to an asymptomatic preocclusive lesion^[18]. This “in stent stenosis” is the result of intimal hyperplasia as a reaction to manipulation and trauma within the lumen of the artery. It involves apoptosis and the invasion of inflammatory cells. Vascular smooth muscle cells are recruited into the subendothelial space *via* signalling cascades involving tyrosin kinase receptors and the ras-pathway^[19]. The same pathways will be induced once more if a restenosis is treated by an endovascular approach. Furthermore endovascular interventions are associated with risk of access site and arterial complications from catheter and wire manipulation, balloon dilation, and stent placement. These risks are higher in the case of a reintervention on the SMA^[18]. In the mesenteric arteries, dissection, thrombosis, embolization, or perforation may result in bowel ischemia or bleeding,

necessitating additional “bail-out” manoeuvres, including emergency conversion to open repair. These complications can be fatal or result in significant morbidity and prolonged hospitalization if not recognized immediately. It is therefore advisable to change to open revascularisation especially in those patients in which endovascular treatment has already failed and who developed “in stent stenoses”.

CLINICAL PRACTISE

Today PTA/stent of the mesenteric artery has surpassed open bypass as the most frequently used option for mesenteric revascularisation, rendering open surgery as method of choice for these with unsuitable anatomy. Schermerhorn *et al*^[20] have reported on a 7 fold increase on mesenteric interventions. This was accompanied by a decrease in mortality from 15% with open bypass surgery to 4% with endovascular treatment. Kougiaris *et al*^[21] compared outcomes of endovascular treatment in a retrospective analysis which included 48 patients (58 vessels) in the endovascular group while open repair was performed in 96 patients (157 vessels). Endovascular treatment offered shorter hospitalization. Both groups had similar morbidity and mortality rates. Patients treated with surgical reconstruction were more likely to experience long-term symptomatic relief compared to endovascular cohorts, possibly due to higher incidence of two-vessel surgical revascularization^[21]. The enthusiasm for endovascular procedures is particularly surprising since no improvement of patency rates has been reported in the literature in the past 20 years^[22].

CONCLUSION

The durability and efficacy of open surgical repair are convincing over time. Therefore, open surgical revascularisation remains the treatment of choice for patients who are fit or whose fitness could be improved before surgery. Endovascular therapy has been proposed for patients at high risk for surgery and post surgical corrections. For these unfit patients, or those with short life expectancy, endovascular treatment is preferable owing to its minimally invasive nature and reduced postoperative mortality and morbidity. All others should be treated by conventional reconstruction especially if endovascular treatment has failed before. Randomized controlled studies or patient registries are needed to compare the long-term durability and efficacy of both procedures

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Challenges in diagnosing mesenteric ischemia

Teun C van den Heijkant, Bart AC Aerts, Joep A Teijink, Wim A Buurman, Misha DP Luyer

Teun C van den Heijkant, Joep A Teijink, Misha DP Luyer, Department of Surgery, Catharina Hospital Eindhoven, 5623 EJ Eindhoven, The Netherlands

Bart AC Aerts, Department of Surgery, Amphia Hospital, 4818 CK Breda, The Netherlands

Wim A Buurman, Institute Nutrim, Maastricht University, 6200 MD Maastricht, The Netherlands

Author contributions: van den Heijkant TC and Aerts BAC drafted the manuscript; Teijink JA, Buurman WA and Luyer MDP authored the manuscript; all authors read, edited and approved the final manuscript.

Correspondence to: Misha DP Luyer, MD, PhD, Department of Surgery, Catharina Hospital Eindhoven, 5623 EJ Eindhoven, The Netherlands. misha.luyer@cze.nl

Telephone: +31-40-2399111 Fax: +31-40-2455035

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Abstract

Early identification of acute mesenteric ischemia (AMI) is challenging. The wide variability in clinical presentation challenges providers to make an early accurate diagnosis. Despite major diagnostic and treatment advances over the past decades, mortality remains high. Arterial embolus and superior mesenteric artery thrombosis are common causes of AMI. Non-occlusive causes are less common, but vasculitis may be important, especially in younger people. Because of the unclear clinical presentation and non-specific laboratory findings, low clinical suspicion may lead to loss of valuable time. During this diagnostic delay, progression of ischemia to transmural bowel infarction with peritonitis and septicemia may further worsen patient outcomes. Several diagnostic modalities are used to assess possible AMI. Multi-detector row computed tomographic angiography is the current gold standard. Although computed tomographic angiography leads to an accurate diagnosis in many cases, early detection is a persistent problem. Because early diagnosis is vital to commence treatment, new diagnostic strategies are needed. A non-invasive simple biochemical test would

be ideal to increase clinical suspicion of AMI and would improve patient selection for radiographic evaluation. Thus, AMI could be diagnosed earlier with follow-up computed tomographic angiography or high spatial magnetic resonance imaging. Experimental *in vitro* and *in vivo* studies show promise for alpha glutathione S transferase and intestinal fatty acid binding protein as markers for AMI. Future research must confirm the clinical utility of these biochemical markers in the diagnosis of mesenteric ischemia.

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Key words: Acute mesenteric ischemia; Diagnosis; Biological markers; Intestinal fatty acid binding protein; Alpha-glutathione S transferase

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INTRODUCTION

Mesenteric ischemia accounts for approximately 1% of acute abdomen hospitalizations and occurs in one in 1000 patients presenting to emergency rooms^[1,2]. Despite growing recognition of this entity and interest in preventing irreversible ischemia, identification and early diagnosis is challenging because early symptoms are non-specific^[3]. Despite major diagnostic and treatment advances over the past decades, low clinical suspicion leads to persistently high mortality rates of 40% to 70% for acute mesenteric ischemia (AMI)^[4]. Early diagnosis is necessary to commence appropriate treatment, whereas diagnostic delay contributes to poor patient outcomes. A 24-h delay decreases survival rates by 20%^[5]. Therefore, development of new diagnostic strategies is of great importance. Ideally, a highly specific and sensitive, non-invasive marker is needed to identify patients earlier.

MESENTERIC ISCHEMIA

Etiology and clinical presentation

In 70% to 80% of cases, intestinal ischemia is caused by an arterial embolus or thrombosis within the superior mesenteric artery. In cases of embolic occlusion, the absence of a well-developed collateral circulation causes earlier ischemia and transmural necrosis compared to other causes of mesenteric ischemia. Less common causes are venous thrombosis and non-thrombotic mechanical causes such as strangulated hernia^[6]. Patients with a history of arterial emboli, vasculitis, deep venous thrombosis, hypercoagulable state, or chronic postprandial pain are at increased risk^[7]. Vasculitis is a common cause of mesenteric ischemia in younger people with auto-immune disease^[8]. Finally, case reports implicate vascular anomalies as a cause of mesenteric ischemia^[9,10].

There are a wide variety of clinical presentations for mesenteric ischemic. Classically, AMI is associated with a dramatic onset of severe abdominal pain disproportionate to physical exam findings. Peritonitis and septicemia develop once the ischemia has progressed transmurally^[3]. Postprandial pain, nausea and weight loss often occur in patients with chronic mesenteric ischemia and superior mesenteric artery thrombosis^[3]. In chronic mesenteric ischemia, the association of pain with meals leads to fear of eating and subsequent weight loss^[7].

Early diagnosis is challenging because of the wide variability in clinical presentation of mesenteric ischemia.

Laboratory findings

Classically, patients with mesenteric ischemia have leukocytosis, metabolic acidosis, an elevated D-dimer and elevated serum lactate^[3]. Although profound leukocytosis with peripheral white blood cell counts exceeding $20 \times 10^9/L$ have been reported, this finding is not useful to distinguish AMI from other diagnoses^[11,12]. In a prospective clinical trial, Acosta *et al.*^[13] investigated the classically described metabolic acidosis. In their study, initial blood gas analysis showed metabolic alkalosis more frequently than metabolic acidosis; this finding results from profound vomiting during early bowel ischemia. D-dimer is also purported as an important tool in diagnosing AMI. However, D-dimer, an enzymatic degradation product of fibrin that is released during intravascular coagulation and fibrin deposition, may be present with AMI as well as several other conditions^[14]. A recent trial demonstrated that serum D-dimer detection does not differentiate patients with AMI from those with non-acute mesenteric ischemia, and that there is no difference in serum D-dimer levels between resectable and unresectable bowel necrosis lesions^[15]. L-lactate is associated with late-stage mesenteric ischemia with extensive transmural intestinal infarction, body tissue hypoperfusion, anaerobic metabolism and death^[16]. Some studies report absence of systemic plasma L-lactate elevation in cases of extensive intestinal ischemia. This absence of L-lactate is probably explained by the liver's capacity to clear large quantities of L-lactate from the porto-mesenteric circulation^[17]. A recent study

Table 1 Sensitivity, specificity and likelihood ratios for laboratory findings classically associated with mesenteric ischemia

| Marker | Sensitivity | Specificity | Positive likelihood-ratio (95%CI) | Positive likelihood-ratio (95%CI) |
|------------------------|-------------|-------------|-----------------------------------|-----------------------------------|
| WBC count ¹ | 0.80 | 0.50 | 1.57 (1.07, 2.27) | 0.41 (0.20, 0.83) |
| pH ¹ | 0.38 | 0.84 | 2.49 (0.82, 7.51) | 0.71 (0.45, 1.14) |
| D-dimer ¹ | 0.89 | 0.40 | 1.48 (1.28, 1.71) | 0.30 (0.14, 0.64) |

¹Evennett *et al.*^[19]. WBC: White blood cell.

by Thuijls *et al.*^[18] confirmed that plasma L-lactate level, base excess and leukocyte count cannot be used as markers of mesenteric ischemia. In that study, fifty consecutive patients suspected of having intestinal ischemia provided blood and urine samples. Plasma L-lactate level, base excess and leukocyte count were nondiscriminatory in determining whether patients had intestinal ischemia or other disease such as stomach perforation, pancreatitis or perforated appendicitis. Leukocyte counts in that study did not differ significantly between groups ($13.9 \times 10^9/L$ in the mesenteric ischemia group versus $12.7 \times 10^9/L$ in the control group).

Table 1 shows that classically described laboratory findings cannot be used as markers for AMI because of their insufficient likelihood ratios^[19]. These laboratory findings are not sensitive or specific enough to establish or exclude the diagnosis of AMI. Elevations in AMI serum markers usually occur only after transmural bowel infarction, and therefore, cannot be used for early diagnosis^[7].

Imaging techniques

The American Gastroenterological Association guideline (2000) states that mesenteric angiography is the gold standard for the diagnosis of mesenteric ischemia^[7]. When the clinician is aware of possible AMI, angiography is accurate and increases survival^[20]. However, catheter angiography is invasive and time consuming. Furthermore, the unavailability of this diagnostic modality at most hospitals leads to a critical delay. At the time of the 2000 guideline, computed tomography (CT)-angiography (CTA) seemed promising, but there was limited experience with this modality then. Over the last decade, however, there has been a major shift toward CTA because it is less invasive, less time- and resource-consuming, and more readily available. Today, CTA has replaced angiography as the gold standard in diagnosing mesenteric ischemia with a sensitivity and specificity of 0.96 and 0.94, respectively^[21,22]. These sensitivity and specificity results were obtained in a dedicated study with structural CT evaluation for all AMI characteristics. Generalizability of these outstanding diagnostic values is questionable because it is unlikely whether all AMI characteristics are evaluated by specialized radiologists at all practice locations.

Wiesner *et al.*^[23] described a group of 291 patients who presented with acute abdomen and underwent multidetector-row computed tomographic scans. All original computed tomographic diagnoses were made

during several radiologists' daily routines. The sensitivity and specificity of multidetector-row CTA for the diagnosis of AMI were 0.79 and 0.98, respectively. These statistical measures may better reflect daily practice. In these cases, no focused structural radiological search for characteristics of intestinal ischemia was performed. There were different examination protocols because of variation in suspected diagnosis. Arterial phase scanning was not performed regularly if there was no specific clinical indication. Based on the current evidence, CTA is acceptable and accurate in diagnosing AMI; however, early identification of patients remains a challenge. Early identification could alert the radiologist to perform structural evaluations of CT scans specific for AMI, so as to increase the sensitivity of CTA^[21].

NEW DIAGNOSTIC STRATEGIES

New diagnostic strategies aim for early identification (*e.g.*, biochemical markers) or seek to optimize accurate diagnosis using existing modalities such as contrast-enhanced magnetic resonance angiography (CE-MRA). CE-MRA of the splanchnic vessels is an evolving technology which is theoretically appealing because it is non-invasive and avoids the nephrotoxicity and allergic risks associated with iodinated contrast agents^[22]. Dynamic CE-MRA yielded a sensitivity and specificity of 0.95 and 1.00, respectively, in a clinical trial designed to diagnose severe stenosis or occlusion of the origins of the celiac axes and superior mesenteric artery^[24]. However, this modality is limited to identification of more distally located occlusions and does not have the same spatial resolution and acquisition time as CTA^[14]. If better spatial resolution becomes available in the future, CE-MRA has the potential to become the diagnostic modality of choice.

Non-contrast-enhanced 7 tesla magnetic resonance imaging (7T-MRI) is a recently developed diagnostic modality. A study using an *in vivo* rat model for mesenteric ischemia demonstrated that 7T-MRI allows for the identification of pathological findings of ischemic colitis and histopathological correlation^[25]. Further research is needed to substantiate these promising results in human clinical situations.

Biochemical markers for early detection

In AMI, ischemia starts at the mucosa and extends toward the serosa^[16]. An ideal biomarker for mesenteric ischemia should originate at the mucosa to detect ischemia at the earliest stage. According to a recent review by Evennett *et al.*^[19], intestinal fatty acid binding protein (I-FABP), alpha-glutathione S transferase (GST) and D-lactate are the most promising plasma markers for mesenteric ischemia. Fatty acid binding proteins comprise a class of low molecular weight (14-15 kDa) cytosolic proteins. I-FABP is a small cytosolic protein found in tissues involved in uptake and consumption of fatty acids. It is highly expressed in cells on the luminal side of small intestinal villi. I-FABP is released into the circulation and

renally cleared upon enterocyte membrane integrity loss. These characteristics, combined with localization at the initial site of destruction in mesenteric ischemia, make I-FABP a useful urinary and plasma marker for enterocyte damage^[26]. Urinary and plasma I-FABP levels are significantly elevated in patients with intestinal ischemia compared to healthy controls^[27]. Furthermore, I-FABP levels were increased in patients with small intestinal necrosis, but not patients without intestinal ischemia in a patient population with suspected ischemia due to small bowel obstruction^[28]. I-FABP has been reported as a specific and sensitive marker for postoperative intestinal necrosis^[25]. A recent clinical trial demonstrated a high sensitivity and specificity of 0.90 and 0.89, respectively, for urinary I-FABP^[18]. The rapid clearance of plasma FABPs (calculated half-life time of eleven minutes^[29]) into urine plus urinary FABP accumulation following intestinal damage make urinary FABP more diagnostically useful compared to plasma FABP^[26]. These findings suggest that further research is needed to confirm the diagnostic value of I-FABP in cases of mesenteric ischemia.

Other potential early markers of AMI are alpha-GSTs, a family of cytosolic enzymes involved in detoxification and released from a variety of cells following cell membrane damage^[30]. GST is involved in detoxification of a range of toxic compounds within the cell by conjugation to glutathione. Alpha-GST is known to be highly active both in the liver and the small intestine mucosa^[16]. Alpha-GST has pooled sensitivity and specificity for diagnosing AMI of 0.68 and 0.85, respectively. A limitation of alpha-GST is that hypotensive patients with multiple organ failure and hepatic ischemia may also have elevated alpha-GST levels with concomitant ASAT and ALAT abnormalities.

A third suggested marker for AMI is D-lactate, which originates from bacteria such as *Escherichia coli* in the intestinal lumen^[17]. It was hypothesized that D-lactate levels increase during mesenteric ischemia due to bacterial translocation and overgrowth following mucosal injury. However, a recent review showed a pooled sensitivity and specificity for D-lactate of only 0.82 and 0.48, respectively. Therefore, D-lactate cannot be used as a marker for AMI, given the superiority of alternatives such as I-FABP and alpha-GST.

CONCLUSION

AMI is a rare condition with a non-specific clinical presentation which makes early diagnosis challenging. Despite technical advances in imaging leading to more accurate diagnosis, AMI is often diagnosed late or even missed due to low clinical suspicion; therefore, a high mortality rate results.

A readily available, simple, highly sensitive and specific marker to identify patients with AMI early would be of great importance in selecting candidates for CTA. Future research must confirm the clinical utility of promising biochemical markers such as I-FABP and GST.

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Hepatitis B and inflammatory bowel disease: Role of antiviral prophylaxis

Pilar López-Serrano, Jose Lázaro Pérez-Calle, Maria Dolores Sánchez-Tembleque

Pilar López-Serrano, Jose Lázaro Pérez-Calle, Department of Gastroenterology, University Hospital Fundación Alcorcón, 28922 Madrid, Spain

Maria Dolores Sánchez-Tembleque, Department of Gastroenterology, University Hospital of Guadalajara, 19002 Guadalajara, Spain

Author contributions: López-Serrano P, Pérez-Calle JL and Sánchez-Tembleque MD contributed equally to this work; all authors approved the final version of the manuscript.

Correspondence to: Dr. Pilar López-Serrano, Department of Gastroenterology, University Hospital Fundación Alcorcón, Calle Budapest 1, 28922 Madrid, Spain. pilarlopezserrano@gmail.com

Telephone: +34-916-219890 Fax: +34-916-219705

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Abstract

Hepatitis B virus (HBV) is a very common infection worldwide. Its reactivation in patients receiving immunosuppression has been widely described as being associated with significant morbidity and mortality unless anti-viral prophylaxis is administered. Treatment in inflammatory bowel disease (IBD) patients has changed in recent years and immunosuppression and biological therapies are now used more frequently than before. Although current studies have reported an incidence of hepatitis B in inflammatory bowel disease patients similar to that in the general population, associated liver damage remains an important concern in this setting. Liver dysfunction may manifest in several ways, from a subtle change in serum aminotransferase levels to fulminant liver failure and death. Patients undergoing double immunosuppression are at a higher risk, and reactivation usually occurs after more than one year of treatment. As preventive measures, all IBD patients should be screened for HBV markers at diagnosis and those who are positive for the hepatitis B surface

antigen should receive antiviral prophylaxis before undergoing immunosuppression in order to avoid HBV reactivation. Tenofovir/entecavir are preferred to lamivudine as nucleos(t)ide analogues due to their better resistance profile. In patients with occult or resolved HBV, viral reactivation does not appear to be a relevant issue and regular DNA determination is recommended during immunosuppression therapy. Consensus guidelines on this topic have been published in recent years. The prevention and management of HBV infection in IBD patients is addressed in this review in order to address practical recommendations

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Key words: Hepatitis B virus; Inflammatory bowel disease; Anti-tumor necrosis factor; Prophylaxis; Immunosuppressants

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INTRODUCTION

Approximately 3 billion people have been exposed to the hepatitis B virus (HBV), and there are an estimated 350 million chronic carriers worldwide^[1,2]. Exposure to HBV can be broadly divided into three categories according to viral load and liver biopsy^[1,3]: active HBV, inactive HBV carriers and resolved HBV (Table 1). Chronic hepatitis B (CHB) may result in cirrhosis in about 5%-25% of infected patients.

Active chronic HBV is defined as HBV DNA levels ≥ 2 IU/L and elevated alanine aminotransferase (ALT) levels, and treatment is indicated in either immunocom-

petent or immunosuppressed patients.

Inactive hepatitis B is defined by HBV DNA levels ≤ 2 IU/L and normal ALT levels, and antiviral therapy is not indicated in immunocompetent patients. However, in patients treated with immunosuppressant drugs, viral reactivation may occur regardless of DNA levels, so antiviral prophylaxis must be established.

Finally, patients with resolved HBV infection do not need antiviral therapy, but they must be monitored during immunosuppressant therapy and considered for prophylactic therapy based on the progression of anti-HBc or HBV DNA levels.

Ulcerative colitis (UC) and Crohn's disease (CD) are chronic inflammatory conditions of the gastrointestinal tract. In the last 10 years, treatment of these inflammatory bowel diseases (IBD) has been markedly changed by the use of immunosuppressants (mainly azathioprine/mercaptopurine and methotrexate) and biological therapies. There is an increasing trend towards earlier and more extensive use of these drugs for longer periods of time. As a result, concerns regarding safety related to immunosuppressive treatment are growing^[4,5] among the professionals involved in providing IBD care. One of these issues is related to the reactivation of hepatitis B; immunomodulator therapy has a clear impact on the natural history of viral hepatitis and there are many unresolved questions concerning the relationship between IBD and HBV that must be resolved. The management of IBD patients with viral hepatitis B is addressed in this review.

PREVALENCE OF HBV INFECTION IN IBD PATIENTS

The first step in addressing the problem is to identify its impact. The prevalence of patients with IBD who are at risk for hepatitis B reactivation has been clarified in a prospective cross-sectional nationwide study in Spain^[6]. In this multicentre study of 2076 IBD patients, the prevalence of biological markers of present and/or past hepatitis B was lower than reported in previous studies and similar to the Spanish general population: less than 1% of IBD patients had positive hepatitis B surface antigen (HBsAg) and less than 10% had positive hepatitis B core antibody (HBcAb), without differences between CD and UC patients (7.1% and 8% respectively). Similar results were also shown in a recent study in France^[7], where HBcAb was reported in only 2.78% and 1.59% of CD and UC patients, respectively; similar rates were observed in the general French population.

In previous studies^[8-10], the prevalence of HBV infection in IBD patients was shown to be comparatively higher. Biancone *et al*^[9] reported positive anti-HBc in 10.9% and 11.5% of CD and UC patients in Italy, respectively, compared to 5.1% in controls ($P < 0.02$). The risk of viral hepatitis in IBD patients has been associated with blood transfusions and surgery^[11,12], suggesting nosocomial transmission of the virus. The decreasing prevalence

of viral hepatitis in IBD patients in current reports from Spain and France suggests that preventative measures such as vaccination, the World Health Organization blood transfusion safety programs, single-use materials, and better aseptic perioperative rules and decontamination procedures in endoscopy^[7], have been effective and explains the diminishing risk for HBV.

EFFECT OF IMMUNOSUPPRESSIVE THERAPY ON HBV INFECTION

Reactivation of HBV infection is a well-described complication of immunosuppression in the setting of organ transplantation or cancer chemotherapy^[13]. The frequency of HBV reactivation depends on the type of immunosuppression and the state of HBV infection when chemotherapy is administered^[14-16]. Cytotoxic chemotherapy for hematologic malignancies appears to involve the greatest risk of HBV reactivation; spontaneous reactivation may occur in up to 22% of inactive HBV carriers, but this may increase up to 60% of patients in the case of cytotoxic chemotherapy for lymphoma, with a mortality rate between 4% and 60% if fulminant liver failure occurs^[17-19].

In patients undergoing treatment with biologic agents, hepatitis B reactivation represents an emerging cause of liver disease^[20]. In particular, the risk of HBV reactivation is greatly increased with the use of monoclonal antibodies such as rituximab (anti-CD20) and alemtuzumab (anti-CD52)^[21-23]. Based on the liver damage that occurs after viral reactivation, three kinds of liver disease^[4] can be distinguished: viral reactivation or replication, acute liver failure, and fulminant liver failure. For hepatitis B, reactivation is defined as a 1.5-2-fold increase in ALT compared with the baseline value plus an increase in HBV DNA levels > 2 IU/L or DNA reappearance in a previously negative patient^[24,25]. Acute liver failure is defined as sudden and severe impairment of liver function (bilirubin > 2 mg/dL, albumin < 34 g/L or prothrombin time $< 50\%$), meanwhile fulminant liver failure is a severe acute failure complicated by hepatic encephalopathy, liver transplantation, or death^[25].

Fatal viral reactivation has also been described in patients with IBD or other autoimmune diseases treated with immunosuppressant drugs^[26-29]. Tumor necrosis factor alpha (TNF- α) is important in regulating hepatitis B replication^[30], and the chimeric anti-tumour necrosis factor alpha monoclonal antibody infliximab has been involved in hepatitis B reactivation following treatment^[31-35]. However, the factors that may increase the risk are not properly defined^[36,37]. Additionally, there are no specific data on prophylaxis in IBD patients, the type and the timing of treatment, and which individuals make the most suitable candidates^[38-40].

Most of the information available comes from case reports about HBV reactivation in patients with CD or UC, both after the use of corticoids, with or without azathioprine and biologic therapy with infliximab^[41].

Table 1 Definitions of hepatitis B virus infection

| | HBsAg | HBsAb | HBcAb | HBV DNA | | ALT/AST | Liver biopsy |
|----------------------|----------|----------|----------|---------------|--------------|----------|--|
| | | | | HBeAg (+) | HBeAg (-) | | |
| Chronic HBV | Positive | Negative | Positive | > 20000 IU/mL | > 2000 IU/mL | Elevated | Chronic hepatitis with necroinflammation |
| Inactive HBV Carrier | Positive | Negative | Positive | < 20000 IU/mL | < 2000 IU/mL | Normal | No significant hepatitis |
| Resolved HBV | Negative | Positive | Positive | Undetectable | Undetectable | Normal | No significant hepatitis |

HBV: Hepatitis B virus; HBsAg: Hepatitis B surface antigen; HBsAb: Hepatitis B surface antibody; HBcAb: Hepatitis B core antibody; ALT: Alanine aminotransferase; AST: Aspartate aminotransferase.

In 2004, Esteve *et al*^[42] reported three CD patients, which were hepatitis B carriers, treated with infliximab. One of them received concomitant lamivudine therapy and experienced no variations in clinical or biochemical liver parameters. However, the other two patients suffered from viral reactivation; one patient died and the other resolved after specific treatment. Reactivation may occur as soon as after the first infusion or as late as 2 years after starting infliximab, indicating that it may develop at any time during anti-TNF therapy, however, the time between the start of TNF- α inhibitors and the occurrence of hepatitis is usually longer than one year^[43].

A relevant characteristic is that almost all cases of infliximab-associated HBV reactivation have occurred in patients receiving concomitant treatment with other immunosuppressants such as corticosteroids or thiopurines, suggesting that more profound immunosuppression may facilitate viral reactivation^[26,38-40]. In a Spanish cooperative study (REPENTINA-2) on this problem^[26], 120 patients with hepatitis B or hepatitis B and C markers who had been treated with immunosuppressive therapy during a median time of 1 year were identified. The authors retrospectively assessed the frequency and severity of liver disease in these patients based on exposure to one or more immunosuppressive drugs. Although they identified 25 HBsAg-positive patients, unfortunately only 6 of them had received antiviral treatment before immunosuppression. Nine of these 25 patients developed liver disease, six developed liver failure, and none had fulminant liver failure. Almost all cases of HBV reactivation associated with infliximab occurred in patients receiving concomitant treatment with other immunosuppressants. REPENTINA-2 also showed that no single immunosuppressant seems to be specifically involved in the development of liver failure, and the risk of hepatitis B reactivation seems to be related to the magnitude of immunosuppression.

Regarding other anti-TNF treatments, reactivation has also been reported with adalimumab or etanercept in patients with rheumatoid arthritis^[44-46], but not from IBD patients or those taking certolizumab pegol. As these are newer TNF antagonists, the risk of hepatitis B reactivation is expected to be similar due to a class effect^[4,15].

Regarding patients with resolved HBV infection, reactivation following chemotherapy has also been reported in up to 40% of patients^[17,47-49], though the risk in IBD seems to be much lower. Although there has been a

report of reactivation after treatment with infliximab in a patient with CD and occult HBV infection^[47], no cases were described in the REPENTINA-2 study. In other autoimmune diseases such as rheumatoid arthritis (RA), this issue has also been examined and reactivation rates have not been relevant^[50,51]. Tamori *et al*^[52] followed 50 patients with RA who were positive for HBcAb for a mean period of 23 mo. All were patients treated with immunosuppressive agents such as methotrexate, prednisolone, and/or TNF- α inhibitors for more than one year. HBV reactivation was observed in two out of five patients with HBsAg, compared with only one of the remaining 45 patients without it.

On the other hand, the results from a retrospective analysis of 62 psoriatic patients^[53] with occult HBV infection, treated with anti-TNF biological agents, and without signs of HBV activation after a period of 4 years, also suggest the overall safety of treatment with anti-TNF and/or immunosuppressants drugs in HBV occult carriers.

SCREENING RECOMMENDATIONS

Screening measures must be instituted in IBD patients^[4,54-56]. Recommendations are based on the potentially fatal consequences of HBV reactivation, as we have seen previously, and the availability of safe and effective anti-HBV drugs to prevent them^[57]. Current guidelines by the American Association for the Study of Liver Diseases (AASLD) recommend HBV screening for populations with an intermediate or high prevalence (> 2%) and those requiring immunosuppression, including IBD patients^[3].

Screening for HBV should be performed at the time of IBD diagnosis rather than delaying until consideration of immunomodulators or TNF antagonist medication^[3,58-60]. The hepatitis B surface antibody (HBsAb), and the HBsAg and HBcAb are recommended as screening tests. Although the utility of HBcAb is controversial, it may represent chronic HBV despite undetectable HBsAg/HBsAb in immunosuppressed patients or those co-infected with hepatitis C or human immunodeficiency virus^[3,58], but it may also be falsely elevated in a low percentage of patients.

ANTIVIRAL PROPHYLAXIS

In accordance with specific guidelines for hepatitis B

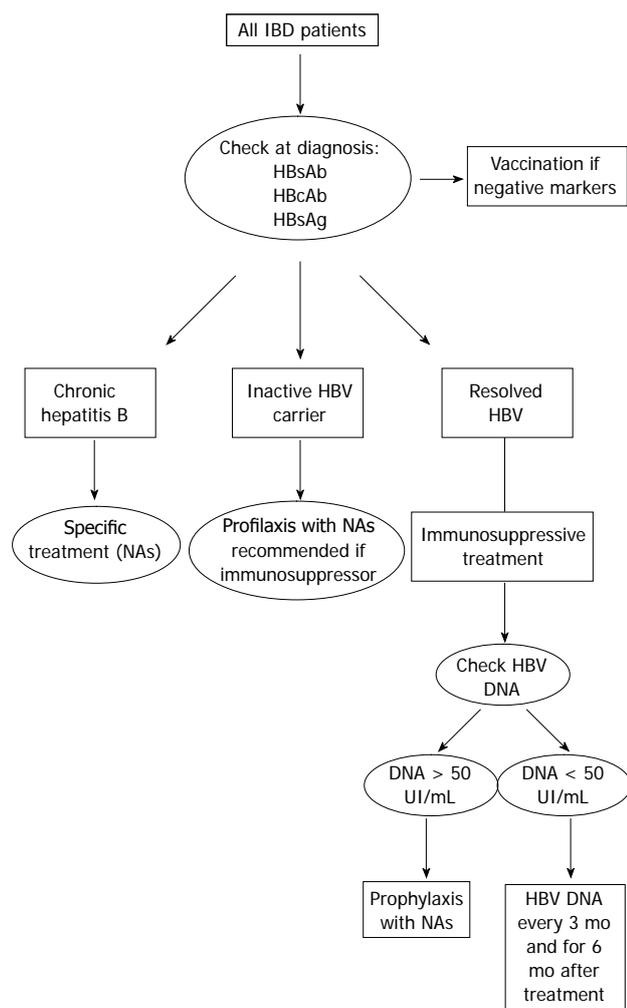


Figure 1 An algorithm for the management of patient with inflammatory bowel disease with different hepatitis B virus infection status. NAs: Nucleos(t)ide analogues; IBD: Inflammatory bowel disease; HBV: Hepatitis B virus; HBsAg: Hepatitis B surface antigen; HBsAb: Hepatitis B surface antibody; HBcAb: Hepatitis B core antibody.

(Figure 1)^[3], patients with high baseline HBV DNA levels (> 2 IU/L) should continue antiviral treatment until the endpoints applicable to non-immunosuppressed patients are reached.

Prophylaxis must be considered in patients with HBsAg without active viral replication prior to undergoing treatment with any immunosuppressant medication, including steroids, immunomodulators or biologicals, in order to prevent reactivation^[3,59].

While there are no data specifically for IBD, recommendations are extrapolated from randomized controlled trials in patients undergoing chemotherapy^[60-62] in which prophylaxis has been shown to be beneficial^[63,64]. Lau *et al.*^[65] randomized 30 lymphoma patients with HBV markers to lamivudine prophylaxis or no prophylaxis before chemotherapy. None of the patients receiving prophylaxis developed HBV reactivation, compared to 53% of those in the no prophylaxis group ($P = 0.002$). A recent meta-analysis^[66] of lamivudine in immunosuppressed patients included 21 studies and showed a mortality benefit

for lamivudine (OR: 0.36; 95%CI: 0.23-0.56).

The AASLD^[3] and the European Association for the Study of the Liver^[67] recommend the early introduction of nucleoside/nucleotide analogues (NAs) for all HBsAg-positive patients requiring immunosuppressive therapy. HBV prophylaxis must be introduced at least 7 d before therapy and it should be maintained for 6 mo to 1 year after completion of chemotherapy, as HBV reactivation may occur after chemotherapy is discontinued^[3,4]. The European Crohn's and Colitis Organisation^[57] also recommend an early introduction of NAs in HBsAg-positive IBD patients requiring immunosuppression, regardless of the number and type of immunosuppressants. However, it must be initiated between one and 3 wk prior to the introduction of immunosuppressive therapy and continued for 6 mo after its cessation^[11,57].

Lamivudine has been the most common drug used in this setting; however, resistance usually develops with prolonged use and has been detected in up to 30% of patients after 1 year and 70% after 5 years of treatment^[66,67]. Resistance has also been described in patients on long-term anti-TNF therapy, which is associated with viral reactivation as reported by Esteve *et al.*^[27] in a CD patient taking lamivudine for more than 5 years. Therefore, this agent may be appropriate for a short course of prophylaxis during chemotherapy but, as immunosuppressive medications for IBD may be required indefinitely, NAs with a lower propensity than lamivudine for generating drug-resistant mutations of HBV DNA must be chosen. Although alternative antiviral medications, such as tenofovir, adefovir, telbivudine and entecavir, have not been evaluated in prophylaxis for IBD patients, tenofovir and entecavir have the lowest rates of resistance with long-term use and should be preferred.

In cases in which lamivudine, adefovir or telbivudine are used, serum aminotransferase levels and HBV DNA levels must be closely followed for signs of drug resistance. Interferon- α must not be used for prophylaxis^[57].

In HBe-positive patients who are lacking HBsAg and have resolved hepatitis B, systematic use of antiviral prophylaxis in IBD patients is not recommended^[1,21,49]. This approach differs from the recommendation for patients undergoing chemotherapy and particularly for rituximab, in which anti-viral prophylaxis is desirable^[21-23,49,68]. Nevertheless, careful and constant monitoring of virological markers, including HBV DNA, is required in these patients during treatment for early recognition of viral reactivation and therapy with NAs at an early stage^[69,70] (Figure 1).

In conclusion, new therapies in IBD patients are increasing the risk for HBV reactivation. All patients should be screened for HBV markers, preferably at diagnosis.

Current guidelines recommend screening with HBsAg and anti-HBs, but anti-HBe must also be considered so as to detect occult HBV. HBsAg positive patients requiring immunosuppressive therapy should receive antiviral treatment, regardless of HBV DNA level. Prophylaxis with nucleos(t)ide analogues must be introduced at least 3 wk before, but as immunosuppressive drugs may be required

indefinitely, NA with a low rate of resistance (tenofovir or entecavir) should be preferred. Patients with positive anti-HBc without HBsAg and undetectable viral DNA should be monitored closely for reactivation.

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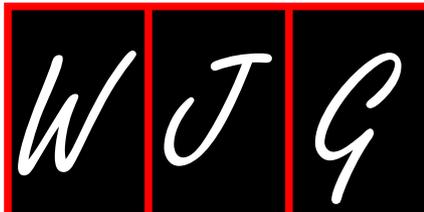
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Efficacy of the vaccination in inflammatory bowel disease

Elisa Carrera, Rebeca Manzano, Elena Garrido

Elisa Carrera, Department of Gastroenterology, University Hospital of Guadalajara, 19002 Guadalajara, Spain

Rebeca Manzano, Department of Gastroenterology, Sureste Hospital, Arganda, 28500 Madrid, Spain

Elena Garrido, Inflammatory Bowel Disease Clinic, Department of Gastroenterology, University Hospital Ramón y Cajal, 28034 Madrid, Spain

Author contributions: Carrera E, Manzano R and Garrido E contributed equally to this work; all authors approved the final version of the manuscript.

Correspondence to: Elisa Carrera, MD, Department of Gastroenterology, University Hospital of Guadalajara, Av. Donantes de sangre sn, 19002 Guadalajara, Spain. ecarrera@hotmail.com
Telephone: +34-94-9209200 Fax: +34-94-9209259

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Abstract

Inflammatory bowel disease (IBD) is associated with conditions that may predispose to infections, such as the lack of an appropriate innate immune response to infectious agents, malnutrition, surgery, and immunosuppressive and biological drugs. Some of these infections may be preventable by vaccination. Therefore, for this particular patient population, the benefits of implementing a well-established immunization protocol in daily clinical practice are potentially even greater than for the general population. In recent years international consensus guidelines have been published, but in spite of these recommendations, studies have shown that a significant number of patients with IBD remain inadequately immunized. Another important issue regarding immunization in this population is that vaccine efficacy among patients receiving immunosuppressive therapies has been variable. In a healthy population, a humoral immune response to hepatitis B vaccination (HBV) is expected in > 90%, whereas a much lower rate is achieved in the IBD patients. Immunosuppressive, anti-tumor necrosis factor therapy and disease activity have been implicated in the impaired efficacy of the

vaccination. The serological response to HBV should be confirmed and patients with an inadequate response should receive a second full series of vaccine. Modified dosing regimens, including doubling the standard antigen dose, might increase the effectiveness. Response to influenza, pneumococcal and tetanus immunization is still not clear, as there are studies that show a normal response to the vaccination while others demonstrate a lack of efficacy. We pose a series of questions on the efficacy of the different vaccinations recommended for IBD patients and attempt to answer them using scientific evidence.

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Key words: Vaccination; Efficacy; Infections; Immunization; Inflammatory bowel disease; Immunosuppressive medications; Hepatitis B vaccines; Influenza vaccines; Pneumococcal vaccines; Tetanus vaccines

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INTRODUCTION

Current therapy for inflammatory bowel disease (IBD) patients often involves agents that suppress the immune system, placing them at an increased risk for developing infections. In recent years the scientific community has made an effort toward promoting preventative and prophylactic strategies against some of these infections^[1-5]. Several guidelines have been published specifically for the vaccination and immunization of patients with IBD^[6-9]. Although a consensus exists on the need to vaccinate these patients, it seems to be underused in clinical practice, and the response to immunizations in this group of patients and the factors influencing this efficacy is unknown.

ARE IBD PATIENTS CORRECTLY VACCINATED IN CLINICAL PRACTICE?

When we try to elucidate the real efficacy of vaccinations in IBD patients, the first question we have to answer is if physicians are following the immunization and vaccination recommendations provided by the guidelines. Several studies have reported on apparent knowledge deficiencies among gastroenterologists, and show that a significant number of patients remain inadequately immunized. Yeung *et al.*^[10] assessed 167 patients and 43 gastroenterologists for immunization attitudes, knowledge and practice. Only 14% of the gastroenterologists surveyed reported taking an immunization history from most of their patients, and the majority of patients felt they did not have enough information about immunizations. Similar data were highlighted in another study, where around a half of the gastroenterologists surveyed remembered asking their patients about their immunization history^[11]. Melmed *et al.*^[12] assessed risk of exposure and immunization status among patients receiving care in an IBD specialty clinic. While about 44% of patients had at least one risk factor for hepatitis B, only 28% had been vaccinated against the infection. Only 45% of the patients in this study recalled receiving a tetanus immunization within the past 10 years, 28% reported regular flu shots and 9% reported having received the pneumococcal vaccine. A Spanish multicenter study detected vaccination against hepatitis B in only 12% of IBD patients^[13].

About 20% of the gastroenterologists surveyed reported that they did not know how important it was for their IBD patients to be up to date on specific immunizations before starting immunomodulating or biological therapy^[10].

In summary, the lack of awareness of vaccination recommendations puts IBD patients at risk of infections which might easily be avoided through a more intensive vaccination program^[14].

HOW EFFECTIVE ARE VACCINATIONS IN IBD PATIENTS?

Vaccine efficacy is defined as percent risk reduction for clinically significant infection in a vaccinated group versus a control group^[15].

Vaccination is a proven and well-established strategy for preventing infectious diseases in the general population. However, immunosuppressive illnesses in general are associated with reduced immunogenicity following vaccination^[16,17]. Moreover, patients receiving immunosuppressive therapy may have a suboptimal serological response after a variety of vaccinations^[18-22].

Hepatitis B virus

A standard 3-dose hepatitis B virus (HBV) vaccination induces protective antibody concentrations in approximately 95% of healthy individuals^[16,23,24]. However the response rate to HBV is lower in patients with IBD, es-

pecially in those receiving immunosuppressive or biological therapies. In a study published by Vida *et al.*^[25], where 43% were on immunosuppressive therapy, only 36% of patients achieved adequate hepatitis B surface antibody (HBsAb) levels (defined as > 10 IU/L). Melmed *et al.*^[12] report similar data in a study where only 33% of the subgroup of patients who were immunized had detectable antibodies to hepatitis B surface antigen (anti-HBs) titers.

According to the World Health Organization, an HBsAb concentration ≥ 10 IU/L is considered a reliable marker of protection against infection^[23,24,26-28].

As time passes, HBsAb titers frequently diminish and become undetectable^[29]. Among immunocompromised patients who respond to the vaccine, clinical HBV infection has been documented in those who do not maintain a HBsAb concentration of ≥ 10 IU/L. Based on this evidence, in the United Kingdom seroprotection against hepatitis B was redefined at ≥ 100 IU/mL, especially in those with chronic diseases or with immunosuppressive therapies^[16,28,30,31]. A recent study published by Altunöz *et al.*^[30] defined the effective immune response as an antibody level > 100 IU/L after comparing the efficacy of the standard 3-dose vaccine in a group of IBD patients and comparing it to a control group. The effective immune response was significantly higher in the control group compared to the IBD group (89% *vs* 53%).

The response rate to the HBV vaccine seems to be quite low in IBD patients. Modified dosing regimens, including doubling the standard antigen dose, might increase response rates in immunocompromised patients^[28,32-35]. Chaparro *et al.*^[36] assessed the efficacy of the HBV vaccine at a double dosage (0, 1 and 2 mo) in IBD patients and found that 60% of the IBD patients had adequate HBsAb titers (≥ 10 IU/mL), but only 34% achieved adequate immunity (≥ 100 IU/mL).

Influenza

Since influenza infection may result in serious illness in immunocompromised individuals, the Advisory Committee on Immunization Practices of the Centers for Disease Control and Prevention recommends influenza vaccination for all immunosuppressed patients^[37]. Several studies have concluded that the influenza vaccine is safe and effective in both children and adults with various chronic diseases^[38-41]. However, the immune response in immunocompromised patients varies. Most studies attempting to identify the efficacy of the influenza vaccine in IBD patients have been carried out in the pediatric population. Mamula *et al.*^[42] compared response to influenza vaccine in children with IBD and observed that the IBD group mounted less effective responses to one-third of antigens compared to controls, while patients on a combination of biologics and immunomodulators had an impaired response to two-thirds of antigens. In contrast, Lu *et al.*^[43] assessed the response rates to influenza vaccinations in IBD children and reported similar responses among IBD patients, regardless of their immunosuppressive status. A recent study performed in adults came to

similar conclusions and demonstrated that IBD patients on immunosuppressive treatment are able to mount an effective immune response^[44].

Pneumococcal

Melmed *et al*^[45] assessed response to pneumococcal vaccine in IBD adults compared to controls. Patients treated with a combination of biologics and immunosuppressants presented with an impaired response to the pneumococcal vaccine compared to controls and patients not receiving immunosuppressive therapy. Dotan *et al*^[44] reported contradictory results in a recent study where they concluded that IBD patients do not have an intrinsic immunodeficiency and display a significant increase in titers for all pneumococcal serotypes.

Tetanus

Response to tetanus immunization in IBD patients is not clear, as there are studies^[44,46] that demonstrate that IBD patients have a normal response to the tetanus vaccine, while others^[47] suggest an impaired anti-tetanus response.

WHAT ARE THE FACTORS THAT AFFECT THE EFFICACY OF VACCINATION IN IBD PATIENTS?

In healthy individuals, several risk factors for non-response to hepatitis B vaccination have been described, such as smoking, older age, male gender and a high body mass index^[24]. Influenza immunization may be less effective for those in the general population with certain medical conditions, such as systemic lupus erythematosus^[48] or patients receiving immunosuppressive treatments^[49].

The lack of response to vaccines by IBD patients may be due to associated immunosuppressive and anti-tumor necrosis factor (TNF) treatments rather than IBD *per se*. In this sense, some authors have demonstrated a suboptimal response to pneumococcal vaccination in patients with IBD under combination therapy with anti-TNF drugs and immunosuppressants^[42], while IBD patients who received non-immunosuppressive therapy exhibited a good response to the vaccine^[45].

Patients on anti-TNF agents have demonstrated a decreased immune response to influenza vaccine in IBD and non-IBD populations^[22,42,43]. HBV vaccination is also influenced by the medication administered. In a study by Chaparro *et al*^[36], 40% of patients had an adequate response to immunization (> 100 IU/L), while the response was much lower in patients undergoing biological therapy (10%), although this difference did not achieve statistical significance, probably due to the small size sample. Altunoz *et al*^[30] assessed immune response to HBV vaccination, and demonstrated in their subgroup analysis that the group of patients not under immunosuppressive treatment achieved an adequate immune response (> 10 IU/L) in 91% and an effective immune response (> 100 IU/L) in 73%, whereas only 61% and 29% of

patients under immunosuppressive therapy, respectively, acquired an adequate and effective immune response. There are contradictory results by Vida *et al*^[25] that could not demonstrate the effect of immunomodulators on the efficacy of the HBV vaccine. The small sample size and corresponding low statistical power may explain the lack of statistically significant differences. In fact, a tendency towards a lower rate of response to vaccine was found in the immunosuppressed group^[28].

Disease activity has also been implicated in impaired responses to vaccination. In this respect, a subanalysis in the study by Altunoz *et al*^[30] concluded that active IBD patients achieved a significantly lower response to vaccination compared to patients in remission (41% *vs* 63%). Thus, these authors recommend HBV vaccination during remission periods.

In summary, patients receiving anti-TNF therapy, immunosuppressants, or with active disease, are at risk of developing an inadequate serologic response. Future studies should attempt to determine if booster immunizations are needed in this group of patients^[43]. Further studies should determine if other factors such as genetics, gender or age could affect the response to immunization in IBD patients.

WHAT CAN WE DO IF REGULAR VACCINATION IS NOT EFFECTIVE?

Although routine serology testing for immunity is not recommended after HBV vaccination in adults, post-vaccination serology is advisable for high-risk individuals such as the immunocompromised, including IBD patients^[16,24,28,50]. Recommended seroprotection against HBV in immunosuppressed patients is defined as HBsAb \geq 100 IU/mL^[16]. In HBV immunization studies, 25%-50% of the patients that do not respond to a primary three-dose vaccine responded to an additional dose, and 44%-100% respond to a second three-dose course. Therefore, those patients that do not achieve an adequate immune response after the primary immunization should be revaccinated with three additional doses^[51]. Moreover, revaccination with a three-dose regimen using a double dose has also been suggested^[52].

Mamula *et al*^[42] propose a second booster dose of influenza vaccine in IBD patients with concomitant immunomodulatory and anti-TNF treatment. There are no other recommendations for the remaining vaccinations.

In conclusion, IBD patients are considered to be at risk for several infections and should therefore be immunized. Response to immunization in this group of patients is still a controversial issue. Further studies are necessary in order to clarify the true efficacy of these vaccinations and to provide recommendations on specific situations such as failure to respond. In spite of the aforementioned recommendations, several studies demonstrate that vaccines are under-prescribed and that intensive educational efforts are required in order to ensure correct adherence to the set guidelines by gastroenterolo-

gists and primary care physicians.

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Vaccines and recommendations for their use in inflammatory bowel disease

María Dolores Sánchez-Tembleque, Carmen Corella, Jose L Pérez-Calle

María Dolores Sánchez-Tembleque, Carmen Corella, Gastroenterology Department, University Hospital of Guadalajara, 19002 Guadalajara, Spain

Jose L Pérez-Calle, Gastroenterology Department, University Hospital Fundación Alcorcón, 28922 Madrid, Spain

Author contributions: Sánchez-Tembleque MD, Corella C, Pérez-Calle JL contributed equally to this work; all authors have approved the final version of the manuscript.

Correspondence to: Dr. María Dolores Sánchez-Tembleque, Gastroenterology Department, University Hospital of Guadalajara, Av. Donantes de sangre sn, 19002 Guadalajara, Spain. loladig@hotmail.com

Telephone: +34-94-9209200 Fax: +34-94-9209259

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Abstract

The patient with inflammatory bowel disease will be predisposed to numerous infections due their immune status. It is therefore important to understand the immune and serologic status at diagnosis and to put the patient into an adapted vaccination program. This program would be applied differently according to two patient groups: the immunocompromised and the non-immunocompromised. In general, the first group would avoid the use of live-virus vaccines, and in all cases, inflammatory bowel disease treatment would take precedence over vaccine risk. It is important to individualize vaccination schedules according to the type of patient, the treatment used and the disease pattern. In addition, patient with inflammatory bowel disease should be considered for the following vaccines: varicella vaccine, human papilloma virus, influenza, pneumococcal polysaccharide vaccine and hepatitis B vaccine.

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Key words: Vaccines; Inflammatory bowel disease; Crohn's

disease; Ulcerative colitis; Immunocompromised

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INTRODUCTION

Inflammatory bowel disease (IBD) is associated with a greater predisposition to infections, many of which are preventable with vaccines^[1]. Many studies show a low rate of seroprotection and adherence to vaccination programs in these patients^[1-4]. Given that at some point in the progression of the disease, 80% of patients will require treatment with corticosteroids, 40% with thiopurines and around 20% with biological drugs, it is important to consider the number of patients who will be at risk for acquiring infections due solely to the medication they receive^[5].

The vaccination plan will be made after thorough study of immunocompetence at the time of diagnosis and prior to any immunomodulating treatment and/or treatment with biological agents^[3]. After immunocompetence has been assessed, we consider the vaccine schedule, serological studies for certain infections and each patient's personal risk in order to adjust the proper vaccination regimen for each case^[2].

IS OUR PATIENT IMMUNOCOMPETENT?

Evaluation of the patient's immunocompetence is essential prior to starting any immunosuppressant treatment or putting the patient in a vaccination program^[5-7]. An IBD patient is considered to be immunocompromised when they meet any of the following criteria: first, pa-

tients on corticosteroid treatment at dosages equal to or greater than 20 mg of prednisone for more than two wk, patients on immunomodulators (azathioprine, mercaptopurine and/or methotrexate), calcineurin inhibitors (cyclosporine, tacrolimus) or anti-tumor necrosis factor (TNF) (infliximab, adalimumab or others); malnourished patients and patients with any associated immunocompromise condition [hyposplenism, asplenia, human immunodeficiency virus (HIV), *etc.*]^[5,6].

STUDIES PRIOR TO VACCINATION

Study of the patient with IBD should be individualized based on age, particular risk factors and previously-administered vaccinations prior to inclusion in a vaccination program.

The medical history should be directed towards both their bacterial (especially urinary tract infections), viral [hepatitis A vaccine, hepatitis B vaccine (HBV), herpes zoster vaccine, herpes simplex virus, HIV, varicella], fungal and tuberculosis infectious disease history. Regarding tuberculosis (TB), study should include possible current or previous contacts with tuberculosis patients as well as a history of travel to endemic areas, both in the past and in the foreseeable future^[5,6]. Careful evaluations for latent TB before the use of anti-TNF therapy is mandatory^[8]. We suspect a latent TB when there is a history of recent exposure to the disease and positive initial tuberculin skin-test (TST) or positive booster TST and no radiological evidence of active TB. Another chapter delves more on this topic^[5].

Laboratory studies will include a hemogram, C-reactive protein, urine analysis (if there is a history of urinary tract infections), the viral serologies mentioned above and a stool culture. The patient's vaccination history will always be useful if the patient has it, as will a study of group risk due to household members or occupational risk (such as teachers or healthcare workers)^[5].

VACCINATION PROGRAM

Ideal vaccination is that which is performed at diagnosis of the disease and/or prior to starting immunosuppressor therapy. In general, all patients should be vaccinated for the following: tetanus, diphtheria and polio, varicella, human papillomavirus, influenza, pneumococcus, HBV, measles, mumps and rubella^[5]. Confirmation of immunization against infections preventable by live-attenuated viruses is recommended since they are contraindicated in cases of immunosuppression, which may apply to the patient if their immune status changes over the course of the disease (Table 1).

Below we mention each of the vaccines used in these patients and the considerations to be taken into account before, during and after their administration.

Flu vaccine and pneumococcal vaccine

Flu is one of the most common vaccine-preventable

Table 1 General vaccination and contraindicated live vaccines in inflammatory bowel disease patients

| |
|--|
| IBD patients |
| General vaccination |
| VZV varicella vaccine |
| Human papilloma virus |
| Influenza (trivalent inactivated vaccine) |
| Pneumococcal polysaccharide vaccine |
| Hepatitis B vaccine in all HBV seronegative patients |
| Contraindicated live vaccines |
| Anthrax vaccine |
| Intranasal influenza |
| Measles-Mumps-rebella |
| Polio live oral vaccine |
| Smallpox vaccine |
| Tuberculosis BCG vaccine |
| Typhoid live oral vaccine |
| Varicella yellow fever |

IBD: Inflammatory bowel disease; HBV: Hepatitis B virus; BCG: Bacille calmette guerin.; VZV: Varicella zoster virus

diseases in adults^[9]. The European Crohn's and Colitis Organisation (ECCO) guidelines recommend the inactivated trivalent vaccine on an annual basis in all patients with IBD with or without immunocompromise^[5]. However, it has been confirmed that the two most common reasons for not receiving the flu vaccine were the patient's lack of awareness (49%) and fear of side effects (18%). Several studies have shown that the inactive trivalent vaccine does not affect IBD activity and caution is only recommended in those patients who have previously had adverse reactions to the vaccine^[9,10]. The Mamula study observed that among patients who received infliximab and immunomodulator treatment, response to two of the vaccine's antigens may be reduced, with significantly lower antibody titers^[11]. Later it was shown that seroconversion after the flu vaccine is not reduced by corticosteroids, methotrexate or anti-TNF treatment^[5,9,12]. However, seroconversion was not guaranteed with combined use of these agents. Thiopurines and cyclosporine did reduce the rate of seroconversion^[13-15].

The polyvalent pneumococcal vaccine is recommended in immune-compromised patients, as is revaccination at 5 years from the first vaccination if immunosuppression persists^[16]. Treatment with methotrexate is associated with a lower seroconversion rate^[5,6].

Hepatitis B vaccine

The prevalence of HBV infection in IBD is similar to the general population. The HBV vaccine is recommended in all seronegative patients^[17]. Patients with IBD may have an increased risk of developing hepatitis B^[18]. Invasive procedures such endoscopies and surgery might be some of the reasons for this. Moreover, the use of immunosuppressors may reactive a latent infection^[19-21]. Effective vaccination is only present in 12% of IBD patients although it is indicated in all with or without immunosuppression. It was observed that the only factor associated with higher efficacy of the vaccine was younger age

and the number of immunomodulators that the patient takes. The use of high antigen doses or administration of a fourth dose is sometimes needed in order to improve response to the vaccine. The response rate in the general population is related to reaching surface antibody levels (HBsAb) that are equal to or greater than 10 mU/mL. The immunological memory conferred by the vaccine produces an anamnestic immune response upon contact with the wild virus that quashes the infection or at least leads to an unapparent infection^[17,18]. Only patients who do not respond to this vaccine should be subjected to intense vaccination schedules such as administration of a fourth dose, complete revaccination and/or the use of high dosages (40 µg) of HBV surface antigen. The conventional HBV vaccination regimen requires three doses at 0, 1 and 6 mo. If faster vaccination is required, the first two doses can be administered at a three or four-week interval (0, 1 and 2 mo)^[18-20]. In another chapter will be explained in more detail the performance against HBV.

Confirmation of HBsAb seroconversion is recommended one or two months after the last dose of the vaccine^[22]. The efficacy of the vaccine is affected by the type and number of immunomodulators, with anti-TNF being the slowest. Several authors suggest that the HBsAb concentration in these patients should be greater, with titers greater than 100 mU/mL considered protective. Annual measurement of HBsAb titers is recommended and administration of a booster is recommended when these patients are older than 10, especially in patients with IBD and immunosuppressor treatment^[5].

Measles, mumps and rubella vaccine

Because this is a live attenuated virus, it is important for the patient to receive it prior to converting to an immunocompromised state^[6,23]. Previous review of the documented medical record or serology at diagnosis and protection of the seronegative group are recommended. In patients with stable IBD who are high risk of contagion, vaccination will be evaluated after discontinuing immunosuppressor therapy for at least 3 mo prior to vaccination^[23,24].

Varicella vaccine

ECCO recommends ensuring immunization against this virus in patients with IBD before their immune status changes^[5,24]. Immunization is performed at diagnosis, at least three weeks before starting immunomodulator therapy or at least 3 mo after discontinuing the immunosuppressor, if the IBD situation permits. Because this is a live attenuated virus vaccine, vaccination is not recommended in patients on immunosuppressor therapy except for seronegative patients who are at a very high risk of contagion (healthcare personnel or teachers) who are on monotherapy with thiopurine drugs. In patients in whom it is decided not to vaccinate, group protection is recommended by adequate household vaccination^[25]. The seroconversion rate in adults and adolescents is less than in children (78% *vs* 97%), so a second dose of the

vaccine is recommended 6-8 mo after the first dose. The Advisory Committee of Immunization Practices of the Centers for Disease Control and Prevention recommends discontinuing 5-aminosalicylic acid (5-ASA) until 6 mo after administering the varicella vaccine. The effects of 5-ASA may theoretically increase the risk of Reye syndrome associated with the use of live virus vaccines such as varicella^[6,23,26].

For patients with increased risk of occupational exposure to varicella (for example, an early childhood teacher or healthcare worker) without prior immunity, careful consideration of the risks of acquiring the infection need to be weighed against the potential risks and benefits of vaccination^[23,24,27]. In cases of active varicella exposure in these patients, postexposure prophylaxis with varicella zoster immunoglobulin is recommended.

Human papillomavirus vaccine

The human papilloma virus (HPV) vaccine is a quadrivalent vaccine that targets the 4 HPV serotypes associated with highest risk of progression to cervical dysplasia and cancer^[28,29]. This vaccine is indicated in all women between 11 and 14 years of age, in addition to strict cytological monitoring, according to the guidelines of each country^[30-32]. There are no studies that defend the use of this vaccine on a routine basis in women over 26 years of age. Because this is not a live virus vaccine, it can be administered to immunocompromised patients with IBD. Interruption of immunomodulator treatment should be considered in patients with extensive cutaneous warts and/or condyloma. However, past HPV infection is not a contraindication for immunomodulator therapy^[10,23].

Other vaccines

Universal administration of the tetanus and diphtheria vaccine and the inactivated polio vaccine is recommended in patients with IBD, including immunocompromised patients^[33,34].

The hepatitis A vaccine is indicated in travelers to medium or highly-endemic areas, groups at high occupational or behavioral risk and immunocompromised individuals^[35,36].

Patients with IBD who wish to travel abroad should consult their physician 4 to 6 mo prior to traveling. There are three types of vaccines in travel guidelines: systematic, recommended and required^[4]. The option of administering live virus vaccines should be considered in all IBD patients based on their immunocompromise status. Infections caused by enteropathogens can cause reactivation of quiescent IBD. The oral cholera vaccine will be indicated in all travelers to highly endemic areas. The oral typhoid vaccine should not be prescribed to patients who have undergone colectomy due to the loss of colonic bacterial colonization, though the parenteral vaccine can be administered^[5,37-40]. Patients who take immunomodulators should be discouraged from traveling to South America or Sub-Saharan Africa where yellow fever is endemic and vaccination with live virus vaccines is

required. Other vaccine-preventable diseases in travelers that should be considered are the following: Japanese encephalitis (inactivated virus), meningococcal meningitis, tick-borne encephalitis, malaria, travelers' diarrhea, tuberculosis and insect-borne diseases^[35,40].

The majority of childhood vaccinations occur in the first two years of life^[34]. The use of live virus vaccines is contraindicated in pediatric patients who receive biological treatment. In addition, children who have been exposed to biological treatment *in utero* should not receive live virus vaccines while the biological agents are still detectable in their blood, generally for the first 6 mo of life^[10,37,41]. Booster immunizations are recommended in the second decade of life at the onset of IBD. Booster recommendations include hepatitis B, diphtheria, polio, tetanus, measles, mumps, rubella and varicella^[6]. The ability to mount an effective immune response will depend on the presence of immunosuppression in the 2 wk after immunization. Adequate immune response is recovered between 3 mo and one year after discontinuing the immunosuppressor^[4,5].

In conclusion, all patients who are recently diagnosed with IBD should have their vaccine serology and immunocompromise status studied thoroughly. If the patient is immunocompromised, they will receive the following vaccines: diphtheria, tetanus, inactivated polio, pertussis, hepatitis B, pneumococcus, human papillomavirus, hepatitis A and flu, among others. If the patient is not immunocompromised, live virus vaccines will be added based on the vaccination schedule in each country and based on the patients behavioral and professional risk. This measure will prevent infectious problems over the course of their disease and reduce morbidity. The patient's disease and its treatment may vary over the course of the disease, which is why we must take prophylactic measures in order to avoid problems in the future when immunocompromise may hinder treatment. The use of immunosuppressants such as immunomodulators and/or biological agents in IBD is becoming more intense and frequent, making it necessary to take prophylactic measures such as the use of vaccines.

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Individualized hepatocellular carcinoma risk: The challenges for designing successful chemoprevention strategies

Cristina Della Corte, Alessio Aghemo, Massimo Colombo

Cristina Della Corte, Alessio Aghemo, Massimo Colombo, AM Migliavacca Center for Liver Diseases, First Division of Gastroenterology, Fondazione IRCCS Ca' Granda Ospedale Maggiore Policlinico, Università degli Studi di Milano, 20122 Milan, Italy

Author contributions: Della Corte C, Aghemo A and Colombo M jointly contributed to this paper.

Correspondence to: Massimo Colombo, MD, AM Migliavacca Center for Liver Diseases, First Division of Gastroenterology, Fondazione IRCCS Ca' Granda Ospedale Maggiore Policlinico, Università degli Studi di Milano, Via F. Sforza 35, 20122 Milan, Italy. massimo.colombo@unimi.it

Telephone: +39-25-5035432 Fax: +39-25-0320410

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Abstract

Hepatocellular carcinoma (HCC) develops in the context of environmental risk factors like chronic viral hepatitis, diabetes and alcohol exposure, often associated to an increased risk of cirrhosis. Antiviral treatments that are effective to counteract hepatitis B and C may also attenuate the risk of tumor development. However, since hepatitis B-related carcinogenesis is promoted independently of the onset of cirrhosis, such antiviral treatments as nucleo(t)side analogs can promote regression of cirrhosis, prevent clinical decompensation and variceal bleeding but not HCC. This means that in successfully treated patients with cirrhosis, HCC is often the consequence of their extended survival. In hepatitis C patients, a sustained virological response to interferon-based therapies can reduce the rate of HCC development, even in patients with cirrhosis who experience histological regression of their liver disease. Future therapies aimed at this endpoint in at risk populations should take into consideration pretreatment patient stratification for host, viral and environmental risk factors. In this context the recent discovery of single nucleotide polymorphisms involved in the immune

system function and tumorigenesis, might permit enrollment of populations of patients enriched with HCC risk factors for targeted chemopreventive therapies. This could finally pave the way to personalized algorithms, as already seen in the diagnosis and treatment schemes for chemoprevention.

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Key words: Hepatocellular carcinoma; Hepatitis C virus; Peginterferon; Hepatitis B virus; Human immunodeficiency virus; Nucleoside analogues; Sustained virological response; Single nucleotide polymorphisms

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INTRODUCTION

Hepatocellular carcinoma (HCC) is the fifth most common cancer in men and seventh in women, accounting for 7% of all cancers, and the third most common cause of cancer related death, worldwide^[1]. HCC is characterized by peculiar biological and clinical features that may be relevant to primary and secondary prevention strategies. Indeed, up to 95% of all HCCs occur in the context of known and preventable risk factors, such as chronic viral hepatitis, alcohol abuse and cirrhosis (Table 1). Secondly HCC is one of the few cancers that can be diagnosed by radiological techniques, hence avoiding invasive diagnostic methods. Lastly, HCC is the only cancer that does not contraindicate patients to organ transplantation but actually benefits from liver transplantation in accurately selected patients. These rather unique features of HCC, in theory, could be used to implement therapeutic

and diagnostic measures to reduce or prevent the rate of HCC development in patients at risk, enforce surveillance programs in at risk patients aimed at early diagnosis and individualize treatment options based upon patient and tumor characteristics once the tumor has developed. Some of these points have been thoroughly investigated by clinical research studies and have led to significant advances in clinical practice that include the development of specific guidelines on surveillance protocols and on individualized treatment algorithms for HCC. Less successful have been attempts to design primary prophylaxis studies in at risk populations, such as those with hepatitis B virus (HBV) or hepatitis C virus (HCV), a fact that is especially worrisome as HCC arising in these patients still represents the core of the HCC epidemic in most countries. Indeed, chronic HBV and HCV infection affect roughly 400 and 200 million people, respectively, worldwide, and are the current leading causes of liver-related death and the main indication for liver transplantation in developed countries^[2].

HCC RISK FACTORS

Hepatic disease status

HCC almost invariably occurs in a histologically abnormal liver, the mere existence of chronic liver disease representing a potential risk for the development of this tumor. Indeed, on the top of specific virus mechanisms that may be directly carcinogenetic to the liver, chronic necro-inflammation and accumulation of reactive oxygen species contribute to carcinogenesis through chromosomal injury^[3]. Cirrhosis, the end stage consequence of hepatic inflammation resulting in nodular transformation of the liver, is considered a premalignant condition, independently of etiology. Whether the association of cirrhosis with HCC reflects a long lasting exposure to carcinogenetic agents capable of causing liver cell inflammation or a carcinogenetic effect of disrupted lobular organization, is a matter of debate^[4].

The prevalence of cirrhosis in persons with HCC is about 80%-90% in autopsied series worldwide, whereas approximately only 10%-20% of HCCs may be encountered in non-cirrhotic patients^[5]. However, only a few non-cirrhotic patients with HCC have absolutely normal liver histology, as the majority show fibrosis stages that range from no fibrosis (F0) to septal (F2) and bridging fibrosis (F3) with necroinflammation, steatosis, and liver cell dysplasia^[6-8].

In a detailed study from Japan involving 490 untreated patients with chronic hepatitis C, the HCC incidence per 100 person years increased with the stage of fibrosis at diagnosis, from 0.4 among patients with stage F0 or F1 to 1.5, 5.1 and 6.9 among those with fibrosis stages F2, F3 and F4, respectively^[9]. The presence of large cell change^[10], irregular regeneration of hepatocytes and macroregenerative nodules have been evaluated as morphologic predictors of HCC in cirrhosis independently of baseline disease^[10-14].

Table 1 Risk factors for hepatocellular carcinoma

| Viral | Environmental | Host related |
|-------|--------------------|--------------|
| HIV | Alcohol exposure | Age |
| HBV | Metabolic syndrome | Male sex |
| HCV | | Genetics |

HIV: Human immunodeficiency virus; HBV: Hepatitis B virus; HCV: Hepatitis C virus.

Cirrhosis is not only driven by viral hepatitis but can also be the consequence of other conditions like hemochromatosis, Wilson's disease, antitrypsin deficiency, primary biliary cirrhosis, and autoimmune hepatitis^[5]. Patients with cirrhosis stemming from genetic hemochromatosis have a 20-fold relative risk of developing HCC, compared to the general population, with an annual incidence of about 3%-4%^[15]. In general, the risk of HCC in patients with cirrhosis increases in parallel to ageing, male gender, disease activity, degree of liver cell proliferation, presence of hepatobiliary phenotype on liver cells and serum level of alfa-fetoprotein. Patients with more advanced Child-Pugh scores are at higher risk of liver cancer, Child-Pugh class B/C patients having a 3-fold increased risk compared to Child Pugh A patients^[16,17].

Viral factors

HBV: The annual incidence of HCC in patients with chronic hepatitis B, ranges from 2% to 5%, in strict correlation with the histological stage of the underlying liver disease, serologic status and geographic area^[18]. Chronic HBV infection is commonly considered a primary risk factor for the development of HCC, exerting its pro-oncogenic properties through both indirect and direct mechanisms^[19-23]. The indirect mechanisms are related to its propensity to induce continuous or recurrent phases of liver necroinflammation and to promote the progression of chronic hepatitis to cirrhosis, often preceding the development of HCC^[24]. Most cases of HBV-related HCC (70%-90%) occur in patients with cirrhosis secondary to chronic necroinflammation, but HBV itself can cause HCC in the absence of cirrhosis through direct carcinogenic mechanisms that have been related to the capacity of HBV to integrate into the host's genome and to produce proteins (X protein and truncated pre-S protein) with potential transforming properties^[25].

In Europe, HBV-related HCC is associated with cirrhosis in the majority of patients^[26,27], whereas this is not true in Asia and Africa where the tumor is common also among carriers with mild hepatic fibrosis, likely as a consequence of long standing infection that is often acquired perinatally^[28-30].

HCC risk may be modulated by viral load^[31-35], even when this predictor is measured years before tumor diagnosis, and can be modified also by serum hepatitis B e antigen (HBeAg) persistence and hepatitis B surface antigen (HBsAg) status^[31,36-38].

Recently, it has been demonstrated that HCC may

also develop in Asian carriers with inactive hepatitis (*i.e.*, persistently normal alanine transaminase and serum HBV DNA < 2000 IU/mL)^[26,27,31,39] and HCC was also found to develop in a significant number of patients from 1 year to more than 10 years following spontaneous clearance of serum HBsAg, with a higher risk among patients who had HBsAg seroclearance after 50 years of age^[40]. Other studies with sensitive amplification assays have shown that HBV DNA persists in serum or liver as an occult HBV infection, even following spontaneous serologic recovery from transient HBV infection (HBsAg-negative status)^[41].

The estimated incidence rates of HCC in subjects with chronic HBV infection in East Asian countries is 0.2 per 100 person-years in inactive carriers, 0.6 per 100 person-years for those with chronic HBV infection without cirrhosis, and 3.7 per 100 person-years for those with compensated cirrhosis; while in Europe/North America the HCC incidence rate was 0.02 per 100 person-years in inactive carriers, 0.3 per 100 person-years in subjects with chronic HBV without cirrhosis, and 2.2 per 100 person-years in subjects with compensated cirrhosis^[42].

Male sex, positive family history and African origins are added risk factors for HCC development in HBV positive patients^[28-30]. Genotype B of the HBV seems to be associated with lower rates of HCC development compared to genotype C of HBV^[43-48], since it is characterized by anticipated HBeAg seroconversion, higher rates of sustained remission after HBeAg seroconversion, less active hepatic necroinflammation and slower progression to cirrhosis. Genotype A infections have a generally more favorable outcome than genotype D infections which predominate in the Mediterranean basin^[49,50].

HCV: Chronic infection with HCV affects more than 170 million individuals, representing a major cause of morbidity and anticipated liver-related death in western countries and Japan. HCC is the dominant, first to appear complication of patients with long standing infection complicated by cirrhosis, which in fact is considered the main risk factor for HCC in patients with HCV infection, as only a minority of HCC cases develop in patients with mild-to-moderate HCV disease. Malignant transformation of hepatocytes occurs through a pathway of increased liver cell turnover, induced by chronic liver injury and regeneration in a context of inflammation and oxidative DNA damage^[51] which facilitates the occurrence of genetic and epigenetic alterations that over decades can lead to the development of HCC. Still there are lines of evidence that support a direct role for HCV in cancer promotion^[52]. Clinically this is supported by the incidence of HCC in HCV-related cirrhosis being higher than that reported in cirrhosis resulting from other liver diseases such as autoimmune hepatitis or metabolic syndrome^[53]. Whether HCV genotype 1b produces a higher risk of HCC development compared to other HCV genotypes, is debatable, yet *de facto* reinforces the concept that HCV itself may promote HCC^[54].

The HCV genome consists of a single stranded positive sense RNA of approximately 9.6 kb in length, that encodes a 327 kDa polyprotein that is processed into 10 mature structural and non structural viral proteins^[55]. HCV proteins have been shown to impact on cell signaling, transcriptional modulation, transformation, apoptosis, membrane rearrangements, vesicular trafficking and translational regulation^[52]. Four of the HCV gene products (core, NS3, NS4B and NS5A) show transformation potential in cell culture systems. Transgenic mouse models support HCV induced carcinogenesis, with transgenic lineages with high level liver specific expression of the core protein and transgenic mice for the two envelop proteins E1 and E2 developing HCC even in the absence of hepatic inflammation^[56,57]. HCV can also induce endoplasmic reticulum (ER) stress, a homeostatic mechanism that regulates cellular metabolism and protein synthesis in response to perturbations in protein folding and biosynthesis^[58]. Persistent ER stress may result in intra- and extra-cellular accumulation of DNA-damaging factors known to predispose cells to mutagenesis.

Human immunodeficiency virus: Human immunodeficiency virus (HIV) infection significantly increases the risk of liver-related morbidity and mortality, primarily because during the highly active antiretroviral therapy (HAART) era an important reduction in HIV-related complications has occurred, leading to the emergence of co-infection with HBV (6%-14%) and HCV (25%-30%) as hepatotoxic factors in addition to excessive alcohol consumption, non-alcoholic fatty liver disease and drug-induced liver injury^[59].

While the MORTAVIC study in 2001 indicated HCC to be responsible for 25% of all liver deaths in HIV patients, in the HAART era there are studies showing HCC developing in co-infected patients to be more aggressive, to present at an earlier age and to less frequently be curable compared to HCC developing in HCV mono-infected patients. A direct oncogenic effect of the virus has therefore been hypothesized by some^[60,61].

Environmental risk factors

Alcohol: Chronic consumption of more than 80 g of ethanol per day for more than 10 years increases the risk of HCC by approximately 5-fold; in women even smaller quantities of alcohol consumption (10 g/d) are associated with a significant (24%) increase of HCC risk^[62]. Alcohol abuse in patients with chronic hepatitis C doubles the risk for HCC, due to a synergism between alcohol and hepatitis C in anticipating HCC onset or causing more severe histological tumor patterns. In a HCC cohort in Austria, alcoholic liver disease was the likely cause of HCC in 35% of subjects^[63], whereas in the United States, the hospitalization rate for HCC related to alcoholic cirrhosis is (8-9)/100 000 people per year compared to about 7/100 000 people per year for hepatitis C^[64]. Heavy alcohol consumption can lead to liver damage by direct liver cell injury and generation of toxic metabolites, thereby

transforming the liver in a mitogenic and mutagenic environment. This not only causes development of liver fibrosis and cirrhosis^[65,66], but also increases conversion of pro-carcinogens to carcinogens through oxidation and metabolism of ethanol in the liver^[67]. Acetaldehyde and oxygen free radicals deriving from ethanol metabolism may also directly induce cell damage by initiating peroxidation of membrane lipids, through oxidative stress^[68].

Metabolic syndrome: Diabetes is an independent risk factor for HCC; a recent surveillance epidemiology and end results based re-analysis has shown a 2-3-fold increase in the risk of HCC, regardless of the presence of other major risk factors^[69]. Further evidence that obesity and diabetes are either jointly or independently associated with an increased risk of HCC is provided by an Italian case control study and by several large-scale epidemiological studies, that have associated the overweight and obesity pandemic in the general population with an increase in HCC risk^[70].

In a cohort of 900 000 American adults, the risk of dying from liver cancer was 4.5 times higher in men with a body mass index of 35 kg/m² or above compared to men with a normal body mass index (18.5-24.9 kg/m²)^[71]. A meta-analysis of case control and cohort studies concluded that the relative risk of liver cancer was 1.17 for overweight subjects and 1.89 for obese patients^[72]. These and other studies contribute to the increased recognition of non-alcoholic steatohepatitis (NASH) as a cause of cirrhosis and HCC, with many patients progressing to liver cancer without histological evidence of advanced fibrosis or cirrhosis^[73,74].

A yearly cumulative incidence of HCC in 2.6% of patients with NASH compared to 4.0% of those with HCV over a median follow-up time of 3.2 years was also demonstrated in patients referred for liver transplant evaluation at Cleveland Clinic in Ohio^[75]. Interestingly, in this cohort of patients, older age at diagnosis of cirrhosis and any alcohol consumption were independently associated with the development of HCC in a NASH-cirrhosis population, suggesting that alcohol intake, even in socially accepted amounts, may potentially increase the risk of HCC development both in NASH- and HCV-cirrhotic patients.

The precise mechanisms through which metabolic factors drive HCC development are complex and beyond the scope of this article; however, major systemic and liver specific molecular mechanisms like insulin resistance, hyperinsulinemia, increased expression of tumor necrosis factor signaling pathways and direct lipotoxicity are major players in the development of HCC.

Host-related risk factors

Age and sex: Among host factors, older age and male sex have consistently been found in longitudinal studies to be associated with an increased risk of HCC among persons with cirrhosis of different etiologies, with the caveat, however, that age might reflect longer duration of

hepatic disease^[76-79].

In patients with cirrhosis, there is a striking gender imbalance in HCC incidence, with a predominance for males independently from geographic area, etiologic factors and ethnicity with a male to female ratio between 2:1 to > 4:1 being reported across studies^[80]. While male preponderance could just reflect the greater incidence of viral hepatitis and alcohol-related disease in males, it could also be related to hormonal diversity. High serum levels of testosterone have been associated with increased HCC risk in nested case-control studies of HBV carriers in Taiwan and Shanghai^[81], while a cross-sectional study in male veterans with chronic HCV in the United States associated higher total serum levels of testosterone with risk of advanced hepatic fibrosis and inflammatory activity, without examining the association with HCC^[82].

Among the complex molecular mechanisms behind gender disparities in HCC, estrogens may play a role in increasing interleukin-6 (IL-6) production and modulation of gene expression through FOXA transcription factors that have been shown to prevent HCC development in experimental models. However, more data are still needed to define the implication of sexual hormones in the molecular pathogenesis of HCC^[83].

Single nucleotide polymorphisms associated with HCC

Genetic host factors play an important role in HCC development. The most common form of genetic variation between individuals is single nucleotide polymorphisms (SNPs) which are a variation in a DNA base at a particular nucleotide locus. A common SNP is defined by having a minor allele frequency of at least 5%. In studies designed with a “candidate gene approach”, a limited number of biologically plausible SNPs were tested. The starting hypothesis is that a given variant in a specific gene involved in a pathway that influences HCC development can sufficiently alter either protein function or expression, and result in the modulation of cancer risk. Another approach to study genetic factors is through “genome wide association studies” (GWAS). These studies are by definition “hypothesis free”, and compare allele/genotype frequency of common variants between cases and unaffected controls and test 100 000 of tag SNPs reflecting common genetic variations across the entire human genome. To reach GWAS significance (P value = 0.05/number of SNPs tested) a P value in the order of $< 10^{-8}$ is typically required^[84].

To identify susceptible genetic variants for HCV- and HBV-related HCC many genetic association studies have been conducted; unfortunately most publications suffer from major methodological drawbacks because of their case-control, retrospective and single center design, mainly involving selected Asian populations. This minimizes the potential importance of ethnic diversity, calling for external validation in populations of different ancestry before effectively translating results to clinical practice. Prospective cohort studies conducted in large homogeneous populations with sufficient number of events

Table 2 Genetic associations with hepatocellular carcinoma at genome wide association studies level

| Study | Patients | n | SNP locus | Strength | Comment |
|--|------------------------|------|-----------|----------------------|---|
| Kumar <i>et al</i> ^[85] | CHC | 1730 | MICA | OR 1.3 | This study considers HCV-negative individuals as the controls, hence it isn't useful in distinguishing HCC high risk population among HCV-related cirrhotic patients |
| | HCV neg | 8376 | | | |
| | CHC-HCC | 2115 | | | |
| Miki <i>et al</i> ^[86] | CHC | 2390 | DEPDC5 | OR 2.2 | Given the relatively small number of cases in the GWAS phase, the statistical power to detect an effect caused by this SNP was only 50%, compared to the 80% recommended to detect an association of the expected effect size |
| | CHC-HCC | 922 | | | |
| Zhang <i>et al</i> ^[88] | CHB | 1790 | KIF1b | 0.6 | Confounding non-genetic HCC risk factors cannot be ruled out in multivariate analysis |
| | CHB-HCC | 2317 | | | |
| Chan <i>et al</i> ^[89] | CHB | 825 | DLC1 | 1.3 | Factors of selection bias cannot be excluded because 11.6% of the "genotyping cohort" had > 60 g alcohol consumption per day, secondly because 16.5% of the controls received antiviral treatment before enrolment |
| | CHB-HCC | 595 | | | |
| Clifford <i>et al</i> ^[123] | HCC ¹ | 386 | MHC II | $p1 \times 10^{-13}$ | The viral infection status of controls was not ascertained with the consequence that there might be hypothetical cases with chronic liver disease |
| | Cirrhosis ² | 86 | | | |
| | Controls ³ | 787 | | | |

¹89% HBV or HCV viral infection; ²76% HBV or HCV viral infection; ³Not known viral status. CHC: Chronic hepatitis C; CHB: Chronic hepatitis B; GWAS: Genome-wide association study; HCC: Hepatocellular carcinoma; HCV: Hepatitis C virus; SNP: Single nucleotide polymorphism.

during follow-up require a long time to be conducted and therefore are still scarce.

The genetic regions found to have a significant statistical association with HCC at a GWAS level in chronic hepatitis C patients are the 5' flanking region of *MICA* (the MHC class I polypeptide-related sequence A gene, on chromosome 6p21.33) which is essential for direct immune system functions^[85] and isoform1 of the *DEPDC5* locus on chromosome, where deletion of the region containing DEPDC has been reported in malignant brain glioblastomas^[86]. Also the MHC class II locus presenting antigen to CD4 p (helper) T cells contains three variants strongly associated with HCC, probably related to altered MHC class II proteins that result in an ineffective T-cell response^[87]. *KIF1B* was identified as a new susceptibility locus for HCC in chronic HBV carriers; the loss of heterozygosity on this locus is a common genetic lesion in a broad range of human cancers^[88]. Variations at chromosome 8p12 may also promote HCC in patients with HBV and risk-associated 8p12 SNPs or haplotypes might have an interacting effect on the DLC1 locus (Deleted in Liver Cancer 1), which becomes more susceptible to deletion or chromosomal loss^[89] (Table 2).

In the HALT-C study cohort a significant association between a polymorphism on epidermal growth factor gene and HCC was detected in a retrospective case-control study, by the use of a candidate gene approach^[90]. With this same approach in HBV patients many other genes involved in immune and tumorigenesis processes were found to be significantly associated with HCC development, among these cytotoxic T-lymphocyte antigen 4 gene^[91], the promoter region of *MCM7* gene and the enhancer II (Enh II)^[92], basal core promoter, and precore regions of HBV^[93].

Still the SNPs identified so far only partly explain the overall variability in HCC susceptibility as they carry a rather low risk ratio for HCC development, have been mostly assessed in well selected patients where HCC tissue was obtained following surgical resection and

therefore at this moment do not permit prediction at the individual and population level. Whole genome sequencing analysis of HCCs nodules are promising approaches; recently Fujimoto *et al*^[94] have identified etiological diverse specific mutation patterns and several mutation of chromatin regulators in 27 HCCs.

In 2008 the International Cancer Genome Consortium was launched with the purpose to coordinate large-scale cancer genome studies in tumors from 50 cancer types and/or subtypes that are of main importance across the globe, including HCC. Systematic studies of more than 25 000 cancer genomes at the genomic, epigenomic and transcriptomic levels are needed to reveal the repertoire of oncogenic mutations, uncover traces of the mutagenic influences, define clinically relevant subtypes for prognosis and therapeutic management, and enable the development of new cancer therapies. Hopefully in the not so distant future, partial or full cancer genomes will routinely be sequenced as part of the clinical evaluation of cancer patients and as part of their on-going clinical management^[95].

CHEMOPREVENTION

Although all host related HCC risk factors are unmodifiable and at this moment do not call for any preventive measures, environmental and viral factors can either be prevented, suppressed or in some cases cured by effective treatments^[96]. For viral hepatitis B and C and for HIV infection the question is whether the effective antiviral treatments that have been developed in the last decade to treat patients with virus induced liver disease can be considered chemopreventive approaches for HCC in parallel.

HBV

Prevention of HCC in patients with a sustained suppression of HBV following interferon or NUC therapy is far from being convincingly demonstrated, especially since this dramatic and life threatening complication in most

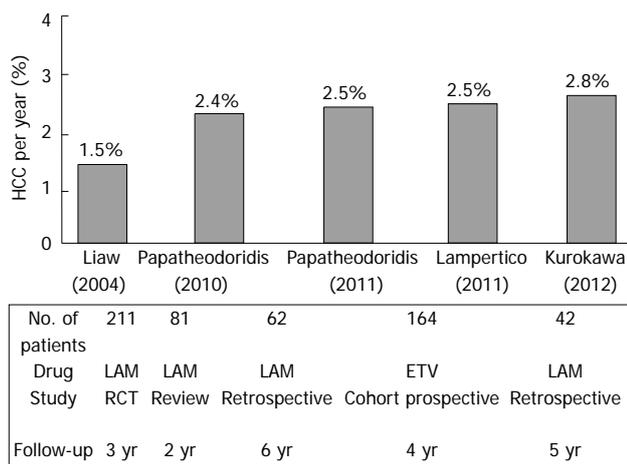


Figure 1 Hepatocellular carcinoma rates in nucleos(t)ide analogs-naive cirrhotic patients with long-term response. Several studies have shown that hepatitis B virus patients who achieve a virological response to lamivudine in some cases still develop hepatocellular carcinoma (HCC) as the only complication. LAM: Lamivudine; ETV: Entecavir; RCT: Randomized controlled trial.

patients occurs many decades after viral infection and many years after diagnosis of hepatitis.

The benefits of a virological suppression in patients with cirrhosis are easier to evaluate as these patients are the closest to developing hepatitis-related complications. Several studies have shown that HBV patients who achieve a virological response to lamivudine are protected against HCC during the precirrhotic phase of infection, in fact this is the only complication arising in virological responders with preexisting cirrhosis^[97-101] (Figure 1). In a systematic review of studies of nucleos(t)ide analog treatment of patients with HBV it was clearly shown that HCC was prevented in patients with chronic hepatitis but not in those with cirrhosis, and in general in patients that could not achieve complete virological suppression^[98]. This was confirmed by a recent cohort study from Greece where long-term cirrhotic patients responding to lamivudine remained at risk of developing liver cancer^[99]. All these studies enrolled patients treated with lamivudine or rescued with adefovir, *i.e.*, regimens characterized by limited potency and low to moderate genetic barrier, which are not recommended any more by International guidelines for treatment of patients with chronic hepatitis B in general, and especially in patients with compensated cirrhosis. This raised the hope that more potent anti-HBV drugs, like entecavir and tenofovir, might confer an advantage in terms of HCC prevention in responders with cirrhosis, however with disappointing results. A multicenter study in Italy conducted in patients with compensated cirrhosis who achieved persistently undetectable serum HBV DNA during 4 years of entecavir monotherapy, showed an annual rate of neoplastic transformation of the liver of approximately 2.5%, that mimics the HCC rates in untreated HBeAg negative patients in Europe^[100]. The reasons for these negative results may be many fold: it should be recalled that development of HCC in successfully treated patients with cirrhosis is often the conse-

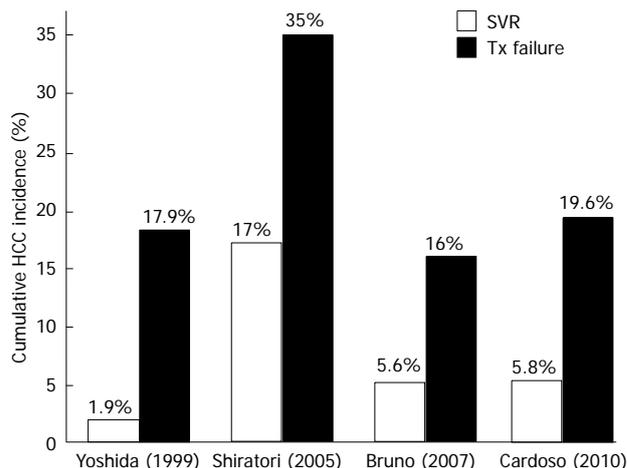


Figure 2 Cumulative incidence of hepatocellular carcinoma in patients with cirrhosis stratified by sustained virological response. Studies in Caucasian patients as well as in patients from Asia have repeatedly shown that a sustained virological response (SVR) following interferon-based therapies can reduce the rate of hepatocellular carcinoma development in hepatitis C virus related cirrhosis. HCC: Hepatocellular carcinoma.

quence of an extended survival provided by nucleot(s)ide analogues (NUCs) preventing clinical decompensation, as it was the case in the Italian multicenter study. Another explanation is that HBV related liver carcinogenesis is likely to be promoted by cellular events that are established early during chronic infection with HBV, independently on the onset of cirrhosis. This would explain why NUCs can determine regression of cirrhosis, protection from clinical decompensation and variceal bleeding but not from HCC development^[102-104].

HCV

With regards to HCV there is conclusive evidence that an sustained virological response (SVR) following interferon (IFN) based therapies can reduce the rate of HCC development, independently of fibrosis stage^[9,105-108]. This has been repeatedly shown both by studies in Caucasian patients as well as in patients from Asia (Figure 2). Interestingly an SVR does not completely negate the risk of HCC, as a small group of patients will still develop HCC in the long term follow-up period, although the annual rate is extremely low and reduced compared to non responders or untreated patients. In an Italian study on 920 patients with compensated cirrhosis who received IFN monotherapy and who were followed-up for a median period of 96 mo after treatment completion, Bruno *et al*^[109] found that SVR patients had significantly lower rates of HCC (0.66 *vs* 2.10 per 100 person-years) and liver-related death (0.19 *vs* 1.44 per 100 person-years, *P* < 0.001) compared to those with treatment failure. Similar findings were also reported by Cardoso *et al*^[110] in a study that analyzed 307 patients with bridging fibrosis or cirrhosis followed-up for 3.5 years after the end of treatment. The authors found significantly lower incidence rates per 100 person-years of liver-related complications, liver-related deaths, and HCC in SVR than in non-SVR patients (0.62

vs 4.16, 0.61 *vs* 3.76 and 1.24 *vs* 5.85, respectively; $P < 0.001$ for all comparisons). A study from France analyzing a cohort of HCV cirrhotics subjected to repeated liver biopsies following an SVR, has linked the protective effect of an SVR on HCC development on regression of cirrhosis^[111]. Indeed the 44% of patients who showed cirrhosis regression had 0% occurrence of HCC during a 118 mo follow-up period, while in the 22 patients without cirrhosis regression 3 patients developed HCC.

Unfortunately the clinical significance of the protective effect of an SVR on HCC development in HCV patients with cirrhosis is limited as the vast majority of HCV cirrhotics fail to achieve a SVR to IFN plus ribavirin therapy. In fact cirrhosis is one of the main factors associated with treatment failure to any regimen based on IFN, hence most of HCV cirrhotics even if treated remain at high risk of HCC. In this context it is important to understand the role played by other clinical and demographic factors in determining treatment outcome to PegIFN plus ribavirin and their interplay with the presence of cirrhosis^[112,113]. Not all cirrhotics show reduced rates of SVR, since the presence of a treatment favorable HCV genotype, HCV-2 and in part HCV-3, or a protective SNP in the IL-28B coding region in HCV-1 and HCV-4 patients can lead to SVR rates in the 70% range^[114-116]. IL-28B has been shown by several studies to be the strongest baseline predictor of treatment outcome; however its clinical utility in terms of individualized chemoprevention strategies is limited by its relatively low negative predictive power that should never lead to treatment deferral, especially considering the significant advantages obtained by an SVR in patients with advanced fibrosis/cirrhosis^[117].

Given that IFN also exerts several important indirect effects in the virus-infected liver that might result in tumor prevention, including immunostimulation and expression of HLA class 1 MC, and inhibition of mutagenic factor beta-UGF, three randomized controlled studies were designed to assess if a long term course of low dose PegIFN therapy could reduce the rate of liver related complications in patients with advanced fibrosis/cirrhosis who did not achieve an SVR to a full course of PegIFN plus ribavirin therapy^[116-120]. Although the three studies are hardly comparable due to differences in the patients characteristics and in the assigned treatment regimens, still they unanimously failed to demonstrate any positive impact of PegIFN maintenance therapy on HCC incidence rates^[121]. A recent extended analysis of the original HALT-C study, performed by Lok *et al.*^[122] and focused on the development of HCC, has partially contradicted these findings as long-term PegIFN maintenance therapy was associated with reduced HCC rates in patients with pre-treatment cirrhosis. The cumulative incidence of HCC at 3, 5 and 7 years was 2.6%, 5.1% and 7.8% in the PegIFN group and 4.0%, 11.1% and 24.2% in the untreated group (log-rank test, $P = 0.009$).

These results are hard to interpret and require caution before suggesting PegIFN maintenance therapy as an ef-

fective chemoprevention strategy in HCV cirrhotics; still they provide important clues and directions for future studies. Indeed, it shows patients enrolled in chemoprevention studies need to be stratified for risk factors at baseline; in this particular case enrolling patients with different disease severity stage might have precluded observation of a protective effect of low dose PegIFN on HCC development. Secondly it suggests that conducting studies on high risk rather than medium risk patients might provide more clinically meaningful data.

HIV

Although a detailed discussion about the effect of antiviral therapy in HIV positive patients on HCC development is beyond the purpose of this study, convincing evidence has been provided that HCC incidence is rising amongst the HIV positive population receiving HAART, almost exclusively in patients with concomitant HCV or HBV infection. The main explanation behind these findings is probably the increased longevity obtained by effective antiviral treatment in this population of patients. An investigation of the Swiss HIV cohort assessed that latest CD4 β cell count and CD4 β cell count percentage were significantly associated with HCC, but no association between HAART use and HCC risk was detected^[123]. Similarly, Sulkowski *et al.*^[124] reported that HCC occurred in many patients despite the use of effective HAART for a median of more than 7 years.

Thus, antiretroviral therapy is unlikely to modify the risk of HCC in HCV-infected patients in whom the risk of cancer is driven by HCV-related cirrhosis and where antiretroviral therapy is also known to have some direct hepatotoxic effects which are worsened by co-infection with HBV or HCV, raising the possibility that HAART *per se* might hasten the progression to cirrhosis and hence HCC^[125]. However the role of HAART on HCC development is controversial because its ability to preserve immune system functions could in theory provide a putatively more active anti-tumor response, since once HCC has developed, chronically low CD4+ and CD8+ lymphocyte counts may result in more rapid growth and spread of disease.

CONCLUSION

HCC develops in the context of readily identifiable environmental risk factors, first of all HBV and HCV, a fact that in theory should enable for successful primary prophylaxis. There are solid treatment endpoints that allow hepatologists to determine the efficacy of an antiviral regimen and its ability to positively modify both the natural course of the disease as well as to reduce the risk of HCC development.

Unfortunately in patients with chronic HBV and HCV infection tumor prevention is an end point quite difficult to assess in patients with early, mild hepatitis whereas it can only partially be achieved in patients with established cirrhosis given they are at higher risk of liver cancer.

In these patients there is increasing need to improve diagnosis rate, ensure equal access to antiviral therapies and develop well tolerated therapies that will finally allow most, if not all, patients with viral hepatitis to benefit from effective antiviral treatment. In fact it is estimated that as few as 35% of patients with chronic HBV and only 25% of HCV patients are aware of their disease in developed countries, with this number obviously shrinking even more in developing countries^[126]. Moreover in Europe and the United States, even if patients are diagnosed correctly only 1%-16% of HBV and HCV patients are estimated to receive treatment^[127-129].

This highlights that one of the key issues for therapies aimed at HCC chemoprevention is to broaden access to therapies by containing costs and increasing disease awareness, or eventually concentrate treatment in those more at risk of HCC development. Performing prospective studies where patients are stratified according to complex models of "genomic risk prediction" that incorporate various panels of specific SNPs, is undoubtedly a bold and possibly unreachable goal, but still it could lead to individualized chemoprevention strategies in the future, while reducing the number of patients needed to be enrolled into chemoprevention studies to assess efficacy of a treatment regimen. Until this is reached, secondary prevention through surveillance of risk populations aiming at early diagnosis is the only approach to improve treatment and survival of patients with HCC.

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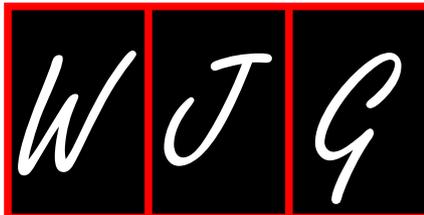
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Endoscopic ultrasonography guided biliary drainage: Summary of consortium meeting, May 7th, 2011, Chicago

Michel Kahaleh, Everson LA Artifon, Manuel Perez-Miranda, Kapil Gupta, Takao Itoi, Kenneth F Binmoeller, Marc Giovannini

Michel Kahaleh, Division of Gastroenterology and Hepatology, Weill Cornell Medical College, New York, NY 10021, United States

Everson LA Artifon, Department of Gastroenterology, University of São Paulo Medical School, São Paulo 05403-000, Brazil

Manuel Perez-Miranda, Endoscopy Unit, Hospital Universitario Rio Hortega, 47012 Valladolid, Spain

Kapil Gupta, Pancreatic Biliary Department, Cedars-Sinai Medical Center, Beverly Hills, CA 90048, United States

Takao Itoi, Department of Gastroenterology and Hepatology, Tokyo Medical University, Tokyo 160-0023, Japan

Kenneth F Binmoeller, California Pacific Medical Center, San Francisco, CA 94115, United States

Marc Giovannini, Department of Gastroenterology, Paoli-calmettes Institute, 13273 Marseille, France

Author contributions: Kahaleh M, Artifon ELA, Perez-Miranda M, Gupta K, Itoi T, Binmoeller KF and Giovannini M designed and organized the meeting and provided summary results; Kahaleh M summarized the data and wrote the paper.

Correspondence to: Michel Kahaleh, MD, FASGE, Professor of Medicine, Chief of Endoscopy, Division of Gastroenterology and Hepatology, Weill Cornell Medical College, 1305 York Avenue, 4th Floor, New York, NY 10021, United States. mkahaleh@gmail.com

Telephone: +1-646-9624000 Fax: +1-646-9620110

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patients with advanced malignancy, seeking minimally invasive interventions and improved quality of life. With the advent of biliary drainage *via* endoscopic ultrasound (EUS) guidance, EUS guided biliary drainage has been used more frequently within the last decade in different countries. As with any novel advanced endoscopic procedure that encompasses various approaches, advanced endoscopists all over the world have innovated and adopted diverse EUS guided biliary and pancreatic drainage techniques. This diversity has resulted in variations and improvements in EUS Guided biliary and pancreatic drainage; and over the years has led to an extensive nomenclature. The diversity of techniques, nomenclature and recent progress in our instrumentation has led to a dedicated meeting on May 7th, 2011 during Digestive Disease Week 2011. More than 40 advanced endoscopists from United States, Brazil, Mexico, Venezuela, Colombia, Italy, France, Austria, Germany, Spain, Japan, China, South Korea and India attended this pivotal meeting. The meeting covered improved EUS guided biliary access and drainage procedures, terminology, nomenclature, training and credentialing; as well as emerging devices for EUS guided biliary drainage. This paper summarizes the meeting's agenda and the conclusions generated by the creation of this consortium group.

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Abstract

Endoscopic retrograde cholangiopancreatography (ERCP) has become the preferred procedure for biliary or pancreatic drainage in various pancreatobiliary disorders. With a success rate of more than 90%, ERCP may not achieve biliary or pancreatic drainage in cases with altered anatomy or with tumors obstructing access to the duodenum. In the past those failures were typically managed exclusively by percutaneous approaches by interventional radiologists or surgical intervention. The morbidity associated was significant especially in those

Key words: Endoscopic ultrasound; Biliary drainage; Endoscopy-guided cholangiopancreatography; Endoscopic ultrasound guided; Pancreatic drainage; Endoscopic retrograde cholangiopancreatography

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INTRODUCTION

Endoscopic retrograde cholangiopancreatography (ERCP) is the procedure of choice for biliary drainage in obstructive jaundice resulting from pancreatobiliary cancer. Although this is successful in more than 90% of the times biliary drainage cannot be achieved *via* ERCP in certain cases^[1,2]. This is usually related to a difficult anatomy from prior surgical interventions or due to locally advanced tumors obstructing access to the duodenum^[3-5]. Traditionally, such patients underwent percutaneous transhepatic cholangiography^[6-8]. However, this method carries a high complication rate and could be associated with fistula formation and recurrent infection^[9]. In 1996, Wiersema used endoscopic ultrasound (EUS)-guided cholangiography to define the biliary anatomy, guiding repeat ERCP^[10]. The initial report of biliary drainage using EUS guidance was published by Giovannini *et al.*^[11], and described a bilio-duodenal anastomosis guided by EUS using a single 10 French plastic stent. Shortly after, the same author published a left hepatico-gastrostomy under EUS-Guidance. A self-expandable metal stent (SEMS) was then placed across the fistula created^[12]. Later a “rendez-vous” technique was demonstrated in a series by Kahaleh *et al.*^[13], describing a total of 13 cases undergoing trans-gastric puncture of the left biliary system. With the combination of techniques increasingly available it became obvious that this technique was destined to grow further. Since then many more papers have been published regarding this technique.

TECHNIQUES APPROACHES IN EUS-GUIDED BILIARY DRAINAGE

EUS-guided biliary drainage (EUS-BD) are divided by access route into EUS-guided intrahepatic bile duct drainage, where the intrahepatic bile duct is punctured from a transesophageal, transgastric or transjejunal approach, and EUS-guided extrahepatic bile duct drainage, where the common bile duct (CBD) is punctured from a transduodenal or transgastric approach (usually from the distal antrum). The overall rationale for performing EUS-BD is threefold: (1) logistic advantage (it can be performed in the same session as the originally failed ERCP without further delay); (2) physiologic advantage (it provides immediate internal biliary drainage without the need for external drains); and (3) anatomic advantage (it can be tailored to the individual patient’s anatomy; the precise imaging provided by EUS resulting in a potentially less invasive procedure than percutaneous transhepatic biliary drainage).

Extrahepatic approach

In addition to the underlying common rationale for EUS-BD, the extrahepatic approach has its own limitation and advantage. In case of obstruction the common bile duct or common hepatic duct are more easily imaged under EUS than the intrahepatic bile ducts, in contrast to what happens under transabdominal ultrasound. It can there-

fore potentially be accessed under EUS with minimal risks. The retroperitoneal location of the CBD makes it also an attractive access site for patients with ascites, in whom fluid around the liver makes transhepatic access (whether percutaneous or transgastric under EUS) more difficult and hazardous.

As explained in more detail, antegrade stent insertion from an extrahepatic access site is challenging and has only been reported in a few series^[14,15]. The real choice between transmural and transpapillary drainage after extrahepatic bile duct access under EUS therefore lies between EUS-guided choledochoduodenostomy (EUS-CDS) and rendezvous. Proponents of rendezvous argue that it may be less invasive than EUS-CDS, since transmural intervention is usually limited to puncture and guidewire passage, then drainage is accomplished in a retrograde fashion *via* ERCP without the need for puncture tract dilation^[16]. However, EUS-BD can fail - even in expert centers - because guidewire passage across the stricture and the papilla can be unsuccessful. The needle does not permit manipulation of the guidewire, across a stricture in the same way as it can be done during ERCP using flexible catheters. EUS-BD by needle-rendezvous may require repeat punctures with different angles often resulting in a prolonged, labor-intensive procedure with the risk of shearing the wire or biliary leakage. The second part of the rendezvous involves exchange of the echoendoscope for a duodenoscope and guidewire retrieval through the duodenoscope. This is also cumbersome and plagued with difficulties. EUS-CDS despite being perhaps more invasive, appears to be a more reproducible approach over transpapillary rendezvous. Nonetheless, both EUS-BD variant approaches can be considered complementary. As we will discuss below, some indications are better suited for one technique versus another. Similarly, even if rendezvous is the intended drainage technique, EUS-CDS can be used as a second line approach to salvage the significant proportion of failed rendezvous cases^[17,18]. This open-ended approach to EUS-BD (*i.e.*, inclusive of both rendezvous and EUS-CDS) results in comparatively higher success rates than that of EUS-BD series limiting their approach to just rendezvous^[16]. Obviously, future prospective studies comparing EUS-BD with PTBD or surgery are necessary.

TERMINOLOGY

Diagnostic and therapeutic ESCP

EUS-guided access to bile and pancreatic ducts under fluoroscopy in order to obtain diagnostic ductograms was termed “endosonography-guided cholangiopancreatography” and acronymized EGCP in 1996. The alternative acronym ESCP stands for the same name. Within ten years, therapeutic procedures building on the ESCP paradigm were reported in 39 patients to attempt duct drainage (26 biliary and 13 pancreatic). Despite seeming differences in technique and a confusing plethora of terms, the 13 reports originating from 9 different institutions which

Table 1 Variant endosonographic cholangiopancrea-tography approaches (n)

| | Transpapillary | | | | Transmural | |
|------------------------|---|----------|------------------------|--|--------------|---|
| | Retrograde ¹ | | Antegrade ² | | Institutions | Patients |
| | Institutions | Patients | Institutions | Patients | Institutions | Patients |
| Pancreatic duct | 4 | 7 | 1 | 2 | 2 | 4 |
| | Bataille <i>et al</i> ^[19] (1) | | | Kahaleh <i>et al</i> ^[23] (2) | | François <i>et al</i> ^[24] (4) |
| | Mallery <i>et al</i> ^[20] (4) | | | | | |
| | Dewitt <i>et al</i> ^[21] (1) | | | | | |
| | Will <i>et al</i> ^[22] (1) | | | | | |
| Intrahepatic bile duct | 1 | 5 | 1 | 1 | 2 | 3 |
| | Kahaleh <i>et al</i> ^[25] (5) | | | Püspök <i>et al</i> ^[14] (1) | | Burmester <i>et al</i> ^[2] (2) |
| | | | | | | Giovannini <i>et al</i> ^[11] (1) |
| Extrahepatic bile duct | 2 | 7 | 1 | 1 | 4 | 9 |
| | Mallery <i>et al</i> ^[20] (2) | | | Püspök <i>et al</i> ^[14] (1) | | Giovannini <i>et al</i> ^[28] (1) |
| | Lai <i>et al</i> ^[26] (1) | | | | | Burmester <i>et al</i> ^[2] (2) |
| | Kahaleh <i>et al</i> ^[27] (4) | | | | | Püspök <i>et al</i> ^[14] (4) |
| | | | | | | Kahaleh <i>et al</i> ^[27] (1) |
| | | | | | | Kahaleh <i>et al</i> ^[25] (1) ³ |

¹18 out of 19 retrograde transpapillary endosonographic cholangiopancrea-tography (ESCP) were carried out via rendezvous; ²None of these four antegrade transpapillary ESCP were “pure” antegrade. Kahaleh *et al*^[23] used a single stent bridging both the papilla and the puncture tract, whereas Püspök *et al*^[14] used dual stenting: a transmural stent together with a transpapillary stent; ³Retrograde cannulation of spontaneous bilio-duodenal fistula developing in the setting of postoperative duct injury, after intrahepatic bile duct injection. Similar to the methylene-blue approach, but using contrast material.

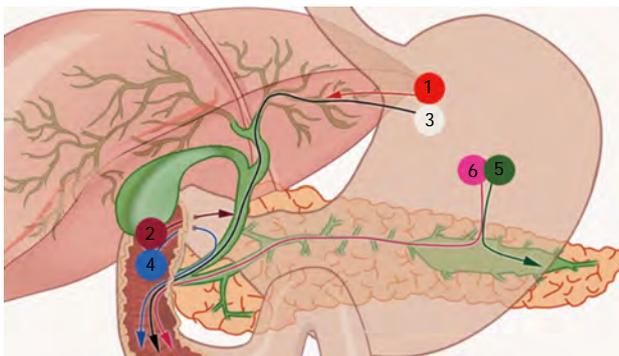


Figure 1 Therapeutic endosonographic cholangiopancreatography: Variant approaches. 1: Transmural drainage, intrahepatic access (hepaticogastrostomy); 2: Transmural drainage, extrahepatic access (choledochoduodenostomy); 3: Transpapillary drainage, intrahepatic access; 4: Transpapillary drainage, extrahepatic access; 5: Transmural drainage, pancreatic access (pancreaticogastrostomy); 6: Transpapillary drainage, pancreatic access. (Reprinted from Perez-Miranda *et al*^[11] with permission).

detail these 39 procedures are strikingly consistent (Table 1)^[2,14,19-28]. In all of them fluoroscopy-guided intervention through ERCP-based techniques was used to provide drainage following EUS-guided ductal access in complex cases not amenable to ERCP. The substantial anatomic variation in this highly selected patient cohort led to different procedural options, which can be classified into 9 subgroups (3 pancreatic and 6 biliary) based on just two variables, access route and drainage route (Figure 1). Access route can be intrahepatic biliary, extrahepatic biliary or pancreatic, whereas drainage route can be either transmural or transpapillary. Transpapillary drainage can be accomplished either antegradely - most commonly by direct stent insertion across the EUS puncture tract into the duct, the stricture and then the papilla, or retrogradely - most commonly *via* rendezvous ERCP.

Nomenclature problem

Determining the essential elements of a given procedure - the common ground to all its potential variant approaches - and the key variables defining those variant approaches, is of critical relevance to successfully name it. An example of a successful name encapsulating the essence of a complex procedure is ERCP. The acronym ERCP has withstood the test of time over four decades, as the procedure itself metamorphosed from diagnostic to therapeutic, branching out to encompass an ever growing range of interventions. The opposite is true for EUS-guided ductal access and drainage interventions. Not a single one of the 13 early reports replicates the name ESCP. It is significant that 5 of them fail to reference it at all when describing their individual variants of ESCP. However, it is even more significant that most will mention it as just one of the many then developing applications of EUS, overall or on the pancreas. A parent role for ESCP is acknowledged only implicitly and occasionally. This tendency to focus on the uniqueness of each novel variation, losing touch with the prior common ground, has worsened in subsequent reports. With a few hundred cases published, over 120 names and 30 different acronyms have been put in circulation to refer to essentially the same procedure or any of its major variants. Table 2 lists the 22 names and 8 different acronyms proposed for an all-variant encompassing procedure. Further name lists could be produced for just biliary overall ($n = 19$), pancreatic ($n = 14$), transmural overall ($n = 22$), transmural biliary ($n = 24$) or transpapillary ($n = 22$) variants.

Key name descriptors

Semantic analysis of names listed in Table 2 under the heading “diagnosis” reveals that descriptors incorporated into the final name include EUS (or endosonography),

Table 2 List of endosonographic cholangiopancreatography names (biliary and panc, *n* = 22)

| | Name | Acronym |
|--|---|----------|
| Diagnosis | Endosonography-guided cholangiopancreatography | EGCP |
| | Endosonographic cholangiopancreatography | ESCP |
| | EUS-guided cholangiopancreatography | EUSCP |
| | EUS-guided cholangiography and pancreaticography | |
| | EUS-assisted cholangiopancreatography | |
| | EUS-guided ductography | |
| | Endo-radio-sono-cholangiopancreatography | ERSCP |
| Therapy | Endoscopic antegrade cholangiopancreatography | EACP |
| | EUS-guided cholangio and pancreatic drainage | ECPD |
| | EUS-guided pancreaticobiliary access and therapy | |
| | EUS-assisted duct access and drainage | |
| | EUS-assisted duct opacification and drainage | |
| | EUS-guided ductal access and drainage | |
| | EUS-guided ductal cannulation and therapy | |
| | EUS-guided pancreatic and biliary ductal drainage | EUS-PBDD |
| | EUS-guided drainage | |
| | EUS-guided drainage of pancreatico-biliary ducts | |
| | EUS-guided pancreatobiliary drainage | EUS-PBD |
| | EUS-guided stent insertion | |
| | Pancreatobiliary drainage by EUS-FNA | |
| Therapeutic EUS-FNA with drainage | | |
| EUS-guided biliary and pancreatic duct puncture and drainage | | |

EUS: Endoscopic ultrasound; FNA: Fine needle aspiration.

the ductal component (allowing differentiation between ESCP and related procedures such as EUS-guided pseudocyst drainage), and the presence of fluoroscopy (expressed in the suffix “-graphy”). Names in Table 2 under the heading “therapy” usually omit the critical element of fluoroscopy and often as well the ductal nature of the target pursued under EUS (which, in turn, is what gives fluoroscopy more prominence in ESCP than in, for example, a pseudocyst drainage procedure). The therapeutic intent is variably described, resulting in exceedingly long names. Inconsistent modifiers such as “guided” or “assisted” (sometimes also “directed”) introduce another source of variability without much added meaning. A simple way to solve the terminology conundrum would be to follow the ERCP paradigm with ESCP, where the latter refers not only to the ductograms it literally designates but also to therapeutic intervention under fluoroscopy to provide bile and/or pancreatic duct drainage. “Endoscopic” and “retrograde” qualify the way ducts are accessed, as would “endosonographic”.

A parent procedure with two major branches

The acronym ESCP could accommodate the term “endosonography-guided cholangiopancreatography” if it proves too ingrained as well as the shorter more specific versions of ESC and ESP, just like ERCP does with ERC and ERP, to designate one ductal system only. ESCP

as a manageable acronym would help frame a unifying concept for a procedure distinct from other EUS-guided interventions and from combined EUS-ERCP arrangements, despite the manifold ways in which it can be carried out. Consistently used terms describing individual ESCP approaches, such as hepaticogastrostomy, choledochoduodenostomy or rendezvous, would be bound by a specific umbrella concept and name rather than standing independently among the growing list of EUS-guided interventions.

Dr. Binmoeller proposed the alternative term “EACP”:

Endoscopic (or EUS-guided) antegrade cholangiopancreatography. He argued that EACP highlights the antegrade route of duct access (relative to the ampulla) in contrast to the retrograde of ERCP, and therefore should be the key distinguishing feature. He noted that “ERCP” does not specify use of a specific imaging modality, in contrast to ESCP. To withstand the test of time, the acronym should be open to imaging modalities that may be used in the future. Choosing an acronym that is familiar will be more likely to achieve adoption, and EACP mirrors ERCP as the antegrade option. Like ERCP, EACP broadly covers a range of diagnostic and therapeutic bilio-pancreatic interventions that will eventually be used under this name. A vote was taken during the conference and the majority favored “ESCP” over “EACP”, however, adequate presentation time was not allowed to discuss the pros and cons of the acronyms. It is important to consider the acceptance of this name worldwide and further discourse is planned.

TRAINING IN JAPAN AND IN THE WORLD: DOES ONE SIZE FIT ALL?

Interventional EUS has become popular. In order to perform EUS-guided pancreatico-biliary drainage, experience of not only endosonographer but also ERCP endoscopist is required.

Current situation of EUS instrument in Japan and in the world

Diagnostic EUS using radial model began in 1980s in Japan. Since then, basically radial EUS has been popular in Japan. In contrast, in United States and Europe, although at first radial EUS was performed, EUS-guided fine needle aspiration (EUS-FNA) showed a rapid increase. The ratio of curved linear array (CLA) echoendoscopes to all EUS scopes is 12% in Japan and 40% in United States and Europe^{29,30}. One of the reasons is the reimbursement of EUS-FNA procedure. The cost of EUS-FNA in Japan is about less than 200 United States dollars.

Current situation of EUS centers in Japan and in the world

There is no dedicated center for interventional EUS using linear EUS since endosonographers usually perform both radial and linear EUS not only for pancreaticobili-

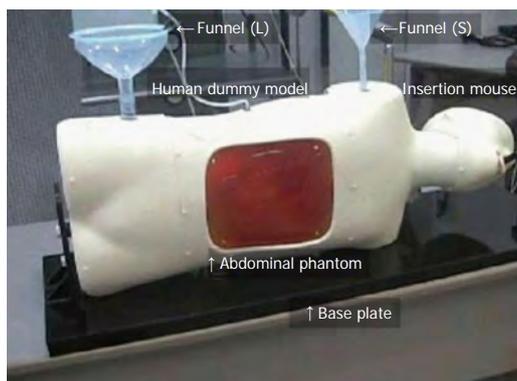


Figure 2 Pancreaticobiliary phantom model (Olympus Medical Systems).

ary diseases but also for gastrointestinal tract diseases, like esophageal or rectal lesions. Most high volume EUS centers have tutorial courses or EUS seminars for endoscopists who want to learn diagnostic EUS, namely EUS imaging including fundamental mode, contrast-enhanced mode and elastography, and EUS-FNA^[31].

In Japan, there are more than 30 EUS centers in which conventional therapeutic EUS including pancreatic pseudocyst drainage and celiac plexus neurolysis are performed. Of these centers, only in about 5 EUS centers, EUS-guided biliary drainage and/or pancreatic duct drainage are performed (more than 20 cases). In United States, there are more than 40 therapeutic EUS centers. In Australia, endosonographers perform interventional EUS in more than 17 centers. In South America, therapeutic EUS is performed in more than 10 EUS centers. In Asia including Japan, there are more than 70 EUS centers. In Europe, around 30 EUS centers provide interventional EUS services.

Current status of EUS training

As many endosonographers described, there is no well designed EUS training system. In terms of United States and Europe, the American Society for Gastrointestinal Endoscopy (ASGE) and the European Society of Gastrointestinal Endoscopy sponsored hands-on EUS workshops using a porcine model is regularly held. We can perform not only diagnostic EUS (imaging) but also EUS-FNA (including cyst puncture). Because EUS training on a swine model is recognized to be the more expensive way for EUS teaching, not all trainees in every country can use the model. In contrast, computer-based simulator (EUS mentor; Symbionix, Tel Hashomer, Israel) has been developed for repeat training and shortening the learning phase of EUS. This model is relatively similar to normal anatomy, but it is also expensive. Recently, Olympus Medical Systems made the pancreaticobiliary phantom for both radial and linear EUS training (Figure 2). It includes not only the parenchymal organs but also middle to large vessels. Training system using this kind of phantom and porcine model will probably become mandatory to establish skills in therapeutic EUS.

EMERGING DEVICES FOR EUS-BD

The technique entails three basic steps using various “off-the-shelf” tools in conjunction with a therapeutic CLA echoendoscope.

EUS-guided ductal puncture

Most cases described in the literature use conventional FNA needle (usually 19-gauge to enable passage of a 0.035 inch wire). The continuous stainless steel needle lends excellent visibility on sonographic and fluoroscopic imaging, as well as excellent transfer of force. Drawbacks of the FNA needle are its relative stiffness, which results in a very tangential angle of puncture. An alternative instrument for access is a diathermic needle knife with removable inner needle. Pure cutting current is applied during puncture to penetrate tissue. The advantage of the needle knife is the ability to immediately exchange the inner needle for a guidewire. The outer catheter can then be easily steered in the bile duct, off-axis from the angle that the duct was punctured. The main drawback of the needle knife is the poor visibility of the needle, limited to the catheter tip, on ultrasound and fluoroscopy. A further drawback is the risk of diathermy trauma to tissue, a particular concern should the needle veer off course during puncture. Whereas a continuous stainless steel needle will maintain the predicted trajectory path as it is advanced, the more flexible needle knife catheter may veer off-axis into a neighboring structure, which may be a major vessel.

Dilation of the bilio-enterostomy tract

Tract dilation is required prior to stenting. As in ERCP, graduated bougies and non compliant balloon catheters can be used. Each has pros and cons. Bougies have the advantage of excellent operator control of the dilation force, as the operator can gauge the amount of resistance encountered during advancement of the bougie. However, the dilation force is axial, which can lead to a separation of tissue planes during bougie advancement. Balloons have the advantage that they can be inserted in a compressed state, thereby minimizing the delivery catheter size to around 5 Fr. The dilation force is radial. However, balloons dilate to a fixed diameter in an “all-or-nothing” fashion which increases the risk of perforation, leakage, and bleeding.

If passage of a balloon or bougie across the bowel wall fails after guidewire access, tract dilation can be facilitated with diathermy using a double lumen needle knife catheter. Alternatively, a catheter with a diathermic ring at the tip can be used. Endoscopists in Europe have used a 6 Fr diathermic ring device, (Endoflex, Voerde, Germany). In the United States, only a 10 Fr diathermic ring device (Cystotome CST10; Cook Medical) is Food and Drug Administration cleared. Due to the large size, the Cystotome is a rigid device that is difficult to advance across the oblique exit of the working channel of the therapeutic CLA echoendoscope.

Stent drainage

Again, as in ERCP, a variety of plastic and metal stents can be used. Pigtail stents are a logical choice to minimize the risk of stent migration (especially into the duct), but the pigtail end makes coaxial stent insertion more difficult. An advantage of a straight stent is the ability to retrieve or exchange the stent over the wire without loss of ductal access. Covered SEMS have been used for transenteric drainage, but may migrate, particularly with shortening^[32]. The covering may block drainage of a secondary duct (*e.g.*, cystic duct or intrahepatic branch). Uncovered SEMS are unsuited for transenteric drainage due to leakage between the struts. However, an uncovered SEMS can be placed in exchange for a temporary plastic stent after the fistula tract has matured^[11].

Current technical challenges

There are three main limitations using current “off-the-shelf” tools. The first is the “step-off” between the wire and device. The device tends to “buckle” where tissue resistance is encountered and may not advance over the wire. The second is the need to exchange multiple devices over-the-wire. This can result in leakage of bile into the periductal space, or leakage of enteric contents into the extraintestinal space. Device exchanges are cumbersome and time consuming, and guidewire access to the bile duct can be lost. The third limitation relates to tubular stents, which are designed for luminal drainage. Whether plastic or metal, tubular stents do not impart the necessary apposition of two nonadherent lumens to prevent leakage. Lacking anchorage, the stent may move or dislocate. The ends of tubular stents may also cause tissue injury.

Compression magnet anastomosis

Jamidar *et al.*^[33] reported on a novel hinged metalloplastic anastomotic device to create a choledochoduodenostomy. The device resembles a 7 Fr stent, but has a central ferrous metallic component. The devices were inserted into the bile duct of pigs using standard ERCP technique over a 0.035-inch guidewire. A magnet was then endoscopically positioned in the duodenum to mate with the bile duct magnet and exert compressive ischemic force. Anastomoses ranging from 5 to 10 mm were successfully accomplished in all survival animals.

Compression coil anastomosis

Chang *et al.*^[34] reported on a novel EUS-guided coil technique using a modified compression coil device with “fin-coil” configuration in dogs. The coil delivery device consisted of a 19 gauge needle pre-loaded with stretched coil in the lumen. EUS-guided needle puncture into the CBD was followed by deployment of 50% of the coil into CBD, and remaining 50% stayed within the duodenal bulb to hold the CBD and duodenum walls together with its compressive force. Immediate drainage was successful in 3/4 animals with overall drainage (normalization of bilirubin) successful in 4/4. Creation of a chronic fistula between CBD and duodenum was achieved in all 4 dogs

and there was no evidence of bile leak or perforation. All coils dislodged successfully into the duodenum.

Exchange-free lumen-apposing device

Binmoeller *et al.*^[35] reported on a catheter-based system (AXT System, Xlumena, Mountain View, CA) that delivers multiple tools in a co-axial fashion without the need for device exchange to secure bile duct access, tract dilation and immediate stent placement for drainage. The AXT device locks to the echoendoscope and is designed for single operator - single hand deployment. The exchange-free system is composed of a unique anchor needle that punctures the walls of the gastrointestinal tract and bile duct and maintains continuous apposition of the two lumens to prevent leakage of contents during instrumentation. A fully covered lumen-apposing metal stent (AXIOS, Xlumena), previously evaluated in porcine studies^[36], is pre-loaded into the AXT System and deployed directly over the anchor needle to maintain tissue apposition and create a tract for drainage. Chronic porcine survival studies were conducted on 3 animals with technical success in creation of a cholecystgastrostomy in all.

CERTIFICATION AND CREDENTIALING

EUS guided biliary drainage is becoming more widely accepted as an alternative to failed ERCP^[13,28,37]. Like any other evolving technology in its developing stages, currently there are no defined guidelines as to who should be performing ESCP and what should be the criteria for credentialing someone to perform this procedure. The limitation of this technique resides in its infrequent use with limited number of cases performed and hence formal training dedicated to this specific procedure can be difficult. Further, having a fixed minimum number of cases required as part of training can be even more challenging. Currently ESCP is also limited by the tools available, which are not dedicated for this type of procedure.

National and International organizations also do not have any recommendations or required criteria for training. ASGE has recommendations for number of procedures required for EUS and ERCP credentialing. ASGE recommends 75 mucosal and pancreatico-biliary exams and 50 EUS with FNA^[38]. For ERCP the number required is 180 with half being therapeutic^[39]. Even these numbers seem suboptimal for comfort of these procedures based on surveys of trainees in advanced training. Definitely the numbers for EUS guided biliary drainage will be much higher based on the significant technical complexity involved. Different variations of the technique with different complexity, are further complicating the credentialing process.

Based on these complexities, in this recent consortium meeting of experts, recommendations on who should be doing ESCP include: (1) Endoscopists routinely performing pancreatico-biliary EUS and FNA; (2) Endoscopists with large ERCP and EUS experience for nearly 4-5 years (at least 200-300 EUS and ERCP each year); (3) Endosco-

pists with 95%-98% success rate for standard ERCP with normal anatomy; and (4) Location into a center with IR and/or pancreatico-biliary surgery back up.

CODING AND REIMBURSEMENT

Optimal reimbursement of a procedure is dependent on appropriate coding. When developing a code for a procedure, components, which are looked at, are: physician work, practice expense and malpractice expense.

Currently no specific codes exist for ESCP. They are billed with combination of EUS codes and ERCP codes. Some of the interventions done don't fall in the realm of either EUS or ERCP and then surrogate codes are used which is not optimal. Some of the interventions performed, which are unique to ESCP are EUS guided contrast injection, guidewire placement, dilation of the transgastric and transduodenal tracts (balloon/bougie or needle knife) and transmural stent placement^[13,37]. CPT specifically states that one should not simply "approximate" coding by using codes that may seem "close enough" but do not accurately describe a service.

The different approaches and drainage routes used make this even more complex. In situations where EUS is used as a mode to access the bile duct (extrahepatic or intrahepatic) and subsequent rendezvous transpapillary ERCP is performed is the simplest situation to code. One can combine EUS with FNA with standard ERCP codes. Even in this case, as there is no specific code for passage of guidewire through the EUS needle, part of the procedure is not clearly defined with currently available codes. Hence the procedure should also be coded with additional *code for unlisted procedure* and *procedure explained in detail*.

The most optimal way for coding these EUS guided bile duct procedures will be to use standard codes for very obvious interventions and along with that use the code for unlisted procedure^[40]. Using *code for unlisted procedure* requires additional work. Supporting documentation with each claim has to be submitted separately which should describe the nature, extent and need for the procedure. Time effort and equipment necessary along with complexity of symptoms, final diagnosis, and patient findings are to be described as well. Manual review by payor on case-by-case review is done and many times individual payor has to be contacted to discuss the procedure.

In conclusion, EUS-guided biliary drainage is a novel procedure destined to be incorporated into our therapeutic arsenal. The ability to offer a tailored minimally invasive solution for patients in whom ERCP fails or is not feasible due to various reasons; led to the creation of a dedicated consortium. This consortium aims to create a registry to catalogue the growing number of ESCP techniques and tools, as well as indications. Ultimately, technical advancements will be driven by dedicated research protocols, while nomenclature, training and credentialing will be formalized. Only this will allow to establish EUS-BD as a standard procedure.

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Hepatic regeneration and the epithelial to mesenchymal transition

Zeng-Fu Xue, Xiu-Min Wu, Ming Liu

Zeng-Fu Xue, Ming Liu, Department of Digestive Diseases, The First Affiliated Hospital of Xiamen University, Xiamen 361003, Fujian Province, China

Xiu-Min Wu, Department of Pharmacy, The First Affiliated Hospital of Xiamen University, Xiamen 361003, Fujian Province, China

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Correspondence to: Ming Liu, Vice Professor, Department of Digestive Diseases, The First Affiliated Hospital of Xiamen University, 10 Shanggu Road, Xiamen 361003, Fujian Province, China. zengfuxue@yahoo.com.cn

Telephone: +86-592-2137708 Fax: +86-592-2137559

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Abstract

Liver injuries are repaired by fibrosis and regeneration. The core stage is the repair response and fibrosis formation as a scar. The cause of overly-responsive scar formation and diminished regeneration, especially in liver fibrosis and cirrhosis, is still unknown. The epithelial to mesenchymal transition (EMT), a previously discovered mechanism, plays an important role in liver fibrosis and tumor metastasis. Recently, EMT has been found to be associated with liver and bile duct cell fibrosis. Analyzing the established models and chronic disease processes, we propose that EMT liver cells may also lose their regenerative capability due to phenotype changes and that the remaining liver cells may quickly lose their regenerative capability in liver fibrosis or cirrhosis. Recognizing these phenotype changes or transition cells may play an important role in targeting therapy to reverse fibrosis not only by disrupting the transition that is necessary to produce the extracellular

matrix but also by restoring the regenerative capacity of EMT-like cells.

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Key words: Epithelial to mesenchymal transition; Hepatocyte; Regeneration; Fibrosis; Transforming growth factor- β ; Liver; Epithelial to mesenchymal transition-like; Hepatocyte stellate cells

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INTRODUCTION

Wound injury and repair is one of the most common pathophysiological processes during human life. In all organ systems, the normal mammalian response to injury occurs in three overlapping but distinct stages: inflammation, new tissue formation, and tissue remodeling^[1]. Therefore, injured organs, such as lung, kidney, liver or skin, will first respond with inflammation when insults, such as those caused by microorganisms and toxins, occur. Then next critical step is repair and regeneration, but during the repair or remodeling process, excessive deposition of extracellular matrix (ECM) collagen 1 and collagen 3 leads to hypertrophic scars, which results in tissue dysfunction^[1]. Fibrosis is an excessive, uncontrolled injury response that occurs in organs, such as the lungs, kidneys, heart or skin, and is described by a persistent deposition of ECM. Fibrosis has been as a leading cause of morbidity and mortality^[2]. There are more similarities between liver and skin regeneration due to the sustained regenerative capabilities of epidermal cells and hepatocytes after injury. Additionally, epidermal cells can be regenerated by

remnant stem cells in hair follicles, and hepatocytes can be regenerated by cells in the canals of Hering, such as oval stem cells or other progenitors^[1]. The core stage for injuries is the repair response and scar formation. As a protective response to insults, regeneration follows injury; therefore, the fibrogenetic source and the mechanism impeding regeneration are essential. In this review, we will explore recent evidence of liver fibrogenesis and possible regenerative capacities, especially in pathways that have been newly targeted to reverse fibrogenesis.

LIVER REGENERATION IN PHYSIOLOGICAL AND PATHOLOGICAL CONDITIONS

Unlike liver regeneration by Prometheus in Greek mythology, we have considered “regeneration” to be a hyperplastic response rather than the actual regeneration of the remnant liver^[3-5]. After a 2/3 partial hepatectomy, most hepatic cells rapidly enter the cell cycle and undergo an average of approximately 1.6 cycles of replication per cell to completely restore the original liver mass^[5,6]. The classic transplantation experiments using fumarylacetoacetate hydrolase (FAH)-deficient mice (FAH knockout mice) and urokinase-type plasminogen activator transgenic mice demonstrated that hepatocytes can replicate at least 69 times^[7,8]. Interestingly, hepatocytes can differentiate into cholangiocytes and form mature bile ducts after bile duct ligation and toxic biliary injury and may even behave as stem cells under select circumstances^[9,10]. However, under physiological conditions, hepatic regeneration only occurs to replace individual, aged hepatocytes—typically those in zone 1 (periportal)^[11]. The regenerative components required for the process may be categorized into three networks: cytokine, growth factor and metabolic^[12]. The innate immune system and cytokines, such as interleukin-6 (IL-6), tumor necrosis factor (TNF), hepatocyte growth factor (HGF) and complement, have been identified and recognized as playing important roles in regenerating the liver^[12-14]. TNF binds its type I receptor, leading to nuclear factor kappa B activation in Kupffer cells, which produce IL-6 and TNF; IL-6 is subsequently released into the serum and binds to its receptor to activate the STAT-3 signaling pathway to initiate hepatocyte regeneration^[12]. Transforming growth factor-beta (TGF- β) is released by the stellate cells, leading to Smad2 and Smad3 phosphorylation. Smad complexes translocate into the nucleus to transactivate target genes to induce epithelial to mesenchymal transition (EMT) and inhibit proliferation^[15]. TGF- β may be an essential factor in liver fibrosis.

Activation of intrahepatic stem cells, such as hepatocyte progenitor cells and oval stem cells, and bone marrow stem cells are the main sources of exceptional regenerative capacity^[16]. The properties of the liver generally allow for complete reconstitution following acute, moderate injuries.

After partial hepatectomy or CCl₄-induced injury, liver regeneration is replicated by remanent hepatocytes. In rare

cases, in the regeneration of the liver that injures induced by other toxins, such as galactosamine, activation of a progenitor cell compartment to replicate and differentiate^[12].

However, the liver does not heal as effectively in response to chronic liver diseases such as liver fibrosis and cirrhosis.

LIVER FIBROSIS AND REGENERATION

Liver fibrosis is an excessive scar response leading to cirrhosis, which is characterized by the formation of regenerative nodules in the liver parenchyma separated by fibrotic septa. Three major mechanisms are involved in the generation of cirrhosis: cell death, aberrant extracellular matrix deposition (fibrosis), and vascular reorganization^[17]. Liver fibrosis mainly represents quantitative and qualitative changes in the ECM, including the deposition of collagens, elastin, and tenascin, which can increase 3-5 fold^[18]. The excessive deposition is accompanied by a shift in the type of ECM in the subendothelial space from the normal low-density, membrane-like, basement matrix to an interstitial-type matrix containing fibril-forming collagens^[18]. Liver fibrosis accelerates to end-stage cirrhosis and shifts from reversible to irreversible. Therefore, most efforts should focus on treating liver fibrosis and understanding early fibrogenesis to control its progression. Activated fibroblasts or myofibroblasts are the key mediators of liver fibrosis^[17,19,20]. Stellate cells, which are located in the subendothelium and constitute approximately 5% of the liver parenchyma, had been considered to represent the entire population of activated fibroblast cells; however, stellate cells have recently been found to possess more multifaceted functions in liver fibrosis, and we now know they are not the sole cause of fibrosis^[19,21]. Such activated fibroblasts can be derived via the activation and proliferation of resident fibroblasts [hepatocyte stellate cells (HSCs) and portal fibroblasts], circulating fibrocytes, bone marrow stem or progenitor cells and epithelium-mesenchymal transition cells^[2,19,22].

The underlying causes of diminished liver regeneration in liver fibrosis and cirrhosis are still a mystery. Using the proliferating cell nuclear antigen to assess proliferation, a cirrhotic liver has more DNA synthesis than a normal liver but also has great variability in the pseudodubules^[23,24]. However, the high proliferation index does not reflect the actual cell division rates. The cell cycle is impaired by anaphase bridges, aberrant mitosis, and arrest in G₂/M, which has been shown in mTR^{-/-} transgenic mice^[25]. Telomere shortening delays liver regeneration and can be restored by telomerase treatment, which has been shown to improve albumin levels in mTR^{-/-} transgenic CCl₄-cirrhosis models. Telomere shortening is thought to be proof that the replicative activity of hepatocytes is diminished in advanced cirrhosis and chronic liver injury in humans^[26-28]. Telomere delivery inhibits the progression of cirrhosis in mice^[25] and leads to a state of “replicative senescence”^[29]. As with wound repair, the

majority of hepatocytes are believed to undergo necrosis or apoptosis, providing space for proliferating cells during chronic insults, such as viral infections, that exhaust the regenerative capability of the liver. Telomere shortening is evidence of these processes. However, there is no direct evidence to support the loss of regenerative capability during chronic insults is entirely due to continuous regeneration and impairments. Therefore, we investigated possible regenerative mechanisms in fibrosis.

HEPATOCTE EMT AND FIBROSIS

The most distinct difference in the injury response is the liver's powerful regenerative capability compared to the kidneys, lungs and others organs. Another difference is the rapid progression in fibrosis due to acute processes, such as hepatitis C virus infection^[30] or drug injury, or a chronic process, such as chronic hepatitis B virus (HBV) infection. Therefore, liver fibrosis is a more complex process relative to many other processes. The EMT is believed to play an important role in fibrosis, which may be reversed or attenuated by antagonizing essential cytokines and growth factors^[31]. Undergoing an EMT refers to the loss of apicobasal polarity in epithelial cells; intercellular adhesion complexes undergo dramatic phenotypic changes, causing them to become nonpolar and thus allowing these cells to move through the ECM like mesenchymal cells^[32-34]. Organ fibrosis can be classified as a type 2 EMT, which is associated with tissue repair and involves secondary epithelial or endothelial cells transitioning to resident tissue fibroblasts in response to persistent inflammation^[33,34]. A type 2 EMT can continue to respond to ongoing inflammation and lead to the expression of mesenchymal markers on cells, which can advance to various extents through an EMT, namely, a partial EMT. The partial EMT refers to an intermediate phenotype as cell transition, with progressive loss of epithelial markers (E-cadherin, ZO-1) and gain mesenchymal markers (vimentin, alpha smooth muscle actin, FSP1 and β -catenin)^[33]. If the cells ultimately shed all of their epithelial markers and gain a complete fibroblastic phenotype, the cells have undergone a complete EMT^[33]. The accumulating evidence has suggested that the EMT contributes to liver fibrosis, similar to processes that occur in other organs, such as the lungs, kidneys, and intestines^[33].

An EMT can be found in response to growth factors, such as epidermal growth factor (EGF) or TGF- β , and dimethyl sulfoxide in rat fetal liver cells^[35,36]. In normal mouse and adult livers, stroma cells can express both mesenchymal (vimentin, collagen I and alpha smooth muscle actin) and epithelial markers (cytokeratins, albumin and E-cadherin) during the hematopoietic but not the nonhematopoietic liver by the end of gestation, indicating that liver stroma cells may be EMT cells that support hematopoiesis^[37]. Chronically damaged livers, as in cirrhosis, in which there are a large number of epithelial progenitors and myofibroblastic HSCs, have cells that undergo an EMT similar to the transition that occurs in

the kidneys under pathological conditions^[38,39]. Indirect evidence of EMT has been observed in HSC culture *in vitro*, in which HSCs were shown to coexpress mesenchymal and epithelial markers^[38]. The primary HSC express stable mRNA levels of two epithelial markers, Mpk (an oval cell marker) and CK-19 (a marker of immature and mature biliary epithelial cells)^[38]. Convincing evidence has shown that TGF- β can induce an EMT in mouse hepatocytes *in vitro*. The mechanism was demonstrated to be the result of TGF- β -induced activation of the Snail transcription factor, which is a key molecule in the EMT, and repression of epithelial markers, such as E-cadherin^[32,40,41]. Further evidence has demonstrated, using AlbCre. R26R-stoplacZ double transgenic mice *in vivo*, that hepatocytes can undergo EMT. Avoiding the double transgenic mice phenotype changes, using lineage-tracing experiments, hepatocytes can transdifferentiate into mesenchymal-like cells that have lost albumin and still have an activated *Laz* gene. After CCl₄-induced liver fibrosis, up to 45% of FSP1-positive fibroblasts were found to be *Laz*(+), which drives the EMT, similar to processes that occur in the kidneys^[39,42]. Undoubtedly, these transition cells have lost their hepatic markers, such as albumin, thus losing its main secreting function. Using collagen I and transferrin costaining demonstrated that half the resident hepatocytes had undergone an EMT phenotype in a TGF- β transgenic mouse model and in samples from patients with HBV, and the key transcription factor Snail was also found in the damaged regions^[43]. Hepatitis C viral protein NS5A also induces hepatic dysplastic alterations of cell morphology with EMT phenotype and participates in oncogenic transformation of primary hepatocyte precursors^[44]. TGF- β induces Snail, activates the Smad2/3 pathway and, finally, mediates the transition to the EMT phenotype^[15]. The Snail-positive cells also consisted of 50% of the remaining hepatocytes in the damaged regions^[43]. The mesenchymal markers vimentin and α -SMA were detected in fibrotic human and rat livers around the fibrotic septa, which indicates the presence of transition hepatocytes and EMT^[45]. EMTs also occur in cirrhotic liver cells derived from murine CCl₄-induced models^[46]. Interestingly, the EMT-like cells express albumin and the mesenchymal marker vimentin and also gain collagen I secretory functions. Cell isolates from cirrhotic livers can exhibit anti-apoptosis effects in contrast to normal hepatocytes under TGF- β treatment^[46]. While untreated normal liver-derived hepatocytes have been shown not to display features of an EMT, they did respond to TGF- β with increased vimentin expression and EMT characteristics^[46]. Therefore, accumulated evidence has shown that EMT-like cells, even EMT cells, exist during chronic liver injury with a partial loss of functions such as albumin and transferrin secretion and a gain of mesenchymal features.

Recently, an EMT was also found in bile ducts in animal models and patients, especially in bile duct ligation, primary biliary cirrhosis and nonalcoholic fat liver diseases, due to hedgehog (Hh) signaling activation^[47-49]. Hedge-

hog signaling is low in the normal adult liver but plays an important role in liver development^[50]. Injured cholangiocytes activate hedgehog signaling through the patched receptor (Ptc) to release glioblastoma (Gli) family transcription factors to Hh-target genes. In Patched-deficient, Patched haplo-insufficient [Ptc(+/-)] and PtcLacZ mouse models, the bile ducts exhibit more EMTs, myofibroblast accumulation and fibrosis^[48,49,51]. Hh activation in bile ducts can contribute to biliary fibrosis and cirrhosis. Different mesenchymal markers, such as vimentin, α -smooth muscle actin and S100A4, are located in the fibrous septa and extend around the nodules of liver parenchyma, which contain transition hepatocytes that have gained the ability to secrete ECM^[43,46,48,49]. Based on the recent discoveries, these transition hepatocytes and cholangiocytes can be remodeled into other fibroblast-like phenotypes upon exposure to different insults and thus can be divided into two stages: partial-EMT or EMT-like and true, or complete, EMT. The partial-EMT can exist in the three stages of wound repair: inflammation, new tissue formation, and remodeling. Inflammation and necrosis can release different cytokines that initiate the clearance of the necrosis area and regeneration. The recognized cells contain EGF, IL-6, HGF, TNF and the important negative regulator TGF- β , especially in liver regeneration^[1,12]. Until now, TGF- β , EGF and others through the TGF- β , Smad or hedgehog pathways initiate the EMT in different liver diseases and liver fibrosis in animal models^[43,46,48-50]. Therefore, various cytokines and growth factors, released by necrotic cells, infiltrating inflammatory cells, HSCs and activated fibroblasts from different sources, can initiate the EMT as an early event during liver fibrosis.

However, these studies have been recently questioned because the use of transgenic-animal models allows for *in vivo* lineage-specific tracking using EMT-specific markers. These studies use a cross between an alpha-fetoprotein (AFP) cre mouse and a ROSA26YFP or ROSA26- β -gal stop mouse to trace cell fate through the expression of AFP^[52,53]. None of the resulting fibrosis cells originated from the genetically marked hepatic and biliary epithelial cells. The challenging EMT results were repeated after inducing liver fibrosis by bile duct ligation (BDL), CCl₄, or 3,5-diethoxycarbonyl-1,4-dihydrocollidine. However, the critical concerns originate from these EMT markers and the absence of such evidence in clinical patients.

FSP1 and Snail are popular markers for detecting the transition. FSP1 was not expressed by HSCs or type I collagen-producing fibroblasts in liver sections from FSP-GFP reporter mice BDL or CCl₄-treated mice. FSP-1 is expressed in inflammatory macrophages, thus FSP1 is not a marker for myofibroblasts or their precursors^[54]. Another important EMT marker, Snail, plays a key role in liver fibrosis progression *in vivo* in a CCl₄ model by triggering the proximal genetic programs that control multiple aspects of fibrogenesis in the Snail transgenic CKO mouse, which promotes growth factor expression and extracellular matrix biosynthesis to the ensuing chronic inflammatory responses in liver fibrosis^[55]. These Snail-

positive cells also lose transferrin expression in the damaged regions, which is a specific liver marker in a complete epithelial to mesenchymal transition. The partially trans-differentiated hepatocytes comprised 50% of the remaining hepatocytes in the damaged regions, although they cannot be distinguished from other immune cells^[43].

Although there is no current evidence that myofibroblasts originate from liver cells, more experimental and clinical liver fibrosis were reported. These models may not reflect the pathophysiology in chronic human liver disease and in the TGF- β mouse model, which requires further study. Clearly, such studies have offered important evidence for the further study of EMT programs in liver fibrosis. Nevertheless, liver fibrosis may be independent of the hepatocyte EMT program and may instead be affected by important factors, such as Snail.

TARGETS OF LIVER FIBROSIS AND RECOVERING THE REGENERATIVE CAPACITY

Stellate cells can be found within the progenitor cell niche in normal and regenerating livers, near the intrahepatic bile ducts, which was recognized as one of the central sources of fibrogenesis^[21]. Recent studies have shown more important roles for immunity in promoting liver fibrogenesis^[19]. Various cytokines and growth factors can affect stellate cells from inflammatory infiltrates and necrotic tissue. Stellate cells can also produce different cytokines or growth factors by paracrine or autocrine transmission to influence the adjacent hepatocytes. TGF- β , a key regulator of fibrogenesis and EMT, can be observed in acute or chronic liver injury in HSCs^[13,56,57]. TGF- β can directly activate adjacent hepatocytes through Smad signaling, induce EMTs, and induce ECM and fibrogenesis. Hedgehog signaling may also be a key regulator in the promotion of EMT and fibrogenesis in bile duct diseases. Blocking these signaling pathways is an important therapeutic target for mediating regeneration (Figure 1).

TGF- β exerts its effects by binding to the TGF- β type II receptor, which causes recruitment and phosphorylation of receptor type I and formation of a complex. The activated receptor type I subsequently recruiting Smad2/3 and Smad4, which are known intracellular mediators of TGF- β . Once phosphorylated by the activated TGF- β receptor, Smad2 and/or Smad3 bind Smad4 and translocate to the nucleus, where they regulate TGF- β target genes^[57]. Smad7, a feedback regulator of TGF- β , can prevent liver fibrosis and HSC activation in rat BDL- and CCl₄-induced liver fibrosis models^[13,58]. Using Smad transgenic mice also attenuates TGF- β signaling and EMTs, thus improving CCl₄-provoked liver damage and fibrosis^[43]. The complex formation of BMP-7 and TGF- β with different types of ALK receptors is mediated by Smad proteins. In renal tubular epithelial cells and mammary ductal epithelial cells, BMP7 has been shown to reverse the TGF- β 1-induced EMT, given that NTN

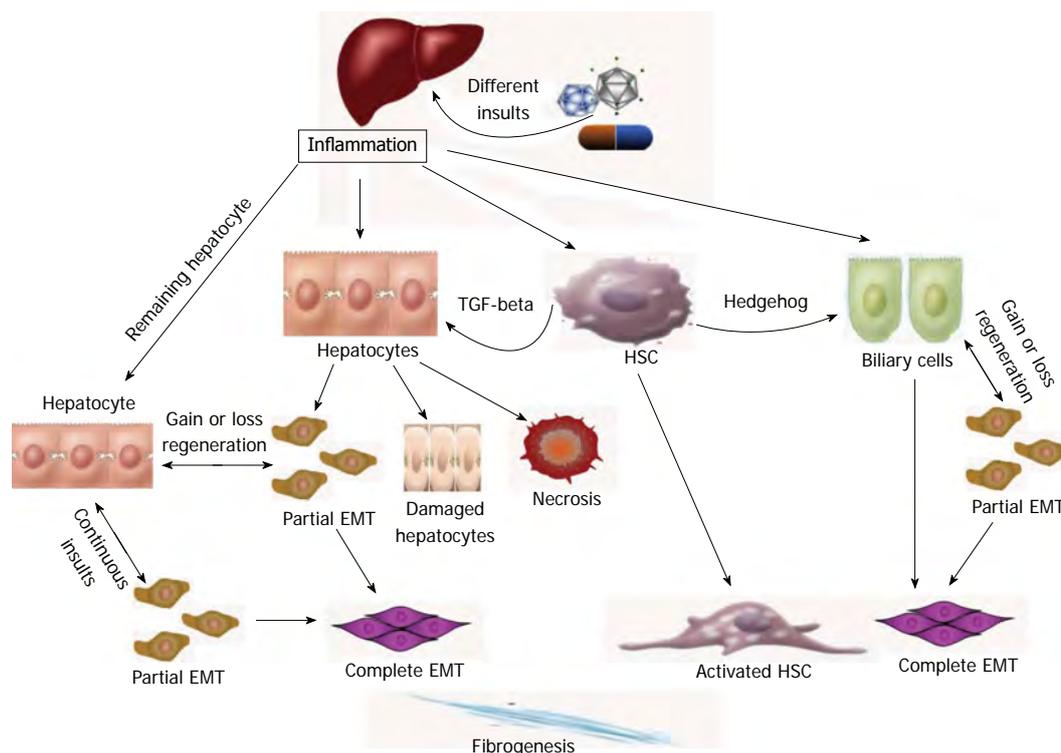


Figure 1 Liver regeneration and the epithelial to mesenchymal transition. The diagram shows the fibrogenesis *via* epithelial to mesenchymal transition (EMT) in the liver. Different insults initiate inflammation and then cause hepatocyte stellate cells (HSCs) activation and hepatocyte and biliary cell damage, necrosis and EMT. Except for the necrotic and damaged cells, the remaining “normal” liver cells can be divided into 2 groups: normal and EMT-like hepatocytes. Continuous insults will shift those EMT-like cells to complete EMT cells and finally myofibroblasts, the main producer of extracellular matrix, which may be one of the main causes of an early loss of regenerative capacity. A similar process also occurs in biliary cells. HSCs play an important role in secreting key cytokines, such as transforming growth factor-beta (TGF-beta) and hedgehog, to affect the adjacent cells and promote EMTs.

mice (nephrotoxic serum nephritis; a chronic nephritis model) treated with recombinant human BMP can re-induce E-cadherin^[59].

The Hh signaling that was observed in bile duct fibrosis and EMTs has provided us with a new mechanism and new therapeutic targets^[47,51]. Hh ligands that interact with the Hh receptor Patched (Ptc) liberate the coreceptor Smoothed, which activates Gli family transcription factors and Hh-target genes^[50]. The Hh pathway can be activated in the liver after BDL and Roux-en-Y hepaticojejunostomy to relieve biliary obstructions. Hh signaling decreased as the duct populations and concomitant fibrosis were observed^[48,60]. The role of the Hh pathway was observed in nonalcoholic fatty liver disease and verified in Ptc transgenic mouse bile duct cells. The Hh inhibitor cyclopamine and the Hh-neutralizing antibody have been shown to reverse the EMT and fibrosis in mouse models and *in vitro* co-culture with cholangiocytes and MF-HSCs and also to reduce TGF-β expression^[48,49].

Similar to TGF-β, activated HSCs can produce an Hh ligand by paracrine and autocrine signaling^[48,61,62]. Therefore, HSC can produce TGF-β and the Hh ligand to affect the adjacent hepatocytes and cholangiocytes to induce EMT and fibrosis. Inhibiting the Hh pathway may reduce TGF-β expression, which may indicate a commu-

nication between them^[49].

If the remaining “normal” liver cells had 40%-50% EMTs, as mentioned above, and continuously were damaged, the hepatocytes capable of regeneration may actually be less than 50%, excluding the damaged or necrotic hepatocytes, which would lose their normal functions and exhaust their regenerative capacity earlier than expected. The remaining hepatocytes, including the newly identified partial EMT or EMT-like cells, have diminished regenerative capacity potentially due to elevated rates of apoptosis followed by repeated regeneration and damage according to the traditional theory^[13]. The newly regenerated hepatocytes and the remaining hepatocytes can continuously transition to mesenchymal cells during fibrosis (Figure 1). Recognizing whether these phenotype changes or the transition cells have a more important role in targeting therapy for controlling the transition phenotype can not only disrupt the transition to produce ECM but can also help to recover the regenerative capacity of the EMT-like cells.

As mentioned above, EMT-targeted therapy can reverse partial EMT cells, not only during fibrosis but also during regeneration. These phenotype changes or transition cells may provide us a new insight in identifying the regenerative capacity of these partial EMT or complete EMT cells and also for targeting new therapies.

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Pre-treatment role of inosine triphosphate pyrophosphatase polymorphism for predicting anemia in Egyptian hepatitis C virus patients

Walaa H Ahmed, Norihiro Furusyo, Saad Zaky, Abeer Sharaf Eldin, Hany Aboalam, Eiichi Ogawa, Masayuki Murata, Jun Hayashi

Walaa H Ahmed, Norihiro Furusyo, Jun Hayashi, Department of Environmental Medicine and Infectious Diseases, Kyushu University Hospital, Fukuoka 812-8582, Japan

Saad Zaky, Abeer Sharaf Eldin, Department of Tropical Medicine and Gastroenterology, Assiut University Hospital, Assiut 71515, Egypt

Hany Aboalam, National Center for Treatment of Chronic Hepatitis C, Ministry of Health, Assiut 71329, Egypt

Norihiro Furusyo, Eiichi Ogawa, Masayuki Murata, Jun Hayashi, Department of General Internal Medicine, Kyushu University Hospital, Fukuoka 812-8582, Japan

Author contributions: Ahmed WH and Furusyo N designed the research; Ahmed WH, Zaky S, Sharaf Eldin A and Aboalam H regularly collected the samples from the patients for laboratory investigations; Sharaf Eldin A and Aboalam H contributed to monitoring of the patients during treatment; Ahmed WH analyzed and interpreted the data with statistical performance; Ahmed WH performed the research and wrote the paper; Furusyo N, Zaky S, Ogawa E, Murata M and Hayashi J reviewed the article critically for important intellectual content.

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Correspondence to: Jun Hayashi, MD, PhD, Department of General Internal Medicine, Kyushu University Hospital, 3-1-1, Maidashi, Higashi-ku, Fukuoka 812-8582,

Japan. hayashij@gim.med.kyushu-u.ac.jp

Telephone: +81-92-6425909 Fax: +81-92-6425916

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METHODS: The human genomic DNA of all patients was extracted from peripheral blood cells in order to determine the single nucleotide polymorphism (SNP) of *ITPA* (rs1127354). SNP genotyping was performed by real time polymerase chain reaction (PCR, ABI TaqMan allelic discrimination kit) for 102 treatment-naive Egyptian patients with chronic HCV. All patients had no evidence of cardiovascular or renal diseases. They received a combination treatment of pegylated interferon α (PEG-IFN α) as a weekly subcutaneous dose plus an oral weight-adjusted dose of ribavirin (RBV). The majority received PEG-IFN α 2a (70.6%) while 29.4% received PEG-IFN α 2b. The planned duration of treatment was 24-48 wk according to the viral kinetics throughout the course of treatment. Pre-treatment liver biopsy was done for each patient for evaluation of fibrosis stage and liver disease activity. The basal viral load level was detected quantitatively by real time PCR while viral load throughout the treatment course was performed qualitatively by COBAS TaqMan assay.

RESULTS: Ninety-three patients (91.2%) had *ITPA* SNP CC genotype and 9 (8.8%) had non-CC genotype (CA and AA). The percentage of hemoglobin (Hb) decline was higher for CC patients than for non-CC patients, particularly at weeks 4 and 8 ($P = 0.047$ and 0.034 , respectively). During the first 12 wk of treatment, CC patients had significantly more Hb decline > 3 g/dL than non-CC patients: 64.5% vs 22.2% at weeks 8 and 12, respectively, ($P = 0.024$ and 0.038). Reduction of the amount of the planned RBV dose was significantly higher for CC patients than non-CC patients during the first 12 wk ($18\% \pm 12.1\%$ vs $8.5\% \pm 10.2\%$, $P = 0.021$). The percentage of CC patients with RBV dose reduction was significantly greater than that of non-CC patients (77.4% vs 44.4% , $P = 0.044$). Multivariate analysis identified only the percentage of RBV dose as a predictor for Hb decline. Platelet decline

Abstract

AIM: To investigate and clarify, for the first time, the role of inosine triphosphate pyrophosphatase (*ITPA*) polymorphism in Egyptian chronic hepatitis C virus (HCV) patients.

was significantly higher in non-CC patients than CC patients at weeks 12, 24 and 48 ($P = 0.018, 0.009$ and 0.026 , respectively).

CONCLUSION: Rs1127354 *ITPA* polymorphism plays a decisive role in protecting against treatment-induced anemia and the need for RBV dose reduction in Egyptian HCV patients.

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Key words: Anemia; Dose reduction; Hepatitis C; Inosine triphosphate; Ribavirin; Rs1127354

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INTRODUCTION

Hepatitis C virus (HCV) infection is considered one of the most horrifying diseases. It affects approximately 3% of the world's total population, more than 170 million people worldwide. It can range in severity from a mild illness lasting a few weeks to a serious lifelong disease that may end with cirrhosis and hepatocellular carcinoma^[1,2]. The prevalence of HCV infection shows considerable differences between countries; the lowest prevalence rate has been estimated in European countries. Parenteral anti-schistosomal treatment that was used in the 1960s and 1970s played a major role in the spread of HCV throughout Egypt^[3,4]. Egypt has been reported as having the highest prevalence rate of HCV worldwide, with HCV antibodies estimated at 10% and 20% of Egyptian adults in urban and rural areas, respectively^[5].

The standard treatment for HCV treatment worldwide, including Egypt, is pegylated interferon α (PEG-IFN α), which acts as an antiviral and an immunomodulatory cytokine, and ribavirin (RBV), a guanosine analogue that interrupts the viral RNA metabolism^[6,7]. Anemia is a well-known, serious side effect associated with the PEG-IFN plus RBV combination treatment that has been reported to occur in 54% of patients during the course of treatment^[8]. It leads to the necessity of dose reduction or premature discontinuation of treatment by 10%-14% of the patients^[6]. Anemia is mainly due to a RBV hemolytic effect on red blood cells (RBCs) or by IFN through either its suppressive effect on bone marrow or an auto-immune hemolytic reaction from excessive apoptosis of erythroid cells^[9-11]. Pretreatment screening of biomarkers is essential for evaluating both the risks and benefits of the available treatment regimen. In the last few years, some genome wide association studies have discussed the impact of host genetic factors on the treatment of hepa-

titis C. Some of these studies targeted the single nucleotide polymorphism (SNP) in the inosine triphosphate pyrophosphatase (*ITPA*) gene of patients mono-infected with HCV^[12-14]. Domingo *et al*^[15] reported a strong association between this polymorphism and induction of anemia in HCV/human immunodeficiency virus (HIV) co-infected patients. This gene is located on chromosome 20, and the SNP occurs at nucleotide 94 in exon 2 of the *ITPA* gene and leads to substitution of proline residue at position 32 to threonine (P32T, 94C \rightarrow A)^[16]. Although HCV is widespread in Egypt, it has not been spotlighted from the standpoint of host genetic studies. Our motivation to carry out this study was to clarify for the first time in Egyptian patients the association between the variants of the *ITPA* gene (encoding ITPase enzyme) and anemia induced during PEG-IFN α and RBV combination treatment, regardless of the impact on treatment outcome.

MATERIALS AND METHODS

Patients

The data of this study were collected from 102 Egyptian chronic hepatitis C patients. All were positive for HCV RNA for more than 6 mo and negative for any other viral infection. The pretreatment demographic data are displayed in Table 1. All patients were mono-infected with chronic hepatitis C with no evidence of HIV or hepatitis B infection. All participants were treatment-naive patients with no evidence of the presence of any associated liver diseases other than chronic viral hepatitis C. Patients with cardiovascular problems or renal impairment were not eligible for the study. They received standard doses of the combination treatment of PEG-IFN α and RBV in the Assiut National Center for the Treatment of Chronic Hepatitis under the supervision of the Egyptian Ministry of Health.

In the first week of treatment, all patients were given the fully recommended dosages of RBV. At week 2 and throughout the course of treatment, the dose of RBV was modified according to the hemoglobin (Hb) level. In the current study, we calculated the values of Hb at weeks 2 and 4, then every 4 wk throughout the treatment course. Informed consent was obtained from all participants for the collection and storage of blood samples for *ITPA* polymorphism testing. The study protocol conforms to the ethical guidelines of the 1975 Declaration of Helsinki.

Clinical endpoints

In relation to the polymorphism of *ITPA* genotyping, we had some clinical endpoints, including the absolute and percentage decline of Hb during the first 12 wk, RBV dose reduction following anemia, and the percentage of platelet (plt) count change.

Treatment regimen

The protocol specified a treatment duration of 24-48 wk depending on viral genotype and the response of patients

Table 1 Basal demographic and biochemistry criteria of all patients (mean \pm SD)

| Criteria | Total |
|------------------------------------|--|
| Number | 102 |
| Age (yr) | 32.5 \pm 7.3 |
| Sex (male/female) | 90/12 |
| BMI | 25.6 \pm 2.7 |
| Baseline Hb (g/dL) | 14.07 \pm 1.1 |
| plt ($\times 10^3/\mu\text{L}$) | 238.5 \pm 62.7 |
| WBCs ($\times 10^3/\mu\text{L}$) | 6.3 \pm 1.4 |
| ALT > ULN, n (%) | 78 (76.5) |
| Creatinine level (mg/dL) | 0.91 \pm 0.44 |
| Baseline HCV RNA (log IU/mL) | 5.4 \pm 0.7 |
| Metavir fibrosis stage, n (%) | |
| F1 | 69 (67.7) |
| F2 | 30 (29.4) |
| F3 | 3 (2.9) |
| Grade of inflammation, n (%) | |
| A1 | 64 (62.7) |
| A2 | 33 (29.4) |
| A3 | 5 (5.4) |
| PEG IFN, n (%) (dose) | |
| $\alpha 2a$ | 72 (70.6) (180 $\mu\text{g}/\text{wk}$) |
| $\alpha 2b$ | 30 (29.4) (1.5 $\mu\text{g}/\text{kg}$ per week) |
| Ribavirin dose (mg/kg per day) | 12.5 (10-15) |
| HCV genotype (G), n (%) | G4, 62 (60.8); G1, 40 (39.2) |

Hb: Hemoglobin; BMI: Body mass index; plt: Platelet; WBCs: White blood cells; ALT: Alanine aminotransferase; ULN: Upper limit of normal of ALT = 12 IU/L; HCV: Hepatitis C virus; PEG-IFN: Pegylated interferon.

during the treatment course. An additional 24 wk of follow-up was done for evaluation of efficacy. The majority of patients (70.6%) received PEG-IFN $\alpha 2a$ (Pegasys) at a dose of 180 μg per week and 29.4% received PEG-IFN $\alpha 2b$ (Peginteron) at a dose of 1.5 $\mu\text{g}/\text{kg}$ per week, given as a subcutaneous injection. All patients received a 15 mg/kg daily oral dose of RBV (Rebetol).

The study had a planned treatment duration of 48 wk. However, the study was designed with a futility-stopping rule that would halt the trial if there was < 2 log₁₀ HCV RNA decline at week 12 or persistent viremia at week 24. The treatment duration was prolonged to 72 wk only for patients who had detectable HCV RNA at week 12 but undetectable at week 24. All patients were followed for 24 wk after cessation of treatment.

HCV RNA measurement and HCV genotyping

The pre-treatment HCV RNA level was detected by quantitative real time polymerase chain reaction (PCR) technique with a lower limit of detection of 12 IU/mL. Qualitative PCR by COBAS TaqMan assay^[17] was used for detection of viral load at weeks 12, 24 and 48. All patients underwent a pretreatment liver biopsy from which the liver disease activity and fibrosis stage were scored by the Metavir scoring system^[18]. HCV genotype determination was by sequence determination in the 5'-nonstructural region of the HCV genome followed by phylogenetic analysis^[7].

SNP genotyping of ITPA

Human genomic DNA was extracted from peripheral

Table 2 Clinical and laboratory data according to rs1127354 inosine triphosphate pyrophosphatase genotypes (mean \pm SD)

| Criteria | CC | CA + AA | P value |
|------------------------------------|------------------|------------------|---------|
| Number, n (%) | 93 (91.2) | 9 (8.8) | |
| Age (yr) | 32.3 \pm 7.3 | 34.4 \pm 7.7 | 0.457 |
| Sex (male/female) | 82/11 | 8/1 | |
| BMI | 25.4 \pm 2.8 | 26.8 \pm 1.6 | 0.046 |
| Baseline Hb (g/dL) | 14 \pm 1.2 | 14.3 \pm 1.03 | 0.441 |
| plt ($\times 10^3/\mu\text{L}$) | 233.2 \pm 62.1 | 292.2 \pm 74.3 | 0.046 |
| WBCs ($\times 10^3/\mu\text{L}$) | 6.2 \pm 1.5 | 6.8 \pm 1.1 | 0.172 |
| ALT > ULN, n (%) | 71 (76.3) | 7 (77.7) | > 0.99 |
| Creatinine level (mg/dL) | 0.9 \pm 0.46 | 0.98 \pm 0.22 | 0.349 |
| Baseline HCV RNA (log IU/mL) | 5.46 \pm 0.7 | 5.4 \pm 0.8 | > 0.99 |

Hb: Hemoglobin; BMI: Body mass index; plt: Platelet; WBCs: White blood cells; ALT: Alanine aminotransferase; ULN: Upper limit of normal of ALT = 12 IU/L; HCV: Hepatitis C virus.

blood. Genotyping of the *ITPA* SNP (rs1127354) was performed using the ABI TaqMan allelic discrimination kit (7500 Real Time PCR System; Applied Biosystems, Carlsbad, CA, United States). Patients were genotyped as CC, CA, or AA at the polymorphic site rs1127354.

Statistical analysis

The statistical analysis of comparisons of the categorical variables of the groups was performed by two tailed Fischer's exact probability test calculated on 2 \times 2 contingency tables. Data are shown as mean \pm SD. Student *t* test was used to compare the direct continuous variables between groups with a significant *P* value of less than 0.05. Odds ratios (OR) for the relative risk and 95%CI in the univariate analysis were estimated by binary logistic regression (program written by Pezzullo JC and Sullivan KM, version 05.07.20). The multivariate regression analysis for the identification of the predictors of Hb decline more than 3 g/dL were performed by JMP software (JMP IN, Version 9.0.2, SAS Institute Inc., Cary, NC, United States).

RESULTS

Baseline characteristics

Baseline demographic and biochemistry criteria for all 102 patients are reported in Table 1. Comparison of the *ITPA* genotyping groups (CC and non-CC) and their relationships to the baseline data are shown in Table 2. Baseline plt count and body mass index were the only significant baseline characters, and both were higher for non-CC than for CC patients (both *P* < 0.05). Hb data were missing for 6 patients from week 16 till end of treatment. Eleven patients stopped the treatment at week 24 due to continuous positivity of HCV RNA, with 91 receiving the complete course of 48 wk.

Distribution of rs1127354 SNP

Rs1127354 SNP genotyping of all participants was successful, and the distribution is as follows: 93 (91.2%) patients were identified as CC and 9 as non-CC (8 with the CA and one with the AA genotype) (8.8%). The minor

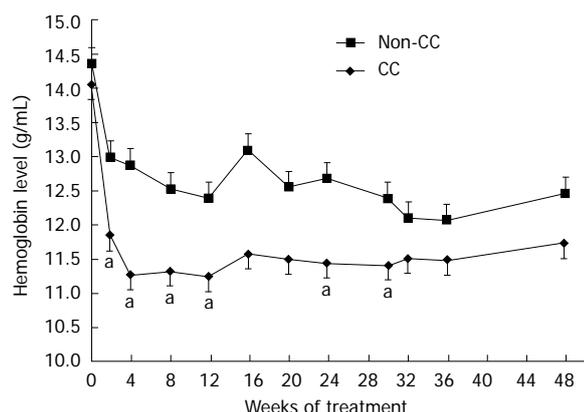


Figure 1 The hemoglobin decline in CC and non-CC inosine triphosphate pyrophosphatase genotypes. The hemoglobin (Hb) levels of the CC group show greater decline than those of the non-CC treatment groups during the course of treatment, with significance at weeks 2, 4, 8, 12, 24 and 30. $P < 0.05$ vs non-CC. The error bars indicate SE of the mean Hb level.

Table 3 Logistic regression analysis during the first 12 wk of combination therapy

| Variable | Univariate | | Multivariate | |
|---------------------------|-------------------|---------|------------------|---------|
| | OR (95%CI) | P value | OR (95%CI) | P value |
| Sex (female) | 0.49 (0.1-2.4) | 0.386 | 4.7 (0.9-26.4) | 0.123 |
| Age (≥ 40 yr) | 0.66 (0.24-1.8) | 0.421 | 1.02 (0.9-1.1) | 0.462 |
| BMI (≤ 24) | 2.03 (0.82-5.01) | 0.124 | 1.2 (1-1.5) | 0.068 |
| Baseline Hb (< 15) | 0.14 (0.04-0.49) | 0.002 | 0.8 (0.5-1.2) | 0.610 |
| Baseline plt (< 200) | 0.26 (0.1-0.65) | 0.004 | 0.9 (0.9-1) | 0.073 |
| <i>ITPA</i> genotype (CC) | 6.36 (1.24-32.41) | 0.025 | 0.4 (0.04-4.08) | 0.671 |
| RBV dose $\geq 80\%$ | 0.08 (0.01-0.3) | 0.0009 | 0.87 (0.82-0.92) | 0.00002 |

OR: Odds ratio; BMI: Body mass index; Hb: Hemoglobin; plt: Platelet; *ITPA*: Inosine triphosphate pyrophosphatase; RBV: Ribavirin.

allele frequency of rs1127354 was 0.04, which means the chance of (A) allele occurring was 4%; the distribution here is consistent with Hardy Weinberg Equilibrium ($P = 0.108$).

Hb decline according to *ITPA* genotypes

In the present study, anemia was defined as an Hb level decreased by > 3 g/dL and severe anemia was reported when Hb level declined to less than 10 g/dL. Figure 1 shows the difference in Hb level between the CC and non-CC groups throughout the treatment course. It was significantly different at weeks 2, 4, 8, 12, 24 and 30 ($P = 0.019, 0.016, 0.015, 0.013, 0.036$ and 0.047 , respectively). Hb decline > 3 g/dL during the first 12 wk was faster and more vigorous in the anemia-susceptible group CC than in the protective non-CC group at weeks 4 and 8 ($P = 0.047$ and 0.034 , respectively). By week 16, Hb had settled in the CC group with no further decrease, in contrast to the non-CC genotype group that showed a sudden increase in Hb followed by a continuous slow decrease until the end of treatment (Figure 1). The Hb decline > 3 g/dL was significantly higher among CC patients than non-CC patients at week 8 [36 of 93 (38.7%) vs none of 9 (0%), $P = 0.024$] and week 12 [60 of 93 (64.5%) vs 2

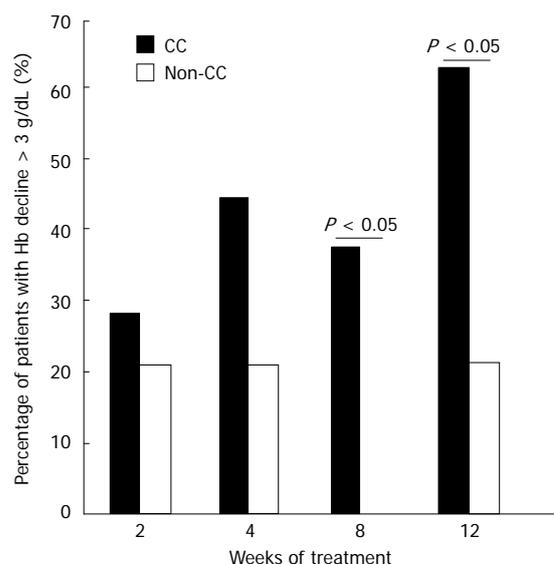


Figure 2 Patients with hemoglobin decline more than 3 g/dL. The percentage of CC and non-CC group patients who had hemoglobin (Hb) decline of more than 3 g/dL during the first 12 wk.

of 9 (22.2%), $P = 0.038$] (Figure 2). This indicated a protective advantage for patients with the minor allele A of rs1127354 SNP of *ITPA* gene against early onset anemia during combination treatment for Egyptian patients with chronic hepatitis C. During the first 12 wk, severe anemia with an Hb level less than 10 g/dL was identified in 29% of the CC patients compared with 11.1% of the non-CC patients ($P = 0.438$). Interestingly, the only patient in this study harboring a homozygous variant for the minor allele (AA) did not suffer anemia at any time during the treatment. In an attempt to identify factors predictive of the incidence of Hb decline more than 3 g/dL during the first 12 wk, we performed both univariate and multivariate regression analyses (Table 3). The univariate logistic regression analysis detected four factors as independent variables: *ITPA* genotype, percentage of received RBV dose and the baseline Hb and plt levels. On the other hand, the percentage of the received RBV dose ($\geq 80\%$ of total dose), was the only significant independent variable in the multivariate analysis.

Adjustment of RBV dose during combination treatment

The dose of RBV was modified according to the presence of treatment side effects. During the first 12 wk, the total number of patients needing an RBV dose reduction was 76 of 102 (74.5%). The percentage of RBV dose reduction was higher for the CC than for the non-CC patients ($18\% \pm 12.1\%$ vs $8.5\% \pm 10.2\%$ of the recommended dose, $P = 0.021$). The percentage of CC patients requiring an RBV dose reduction was greater than that of non-CC patients (77.4% vs 44.4%, respectively, $P = 0.044$) (Figure 3). Calculation of the time-point of reduction showed a significant difference between CC and non-CC patients at week 4, at which time none of the non-CC patients required an RBV dose reduction compared to 35 CC patients having a high reduction of RBV ($P =$

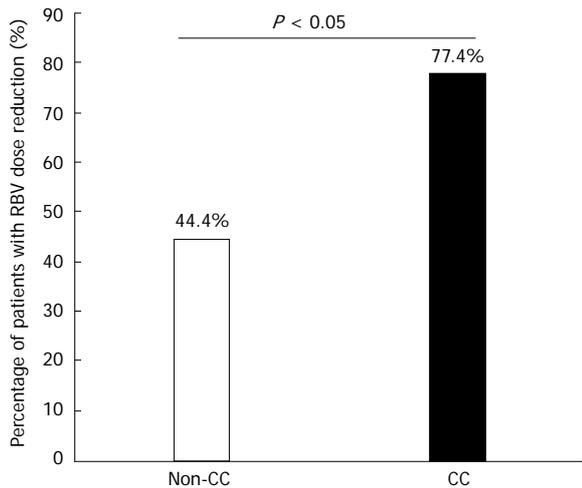


Figure 3 Patients with ribavirin dose reduction. Percentage of patients who had ribavirin (RBV) dose reduction during the first 12 wk of the combination treatment: It was significantly higher in the CC than in the non-CC genotype groups.

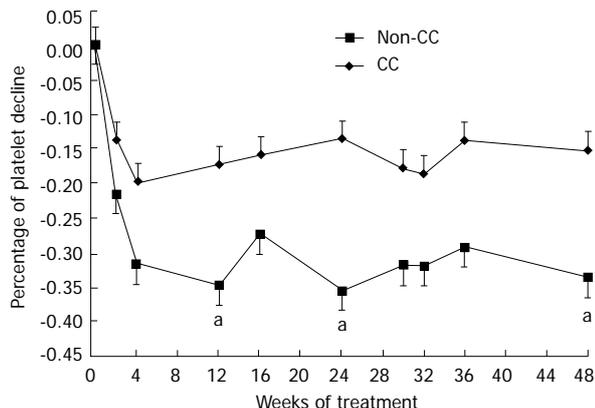


Figure 4 Decline of platelets of patients with different inosine triphosphate pyrophosphatase genotypes. The percentages of platelet decline of CC and non-CC group patients throughout the 48 wk of combination treatment. It indicates a greater decline of platelet count in the non-CC than CC variants. ^a*P* < 0.05 at weeks 12, 24 and 48. Error bars indicate standard error.

0.025). Although the reduction of RBV started earlier for patients with the CC than with the non-CC genotype (6.1 ± 5.3 wk *vs* 10 ± 8.5 wk, respectively), this did not reach statistical significance (*P* = 0.325). After the first 12 wk, 11 CC patients stopped the treatment due to non-responsiveness, whereas all non-CC patients completed the full course of treatment. Table 4 shows a comparison of the average RBV dose during the first 12 wk with the following 36 wk of treatment course between CC and non-CC patients. A significant difference was found (*P* = 0.035 and 0.029, respectively).

Protocol of RBV dose reduction

Almost all of the patients in this study had an RBV dose reduction in the first 12 wk of treatment, with 200 mg the initial reduction when Hb declined by more than 3 g/dL. Patients with severe anemia had a higher reduction when their Hb reached less than 10 g/dL. Usually, dis-

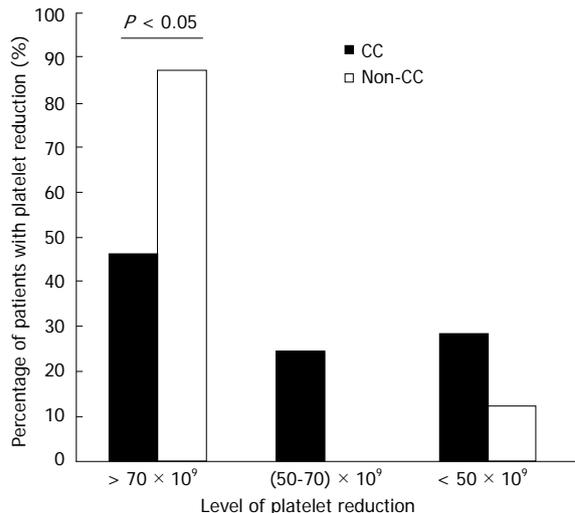


Figure 5 Patients with 3 different levels of platelet decline. The percentages of patients according to 3 different levels of platelet decline at week 4 of combination treatment in both groups of inosine triphosphate pyrophosphatase genotypes. Statistical significance is shown with greater level of platelet decline.

Table 4 The average ribavirin dose during 2 different time-points among inosine triphosphate pyrophosphatase CC and non CC genotypes

| | During first 12 wk | | | 12-48 wk of treatment | | |
|------------------------------|--------------------|-------------|--------------|-----------------------|---------------|--------------|
| | Total | CC | CA + AA | Total | CC | CA + AA |
| No. of patients | 102 | 93 | 9 | 91 | 82 | 9 |
| RBV dose (mg/ wk), mean ± SD | 947 ± 151.4 | 936 ± 148.3 | 1059 ± 144.1 | 943 ± 153.2 | 930.5 ± 149.6 | 1059 ± 144.1 |
| <i>P</i> value | | 0.035 | | | 0.029 | |

RBV: Ribavirin.

continuation of treatment is considered when the Hb declines to less than 8.5 g/dL; however, in this study, none of the patients had treatment discontinued due to this cause. During the first 12 wk, 74.2% of the CC patients (69 of 93) received 80% or more of the initially recommended dosage of RBV, whereas all non-CC patients received more than 80% of the dosage (*P* = 0.112).

Impact of ITPA polymorphism on platelet count change

Calculation of plt count change throughout the 48 wk of treatment showed a difference in the percentage of plt reduction between the CC and non-CC patients (Figure 4). Significant differences were found at weeks 12, 24 and 48 (*P* = 0.018, 0.009 and 0.026, respectively). Although CC patients had a lower baseline median plt count than non-CC patients (233.2 *vs* 292.2, respectively), they had a lower percentage of plt reduction during the early weeks of treatment (17.4% *vs* 28.5%, respectively). Figure 5 shows the difference between *ITPA* genotypes according to three levels of maximum plt reduction at week 4 (plt reduction more than $70 \times 10^9/L$, $(50-70) \times 10^9/L$, and less than $50 \times 10^9/L$). The percentage of patients was significantly different among those with plt reduction more than $70 \times 10^9/L$: 87.5% of non-CC *vs* 46.4% of CC (*P* =

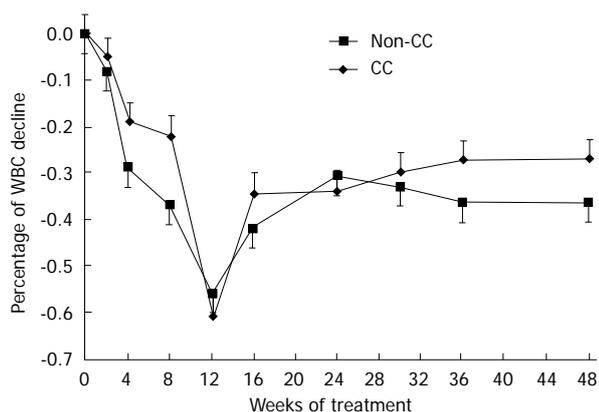


Figure 6 White blood cell decline of both inosine triphosphate pyrophosphatase genotypes. The change in white blood cells (WBCs) in the CC and non-CC inosine triphosphate pyrophosphatase genotype groups during the 48 wk of combination treatment: No significant difference between the 2 groups was found. Error bars indicate standard error.

0.029). This result indicates that the anemia-susceptible patients of group CC are less likely to develop a higher degree of plt reduction than non-CC patients. Notably, the percentage of the relative reactive increase in the plt count during weeks 1-4 was higher for CC patients than for non-CC patients but did not reach statistical significance (34.4% *vs* 11%, respectively, $P = 0.264$). However, the reactive plt increase of the CC and non-CC patients did not correlate with either an Hb reduction > 3 g/dL or with the baseline plt count of weeks 1-4.

No significant difference in the white blood cells was found between the CC and non-CC patients during the early weeks of treatment ($P > 0.05$) (Figure 6). Also, there was no significant association between *ITPA* genotype and early virological response at week 12.

DISCUSSION

To our knowledge, this is the first study to assess the impact of the rs1127354 *ITPA* genotype on the anemia of Egyptian patients infected with chronic hepatitis C, regardless of the outcome of treatment. Marsh *et al.*^[19] and Cao *et al.*^[20] reported the distribution of *ITPA* genotypes in multiple populations, which reached the highest rate among Asians (11%-19%) and lowest in Central/South Americans (1%-2%), while in Caucasian and African populations the distributions were constant (5%-7%). In our study, the minor allele distribution of *ITPA* polymorphism was 4%, which is almost comparable with previous reports. Previous studies have demonstrated the protective benefit of the minor allele A of the rs1127354 *ITPA* SNP against RBV-induced anemia during the first 12 wk of combination treatment^[14,15,21-23]. Similarly, Hb decline > 3 g/dL was detected in about 64.5% of the anemia-susceptible group CC, which means that about 35.5% did not develop anemia.

The difference of Hb decline between the CC and non-CC groups throughout the treatment is comparable with previous studies^[14,15,21,24]. Hb decline in the first 4

wk has been reported to be an independent predictor of development of anemia at some time in the course of treatment^[25-29]. This indicates the value of early monitoring of Hb and the consequence of RBV dose adjustment to obviate further hazards of anemia. Anemia is a major cause of RBV dose reduction and premature withdrawal from treatment by 10%-14% of HCV-infected patients in the first 12 wk^[6]. RBV dose reduction was obviously more necessary for patients in the CC group than for those in the protective non-CC group in the early weeks of treatment, which may explain the relative stability of the Hb level by week 16 in the CC group in comparison with the non-CC group. Since the mean age of all participants in this study was 35.5 years (range 21-50), their mean baseline Hb was 14 ± 1.1 g/dL and almost of them showed mild liver disease, thus there was no need for pretreatment reduction of the RBV dose. There are two explanations for the lack of statistical significance for the *ITPA* genotypes in multivariate analysis. First, the RBV dose in the univariate analysis showed higher significance than the *ITPA* genotype (0.0009 *vs* 0.025, respectively). Second, the colinearity between RBV dose reduction and hemoglobin decline makes the variable of RBV dose reduction strong enough to prevent the impact of *ITPA* genotypes in the multivariate analysis. This interpretation was previously confirmed by Domingo *et al.*^[15]. In previous studies^[12-14,21-23], the *ITPA* genotypes were statistically significant in their multivariate analysis; this may be due to the different ethnic cohorts and the greater number of patients included in their studies. Accumulation of the RBV metabolite (triphosphorylated RBV) in RBCs causes a relative deficiency of ATP, and hence antioxidative damage of the cells with erythrophagocytosis^[30]. Additionally, the phosphorylation of RBV is reversible in nucleated cells, and the half-life of RBV elimination from RBCs has been reported to be greater than from plasma, 40 d *vs* only 24 h respectively^[2,11], which in turn enhances the destructive effect on RBCs. Recently, it has been reported that the accumulated ITP in RBCs starts to substitute guanosine triphosphate, which has already been depleted by RBV, for biosynthesis of ATP^[31]. According to the classification of predicted ITPase deficiency by Thompson *et al.*^[14], we can classify our results of rs1127354 *ITPA* genotypes into -, ++, +++ (CC, CA, and AA, respectively). The wild type CC usually shows no deficiency (-) with 100% ITPase activity, while the heterozygous genotype CA was predicted to have 25% of ITPase activity. The mutant homozygous AA genotype represents 0% activity (the highest level of deficiency +++). This deficiency in turn leads to accumulation of ITP inside RBCs, instead of RBV triphosphate^[32].

There is a robust point in this study; we demonstrated the association between *ITPA* polymorphism (rs1127354) and RBV-induced anemia without resorting to checking the other SNP, rs7270101, of the same gene (a splice altering single-nucleotide polymorphism in intron 2). This was previously confirmed by Suzuki *et al.*^[33]. In our study, the difference in plt change of CC and non-CC genotype

patients supported, to some extent, the previous results of Tanaka *et al.*^[34]; however, it was different than those reported by Thompson *et al.*^[14]. Tanaka *et al.*^[34] identified a significant difference in their population at a maximum plt reduction of $< 30 \times 10^9/L$, but in the present study a significant difference was determined at plt count reduction of $> 70 \times 10^9/L$. In the current study, we did not find a direct association between the *ITPA* genotype and plt count change. On the other hand, the association is thought to be indirect through the role of endogenous erythropoietin (EPO), which may increase to confront the reduction of Hb during treatment^[25,34,36]. The later study showed sequence homology between EPO and thrombopoietin^[36], which may lead to similarity in their action. Actually, the effect of EPO on the plt count is controversial. In the current study, EPO was not prescribed for any patient. Although EPO prescription is not stated in the established protocol of the Egyptian Ministry of Health for the treatment of hepatitis C in Egypt, some cases may require the addition of EPO in other studies.

There are some limitations in the present study: (1) the small number of female participants did not enable us to evaluate possible associations with gender; (2) the impact of the *ITPA* polymorphism on the treatment outcome has not been assessed. This may be attributed to the lower distribution rate of the *ITPA* protective minor allele among populations, which reflects its lack of significance to change the treatment outcome^[12,14]; and (3) the unavailability of data to evaluate the endogenous serum level of EPO to determine if there was any association between it and the reactive increase in the plt count among the two *ITPA* groups.

Further research is needed to cover the following points: First, efficacy of the clinical use of this approach and the elucidation of its cost effectiveness. This may enable the physician to take precautions before starting therapy of those patients who are likely to develop anemia during therapy (*ITPA* CC genotype). These precautions may include pretreatment initial doses of EPO, initial reduction of RBV doses or even postponement of combined treatment in susceptible patients with no or mild liver disease. Second, verification is needed of the correlation between *ITPA* genotypes and RBV-induced hemolytic anemia by use of the new Specifically Targeted Antiviral Therapy for hepatitis C among Egyptian HCV patients. Third, the impact of *ITPA* polymorphism should be determined in targeted cohorts of Egyptian HCV patients, including old age populations and those suffering from advanced liver disease or chronic kidney disease. Finally, the association between *ITPA* variants and the plasma concentration of RBV in Egyptian patients should be investigated.

In summary, the minor allele in *ITPA* rs1127354 variants (CA/AA) plays a decisive role in protection against treatment-induced anemia and RBV dose reduction of Egyptian HCV patients. Additionally, pretreatment clinical decisions regarding RBV dose adjustment can be bolstered by identifying such polymorphisms.

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COMMENTS

Background

Hepatitis C infection is a widespread disease caused by hepatitis C virus (HCV). It infects the liver, mainly causing acute disease developing to chronic if left without treatment. Its current standard therapy is a combination therapy of pegylated interferon injection plus oral ribavirin drug. The treatment is effective in more than half of patients but has moderate to severe side effects. Anemia is a common side effect which may be a leading cause of decreasing the doses of therapy or even premature withdrawal of the treatment. Herein, the authors reported a correlation between host genetic polymorphisms and pre-treatment prediction of anemia in Egyptian HCV patients.

Research frontiers

Due to the multiple side effects associated with the therapy of hepatitis C disease, many researchers are focusing on studying the pretreatment predictors of these side effects. This will help in obviating the dose reduction and the premature withdrawal of therapy. Although Egypt has the highest rate of HCV infection, no research has been done on its HCV patients in that field. Therefore, the authors of this study targeted this cohort.

Innovations and breakthroughs

The authors reported, for the first time, the implication of inosine triphosphate pyrophosphatase (*ITPA*) single nucleotide polymorphism (SNP) genotypes in predicting the incidence of anemia in Egyptian HCV patients during the combination therapy. The mutant genotype of this polymorphism has a crucial role in protection against treatment-induced anemia and ribavirin (RBV) dose reduction in Egyptian HCV patients. Further studies are needed to elucidate the cost effectiveness of this approach.

Applications

ITPA genotyping is a promising pretreatment predictor of treatment-induced anemia before starting the combination therapy of HCV disease. This will enhance the pretreatment decision of early adjustment of RBV dose in patients with the unfavorable genotype.

Terminology

SNP is the most common type of genetic variation of the human DNA. There are millions of human SNPs. They occur when a single nucleotide in a genome differs among members of the same species. This difference is due to substitution, insertion or deletion of one nucleotide within the genetic structural unit of human DNA. Many studies have found a robust correlation between SNPs and different diseases.

Peer review

The present study provides interesting results and novelty to genetic factors related to ribavirin-induced anemia during HCV therapy in an Egyptian HCV cohort. The manuscript is well written and the conclusion drawn in the data is appropriate.

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Influence of Kupffer cells and platelets on ischemia-reperfusion injury in mild steatotic liver

Koichi Ogawa, Tadashi Kondo, Takafumi Tamura, Hideki Matsumura, Kiyoshi Fukunaga, Tatsuya Oda, Nobuhiro Ohkohchi

Koichi Ogawa, Tadashi Kondo, Takafumi Tamura, Hideki Matsumura, Kiyoshi Fukunaga, Tatsuya Oda, Nobuhiro Ohkohchi, Department of Surgery, Graduate School of Comprehensive Human Sciences, University of Tsukuba, Tsukuba 305-8575, Japan

Author contributions: Ogawa K and Kondo T contributed equally to this work; Ogawa K, Kondo T, Tamura T and Matsumura H designed the study; Ogawa K and Tamura T performed the experiments; Fukunaga K, Oda T and Ohkohchi N contributed the analysis of the data; Ogawa K, Kondo T, Fukunaga K and Ohkohchi N wrote the manuscript.

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Correspondence to: Nobuhiro Ohkohchi, MD, PhD, Professor, Department of Surgery, Graduate School of Comprehensive Human Sciences, University of Tsukuba, 1-1-1 Tennodai, Tsukuba 305-8575, Japan. nokochi3@md.tsukuba.ac.jp

Telephone: +81-29-8533221 Fax: +81-29-8533222

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Abstract

AIM: To investigate the effect of mild steatotic liver on ischemia-reperfusion injury by focusing on Kupffer cells (KCs) and platelets.

METHODS: Wistar rats were divided into a normal liver group (N group) and a mild steatotic liver group (S group) induced by feeding a choline-deficient diet for 2 wk. Both groups were subjected to 20 min of warm ischemia followed by 120 min of reperfusion. The number of labeled KCs and platelets in sinusoids and the blood perfusion in sinusoids were observed by intravital microscopy (IVM), which was performed at 30, 60 and 120 min after reperfusion. To evaluate serum alanine aminotransferase as a marker of liver deterioration, blood samples were taken at the same time as IVM.

RESULTS: In the S group, the number of platelets adhering to KCs decreased significantly compared with the N group (120 after reperfusion; 2.9 ± 1.1 cells/acinus vs 4.8 ± 1.2 cells/acinus, $P < 0.01$). The number of KCs in sinusoids was significantly less in the S group than in the N group throughout the observation periods (before ischemia, 19.6 ± 3.3 cells/acinus vs 28.2 ± 4.1 cells/acinus, $P < 0.01$ and 120 min after reperfusion, 29.0 ± 4.3 cells/acinus vs 40.2 ± 3.3 cells/acinus, $P < 0.01$). The blood perfusion of sinusoids 120 min after reperfusion was maintained in the S group more than in the N group. Furthermore, elevation of serum alanine aminotransferase was lower in the S group than in the N group 120 min after reperfusion (99.7 ± 19.8 IU/L vs 166.3 ± 61.1 IU/L, $P = 0.041$), and histological impairment of hepatocyte structure was prevented in the S group.

CONCLUSION: Ischemia-reperfusion injury in mild steatotic liver was attenuated compared with normal liver due to the decreased number of KCs and the reduction of the KC-platelet interaction.

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Key words: Steatotic liver; Mild steatotic liver; Kupffer cell; Platelet; Ischemia-reperfusion; Intravital microscopy

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INTRODUCTION

It is widely accepted that the steatotic liver is more sus-

ceptible to hepatic ischemia-reperfusion (IR) injury. Increased microcirculatory deterioration is suggested as a reason for this increased liver damage in the steatotic liver^[1-3]. Morphologically, the steatotic liver is characterized by a demonstrable deposition of large, macronodular fatty droplets in liver parenchymal cells^[4]. In addition, narrow and distorted lumens of sinusoids, resulting from the swelling of hepatic parenchymal cells due to accumulated lipid, cause a decrease in sinusoidal perfusion^[2]. These changes to microcirculation are exacerbated by leukocytes, either mechanically trapped in the narrowed sinusoids or adhering to activated Kupffer cells (KCs) with the release of cytokines, in addition to free radicals^[2,5].

Thirty percent of the population in Japan and Western countries suffers from steatotic liver, and the percentage is still increasing^[3,6]. Most of these patients have been diagnosed by abdominal ultrasonography screening, even though elevation of serum liver enzyme levels was detected in a lesser percentage^[6]. Steatosis of the liver is classified clinically into three grades according to the proportion of hepatocytes with fatty droplets: mild (< 30%), moderate (30%-60%) and massive (> 60%)^[7]. Fishbein *et al.*^[8] reported that liver enzyme levels were not elevated in the steatotic liver in which the proportion of hepatocytes with fat deposition was less than 18%. Steatotic liver even in a mild degree is a risk factor for complication after liver resection^[9]. Most of the reports describing IR injury of the steatotic liver were investigations of moderate and severe steatosis. It is unclear whether the intensity of the hepatic IR injury depends on the degree of fatty change.

Previously, we reported that liver ischemia induced the adhesion of platelets to KCs in the early period after reperfusion, and that interaction between KCs and platelets played a key role in reperfusion injury of the liver^[10,11]. In this study, we have focused on the interaction between KCs and platelets in the mild steatotic liver with intravital microscopy (IVM). We hypothesized that tolerance to hepatic IR injury differs according to the degree of steatosis. The aim of this study was to clarify the hepatic dysfunction after IR in the mild steatotic liver compared with the normal liver.

MATERIALS AND METHODS

Animals

Male Wistar rats were obtained from CLEA Japan, Inc. (Tokyo, Japan). We prepared two model types, normal liver and mild steatotic liver. In the normal liver model, rats weighting 250 g to 300 g were used. In the steatotic model, the rats' weights were adjusted to the same weight range after they were fed a choline-deficient diet (CDD) (Oriental Bio Service Kanto Inc., Ibaraki, Japan) for 2 wk. Animal experiments were carried out in a humane manner after receiving approval from the Institutional University Experiment Committee of the University of Tsukuba, and in accordance with the Regulation for Animal Experiments in our university and the Fundamental Guideline for Proper Conduct of Animal Experiment

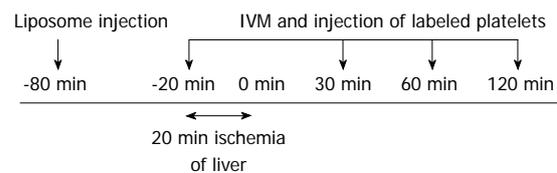


Figure 1 Experimental design. In all groups, total warm hepatic ischemia was induced for 20 min by clamping the portal triad. A total of 1×10^8 fluorescence-labeled platelets, approximately 1% of all circulating platelets in the recipient rat, were injected *via* the left carotid artery 5 min before intravital microscopy (IVM).

and Related Activities in Academic Research Institutions under the jurisdiction of the Japanese Ministry of Education, Culture, Sports, Science, and Technology.

Experimental design

Animals were divided into two groups as follows: (1) the normal liver group (N group; $n = 6$); and (2) the mild steatotic liver group (S group; $n = 6$). In both groups, total normothermic hepatic ischemia was induced for 20 min by clamping the portal triad. The hepatic microcirculation and dynamics of platelets and KCs were observed just before ischemia and at 30, 60 and 120 min after reperfusion (Figure 1).

Surgical procedure

Under anesthesia using isoflurane, the animals were tracheotomized. To reduce spontaneous breathing, animals were ventilated mechanically (MK-V100; Muromachi Kikai Co. Ltd, Tokyo, Japan). The animals were placed in a supine position on a heated pad to maintain the rectal temperature at 37 °C. To monitor arterial blood pressure and allow continuous infusion of Ringer's solution, polyethylene catheters (PE-50, 0.58/0.96mm internal/external diameter; Becton Dickinson, Sparks, MD) were inserted into the left carotid artery and left jugular vein, respectively. After performing laparotomy *via* a transverse incision, the ligaments around the liver were dissected to mobilize the left lobe. At the same time, the hepatoduodenal ligament was taped for clamping later. The left hepatic lobe was exteriorized on a plate specially designed to minimize movements caused by respiration and covered with cover glass. Surgical procedures were performed using sterile techniques. After 60 min of normal saline continuous infusion, IVM was performed as a pre ischemia study. Then, hepatic ischemia was induced by clamping the portal triad (the hepatic artery, portal vein, and bile duct) with a microclip (B. Braun Aesculap Japan Co., Ltd, Tokyo, Japan) for 20 min. IVM was performed at 30, 60 and 120 min after reperfusion. Blood samples were taken for analysis of enzyme activities from a catheter placed in the left carotid artery at the same time as IVM. Alanine aminotransferase (ALT) was evaluated as one of the liver enzyme. At 120 min of reperfusion, total body blood was taken for euthanasia. At the end of the experiments, liver tissue was taken for histological examination.

Platelet preparation

Platelets were isolated from whole blood samples of syngeneic rats and labeled with rhodamine-6G (50 μ L/mL whole blood: R-4127; Sigma, St. Louis, MO, United States), as described by Massberg *et al.*^[12]. Briefly, the collected blood was diluted with buffer after the addition of prostaglandin E1 and rhodamine 6G. After two-cycle centrifugation, fluorescent platelets were resuspended in phosphate-buffered saline. In this study, a total of 1×10^8 fluorescence-labeled platelets, approximately 1% of all circulating platelets in the recipient rat, were injected through the left carotid artery at 5 min before IVM.

Liposome entrapment method (fluorescence labeling of KCs)

Fluorescently labeled phosphatidylcholine (PC) was incorporated into liposomes, as described by Watanabe *et al.*^[13]. The fluorescent pigment used was 2-(12-(7-nitrobenz-2-oxa 1,3-diazol-4-yl) amino) dodecanoyl-1-hexadecanoly-*sn*-glycero-3-phosphocholine (NBD-C₁₂-HPC; Molecular Probes; Eugene, United States). After intra-arterial injection, KCs in the rat livers were stained and were clearly delineated under the fluorescence image in the IVM. Phagocytic activity of KCs after the administration of liposomes was reported by measuring the amount of hepatic uptake of intravenously administered fluorescent microspheres; no detrimental influence of the liposomes on the phagocytic activity was observed. Additionally, no histopathological changes were found in the livers from liposome-treated rats^[13]. Sixty minutes before hepatic ischemia, liposome-encapsulated fluorescent liposomes (4 mL/kg) were administered *via* the carotid artery catheter.

Intravital microscopy

IVM was performed using a modified microscope (BX30 FLA-SP; Olympus Co., Tokyo, Japan) with a 100 W mercury lamp attached to a filter block. The hepatic microcirculation was recorded by means of a CCD camera (C5810; Hamamatsu Photonics, Hamamatsu, Japan) and a digital video recorder (GV-HD700/1; Sony, Tokyo, Japan) for offline analysis. Using objective lenses (10 \times 0.3 to 20 \times 0.7; Olympus Co., Tokyo, Japan), a final magnification from x325 to x650 was achieved on the video screen. To assess sinusoidal perfusion, sodium fluorescein (2×10^{-3} M/kg, F-6377; Sigma, St. Louis, MO, United States) was injected *via* the jugular catheter. Rhodamine-6G labeled platelets were infused intra-arterially just before ischemia and at 30, 60 and 120 min after reperfusion, and 10 randomly chosen acini were visualized. Quantitative assessment of the microcirculatory parameters was performed offline using WinROOF imaging software (version 5.0; Mitani Shoji, Tokyo, Japan).

Microcirculatory analysis

The following parameters were analyzed: (1) the number of adherent platelets, *i.e.*, platelets firmly attached to the sinusoidal endothelium for longer than 20 s [the number of adherent platelets in the scanned acini was counted

and results were expressed as the number of adherent platelets per field (1 field = approximately 0.2 mm²); (2) the number of adherent platelets adhering to KCs; (3) the number of KCs; and (4) the sinusoidal perfusion failure rate (%) as an index of microcirculatory disturbance, calculated as the ratio of non-perfused sinusoids among the sinusoids observed in one acinus after 120 min of reperfusion.

Immunohistochemical study of KCs

We immunohistochemically assessed the number of KCs in the acini. To compare the differences between the normal liver group and the mild steatotic liver group before ischemia and after reperfusion, liver tissues were obtained from each group both before ischemia and at the end of the surgical procedure, and from another animal before ischemia. The tissues were fixed in 10% formalin and embedded in paraffin and cut into 4 μ m-thick sections. It was immersed in 0.03% hydrogen peroxidase to block endogenous peroxidase activity, and then blocked with 2% bovine serum albumin to reduce background staining. To specifically recognize KCs, mouse anti-rat ED2 antibody (Santa Cruz Biotechnology, Santa Cruz, CA, United States) was used as the primary antibody. The sections were incubated with primary diluted antibodies (1:50) at room temperature for 60 min. Primary antibody reactions were enhanced using horseradish peroxidase EnVision (Dako Japan, Tokyo, Japan). The immunoreaction was visualized with 0.05% 3,3-diaminobenzidine solution. After washing in distilled water, specimens were counterstained with hematoxylin then mounted. The number of ED2-positive cells per acinus was counted in five randomly chosen acini.

Biochemical assays

As a marker of liver deterioration, serum ALT levels were measured using a Drychem 7000V autoanalyzer (Fuji Film, Tokyo, Japan). Serum was stored at -80 $^{\circ}$ C until use for cytokine determination. Levels of interleukin (IL)-6 were measured using commercial enzyme-linked immunosorbent assay kits (R and D Systems, Minneapolis, MN, United States).

Histological analysis

Liver tissue was obtained before ischemia from the S group to assess the degree of steatosis and after 120 min of reperfusion from each group to assess the histological changes due to IR. The samples were fixed with 10% formalin, and embedded in paraffin. Thin sections (4 μ m) were prepared and stained with hematoxylin and eosin (HE). Tissue damage was evaluated in 5 randomly selected high-power fields (\times 200).

Statistical analysis

All data are expressed as mean \pm SD. The Mann-Whitney *U* test and analysis of variance were used, followed by Scheffe's test. *P* values < 0.05 were considered statistically significant.

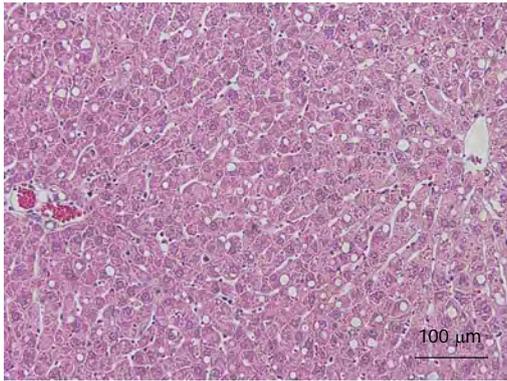


Figure 2 Mild steatotic liver model. Hematoxylin and eosin staining of liver specimens after 2 wk of a choline deficient diet demonstrated that between 10% and 20% of hepatocytes had deposition of microvesicular lipid droplets.

RESULTS

Mild steatotic liver model

Mild steatosis of the liver was induced by feeding a CDD. The CDD-induced steatotic liver is an established experimental model, in which morphological and functional features are very similar to those of the clinical steatotic liver^[14]. The rats fed on CDD for 2 wk developed mild steatotic livers. They were characterized by microvesicular lipid droplet filtration in 10% to 20% of hepatocytes (HE stain) (Figure 2). These findings were identified as a mild degree of steatosis of the liver. We preoperatively confirmed similar findings by liver biopsy in several animals.

Number of adherent platelets in acini

The number of adherent platelets in sinusoids increased along with the reperfusion time both in the N group and in the S group (Figure 3A). In the S group, the number of adherent platelets adhering to KCs was significantly less than in the N group at 30 min after reperfusion and concomitant with the reperfusion period (Figure 3B and C). In addition, there was no significant difference between the two groups in the number of blood platelets (data not shown).

Number of KCs in acini

The mild steatotic change in the liver significantly decreased the number of KCs in sinusoids compared with the normal liver at any point in time before and after ischemia reperfusion with IVM study (Figure 4A). In the IVM observation, the counted KCs represented only KCs labeled by liposome entrapment methods. In addition, the number of KCs was verified immunohistochemically. In immunohistochemical staining, the numbers of ED2-positive cells were lower in the S group than in the N group already at the time before ischemia and after 120 min of reperfusion (Figure 4B).

Sinusoidal perfusion failure rate

Vollmar *et al.*^[15] reported that the sinusoidal perfusion rate was one of the indexes of reperfusion injury. After 120 min of reperfusion, the rate of sinusoidal perfusion

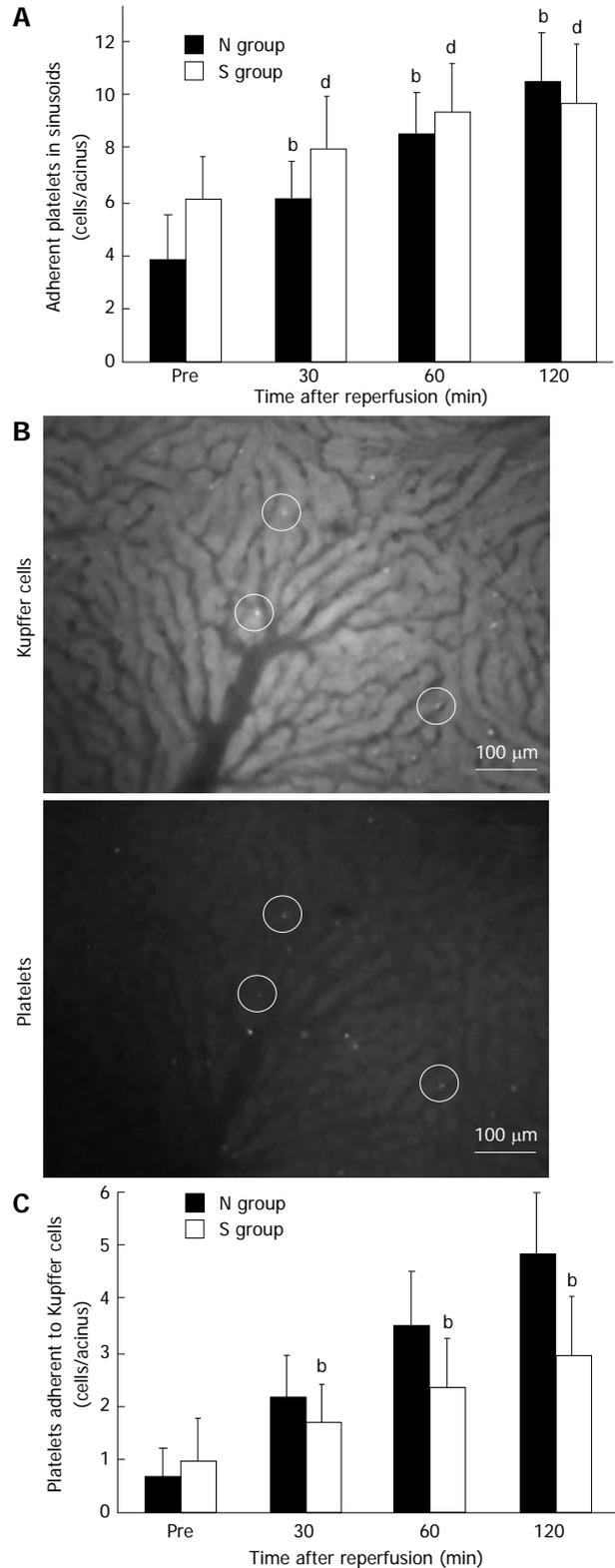


Figure 3 Platelets and Kupffer cell dynamics in sinusoids. A: The number of adherent platelets in sinusoids increased along with the reperfusion time in both groups. Shown as mean ± SD; $n = 6$. ^b $P < 0.01$ vs pre-ischemia in the normal liver (N) group, ^d $P < 0.01$ vs pre-ischemia in the mild steatotic liver (S) group; B: Video images of Kupffer cells (KCs) and platelets in acini 30 min after reperfusion. The field is approximately 0.2 mm². The upper figure shows the acini of fluorescently stained KCs, and in the lower figure, the acini of fluorescently stained platelets. White circles indicate adhesion to KCs and platelets in the same place; C: In the S group, the number of adherent platelets adhering to KCs was significantly less than in the N group at 30 min after reperfusion and concomitant with the duration of reperfusion. Shown as mean ± SD; $n = 6$. ^b $P < 0.01$ vs the N group.

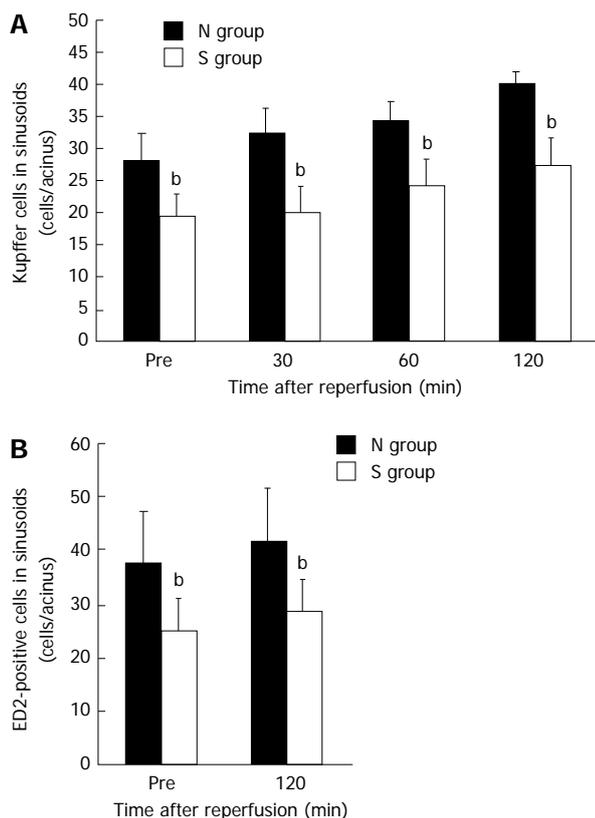


Figure 4 The number of Kupffer cells in sinusoids (mean ± SD). A: The number of Kupffer cells in sinusoids observed in the intravital microscopy system was decreased significantly in the mild steatotic liver (S) group compared to the normal liver (N) group at any point in time before and after ischemia reperfusion. $n = 6$. ^a $P < 0.01$ vs the N group; B: In immunohistochemical staining, the number of ED2-positive cells was significantly less in the S group than in the N group. $n = 6$. ^b $P < 0.01$ vs the N group.

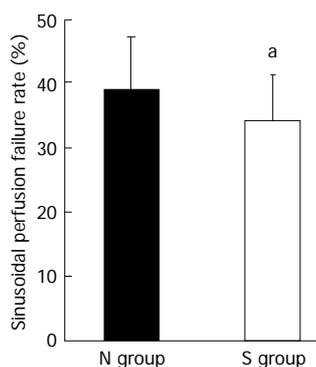


Figure 5 Sinusoidal perfusion failure rate (mean ± SD). The sinusoidal perfusion failure rate after 120 min of reperfusion was lower in the mild steatotic liver (S) group than in the normal liver (N) group. $n = 6$. ^a $P < 0.05$ vs the N group.

failure was significantly higher in the N group than in the S group (Figure 5). The perfusion of sinusoids in which KCs and platelets adhered had a failure rate of about 50% in both groups (data not shown). In contrast, the perfusion failure rate of sinusoids in which KCs adhering to platelets was not observed remained at approximately 30% in both groups (data not shown). This indicated that

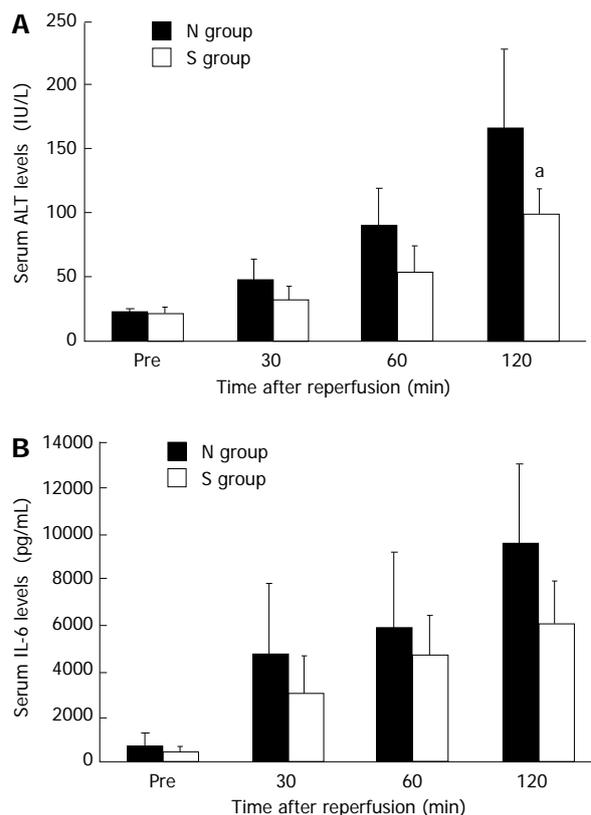


Figure 6 Serum alanine aminotransferase and interleukin-6 levels (mean ± SD). A: Serum alanine aminotransferase (ALT) levels were significantly lower in the mild steatotic liver (S) group than in the normal liver (N) group at 120 min after reperfusion. $n = 6$. ^a $P < 0.05$ vs the N group; B: The concentration of serum interleukin-6 (IL-6) tended to be lower in the S group compared with in the N group but there was no significant difference (after 120 min of reperfusion, $P = 0.09$ vs the N group). $n = 6$.

the interaction between KCs and platelets was associated with sinusoidal perfusion failure.

Serum ALT and interleukin-6 levels

Serum ALT level as a measure of hepatic parenchymal impairment was significantly lower in the S group compared with the N group after 120 min of reperfusion (Figure 6A). The concentration of serum IL-6 had a tendency to be lower in the S group than in the N group, but there was no significant difference (Figure 6B).

Histological findings

In the S group, histological damage to the liver, such as disturbance of the sinusoidal structure and sinusoidal narrowing, was slightly greater than in the N group. Necrotic changes were not observed in either group (Figure 7).

DISCUSSION

Steatosis of the liver is a common disorder with several different etiologies. This disorder is one of the most common obstacles in liver surgery, since steatotic livers are susceptible to some stressful loads, especially IR injury^[1,16]. With the increase in steatotic liver patients in the

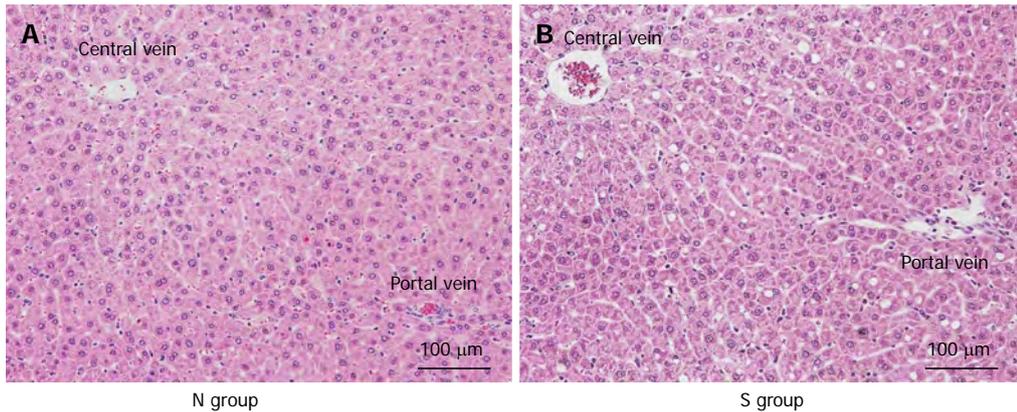


Figure 7 Histological findings after 120 min of reperfusion. In the normal liver (N) group, hepatocyte structure was strongly impaired compared with the mild steatotic liver (S) group, and sinusoidal narrowing was observed.

world, the number of mild steatotic liver cases is also increasing^[17]. A recent analysis of liver transplants, in which the graft liver suffered from cold ischemia, reported that the degree of liver graft steatosis is an important determinant of IR injury and correlated with the rate of postoperative complications and mortality^[18,19]. In warm ischemia, however, the influence of the degree of hepatic steatosis on liver dysfunction is unclear. In this study, we demonstrated that IR injury in mild steatotic liver was attenuated compared with that in normal liver, and that it resulted from the reduction in the interaction between KCs and platelets due to the decreased number of KCs.

It was reported that there are two distinct periods of liver injury after warm IR^[20,21]. The early period of IR injury, which occurs within 120 min after reperfusion, is characterized by KC-induced reactive oxygen species (ROS)^[21,22]. KC production and the release of ROS result in acute hepatocellular injury. In addition, in response to the exposure to activated KCs, neutrophils accumulate in the post-ischemic liver. In the late period of IR injury, inflammatory responses from accumulating neutrophils induce hepatocyte injury, which appears more than 6 h after reperfusion^[22,23]. Elevated liver enzymes and apoptosis of hepatocytes and sinusoidal endothelial cells (SECs) can already be observed in the early period^[10,24]. A reduction in the early period of IR injury, for instance KC depletion, also leads to inhibition of injury in the late period^[25]. Recently, some studies have focused on the function of platelets in hepatic IR injury^[26,27]. Sindram *et al.*^[24] reported that platelets caused SEC apoptosis and significantly contributed to IR injury. Khandoga *et al.*^[28] have shown that warm hepatic IR induced rolling and adhesion of platelets to SECs as well as accumulation of platelets in sinusoids. We previously reported that platelet-SEC interactions occur earlier than leukocyte responses after reperfusion, and that adhesion of platelets requires the presence of activated KCs^[11]. In addition, based on our study using the IVM system and electron microscopy, we also reported that platelet-KC interaction as well as platelet-SEC interaction contributes to early-period hepatic

IR injury^[10,24,28]. Most of the events that determine the extent of IR injury, such as KC activation, platelet adhesion to KCs or SECs, and neutrophil accumulation, occur in the early period of IR injury. Therefore, we focused on observation until 120 min after reperfusion. In this study, we demonstrated that the adherence of platelets to KCs decreased and reperfusion injury was reduced in the mild steatotic liver. The number of adherent platelets in sinusoids increased along with the reperfusion time both in the normal liver group and in the mild steatotic liver group. This suggests that the adherent ability of platelets was not reduced in the mild steatotic liver group. Therefore, we considered that the reduction in KC-platelet interaction was a result of the decreased number of KCs in the mild steatotic liver.

KCs are more likely to be activated in the steatotic liver^[5,29]. In addition, hepatic IR activates KCs^[30]. However, it is unknown whether KC activation after IR increases in steatotic liver more than in normal liver. After IR, KCs secrete pro-inflammatory cytokines including IL-6^[31,32]. Moreover, as described above, activated KCs cause adhesion of platelets to KCs, and lead to later leukocyte accumulation^[11]. We consider that serum IL-6 levels reflect the degree of the interaction between KCs and platelets according to the activity of KCs. In the present study, elevation of serum IL-6 levels after IR was less in mild steatotic liver than in normal liver. Our results indicated that in the mild steatotic liver, IL-6 secretion was suppressed because of the decreased number of KCs, even if KCs were activated after IR.

In our present study, we demonstrated that there were fewer KCs in the sinusoids in our mild steatotic liver than in the normal liver. The results were confirmed by both the IVM study and immunohistochemical examination. Several studies reported on the change in the number of KCs in steatotic liver models. Shono *et al.*^[33] reported that the number of KCs in a particular subgroup could change, for example, the proportion of CD68 positive KCs decreased in their steatotic model induced by a high-fat diet and a high-cholesterol diet. Veteläinen *et al.*^[34]

investigated the difference between a methionine-choline deficient diet (MCD) model and a CDD model in their effect on the number of KCs, and reported that the number of KCs increased in the MCD model, but did not change in the CDD model. The degree of steatosis was moderate in their CDD model, and severe in their MCD model. These investigators indicated that the difference in the method of inducing steatosis of the liver resulted in a change in the number of KCs in the sinusoids. In addition, Guo *et al.*^[35] reported that the number of KCs was reduced in their steatotic liver model induced by palmitoleate, a monounsaturated fatty acid. Thus, differences in nutrient factors may influence the number of KCs in the steatotic liver. We supposed that a change in nutrient conditions induced by CDD might lead to a decrease in KCs as well as mild steatosis of the liver. The relationship between KCs and steatotic liver has not been well established yet. Further research using various steatotic liver models and various degrees of steatosis will be necessary to elucidate the impact of steatotic liver on KCs.

Steatotic liver patients in the clinical setting of hepatic surgery have tended to increase^[17]. IR injury is closely involved with complications in the steatotic liver after hepatic resection^[36]. It is known that the steatotic liver is a risk factor for postoperative complications^[9,36]. Some investigators reported that patients with a steatotic liver who received a major hepatectomy were more likely to suffer from infective, wound-related, hepatobiliary and gastrointestinal postoperative complications^[37]. The decreased tolerance of steatotic liver to IR injury is a result of impaired microcirculation due to hepatocytes with fat deposition^[38]. Between the mild and severe steatotic liver, there are differences in microcirculatory disturbances due to differences in the degree of fat deposition. A recent meta-analysis revealed a significant association between the degree of steatosis and increased risk of postoperative complications and mortality^[9]. Several investigators reported that postoperative complications, especially infectious complications, and mortality increased in patients with severe steatotic liver compared with those with mild steatotic liver^[9,37]. It will be necessary to evaluate IR injury in the moderate to severe steatotic liver in a similar experimental model in the future.

On the other hand, there is a report that postoperative liver failure was slight in the mild steatotic liver, and so mild steatotic liver can be an indication for hepatic surgery^[39]. Moreover, mild to moderate steatotic livers have been accepted as a marginal graft in transplantation^[18,19]. Our study suggested that IR injury does not depend on the degree of fat deposition. In addition, our results provide some evidence that postoperative outcomes after liver resection or transplantation are not aggravated in mild steatotic liver.

In conclusion, in mild steatosis liver induced by CDD, hepatic IR injury was attenuated compared with the normal liver. The small number of KCs in the sinusoids decreased the number of KCs adhering to platelets, and re-

sulted in decreased interaction between KCs and platelets.

COMMENTS

Background

The steatotic liver is well known to be sensitive to ischemia-reperfusion (IR) injury. Increased microcirculatory deterioration is suggested as a reason for this increased liver damage in the steatotic liver. However, it is unclear whether IR injury increases in the mild steatotic liver. Previously, authors reported that the interaction between Kupffer cells (KCs) and platelets played a key role in hepatic IR injury. This study investigated the effect of mild steatotic liver on IR injury, focusing on Kupffer cells and platelets.

Research frontiers

KC activation plays a pivotal role in hepatic IR injury. Upon activation by hepatic ischemia, KCs release reactive oxygen species and inflammatory mediators, such as cytokines and chemokines. In addition, KC activation leads to platelet and neutrophil accumulation in hepatic sinusoids. On the other hand, KCs are more likely to be activated in the steatotic liver.

Innovations and breakthroughs

This is the first study focusing on KCs and platelets in IR injury in the mild steatotic liver. The authors demonstrated that hepatic IR injury in the mild steatotic liver was attenuated compared with normal liver. The small number of KCs in the sinusoids decreased the number of KCs adhering to platelets, and resulted in decreased interaction between KCs and platelets.

Applications

The authors suggest that IR injury in the steatotic liver does not depend on the degree of fat deposition. The results of this study provide some evidence that the postoperative outcomes after liver resection or transplantation are not aggravated in the mild steatotic liver.

Terminology

Intravital fluorescence microscopy (IVM) of the rat liver is a high-resolution real-time technique that allows authors visualize hepatic sinusoidal perfusion. The availability of an enormous number of different fluorescent markers for *ex vivo* and *in vivo* staining has extended the possibilities of IVM from purely morphological analysis to the study of complex physiological and pathological events.

Peer review

The manuscript demonstrated a decrease in IR injury in mild steatotic liver in Wistar rats by feeding with a choline-deficient diet compared to normal rats. In addition, the results also showed that the attenuation of IR injury was associated with a decrease in KC number and platelet-KC adhesion in acini. The study is well designed and the results are interesting and conceivable.

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Exogenous bone morphogenetic protein-7 reduces hepatic fibrosis in *Schistosoma japonicum*-infected mice via transforming growth factor- β /Smad signaling

Bo-Lin Chen, Jie Peng, Qing-Fu Li, Min Yang, Yuan Wang, Wei Chen

Bo-Lin Chen, Jie Peng, Qing-Fu Li, Yuan Wang, Department of Gastroenterology, Xiangya Hospital, Central South University, Changsha 410008, Hunan Province, China

Min Yang, Department of Respiration, Hunan Children's Hospital, Changsha 410011, Hunan Province, China

Wei Chen, Department of Gastroenterology, Changsha Central Hospital, Changsha 410004, Hunan Province, China

Author contributions: Chen BL designed the research, performed the majority of the experiments and wrote the manuscript; Peng J supervised the research; Yang M was involved in revising the manuscript; Li QF, Wang Y and Chen W helped perform the research and participated in data analysis.

Correspondence to: Dr. Jie Peng, Department of Gastroenterology, Xiangya Hospital, Central South University, Changsha 410008, Hunan Province, China. pengjie2@medmail.com.cn

Telephone: +86-731-84327321 Fax: +86-731-84327321

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Abstract

AIM: To investigate the antifibrotic effects of bone morphogenetic protein-7 (BMP-7) on *Schistosoma japonicum* (*S. japonicum*)-induced hepatic fibrosis in BALB/C mice.

METHODS: Sixty BALB/C mice were randomly divided into three groups, including a control group (group A, $n = 20$), model group (group B, $n = 20$) and BMP-7 treated group (group C, $n = 20$). The mice in group B and group C were abdominally infected with *S. japonicum* cercariae to induce a schistosomal hepatic fibrosis model. The mice in group C were administered human recombinant BMP-7. Liver samples were extracted from mice sacrificed at 9 and 15 wk after modeling. Hepatic histopathological changes were assessed using Masson's staining. Transforming growth factor-beta 1 (TGF- β 1), alpha-smooth muscle actin (α -SMA), phosphorylated

Smad2/3 (pSmad2/3) and Smad7 protein levels and localization were measured by Western blotting and immunohistochemistry, respectively, and their mRNA expressions were detected by reverse transcription-polymerase chain reaction (RT-PCR).

RESULTS: The schistosomal hepatic fibrosis mouse model was successfully established, as the livers of mice in group B and group C showed varying degrees of typical schistosomal hepatopathologic changes such as egg granuloma and collagen deposition. The degree of collagen deposition in group C was higher than that in group A (week 9: 22.95 ± 6.66 vs 2.02 ± 0.76 ; week 15: 12.84 ± 4.36 vs 1.74 ± 0.80 ; $P < 0.05$), but significantly lower than that in group B (week 9: 22.95 ± 6.66 vs 34.43 ± 6.96 ; week 15: 12.84 ± 4.36 vs 18.90 ± 5.07 ; $P < 0.05$) at both time points. According to immunohistochemistry data, the expressions of α -SMA, TGF- β 1 and pSmad2/3 protein in group C were higher than those in group A (α -SMA: week 9: 21.24 ± 5.73 vs 0.33 ± 0.20 ; week 15: 12.42 ± 4.88 vs 0.34 ± 0.27 ; TGF- β 1: week 9: 37.00 ± 13.74 vs 3.73 ± 2.14 ; week 15: 16.71 ± 9.80 vs 3.08 ± 2.35 ; pSmad2/3: week 9: 12.92 ± 4.81 vs 0.83 ± 0.48 ; week 15: 7.87 ± 4.09 vs 0.90 ± 0.45 ; $P < 0.05$), but significantly lower than those in group B (α -SMA: week 9: 21.24 ± 5.73 vs 34.39 ± 5.74 ; week 15: 12.42 ± 4.88 vs 25.90 ± 7.01 ; TGF- β 1: week 9: 37.00 ± 13.74 vs 55.66 ± 14.88 ; week 15: 16.71 ± 9.80 vs 37.10 ± 12.51 ; pSmad2/3: week 9: 12.92 ± 4.81 vs 19.41 ± 6.87 ; week 15: 7.87 ± 4.09 vs 13.00 ± 4.98 ; $P < 0.05$) at both time points; the expression of Smad7 protein in group B was higher than that in group A and group C at week 9 (8.46 ± 3.95 vs 1.00 ± 0.40 and 8.46 ± 3.95 vs 0.77 ± 0.42 ; $P < 0.05$), while there were no differences in Smad7 expression between the three groups at week 15 (1.09 ± 0.38 vs 0.97 ± 0.42 vs 0.89 ± 0.39 ; $P > 0.05$). Although minor discrepancies were observed, the results of RT-PCR and Western blotting were mainly consistent

with the immunohistochemical results.

CONCLUSION: Exogenous BMP-7 significantly decreased the degree of hepatic fibrosis in both the acute and chronic stages of hepato-schistosomiasis, and the regulatory mechanism may involve the TGF- β /Smad signaling pathway.

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Key words: Bone morphogenetic protein-7; *Schistosoma japonicum*; Hepatic fibrosis; Smad; BALB/C mice

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INTRODUCTION

Schistosomiasis japonica, a chronic and debilitating disease caused by the trematode *Schistosoma japonicum* (*S. japonicum*), is one of the major public health problems in China and other tropical countries such as the Philippines and Indonesia. It seriously impacts the health of residents within endemic areas as well as social and economic development^[1-3]. Human immune response to schistosome eggs deposited in the liver and the granulomatous inflammation they evoke are the initial factors of hepato-schistosomiasis, while the subsequent hepatic fibrosis represents a wound-healing response to previous liver damage^[4-8]. The primary cell type involved in schistosomal hepatic fibrosis is the hepatic stellate cell (HSC); HSCs are activated in response to inflammatory injury and converted from vitamin A-storing cells into myofibroblast-like cells, characterized by the expression of alpha-smooth muscle actin (α -SMA), the secretion of excessive collagens and other extracellular matrix components, and the production of various pro-fibrosis cytokines such as transforming growth factor-beta (TGF- β)^[9-11]. TGF- β not only maintains the progressive activation of myofibroblasts, but also activates other silent HSCs^[12,13]. This positive feedback cascade reaction always causes continuous schistosomal hepatic fibrosis even when timely and effective anti-helminthic treatment has been given. In addition, praziquantel resistance has become common because of a long term dependence on this single anthelmintic^[14,15]. As etiological therapy alone is not enough to treat hepatic fibrosis, finding other strategies that can block the activation of HSCs and suppress the progression of collagen deposition is important. Considering the dominant role of the cytokine system in hepatic fibrosis, research on cytokine regulators (antagonist or promoter) has become a new focus and has very promising value.

Among the numerous cytokines and growth factors that are involved in hepatic fibrosis, TGF- β , especially

TGF- β 1, is an acknowledged critical fibrogenic stimulus to HSCs^[16,17]. TGF- β performs its functional role mostly *via* the TGF- β /Smad signaling pathway, which is implicated in a wide range of physiological and pathological events, including embryogenesis, inflammation and fibrosis. In this pathway, phosphorylated Smad2/3 (pSmad2/3) proteins act as pivotal downstream effectors of TGF- β which convey signals from TGF- β receptors to the nucleus, while Smad7 seems to be antagonistic to TGF- β as a negative feedback mediator^[18-21]. Bone morphogenetic protein-7 (BMP-7), a member of the TGF- β superfamily, has been studied extensively due to its essential roles during morphogen formation and cell differentiation^[22,23]. Recently, its therapeutic potential in the regulation of fibrosis was recognized based on the counteractive effect of BMP-7 against the TGF- β /Smad signaling pathways. For instance, Zeisberg *et al.*^[24] demonstrated the Smad-dependent reversal of TGF- β 1-induced epithelial-to-mesenchymal transition (EMT) by BMP-7 to renal tubular epithelial cells, while EMT is recognized as an important event in fibrogenesis. Moreover, varying degrees of inhibition of thioacetamide- and CCL₄-induced liver fibrosis by BMP-7 has been respectively observed in recent research^[25]. These limited findings led us to hypothesize that BMP-7 may have a similar effect on schistosomal hepatic fibrosis. Therefore, in the current study, we set TGF- β 1 and Smads as our intervention targets to investigate the potential therapeutic effect of BMP-7 in a mouse model of schistosomal hepatic fibrosis.

MATERIALS AND METHODS

Animals and parasite

Six-week-old SPF BALB/C female mice, weighing 12-16 g, were obtained from the Experimental Animal Center, Central South University, Changsha, China. All animal experiments were performed under the control of the Animal Care Committee of Central South University in accordance with the Guidelines on Animal Experiments in Central South University. *Oncomelania hupensis* harboring *S. japonicum* cercariae were purchased from the Institute of Schistosomiasis Control Center (Yueyang, Hunan, China) and the vitality of cercariae was confirmed by microscopy.

Animal treatment

Sixty BALB/C mice were randomly divided into three groups, including a control group (group A), model group (group B) and BMP-7 treated group (group C) ($n = 20$ in each group). All animals were maintained under specific pathogen-free conditions, kept at 20 °C-25 °C in a 12-h light/12-h dark cycle and had free access to standard laboratory water and chow. The mice in group B and group C were percutaneously infected with *S. japonicum* by placing a coverslip carrying 15 ± 1 cercariae in non-chlorine water on their abdomen for 30 min. The mice in group A were treated with non-chlorine water containing no cercariae. Six weeks after infection, the initial phase of hepatic schistosomiasis where, according to our previous

studies^[26], schistosome eggs reached the liver, the mice in group C were administered recombinant human BMP-7 (Peprotech, United States, Catalog Number: 120-03), 300 pg/g intraperitoneally, every other day for a period of four weeks. At 9 wk and 15 wk after infection, which are the extreme and stationary phases of schistosomal hepatic fibrosis according to our previous studies^[26], 10 mice from each group were randomly selected and sacrificed. Liver tissues were obtained and divided into two parts: the left lobes were fixed in a 4% paraformaldehyde solution for 12 h and the remainder was preserved at -80 °C until use.

Histological examination

After a graded alcohol series, dehydration and xylene treatment, the liver specimens were embedded in paraffin blocks and cut into 5- μ m thick sections. The degree of collagen deposition was assessed using Masson's staining according to standard procedures. A pathologist who was blinded to the research design checked all the sections and described the pathological changes mainly concerning hepatic fibrosis. In addition, a medical color image analysis system (Image-Pro Plus 6.0) was used to scan and sum the collagen deposition areas then calculate the percentage of collagen, a relative objective index to assess the degree of hepatic fibrosis, expressed as the ratio of the fibrotic area to the whole area. The field examined at 100 \times magnification contained at least a granuloma, portal area, or a centrilobular vein, and the results are presented as the mean of ten different fields in each section.

Immunohistochemistry

Immunohistochemical staining was performed with an HRP-Polymer anti-Mouse/Rabbit IHC Kit (MAIXIN-BIO, China, Catalog Number: KIT-5020). The sections were dewaxed, dehydrated, washed in phosphate-buffered saline (PBS, 0.01 mol/L, pH 7.2) 3 \times 5 min, heated at 100 °C in a microwave oven 6 \times 2 min, incubated in 3% H₂O₂ in deionized water for 10 min to block endogenous peroxidases activity, and washed 3 \times 5 min with PBS. The sections were then incubated overnight at 4 °C with primary antibodies (rabbit TGF- β 1 antibody, sc-146, Santa Cruz Biotechnology, Inc., 1:500; goat p-Smad2/3 antibody, sc-11769, Santa Cruz Biotechnology, Inc., 1:300; mouse Smad7 antibody, sc-365846, Santa Cruz Biotechnology, Inc., 1:300; rabbit α -SMA antibody, ab5694, Abcam, Inc., 1:400). After washing 3 \times 5 min with PBS, the appropriate HRP-polymer anti-mouse/rabbit immunoglobulin G (IgG) was added to the sections and incubated at 37 °C for 20 min. The sections were then washed 3 \times 5 min with PBS, and the color was developed with DAB for 3-5 min. The nuclei were lightly counterstained with hematoxylin. Negative controls were incubated with PBS without the primary antibody. The integral optical density (IOD) of the target protein was measured with Image-Pro Plus 6.0, and the result was determined as the sum of five different fields (one in the center and four in the periphery) of each section. IOD was defined as the sum of the optical densities of all the positive pixels in the image,

which represents the quantity of the targeted protein.

Reverse transcription-polymerase chain reaction

Total RNA was extracted from preserved liver tissue with TRIZOL Reagent (Invitrogen, United States) then reverse-transcribed into cDNA by polymerase chain reaction (PCR). Mix Reagent kits (Fermentas, Canada) were used according to the manufacturer's protocol. The housekeeping gene, glyceraldehyde-3-phosphate dehydrogenase (GAPDH), was used as an internal control to calculate relative quantification of target gene expression. The primer sequences were as follows: TGF- β 1 (168 bp) forward 5'-AGGGCTACCATGCCAACTTC-3' and reverse 5'-CCACGTAGTAGACGATGGGC-3'; Smad2 (148 bp) forward 5'-CTGTGACGCATGGAAGGTCT-3' and reverse 5'-CCACGTAGTAGACGATGGGC-3'; Smad3 (135 bp) forward 5'-CAGCGAGTTGGGGAGACATT-3' and reverse 5'-TGTAAGTTCCACGGCTGCAT-3'; Smad7 (230 bp) forward 5'-GCACTCGGTGCTCAAGAAAC-3' and reverse 5'-CCGAGGAATGCCTGAGATCC-3'; α -SMA (300 bp) forward 5'-AAGAGCATCCGACACTGCTG-3' and reverse 5'-AATAGCCACGCTCAGTCAGG-3'; GAPDH (132 bp) forward 5'-AATTTGGCATTGTGGAAGG-3' and reverse 5'-GGATGCAGGGATGATGTTCT-3'. In the RT step, a 20 μ L reaction volume contained the following components: 1 μ L RNA sample (1 μ g/ μ L), 1 μ L Oligo (dT) (10 pmol/ μ L), 10 μ L DEPC-water, 4 μ L 5 \times buffer, 2 μ L dNTP mixture (10 mmol/L), 1 μ L RNase inhibitor (10 U/ μ L) and 1 μ L ReverTra Ace. The reaction was performed at 25 °C for 5 min, followed by 42 °C for 60 min, 70 °C for 5 min, and 4 °C for 5 min. In the PCR step, a 25 μ L reaction volume contained the following components: 12.5 μ L 2 \times Master Mix, 10.5 μ L nuclease-free water, 1 μ L primer, and 1 μ L cDNA. The PCR protocol was as follows: denaturation at 94 °C for 3 min; 35 cycles of denaturation at 94 °C for 30 s, annealing at 59 °C (TGF- β 1, Smad2, Smad3 and GAPDH)/58 °C (Smad7 and α -SMA) for 30 s, and elongation at 72 °C for 45 s; and final elongation at 72 °C for 5 min. The amplified products were separated by electrophoresis on 1.5% agarose gels (volume: 8 μ L samples plus 2 μ L buffer; voltage: 100 V), visualized with ethidium bromide staining and photographed using an ultraviolet imaging system (Kodak, United States). We used gel analysis software (Gel-Pro 4.0) to scan and calculate the IOD of strips. The relative mRNA expression of the target gene was represented as the ratio of target gene IOD and GAPDH IOD.

Western blotting

Liver tissues (0.5 g) were homogenized on ice in 1 mL lysis buffer prepared from a Total Protein Extraction kit (ProMab, United States) for about 20 min and then ultrasonicated for 3 \times 3 s. The homogenates were centrifuged at 9000 \times g for 10 min at 4 °C and the supernatants were then extracted to obtain the gel sample by mixing it with sampling buffer. Following heat denaturation at 100 °C for 3 min, the samples (20 μ g protein each lane) were separated by sodium dodecyl sulfate-polyacrylamide

gel electrophoresis in running buffer and subsequently transferred to nitrocellulose membrane (Pierce, United States) in precooled transfer buffer at 300 mA constant current for 70 min. Non-specific binding site sealing was performed by incubating in PBS containing 5% non-fat milk for 2 h at room temperature. The primary antibodies (rabbit TGF- β 1 antibody, sc-146, Santa Cruz Biotechnology, Inc., 1:400; goat p-Smad2/3 antibody, sc-11769, Santa Cruz Biotechnology, Inc., 1:500; mouse Smad7 antibody, sc-365846, Santa Cruz Biotechnology, Inc., 1:800; rabbit α -SMA antibody, ab5694, Abcam, Inc., 1:500; mouse GAPDH antibody, SC-365062, Santa Cruz Biotechnology, Inc., 1:800) were incubated with the membrane overnight at 4 °C. After being washed 5 \times 4 min with PBS-Tween 20 (PBST), the secondary antibody (goat anti-rabbit IgG HRP for TGF- β 1 and α -SMA, 1:40 000; rabbit anti-goat IgG HRP for p-Smad2/3, 1:40 000; goat anti-mouse IgG HRP for Smad7 and GAPDH 1:80 000) was incubated with these membranes for 1 h at room temperature. After being washed 5 \times 4 min with PBST, enhanced chemiluminescence detection of the target protein was performed. The film was scanned and the image was analyzed with Gel-Pro 4.0. The relative expression of target protein was represented by the ratio of target protein IOD and GAPDH IOD.

Statistical analysis

Statistical analysis was performed using SPSS 13.0 software. Comparisons between groups were performed using one-way analysis of variance (homogeneity of variance: S-N-K; heterogeneity of variance: Dunnett's T3). Comparisons between time points were performed using independent-samples *t* test. *P* values less than 0.05 were considered statistically significant.

RESULTS

Schistosomal hepatopathology

Typical schistosomal hepatopathological characteristics include mainly egg granuloma and collagen deposition and were observed using Masson's staining in group B and group C at both time points (Figure 1C-F), while group A showed normal hepatocyte morphology (Figure 1A and B). At week 9, in group B, a dense mass of collagen fibers surrounded the egg granulomas, and spread to the space around them, or extended to neighboring lobules (Figure 1C); in group C, there were still numerous collagen fibers around the granulomas, but these were fewer (Figure 1E). At week 15, compared to week 9, a reduction in collagen deposition in group B was observed (Figure 1D), while there were only a few collagen fibers wrapped around disintegrated granulomas in group C (Figure 1F). Data of the percentage of collagen fibers in the different groups and at the two time points are expressed as the mean \pm SD and are shown in Figure 1G.

Expression of TGF- β 1, α -SMA, pSmad2/3 and Smad7 (immunohistochemistry)

Wispy traces of TGF- β 1 positive staining were sparsely

distributed in sections of group A (Figure 2A and D). At week 9, in group B, densely TGF- β 1-stained cells which could be distinguished by their yellow, brownish-yellow or snuff color surrounded and infiltrated the granulomas, and accumulated in fibrotic lesions or stretched along the fibrous septum (Figure 2B); in group C, the quantity and intensity of positive traces were reduced compared to group B (Figure 2C) (*P* < 0.05). At week 15, in group B, there were still some TGF- β 1-stained cells wrapped around the fibrotic granulomas or scattered around them (Figure 2E); however, only a few dispersed yellow traces were seen in group C (Figure 2F) (*P* < 0.05). The variation in α -SMA and pSmad2/3 expressions between the time points and groups were similar to TGF- β 1, although discrepancies were observed (Figure 2G-L and Figure 3A-F). It is worth mentioning that pSmad2/3 was mainly located in the nuclei not only in fibrocytes and inflammatory cells, but also in normal hepatocytes (Figure 3A-F). The expression of Smad7 in the three groups was different, and was only observed at week 9 in group B. At this point, brownish-yellow traces were distributed around the granulomas and scattered in the surrounding normal hepatic tissue (Figure 3H), but no positive staining was observed in other cells (Figure 3G and I-L). Figure 2M and N, Figure 3M and N show the IODs of each target protein in the different groups and time points. These results are expressed as IOD ($\times 10^2$ or $\times 10^3$) and as the mean \pm SD.

Expression of TGF- β 1, α -SMA, pSmad2/3 and Smad7 mRNA (RT-PCR) and protein (Western blotting)

The experimental data on target mRNAs (RT-PCR) and proteins (Western blotting) were all roughly consistent with the immunohistochemical results (Figures 4 and 5). In summary, the expressions of TGF- β 1, pSmad2/3 and α -SMA mRNA and protein in group C were higher than or similar to those in group A, but significantly decreased compared to group B at both time points. With regard to the expressions of Smad7 mRNA and protein, there were no significant differences between group A and group C at both time points or group B at week 15, but they were all lower than those in group B at week 9. All data are shown in Figures 6 and 7.

DISCUSSION

The molecular components and regulatory mechanism of the TGF- β /Smad signaling pathway are more or less diverse under different pathologic processes and environmental conditions^[27-30]. During acute liver injury, especially in toxicopathic hepatitis, the principal components and the canonical progression of this signaling are as follows: catalytically active TGF- β type I receptor phosphorylates Smad2 and the highly similar protein Smad3 to create their phosphorylated (p) isoforms, then TGF- β promotes collagen synthesis in activated HSCs *via* pSmad2/3 pathways^[31]. In the recovery stage of acute liver injury, to avoid excessive collagen deposition, TGF- β also initiates the expression of antagonistic Smad7 which functions in a negative-feedback loop to reduce the fibro-

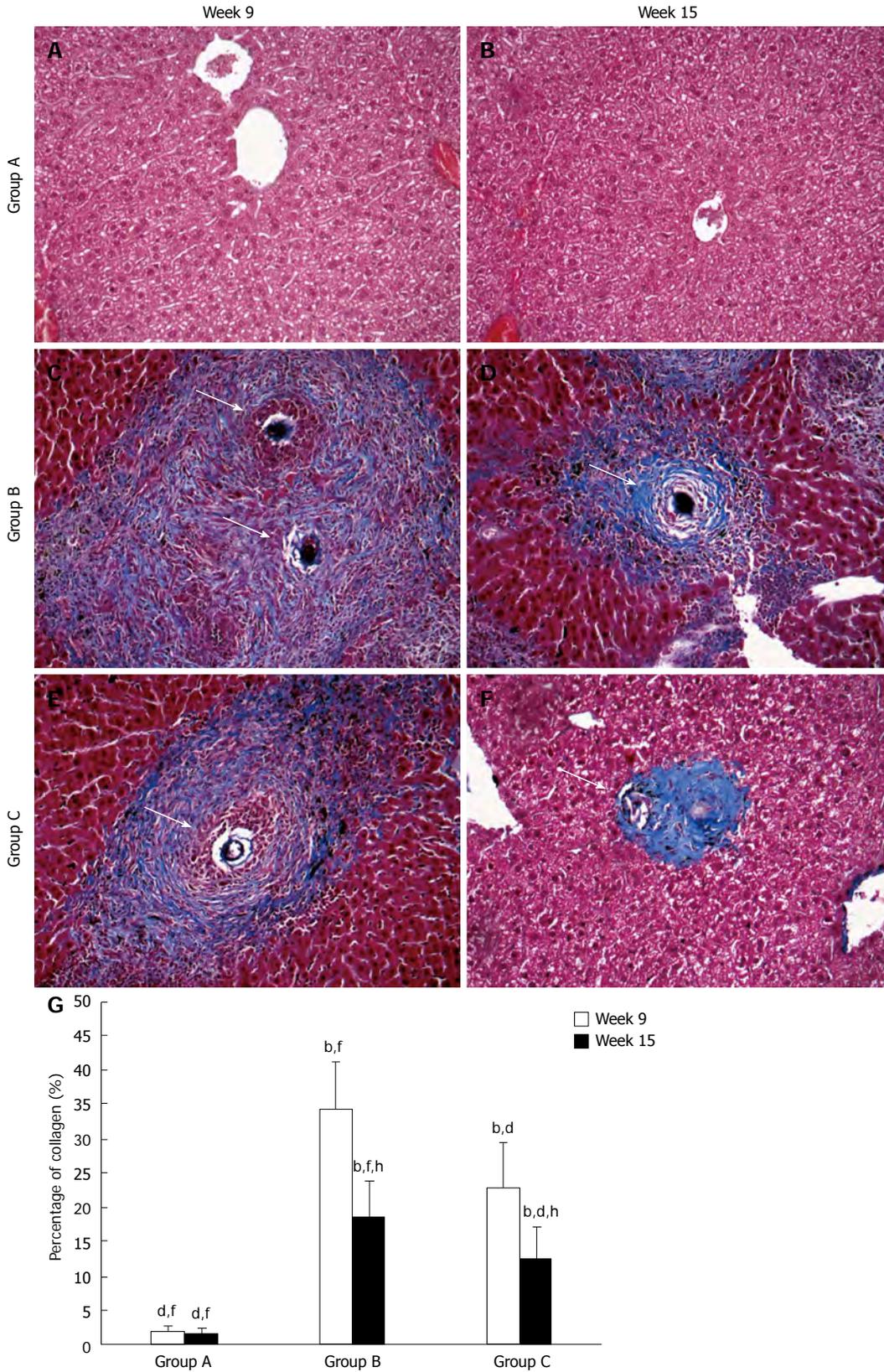


Figure 1 Representative images of schistosomal hepatic fibrosis in the groups over time. Collagen fibers are stained blue and arrows show egg granulomas. Original magnification 100 \times . Histogram shows the percentage of collagen which represented the degree of hepatic fibrosis. ^b $P < 0.01$ vs group A; ^d $P < 0.01$ vs group B; ^f $P < 0.01$ vs group C; ^h $P < 0.01$ vs week 9.

genic strength of the signal^[32,33]. However, the negative regulation of TGF- β /Smad signaling is insufficient and

transitory; when liver injury and fibrosis enter the chronic phase, the induction of Smad7 gradually ceases, while

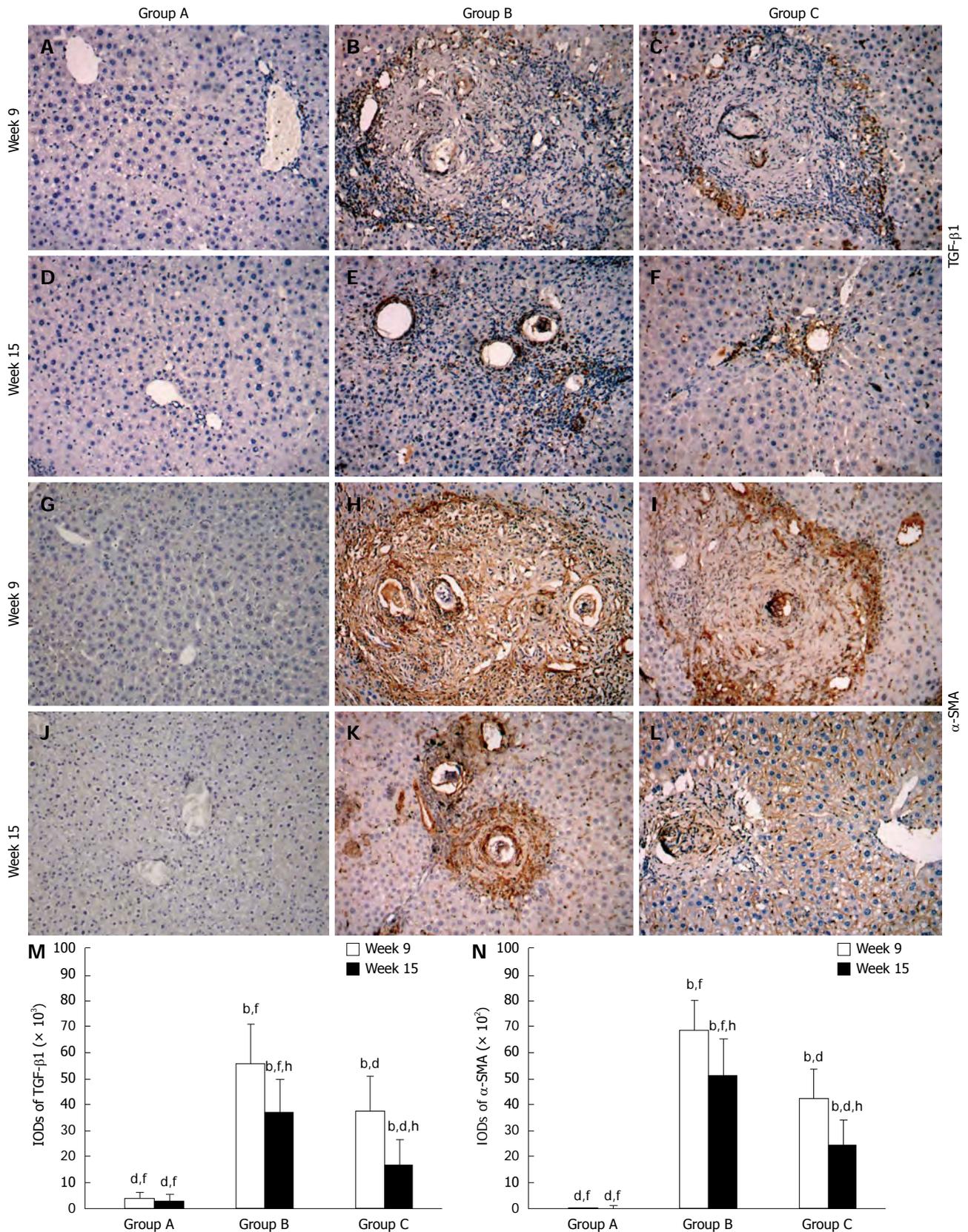


Figure 2 Representative images of immunostaining for transforming growth factor-beta 1 and alpha-smooth muscle actin in the groups over time. Target protein positive staining is yellow, brownish-yellow or snuff. Original magnification 100 \times . Histogram shows integral optical densities of target proteins. TGF- β 1: Transforming growth factor-beta 1; α -SMA: Alpha-smooth muscle actin; IOD: Integral optical density. ^b $P < 0.01$ vs group A; ^d $P < 0.01$ vs group B; ^f $P < 0.01$ vs group C; ^h $P < 0.01$ vs week 9.

other promotive factors continue to work^[34]. That is why an appropriate exogenous cytokine regulator is so attrac-

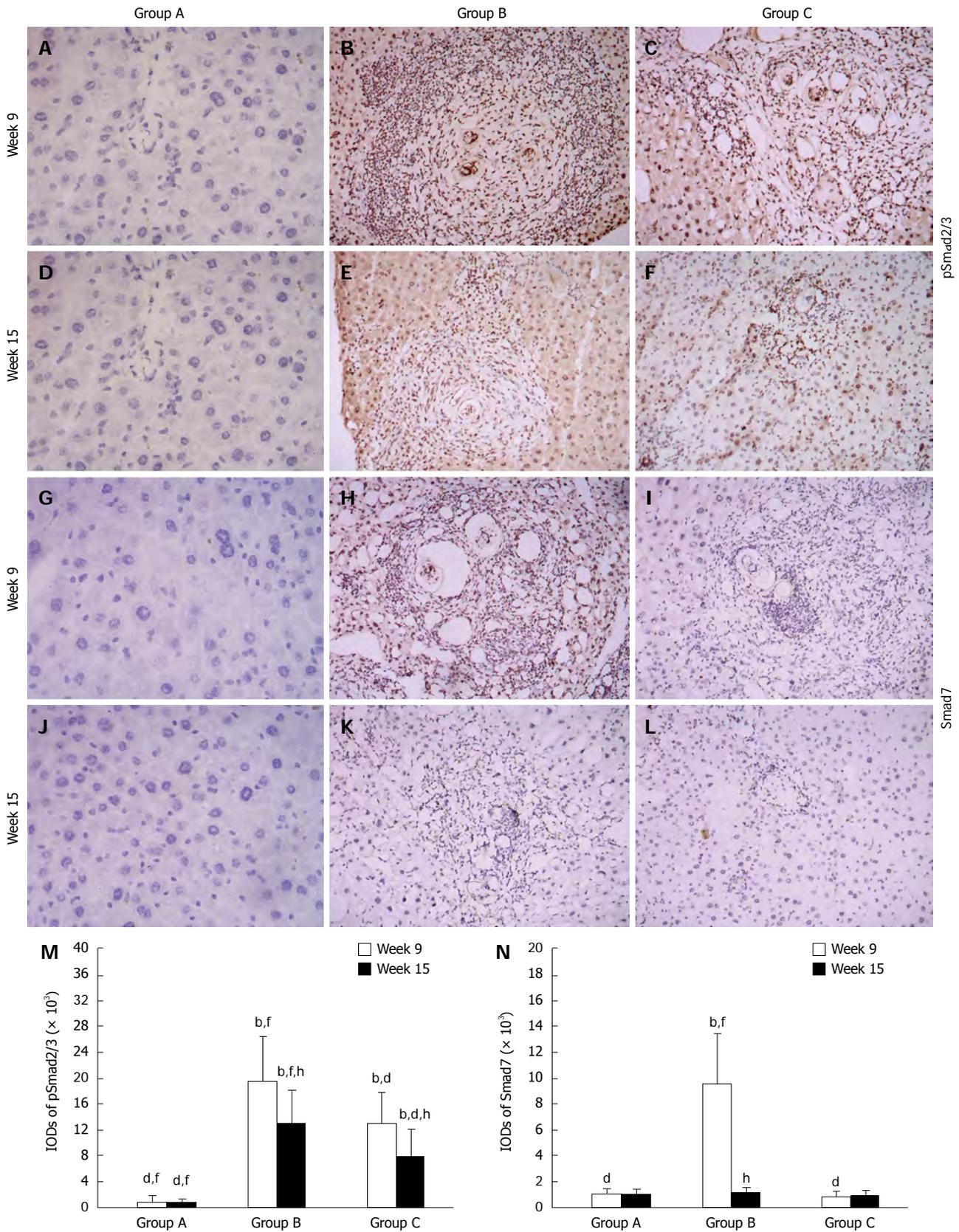


Figure 3 Representative images of immunostaining for pSmad2/3 and Smad7 in the groups over time. Target protein positive staining is yellow or brownish-yellow. Original magnification 100 \times . Histogram shows integral optical densities of target proteins. ^b $P < 0.01$ vs group A; ^d $P < 0.01$ vs group B; ^f $P < 0.01$ vs group C; ^h $P < 0.01$ vs week 9. IOD: Integral optical density.

tive due to its potential therapeutic effect in blocking or reversing hepatic fibrosis. Although BMP-7 belongs to

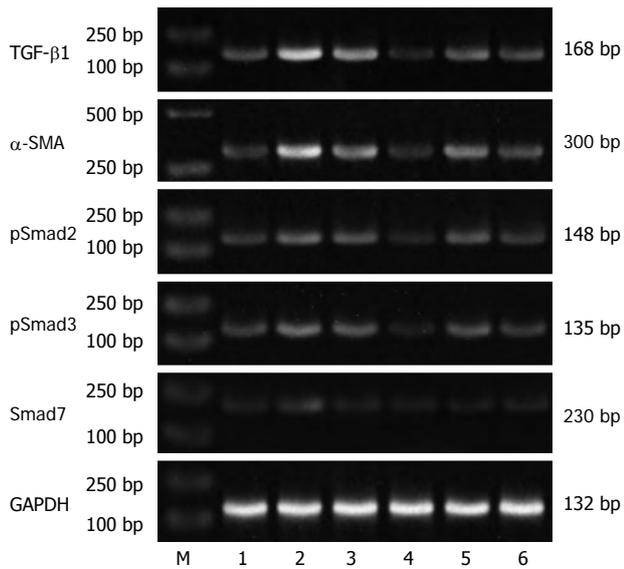


Figure 4 Representative electrophoretic bands of transforming growth factor-beta 1, alpha-smooth muscle actin, pSmad2, pSmad3 and Smad7 mRNA (reverse transcription-polymerase chain reaction). Numbers 1-3 represent group A, B and C, respectively, at 9 wk; Numbers 4-6 represent group A, B and C, respectively, at 15 wk. The 132 bp glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA fragment was used as an internal control. M: Marker, TGF-β1: Transforming growth factor-beta 1; α-SMA: Alpha-smooth muscle actin.

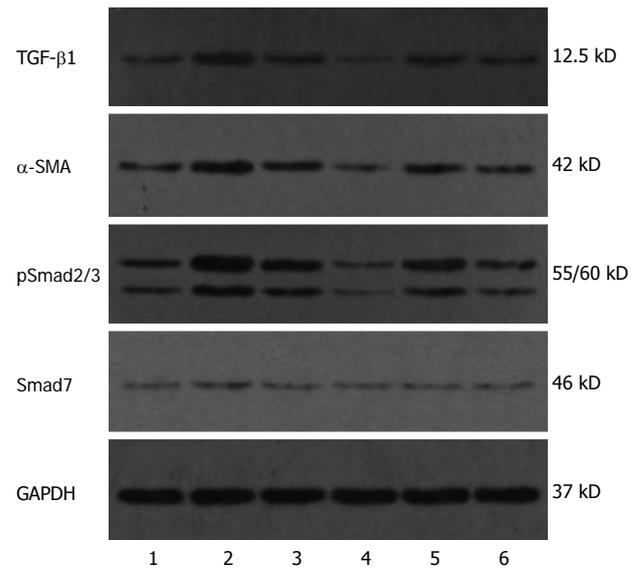


Figure 5 Representative electrophoretic bands of transforming growth factor-beta 1, alpha-smooth muscle actin, pSmad2/3 and Smad7 protein (Western blotting). Numbers 1-3 represent group A, B and C, respectively, at 9 wk. Numbers 4-6 represent group A, B and C, respectively, at 15 wk. The 37 kD glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as an internal control. TGF-β1: Transforming growth factor-beta 1; α-SMA: Alpha-smooth muscle actin.

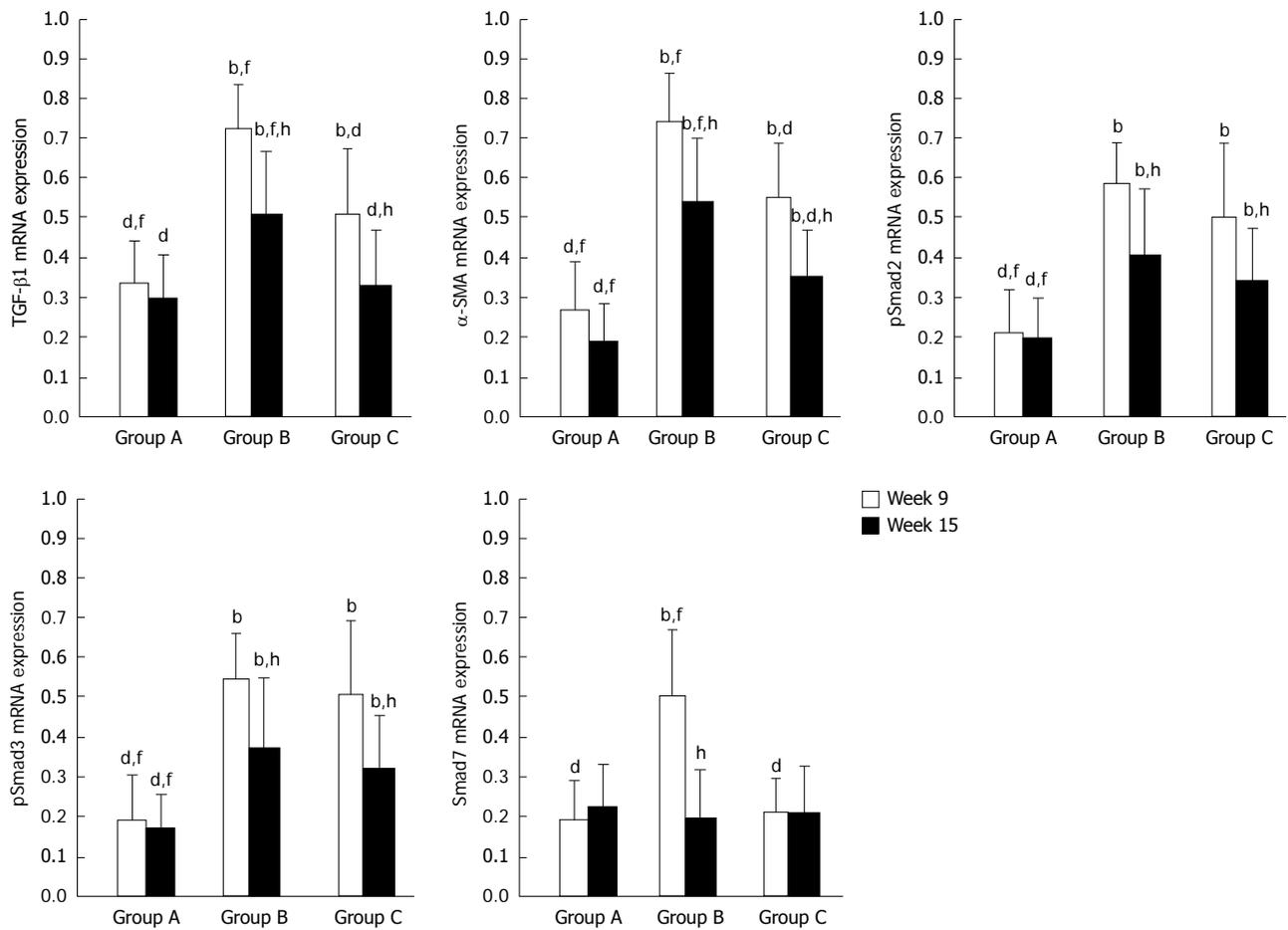


Figure 6 Relative mRNA expressions (target gene integral optical density/glyceraldehyde-3-phosphate dehydrogenase integral optical density, reverse transcription-polymerase chain reaction) of transforming growth factor-beta 1, alpha-smooth muscle actin, pSmad2, pSmad3 and Smad7. TGF-β1: Transforming growth factor-beta 1; α-SMA: Alpha-smooth muscle actin. ^a*P* < 0.01 vs group A; ^b*P* < 0.01 vs group B; ^c*P* < 0.01 vs group C; ^d*P* < 0.01 vs week 9.

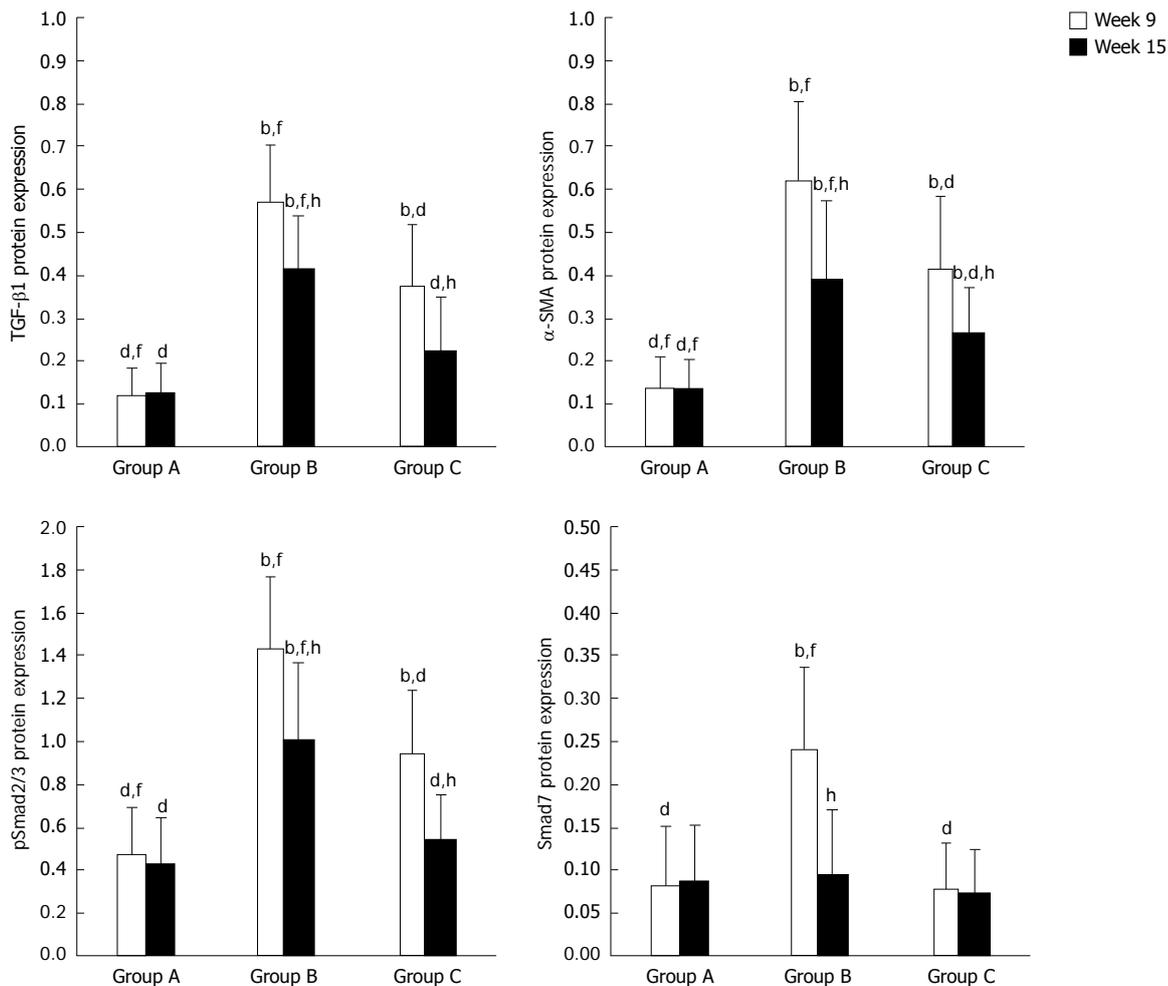


Figure 7 Relative protein expressions (target protein integral optical density/glyceraldehyde-3-phosphate dehydrogenase integral optical density, Western blotting) of transforming growth factor-beta 1, alpha-smooth muscle actin, pSmad2/3 and Smad7. TGF-β1: Transforming growth factor-beta 1; α-SMA: Alpha-smooth muscle actin. ^bP < 0.01 vs group A; ^cP < 0.01 vs group B; ^dP < 0.01 vs group C; ^eP < 0.01 vs week 9.

the TGF-β superfamily due to their shared morphological characteristics, it has an almost contrary biological function compared to TGF-β. An increasing number of reports indicate that BMP-7 may be a new antagonist of organ fibrosis because of its counteractive effect on the TGF-β/Smad signaling pathway; however, the role of BMP-7 in schistosomal hepatic fibrosis and the underlying regulatory mechanism remains a mystery. The pathogenic progression and prognosis of hepatic fibrosis induced by *S. japonicum* infection are different to other types of hepatic fibrosis, and correlative studies are necessary.

In the present study, we administered recombinant human BMP-7 at the initiation of hepatic schistosomiasis and extended the treatment period to 3 wk to ensure an adequate biological effect. The data showed that both the acute and chronic phases of liver injury and collagen deposition in the model group were accompanied by high expressions of protein and mRNA of TGF-β1, pSmad2/3 and α-SMA compared to the normal group, indicating that the “TGF-β1 active HSCs *via* pSmad2/3” classic pathway is still active in *S. japonicum*-induced hepatic fibrosis. Following treatment with BMP-7, the degree

of collagen deposition significantly reduced at both time points as well as the expressions of TGF-β1, pSmad2/3 and α-SMA, indicating that BMP-7 had an inhibitory effect on schistosomal hepatic fibrosis, at least partly *via* down regulation of the expressions of TGF-β1 and pSmad2/3 and then suppression of HSC activation. Although Smad2 and Smad3 are activated only in response to TGF-β, there are still other Smads through which BMP-7 can promote fibrosis without TGF-β. For instance, Kinoshita found that BMP-7 utilized Smad1/5/8 as signaling intermediates and decreased the expression of type I collagen and α-SMA in primary cultured HSCs independent of the presence of TGF-β^[35]. Whether the above cytokines act in schistosomal hepatic fibrosis requires further research.

Smad7, known as a negative feedback regulator to profibrotic TGF-β1, seems only to act in the acute phase of schistosomal liver injury. In this stage, hepatic damage caused by schistosome eggs induces severe inflammation; to prevent further acute injury, reparative fibrosis starts and numerous collagen fibers are secreted. We speculate that the upregulation of Smad7 is decided by the inten-

sity of hepatic fibrosis, that is, only an extremely high degree of TGF- β 1 activity and collagen secretion can initiate the negative feedback effect of Smad7. This assumption is based on the following two reasons: firstly, at 15 wk after infection in the model group, hepatic fibrosis was present, but at a lower degree than previously, however, the expression of Smad7 was almost down to normal levels; secondly, after the administration of BMP-7, the degree of hepatic fibrosis at 9 wk after infection was markedly alleviated, accompanied by a lack of Smad7 induction. Interestingly, a previous report on an animal model of CCl₄-induced liver fibrosis showed that Smad7 levels (both mRNA and protein) were up-regulated in the model group in a time-dependent manner which lasted 12 wk after modeling compared to the control group, and at week 12 Smad7 was significantly lower in the BMP-7 treatment group than in the model group and control group^[36]. Thus, our speculation regarding the expression pattern of BMP-7 remains controversial and needs further verification.

In conclusion, the role of BMP-7 as an antagonist to the TGF- β 1/Smads signaling pathway and its antifibrotic effect during both the extreme and stationary phases of schistosomal hepatic fibrosis were confirmed in this study. This provides a new research strategy and offers therapeutic potential in the treatment of hepatic schistosomiasis, although the detailed intervention mechanism still requires more research. In addition, the preparatory work for the clinical application of BMP-7 is a long, arduous task.

COMMENTS

Background

There are currently few effective therapies for schistosomal hepatic fibrosis due to a lack of appropriate intervention targets. Research on antagonists which counteract key pro-fibrosis cytokines or signaling pathways has become a new attractive focus.

Research frontiers

The therapeutic potential of bone morphogenetic protein-7 (BMP-7) as an antagonist target of transforming growth factor-beta (TGF- β)/Smad signaling has been recognized recently based on research on renal fibrosis and thioacetamide/CCl₄-induced liver fibrosis. However, its role in schistosomal hepatic fibrosis is still unclear and urgently needs to be explored, as the global epidemic of this disease is becoming more serious.

Innovations and breakthroughs

This study firstly observed the role of BMP-7 as an antagonist to TGF- β 1/Smads signaling in schistosomal hepatic fibrosis, and then confirmed the antifibrotic effect of exogenous BMP-7 during both the extreme and stationary phase of this pathological process.

Applications

This study indicates the therapeutic potential of BMP-7 in the treatment of schistosomal hepatic fibrosis and provides a new research strategy related to cytokine regulators.

Peer review

The authors are to be congratulated for confirming that BMP-7 has an inhibitory effect during both the extreme and stationary phase of schistosomal hepatic fibrosis, "at least partly" *via* down regulating the expressions of TGF- β 1 and pSmad2/3 then suppressing the activation of hepatic stellate cell. This study provides a new research strategy and offers therapeutically potential for the treatment of hepatic schistosomiasis.

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Corticotropin-releasing factor receptor subtype 2 in human colonic mucosa: Down-regulation in ulcerative colitis

Ekaterini Chatzaki, Peter A Anton, Mulugeta Million, Maria Lambropoulou, Theodoros Constantinidis, George Kolios, Yvette Taché, Dimitri E Grigoriadis

Ekaterini Chatzaki, Dimitri E Grigoriadis, Neurocrine Biosciences Inc., San Diego, CA 92121, United States

Ekaterini Chatzaki, George Kolios, Laboratory of Pharmacology, Faculty of Medicine, Democritus University of Thrace, 68100 Alexandroupolis, Greece

Peter A Anton, Center for HIV Prevention Research, Division of Digestive Diseases, University of California at Los Angeles, and VA Greater Los Angeles Healthcare System, Los Angeles, CA 90073, United States

Peter A Anton, Mulugeta Million, Yvette Taché, Digestive Diseases Research Center and Center for Neurobiology of Stress, Division of Digestive Diseases, University of California at Los Angeles, and VA Greater Los Angeles Healthcare System, Los Angeles, CA 90073, United States

Maria Lambropoulou, Laboratory of Histology-Embryology, Faculty of Medicine, Democritus University of Thrace, 68100 Alexandroupolis, Greece

Theodoros Constantinidis, Laboratory of Hygiene and Environmental Protection, Faculty of Medicine, Democritus University of Thrace, 68100 Alexandroupolis, Greece

Author contributions: Chatzaki E, Taché Y and Grigoriadis DE designed the research; Chatzaki E, Million M and Lambropoulou M performed the research; Anton PA contributed new reagents/analytic tools/tissues; Constantinidis T and Kolios G analyzed the data; Chatzaki E and Taché Y wrote the paper.

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Correspondence to: Ekaterini Chatzaki, PhD, Associate Professor, Laboratory of Pharmacology, Faculty of Medicine, Democritus University of Thrace, Dragana, 68100 Alexandroupolis, Greece. achatzak@med.duth.gr

Telephone: +30-255-1030533 Fax: +30-255-1030533

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patients with ulcerative colitis (UC).

METHODS: We examined CRF₂ gene and protein expression in the distal/sigmoid colonic mucosal biopsies from healthy subjects and patients with UC (active or disease in remission), human immunodeficiency virus (HIV) and functional bowel disease (FBD) by reverse transcription-polymerase chain reaction and immunofluorescence.

RESULTS: Gene expression of CRF₂ was demonstrated in the normal human colonic biopsies, but not in the human colorectal adenocarcinoma cell line Caco2. Receptor protein localization showed immunoreactive CRF₂ receptors in the lamina propria and in the epithelial cells of the distal/sigmoid biopsy samples. Interestingly, CRF₂ immunoreactivity was no longer observed in epithelial cells of patients with mild-moderately active UC and disease in remission, while receptor protein expression did not change in the lamina propria. No differences in CRF₂ expression profile were observed in distal/sigmoid intestinal biopsies from HIV infection and FBD patients, showing no signs of inflammation.

CONCLUSION: The down-regulation of the CRF₂ receptor in the distal/sigmoid biopsies of UC patients is indicative of change in CRF₂ signalling associated with the process of inflammation.

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Key words: Colonic mucosa; Corticotropin-releasing factor; Corticotropin-releasing factor receptor; Human immunodeficiency virus; Ulcerative colitis; Urocortin

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Abstract

AIM: To assess corticotropin-releasing factor receptor 2 (CRF₂) expression in the colon of healthy subjects and

INTRODUCTION

Ulcerative colitis (UC) is one of the main inflammatory bowel diseases (IBD) which affect primarily the colonic segment of the gut. Inflammation is continuous and limited to the mucosa, accompanied by ulceration, pseudopolyps, oedema, crypt abscesses and spontaneous haemorrhage. Patients present urgent diarrhoea with blood and mucus, abdominal pain, fever, continual tiredness, anaemia, weight loss and potentially serious complications, such as megacolon toxicum. Symptoms often require steroid therapy and are characterised by alternating acute and remission periods. Although its etiology is unknown, its development and acute exacerbation have been related to both physical and mental stress^[1-9].

It is well established that corticotropin-releasing factor (CRF) and its homologue peptides urocortin 1, 2 and 3, are the key mediators of the endocrine, behavioural, autonomic and visceral responses to stress^[10-12]. These neuropeptides exert their multiple actions through activation of two distinct receptor types, CRF₁ and CRF₂, both belonging to the class B G-protein coupled receptor superfamily^[13,14]. CRF₁ is expressed primarily in the brain and the pituitary^[15-17] whereas expression of CRF₂ has been reported in the central nervous system but also in various peripheral tissues such as heart, skeletal muscle, gut and testis^[18-22]. CRF and urocortin 1 display equal affinity for the CRF₁ receptor while urocortin 1 is 40 times more potent than CRF in binding CRF₂^[23]. In contrast, urocortins 2 and 3 bind selectively to CRF₂ and have been established as the endogenous ligands for this receptor subtype^[13].

The gastrointestinal tract is one of the primary peripheral systems affected by stress and there is accumulating evidence that the CRF signalling pathways are responsible for mediating these effects *via* both central and peripheral routes^[24-26]. Previous reports indicate that CRF, urocortin 1 and urocortin 2 are elevated in the colonic mucosa of patients with active UC^[27,28] and are involved in the pathogenesis of this disease, participating in the regulation of motility and/or inflammatory process *via* autocrine/paracrine actions^[29-31]. Local expression of CRF receptors is a prerequisite for mediation of these effects and understanding their histological distribution would provide anatomical support to the physiological and pathophysiological condition/phenomena. Furthermore, they could provide targets for new strategies of pharmacotherapy^[32-34].

We have recently reported that CRF₁ is up-regulated in the colonic mucosa of UC patients, particularly in macrophages supporting its involvement in the immune/inflammatory process^[35]. However, recent studies in a rat model of chemically induced colitis showed that CRF₂ rather than CRF₁ is pivotal for macroscopic spread of colitis and resolution of edema^[36]. Here, we investigated the expression pattern of CRF₂ in the distal/sigmoid colonic mucosal biopsies of healthy human subjects and compared it to inflamed colonic tissues from patients with UC or with human immunodeficiency virus (HIV)

infection and functional bowel diseases (FBD), without signs of inflammation.

MATERIALS AND METHODS

Tissues

All sigmoid colonic mucosal biopsy specimens were obtained from the Mucosal Immunology Core (UCLA AIDS Institute Center for AIDS Research). Approval to conduct the study was obtained from the UCLA Human Subjects Protection Committee. All participants provided written consent at the time they presented for scheduled screening endoscopy. Sigmoid colonic biopsies were collected by flexible sigmoidoscopy, 10-20 cm from the anal verge, from 6 healthy controls (33-65 years; 1 male, 5 females) and 10 UC patients (32-83 years; 6 males and 4 females). Patients had UC for > 10 years, based on history, endoscopic and pathology reports over the years. Four patients had active clinical disease presentation and met the criteria for mild-moderately active disease (grade as 2-3) and 6 UC patients met the criteria for minimal to no active inflammation with markers of quiescent disease (grade as 1-2). Grades were based on Matts UC classifications as used in other studies^[35]. No participants were taking steroids and one was taking a low dose of the immunosuppressive agent, 6-mercaptopurine (25 mg/d). All were treated with 5-aminosalicylate except one patient who was taking only omega-3 fatty acid gel caps. The mucosal biopsies of patients with HIV (*n* = 4) or with IBD (*n* = 7) revealed no inflammation at pathological examination. All biopsies were fixed in formalin, embedded in paraffin and stored at room temperature until further use.

Cell line

The human colorectal adenocarcinoma cell line Caco2 was obtained by the American Type Culture Collection (ATCC HTB-37, Manassas, VA, United States). Cells were maintained in ATCC-formulated Eagle's Minimum Essential Medium (Invitrogen, Grand Island, NY, United States), supplemented with 1% antibiotic-antimycotic solution and 20% fetal bovine serum (Invitrogen) in tissue culture flasks (Nunclon, Rochester, NY) at 37 °C in 5% CO₂. Cell culture medium was replaced every 2-3 d and cells were passaged when subconfluent.

Reverse transcription polymerase chain reaction

Universal reference total RNAs from normal human colon and from whole human brain were purchased from Clontech (Mountain View, CA, United States). RNA from Caco2 cells was extracted using Trizol Reagent, according to the manufacturer's instructions. Reverse transcription (RT) was performed using the SuperScript Preamplification System (Invitrogen, Paisley, United Kingdom) and random hexamers in a total volume of 20 µL. Two microliter of the RT product was used as a template, amplified by polymerase chain reaction (PCR) using 2 mol/L MgCl₂, PCR buffer, 0.2 mol/L of sense and antisense

primers, 0.2 mol/L dNTPs and 2.5 U Taq polymerase (Invitrogen) in a final reaction volume of 50 μ L. PCR was performed in a Perkin-Elmer DNA Thermal Cycler with the following cycling parameters: a pre-amplification cycle (denaturation for 5 min at 96 °C), 35 cycles of amplification (denaturation for 30 s at 96 °C, annealing for 40 s at 56 °C and extension for 50 s at 72 °C) and a final extension step for 7 min at 72 °C. The primers were designed to amplify specifically the human CRF₂ (sense AAGCTTGCCATGGACGCGGCACTGCTC antisense AAGGGCGATGCGGTAGTGC, in the area of the gene encoding the transmembrane part of the receptor and thus targeting all splice variants) according to the GenBank published sequences. The size of the amplified products was expected to be 308 bp. Negative control samples where no RT enzyme was added in a total brain RNA sample (no RT) or without DNA template (no DNA) were included in every assay in order to exclude the possibility of genomic or other DNA contamination. RT-PCR for β -actin, with expected PCR product size of 175 bp, was also performed for every sample in order to assure RNA quality. The amplified PCR products were fractionated by 1.5% agarose gel electrophoresis and detected by ethidium bromide staining under UV.

Indirect immunofluorescence

Immunofluorescence was conducted as previously described^[37]. Briefly, four-micron distal/sigmoid colonic tissue sections (4 μ m) were cut, deparaffinized, and rehydrated by standard procedures. Sections were then incubated with Antigen Retrieval Solution (Dako, Glostrup, Denmark) for 15 min, pre-blocked in 1% normal goat serum (Vector Laboratories, Burlingame, CA, United States) in phosphate buffered saline (PBS). They were then incubated with the primary antisera 4392a-CRF_{1and2} (1:2000) (raised against a synthetic peptide corresponding to amino acids 381-415 of the human/rat CRF_{1/2} C-terminus) and 2064a-CRF₂ (1:500) (raised against a synthetic peptide corresponding to amino acids 404 to 438 of the human/rat CRF₂ C-terminus)^[38,39], diluted in 1% normal goat serum in PBS for 45 min at room temperature in a humid chamber, in parallel with negative controls using normal rabbit IgG instead of the primary antiserum. Following washing in PBS, secondary antibody conjugated to a fluorescent dye, goat anti-rabbit AlexaFluor594 (Molecular Probes, Eugene, OR, United States; 1:500 or 4 μ g/mL) was added for 30 min at room temperature. Slides were subsequently mounted in anti-fade mounting media (Molecular Probes) and visualized by standard fluorescence microscopy. Replacement of the antisera by non-specific rabbit IgG (negative) was used as negative control. The number of immunoreactive cells were counted and quantified in an average number of 5 fields (340 μ m \times 260 μ m/field) from each specimen in a blinded fashion such that the information on the clinical, endoscopic and pathological findings were unknown until all counting was completed.

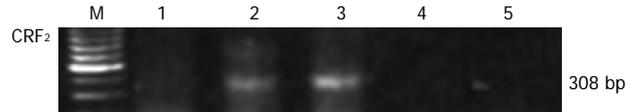


Figure 1 Reverse transcription polymerase chain reaction for corticotropin-releasing factor 2 receptor gene expression in total RNA isolated from the human colon cancer cell line Caco2 (1), human colon (2), and human brain (3) from healthy subjects. The predicted size products (308 bp) were amplified in the human colon and brain samples. Negative controls in the absence of reverse transcription enzyme (4) or DNA template (5) are also presented. CRF: Corticotropin-releasing factor; M: Marker.

Statistical analysis

Statistical significance was assessed by Fisher's exact method for small samples using the SPSS 17.0 statistical software (SPSS Inc. Chicago, Illinois, United States). Significance was set at a *P* value < 0.05.

RESULTS

CRF₂ receptor gene expression in the distal/sigmoid colon of healthy human subjects and cell lines

The expression of CRF₂ receptor gene was investigated by RT-PCR in total RNA preparations from normal human distal/sigmoid colon tissue or whole cellular extract isolated from the human colon cancer cell line Caco2. A unique RT-PCR product was amplified in RNA preparations from human distal/sigmoid colon tissue but not from the Caco2 cells (Figure 1). The size of the product was as the expected size for CRF₂ transcripts and was identical to that amplified from human brain mRNA extracts. No PCR product was amplified in the human mRNA sample when reverse transcriptase enzyme was not added in the RT reaction, or when PCR was performed in the absence of DNA template, excluding the possibility of genomic or other DNA contamination.

CRF₂ receptor protein expression in the distal/sigmoid colon biopsies of healthy human subjects

Serial tissue sections from 10 human colonic biopsies from healthy subjects were stained by immunofluorescence for CRF₂ receptor protein, using two specific polyclonal antisera (Figure 2), one of them selective for the CRF₂ receptor type. Both antibodies revealed membranous staining and similar patterns. CRF₂ positive cells were localized in the lamina propria of the colonic mucosal and in the epithelial cells of the intestinal crypts. Replacement of the antisera by non-specific rabbit IgG abolished all specific staining (although there is still some background due to non-specific absorption of secondary antibody). There were no significant differences on the pattern of distribution of receptor expression in the serial sections of the colon biopsy samples examined.

Intestinal CRF₂ receptor protein expression in distal/sigmoid colonic biopsies of patients with ulcerative colitis, HIV and FBD

CRF₂ receptor protein expression was compared in tissue

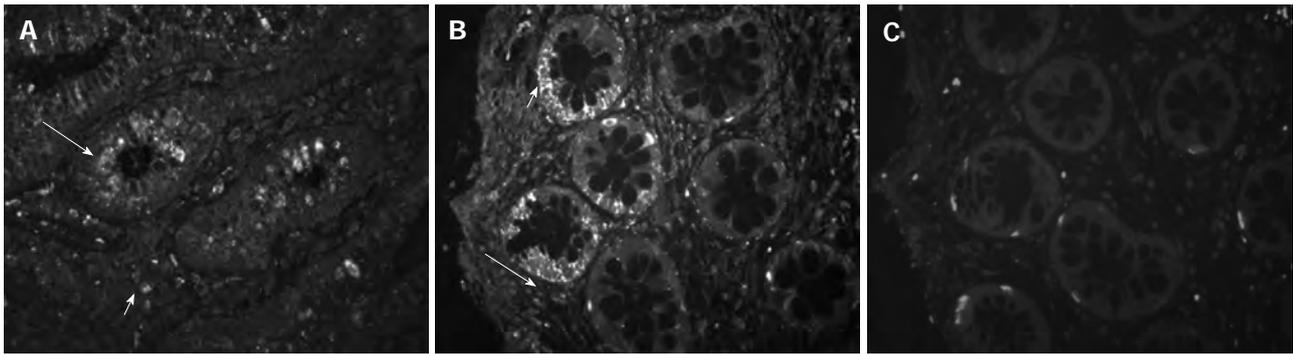


Figure 2 Mapping of corticotropin-releasing factor 2 receptor protein expression in distal/sigmoid colon biopsies of healthy human subjects. Tissue sections from normal mucosal biopsies were stained by immunofluorescence using specific antisera against corticotropin-releasing factor 2 (CRF₂) (A, 2064a-CRF₂) and both CRF₁ and CRF₂ (B, 4392a-CRF₁ and 2). Immunoreactivity was localized in isolated structures of the lamina propria (long arrows) and in the epithelial cells of the intestinal crypts (short arrows). Replacement of the antiserum by non-specific rabbit IgG (C, negative) abolished all specific staining. Original magnification 100 \times .

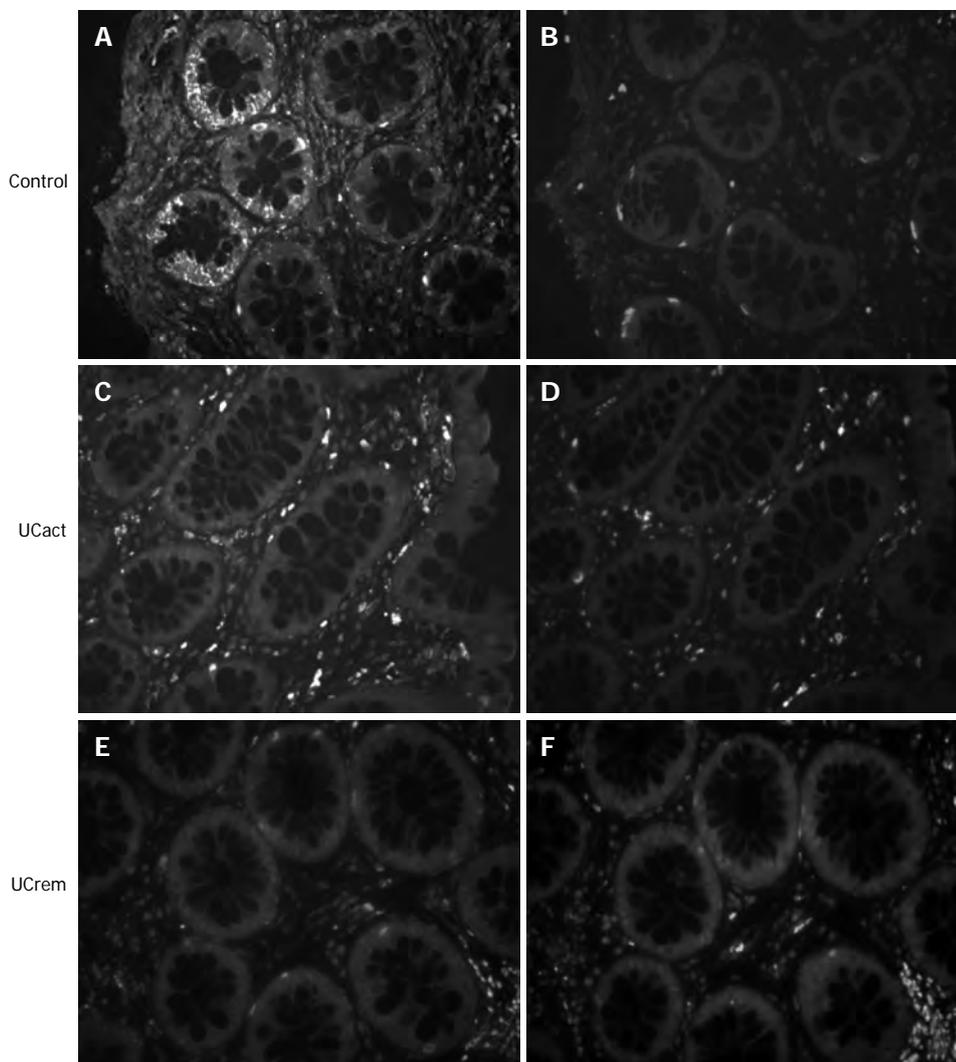


Figure 3 Comparison of corticotropin-releasing factor 2 receptor immunoreactivity in distal/sigmoid biopsies from healthy human subjects and patients with moderate ulcerative colitis and patients in remission. Tissue sections from mucosal biopsies were stained by immunofluorescence using specific antisera 2064a-corticotropin-releasing factor 2 (CRF₂) (A, C, E). Immunoreactivity for CRF₂ observed in the epithelial cells of the intestinal crypts in the control group, was not found in the UCact and UCrem groups. Replacement of the antiserum by non-specific rabbit IgG (B, D, F) abolished all specific staining. Original magnification 100 \times .

sections from five groups: A: healthy subjects (control, $n = 10$), B: UCact ($n = 4$), C: UCrem ($n = 6$), D: HIV ($n = 4$), and E: FBD ($n = 4$). The results are presented in Figures 3 and 4. CRF₂ expression in the colonic epithelial cells was down regulated in both UC patient groups, those with moderately active UC and those with disease in remission. In particular, CRF₂ immunoreactivity was

detected in the epithelial cells of the crypts in 6/10 (60%) of healthy subjects, 2/4 (50%) in the HIV group and 3/4 (75%) in the FBD group, but in none of the UCact (0/5, 0%) and UCrem group (0/6, 0%) and these differences were statistically significant ($P < 0.05$). Positive CRF₂ staining in the lamina propria was seen in all the tissues examined.

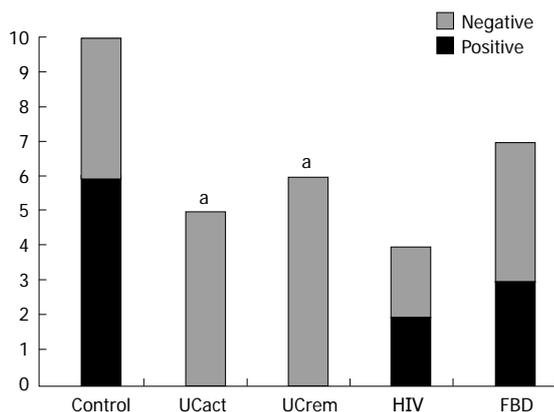


Figure 4 Corticotropin-releasing factor 2 receptor protein expression in epithelial cells in distal/sigmoid biopsies from five groups: Healthy subjects, patients with moderately active ulcerative colitis, patients with ulcerative colitis in remission, patients with human immunodeficiency virus and functional bowel disease. UCact: Active ulcerative colitis; UCrem: Ulcerative colitis in remission; HIV: Human immunodeficiency virus; FBD: Functional bowel disease. ^a*P* < 0.05 vs control group.

DISCUSSION

In the present study, we describe the expression pattern of CRF₂ in the distal/sigmoid colonic mucosa of patients with colonic inflammation (UC) or disease without inflammation (HIV infection and FBD) and compare it to healthy control subjects. Receptor protein was prominent in both the lamina propria and in epithelial cells of the intestinal crypts in tissues from healthy subjects. Interestingly, in patients with UC either moderately active or with disease in remission, expression was limited only to the lamina propria of the mucosa, whereas in the intestinal crypts it was found to be down regulated. No such difference was observed in tissues from the HIV and FBD patients.

Gene expression was confirmed by RT-PCR in total RNA preparations from full thickness intestinal tissues in healthy human subjects. Previous studies also showed CRF₂ gene expression in isolated lamina propria mononuclear cells and lower levels in the epithelial cells fraction isolated macroscopically from normal colorectal biopsies of patients undergoing surgery for non-obstructive colorectal cancer.^[28,40] More recently Wallon *et al*^[41] also found CRF₂ in subepithelial cells of human sigmoid biopsies, proven to be mast cells by colocalization with tryptase. In experimental studies, CRF₂ receptors have also been localized in the colonic enteric plexus^[38,42,43], the endothelium and vascular smooth muscle, and closely resemble human colonic expression patterns of urocortin 1 and urocortin 3^[44]. These results complement our present findings on the receptor protein histological localization, showing the respective/predicted expression pattern. By contrast, we found that the human colorectal adenocarcinoma cell line Caco2 did not express any receptor gene. These data seem specific to this cell line since previous studies showed that adenocarcinoma HT-29 cells expressed mRNA for CRF_{2x}^[45] and the non-transformed human, NCM460 colonocyte^[46] also express CRF_{2x} al-

though at a low level^[45].

Saruta *et al*^[28] showed CRF₂ mRNA expression in the lamina propria macrophages of UC patients without glucocorticoid treatment, along with an upregulation of urocortin 1 within the same cells in proportion to the severity of inflammation. We could also confirm these findings, observing an increase in urocortin 1 immunoreactivity in the colonic mucosa of UC patients (moderately active or in remission) although not a thorough quantification was performed (results not shown). Another report indicates that there is an increased gene expression of both urocortin 2 and CRF₂ in biopsies from UC patients with moderately active disease undergoing colonoscopy^[30]. The differential up-regulation of CRF₂ mRNA levels previously reported in UC and the decreased CRF₂ immunoreactivity in colonic biopsies of UC patients (present study) is not clear at the present time and may reflect alterations in CRF₂ transcription under UC conditions. However experimental studies in a rat model of chemically-induced colitis showed increased urocortin 2 accompanied by down-regulation of CRF₂ expression. CRF was also enhanced in the colonic mucosa of UC patients, in both inflammatory (namely macrophages and eosinophils) and epithelial cells^[27,47]. It is possible that the increase of peptide ligands related to the local inflammatory process, accounts for the down regulation of CRF₂ receptor protein expression reported here (being either a cause or a resultant effect). Regulation of G-protein receptor expression by its ligands is a frequent homeostatic mechanism observed in many endocrine/paracrine pathways^[48].

The down regulation of CRF₂ protein expression in the sigmoid observed in the UC patients seems to be specific to UC. This notion is enforced by our findings from a small number of two more patient groups, with HIV infection or FBD, where CRF₂ protein expression in colonic mucosa was not down-regulated. Thus, we could conclude that CRF₂ down-regulation is specific to acute or chronic inflammation of UC patients with moderately active disease and persist in colitis in remission (also inflamed) in patients receiving conventional, non-steroid therapy.

Data from experimental animal models point to a prominent role for CRF₂ in colitis-related inflammation. In a 2,4,6-trinitrobenzenesulfonic acid-induced colitis model in rats, CRF₂ expression, present on myenteric neurons and macrophages, decreased in the early phase (day 1-3) of inflammation^[49]. However a study by Moss *et al*^[30] performed on a graft of human fetal small intestinal and colonic tissues in immunodeficient mice and in which inflammation is induced by *C. difficile* toxin A 12 wk later, demonstrated increased mucosal urocortin 2 mRNA expression and up-regulation of mucosal CRF₂ expression (after 6 h), as was also the case in the murine toxin A model^[50]. Taken together, while the experimental and clinical studies provide convergent data showing an up regulation of CRF ligands including CRF, urocortin 1 and urocortin 2 under conditions of colonic inflamma-

tion or UC, this can be associated with either up or down regulation of CRF₂ receptor.

A number of studies involve the expression of the CRF system (ligands and receptors) in the regulation of local inflammation in many different tissues. In particular, local expression of urocortin 1 has been reported to act as a proinflammatory factor in rheumatoid arthritis^[29,51] or having anti-inflammatory actions in *Helicobacter pylori*-related gastritis^[52]. It seems likely that the two receptor types CRF₁ and CRF₂, being distributed in different cellular types, could mediate distinct, even opposite effects in the process of local inflammatory phenomena. Opposite signalling of the two receptor types and CRF neuropeptides has been previously reported^[53-55]. This is further supported by our data showing increased CRF₁ positive macrophages in the colonic mucosa of UC patients.

In conclusion, we report the expression of CRF₂ in the mucosal epithelium of normal human colon at gene and protein levels, and its histological mapping in the colonic mucosal and lamina propria cells. Moreover, we show down regulation of the CRF₂ protein receptor in tissues from UC patients either with moderately active disease or in remission that were not treated with glucocorticoids. These findings along with our recent studies showing the upregulation of CRF₁ receptor protein expression in macrophages of the lamina propria from sigmoid biopsies in UC patients suggest the involvement of CRF receptors in the modulation of colonic mucosa inflammation which needs further investigation. These results along with existing evidence^[32] point to the potential therapeutic use of drugs targeting CRF signaling systems to interfere with UC pathophysiology.

COMMENTS

Background

An established etiological factor for the development of ulcerative colitis (UC) is stress acting *via* initial nervous disturbance and subsequent immune dysfunction through brain-gut interactions. Activation of corticotropin-releasing factor (CRF) receptors is the principal mediator of the neuroendocrine stress responses.

Research frontiers

Previous reports indicate that CRF neuropeptides are elevated in the colonic mucosa of patients with active UC and are involved in the pathogenesis of this disease. Local expression of CRF receptors is a prerequisite for mediation of these effects and understanding their histological distribution would provide anatomical support to the physiological and pathophysiological condition/phenomena. Furthermore, they could provide targets for new strategies of pharmacotherapy.

Innovations and breakthroughs

The authors have recently reported that CRF₁ is up-regulated in the colonic mucosa of UC patients, particularly in macrophages supporting its involvement in the immune-inflammatory process. However, recent studies in a rat model of chemically induced colitis showed that CRF₂ rather than CRF₁ is pivotal for macroscopic spread of colitis and resolution of edema. In order to elucidate the molecular mechanism underlying the stress-related activation of UC symptomatology, here, we investigated the expression pattern of CRF₂ in the distal/sigmoid colonic mucosal biopsies of healthy human subjects and compared it to inflamed colonic tissues from patients with UC or with human immunodeficiency virus infection and functional bowel diseases, without signs of inflammation.

Applications

The results suggest an involvement of CRF₂ receptor in the process of inflammation in the colon and that alterations in CRF receptor expression might

participate in the pathophysiology of UC, by arresting direct stress effects on the colonic tissue. These findings could be exploited for the development of effective drugs against colitis.

Terminology

UC is one of the main inflammatory bowel diseases which affect primarily the colonic segment of the gut. Although its aetiology is unknown, its development and acute exacerbation have been related to both physical and mental stress. The hypothalamic neuropeptide CRF and its homologues urocortin 1, 2 and 3, are the key mediators of the endocrine, behavioural, autonomic and visceral responses to stress, acting *via* 2 G-protein coupled receptors, CRF₁ and CRF₂.

Peer review

The work is well written. Authors should better specify the expression of CRF₂ in response to therapy and how it changes in activity or remission state of disease.

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Endoscopic and surgical resection of T1a/T1b esophageal neoplasms: A systematic review

George Sgourakis, Ines Gockel, Hauke Lang

George Sgourakis, Ines Gockel, Hauke Lang, Department of General and Abdominal Surgery, Johannes Gutenberg University Hospital of Mainz, D-55131 Mainz, Germany
George Sgourakis, 2nd Surgical Department and Surgical Oncology Unit of "Korgialenio-Benakio", Red Cross Hospital of Athens, 11526 Athens, Greece

Author contributions: Sgourakis G designed the research and performed the statistical analysis; Gockel I acquired the data, and analyzed and interpreted the data; Lang H revised the manuscript critically for important intellectual content; Sgourakis G and Gockel I contributed equally to this manuscript.

Correspondence to: George Sgourakis, MD, PhD, FACS, 2nd Surgical Department and Surgical Oncology Unit of "Korgialenio-Benakio", Red Cross Hospital of Athens, 11 Mantzarou Street, Neo Psychiko, 11526 Athens, Greece. gsgourakis@yahoo.gr

Telephone: +30-210-6716015 Fax: +30-210-6716015

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Abstract

AIM: To investigate potential therapeutic recommendations for endoscopic and surgical resection of T1a/T1b esophageal neoplasms.

METHODS: A thorough search of electronic databases MEDLINE, Embase, Pubmed and Cochrane Library, from 1997 up to January 2011 was performed. An analysis was carried out, pooling the effects of outcomes of 4241 patients enrolled in 80 retrospective studies. For comparisons across studies, each reporting on only one endoscopic method, we used a random effects meta-regression of the log-odds of the outcome of treatment in each study. "Neural networks" as a data mining technique was employed in order to establish a prediction model of lymph node status in superficial submucosal esophageal carcinoma. Another data mining technique, the "feature selection and root cause analysis", was used to identify the most impor-

tant predictors of local recurrence and metachronous cancer development in endoscopically resected patients, and lymph node positivity in squamous carcinoma (SCC) and adenocarcinoma (ADC) separately in surgically resected patients.

RESULTS: Endoscopically resected patients: Low grade dysplasia was observed in 4% of patients, high grade dysplasia in 14.6%, carcinoma *in situ* in 19%, mucosal cancer in 54%, and submucosal cancer in 16% of patients. There were no significant differences between endoscopic mucosal resection and endoscopic submucosal dissection (ESD) for the following parameters: complications, patients submitted to surgery, positive margins, lymph node positivity, local recurrence and metachronous cancer. With regard to piecemeal resection, ESD performed better since the number of cases was significantly less [coefficient: -7.709438, 95%CI: (-11.03803, -4.380844), $P < 0.001$]; hence local recurrence rates were significantly lower [coefficient: -4.033528, 95%CI: (-6.151498, -1.915559), $P < 0.01$]. A higher rate of esophageal stenosis was observed following ESD [coefficient: 7.322266, 95%CI: (3.810146, 10.83439), $P < 0.001$]. A significantly greater number of SCC patients were submitted to surgery (log-odds, ADC: -2.1206 ± 0.6249 vs SCC: 4.1356 ± 0.4038, $P < 0.05$). The odds for re-classification of tumor stage after endoscopic resection were 53% and 39% for ADC and SCC, respectively. Local tumor recurrence was best predicted by grade 3 differentiation and piecemeal resection, metachronous cancer development by the carcinoma *in situ* component, and lymph node positivity by lymphovascular invasion. With regard to surgically resected patients: Significant differences in patients with positive lymph nodes were observed between ADC and SCC [coefficient: 1.889569, 95%CI: (0.3945146, 3.384624), $P < 0.01$]. In contrast, lymphovascular and microvascular invasion and grade 3 patients between histologic types were comparable, the respective rank order of the predictors of lymph node positivity was: Grade 3, lymphovascular invasion (L+), microvascular

invasion (V+), submucosal (Sm) 3 invasion, Sm2 invasion and Sm1 invasion. Histologic type (ADC/SCC) was not included in the model. The best predictors for SCC lymph node positivity were Sm3 invasion and (V+). For ADC, the most important predictor was (L+).

CONCLUSION: Local tumor recurrence is predicted by grade 3, metachronous cancer by the carcinoma in-situ component, and lymph node positivity by L+. T1b cancer should be treated with surgical resection.

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Key words: Superficial esophageal cancer; Endoscopic resection; Mucosal infiltration; Submucosal involvement; Recurrent tumor; Controversies in treatment; Squamous cell carcinoma; Adenocarcinoma; Lymphatic invasion; Vascular invasion; Submucosal layer; Superficial submucosal layer; Middle third submucosal layer; Deep third submucosal layer; Esophageal cancer; Endoscopic gastrointestinal surgical procedures; Endoscopic gastrointestinal surgery; Lymph node dissection; Dysplasia

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INTRODUCTION

Endoscopic mucosal resection (EMR) and endoscopic submucosal dissection (ESD), in addition to local ablation techniques, are now more extensively employed for the management of early adenocarcinoma (ADC) or squamous cell carcinoma (SCC) of the esophagus. The aim of endoscopic resection is to maintain the integrity of the esophagus and avoid the considerable morbidity and mortality of esophagectomy^[1].

Several cohort studies^[2-5] suggest the use of EMR or ESD for T1a esophageal neoplasia (including high grade dysplasia, adenocarcinoma, or squamous-cell carcinoma) confined to the superficial mucosa and not extending into the muscularis mucosa. Other studies contemplate endoscopic resection, even in muscularis mucosa invasion and in selected cases where upper third submucosal involvement is present^[6]. T1b disease may be treated by esophagectomy.

At present, there are no reliable pre-excision molecular, biological or immunohistochemical predictive markers of lymph node metastasis in T1 esophageal cancer. Moreover, the current diagnostic workup has a low diagnostic performance for N1-disease which is considered the most influential predictor of long term prognosis^[7].

The pros and cons of each endoscopic resection method have yet to be established, and level I evidence of their safety and efficacy is missing from the literature. Predictive markers of lymph node metastasis in mucosal and

submucosal esophageal cancer are also an unsolved issue.

Answers to the aforementioned issues might enable researchers to formulate curative treatment strategies and considerations for neoadjuvant referral in early esophageal carcinoma cases.

The objectives of this study were: (1) to compare the safety and efficacy of EMR and ESD in the management of early esophageal neoplasia; (2) to investigate their role as part of the diagnostic workup; (3) to establish predictors of lymph node status, local recurrence and metachronous cancer development in superficial esophageal carcinoma; and (4) to investigate potential therapeutic recommendations.

MATERIALS AND METHODS

Literature search

Medline, Embase, Pub Med and the Cochrane Library databases were searched for articles in the English language from 1997 up to 2011. The following search terms were used: Early esophageal cancer, esophageal dysplasia, high grade dysplasia, low grade dysplasia, intraepithelial neoplasia, Barrett's esophagus, superficial esophageal cancer, mucosal esophageal cancer, submucosal esophageal cancer, intramucosal/submucosal carcinoma of the esophagus, esophageal adenocarcinoma, esophageal squamous cell carcinoma, adjuvant treatment, T1a, T1b, T1m and T1sm. Terms were combined with "and/or" and asterisks. References from included studies were examined for additional studies. The main reasons for initial exclusion included animal studies, non-English literature, case reports, reviews and double publications. Figure 1 shows the process and stages throughout the review of the studies included.

Inclusion and exclusion criteria for the endoscopic database

Inclusion: (1) Application of EMR and/or ESD for early esophageal cancer; (2) Low-grade dysplasia or high grade dysplasia (HGD) in the setting of Barrett's esophagus as well as early esophageal cancer; and (3) Siewert I and II tumors.

Exclusion: (1) Studies involving previously untreated patients (no neoadjuvant therapy); (2) Studies including patients with Siewert type III, and with metastatic disease; and (3) Studies including patients with tumors other than ADC/SCC.

Inclusion and exclusion criteria for the surgically resected patients' database

Inclusion: (1) Information from the pathology reports after esophagectomy for submucosal carcinoma with curative intent; (2) Studies including patients with esophago-gastric junction carcinoma were eligible for analysis; and (3) Studies providing separate data for SCC and ADC.

Exclusion: (1) Studies administering neo-adjuvant treatment; (2) Studies involving patients with distant metastasis.

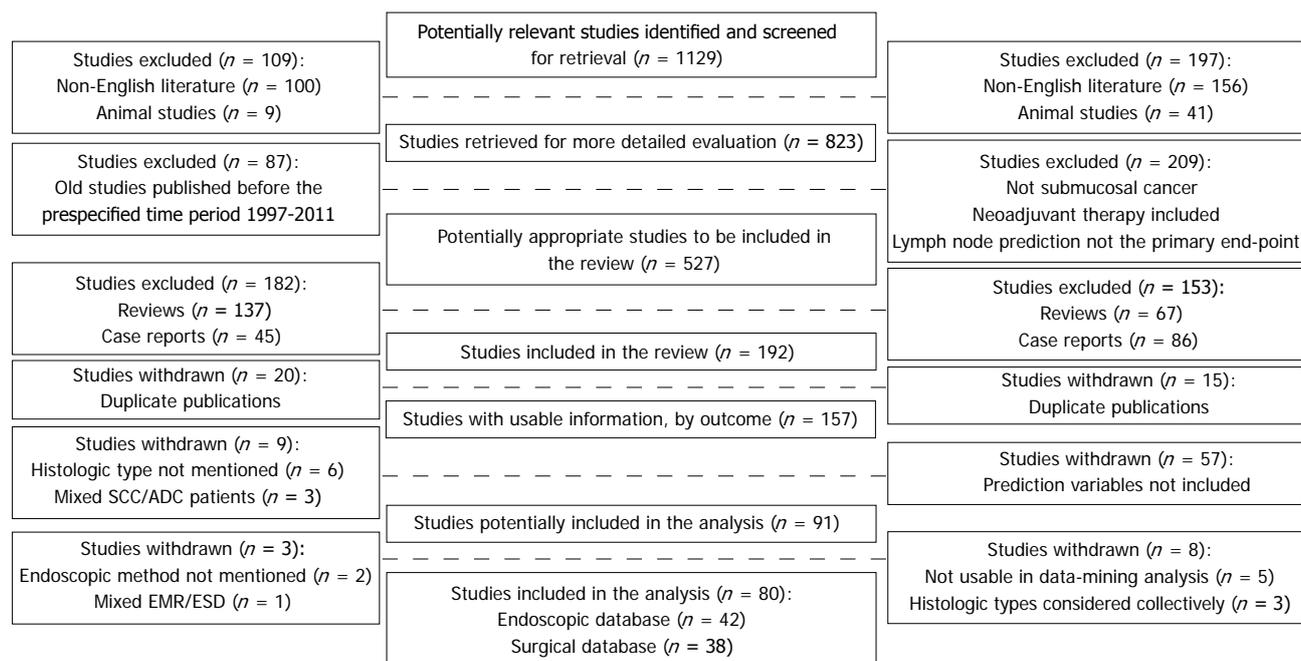


Figure 1 Progress through the stages of study review included. SCC: Squamous cell carcinoma; ADC: Adenocarcinoma; EMR: Endoscopic mucosal resection; ESD: Endoscopic submucosal dissection.

sis; (3) Case reports; (4) Mixed data for SCC and ADC; and (5) Mixed data for T1a and T1b tumors and/or surveillance of patients with dysplasia.

Data extraction

The two authors independently selected studies for inclusion and exclusion and reached a consensus when there was initial disagreement. The parameters ascertained included authors, journal and year of publication, total number of patients, type of estrogen receptor (ER) involved, final pathology results, histological type, tumor diameter, tumor location, pattern of growth, degree of differentiation, depth of tumor invasion, lymph node status, presence of lymphatic or venous invasion, as well as positive resection margins on the pathology specimen, number of patients with local recurrence, presence of metachronous lesions, and additional therapies necessary beyond ER, including surgery.

Definitions

Submucosal lesions were classified as Sm1 for tumors invading the more superficial layer of the submucosa (corresponding to one-third of its thickness), Sm2 for those invading the middle third, and Sm3 for those invading the deeper submucosal layer^[8].

Statistical analysis

For comparisons across studies, each reporting on only one treatment/histologic type, we used a random effects meta-regression of the log-odds of the outcome of treatment in each study. In this case, we estimated the variance of each study-specific log-odds as the sum of the reciprocals of the number of successes and failures. Counts of 0 were replaced by 0.5.

Statistical analysis for comparisons across studies was performed using the “metareg command” of STATA/SE 11. To address multiple testing (calculate *P* values for covariates) the “permute option” based on a Monte Carlo permutation test of STATA/SE 11 was used.

“Neural networks” as a data mining technique^[9] was employed in order to establish a prediction model of lymph node status in superficial submucosal esophageal carcinoma and find a simple model to fit the data better. The definition of a linear network was followed by training of the network. The data set was divided into three subsets: training, selection, and test cases in the proportions 3:1:1 between the training, selection, and test subsets.

Another data mining technique, the “feature selection and root cause analysis”, was used to identify the most important predictors of local recurrence and metachronous cancer development in endoscopically resected patients, and lymph node positivity in SCC and ADC separately in surgically resected patients.

In brief, this test provides extremely useful shortcuts for identifying root causes for the values observed in the outcome variables under investigation (*e.g.*, an indicator of quality or process yield); final selections of predictors are not biased in favor of any particular model (fitted to the data for the selected predictors).

The statistical programs used were: STATA/SE 11 (Statacorp LP 4905 Lakeway Drive College Station TX 77845, United States), the NCSS 2007 and GESS 2006 version 07.1.13, (Kaysville, Utah, United States) and Statistica release 7 (Stat Soft Inc., Tulsa, United States).

Table 1 Forty-two studies were included in the analysis of endoscopically resected patients

| Author | EMR/ESD | Patients | Surgery | ADC/SCC | Positive resection margin | Other therapy | Local recurrence | Meta-chronous | N (+) | L (+) | Re-classification | Grade 3 | In situ | Piecemeal resection |
|---|---------|----------|---------|---------|---------------------------|---------------|------------------|---------------|-------|-------|-------------------|---------|---------|---------------------|
| Buttar <i>et al</i> ^[10] | EMR | 17 | 0 | ADC | 3 | PDT | | | | | 8 | | 0 | |
| Chaves <i>et al</i> ^[11] | ESD | 5 | | SCC | 0 | | 0 | | | | | | 3 | 1 |
| Chennat <i>et al</i> ^[12] | EMR | 49 | 3 | ADC | | | 0 | | | | 22 | | 0 | |
| Ciocirlan <i>et al</i> ^[13] | EMR | 51 | 2 | SCC | 14 | CHEMO | 8 | 2 | | | | 0 | 4 | 36 |
| Conio <i>et al</i> ^[14] | EMR | 39 | 3 | ADC | | | 0 | 1 | | 2 | 10 | 5 | 0 | |
| Ell <i>et al</i> ^[15] | EMR | 64 | 5 | ADC | | PDT/APC | 6 | 3 | | | 6 | 6 | 0 | |
| Espinel <i>et al</i> ^[16] | EMR | 4 | 1 | ADC | | | | | | | 1 | 0 | 0 | |
| Fujishiro <i>et al</i> ^[17] | ESD | 43 | | SCC | 7 | | 1 | 1 | | 1 | | | 24 | 0 |
| Gerke <i>et al</i> ^[18] | EMR | 41 | | ADC | 9 | RFA | 3 | 0 | | | | | 0 | |
| Goda <i>et al</i> ^[19] | EMR | 58 | 1 | SCC | | CRT | | | | 1 | | | 0 | |
| Higuchi <i>et al</i> ^[20] | EMR | 20 | 0 | SCC | 6 | CRT/APC | 0 | 0 | 0 | 6 | | 1 | 0 | |
| Hull <i>et al</i> ^[21] | EMR | 10 | | ADC | | | | | | | 2 | | 0 | |
| Iguchi <i>et al</i> ^[22] | EMR | 8 | 1 | SCC | | | | | | | | 0 | 4 | |
| Ishihara <i>et al</i> ^[22] | EMR/ESD | 70 | | SCC | | CRT | 12 | | | 0 | | | 40 | |
| Ishii <i>et al</i> ^[23] | ESD | 35 | 1 | SCC | 2 | CHEMO | 0 | | | 1 | | | 28 | 0 |
| Larghi <i>et al</i> ^[24] | EMR | 40 | 5 | ADC | | PDT/APC | 0 | | | | 6 | | 19 | |
| Lewis <i>et al</i> ^[25] | EMR | 100 | 1 | ADC | 1 | PDT | | | 1 | | 8 | | | |
| Lin <i>et al</i> ^[26] | EMR | 9 | 1 | SCC | 0 | | 1 | | 0 | 0 | 1 | 0 | 7 | |
| Lopes <i>et al</i> ^[27] | EMR | 41 | 1 | ADC | | APC/CRT | 4 | 2 | | | 14 | | 2 | |
| Maish <i>et al</i> ^[28] | EMR | 7 | 7 | ADC | 1 | | | | 0 | | 4 | | 0 | |
| Manner <i>et al</i> ^[6] | EMR/ESD | 21 | 1 | ADC | 27 | APC | 3 | 2 | | 0 | | 0 | 0 | |
| Naritaka <i>et al</i> ^[29] | EMR | 13 | 1 | SCC | 2 | RT | 1 | | | | | | 7 | 9 |
| Nijhawan <i>et al</i> ^[30] | EMR | 25 | 2 | ADC | | PDT | 0 | | | | 11 | | | |
| Noguchi <i>et al</i> ^[31] | EMR | 33 | 5 | SCC | | CRT | | | 0 | 5 | 14 | | 15 | |
| Nomura <i>et al</i> ^[32] | EMR | 51 | 1 | SCC | | CRT | 4 | | | | | | 30 | 41 |
| Nonaka <i>et al</i> ^[33] | ESD | 25 | 1 | SCC | 3 | RT/CRT | 0 | | | | 10 | | 0 | 0 |
| Ohashi <i>et al</i> ^[9] | EMR | 179 | | SCC | | | | | | 13 | | | 68 | |
| Ono <i>et al</i> ^[34] | ESD | 84 | 9 | SCC | 7 | CRT | 1 | 2 | 2 | | | | 0 | 0 |
| Ota <i>et al</i> ^[35] | EMR | 18 | 0 | SCC | 5 | CRT | 0 | | 4 | 11 | | 3 | 0 | |
| Pech <i>et al</i> ^[5] | EMR/ESD | 39 | | SCC | 20 | PDT/CHEMO | 5 | | 2 | 7 | | 1 | 10 | |
| Peters <i>et al</i> ^[33] | EMR/ESD | 141 | | ADC | 37 | | | | | 1 | 73 | 14 | | |
| Pouw <i>et al</i> ^[37] | EMR/ESD | 34 | 1 | ADC | | APC | 3 | | | | 14 | | 10 | |
| Prasad <i>et al</i> ^[39] | EMR | 25 | 25 | ADC | 17 | | | | 5 | | 16 | | | |
| Repici <i>et al</i> ^[51] | ESD | 20 | 2 | SCC | 1 | | 0 | 0 | 1 | | | 2 | 3 | 0 |
| Scheil-Bertram <i>et al</i> ^[40] | EMR | 16 | 16 | ADC | | | | | 16 | | | | 1 | |
| Schröder <i>et al</i> ^[41] | EMR | 16 | | ADC/SCC | 9 | | | | 3 | 13 | | 1 | | |
| Shimizu <i>et al</i> ^[42] | EMR | 82 | | SCC | | APC | 2 | 12 | | | | | 16 | |
| Takeo <i>et al</i> ^[44] | EMR | 29 | 5 | | | | 0 | | 0 | | 15 | | 10 | |
| Tanabe <i>et al</i> ^[46] | EMR | 85 | 0 | SCC | 15 | APC/CRT | 5 | | | | | | 0 | 41 |
| Teoh <i>et al</i> ^[47] | EMR/ESD | 28 | | SCC | 6 | RT/CRT | 1 | | | 1 | | | | |
| Urabe <i>et al</i> ^[48] | EMR/ESD | 122 | | SCC | | | 6 | | | | | | 56 | |
| Vieth <i>et al</i> ^[54] | EMR | 295 | | ADC | 210 | | | | | 10 | | 22 | | |
| Yokoyama <i>et al</i> ^[49] | EMR | 17 | 0 | SCC | | RT | | | | | | | 7 | |
| Zehetner <i>et al</i> ^[50] | EMR | 28 | 3 | ADC | 0 | RFA | 5 | 3 | | 2 | | 2 | | |

EMR: Endoscopic mucosal resection; ESD: Endoscopic submucosal dissection; SCC: Squamous carcinoma; ADC: Adenocarcinoma; APC: Argon plasma coagulation; PDT: Photodynamic therapy; CRT: Chemoradiation therapy; RFA: Radiofrequency ablation; RT: Radiology; CHEMO: Chemotherapy.

RESULTS

Endoscopically resected patients

Forty-two studies^[6,10-50] were selected (Table 1) which included a total of 2092 patients. Low grade dysplasia was observed in 4% of patients, high grade dysplasia in 14.6%, carcinoma *in situ* in 19%, mucosal cancer in 54%,

and submucosal cancer in 16% of patients. Histologic types were SCC in 23 studies and ADC in 19 studies.

EMR was employed in 29 studies and ESD in 6 studies. Both EMR and ESD were used in 7 studies. Lymphovascular invasion was found to range from 0%-30%, microvascular invasion was observed in 0%-33% of patients, and 7.4% of patients were poorly differentiated.

Table 2 Meta-regression analysis of the methods of endoscopic resection according to the published studies (the random effects model was used)

| EMR <i>vs</i> ESD | Coefficient | 95%CI | P value | Favors |
|----------------------------------|-------------|----------------------|---------|--------|
| Patients submitted to surgery | 0.401 | -2.912964, 3.714436 | 0.806 | None |
| Positive margins | -0.741 | -3.362995, 1.881024 | 0.558 | None |
| Local recurrence | -1.713 | -4.420582, 0.9937198 | 0.201 | None |
| Lymph node metastasis | 0.905 | -5.762587, 7.573427 | 0.762 | None |
| Metachronous cancer | -1.804 | -4.350273, 0.7420371 | 0.143 | None |
| Procedural complications | 1.397 | -1.264597, 4.058631 | 0.289 | None |
| Stenosis | 7.322 | 3.810146, 10.83439 | < 0.001 | EMR |
| Piecemeal resection ¹ | | | | |
| Number of cases | -7.709 | -11.03803, -4.380844 | < 0.001 | ESD |
| Local recurrence | -4.034 | -6.151498, -1.915559 | < 0.01 | ESD |
| Resection margins | 0.837 | -3.725993, 5.39999 | 0.678 | None |

¹Data available only for squamous cell carcinoma studies. EMR: Endoscopic mucosal resection; ESD: Endoscopic submucosal dissection.

Argon plasma coagulation (APC) as the only modality was used in 3 studies^[6,37,42]. In addition to APC, 2 studies^[15,24] also utilized photodynamic therapy (PDT) and 3 studies added chemoradiation therapy (CRT)^[20,27,46]. Adjuvant only CRT was administered in 6 studies^[19,22,31,32,34,35], radiotherapy only in 2^[29,49], radiotherapy and CRT in 2^[33,47], PDT only in 3^[10,25,30], chemotherapy only in 2^[13,23], and PDT/chemotherapy in one study^[4]. Radiofrequency ablation was used in 2 studies^[18,50]. Mean follow-up time varied from 12 to 62 mo and median follow-up time ranged from 7 to 39 mo.

Lymph node metastasis

Eleven studies^[4,20,25,26,28,31,34,35,38,40,51] provided data on lymph node metastasis. Thirty-one patients out of 371 were node-positive. The overall increase in the odds was 5% for ADC and approximately 1% for SCC. No significant differences were observed between either ADC *vs* SCC or EMR *vs* ESD patients (Tables 2 and 3). Lymphovascular invasion was found to be the only predictor of lymph node metastasis (*F* value: 416.45, *P* < 0.001).

Differences between pre- and post-endoscopic resection tumor staging

Eighteen studies^[10,12,14-16,24-28,30,31,33,37,38,44,52,53] including 685 patients reported differences between pre- and post-endoscopic resection tumor staging in 235 cases. These differences were mainly due to either the histological assessment (HGD *vs* carcinoma) and/or tumor depth of invasion (Table 3). Patients treated with both endoscopic methods and subsequently submitted to surgery due to unfavorable tumor characteristics did not differ significantly (Figure 2A), although SCC patients were statistically more likely to be referred for surgery. The combined odds were 53% and 39% for ADC and SCC, respectively.

Piecemeal resection

Piecemeal resection was accomplished in 48% (732/1516)

of cases. Ten studies^[11,13,17,23,29,32-34,46,51] reporting piecemeal resection cases (*n* = 412) additionally provided the number of lesions (*n* = 466) per patient, number of patients with positive margins (*n* = 36) and local recurrence rates (*n* = 20 patients). All the aforementioned 10 studies enrolled SCC patients. Piecemeal resection and local recurrence rates were statistically significantly lower when performing ESD (Tables 2 and 3; Figure 2B). In contrast, positive margins did not differ significantly between the two endoscopic methods.

Resection margins

Eighteen studies^[10,11,13,17,20,23,25,26,28,29,33-35,38,46,50,51,54] reported outcomes concerning specimen margin status. Thirty-three per cent (294/880) of cases demonstrated positive margins. Positive margin data were from primary endoscopic resection. The overall increase in the odds was 9% for ADC and approximately 7% for SCC. No significant differences on positive resection margins were observed between either ADC *vs* SCC or EMR *vs* ESD patients (Tables 2 and 3; Figure 2C).

Monte Carlo permutation adjusted testing for meta-regression disclosed that local recurrence in patients with positive resection margins was independent of endoscopic resection modality (EMR/ESD, *P* = 1.000), histologic type (ADC/SCC, *P* = 0.972) and type of adjuvant therapy (chemo/CRT/APC/RT/PDT, *P* = 0.899). Data mining showed that grade 3 was an independent predictor of local recurrence in cases with positive margins (*P* < 0.001).

Local recurrence

Local recurrence among 30 studies^[3,6,11-15,17,20,22-24,26,27,29,30,32-35,37,42,46-48,50,51] which provided relevant data ranged from 0-17%. The combined odds were 0.8% and 1% for ADC and SCC, respectively. No significant differences were observed between either ADC *vs* SCC or EMR *vs* ESD patients (Tables 2 and 3; Figure 2D). Data mining showed that grade 3 was an independent predictor of local recurrence (*F* value: 16.2, *P* < 0.05). In cases of piecemeal resection, local recurrence was significantly higher when performing EMR (*F* value: 5.39, *P* < 0.01).

Development of metachronous lesions

Development of metachronous lesions ranged from 2%-14% in 10 studies^[6,13-15,17,20,27,34,42,50,51]. The combined odds were 6% and 1% for ADC and SCC, respectively. No significant differences were observed between either ADC *vs* SCC or EMR *vs* ESD patients (Tables 2 and 3; Figure 2E). Data mining showed that the presence of carcinoma *in situ* was an independent predictor of metachronous lesion development (*F* value: 62.5, *P* < 0.01).

Procedural and late morbidity

Twenty-five studies^[10-17,23,24,26-31,33-35,41,43-46,51] provided satisfactory data on procedural morbidity and late complications. Procedural morbidity included bleeding managed conservatively in 5.8%, bleeding requiring intervention in 0.6%, perforation 1.8% and pain in 4.2% of patients.

Table 3 Meta-regression analysis of the outcomes of endoscopic resection according to the histologic type of early esophageal cancer (the random effects model was used)

| Outcome | Histologic type | Log-odds ratio | SE | 95.0% lower confidence limit | 95.0% upper confidence limit | Odds | Favors |
|-------------------------------------|-----------------|----------------|--------|------------------------------|------------------------------|-------|------------|
| Patients submitted to surgery | ADC | -2.1206 | 0.6249 | -3.3454 | -0.8958 | 12% | ADC |
| | SCC | 4.1356 | 0.4038 | -4.9271 | -3.3440 | 37% | $P < 0.05$ |
| Positive margins | ADC | -2.3761 | 1.0181 | -4.3716 | -0.3806 | 9% | None |
| | SCC | -2.5689 | 0.6973 | -3.9357 | -1.2022 | 7% | |
| Local recurrence | ADC | -4.8189 | 0.1469 | -5.1068 | -4.5309 | 0.80% | None |
| | SCC | -4.3347 | 0.2792 | -4.8819 | -3.7874 | 1% | |
| Lymph node metastasis | ADC | -3.0565 | 0.7714 | -4.5685 | -1.5445 | 5% | None |
| | SCC | -4.7682 | 0.4413 | -5.6332 | -3.9032 | 0.90% | |
| Metachronous cancer | ADC | -2.8017 | 0.2384 | -3.2690 | -2.3344 | 6% | None |
| | SCC | -4.6030 | 0.6059 | -5.7905 | -3.4155 | 1% | |
| Pre- vs post-endoscopic tumor stage | ADC | -0.5449 | 0.4316 | -1.3909 | 0.3011 | 53% | - |
| | SCC | -0.8267 | 0.3324 | -1.4782 | -0.1752 | 39% | |

SCC: Squamous cell carcinoma; ADC: Adenocarcinoma.

Esophageal stenosis was experienced by 12.2% of patients. No significant differences in procedural complications were observed between EMR vs ESD patients. In contrast, esophageal stenosis was statistically more prevalent among patients managed with ESD ($P < 0.001$) (Tables 2 and 3; Figure 2F).

Surgically resected patients

Of 677 screened studies, 38 studies comprising a total of 2149 participants were finally included^[20,31,40,55-86].

The magnitude of kappa (0.86) reflected adequate agreement between the two reviewers. All 38 studies provided data on lymph node metastasis. The histological parameters of patients are presented in Table 4. Eight-hundred and eighty-eight (888) patients among 2149 were node-positive. Significant differences in patients with positive lymph nodes were observed between ADC and SCC ($P < 0.01$). In contrast, lymphovascular and microvascular invasion and grade 3 patients between histologic types were comparable (Table 5). Grade 3 patients were seen in 24% (158/663) with SCC and in 49% (267/541) with ADC.

Setting up a model for prediction of lymph node metastasis

In an endeavor to set up a model to predict lymph node metastasis, we applied Neural Networks as a data mining technique. All included studies provided sufficient information on depth of tumor invasion (Sm1, Sm2, Sm3), lymphatic invasion, vascular invasion, histologic differentiation, and histologic type (SCC, ADC) (Table 6).

The number of patients with positive lymph nodes was set as the dependent variable, while the respective number of patients with Sm1, Sm2, Sm3 invasion, lymphatic invasion, vascular invasion, and poor differentiation were used as continuous independent variables. The histologic type of esophageal cancer was set as a categorical variable. The linear model 5:5-1:1 emerged as the best neural network model according to its regression statistics, with the smallest error: data standard deviation ratio (0.07506; an SD ratio closer to 0.1 generally indicates very

good regression performance). This was also true for the close correlation between the prediction of the independent and dependent variables (0.99774). Its format was $\langle \text{type} = \text{Linear} \rangle \langle \text{inputs} = 5 \rangle : \langle \text{layer 1} = 5 \rangle - \langle \text{layer 2} = 1 \rangle : \langle \text{outputs} = 1 \rangle$, with two layers. Missing values were patched using the mean variable value.

The rank order of importance of the predictors of lymph node positivity was: Grade 3, Sm3 invasion, L(+), V(+), Sm2 invasion and Sm1 invasion, respectively. Histologic type (ADC/SCC) had a ratio network error ≤ 1 , and thus should not be considered as a predictor.

Validation of the model

The data set was divided into three subsets: the training, selection, and test cases (3:1:1 in our model) in order to preclude the predictive performance of the linear model being attributed to a data over-fitting phenomenon. The predicted number of patients in various studies with positive lymph nodes by the linear model was almost identical to that observed by the authors.

Predictors of lymph node metastasis in SCC and ADC

Considering only the predictors of lymph node metastasis defined by the aforementioned linear model in each of the two histological entities (SCC, ADC), we applied another data mining technique (Feature selection and root cause analysis).

The best predictors of lymph node positivity in SCC were Sm3 invasion ($P < 0.001$) and microvascular invasion ($P < 0.01$). In relation to ADC, the most important predictor was lymphovascular invasion ($P < 0.05$).

DISCUSSION

According to NCCN guidelines version 1.2011 for esophageal and esophagogastric junction cancers, in the absence of evidence of lymph node metastases, lymphovascular invasion or poor differentiation grade, T1a disease can be treated with full EMR. In cases of unfavorable characteristics, the choice lies between EMR plus ablation or esophagectomy. T1b disease may be treated by esophagectomy.

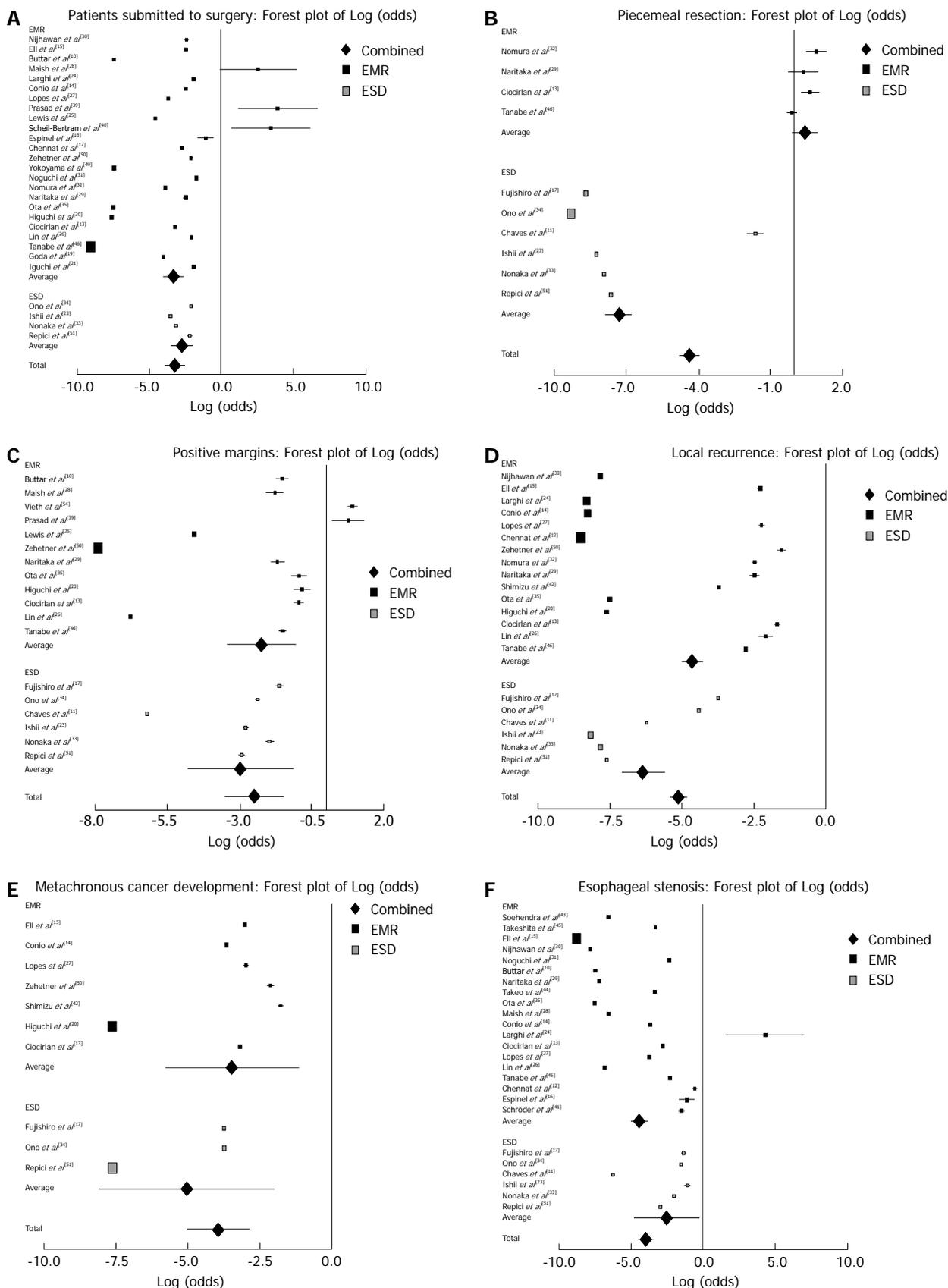


Figure 2 Forest plot of log-odds in both groups (endoscopic mucosal resection and endoscopic submucosal dissection): No statistically significant differences were observed. A: Forest plot of log-odds patients submitted to surgery; B: Forest plot of log-odds of piecemeal resected patients; C: Forest plot of log-odds of positive resection margins patients; D: Forest plot of log-odds of local recurrence in both groups [endoscopic mucosal resection and endoscopic submucosal dissection (EMR-ESD)]; No statistically significant differences were observed, with the exception of piecemeal resected patients. In this last instance ESD was more efficient; E: Forest plot of log-odds of metachronous cancer development; F: Forest plot of log-odds of esophageal stenosis in both groups (EMR-ESD): EMR was less destructive.

Table 4 Thirty-eight studies were included in the analysis of surgically resected patients

| Study | SCC/ADC | sm | sm1 | sm2 | sm3 | N+sm | N+sm1 | N+sm2 | N+sm3 | L+sm | L+sm1 | L+sm2 | L+sm3 | V+sm | V+sm1 | V+sm2 | V+sm3 | Grade III |
|---|---------|-----|-----|-----|-----|------|-------|-------|-------|------|-------|-------|-------|------|-------|-------|-------|-----------|
| Amano <i>et al.</i> ^[51] | SCC | 83 | 10 | 10 | 63 | 47 | 4 | 4 | 39 | 15 | 0 | 4 | 8 | 23 | 0 | 2 | 4 | 6 |
| Araiki <i>et al.</i> ^[56] | SCC | 58 | 12 | 18 | 28 | 15 | 1 | 4 | 10 | 12 | 0 | 4 | 8 | 6 | 0 | 2 | 4 | 5 |
| Bollschweiler <i>et al.</i> ^[57] (ADC) | ADC | 22 | 9 | 4 | 9 | 9 | 2 | 0 | 7 | | | | | | | | | 8 |
| Bollschweiler <i>et al.</i> ^[57] (SCC) | SCC | 22 | 3 | 6 | 13 | 11 | 1 | 1 | 9 | | | | | | | | | 17 |
| Buskens <i>et al.</i> ^[58] | ADC | 42 | 16 | 13 | 13 | 12 | 0 | 3 | 9 | 16 | 0 | 4 | 12 | | | | | 20 |
| Cen <i>et al.</i> ^[59] | ADC | 51 | | | | 12 | | | | 14 | | | | | | | | |
| Chino <i>et al.</i> ^[61] | SCC | 22 | 5 | 8 | 9 | 11 | 1 | 6 | 4 | 17 | 5 | 6 | 6 | 6 | 0 | 2 | 4 | |
| Eguchi <i>et al.</i> ^[61] | SCC | 364 | 32 | | | 196 | 17 | | | 11 | 11 | | | | | | | |
| Endo <i>et al.</i> ^[62] | SCC | 121 | 18 | 48 | 55 | 51 | 2 | 15 | 34 | | | | | 95 | 6 | 35 | 54 | |
| Gockel <i>et al.</i> ^[63] | ADC | 15 | 8 | 2 | 5 | 3 | 1 | 1 | 1 | | | | | | | | | |
| Gockel <i>et al.</i> ^[63] | SCC | 15 | 7 | 4 | 4 | 2 | 1 | 1 | 0 | | | | | | | | | |
| Goseki <i>et al.</i> ^[64] | SCC | 30 | | | | 15 | | | | 21 | | | | 22 | | | | 8 |
| Higuchi <i>et al.</i> ^[60] | SCC | 15 | 15 | | | 3 | 3 | | | 14 | 14 | | | 7 | 7 | | | |
| Ide <i>et al.</i> ^[65] | SCC | 85 | | | | 26 | | | | 54 | | | | 23 | | | | |
| Ikeeda <i>et al.</i> ^[66] | SCC | 45 | | | | 19 | | | | 23 | | | | 23 | | | | |
| Kim <i>et al.</i> ^[67] | SCC | 133 | 36 | 27 | 69 | 39 | 6 | 5 | 28 | 30 | | | | | | | | 18 |
| Kimura <i>et al.</i> ^[68] | SCC | 26 | | | | 9 | | | | 11 | | | | 5 | | | | |
| Kuwano <i>et al.</i> ^[63] | SCC | 26 | 4 | 2 | 20 | 10 | | | | 18 | | | | 10 | | | | 6 |
| Liu <i>et al.</i> ^[71] | ADC | 37 | | | | 10 | | | | 15 | | | | | | | | 0 |
| Makuuchi <i>et al.</i> ^[72] | SCC | 81 | 18 | 25 | 38 | 33 | 4 | 11 | 18 | 60 | 13 | 19 | 28 | 31 | 2 | 9 | 20 | |
| Matsumoto <i>et al.</i> ^[73] | SCC | 87 | 15 | 26 | 46 | 41 | | | | 48 | | | | 26 | | | | |
| Nakajima <i>et al.</i> ^[74] | SCC | 84 | 9 | 29 | 46 | 33 | 0 | 5 | 28 | 60 | | | | 42 | | | | 9 |
| Natsugoe <i>et al.</i> ^[75] | SCC | 92 | 21 | 28 | 43 | 42 | 6 | 11 | 25 | 51 | | | | 25 | | | | 21 |
| Noguchi <i>et al.</i> ^[31] | SCC | 38 | 6 | 10 | 22 | 20 | 1 | 3 | 16 | 31 | 4 | 8 | 19 | 10 | 1 | 1 | 8 | |
| Ohno <i>et al.</i> ^[76] | SCC | 16 | | | | 2 | | | | 6 | | | | 4 | | | | 5 |
| Paraf <i>et al.</i> ^[77] | ADC | 12 | | | | 1 | | | | | | | | 5 | | | | |
| Rice <i>et al.</i> ^[78] | ADC | 24 | | | | 5 | | | | | | | | | | | | 123 |
| Rice <i>et al.</i> ^[78] | SCC | 3 | | | | 1 | | | | | | | | | | | | 13 |
| Scheil-Bertram <i>et al.</i> ^[60] | ADC | 21 | 7 | 2 | 12 | 5 | 1 | 0 | 4 | 9 | 2 | 0 | 7 | 1 | 1 | 0 | 0 | 12 |
| Schmidt <i>et al.</i> ^[79] | SCC | 5 | | | | 2 | | | | 3 | | | | | | | | |
| Shiozaki <i>et al.</i> ^[81] | SCC | 180 | 21 | 73 | 86 | 92 | 8 | 37 | 47 | 119 | 11 | 51 | 57 | 45 | 3 | 18 | 24 | 54 |
| Soga <i>et al.</i> ^[82] | SCC | 4 | | | | 2 | | | | 3 | | | | | | | | 2 |
| Tomita <i>et al.</i> ^[83] | SCC | 89 | 11 | 10 | 68 | 51 | 5 | 4 | 42 | 32 | | | | 44 | | | | 7 |
| Tsutsui <i>et al.</i> ^[84] | SCC | 38 | | | | 8 | | | | | | | | 17 | | | | |
| Westertep <i>et al.</i> ^[85] | ADC | 66 | 25 | 23 | 18 | 18 | 0 | 6 | 12 | | | | | | | | | 59 |
| Yoshikane <i>et al.</i> ^[86] | SCC | 17 | | | | 12 | | | | 11 | | | | 4 | | | | 1 |
| Sepesi <i>et al.</i> ^[80] | ADC | 29 | 14 | 11 | 4 | 9 | 3 | 4 | 2 | | | | | | | | | |
| Leers <i>et al.</i> ^[70] | ADC | 51 | 19 | 9 | 23 | 11 | 4 | 1 | 6 | | | | | | | | | |

SCC: Squamous cell carcinoma; ADC: Adenocarcinoma; L+: Lymphovascular invasion; V+: Microvascular invasion; sm: Submucosal layer.

The present meta-analysis: (1) investigated the particular role of each of the two endoscopic modalities in treating early esophageal cancer; (2) analyzed the issue of local recurrence and metachronous cancer development in patients treated endoscopically; and (3) analyzed for potential unfavorable tumor characteristics (besides those found by

Table 5 Meta-regression analysis of histologic parameters between adenocarcinoma and squamous cell carcinoma patients according to the published studies (the random effects model was used)

| Comparison of ADC <i>vs</i> SCC | Coefficient | 95%CI | P value | Better status |
|---------------------------------|-------------|----------------------|---------|---------------|
| Positive lymph nodes | 1.890 | 0.3945146, 3.384624 | < 0.01 | ADC |
| Lymphovascular invasion | 0.626 | -0.7032339, 1.956155 | 0.340 | None |
| Microvascular invasion | 1.114 | -0.2682334, 2.496538 | 0.108 | None |
| Grade 3 | 0.305 | -1.584654, 2.195142 | 0.731 | None |

SCC: Squamous cell carcinoma; ADC: Adenocarcinoma.

Table 6 Number of patients with lymph node metastasis and lymphatic and vascular invasion according to the depth of tumor in the submucosal layer

| Patients with diseases | | | | | | | | |
|---|--------------|-----------------------|------------|-----------------------|-------------|-----------------------|-------------|--|
| Lymph node metastasis | | | | | | | | |
| sm (38 studies: <i>n</i> = 2149) ¹ | | sm1 (<i>n</i> = 308) | | sm2 (<i>n</i> = 349) | | sm3 (<i>n</i> = 624) | | |
| SCC | ADC | SCC | ADC | SCC | ADC | SCC | ADC | |
| 793/1779 (45%) | 95/370 (26%) | 60/224 (27%) | 8/84 (10%) | 107/296 (36%) | 11/53 (21%) | 301/544 (55%) | 39/80 (49%) | |
| Lymphovascular invasion | | | | | | | | |
| sm (<i>n</i> = 1286) ¹ | | sm1 (<i>n</i> = 134) | | sm2 (<i>n</i> = 150) | | sm3 (<i>n</i> = 209) | | |
| 627/1090 (56%) | 76/196 (39%) | 58/111 (52%) | 2/23 (9%) | 88/135 (65%) | 4/15 (27%) | 118/184 (64%) | 19/25 (76%) | |
| Microvascular invasion | | | | | | | | |
| sm (<i>n</i> = 1194) ¹ | | sm1 (<i>n</i> = 104) | | sm2 (<i>n</i> = 185) | | sm3 (<i>n</i> = 251) | | |
| 468/1161 (40%) | 6/33 (18%) | 19/97 (20%) | 1/7 (14%) | 67/183 (37%) | 0/2 (0%) | 114/239 (48%) | 0/12 (0%) | |

¹Total numbers of patients differ since not all studies provide relevant information. sm: Submucosal layer; SCC: Squamous cell carcinoma; ADC: Adenocarcinoma.

imaging) that obviate the need for neoadjuvant or peri-operative therapy. To our knowledge, level I evidence related to these issues is missing from the literature. The only published meta-analysis based on retrospective studies (seven full-text and eight abstracts) compares EMR *vs* ESD for esophageal, gastric, and colorectal neoplasms jointly^[87].

In addition to a variety of local ablation techniques, EMR and ESD are now extensively used for the treatment of stage Tis (high-grade dysplasia) and T1a ADC or SCC, aiming to reduce the considerable morbidity and mortality associated with esophagectomy.

The possibility of lymph node metastases, completeness of endoscopic resectability, early and late complications, local recurrence and development of a metachronous cancer, are concerns that should be measured when deciding whether to proceed with EMR, ESD or surgery.

According to our pooled analysis there were no significant differences between EMR and ESD for the following parameters: procedural complications, number of patients submitted to surgery, positive specimen margins, lymph node positivity, local recurrence rates and metachronous cancer development. In instances of piecemeal tumor resection, in particular, ESD performed better since the number of cases was significantly less ($P < 0.001$); hence, local recurrence rates were significantly lower ($P < 0.01$). An important point that should be kept in mind is the higher rate of esophageal stenosis observed following ESD ($P < 0.001$). Data on circumferential spread and tumor size were scarce among the studies.

There were no considerable differences in the appli-

cation of endoscopic methods to each of the main histologic types of early esophageal cancer, other than the fact that a significantly greater number of SCC patients were submitted for surgery ($P < 0.05$).

Another significant finding was the high percentage of patient restaging after endoscopic intervention. EUS staging prior to proceeding with mucosal resection in the setting of carcinoma is recommended. In a recent meta-analysis^[7], the pooled sensitivity (95%CI) and specificity (95%CI) for regional lymph node metastases was 0.764 (0.741-0.785) and 0.724 (0.693-0.753), respectively. The pooled diagnostic odds ratio (95%CI) was 8.001 (6.369-10.051). Although EUS has a better diagnostic performance compared to computed tomography (CT) scanning and positron emission tomography CT, the question of regional lymph node detection has yet to be satisfactorily addressed.

With regard to preoperative staging, endoscopic resection after endoscopic biopsy plays a key role. The odds for re-classification of tumor stage after endoscopic resection were 53% and 39% for ADC and SCC, respectively. This was possibly due to biopsy sampling failure, lack of adequate specimen and pathologist misinterpretation of the muscular anatomy. This obviates the need to optimize pre-treatment diagnostics and reconsider treatment strategies. The introduction of endoscopic resection in selected cases as part of the diagnostic workup should be strongly taken into consideration. This particular issue is supported by our data mining analysis: local tumor recurrence was best predicted by grade 3 differentiation and piecemeal resection, metachronous cancer development by the car-

cinoma *in situ* component and lymph node positivity by lymphovascular invasion. All the aforementioned predictors/histologic features can easily be retrieved from the EMR/ESD sample.

However, ESD is a technically demanding procedure that is not widely available. Although we were not able to perform a direct comparison of the outcomes of ESD *vs* surgery due to lack of relevant data, the likelihood of lymph node metastases and endoscopic resectability being factors that should be considered in deciding whether to pursue ESD or surgery is high, as stated by some authors^[17]. According to our results, the presence of grade 3, piecemeal resection, the carcinoma *in situ* component and lymphovascular invasion would prompt surgical resection.

Available evidence from our esophagectomy series with radical lymph node dissection database suggests that the frequency of lymph node metastasis increases in proportion with tumor depth.

The diagnostic performance of sentinel lymph node biopsy for esophageal and gastric cardia cancer provides sensitivity between 75%-100% and accuracy between 78%-100%. Albeit applied in only a small number of patients, CT-lymphography seems to be the most promising method^[7].

Considering the low incidence of lymph node metastasis (the odds are 5% for ADC and approximately 1% for SCC) and the absence of lymphovascular invasion in neoplasms limited to the mucosa, endoscopic resection is oncologically adequate for well-differentiated cancers, resected completely and lacking *in situ* foci. With regard to Barrett's patients in particular, close endoscopic surveillance should be life-long and requires the commitment of both the patient and the physician since according to our results, the odds for lymph node metastasis are 5% and for metachronous cancer development 6%.

When endoscopic therapy for early esophageal cancer is considered, EMR or ESD should be applied first prior to the use of ablative techniques. According to our analysis, the application of ablative techniques has not gained significance as an independent predictor of local recurrence or metachronous cancer development.

Considering studies including surgically resected patients, lymph node positivity was statistically greater in SCC, while lymphovascular and microvascular invasion and grade 3 percentages were comparable between ADC and SCC patients. In rank order of importance, the predictors of lymph node metastasis in the prediction model were: Grade 3, Sm3 invasion, lymphovascular invasion, microvascular invasion, Sm2 invasion and Sm1 invasion, respectively. The best predictors of lymph node positivity in SCC were Sm3 invasion and microvascular invasion. For ADC, the most important predictor was lymphovascular invasion. According to the above, the present study supports the surgical rather than the endoscopic resection of T1b esophageal cancer, since even Sm1 invasion was included in our model. In consequence, Sm1 lesions should not be removed endoscopically. Interestingly, the presence of specific histologic features should prompt

consideration of a more aggressive management, such as the use of neoadjuvant or perioperative treatment. This perception also poses the question as to the endorsement of EMR/ESD as part of the diagnostic workup.

Since there is a lack of apposite molecular-biological markers that can predict lymphatic spread in T1a and T1b-esophageal carcinoma with high diagnostic yield and the inconsistent success of the diagnostic work-up, the predictors found in our data mining analysis would possibly be of relevance in clinical decision making.

The analysis of surgically only resected patients is an updated version of an already published study by our group^[88]. Although more studies have been included, the results were identical.

The current work is not without its limitations: (1) The report included studies of retrospective case series; thus, a formal meta-analysis could not be applied; (2) Parameters, such as dysplasia grade, segment length of Barrett's and small areas of intestinal metaplasia hidden underneath neosquamous mucosa, the so-called "buried Barrett's", could not be analyzed due to paucity of data; (3) Overall patient survival and disease-free survival could not be assessed due to data inconsistency; (4) the type of resection (*en-bloc*, transhiatal, Ivor Lewis, minimally invasive) and differences according to the location of the tumor, with regard to lymph node, L and V invasion, may have influenced, to a degree, the prevalence of node positivity; and (5) in some studies, the stratification of data for distribution of the lymphovascular involvement according to the depth of tumor infiltration, and similar stratification for nodal involvement (m1, m2, m3, sm1, sm2 and sm3), were not available.

The value of patient data mining has already been established by The Medical Quality Improvement Consortium^[89]. This large clinical data warehouse contains patient data including their problem lists, test results, procedures and medication lists, all of which help identify valid associations.

Currently, the National Comprehensive Cancer Network recommends an esophagectomy over endoscopic therapy for fit patients with T1b cancer. This study suggests the option of neoadjuvant treatment for those patients with unfavorable histological characteristics in terms of tumor histologic entity, aiming at a R0 resection.

In summary, according to this study, there were no significant differences between EMR and ESD concerning procedural complications, number of patients submitted to surgery, positive specimen margins, lymph node positivity, local recurrence rates and metachronous cancer development. In instances of a predicted piecemeal tumor resection, ESD performed better since the number of cases was significantly less and local recurrence rates were therefore significantly lower. A higher rate of esophageal stenosis was observed following ESD.

Local tumor recurrence after endoscopic resection was best predicted by grade 3 differentiation, metachronous cancer development by the carcinoma *in situ* component, and lymph node positivity by lymphovascular

invasion.

T1b esophageal cancer should be managed with surgical resection and systematic lymphadenectomy since even Sm1 invasion was in the constructed model, while the histologic type and presence of specific predictors could likely alter the surgeon's policy and perspective of multimodality management. The best predictors of lymph node positivity in SCC were Sm3 invasion and microvascular invasion. For ADC, the most important predictor was lymphovascular invasion. Prospective studies, or preferably randomized controlled trials, are needed in order to validate the refinements for patient selection made by this study.

COMMENTS

Background

Endoscopic mucosal resection (EMR) and endoscopic submucosal dissection (ESD) are frequently used to treat early esophageal cancer. Esophagectomy remains the standard of treatment especially in submucosal invasion. However, there is controversy between surgeons and endoscopists as to which is the best treatment option. The literature lacks a satisfactory level of evidence with respect to T1a and T1b esophageal cancer management.

Research frontiers

The present meta-analysis: (1) Investigated the particular role of each of the two endoscopic modalities in the treatment of early esophageal cancer; (2) Analyzed the issue of local recurrence and metachronous cancer development in patients treated endoscopically; and (3) Analyzed for potential tumor lymph node positivity.

Innovations and breakthroughs

Level I evidence related to the endoscopic management of early esophageal cancer is missing from the literature. The only published meta-analysis based on retrospective studies (seven full-text and eight abstracts) compares EMR vs ESD for esophageal, gastric, and colorectal neoplasms jointly.

Applications

Potential unfavorable tumor characteristics as documented in this systematic review and meta-analysis (besides those found by imaging) may obviate the need for neoadjuvant or perioperative therapy.

Terminology

Meta-regression is a tool used in meta-analysis to examine the impact of moderator variables on study effect size using regression-based techniques. Meta-regression is more effective at this task than standard regression techniques. The random or mixed effects model allows for within study variation and between study variation and is therefore the most appropriate model to choose. A neural network is a system of programs and data structures that approximates the operation of the human brain. A neural network generally involves a large number of processors operating in parallel, each with its own small sphere of knowledge and access to data in its local memory. Typically, a neural network is initially "trained" or fed large amounts of data and rules about data relationships.

Peer review

The authors reviewed endoscopic and surgical resection of superficial esophageal neoplasms. The review was well conducted in the topic is very interesting in order to identify selection of treatment implicated in the superficial esophageal cancer.

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CD24 expression predicts distant metastasis in extrahepatic bile duct cancer

Kyubo Kim, Hye Sook Min, Eui Kyu Chie, Jin-Young Jang, Sun Whe Kim, Sae-Won Han, Do-Youn Oh, Seock-Ah Im, Tae-You Kim, Yung-Jue Bang, Ja-June Jang, Sung W Ha

Kyubo Kim, Eui Kyu Chie, Sung W Ha, Department of Radiation Oncology, Seoul National University College of Medicine, Seoul 110-744, South Korea

Hye Sook Min, Ja-June Jang, Department of Pathology, Seoul National University College of Medicine, Seoul 110-744, South Korea

Jin-Young Jang, Sun Whe Kim, Department of Surgery, Seoul National University College of Medicine, Seoul 110-744, South Korea

Sae-Won Han, Do-Youn Oh, Seock-Ah Im, Tae-You Kim, Yung-Jue Bang, Department of Internal Medicine, Seoul National University College of Medicine, Seoul 110-744, South Korea

Eui Kyu Chie, Sung W Ha, Institute of Radiation Medicine, Medical Research Center, Seoul National University, Seoul 151-742, South Korea

Author contributions: Kim K and Min HS contributed equally to this work; Kim K, Min HS and Chie EK designed the research; Kim K, Min HS, Chie EK, Jang JY, Kim SW, Han SW, Oh DY, Im SA, Kim TY, Bang YJ, Jang JJ and Ha SW performed the research; Kim K analyzed the data; and Kim K, Min HS and Chie EK wrote the paper.

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Correspondence to: Eui Kyu Chie, MD, Department of Radiation Oncology, Seoul National University College of Medicine, 101 Daehak-ro, Jongno-gu, Seoul 110-744, South Korea. ekchie93@snu.ac.kr

Telephone: +82-2-20723705 Fax: +82-2-7653317

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Abstract

AIM: To evaluate the prognostic significance of CD24 expression in patients undergoing adjuvant chemoradiotherapy for extrahepatic bile duct (EHBD) cancer.

METHODS: Eighty-four patients with EHBD cancer who underwent curative resection followed by adjuvant chemoradiotherapy were enrolled in this study. Postoperative radiotherapy was delivered to the tumor bed and regional lymph nodes up to a median of 40

Gy (range: 40-56 Gy). All patients also received fluoropyrimidine chemotherapy for radiosensitization during radiotherapy. CD24 expression was assessed with immunohistochemical staining on tissue microarray. Clinicopathologic factors as well as CD24 expression were evaluated in multivariate analysis for clinical outcomes including loco-regional recurrence, distant metastasis-free and overall survival.

RESULTS: CD24 was expressed in 36 patients (42.9%). CD24 expression was associated with distant metastasis, but not with loco-regional recurrence nor with overall survival. The 5-year distant metastasis-free survival rates were 55.1% and 29.0% in patients with negative and positive expression, respectively ($P = 0.0100$). On multivariate analysis incorporating N stage, histologic differentiation and CD24 expression, N stage was the only significant factor predicting distant metastasis-free survival ($P = 0.0089$), while CD24 expression had borderline significance ($P = 0.0733$). In subgroup analysis, CD24 expression was significantly associated with 5-year distant metastasis-free survival in node-positive patients (38.4% with negative expression *vs* 0% with positive expression, $P = 0.0110$), but not in node-negative patients (62.0% with negative expression *vs* 64.0% with positive expression, $P = 0.8599$).

CONCLUSION: CD24 expression was a significant predictor of distant metastasis for patients undergoing curative resection followed by adjuvant chemoradiotherapy especially for node-positive EHBD cancer.

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Key words: CD24; Tissue microarray; Extrahepatic bile duct cancer; Adjuvant chemoradiotherapy; Distant metastasis

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INTRODUCTION

Extrahepatic bile duct (EHBD) cancer is a rare malignancy with a poor prognosis^[1]. Surgical resection is considered as the only curative modality in the management of EHBD cancer, but a significant number of patients have loco-regional recurrences after curative resection alone^[2-4]. Therefore, adjuvant radiotherapy with or without chemotherapy has been used for these patients, resulting in the improved loco-regional control and survival^[5-9]. As a result of the increased loco-regional control, however, the distant metastasis rate has increased^[5,6,10]. In our previous report, only 11.6% of patients had isolated loco-regional recurrences, whereas 44.2% of patients had distant metastases with or without loco-regional failure^[10]. Likewise, further technical advances in the delivery of radiotherapy may further increase the proportion of distant metastasis^[11].

Recently, metastasis-associated protein CD24 has been reported to be associated with shorter survival in various malignancies^[12-20]. As for bile duct cancer, Agrawal *et al*^[21] noted that CD24 expression was predictive of poor survival and possibly poor response to chemotherapy or radiotherapy. However, the patient number was small and the treatment details were heterogeneous.

In this study, we evaluated CD24 expression as a potential prognostic factor for predicting distant metastasis in patients undergoing curative surgery followed by adjuvant chemoradiotherapy for EHBD cancer.

MATERIALS AND METHODS

Study population

Between January 2000 and August 2006, 108 patients underwent adjuvant radiotherapy after curative resection for EHBD cancer. Of these, 6 patients who did not receive concomitant chemotherapy and 18 patients whose paraffin-embedded tissue was unavailable were excluded from the analysis. Therefore, 84 patients were the subjects of this study. This study was approved by Institutional Review Board, and all patients gave informed consent prior to treatment.

Adjuvant chemoradiotherapy

All patients underwent adjuvant chemoradiotherapy. In 69 patients, a total dose of 40 Gy was delivered using 2 Gy/fraction, 5 d/wk with 2 wk of planned rest after 20 Gy. Concomitant 5-fluorouracil (5-FU, 500 mg/m² per day *iv* bolus) was administered for the first 3 d of each 2 wk course of radiotherapy. Fifteen patients received a continuous course of radiotherapy, and the total dose ranged from 50 to 56 Gy in conventional fractionation. Of 15 patients, 13 patients received concomitant 5-FU

(500 mg/m² per day *iv* bolus for 3 d) on weeks 1 and 5 of radiotherapy. Capecitabine was prescribed for the 2 remaining patients during radiotherapy.

Fluoropyrimidine-based maintenance chemotherapy was administered to 68 patients after the completion of concurrent chemoradiotherapy. The scheduled duration of maintenance chemotherapy was 6-12 mo.

Tissue microarray and immunohistochemistry

All 84 cases diagnosed as adenocarcinoma of EHBD were retrieved from the archives in Seoul National University Hospital, which contained enough paraffin-embedded tissue for the study. All the hematoxylin and eosin-stained slides were reviewed and confirmed as adenocarcinoma. Representative paraffin blocks were selected and the tissue microarray of 4 mm core was produced. Immunohistochemical staining was done on all 84 cases using antibody to CD24 (clone SN3b, Thermo Scientific, Fremont, CA, United States; 1:200) automatically, according to the manufacturer's protocol based on the conventional streptavidin-biotin-peroxidase method. For statistical analysis, CD24 expression was scored in 4 tiers: 0, no staining; 1, staining in less than 20% of the cells; 2, staining in 20%-50% of the cells; and 3, staining in more than 50% of the cells.

Statistical analysis

Survival was calculated from the date of surgical resection. Statistical analysis was performed using SPSS software (release 12.0.1. SPSS Inc. Chicago, IL, United States). Differences in categorical variables between the parameters were compared with the standard χ^2 test or Fisher's exact test. The actuarial survival rates were calculated using the Kaplan-Meier method, and statistical significance between the actuarial survival rates was evaluated by the log-rank test. The Cox proportional hazard model was used for multivariate analysis.

RESULTS

Patient characteristics

There were 62 males and 22 females. The median age of all patients was 62 years (range: 36-86 years). Sixty-two patients had no residuum (R0), whereas 22 patients had microscopic residual disease (R1). As for the location of the tumor, 59 patients had hilar or proximal tumors, and 22 patients had intrapancreatic (distal) tumors. Three patients had tumors extending from the proximal to the distal EHBD. Stage was determined according to the American Joint Committee on Cancer staging system, 6th edition^[22]. For T classification, 3 patients had T1, 30 patients had T2, 40 patients had T3, and 11 patients had T4 disease. Thirty-three patients had lymph node involvement, whereas 48 patients did not. Lymph node dissection was not performed in 3 patients. The degree of histologic differentiation was as follows; well differentiated in 15, moderately differentiated in 59, and poorly differentiated in 8 patients. Histologic differentiation in-

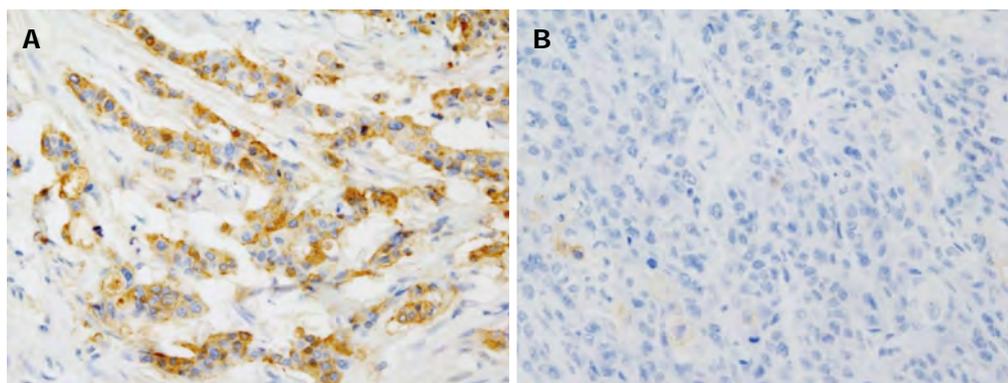


Figure 1 Immunohistochemical staining of CD24. A: Positive in the cytoplasm; B: Negative. Original magnification $\times 400$.

Table 1 Clinicopathologic variables according to expression of CD24

| Variables | Patients | | P value |
|---|----------------------|----------------------|---------|
| | CD24 (-) (n = 48) | CD24 (+) (n = 36) | |
| Age | | | |
| ≤ 60 yr | 21 | 16 | 0.949 |
| > 60 yr | 27 | 20 | |
| Residual disease | | | |
| R0 | 37 | 25 | 0.431 |
| R1 | 11 | 11 | |
| Tumor location ¹ | | | |
| Proximal | 30 | 29 | 0.077 |
| Distal | 16 | 6 | |
| T stage | | | |
| T1-2 | 19 | 14 | 0.949 |
| T3-4 | 29 | 22 | |
| N stage ² | | | |
| N0 | 33 | 15 | 0.036 |
| N1 | 15 | 18 | |
| Histologic differentiation ³ | | | |
| W/D | 13 | 2 | 0.041 |
| M/D | 30 | 29 | |
| P/D | 4 | 4 | |
| Use of maintenance chemotherapy | | | |
| No | 9 | 7 | 0.936 |
| Yes | 39 | 29 | |

¹3 patients with tumor extending from the proximal to distal bile duct were excluded; ²In 3 patients, lymph node dissection was not performed; ³In 2 patients, the information on histologic differentiation was unavailable. R0: No microscopic residual disease; R1: Microscopic residual disease; W/D: Well differentiated; M/D: Moderately differentiated; P/D: Poorly differentiated.

formation was missing for 2 patients.

CD24 immunohistochemical staining

CD24 was expressed in 36 patients (42.9%) and not in 48 patients (score = 0, 57.1%) (Figure 1). The percentage of positive cells was variable and the staining intensity was more than intermediate in most cases. Twenty-nine cases expressed CD24 in less than 20% of the tumor cells (score = 1, 80.6%), 6 cases expressed in 20%-50% of the tumor cells (score = 2, 16.7%) and only one case was diffusely

positive for CD24 (score = 3, 2.8%).

Correlation between CD24 expression and other variables

Patients with CD24 expression were more likely to have node-positive tumors ($P = 0.0360$), and less likely to have well differentiated tumors ($P = 0.0405$). Also, patients with CD24 expression tended to have proximal tumors, but the correlation was statistically marginal ($P = 0.0770$, Table 1). As for a treatment-related factor, there was no association between CD24 expression and the use of maintenance chemotherapy.

Prognostic factors

CD24 expression had a significant impact on the 5-year distant metastasis-free survival (29.0% with positive expression *vs* 55.1% with negative expression, $P = 0.0100$, Figure 2A), but not on loco-regional recurrence-free or overall survival. N stage and histologic differentiation were also significantly correlated with distant metastasis-free survival ($P = 0.0003$ and 0.0394 , respectively), while tumor location had marginal significance ($P = 0.0623$, Table 2). On multivariate analysis incorporating N stage, histologic differentiation, and CD24 expression, N stage was the only significant prognostic factor predicting distant metastasis-free survival ($P = 0.0089$, Figure 2B), while CD24 expression had borderline significance ($P = 0.0733$). When the use of maintenance chemotherapy was added in this model, the statistical significance of N stage and CD24 expression was similarly maintained ($P = 0.0100$ and 0.0683 , respectively).

In the subgroup analysis according to nodal involvement, CD24 expression was significantly associated with 5-year distant metastasis-free survival in node-positive patients (38.4% with negative expression *vs* 0% with positive expression, $P = 0.0110$), but not in node-negative patients (62.0% with negative expression *vs* 64.0% with positive expression, $P = 0.8599$, Figure 2C).

As for loco-regional relapse-free survival, residual disease status was the only significant prognostic factor on univariate analysis ($P = 0.0356$). Tumor location ($P =$

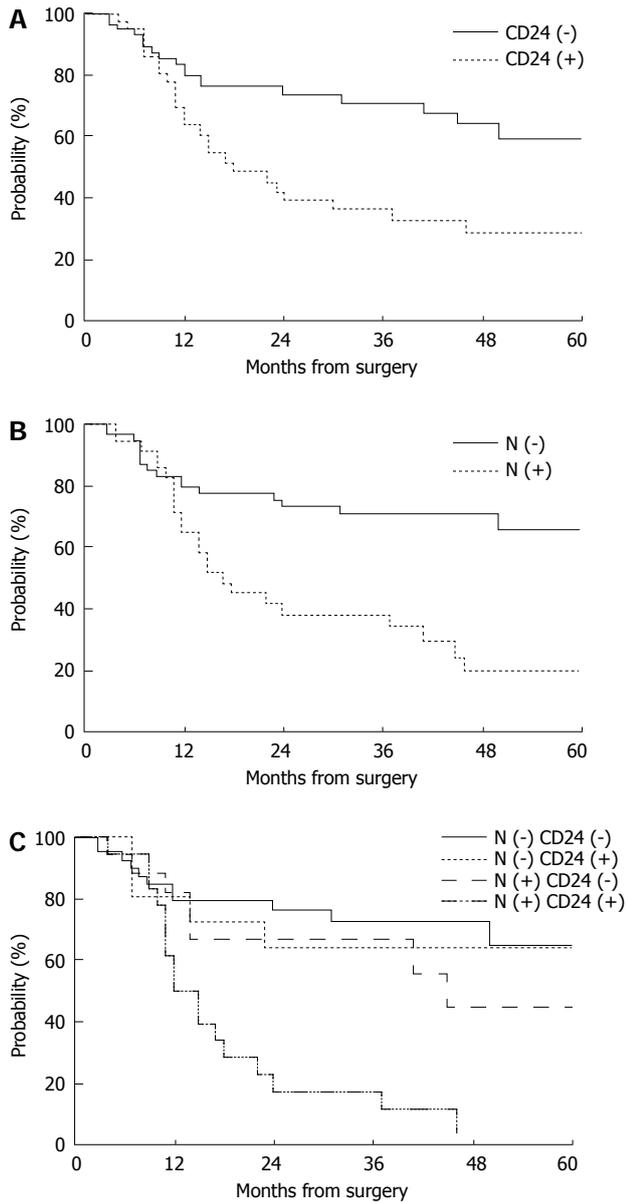


Figure 2 Distant metastasis-free survival curves. A: CD24 expression; B: Nodal involvement; C: Nodal involvement and CD24 expression.

0.0288) and N stage ($P = 0.0514$) were correlated with overall survival on univariate analysis.

DISCUSSION

This study indicates that CD24 expression is associated with poorer distant metastasis-free survival in patients undergoing curative surgery followed by adjuvant chemoradiotherapy for node-positive EHBD cancer.

CD24 is reported to be expressed in various malignancies including tumors of the ovary, lung, breast, *etc.* Because CD24 is known to be a ligand for P-selectin, which is expressed on platelets and endothelial cells, CD24-positive tumor cells can attach more easily to activated platelets and endothelial cells, and are involved in cell adhesion and metastatic tumor spread. The prognos-

Table 2 Univariate analysis for loco-regional relapse-free, distant metastasis-free and overall survival

| Variables | n | 5-yr LRRFS | P value | 5-yr DMFS | P value | 5-yr OS | P value |
|---|----|------------|---------|-----------|---------|---------|---------|
| Age | | | | | | | |
| ≤ 60 yr | 37 | 62.1% | 0.3615 | 53.6% | 0.6837 | 35.1% | 0.3495 |
| > 60 yr | 47 | 63.0% | | 37.9% | | 51.4% | |
| Residual disease | | | | | | | |
| R0 | 62 | 76.3% | 0.0356 | 49.6% | 0.7748 | 53.1% | 0.2421 |
| R1 | 22 | 33.4% | | 25.6% | | 27.3% | |
| Tumor location ¹ | | | | | | | |
| Proximal | 59 | 54.1% | 0.1236 | 34.8% | 0.0623 | 38.7% | 0.0288 |
| Distal | 22 | 79.9% | | 67.0% | | 59.7% | |
| T stage | | | | | | | |
| T1-2 | 33 | 59.2% | 0.6311 | 48.3% | 0.4501 | 49.4% | 0.9502 |
| T3-4 | 51 | 64.3% | | 40.4% | | 42.2% | |
| N stage ² | | | | | | | |
| N0 | 48 | 67.5% | 0.7606 | 64.2% | 0.0003 | 51.7% | 0.0514 |
| N1 | 33 | 54.4% | | 15.8% | | 30.8% | |
| Histologic differentiation ³ | | | | | | | |
| W/D | 15 | 69.8% | 0.2003 | 66.0% | 0.0394 | 35.0% | 0.3538 |
| M/D | 59 | 60.7% | | 41.4% | | 50.2% | |
| P/D | 8 | 36.5% | | 0% | | 25.0% | |
| Use of maintenance chemotherapy | | | | | | | |
| No | 16 | 59.5% | 0.5444 | 35.2% | 0.1976 | 33.7% | 0.1435 |
| Yes | 68 | 62.7% | | 45.2% | | 47.3% | |
| CD24 expression | | | | | | | |
| Negative | 48 | 68.9% | 0.3519 | 55.1% | 0.0100 | 50.3% | 0.1873 |
| Positive | 36 | 49.9% | | 29.0% | | 37.2% | |

¹3 patients with tumor extending from the proximal to distal bile duct were excluded; ²In 3 patients, lymph node dissection was not performed; ³In 2 patients, the information on histologic differentiation was unavailable. LRRFS: Loco-regional relapse-free survival; DMFS: Distant metastasis-free survival; OS: Overall survival; R0: No microscopic residual disease; R1: Microscopic residual disease; W/D: Well differentiated; M/D: Moderately differentiated; P/D: Poorly differentiated.

tic value of CD24 has also been evaluated in the aforementioned tumors, and CD24 expression was demonstrated to be associated with poor survival^[12-20].

As for cholangiocarcinoma, Riener *et al.*^[23] reported that CD24 expression was observed in 21% of intrahepatic cholangiocarcinoma, 58% of extrahepatic cholangiocarcinoma, and 42% of gallbladder carcinoma. Regarding the prognostic value of CD24, Agrawal *et al.*^[21] evaluated CD24 expression in 22 patients with cholangiocarcinoma by immunohistochemical staining, but information on the tumor, such as location, was not described in detail. CD24 was expressed in 81.8% of patients, and the median survival times of patients with low and high CD24 expression were 36 and 8 mo, respectively ($P = 0.02$). They also tried to correlate CD24 expression with the response to chemotherapy or radiotherapy. Better survival was observed in patients with low CD24 expression in the subgroup analyses, which included patients treated with either chemotherapy or radiotherapy. However, the patient number was small, and the treatment details were heterogeneous. Moreover, the patterns of failure were not given, and therefore the prognostic value of CD24 as a metastasis-associated protein could not be fully evaluated. Su *et al.*^[24] also reported that CD24

was expressed in 36 of 70 patients (51%) with resected intrahepatic cholangiocarcinoma, and that the median survival times of patients with CD24 positive and negative tumors were 8.1 and 17.2 mo, respectively ($P = 0.028$). However, the association between CD24 expression and distant metastasis was also not reported in their study.

In the current study, CD24 expression was observed in 42.9% of patients, and the 5-year distant metastasis-free survival rate was significantly inferior in patients with CD24 positive tumors. CD24 expression was correlated with nodal involvement and histologic differentiation, all of which are known to be predictive of distant metastasis^[10,25]. On multivariate analysis incorporating these risk factors as well as CD24 expression, however, CD24 expression still showed a borderline significance. Keeratichamroen *et al*^[26] observed similar findings in 34 patients with resected cholangiocarcinoma, that is, less nodal involvement and more well differentiated tumors in patients with low CD24 expression. Multivariate analysis in the aforementioned study showed that CD24 expression was the only independent risk factor for survival. However, details on adjuvant treatment were unavailable. In the present study, CD24 expression was not associated with maintenance chemotherapy, and moreover, the statistical significance of CD24 expression remained even after the use of maintenance chemotherapy was added in the multivariate analysis. Therefore, the correlation between CD24 expression and distant metastasis was not confounded by maintenance chemotherapy.

Several strengths of our study are the relatively large sample size including only EHBD cancer patients ($n = 84$), the relatively homogeneous treatment (curative resection followed by adjuvant chemoradiotherapy), and the relevant endpoint (distant metastasis). As previously mentioned, the reported proportion of distant metastasis was increased in resected EHBD cancer as the result of increased loco-regional control with the use of adjuvant chemoradiotherapy^[5,6,10]. In addition to nodal involvement, CD24 expression can be used as a relevant predictor of distant metastasis in these populations. A distinct finding of our study is that the prognostic significance of CD24 was limited to those patients with nodal involvement. There was no difference in 5-year distant metastasis-free survival between CD24 positive and negative expression in node-negative patients. Therefore, those patients with higher risk of distant metastasis, that is, nodal involvement and CD24 expression, should be considered as potential candidates for more intensive systemic therapy.

However, due to the retrospective nature of our study, conclusions drawn from this study are limited and need further validation through another patient cohort, and possibly, through a prospective trial. In addition, it is unknown why the prognostic significance of CD24 was limited to those patients with nodal involvement. Further studies are needed to confirm and elucidate this observation.

In conclusion, CD24 expression was a relevant predictor of distant metastasis in patients undergoing curative resection followed by adjuvant chemoradiotherapy

for node-positive EHBD cancer. CD24 expression may be used as an additional index for selecting patients with higher risk of distant metastasis in future trials.

COMMENTS

Background

Extrahepatic bile duct (EHBD) cancer is a rare malignancy with a poor prognosis. The major pattern of failure after surgical resection has been shifted from loco-regional recurrence to distant metastasis as a result of increased loco-regional control with the use of adjuvant chemoradiotherapy. Given these observations, a prognostic factor predicting distant metastasis needs to be evaluated to select patients who need more intensive systemic therapy.

Research frontiers

There are a number of studies demonstrating that CD24 was associated with shorter survival in various malignancies, but only a few for EHBD cancer. In the current study, the prognostic significance of CD24 expression in EHBD cancer patients who underwent adjuvant chemoradiotherapy after curative resection was evaluated.

Innovations and breakthroughs

There were a few reports on the prognostic value of CD24 in patients with cholangiocarcinoma. However, patient numbers were small and tumor location and treatment details were heterogeneous. Moreover, the patterns of failure were not given, and therefore the prognostic value of CD24 as a metastasis-associated protein could not be fully evaluated. Several strengths of the present study are the relatively large sample size including only EHBD cancer patients, relatively homogeneous treatment, and a relevant endpoint, that is, distant metastasis. The authors found that CD24 expression was associated with poorer distant metastasis-free survival in node-positive EHBD cancer patients who underwent adjuvant chemoradiotherapy after curative resection.

Applications

From these results, CD24 expression along with nodal involvement can be used as a relevant predictor of distant metastasis and as a potential indicator for more intensive systemic therapy.

Terminology

CD24, metastasis-associated protein, is reported to be expressed in various malignancies including tumors of ovary, lung and breast. Because CD24 is known to be a ligand for P-selectin, which is expressed on platelets and endothelial cells, CD24-positive tumor cells can attach more easily to activated platelets and endothelial cells and are involved in cell adhesion and metastatic tumor spread.

Peer review

The topic and the results are interesting, well presented and of great clinical importance. The number of involved patients is also notable considering the observed type of cancer. The main conclusion of the study, namely that the timorous CD24 expression is a useful predictor of distant metastasis in patients undergoing curative resection followed by adjuvant chemoradiotherapy, especially for node-positive cases, is important both clinically and therapeutically, as CD24 immunohistochemistry (IHC) may provide a basis for patient selection for perioperative bile duct cancer treatment. The methodology is clear, tissue microarray analysis scores are relevant, and the IHC figures are representative.

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Effect of thienorphine on intestinal transit and isolated guinea-pig ileum contraction

Pei-Lan Zhou, Yu-Lei Li, Ling-Di Yan, Zheng Yong, Gang Yu, Hua-Jin Dong, Hui Yan, Rui-Bin Su, Ze-Hui Gong

Pei-Lan Zhou, Yu-Lei Li, Ling-Di Yan, Zheng Yong, Gang Yu, Hua-Jin Dong, Hui Yan, Rui-Bin Su, Ze-Hui Gong, Beijing Institute of Pharmacology and Toxicology, Beijing 100850, China
Author contributions: Zhou PL and Li YL performed the majority of experiments; Yan LD and Yong Z provided vital reagents and analytical tools; Yu G, Dong HJ and Yan H conducted the isolated guinea-pig ileum experiment; Su RB and Gong ZH designed the study; Zhou PL and Su RB wrote the manuscript.
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Correspondence to: Ze-Hui Gong, Professor, Beijing Institute of Pharmacology and Toxicology, No. 27, Taiping Road, Beijing 100850, China. gongzeh@yahoo.com.cn

Telephone: +86-10-66931620 Fax: +86-10-68211656

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Abstract

AIM: To evaluate the effect of thienorphine on small intestinal transit *in vivo* and on guinea-pig ileum (GPI) contraction *in vitro*.

METHODS: The effects of thienorphine on intestinal transit were examined in mice and in isolated GPI. Buprenorphine and morphine served as controls. The distance traveled by the head of the charchol and the total length of the intestine were measured *in vivo*. Gastrointestinal transit was expressed as a percentage of the distance traveled by the head of the marker relative to the total length of the small intestine. The isolated GPI preparations were connected to an isotonic force transducer and equilibrated for at least 1 h before exposure to drugs. Acetylcholine was used for muscle stimulation.

RESULTS: Thienorphine (0.005-1.0 mg/kg, *ig*) or bu-

prenorphine (0.005-1.0 mg/kg, *sc*) dose-dependently significantly inhibited gut transit compared with saline. Thienorphine inhibited gut transit less than buprenorphine. The maximum inhibition by thienorphine on the intestinal transit was 50%-60%, whereas the maximum inhibition by morphine on gut transit was about 100%. Thienorphine also exhibited less inhibition on acetylcholine-induced contraction of GPI, with a maximum inhibition of 65%, compared with 93% inhibition by buprenorphine and 100% inhibition by morphine. Thienorphine induced a concentration-dependent decrease in the basal tonus of spontaneous movement of the GPI, the effect of which was weaker than that with buprenorphine. The duration of the effect of thienorphine on the GPI was longer than that with buprenorphine.

CONCLUSION: Thienorphine had less influence, but a longer duration of action on GPI contraction and moderately inhibited intestinal transit.

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Key words: Thienorphine; Buprenorphine; Guinea-pig ileum; Gut transit; Contraction

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INTRODUCTION

Opioids have a wide range of effects on the body, the most important of which is the treatment of moderate-to-severe pain. However, the utility of opioid agonists is limited by a number of well-known side effects, including tolerance, physical dependence, respiratory depres-

sion and gastrointestinal effects. In order to avoid addiction and other side effects, researchers have focused on the modification of oripavine. Buprenorphine, a semi-synthetic opioid derived from the opium alkaloid, thebaine, has been widely used for the treatment of opioid dependence. However, buprenorphine can only be administered sublingually in the clinic^[1,2] due to its low oral bioavailability^[3]. Thienorphine, N-Cyclopropylmethyl-7-[1-(R)-1-hydroxy-1-methyl-3-(thien-2-yl) propyl]-6,14-endo-ethanotetrahydro-oripavine (Figure 1), is a new oripavine derivative designed by our institute through structural modification of buprenorphine^[4]. Thienorphine exhibits higher oral bioavailability and has a stronger antinociceptive effect than buprenorphine, which is considered to be mediated by μ -opioid receptor agonism^[5]. Furthermore, thienorphine has been proved to be a long-acting κ -opioid receptor agonist^[6]. Although its efficacy in a rhesus monkey analgesic model was low, the antinociceptive effect of thienorphine (0.32 mg/kg) lasted for a week in monkeys^[6] and the protective effect of thienorphine on morphine-induced lethality was as long as 15 d in mice^[5]. Thienorphine also inhibited morphine-induced behavioral sensitization in mice^[7] and has been used for the prevention of psychological dependence induced by morphine. Therefore, thienorphine, the new analog of buprenorphine, has several advantages over buprenorphine and may have wider application in the treatment of pain and opioid dependence.

Opiates can influence the autonomic outflow to the gut through their effect on the central nervous system^[8] and have a direct effect on the bowel^[9], and therefore induce changes in gastrointestinal motility and propulsion, leading to adverse effects on gastrointestinal function. To evaluate the effect of thienorphine on the intestinal tract, *in vivo* small intestinal transit in mice and *in vitro* guinea-pig ileum (GPI) assays were performed in the present study. Hopefully these assays may provide further evidence to understand the peripheral action and the possible adverse effects of thienorphine.

MATERIALS AND METHODS

Animals

Male guinea-pigs (300-400 g) and male Kunming mice (18-22 g) were obtained from Beijing Animal Center (Beijing, China). Animals were housed in a temperature-controlled room (25 °C \pm 1 °C) and maintained on a 12-h/12-h light/dark cycle. Animals had free access to food and water. Animal care and procedures were strictly in accordance with the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health, and this study was approved by the Animal Care Committee of Beijing Institute of Pharmacology and Toxicology.

Chemicals

Thienorphine HCl and buprenorphine HCl were synthesized in our institute^[4]. Naloxone and acetylcholine (Ach) were purchased from Sigma (St. Louis, MO, United States). NaCl, KCl, CaCl₂, KH₂PO₄, NaHCO₃, MgSO₄,

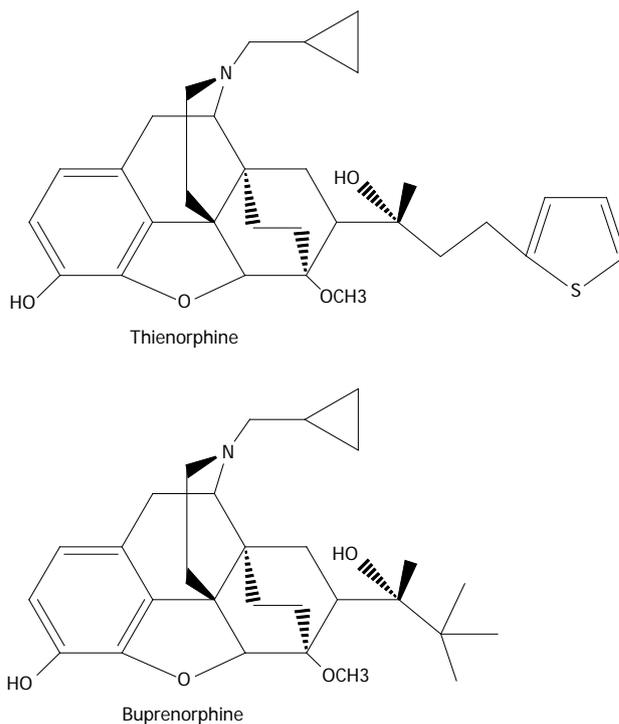


Figure 1 Chemical structure of thienorphine and buprenorphine.

glucose and charcoal were provided by Beijing Chemical Plant. Morphine was produced by Qinghai Pharmaceutical Factory (Xining, China).

Effect of thienorphine on gastrointestinal transit

Mice were randomly divided into 29 groups with 10 animals in each group. The groups were treated with saline, thienorphine (0.005, 0.05, 0.5, 1.0, 2.5, 5.0, 10 and 20 mg/kg, *ig*), buprenorphine (0.005, 0.05, 0.5, 1.0, 2.5, 5.0, 10 and 20 mg/kg, *sc*), morphine (0.5, 1.0, 2.5, 5.0, 10 and 20 mg/kg, *sc*), and naloxone (0.1, 0.5, 1.0, 5.0, 10 and 20 mg/kg, *sc*), respectively. Charcoal, used as a marker, was administered orally to mice at 0.3 mL (5 g of charcoal in 100 mL of 0.5% methylcellulose). The mice were subcutaneously treated with saline (10 mL/kg), a single dose of buprenorphine, morphine or naloxone 15 min before administration of the marker. Thienorphine was administered intragastrically 30 min before the marker. At 15 min after charcoal administration, the mice were sacrificed by cervical dislocation, the abdomen was dissected and the intestine removed from the pyloric junction to the cecal end. The distance traveled by the head of the marker and the total length of the intestine were measured. Gastrointestinal transit was expressed as a percentage of the distance traveled by the head of the marker relative to the total length of the small intestine.

Effect of naloxone on the inhibition of gastrointestinal transit by thienorphine

To determine the effects of an opioid receptor antagonist on the inhibition of intestinal transit by thienorphine (0.5 mg/kg), buprenorphine (0.5 mg/kg) and morphine (10 mg/kg), the animals were pretreated with the non-

selective opioid antagonist, naloxone (10 mg/kg, *sc*), 15 min prior to the administration of these chemicals.

Effect of thienorphine on isolated GPI contraction

All experiments were performed on isolated ileum from male guinea-pigs weighing 300-400 g. The animals were stunned and decapitated, and the ileum was quickly isolated about 10 cm from the ileo-cecal junction. The myenteric plexus-longitudinal muscle (MPLM) was prepared using the method of Rang^[10]. A glass rod was inserted into the lumen of an intestinal segment and the MPLM was removed by rubbing with a cotton swab soaked in Krebs' solution. The preparations (2.0-2.5 cm length) were suspended under 1.0 g tension in a 10 mL organ bath containing Krebs' solution (KCl 4.69 mmol/L, CaCl₂ 2.52 mmol/L, KH₂PO₄ 1.18 mmol/L, MgSO₄ 1.22 mmol/L, NaHCO₃ 25.0 mmol/L, NaCl 118.06 mmol/L, Glucose 10.0 mmol/L, pH = 7.4), at 37 °C and bubbled with 95% O₂ and 5% CO₂. The preparations were connected to an isotonic force transducer linked to eight channel organ baths (Medlab6, Meiyi Ltd., Nanjing, China). All the tissues were stimulated by Ach^[11,12]. Only the tissue preparations which responded to Ach (1 μmol/L) and produced contractions of more than 1.5 g tension were used. Preparations were equilibrated for at least 1 h with washes every 15 min before exposure to drugs. At the start of each experiment, a maximum response to Ach (1 μmol/L) was obtained in each tissue to confirm its suitability. After washing the preparation, the opioid agonists or antagonist were added to the organ bath for 10 min after which the second contraction with Ach was obtained. Maximal phasic responses were calculated as a percentage of the primary Ach-induced contraction, which was taken as 100% in each experiment. Each experiment was repeated with at least four separate tissue preparations obtained from different animals.

Effect of thienorphine on morphine-induced GPI contraction

To determine the effects of naloxone, thienorphine or buprenorphine on the inhibition by morphine on MPLM preparations of GPI, the MPLM preparations were treated with morphine (2.4 mmol/L) for 10 min, and then washed twice with Krebs' solution within 30 min. Before the second morphine (2.4 mmol/L) application, the preparations were treated with naloxone (0.5 mmol/L), thienorphine (32 μmol/L) or buprenorphine (32 μmol/L) for 10 min in the organ baths.

Recovery of GPI contraction after thienorphine treatment

To study the influence of thienorphine on the recovery of GPI contraction, thienorphine (0.1 mmol/L), morphine (3.2 mmol/L), buprenorphine (0.1 mmol/L) or naloxone (1.0 mmol/L) were added to the organ baths containing the tissue preparations. After 10 min treatment, the preparations were washed with Krebs' solution. The recovery of GPI contraction was evaluated based on stimulation with Ach (1 μmol/L) at different times during the pro-

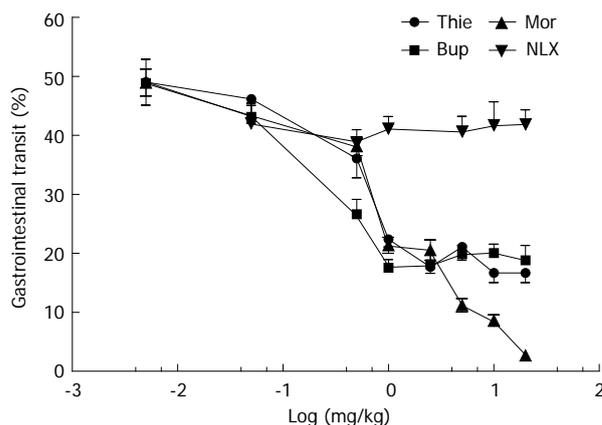


Figure 2 Effects of thienorphine, buprenorphine, morphine and naloxone on gastrointestinal propulsive activity in Kunming mice. Gastrointestinal transit was expressed as % of the distance traveled by an orally administered marker relative to the total length of the small intestine over 15 min after marker administration. Each column and vertical bar represent the mean \pm SE of 9-10 mice. Thie: Thienorphine; Bup: Buprenorphine; Mor: Morphine; NLX: Naloxone.

longed washing process, and the recovery time-course curves were generated based on GPI contraction.

Statistical analysis

Statistical and curve-fitting analysis were performed using PRISM 5.0 (GraphPad Software Inc., La Jolla, CA, United States). The data were expressed as mean \pm SE. Student's *t* test was used to compare single treatment means with control means. Analysis of variance followed by Newman-Keuls *post hoc* test was used for analysis of multiple treatment means. *P* < 0.05 was considered statistically significant.

RESULTS

Effect of thienorphine on gastrointestinal transit

Charcoal (0.3 mL, charcoal in 100 mL 0.5% methylcellulose) did not induce diarrhea in the mice. Stools colored by the marker were of the same form as normal stools, but could easily be distinguished by their black color. Gastrointestinal transit over 15 min was approximately 50% in the saline treated mice. Morphine (0.05-20.0 mg/kg, *sc*) significantly inhibited gut transit (Figure 2). Thienorphine (0.005-1.0 mg/kg, *ig*) dose-dependently inhibited gut transit, however, the inhibitory effect was not as strong as that of buprenorphine (0.005-1.0 mg/kg, *sc*). At higher doses, both thienorphine (1.0-20.0 mg/kg, *ig*) and buprenorphine (1.0-20.0 mg/kg, *sc*) inhibited intestinal transit by about 50%-60%. Naloxone (0.5-20.0 mg/kg, *sc*) had no effect on gut transit compared with the saline treated group. However, naloxone (10.0 mg/kg, *sc*) antagonized the inhibitory effect of thienorphine (0.5 mg/kg, *ig*), buprenorphine (0.5 mg/kg, *sc*) and morphine (10.0 mg/kg, *sc*) on gastrointestinal transit when it was administered 15 min prior to these chemicals (Figure 3).

Effect of thienorphine on GPI contraction

The GPI displayed regular spontaneous rhythmic contrac-

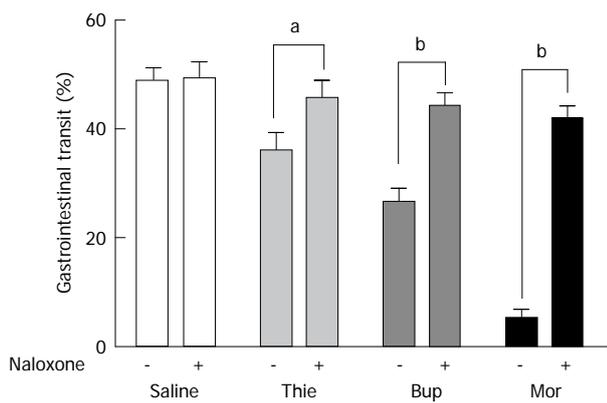


Figure 3 Effects of naloxone on the gastrointestinal propulsive activity in Kunming mice treated with thienorphine (0.5 mg/kg), buprenorphine (0.5 mg/kg), or morphine (10 mg/kg). Gastrointestinal transit was expressed as % of the distance traveled by an orally administered marker relative to the total length of the small intestine over 15 min after marker administration. Each column and vertical bar represent the mean \pm SE of 9-10 mice. ^a $P < 0.05$, ^b $P < 0.001$ vs intestinal transit without naloxone treatment. Thie: Thienorphine; Bup: Buprenorphine; Mor: Morphine.

tions following the equilibration period of 45-60 min. A contraction lasted without fading for up to several hours in the control state (Figure 4A). Thienorphine (0.32-32.0 $\mu\text{mol/L}$) or buprenorphine (0.32-32.0 $\mu\text{mol/L}$) decreased the basal tonus of GPI contraction in a concentration-dependent manner. The basal tonus of GPI was decreased by 10.0 $\mu\text{mol/L}$ of thienorphine or buprenorphine (Figure 4B and C). The maximum decrease in the basal tonus was 0.02 g by thienorphine (32.0 $\mu\text{mol/L}$) and 0.03 g by buprenorphine (32.0 $\mu\text{mol/L}$), with no difference between thienorphine and buprenorphine (Figure 5A). In comparison, morphine (0.4-3.2 mmol/L) concentration-dependently increased the basal tonus and spontaneous movement of GPI, and the maximum increase in basal tonus was about 0.65 g at 2.4 mmol/L (Figure 5B). The basal tonus and spontaneous movement of GPI was increased after the application of morphine 1.6 mmol/L (Figure 4E). Naloxone did not influence the basal contractile tonus, but increased the spontaneous movement of GPI at higher concentrations (Figure 4D).

Morphine (2.4 mmol/L) increased the basal tonus of GPI from 0.60-0.75 g (Figure 5C). After a 30 min resting period in Krebs' solution, naloxone (0.5 mmol/L), thienorphine (32.0 $\mu\text{mol/L}$) or buprenorphine (32.0 $\mu\text{mol/L}$) was added to the organ baths, the increases in basal tonus following the second addition of morphine (2.4 mmol/L) were all significantly inhibited by about 50%-60% (Figure 5C). Therefore, thienorphine, as well as buprenorphine, showed a potent antagonizing effect against morphine.

Effect of thienorphine on Ach-induced GPI contraction

The effects of thienorphine, buprenorphine, morphine and naloxone on GPI contraction are shown in Figure 6. All three drugs inhibited ileal muscle contraction induced by Ach (1 $\mu\text{mol/L}$) in a concentration-dependent manner. Thienorphine exhibited a moderate inhibition of

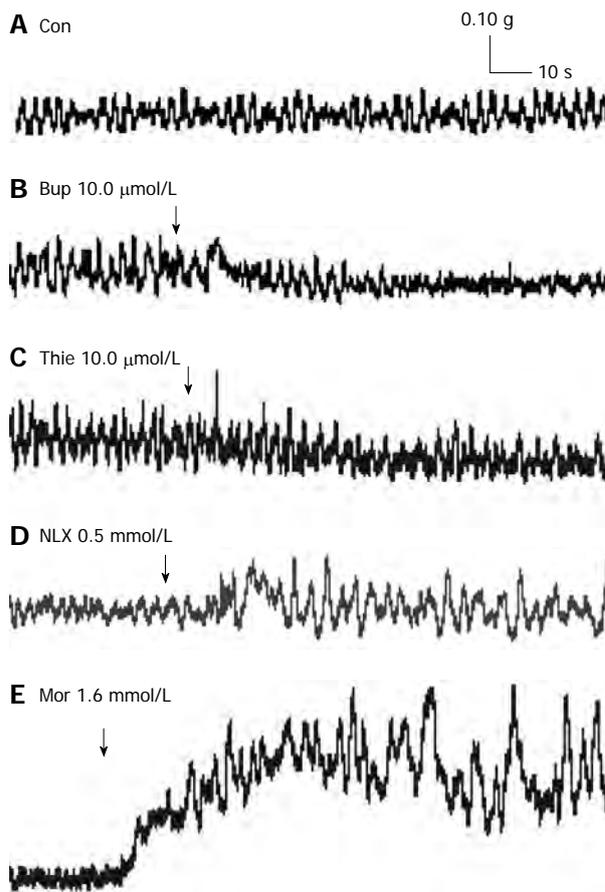


Figure 4 Typical trace of thienorphine, buprenorphine, naloxone and morphine on guinea-pig ileum. A: Spontaneous movement of the guinea-pig ileum without chemical; B: The basal tonus decreased after buprenorphine (10.0 $\mu\text{mol/L}$) treatment; C: The basal tonus decreased after thienorphine (10.0 $\mu\text{mol/L}$) treatment; D: Spontaneous movement increased after naloxone (0.5 mmol/L) treatment; E: The basal tonus and spontaneous movement increased after morphine (1.6 mmol/L) treatment. The arrow indicates the addition of chemicals. Con: Control; Thie: Thienorphine; Bup: Buprenorphine; Mor: Morphine; NLX: Naloxone.

65% ($P < 0.01$) at the highest concentration (100 $\mu\text{mol/L}$). However, buprenorphine exhibited a highly significant inhibition rate of 93% ($P < 0.01$) at 100 $\mu\text{mol/L}$. Morphine also showed a significant inhibition rate of 100% ($P < 0.01$) at the highest dose of 6.4 mmol/L. Naloxone exhibited an inhibition rate of 70% ($P < 0.01$) at the highest concentration of 1.0 mmol/L.

Recovery of GPI contraction after thienorphine treatment

When GPI was treated with a single concentration of thienorphine 100 $\mu\text{mol/L}$, buprenorphine 100 $\mu\text{mol/L}$, morphine 3.2 mmol/L or naloxone 1.0 mmol/L, the contractile amplitude response to Ach (1 $\mu\text{mol/L}$) was 27.53% \pm 4.21%, 9.60% \pm 2.43%, 21.78% \pm 4.78% and 31.75% \pm 5.08% with respect to the amplitude before treatment, respectively. The ileal muscle strip was washed with Krebs' solution. During the washing process, the ileal muscle contractile activity response to Ach (1 $\mu\text{mol/L}$) recovered more slowly in the thienorphine treated group than in the buprenorphine treated group. In the morphine and naloxone treated groups, the response of

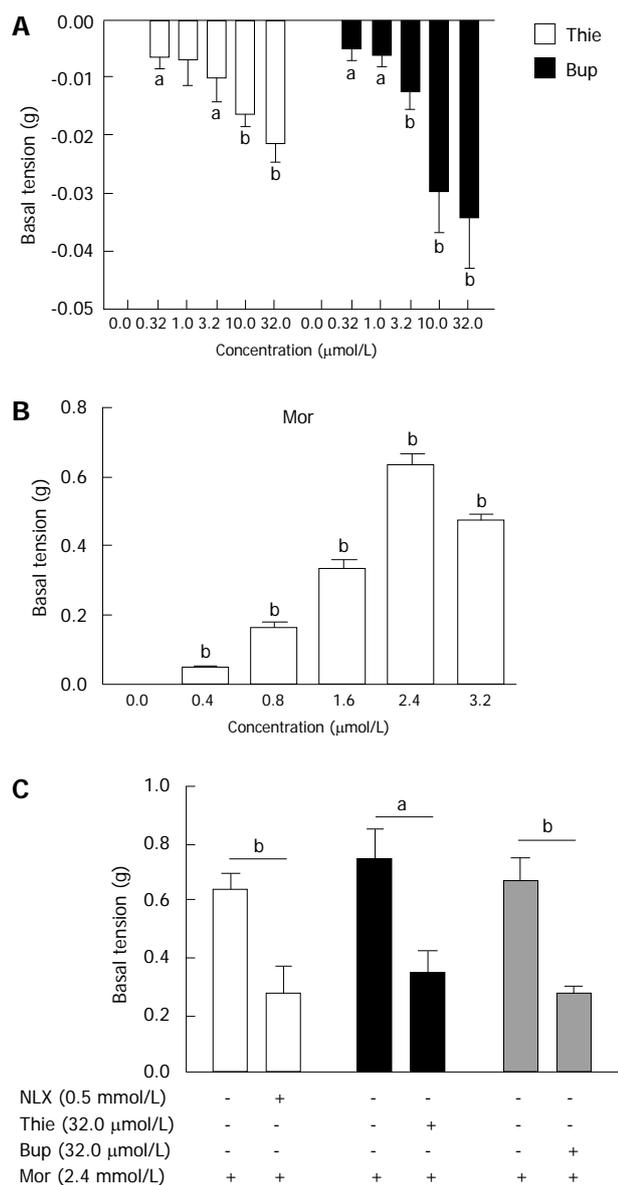


Figure 5 Effects of thienorphine, buprenorphine and morphine on the basal tonus of spontaneous movement of the guinea-pig ileum. Each point represents the mean ± SE (*n* = 5 or 6 preparations). A: Thienorphine (Thie) or buprenorphine (Bup) concentration-dependently decreased the basal tonus of guinea-pig ileum (GPI); B: Morphine (Mor) concentration-dependently increased the basal tonus of GPI; C: Naloxone (NLX, 0.50 mmol/L), Thie (32.0 μmol/L) or Bup (32.0 μmol/L) antagonized the elevation of basal tonus of GPI induced by Mor (2.4 mmol/L). ^a*P* < 0.05, ^b*P* < 0.001 vs the basal tonus before chemical application.

ileal muscle to Ach (1 μmol/L) recovered completely within 15 min. These results suggested that the effects of thienorphine on GPI lasted longer than those of the other opioids. At 60 min after the washout, the contractile amplitude of the ileal muscle after buprenorphine application recovered to 92.85% ± 3.89% with respect to the amplitude before treatment, whereas in the thienorphine treated preparation, the contractile amplitude remained at 51.25% ± 10.81%, which was significantly lower than that of the buprenorphine treated groups (*P* < 0.01). At 180 min after the washout, the response to Ach (1 μmol/L) of ileal muscle treated with thienorphine recovered to

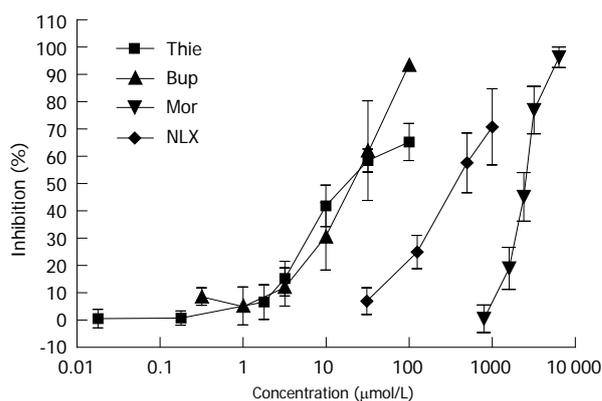


Figure 6 Effects of thienorphine, buprenorphine, morphine and naloxone on Ach-induced guinea-pig ileum contraction. Values represent mean ± SE for 4-6 preparations. Thie: Thienorphine; Bup: Buprenorphine; Mor: Morphine; NLX: Naloxone.

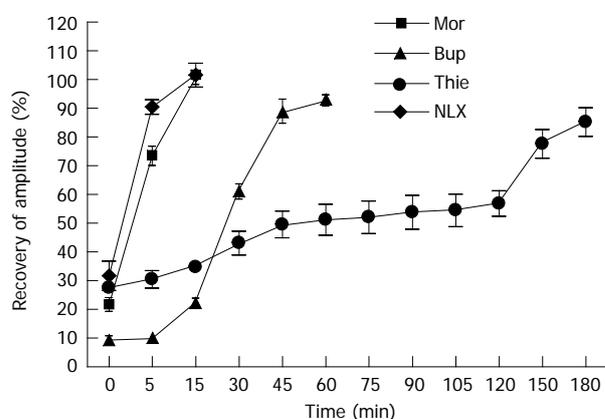


Figure 7 Time course of the amplitude inhibited by thienorphine (100 μmol/L), buprenorphine (100 μmol/L), morphine (3.2 mmol/L) or naloxone (1.0 mmol/L) on guinea-pig ileum contraction induced by Ach (1 μmol/L) during the prolonged washing process. The data are expressed as % amplitude with respect to the amplitude before chemical application, and each point represents the mean ± SE (*n* = 4). Thie: Thienorphine; Bup: Buprenorphine; Mor: Morphine; NLX: Naloxone.

85.25% ± 9.97% (Figure 7).

DISCUSSION

This study examined the effects of thienorphine on intestinal transit in mice and isolated GPI. The results indicated that thienorphine had a moderate inhibitory effect on intestinal transit in mice through the activation of opioid receptors. Compared with buprenorphine, thienorphine exhibited less inhibition, but the inhibitory effect on the contractility of GPI by direct or indirect activation of opioid receptors lasted longer. Therefore, thienorphine may have fewer adverse gastrointestinal effects when used for the treatment of opioid dependence.

The effects of opioids on gastrointestinal motility and transit have been attributed to the blockade of intestinal propulsion, resulting in slower elimination of the intestinal contents^[13]. Similar to buprenorphine, thienorphine seemed to be a partial μ-opioid receptor agonist, and had

weaker inhibition than morphine in the *in vivo* intestinal transit test. The present study showed 26.5% inhibition of gut transit by 0.5 mg/kg thienorphine and 6.1% inhibition by 0.05 mg/kg thienorphine. The K_i values of morphine for μ -, κ - and δ -opioid receptors were about 100-1000 times that of thienorphine. In the [35 S]-GTP γ S binding assay, the EC_{50} value for the stimulatory effects of thienorphine and morphine on μ -opioid receptors was 0.009 nmol/L and 18.24 nmol/L, and the maximum stimulatory values were 62.42% and 100%, respectively^[5]. In the present study, the maximum inhibition by thienorphine on intestinal transit was 50%-60%, whereas the maximum inhibition by morphine on gut transit was approximately 100%. The *in vivo* results of thienorphine were in accordance with the [35 S]-GTP γ S binding assay, which indicated that the adverse gastrointestinal effects of thienorphine would not be as serious as those of morphine.

Furthermore, the inhibition by thienorphine or morphine on intestinal transit was antagonized by naloxone (10.0 mg/kg). The inhibitory effects of morphine on small intestinal transit in mice were associated with the contractile effects on circular muscle in the ileum through binding to μ -opioid receptors, resulting in the inhibition of descending peristalsis relaxation^[14]. It was reported that few μ -opioid receptors were expressed in the mouse ileum, where δ - and κ -opioid receptors are dominant, and electrically induced contractions could be inhibited by δ - and κ -opioid receptor agonists, but not morphine^[15]. Thienorphine displayed high affinities for both μ -, κ - and δ -opioid receptors and produced a maximum stimulation of 74.5% on κ -opioid receptors (in comparison with U69, 593), and 19.3% on μ -opioid receptors (in comparison with DAMGO)^[6]. As a partial agonist of μ - and κ -opioid receptors, thienorphine also inhibited intestinal transit, but the effect was not as strong as that of morphine.

The MPLM from the GPI contains opioid peptide innervation, and enteric neurons modulate the release of enteric neurotransmitters^[16], therefore, MPLM has been used to investigate the effects of opioids on intestinal motility. Stimulation of the ileum or the MPLM preparation caused rapid contractions which were abolished by atropine, suggesting the participation of cholinergic neurons. Morphine can decrease the spontaneous release of Ach from the GPI and inhibit the electric-evoked contraction of MPLM from the GPI^[17]. Buprenorphine was approximately 100-fold more potent in inhibiting the electric-evoked contraction of MPLM than morphine, and the inhibition produced by buprenorphine was not eliminated by naloxone^[18]. In addition, naloxone itself inhibited these contractions^[18]. These results suggest that the effect of opioids on GPI contraction involves both a direct opioid receptor mechanism and indirect non-opioid receptor mechanisms. In the present study, MPLM preparations were stimulated by Ach instead of electrical stimulation. The inhibition of opioids on the release of Ach from the MPLM preparation could be ignored due to the application of Ach (1 μ mol/L). Through activation of M2 and M3 acetylcholine receptors, Ach induced voltage-dependent and voltage-independent Ca^{2+} entry

and intracellular Ca^{2+} release, leading to the contraction of intestinal smooth muscle^[19]. Thienorphine and buprenorphine (0.01-100 μ mol/L) induced the relaxation of contractile GPI under Ach stimulation in a concentration-dependent manner, which was similar to morphine and naloxone. Thienorphine was approximately 100-fold more potent than morphine, and the inhibition was not eliminated by naloxone. These results further proved the participation of indirect non-opioid receptors mechanisms. Activation of μ -opioid receptors led to an outward potassium conductance, and therefore produced membrane hyper-polarization and an increase in conductance, plus indirect inhibition of calcium entry^[20]; voltage-clamp studies showed that a κ opioid receptor agonist directly reduced calcium currents in mouse DRG cells^[21,22]; acute opioid exposure decreases calcium fluxes in neurons^[23,24]; loperamide, a peripheral agonist of μ opioid receptors, was found to induce intestinal relaxation by opening K_{ATP} channels *via* the cAMP-PKA pathway^[25] which further induced hyper-polarization of the cell membrane leading to relaxation of smooth muscle. Based on these studies, the inhibition by opioids on Ach-induced GPI contraction at higher concentrations further suggested the participation of other indirect non-opioid receptors or channels.

Thienorphine or buprenorphine induced a concentration-dependent decrease in the basal tonus and spontaneous movement of MPLM, whereas morphine concentration-dependently increased the basal tonus and spontaneous contraction of MPLM. The effect of thienorphine and buprenorphine was not antagonized by naloxone, while morphine-induced contraction was partially inhibited by naloxone or thienorphine. Similar results for morphine on the isolated circular muscle of mouse ileum have also been reported^[14]. Therefore, the effect of thienorphine on the MPLM simulates neither naloxone nor morphine, the mechanisms of which still require further study.

In our previous study, the contractile activity of uterine strips after thienorphine treatment recovered more slowly than after buprenorphine treatment during the washing process. In the present study, the contractile activity of isolated guinea-pig ileum also recovered more slowly after thienorphine treatment compared with buprenorphine or morphine treatment. Therefore, in the *in vitro* isolated tissue assay, the effects of thienorphine lasted longer, which was consistent with its *in vivo* effects.

In this study, we demonstrated that thienorphine, a new derivative of buprenorphine, moderately inhibited intestinal transit, showed less inhibition on contractile amplitude and a longer duration of action on guinea-pig isolated ileum.

COMMENTS

Background

Thienorphine, the new analog of buprenorphine, had several advantages over buprenorphine and may have wider application in the treatment of pain and opioid dependence. However, the utility of opioids is limited by a number of well-known side effects, including tolerance, physical dependence, respiratory depression and gastrointestinal effects.

Research frontiers

Opioids exhibit their effects through μ , κ and δ receptors both in the central and peripheral nervous system. Opiates can influence the autonomic outflow to the gut through their effects on the nervous system and have a direct effect on the bowel, and therefore induce changes in gastrointestinal motility and propulsion.

Innovations and breakthroughs

Thienorphine has several advantages over buprenorphine, such as stronger antinociceptive effects, potent rate-decreasing effects and better oral bio-availability than buprenorphine. Therefore, thienorphine, the new analog of buprenorphine, may have wider application in the treatment of pain and opioid dependence. This study reported on thienorphine, a new partial agonist of opioid receptors, which has fewer effects on intestinal transit and isolated guinea-pig ileum contraction.

Applications

Thienorphine has less influence and a longer duration of action on intestinal motility, and may be a new candidate for the treatment of pain and opioid dependence.

Terminology

In vivo intestinal transit tests and isolated guinea-pig ileum assays are well reported methods used to study the effects of chemicals on smooth muscle contraction.

Peer review

This is an interesting and important paper, which used well known tests for studying the effect of a new drug on motility. Thienorphine had moderate inhibition on intestinal transit, less inhibition on the contractile amplitude and longer duration of action on the guinea-pig isolated ileum. Thienorphine has less influence and longer duration on motility which may be a new useful candidate for the treatment of pain and opioid dependence.

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Decision making for pancreatic resection in patients with intraductal papillary mucinous neoplasms

Bin Xu, Wei-Xing Ding, Da-Yong Jin, Dan-Song Wang, Wen-Hui Lou

Bin Xu, Wei-Xing Ding, Department of General Surgery, Shanghai Tenth People's Hospital, Tongji University School of Medicine, Shanghai 200072, China

Da-Yong Jin, Dan-Song Wang, Wen-Hui Lou, Department of General Surgery, Zhong Shan Hospital, Fudan University, Shanghai 200032, China

Author contributions: Xu B and Ding WX contributed equally to this work; Xu B and Lou WH designed the research; Xu B, Ding WX, Jin DY, Wang DS and Lou WH performed the research; Ding WX, Jin DY and Lou WH analyzed the data; Xu B wrote the paper.

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Correspondence to: Wen-Hui Lou, MD, Department of General Surgery, Zhong Shan Hospital, Fudan University, Shanghai 200032, China. wenhuilou@yahoo.com.cn

Telephone: +86-21-64041990 Fax: +86-21-64038472

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Abstract

AIM: To identify a practical approach for preoperative decision-making in patients with intraductal papillary mucinous neoplasms (IPMNs) of the pancreas.

METHODS: Between March 1999 and November 2006, the clinical characteristics, pathological data and computed tomography/magnetic resonance imaging (CT/MRI) of 54 IPMNs cases were retrieved and analyzed. The relationships between the above data and decision-making for pancreatic resection were analyzed using SPSS 13.0 software. Univariate analysis of risk factors for malignant or invasive IPMNs was performed with regard to the following variables: carcinoembryonic antigen, carbohydrate antigen 19-9 (CA19-9) and the characteristics from CT/MRI images. Receiver operating characteristic (ROC) curve analysis for pancreatic

resection was performed using significant factors from the univariate analysis.

RESULTS: CT/MRI images, including main and mixed duct IPMNs, tumor size > 30 mm or a solid component appearance in the lesion, and preoperative serum CA19-9 > 37 U/mL had good predictive value for determining pancreatic resection ($P < 0.05$), but with limitations. Combining the above factors (CT/MRI images and CA19-9) improved the accuracy and sensitivity for determining pancreatic resection in IPMNs. Using ROC analysis, the area under the curve reached 0.893 ($P < 0.01$, 95%CI: 0.763-1.023), with a sensitivity, specificity, positive predictive value and negative predictive value of 95.2%, 83.3%, 95.2% and 83.3%, respectively.

CONCLUSION: Combining preoperative CT/MRI images and CA19-9 level may provide useful information for surgical decision-making in IPMNs.

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Key words: Intraductal papillary mucinous neoplasms; Surgical decision-making; Carbohydrate antigen 19-9; Computed tomography/magnetic resonance imaging; Predictor

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INTRODUCTION

There is a controversy between pancreatic resection and close follow-up for the treatment of intraductal papillary mucinous neoplasms (IPMNs). The prognosis of non-invasive IPMNs is better than that of invasive IPMNs.

The overall prognosis of IPMNs is better than that of pancreatic adenocarcinomas^[1,2]. When noninvasive IPMNs transform into invasive IPMNs, the prognosis of this disease is as poor as that of pancreatic ductal adenocarcinomas^[3,4]. There is no good way of monitoring this malignant transformation. Considering the nature of potential malignant transformation, some surgeons think that all IPMNs should be resected. However, for patients with benign lesions, close follow-up may be reasonable, as pancreatectomy may have severe adverse consequences due to its high mortality (15% at immature clinics) and high rate of postoperative complications (49%)^[5]. It is increasingly clear that not all IPMNs should be resected immediately^[6,7]. Wang *et al*^[8] reported that there was no significant difference in the 5-year survival rate between resected IPMNs and non-resected IPMNs. He also suggested that resection is not justified in elderly patients with high surgical risks, especially in asymptomatic and aged patients. A prospective study on the management of 109 asymptomatic patients also indicated that careful non-operative management seemed to be safe and effective^[9]. Therefore, it is crucial to establish a protocol to determine which IPMNs should be resected.

According to the Sendai consensus guidelines^[10], all main duct IPMNs, symptomatic branch duct IPMNs and IPMNs > 3 cm in size accompanying mural nodules should be resected. The presence of mural nodules is a very reliable malignant predictor in IPMNs. However, there is controversy regarding lesion size > 3 cm as a reliable indicator^[11]. The Sendai Criteria also showed that more data are needed to determine whether all branch duct IPMNs > 30 mm in size should be resected immediately^[10]. The Sendai criteria have a very high negative predictive value (NPV) and low positive predictive value (PPV)^[12], which may miss some malignant cases^[12]. Moreover, the management of some asymptomatic branch duct IPMNs < 30 mm in size is not clear using these criteria^[10]. Therefore, preoperative guidelines for surgical decision-making in IPMN patients need to be investigated^[13].

However, much work have been done to clarify the characteristics of malignant or invasive IPMNs. Both computed tomography (CT) and magnetic resonance imaging (MRI) examinations have good predictive value for invasive IPMNs. They have similar functions in pancreatic diagnosis^[14], evaluating the extent of tumor invasion and judging the resectability of pancreatic tumors^[15]. In addition, a high serum carbohydrate antigen 19-9 (CA19-9) level is associated with malignant pancreatic disease^[16-19]. Because not all lesions predicted to be malignant or invasive are suitable for surgery (*e.g.*, advanced malignancy or those wrongly predicted to be malignant), and some lesions predicted to be benign may require surgery (*e.g.*, borderline tumors or those wrongly predicted to be benign), there are differences between the prediction for malignant or invasive IPMNs and the prediction for surgical decision-making. A pragmatic approach for pancreatic resection decision-making in IPMN patients is

lacking. Serum CA19-9 and CT/MRI scans are useful in predicting malignant/invasive IPMNs, but their function in surgical decision-making for IPMNs is still unclear. Moreover, the value of the combination of preoperative serum CA19-9 and dynamic enhanced thin-slice CT/MRI scanning in operative decision-making is also unknown. Therefore, in this study, the above problems were investigated and a simple and pragmatic approach for surgical decision-making in IPMNs was designed.

MATERIALS AND METHODS

Clinical data

Fifty-four patients with IPMNs of the pancreas observed from March 1999 to November 2006 were retrospectively reviewed. Clinical data and tumor marker levels, including carcinoembryonic antigen and CA19-9, were collected. All patients underwent surgery and had a confirmed pathological diagnosis. Dynamic enhanced thin-slice CT/MRI images were objectively reevaluated by two radiologists and the final decision was made by consensus. The parameters of CT/MRI images included the type of tumor, the morphology of the lesion, the size of the lesion, the diameter of the main pancreatic duct, the diameter of the common bile duct, the septum appearance of the lesion, the solid component appearance in the lesion, and the papulous intraductal papilla.

Premise and hypothesis: Gold standard for pancreatic resection in IPMNs

IPMNs include adenomas, borderline tumors, adenocarcinoma *in situ*, and invasive IPMNs. Malignant IPMNs include adenocarcinoma *in situ* and invasive IPMNs. Benign IPMNs include adenomas and borderline tumors. By analyzing the postoperative pathological diagnosis and the clinicopathological stage, we found that some of the 54 patients required surgery, while other patients with benign diseases or more advanced stages were not suitable for pancreatic resection. Because borderline tumors have a high potential to change to malignant tumors, borderline tumors or malignant tumors with stage 0, I and II (UICC 6th edition, 2002) at pathological diagnosis were the gold standard (criterion-G) for surgical resection of IPMNs. According to criterion-G, the 54 cases were divided into two groups: the operative group who required surgical resection and the observational group who required close follow-up. The predictive value of preoperative serum CA19-9 level, CT/MRI scan, or their combination for determining surgical resection were then investigated.

Statistical analysis

Statistical analysis was carried out using SPSS 13.0 for Windows. All continuous data were presented as mean \pm SD. Categorical variables were compared by the χ^2 test or Fisher's exact test. The independent-samples *t* test were used to compare the means of the two groups. Logistic regression analysis was performed to identify independent risk factors; receiver operating characteristic (ROC)

Table 1 Univariate predictors of invasive intraductal papillary mucinous neoplasms

| Factors | Noninvasive | Invasive | d/χ^2 | P value |
|---------------------------------------|--------------|--------------|------------|---------|
| Gender | | | | |
| Male | 12 | 24 | | |
| Female | 6 | 12 | 0.00 | 1.00 |
| Age (yr) | 60.00 ± 8.48 | 61.94 ± 7.76 | -0.84 | 0.40 |
| Size of lesion (mm) ¹ | | | | |
| ≤ 30 | 16 | 2 | | |
| > 30 | 2 | 20 | 25.47 | < 0.01 |
| Type | | | | |
| Non-branch duct | 10 | 36 | | |
| Branch duct | 8 | 0 | 18.78 | < 0.01 |
| Morphology of the lesion ¹ | | | | |
| Unilocular cystic | 4 | 0 | | |
| Multilocular cystic | 14 | 6 | | |
| Cystic and solid | 0 | 16 | 23.03 | < 0.01 |
| Septum | | | | |
| Yes | 12 | 6 | | |
| No | 6 | 30 | 20.00 | 0.072 |
| Solid appearance | | | | |
| Yes | 1 | 26 | | |
| No | 17 | 10 | 21.33 | < 0.01 |
| Diameter of main pancreatic duct (mm) | | | | |
| ≤ 8.25 | 18 | 20 | | |
| > 8.25 | 0 | 16 | 11.36 | < 0.01 |
| Diameter of common bile duct (mm) | | | | |
| ≤ 8.7 | 18 | 12 | | |
| > 8.7 | 0 | 24 | 21.60 | < 0.01 |

¹Not including main-duct intraductal papillary mucinous neoplasms.

curve analysis was used to observe predictive values. $P < 0.05$ was considered statistically significant.

RESULTS

Clinical and pathological results

There were 36 male and 18 female patients in this study. The average age was 61.43 ± 8.24 years (range, 43-81 years). Conventional pancreatoduodenectomy was performed in 40 patients, total pancreatectomy was performed in 4 cases, distal pancreatectomy with splenectomy was performed in 5 cases, and distal pancreatectomy without splenectomy was performed in 1 case. Biopsy was carried out in 4 cases due to superior mesenteric artery (SMA) invasion, celiac trunk invasion or liver metastasis. The perioperative mortality rate was zero. Postoperative pathological diagnosis of IPMNs showed that there were 8 cases of adenoma, 8 cases of borderline tumors, 2 cases of adenocarcinoma *in situ*, and 36 cases of invasive IPMNs. There were 6 IPMN cases with a positive margin status, including 5 cases with pancreatic intraepithelial neoplasia 1 (PanIN-1) and 1 case with PanIN-3. According to the stage of pancreatic malignant tumor (UICC, 6th edition), there were 2 Stage 0, 3 Stage I A, 4 Stage I B, 20 Stage II A, 5 Stage II B, 3 Stage III and 1 Stage IV in 38 malignant IPMNs.

Decision-making for pancreatic resection by CT/MRI scans

In 54 patients, 42 patients required surgery according to

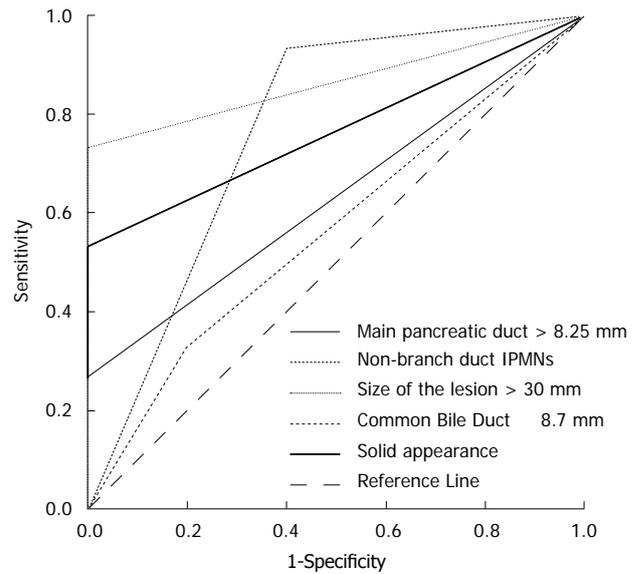


Figure 1 Characteristics of computed tomography/magnetic resonance imaging. Receiver operating characteristic analysis showed that non-branch duct intraductal papillary mucinous neoplasms (IPMNs), lesion size > 30 mm and a solid component appearance in the lesion had great significance for predicting pancreatic resection, and the area under the curve reached 0.76 ($P = 0.012$, 95%CI: 0.569-0.964), 0.867 ($P = 0.001$, 95%CI: 0.758-0.976) and 0.76 ($P < 0.01$, 95%CI: 0.623-0.910), respectively.

criterion-G. Following univariate analysis, non-branch duct IPMNs (main-duct and mixed IPMNs), lesions > 30 mm, the cystic and solid appearance of the lesion, the extended main pancreatic duct > 8.25 mm, the dilated common bile duct > 8.7 mm and the solid component appearance in the lesion from CT/MRI images were associated with pathologic findings for invasive IPMNs (Table 1). These features which were considered invasive characteristics from CT/MRI images were then further evaluated to predict surgical decision-making in IPMNs using ROC analysis. This evaluation showed that lesions > 30 mm, non-branch duct IPMNs and the solid component appearance in the lesion were good predictors for determining surgical resection (Figure 1). The patulous intraductal papilla was not analyzed due to a limited number of cases. However, all five cases with patulous intraductal papilla (3 malignant and 2 borderline tumors) harbored malignant potential, and many previous studies have indicated that patulous intraductal papilla is highly associated with invasive IPMNs. Thus, patulous intraductal papilla was also regarded as one of the invasive characteristics in IPMNs in our study. Next, our hypothesis (criterion-CT/MRI) was examined: pancreatic resection would be performed if the patient had any of the following factors on CT/MRI images: lesion size > 30 mm, non-branch duct IPMNs (main-duct and mixed), a solid component appearance in the lesion or patulous intraductal papilla, but no involvement of crucial vessels, such as SMA or celiac trunk, and definitive distant metastasis. According to criterion-CT/MRI, 95% of patients were correctly judged as requiring surgery. Using Fisher's exact test, it was confirmed that criterion-CT/MRI had good predictive value for determining pancreatic resec-

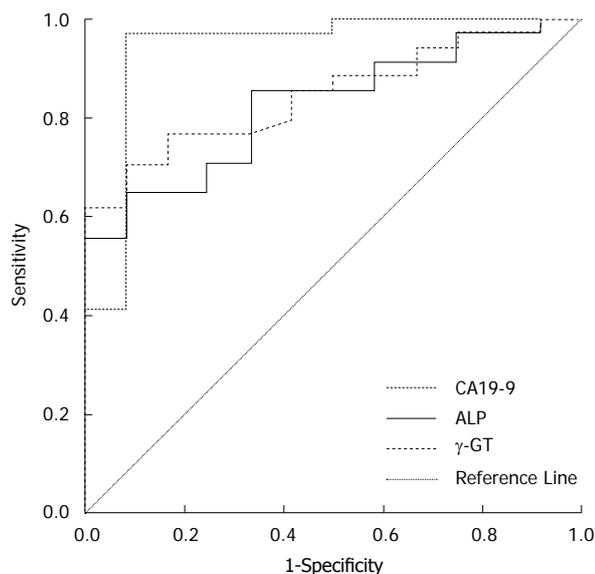


Figure 2 The predictors of malignant intraductal papillary mucinous neoplasms. Receiver operating characteristic analysis showed that γ -GT > 50 U/L, alkaline phosphatase (ALP) > 115 U/L or carbohydrate antigen 19-9 (CA19-9) > 37 U/mL effectively predicted malignant intraductal papillary mucinous neoplasms. However, the area under the curve for CA19-9 > 37 U/mL was the largest among the three indices and reached 0.939 ($P < 0.01$, 95%CI: 0.843-1.035).

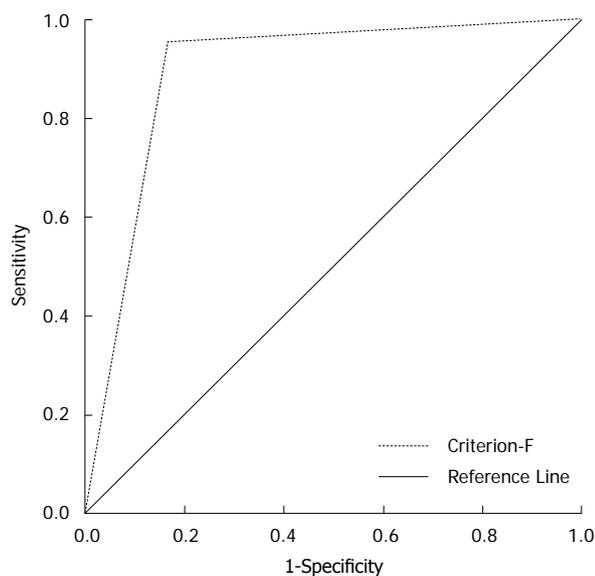


Figure 3 The predictive value of the combination of carbohydrate antigen 19-9 and computed tomography/magnetic resonance imaging (criterion-F). The area under the curve by receiver operating characteristic analysis reached 0.893 ($P < 0.01$, 95%CI: 0.763-1.023).

tion in IPMNs ($P < 0.05$). The sensitivity of criterion-CT/MRI was 95% and the specificity was 50%. The PPV and NPV were 91.3% and 100%, respectively.

Decision-making for pancreatic resection by preoperative serum CA19-9 levels

Thirty-eight cases were malignant and 16 cases were non-malignant IPMNs. Following univariate analysis, jaundice, direct bilirubin > 6.0 μ mol/L, γ -GT > 50 U/L,

Table 2 Univariate predictors of malignant intraductal papillary mucinous neoplasms

| Factors | Benign (n = 16) | Malignant (n = 38) | $d\chi^2$ | P value |
|---------------------------------|------------------|--------------------|-----------|---------|
| Gender | | | | |
| Male | 10 | 26 | | |
| Female | 6 | 12 | 0.18 | 0.67 |
| Age (yr) | 61.75 \pm 7.22 | 61.29 \pm 8.72 | -0.18 | 0.85 |
| Lumbodorsal pain | | | | |
| Yes | 0 | 3 | | |
| No | 10 | 31 | 1.50 | 0.22 |
| Jaundice | | | | |
| Yes | 0 | 19 | | |
| No | 16 | 19 | 12.34 | < 0.01 |
| Direct bilirubin (μ mol/L) | | | | |
| \leq 6.0 | 14 | 12 | | |
| > 6.0 | 2 | 26 | 14.10 | < 0.01 |
| ALP (U/L) | | | | |
| \leq 115 | 13 | 12 | | |
| > 115 | 3 | 26 | 9.26 | 0.02 |
| γ -GT (U/L) | | | | |
| \leq 50 | 13 | 10 | | |
| > 50 | 3 | 28 | 13.80 | < 0.01 |
| CA19-9 (U/mL) | | | | |
| \leq 37 | 14 | 8 | | |
| > 37 | 2 | 30 | 20.59 | < 0.01 |
| CEA (ng/mL) | | | | |
| \leq 5 | 15 | 33 | | |
| > 5 | 1 | 5 | 0.54 | 0.65 |

ALP: Alkaline phosphatase; CEA: Carcinoembryonic antigen; CA19-9: Carbohydrate antigen 19-9.

alkaline phosphatase > 115 U/L and CA19-9 > 37 U/mL were significantly associated with malignancy (Table 2). Using multivariate analysis, only CA19-9 > 37 U/mL was identified as an independent predictor of malignant IPMNs. The ROC curve also showed that CA19-9 had much better predictive potential for malignant IPMNs than the other factors (Figure 2). The relationship between the high level of serum CA19-9 and surgical decision-making was investigated. The higher the level of serum CA19-9, the higher the possibility of surgery for IPMNs ($P = 0.013$). The sensitivity of CA19-9 > 37 U/mL for determining surgery was 57% and the specificity was 84%. The PPV and NPV were 100% and 33%, respectively.

Operative decision-making by the combination of serum CA19-9 and CT/MRI scans

Serum CA19-9 alone had a low sensitivity and NPV for predicting surgical decision-making for pancreatic resections. CT/MRI scans alone had a low specificity. High sensitivity, specificity, PPV and NPV are necessary for pancreatic resection decision-making. Therefore, the predictive value of combining CA19-9 level and CT/MRI scan images was evaluated. Our final approach (criterion-F) was as follows: patients with negative criterion-CT/MRI and serum CA19-9 \leq 37 U/mL would be watched, and the remaining patients would undergo pancreatic resection. The value of criterion-F was analyzed by ROC curve analysis (Figure 3). The area under the curve was

0.893 (95%CI: 0.763-1.023), and its sensitivity was 95.2%, specificity was 83.3%, PPV was 95.2% and NPV was 83.3%.

DISCUSSION

Pancreatic resection is necessary for invasive IPMNs, while close follow-up is also feasible for definitive benign IPMNs^[20,21]. Until now, it has not been possible to effectively identify the nature of IPMNs preoperatively, therefore, it is difficult to accurately judge which IPMNs should be resected. Cytological biopsy from ERCP or fine needle aspiration has a high specificity for differentiation between benign and malignant IPMNs, however, their sensitivity is very low^[22] and are less feasible and convenient than serum CA19-9 or CT/MRI scans. The value of CT/MRI scans in pancreatic diseases has been demonstrated^[23]. Moreover, many predictive factors of malignant or invasive IPMNs were reported, including serum CA19-9^[19,24], jaundice^[25], tumor size > 3 cm^[21], dilatation of the main pancreatic duct^[26], and the presence of patulous papilla^[27,28]. Predictors of malignant or invasive IPMNs may help surgical decision-making. However, their value in pancreatic resection decision-making for IPMNs, especially the value of combining preoperative serum CA19-9 and CT/MRI scans, have not been evaluated.

According to criterion-G, 54 patients were divided into the operative group (42 cases) and the watched group (13 cases). In the univariate analysis, main and mixed duct IPMNs, tumor size > 30 mm, an extended main pancreatic duct > 8.25 mm, common bile duct > 8.7 mm and the solid component appearance in the tumor from CT/MRI images were associated with pathologic findings of invasive IPMNs. However, only three parameters had good predictive value for surgical decision-making: main and mixed duct IPMNs, tumor size > 30 mm and the solid component appearance in the lesion. The patulous intraductal papilla has been reported to be a malignant sign in IPMNs by many researchers^[7,29] and our all IPMN patients with patulous intraductal papilla required pancreatic resections, thus we combined it with the above three parameters to form criterion-CT/MRI.

Our data showed that criterion-CT/MRI or CA19-9 > 37 U/mL alone showed good predictive value for determining pancreatic resection in IPMNs ($P < 0.05$), however, they both had limitations. The specificity of criterion-CT/MRI was only 50%. The sensitivity of CA19-9 > 37 U/mL was just 57% and the NPV of CA19-9 > 37 U/mL was just 33%. Thus, CA19-9 level and criterion-CT/MRI were combined to improve the accuracy and sensitivity. This led to our final approach (criterion-F): patients with negative criterion-CT/MRI and normal CA19-9 level (≤ 37 U/mL) would be observed; while the remaining patients would undergo pancreatic resection. Criterion-F had higher accuracy for pancreatic resection decision-making in IPMNs, with acceptable sensitivity, specificity, PPV and NPV (95.2%, 83.3%, 95.2% and 83.3%, respectively).

The Sendai criteria^[10] have a high NPV, but PPV is low (14%-22%)^[30]. Moreover, the management of some IPMNs, such as lesions > 3 cm without main pancreatic duct dilation or mural nodules, is not very clear using the Sendai criteria. The management of some asymptomatic branch duct IPMNs < 30 mm in size is also not clear using the Sendai criteria^[10]. Our criterion-F can be applied in all patients, including the patients mentioned above. According to our criterion-F, a very small number of patients would be misjudged. This small number may be less than 3% of all patients, which is acceptable considering the high risk of mortality due to pancreatic resection (3%-15%)^[5]. For elderly patients, there is controversy over operative therapy. The implementation of our criterion-F should be appropriately adjusted, especially for noninvasive IPMNs, because the time required for the noninvasive type to develop into the invasive type might be longer than an elderly patient's life expectancy^[31].

In conclusion, a combination of serum CA19-9 and CT/MRI findings may be necessary for surgical decision-making in IPMNs. Criterion-F is effective for making the correct surgical decision, and can be used in all IPMNs. Of course, this study has limitations due to its retrospective design and relatively small sample size. Our findings should be further audited and improved by prospective large-scale clinical trials.

COMMENTS

Background

Due to the potential of malignant transformation from benign intraductal papillary mucinous neoplasms (IPMNs), all patients with IPMNs were previously recommended to undergo pancreatic surgery. There is currently more data available to support non-operative management of some benign IPMNs which is reasonable and feasible. However, it is difficult for surgeons to distinguish between benign and malignant IPMNs preoperatively and make decisions regarding pancreatic resection for IPMNs.

Research frontiers

Although there have been several articles on the malignant or invasive predictive factors for IPMNs, accurate preoperative criteria have not been available to determine pancreatic resection in IPMNs. Surgical decision-making for individual patients with IPMNs is still difficult. It is important to identify a practical approach for preoperative decision-making in IPMNs.

Innovations and breakthroughs

The combined strategy of serum carbohydrate antigen 19-9 level and computed tomography/magnetic resonance imaging provided useful information for preoperative surgical decision-making in IPMNs, with acceptable sensitivity, specificity, positive predictive value and negative predictive value of 95.2%, 83.3%, 95.2% and 83.3%, respectively.

Applications

It would be helpful for surgeons to determine which IPMNs should be resected.

Terminology

IPMN: This is a type of neoplasm which grows within the pancreatic ducts (intraductal) and is characterized by the production of thick fluid by the tumor cells.

Peer review

The paper provides a practical approach for preoperative surgical decision-making for IPMNs. The topic is very interesting, the results are applicable and the conclusions are valuable.

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Middle segmental pancreatectomy: A safe and organ-preserving option for benign and low-grade malignant lesions

Zhi-Yong Du, Shi Chen, Bao-San Han, Bai-Yong Shen, Ying-Bing Liu, Cheng-Hong Peng

Zhi-Yong Du, Ying-Bing Liu, Department of General Surgery, Xinhua Hospital, Shanghai Jiaotong University School of Medicine, Shanghai 200092, China

Zhi-Yong Du, Shi Chen, Bao-San Han, Bai-Yong Shen, Cheng-Hong Peng, Department of General Surgery, Ruijin Hospital, Shanghai Jiaotong University School of Medicine, Shanghai 200025, China

Shi Chen, Department of Hepatobiliary Surgery, Fujian Provincial Hospital, Fuzhou 350001, Fujian Province, China

Author contributions: Chen S, Shen BY and Peng CH performed the clinical surgery; Du ZY, Chen S and Peng CH designed the research; Du ZY, Chen S and Han BS collected and analyzed data; Du ZY and Chen S wrote the manuscript; and Liu YB and Peng CH revised and finally approved the article to be published.

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Correspondence to: Cheng-Hong Peng, MD, FACS, Department of General Surgery, Ruijin Hospital, Shanghai Jiaotong University School of Medicine, Shanghai 200025, China. chhpeng@188.com

Telephone: +86-21-64370045 Fax: +86-523-87636908

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Abstract

AIM: To study the feasibility and safety of middle segmental pancreatectomy (MSP) compared with pancreaticoduodenectomy (PD) and extended distal pancreatectomy (EDP).

METHODS: We studied retrospectively 36 cases that underwent MSP, 44 patients who underwent PD, and 26 who underwent EDP with benign or low-grade malignant lesions in the mid-portion of the pancreas, between April 2003 and December 2009 in Ruijin Hospital. The perioperative outcomes and long-term outcomes of MSP were compared with those of EDP and PD. Periop-

erative outcomes included operative time, intraoperative hemorrhage, transfusion, pancreatic fistula, intra-abdominal abscess/infection, postoperative bleeding, reoperation, mortality, and postoperative hospital time. Long-term outcomes, including tumor recurrence, new-onset diabetes mellitus (DM), and pancreatic exocrine insufficiency, were evaluated.

RESULTS: Intraoperative hemorrhage was 316.1 ± 309.6 , 852.2 ± 877.8 and 526.9 ± 414.5 mL for the MSP, PD and EDP groups, respectively ($P < 0.05$). The mean postoperative daily fasting blood glucose level was significantly lower in the MSP group than in the EDP group (6.3 ± 1.5 mmol/L vs 7.3 ± 1.5 mmol/L, $P < 0.05$). The rate of pancreatic fistula was higher in the MSP group than in the PD group (42% vs 20.5%, $P = 0.039$), all of the fistulas after MSP corresponded to grade A (9/15) or B (6/15) and were sealed following conservative treatment. There was no significant difference in the mean postoperative hospital stay between the MSP group and the other two groups. After a mean follow-up of 44 mo, no tumor recurrences were found, only one patient (2.8%) in the MSP group vs five (21.7%) in the EDP group developed new-onset insulin-dependent DM postoperatively ($P = 0.029$). Moreover, significantly fewer patients in the MSP group than in the PD (0% vs 33.3%, $P < 0.001$) and EDP (0% vs 21.7%, $P = 0.007$) required enzyme substitution.

CONCLUSION: MSP is a safe and organ-preserving option for benign or low-grade malignant lesions in the neck and proximal body of the pancreas.

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Key words: Middle segmental pancreatectomy; Pancreaticoduodenectomy; Extended distal pancreatectomy; Pancreatic fistula; Pancreatic endocrine function; Pancreatic exocrine function

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INTRODUCTION

Clinically, surgical treatment for benign or low-grade malignant lesions of the pancreatic neck and body is often performed using “traditional” procedures such as pancreaticoduodenectomy (PD) or extended distal pancreatectomy (EDP). However, these approaches result in a significant and unnecessary loss of normal pancreatic parenchyma, with subsequent impairment of exocrine and endocrine functions, and the loss of the upper gastrointestinal and biliary anatomy in PD, and the spleen in EDP. In addition, tumor enucleation is only suitable for small and superficial lesions that do not involve the main pancreatic duct. Furthermore, enucleation is associated with high risk for major complications, including pancreatic leakage, and tumor recurrence cannot be ignored because of incomplete removal. Therefore, preventing unnecessary loss of pancreatic tissue and avoiding further deteriorations in the endocrine and exocrine pancreatic functions are huge challenges for surgeons managing benign and low-grade malignant lesions in the neck or the proximal body of the pancreas that cannot be managed by enucleation. Furthermore, it would be beneficial for the patient if PD or DP could be avoided.

Since it was first reported by Guillemin *et al*^[1], middle segmental pancreatectomy (MSP) has increasingly been applied for some lesions, including chronic pancreatitis, traumatic injury, and benign and borderline lesions localized at the neck and body of pancreas^[2-10]. Several recent reports have compared the morbidity, quality of life, and other outcomes in patients with chronic pancreatitis, benign, and low-grade malignant pancreatic tumors after MSP or traditional surgical procedures^[5,11-18]. The purpose of our study was to compare the perioperative safety and the long-term effects, including the preservation of pancreatic endocrine and exocrine function, following MSP, PD or EDP in patients with benign, borderline or low-grade malignant lesions.

MATERIALS AND METHODS

Ethics

The Ethics Committee of Ruijin Hospital Shanghai Jiao-tong University School of Medicine approved the study. All patients provided informed written consent.

Patient tissue

One hundred and six patients with benign or low-grade malignant tumors (without chronic pancreatitis) in the neck or proximal body of pancreas underwent MSP (n

= 36), PD ($n = 44$) or EDP ($n = 26$) between April 2003 and December 2009 in Department of General Surgery, Ruijin Hospital (affiliated to Shanghai Jiao Tong University School of Medicine). The same surgeons performed all the surgical procedures. All patients received computed tomography (CT) scans and abdominal ultrasonography before surgery to determine the location of the lesion and its relationship with mesenteric vessels. The indication for MSP was a lesion localized in the neck or proximal body of the pancreas without evidence of high-grade malignancy. Intraoperative frozen tissue sections were analyzed in all patients to exclude pancreatic adenocarcinoma and to ensure that the resection margins were clear, which was subsequently confirmed by histopathological examination.

Patient characteristics retrieved from medical records included their age, sex, presence of diabetes mellitus (DM) and mean preoperative blood glucose levels. DM was diagnosed based on abnormal fasting blood glucose levels or positive results following an oral glucose tolerance test. Tumor variables included tumor size, pathology and specific position. The location of the tumor was divided into four categories: head-neck, neck, neck-body, and body of the pancreas (Figure 1).

We compared the perioperative survival state and long-term changes in exocrine and endocrine function between MSP patients and patients with PD or EDP using matched-pairs analyses. Patients were matched for age, sex, preoperative DM, as well as tumor histopathology, size, and position (Table 1).

Surgical procedures for MSP

After making a bilateral subcostal incision with an upper midline extension to the xiphoid, the gastrocolic ligament was divided to open the lesser sac and expose the pancreas. The posterior peritoneum along the inferior and superior margins of the gland was dissected and the superior mesenteric vein was identified under the neck of the pancreas. The splenic vein was carefully divided away from the pancreas and all of the small branches of the pancreas draining into the splenic vein were ligated. The involved pancreatic segment was mobilized on both cephalic and caudal sides. The pancreas was then sectioned with an electroscalpel with a gap of at least 1-cm away from the tumor, while the limits for the cephalic and caudal sides were the gastroduodenal artery and a ≥ 5 cm gap from the distal pancreas, respectively. The two resection margins were frozen and prepared for imaging to confirm tumor-free resection. Hemostasis of the cephalic and caudal stumps of pancreas was performed with interrupted 4-0 non-absorbable stitches, and the cephalic pancreatic cut surface was closed by Endo-GIA™ 60-2.5 auto sutures (Johnson Medical Ltd., China) or continuous suture using 4-0 prolene (Figure 2). A small catheter was inserted to maintain the patency of the pancreatic duct on the distal side. The distal side stump was reconstructed by Roux-en-Y pancreaticojejunostomy (PJ, $n = 22$) or pancreaticogastrostomy (PG, $n = 14$) (Figure 3). Two

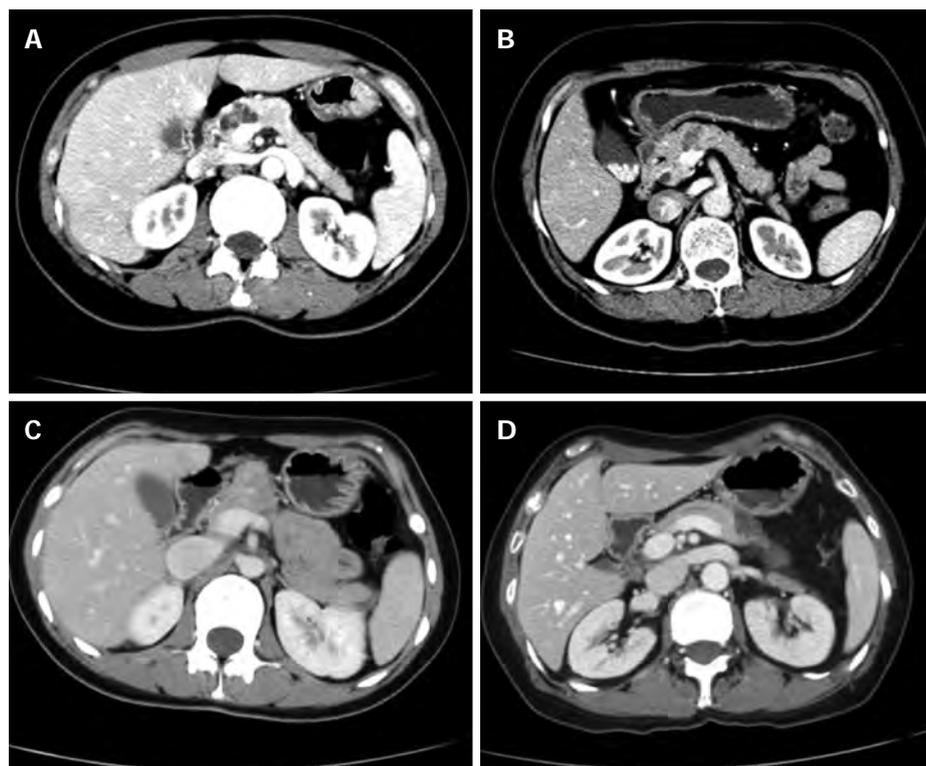


Figure 1 Location of lesions in the pancreas. A: Head-neck; B: Neck; C: Neck-body; D: Body.

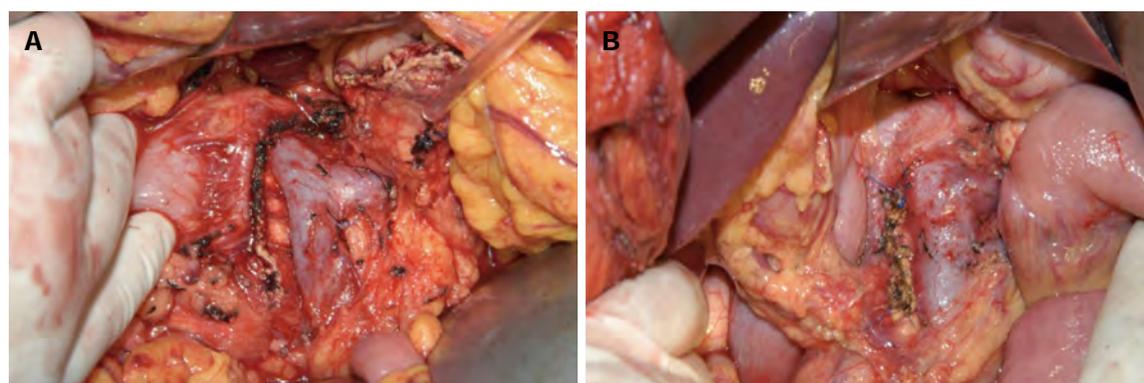


Figure 2 The surgical approach of the cephalic pancreatic cut surface. A: Closed by Endo-GIA™ 60-2.5 auto suture; B: Continuous suture using 4-0 prolene;

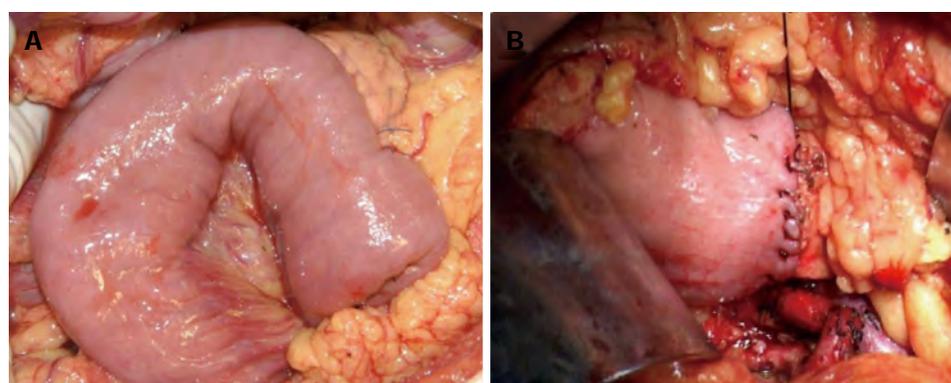


Figure 3 The reconstruction of the distal side stump. A: Pancreaticojejunostomy; B: Pancreaticogastrostomy.

drainage tubes were placed close to the closed cephalic stump and the region of the pancreatic anastomosis. The drains were not removed until the drainage fluid volume was < 10 mL/d and the amylase value was < 300 U/L.

Perioperative outcomes

Perioperative outcomes included operative time, intraoperative hemorrhage, transfusion, pancreatic fistula, intra-abdominal abscess/infection, postoperative bleeding,

Table 1 Matched-pairs analysis of patient characteristics between patients who underwent middle segmental pancreatectomy, pancreaticoduodenectomy or extended distal pancreatectomy

| Variable | PD (n = 44) | MSP (n = 36) | EDP (n = 26) | P value | |
|-------------------------|-----------------------|-----------------------|-----------------------|-----------|------------|
| | | | | PD vs MSP | MSP vs EDP |
| Patient characteristics | | | | | |
| Gender (M/F) | 16/28 | 9/27 | 11/15 | NS | NS |
| Age (yr) | 50 ± 14 (range 18-71) | 49 ± 15 (range 23-76) | 49 ± 12 (range 22-79) | NS | NS |
| Tumor type | | | | | |
| Cyst adenomas | 13 | 16 | 16 | NS | NS |
| Endocrine | 1 | 3 | 2 | | |
| IPMN | 7 | 3 | 2 | | |
| SPT | 16 | 10 | 1 | | |
| Others | 7 | 4 | 5 | | |
| Tumor size (mm) | 30.8 ± 9.6 | 26.5 ± 14 | 31.3 ± 13.5 | NS | NS |
| Tumor location | | | | | |
| Head-neck | 23 | 8 | 0 | NS | NS |
| Neck | 16 | 10 | 0 | | |
| Neck-body | 5 | 9 | 15 | | |
| Body | 0 | 9 | 11 | | |
| Preoperative DM | 5 | 2 | 4 | NS | NS |

MSP: Middle segmental pancreatectomy; PD: Pancreaticoduodenectomy; EDP: Extended distal pancreatectomy; IPMN: Intraductal papillary mucinous neoplasms; SPT: Solid pseudopapillary tumor; DM: Diabetes mellitus; NS: Not significant.

reoperation, mortality, and postoperative hospital time. Pancreatic fistulae were graded based on the International Study Group for Pancreatic Fistula (ISGPF) criteria^[19]. Pre- and postoperative nutritional status (total protein, albumin, and hemoglobin) was evaluated using the formula: (discharge numerical value - preoperative numerical value)/preoperative numerical value × 100 (%). Nutritional parameters were measured on the day of admission and the seventh postoperative day.

For postoperative glycemic control, the following approach was used in all patients. First, insulin was added to the glucose solution (1 U insulin/4-6 g glucose). Second, blood glucose was monitored every 6 h and additional insulin was given if blood glucose exceeded 10 mmol/L. Third, long-acting insulin was given when blood sugar was not well controlled using the above method. Glycemic control was determined as the mean daily fasting blood glucose.

Long-term outcomes

All patients were evaluated after a median follow-up of 44 mo (range 4-72 mo). Our aim was to evaluate the long-term changes in endocrine and exocrine functions, body weight change, and tumor recurrence, based on radiological, clinical and laboratory assessments. New-onset DM was diagnosed according to the criteria of the World Health Organization^[20]. Pancreatic exocrine insufficiency was defined as diarrhea and steatorrhea, and was treated by daily enzyme administration.

Statistical analysis

SPSS software (SPSS Inc., Chicago, IL, United States) was used for all statistical analysis. Continuous variables were summarized as mean ± SD and categorical variables as frequency and percentage. Two-sample *t* tests and Pearson's χ^2 test or Fisher's exact test were used to com-

pare continuous and categorical variables, respectively. The Wilcoxon rank sum test was used to compare body weight change. *P* values < 0.05 were considered statistically significant.

RESULTS

Perioperative outcomes

The perioperative outcomes are shown in Table 2. No in-hospital death occurred after the operation in any group. A statistically significant difference in operation time was found between the PD and MSP groups (333.5 ± 97 min vs 222.1 ± 62.1 min, respectively; *P* < 0.001). Intraoperative hemorrhage was 316.1 ± 309.6, 852.2 ± 877.8 and 526.9 ± 414.5 mL for the MSP, PD and EDP groups, respectively (*P* < 0.05). The splenic artery and vein, as well as the spleen, were preserved in all patients treated with MSP and PD; however, these were removed in all patients who underwent EDP. There were no differences in postoperative hospital time between the MSP group and the PD and EDP groups. The duration of gastrointestinal recovery was shorter in the MSP group than in the PD group (*P* < 0.05). The rate of pancreatic fistula was higher in the MSP group than in the other groups, reaching statistical significance between the MSP and PD groups (*P* < 0.05). However, the pancreatic fistulas in the MSP group corresponded to ISGPF Grade A (*n* = 9/15) or B (*n* = 6/15), and there was no significant difference in the pancreatic fistulas rate between PJ (9/22) and PG (6/14) reconstruction methods in MSP group (*P* > 0.05). All of the fistulas after MSP were sealed following conservative treatment. Two patients in the PD group underwent reoperation, one because of postoperative bleeding and the other as a result of intra-abdominal infection.

There were no differences in preoperative mean blood glucose levels between the MSP group and the PD and

Table 2 Perioperative outcomes for middle segmental pancreatectomy, pancreaticoduodenectomy and extended distal pancreatectomy *n* (%)

| Variable | PD (<i>n</i> = 44) | MSP (<i>n</i> = 36) | EDP (<i>n</i> = 26) | <i>P</i> value | |
|-----------------------------|---------------------|----------------------|----------------------|------------------|-------------------|
| | | | | PD <i>vs</i> MSP | MSP <i>vs</i> EDP |
| Operation time (min) | 333.5 ± 97 | 222.1 ± 62.1 | 202.0 ± 60.7 | < 0.001 | > 0.05 |
| IPH (mL) | 852.2 ± 877.8 | 316.1 ± 309.6 | 526.9 ± 414.5 | < 0.001 | 0.025 |
| PT (d) | 31 ± 29 | 29 ± 23 | 22 ± 10 | NS | > 0.05 |
| Reoperation | 2 (4.5) | 0 (0.0) | 0 (0.0) | NS | |
| DGR (d) | 5.5 ± 2.1 | 4.4 ± 2.0 | 3.2 ± 1.5 | 0.031 | 0.008 |
| Bleeding | 2 (4.5) | 1 (2.8) | 1 (3.8) | NS | NS |
| Intra-abdominal infection | 4 (9.1) | 1 (2.8) | 1 (3.8) | NS | NS |
| Pancreatic fistula | 9 (20.5) | 15 (42) | 8 (31) | 0.039 | NS |
| Nutritional status | | | | | |
| Change in TP (%) | -0.5 ± 14.3 | 3.1 ± 12.4 | -6.6 ± 9.5 | NS | 0.002 |
| Change in Alb (%) | -3.6 ± 15.6 | 0.8 ± 14.3 | -8.2 ± 14.7 | NS | 0.019 |
| Change in Hb (%) | -13.3 ± 12.6 | -11.9 ± 13.2 | -14.3 ± 8.0 | NS | NS |
| Mean blood glucose (mmol/L) | | | | | |
| Preoperative | 5.0 ± 1.1 | 4.9 ± 0.6 | 5.2 ± 1.6 | NS | NS |
| Postoperative | 6.7 ± 1.8 | 6.3 ± 1.5 | 7.3 ± 1.5 | NS | 0.013 |

IPH: Intraoperative hemorrhage; PT: Postoperative time; DGR: Duration of gastrointestinal recovery; TP: Total protein; Alb: Albumin; Hb: Hemoglobin; MSP: Middle segmental pancreatectomy; PD: Pancreaticoduodenectomy; EDP: Extended distal pancreatectomy; NS: Not significant.

EDP groups. However, the mean postoperative daily fasting blood glucose level was significantly lower in the MSP group than in the EDP group (*P* < 0.05). Serum protein is an important clinical and biochemical marker of the patients' nutritional status. Serum total protein and albumin levels were significantly higher in the MSP group than in the EDP group (*P* < 0.01 and *P* < 0.05, respectively).

Long-term outcomes

The long-term outcomes of patients were assessed by telephone interview until March 2010. The median follow-up time was 44 mo (range 4-72 mo). Two and three patients were lost to follow-up in the PD and EDP groups, respectively. The long-term outcomes are shown in Table 3. No tumor recurrence occurred in any of the patients. There was no significant difference in body-weight change between groups MSP and PD (*P* = 0.701), or MSP and EDP (*P* = 0.568). Only one patient (2.8%) in the MSP group *vs* five (21.7%) in the EDP group developed new-onset insulin-dependent DM postoperatively, which was statistically significant (*P* < 0.05). Moreover, significantly fewer patients (0%) in the MSP group than in the PD (33.3%, *P* < 0.001) and EDP (21.7%, *P* < 0.01) required enzyme substitution, indicating superior preservation of the pancreatic exocrine function with MSP than with other approaches.

DISCUSSION

In recent years, benign and borderline pancreatic lesions have been diagnosed more frequently because of the increased use of high-resolution cross-sectional imaging modalities, such as CT and magnetic resonance imaging^[21,22]. As many of these lesions are noninvasive at the time of discovery, parenchymal-sparing techniques may be beneficial to preserve endocrine and exocrine pan-

creatic function^[23-25]. Enucleation is generally appropriate for small lesions that do not involve the main pancreatic duct^[26,27]. However, it is associated with a high incidence of pancreatic fistula and pseudocyst formation^[28]. Therefore, traditional resection methods (*e.g.*, PD and EDP) are still used to treat lesions in the pancreatic neck or proximal body. These resection methods sacrifice a considerable portion of the normal pancreatic tissue, leading to marked postoperative deteriorations in exocrine and endocrine pancreatic functions^[29-31]. Moreover, the loss of the duodenum alters the natural passage of food, leading to an abnormal digestive process. Similarly, bilio-digestive anastomosis increases the risk of ascending cholangitis and subsequently intrahepatic abscesses in PD^[32]. Surgeons performing EDP often remove a large amount of normal pancreatic tissue and sometimes the spleen. Splenectomy carries some risks, including portal vein thrombosis, postsplenectomy sepsis, and reduced immune function^[3-5,33,34]. MSP avoids extensive loss of normal pancreatic tissue as compared with PD and EDP. Theoretically, MSP also preserves digestive tract continuity, as well as the spleen, potentially reducing postoperative morbidity as compared with PD or EDP. However, MSP appears to be associated with a higher incidence of pancreatic fistula compared with both PD and EDP^[11-14,16,35]. This is possibly caused by the need to manage two pancreatic remnants by anastomosis or closure. In this study, the perioperative survival and long-term outcomes were examined in patients with lesions in the neck and proximal body of the pancreas treated by PD, EDP or MSP.

In this study, we found that total blood loss during surgery was less in the MSP group than in the PD and EDP groups. The greater blood loss in the PD group may be caused by the large volume of tissue removed and the complexity of the surgical procedure. Similarly, EDP may be complicated in patients where the surgeon experiences

Table 3 Long-term outcomes following middle segmental pancreatectomy, pancreaticoduodenectomy and extended distal pancreatectomy *n* (%)

| Variable | PD (<i>n</i> = 42) | MSP (<i>n</i> = 36) | EDP (<i>n</i> = 23) | <i>P</i> value | |
|-----------------------------------|---------------------|----------------------|----------------------|----------------|------------|
| | | | | PD vs MSP | MSP vs EDP |
| Exocrine and endocrine function | | | | | |
| New-onset DM | 6 (14.3) | 1 (2.8) | 5 (21.7) | NS | 0.029 |
| NIDDM | 4 | 1 | 3 | | |
| IDDM | 2 | 0 | 2 | | |
| Enzyme substitution | 14 (33.3) | 0 | 5 (21.7) | < 0.001 | 0.007 |
| Anorexia | 9 (21.4) | 3 (8.3) | 1 (4.3) | NS | NS |
| Nausea and vomiting | 5 (11.9) | 2 (5.6) | 0 | NS | NS |
| Abdominal distention and diarrhea | 16 (38.1) | 3 (8.3) | 2 (8.7) | 0.005 | NS |
| Tumor recurrence | 0 | 0 | 0 | | |
| Body weight change ¹ | | | | | |
| Weight gain | 16 | 15 | 8 | | |
| No change | 14 | 12 | 8 | NS | NS |
| Weight reduction | 12 | 9 | 7 | | |

¹Wilcoxon rank sum test. DM: Diabetes mellitus; NIDDM: Non insulin dependent diabetes mellitus; IDDM: Insulin dependent diabetes mellitus; MSP: Middle segmental pancreatectomy; PD: Pancreaticoduodenectomy; EDP: Extended distal pancreatectomy; NS: Not significant.

difficulties in exposing and isolating the spleen, which may result in injury to the spleen parenchyma or splenic vein and artery, leading to significant blood loss. Blind suturing and electric coagulation can also lead to potentially serious consequences. In such cases, the spleen must also be removed. Notably, we found that MSP was easier to perform compared with PD and EDP, because of the smaller volume of tissue to be removed, excellent exposure, and ease of handling the splenic artery and vein branches. Reconstruction *via* Roux-en-Y PJ or PG should not increase the risk of bleeding when performed by experienced surgeons. A further advantage of MSP was its shorter operation time compared with PD. Interestingly, operation time was not different between MSP and EDP, even though PJ or PG was necessary with MSP.

Compared with PD and EDP, MSP was not associated with increased mortality, but was associated with greater postoperative morbidity, notably a higher frequency of pancreatic fistula noted in previous studies^[5,11-18]. Similar to these reports, the fistula rate after MSP in our study was 42% (15/36), higher than that after PD (20.5%), but was not significantly different to that after EDP (31%). There was no significant difference in pancreatic fistulas rate between PJ (9/22) and PG (6/14) reconstruction methods in MSP group. However, all of the pancreatic fistulae in our series corresponded to ISGPF grade A or B, and were sealed by conservative measures. This may explain why the postoperative hospital time was not extended following MSP as compared with PD or EDP. Based on these data, we believe that MSP is a safe operation with morbidity and mortality rates comparable with those of PD or EDP.

PD and EDP result in marked deteriorations in pancreatic exocrine and endocrine functions. For example, Shikano *et al*^[17] reported that the incidence of DM ranged from 10% to 24% after PD and from 8% to 60% after EDP in patients with normal pancreatic parenchyma. However, in patients with chronic pancreatitis, the inci-

dence of DM increases to 40% and 85% after PD and DP, respectively. Meanwhile, the incidence of impaired pancreatic exocrine function ranged from 30% to 60%, even in the absence of chronic pancreatitis. However, the major advantage of MSP is the potential to retain more pancreatic exocrine and endocrine function than with PD and EDP. In their literature review, Allendorf *et al*^[6] reported no cases of impaired exocrine function and only two cases of abnormal endocrine function among 26 patients who underwent MSP. In another literature review, the rate of exocrine insufficiency was 5% and the rate of endocrine insufficiency was 4% among 100 patients who underwent MSP^[10]. In the current series, one of 36 patients developed new-onset DM following MSP, *vs* 21.7% of patients (5/23) after EDP and 14.3% of patients (6/42) after PD. Moreover, none of the patients in the MSP group required pancreatic enzyme substitution, *vs* 33.3% (14/42) and 21.7% (5/23) of patients after PD and EDP, respectively. Matched-pairs analysis comparing MSP with PD or EDP confirmed the superiority of this organ-preserving procedure. Furthermore, long-term exocrine and endocrine pancreatic function was significantly better preserved after MSP than after PD or EDP.

It is important to consider that MSP provides inadequate tissue resection for cancers because of incomplete dissection of soft tissue and nodes. Furthermore, it does not remove the putative lymphatic and venous drainage bed along the distal pancreas and at the splenic hilum from which many malignant pancreatic tumors are thought to spread. Therefore, MSP is contraindicated for invasive pancreatic tumors and is currently limited to benign and low-malignant potential conditions, such as intraductal papillary mucinous neoplasms, mucinous cystic neoplasms, serous cystadenomas, solid pseudopapillary neoplasms, endocrine tumors and other less frequent benign lesions. We have performed this procedure in two patients with ductal adenocarcinoma who could not tolerate a long surgical procedure, and the tumor recurred

3 and 6 mo after surgery in these patients. However, no recurrence was observed in our current series of patients with benign and low malignant potential lesions. Similarly, Adham *et al*^[9] reported no recurrence in 50 patients with non-invasive lesions who underwent MSP. The highest frequency of tumor recurrence was found in Sauvanet *et al*^[7] review in which four of 53 patients developed recurrence. We believe that providing an adequate tumor-free margin is necessary to avoid recurrence, even in benign and low malignant potential lesions.

In conclusion, MSP is a safe and organ-preserving option for benign or low-grade malignant lesions in the neck and proximal body of the pancreas. MSP offers naturally better long-term preservation of pancreatic exocrine and endocrine functions.

COMMENTS

Background

Whether patients with focal pancreatic lesions of benign or low-grade malignant pathology should be treated by middle segmental pancreatectomy (MSP) rather than by classic procedures, such as pancreaticoduodenectomy (PD) and extended distal pancreatectomy (EDP), is controversial. This study evaluated the feasibility and safety of MSP compared with PD and EDP.

Research frontiers

Clinically, surgical treatment for benign or low-grade malignant lesions of the pancreatic neck and body is often performed using 'traditional' procedures such as PD or EDP. However, these approaches result in a significant and unnecessary loss of normal pancreatic parenchyma with subsequent impairment of exocrine and endocrine functions. In the surgical treatment for benign or low-grade malignant lesions of the pancreatic neck and body, the research hotspot is how to ensure the perioperative safety and improve the long-term effects, including the preservation of pancreatic endocrine and exocrine function.

Innovations and breakthroughs

MSP has increasingly been applied for some lesions, including chronic pancreatitis, and benign and borderline lesions localized at the neck and body of pancreas. Several recent reports have compared the morbidity and quality of life in patients with chronic pancreatitis, and benign and low-grade malignant pancreatic tumors after MSP or traditional surgical procedures. The study focuses not only on the long-term effects, but also on the perioperative safety and the early postoperative patients' nutritional status after MSP. Compared with PD or EDP, MSP provided better nutritional status and postoperative preservation of pancreatic exocrine and endocrine functions.

Applications

MSP is a safe and organ-preserving option for benign or low-grade malignant lesions in the neck and proximal body of the pancreas.

Terminology

MSP: The head and tail of the pancreas were preserved, only the middle pancreas was removed when the lesions were located in the neck and proximal body of the pancreas.

Peer review

This is an interesting study that evaluated the feasibility and safety of MSP compared with PD and EDP. The results suggest that MSP is a safe and organ-preserving option for appropriate lesions in the neck and proximal body of the pancreas. MSP also provided better postoperative preservation of pancreatic exocrine and endocrine functions.

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Five-year follow-up of 263 cases of functional bowel disorder

Yu-Rong Tang, Ping Wang, Rui Yin, Jian-Xin Ge, Guo-Pin Wang, Lin Lin

Yu-Rong Tang, Ping Wang, Rui Yin, Jian-Xin Ge, Guo-Pin Wang, Department of Gastroenterology, Nanjing Jiangbei People's Hospital, Medical School, Southeast University, 552 Geguan Road, Nanjing 210048, Jiangsu Province, China

Yu-Rong Tang, Lin Lin, Department of Gastroenterology, The First Affiliated Hospital of Nanjing Medical University, 300 Guangzhou Road, Nanjing 210029, Jiangsu Province, China

Author contributions: Tang YR and Wang P analyzed the data and wrote the manuscript; Lin L designed the research and approved the final paper; Yin R collected and input the data; Ge JX and Yin R analyzed the data; Wang GP approved the final paper.

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Correspondence to: Lin Lin, PhD, Department of Gastroenterology, the First Affiliated Hospital of Nanjing Medical University, Nanjing 210029, Jiangsu Province, China. lin9100@yahoo.com.cn

Telephone: +86-25-83718836 Fax: +86-25-68136920

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Abstract

AIM: To determine the mortality associated with functional bowel disorders (FBDs) and their possible relationship with organic bowel disease.

METHODS: Patients who satisfied the Rome III criteria for FBD (retrospective diagnosis) were followed up by telephone interview and/or outpatient review at 5 years after their first attendance. The patients were divided into the following groups: irritable bowel syndrome, functional abdominal bloating, functional constipation, functional diarrhea and unspecified FBD. The survival of the FBD patients overall and of those with each FBD were compared with data obtained from the Guangzhou population in 2005. The incidences of colonic cancer overall and for each FBD were compared with data from

the Chinese population obtained from 56 cancer registries in 19 provinces of the country in 2008.

RESULTS: Two hundred and sixty-three patients were followed-up. Five patients died, which was not significantly different from the expected survival rate. No differences in mortality among the FBDs were found. There were nine cases of organic bowel disease: three colonic cancers and six colonic polyps. The incidence of colonic cancer in FBD patients was higher than that in the general Chinese population (0.23% vs 0.03%, $P < 0.05$). There were significant differences in the incidence of colonic cancer among the FBDs (0/134, 0/24, 2/29, 1/66, 0/10, respectively, $P < 0.05$); functional constipation was the most common. The incidence of colonic polyps was similar among the FBDs. The baseline age of patients who died was greater than that of those who survived (66.60 ± 6.84 years vs 45.14 ± 10.34 years, $P < 0.05$). The baseline age of patients who had colonic cancer or polyps during follow-up was greater than that of those without colonic cancer or polyps (60.33 ± 1.53 years vs 45.38 ± 10.62 years; 54.50 ± 6.47 years vs 45.34 ± 10.68 years, $P < 0.05$).

CONCLUSION: FBDs do not increase the risk of death. The incidence of colonic cancer in patients with FBDs may be increased, especially in those with functional constipation and in the elderly.

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Key words: Functional bowel disorders; Follow-up; Mortality; Colonic cancer; Colonic polyps

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INTRODUCTION

Functional gastrointestinal disorders (FGIDs) are common in populations of various ethnicities and can seriously affect patients' quality of life^[1,2]. Functional bowel disorders (FBDs) are the most common FGIDs and include irritable bowel syndrome (IBS), functional abdominal bloating, functional constipation, functional diarrhea and unspecified functional bowel disorder^[3,4]. FBDs are common in the Chinese population. Epidemiological investigations have found the prevalence of IBS to be 5.7% and 10.5% in Guangzhou and Beijing, respectively^[5,6], IBS patients account for 11.3% of gastroenterology department outpatients^[7], and the prevalence of functional constipation is about 3.0% in Guangzhou residents^[8]. The extent and outcomes of FBD in the Chinese population are a cause of widespread concern.

FBDs are defined by symptom-based diagnostic criteria that combine chronic or recurrent symptoms attributable to the gastrointestinal (GI) tract in the absence of other, pathologically based disorders according to the Rome III criteria^[3]. In recent years, technological advances and new research have revealed pathophysiologic changes in FBDs, including bowel motility disorder, visceral hypersensitivity, changes in the intestinal flora and psychologic abnormalities^[9-13]. It has been thought that these do not affect patient survival, and a recent study reported that functional dyspepsia was not associated with increased mortality in the community, though the data for any effect of IBS on survival were less clear^[14]. Research from Europe and the United States suggest that FBDs are closely related to certain organic bowel diseases; for example, functional constipation and colorectal cancer, or IBS and inflammatory bowel disease (IBD)^[15-18]. However, such views are controversial.

No study has shown whether FBDs increase the risk of death or whether there is a link between FBDs and organic bowel disease in the Chinese population. In the present study, we followed up 263 outpatients with FBDs for 5 years to determine their survival and incidence of organic bowel disease.

MATERIALS AND METHODS

Subjects

As shown in Figure 1, data were collected from outpatients who attended the Department of Gastroenterology at Nanjing Jiangbei People's Hospital between January, 2005 and December, 2006 and reported mainly bowel symptoms, such as abdominal pain or discomfort, bloating, swelling or abnormal bowel movements. Patients retrospectively diagnosed with FBD according to the Rome III criteria^[3] and who agreed to follow-up were included in the study. All patients underwent routine blood, urine and stool hemocult tests, stool form examination and endoscopy of the GI tract. Patients younger than 18 years or with a structural bowel disease or a history of abdominal surgery were excluded. Pregnant patients were not included. All patients included in the study were fol-

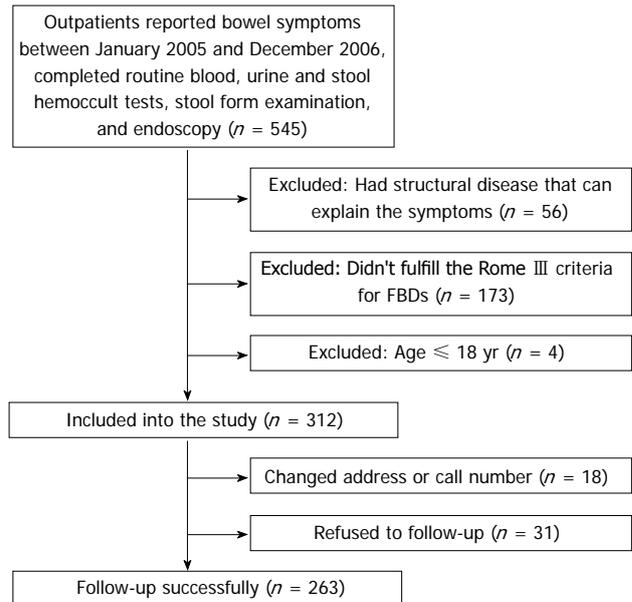


Figure 1 Patient status. FBD: Functional bowel disorder.

lowed up in June, 2012.

Measures

Two hundred and sixty-three patients with FBDs were followed up for more than 5 years and their survival and incidence of organic bowel disease recorded.

Patient groups: Based on the Rome III diagnostic criteria, the patients were divided into the following groups: IBS, functional abdominal bloating, functional constipation, functional diarrhea and unspecified FBD^[3].

Baseline data obtained: Baseline data for each subject were obtained from outpatient medical records, including age, gender, place of residence, and the duration and nature of symptoms.

Follow-up methods and content: All patients were followed up by telephone to ascertain: (1) survival (in the case of patients who had died, details of the cause and date of death were obtained); and (2) the presence of organic bowel disease and date of diagnosis. All surviving patients were asked to undergo review routine blood, urine and stool hemocult tests, stool form examination, and endoscopy or radiographic examination of the GI tract.

Statistical analysis

All data were analyzed using SPSS version 19.0. Statistical significance was set at $P < 0.05$. Categorical data were analyzed using the χ^2 test. All measurement data were reported as the mean \pm SD and analyzed using the t test (between two groups) or one-way analysis of variance (among more than two groups). Kaplan-Meier estimates of overall survival observed *vs* expected for the whole cohort. Expected survival was based on the whole cohort using survival characteristics of the Guangzhou popula-

Table 1 Baseline clinic data for each functional bowel disorder

| FBD | n (%) | Age (yr) (mean ± SD) | Gender (F/M) | Residence (urban/rural) | Duration of FBD |
|-------------------------------|------------|-------------------------|-----------------|----------------------------|--------------------|
| IBS | 134 (47.1) | 45.44 ± 10.32 | 63/71 | 68/66 | 6 mo-20 yr |
| Functional abdominal bloating | 24 (9.1) | 43.17 ± 11.86 | 10/14 | 13/11 | 6 mo-17 yr |
| Functional constipation | 29 (11.0) | 46.59 ± 13.22 | 10/19 | 12/17 | 6 mo-20 yr |
| Functional diarrhea | 66 (25.1) | 46.56 ± 9.34 | 41/25 | 34/32 | 6 mo-20 yr |
| Unspecified FBD | 10 (3.8) | 41.90 ± 13.40 | 4/6 | 4/6 | 6 mo-10 yr |

No differences were found in age, gender, place of residence (urban or rural) or duration of functional bowel disorder (FBD) among groups (all $P > 0.05$). F: Female; M: Male; IBS: Irritable bowel syndrome.

Table 2 Details of patient deaths during follow-up

| Details | n/N (%) |
|-------------------------------|---------------------------|
| Age (yr), mean ± SD | |
| Baseline | 66.60 ± 6.84 ^a |
| Death | 70.40 ± 5.86 |
| Gender | |
| Female | 2/135 (1.48) |
| Male | 3/128 (2.34) |
| Residence | |
| Urban | 3/133 (2.26) |
| Rural | 2/130 (1.54) |
| Cause of death | |
| Cardiovascular | 2 |
| Gastric cancer | 1 |
| Colonic cancer | 2 |
| Diagnosis at baseline | |
| IBS | 1/134 (0.75) |
| Functional abdominal bloating | 1/24 (4.17) |
| Functional constipation | 2/29 (6.90) |
| Functional diarrhea | 1/66 (1.52) |
| Unspecified FBD | 0/10 (0) |

^a $P < 0.001$ vs baseline in patients who survived; no differences in survival were found between genders, places of residence or functional bowel disorder (FBD) groups (all $P > 0.05$). IBS: Irritable bowel syndrome.

tion from 2005^[19]. The incidence of colonic cancer in FBD patients was compared with data from the Chinese population obtained from 56 cancer registries in 19 provinces of the country in 2008^[20], using Fisher's exact test. Survival and the incidence of colonic cancer and polyps in different age, gender and residence groups and in each FBD group were compared using Fisher's exact test.

RESULTS

Baseline data

As shown in Figure 1, 312 patients were included in the study and 263 FBD patients were followed up; a drop-out rate of 15.71%. At baseline, these patients were aged 20-74 years (45.55 ± 10.68 years) and comprised 128 (48.66%) males and 135 (51.33%) females. The duration of FBD ranged from 6 mo to 20 years, with a median du-

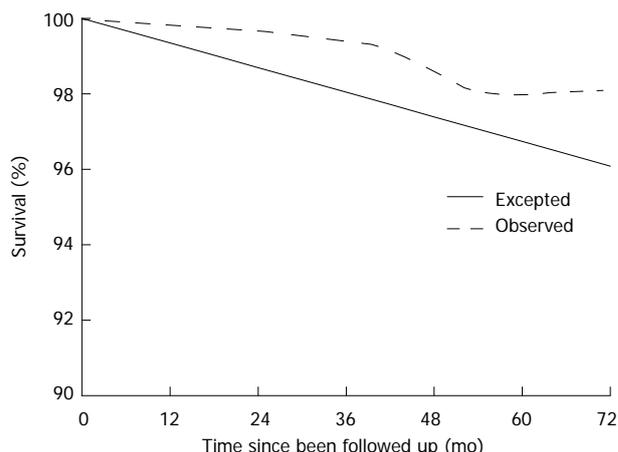


Figure 2 Observed vs expected survival for all functional bowel disease patients. There was no significant difference between the observed and expected survival ($P > 0.05$).

ration of 1 year.

Clinical data for each FBD group are shown in Table 1. IBS was the most common FBD in these patients, followed by functional diarrhea, functional constipation, functional abdominal bloating and unspecified FBD. There were no differences in age, gender, place of residence (urban or rural) or duration of FBD between groups (all $P > 0.05$).

Follow-up data

Survival: Five (1.90%) deaths had been reported in the cohort by the time of the last follow-up interview in June, 2012. Table 2 provides details of the deaths. The average age of the patients who died was greater at baseline than that of those who survived (66.60 ± 6.84 years vs 45.14 ± 10.34 years, $t = 4.617$, $P < 0.01$). There were no differences in mortality between genders or with place of residence (both $P > 0.05$). No differences of mortality in the various FBDs were found ($P > 0.05$). Expected survival was based on the whole cohort using survival characteristics of the Guangzhou population from 2005^[19]. Overall observed vs expected survival for the whole set of respondents is illustrated in Figure 2; the data suggest there was no significant difference between observed and expected ($P > 0.05$).

Detection of organic bowel disease: Organic bowel disease was detected in nine cases (3.42%): colonic cancer in three (1.14%) and colonic polyps in six (2.28%). Table 3 gives the details of the organic bowel diseases observed. The average age of patients with colonic cancer or colonic polyps was greater at baseline than that of the other patients (60.33 ± 1.53 years vs 45.38 ± 10.62 years, $t = 13.582$, $P < 0.01$; 54.50 ± 6.47 years vs 45.34 ± 10.68 years, $t = 2.089$, $P < 0.05$). There were no differences in the incidence of colonic cancer or polyps between genders or with place of residence (all $P > 0.05$). There were significant differences in the incidence of colonic cancer between the various FBDs ($P < 0.05$, Fisher's exact test); the incidence was highest in the functional constipation

Table 3 Details of organic bowel diseases observed in functional bowel disorder patients

| Details | n/N (%) |
|-------------------------------|---------------------------|
| Colonic cancer | |
| Age (yr), mean ± SD | |
| Baseline | 60.33 ± 1.53 ^b |
| Cancer observed | 63.67 ± 2.08 |
| Gender | |
| Female | 1/135 (0.74) |
| Male | 2/128 (1.56) |
| Residence | |
| Urban | 2/133 (1.50) |
| Rural | 1/130 (0.77) |
| Diagnosis at baseline | |
| IBS | 0/134 (0) |
| Functional abdominal bloating | 0/24 (0) |
| Functional constipation | 2/29 (6.70) ^c |
| Functional diarrhea | 1/66 (1.52) |
| Unspecified FBD | 0/10 (0) |
| Colonic polyps | |
| Age (yr), mean ± SD | |
| Baseline | 54.50 ± 6.47 ^c |
| Polyps observed | 57.83 ± 6.52 |
| Gender | |
| Female | 2/135 (1.48) |
| Male | 4/128 (3.13) |
| Residence | |
| Urban | 2/133 (1.50) |
| Rural | 4/130 (3.08) |
| Diagnosis at baseline | |
| IBS | 5/134 (3.62) |
| Functional abdominal bloating | 0/24 (0) |
| Functional constipation | 0/29 (0) |
| Functional diarrhea | 1/66 (1.52) |
| Unspecified FBD | 0/10 (0) |

^b $P < 0.01$ vs baseline in patients without colonic cancer; ^c $P < 0.05$ vs baseline in patients without colonic polyps; ^e $P < 0.05$ vs IBS but not other FBD groups; no differences in the incidence of colonic cancer or polyps were found between genders and places of residence and no differences in the incidence of colonic polyps were found among FBD groups (all $P > 0.05$). FBD: Functional bowel disorder; IBS: Irritable bowel syndrome.

group, being significantly higher than in the IBS group ($P < 0.05$, Fisher's exact test), though not significantly different from the incidence in the other FBD groups ($P > 0.05$). The incidence of colonic polyps was similar between the various FBDs ($P > 0.05$). Compared with data from the general Chinese population obtained from 56 cancer registries in 19 provinces of the country, the incidence of colonic cancer was significantly increased in patients with FBDs ($P < 0.01$, Fisher's exact test; Figure 3).

DISCUSSION

FBDs have long been considered a group of functional diseases in patients without any pathologically based disorder^[3] that do not affect survival and are not associated with organic disease. Recent research from Europe and the United States, however, suggests that FBDs are closely related to certain organic bowel diseases - for example, functional constipation and colorectal cancer, or IBS and IBD^[15-18] - and do have an effect on survival. No

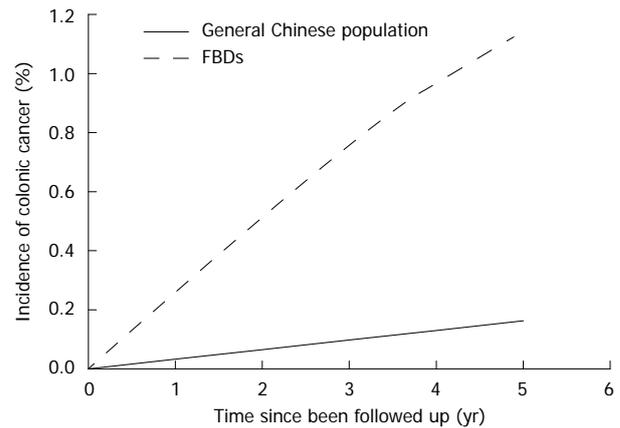


Figure 3 Incidence of colonic cancer in functional bowel disorder patients and in the general Chinese population. Compared with data from the general Chinese population obtained from 56 cancer registries in 19 provinces of the country, the incidence of colonic cancer was significantly increased in patients with functional bowel disorders (FBDs) ($P < 0.01$, Fisher's exact test).

study has investigated the association between FBDs and organic bowel disease, or whether FBDs increase the risk of death, in the Chinese population. In the present study, we followed up 263 outpatients with FBDs for 5 years, revealing new information.

The mortality of the FBD patients was 1.90% over the 5-year follow-up period, equivalent to an average annual mortality of approximately 0.38%. A death spectrum and potential life lost investigation found a crude death rate of 652.3/100 000 (approximately 0.65%) in the Guangzhou population in 2005^[19]. Compared with this population, our FBD patients showed no trend towards increased mortality. We can conclude that FBD does not increase the risk of death in Chinese patients. We found no differences in mortality among the various FBDs.

Data from 56 cancer registries in 19 provinces of China from 2008 shows the incidence of colorectal cancer to be 31.39/100 000 (approximately 0.03%) in the Chinese population^[20]. In this study, three cases (1.14%) of colonic cancer were detected, equivalent to an average annual mortality of approximately 0.23%. The incidence of colonic cancer in patients with FBDs was thus significantly increased.

Significant differences in the incidence of colonic cancer were found between the FBDs; that of functional constipation was up to 6.70%, equivalent to an average annual incidence of approximately 1.34%. This was significantly higher than in the other FBDs, and also higher than in the general Chinese population (approximately 0.03%). A study based on the Japanese population suggests that the number of bowel movements is closely related to the incidence of colorectal cancer^[21]. However, European and American studies have shown that constipation itself does not induce colorectal cancer, but use of cathartics (especially anthraquinones or phenolphthalein) may increase the incidence of both colorectal adenomas and colorectal cancer^[22,23].

During the 5 years of follow-up in the present study,

six cases (2.28%) of colonic polyps were observed. Several studies have reported the detection rate of colonic polyps in outpatients under colonoscopy, but no data are available regarding the annual incidence of colonic polyps in the Chinese population. Thus, we are unable to assess the risk of colon polyps in FBDs.

There were no differences in mortality or in the incidence of colonic polyps or cancer between genders or with place of residence. The average age at baseline of the patients who died during follow-up or who were found to have colonic cancer and polyps was greater than the average age at baseline of the patients who survived or did not have colonic cancer or polyps. We therefore suggest that the possibility of organic bowel disease should be considered in FBD patients over the age of 50 years, and that repeated review by colonoscopy is necessary after the diagnosis of FBD is established.

In conclusion, FBDs did not in general increase the risk of death assessed over 5 years in a Chinese population. Compared with the general population, the incidence of colonic cancer may be increased in FBD patients, especially those with functional constipation. These conclusions are based on the follow-up investigation of a small sample of FBD patients. A larger sample needs to be observed, control data should be collected from the general population over the same period, and a rigorous prospective study must be designed to verify our results.

COMMENTS

Background

Functional gastrointestinal disorders (FGIDs) are common in populations of various ethnicities and can severely affect patients' quality of life. Functional bowel disorders (FBDs) are the most common FGIDs and are common in the Chinese population. The extent and outcomes of FBD in the Chinese population are a cause of widespread concern.

Research frontiers

In recent years, research from Europe and the United States suggests that FBDs are closely related to certain organic bowel diseases; for example, functional constipation and colorectal cancer, or irritable bowel syndrome and inflammatory bowel disease. Whether FBDs increase the risk of death is controversial.

Innovations and breakthroughs

No study has shown whether FBDs increase the risk of death or whether there is a link between FBDs and organic bowel disease in the Chinese population. In the present study, authors followed up 263 outpatients with FBDs over 5 years to determine their survival and the incidence of organic bowel disease.

Applications

The study results suggest FBDs did not increase the risk of death over 5 years, though the incidence of colonic cancer in patients with FBDs may be increased, especially in those with functional constipation. The authors therefore suggest that the possibility of organic bowel disease should be considered in FBDs, especially in functional constipation, and repeated review by colonoscopy is necessary after the diagnosis of FBD is established.

Terminology

FBDs are defined by symptom-based diagnostic criteria that combine chronic or recurrent symptoms attributable to the gastrointestinal tract in the absence of other, pathologically based disorders according to the Rome III criteria. FBDs are the most common functional gastrointestinal disorders and include irritable bowel syndrome, functional abdominal bloating, functional constipation, functional diarrhea and unspecified functional bowel disorder.

Peer review

This is an interesting and well written manuscript examining the morbidity and

mortality of FBDs in China. This has not really been examined before and is important to the extent that certain patients with FBDs may benefit from improved cancer surveillance. This manuscript describes a small, preliminary epidemiologic study of mortality and the development of organic bowel disease in Chinese patients diagnosed with functional bowel disorders over 5 years.

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3.0 T proton magnetic resonance spectroscopy of the liver: Quantification of choline

Li Xu, Bo Liu, Yan Huang, Xian Liu, Si-Wei Zhang, Xue-Gang Xin, Jin-Zhi Zheng

Li Xu, Bo Liu, Xian Liu, Si-Wei Zhang, Ji-Zhi Zheng, Department of Radiology, Guangdong Provincial Traditional Chinese Medicine Hospital and Postdoctoral Mobile Research Station of Guangzhou University of Traditional Chinese Medicine, Guangzhou 510120, Guangdong Province, China

Yan Huang, Department of Neurology, Guangdong Provincial Traditional Chinese Medicine Hospital and Postdoctoral Mobile Research Station of Guangzhou University of Traditional Chinese Medicine, Guangzhou 510120, Guangdong Province, China

Xue-Gang Xin, School of Biomedical Engineering, Southern Medical University, Guangzhou 510515, China

Author contributions: Xu L, Liu B, Huang Y, Liu X and Zhang SW contributed equally to this work; Xu L performed the majority of experiments and wrote the manuscript; Liu B, Huang Y, Liu X, Zhang SW, Xin XG and Zheng JZ provided vital reagents and analytical tools and were also involved in revising the manuscript; Xu L designed the study.

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Correspondence to: Li Xu, MD, Department of Radiology, Guangdong Provincial Traditional Chinese Medicine Hospital and Postdoctoral Mobile Research Station of Guangzhou University of Traditional Chinese Medicine, 111 Da De Lu, Guangzhou 510120, Guangdong Province, China. 985592610@qq.com
Telephone: +86-20-81887233 Fax: +86-20-81887233

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Abstract

AIM: To investigate the normal hepatic magnetic resonance spectroscopy findings choline/lipid2 (Cho/Lip2) associated with age and body mass index (BMI).

METHODS: A total of 58 single-voxel proton spectra of the liver were acquired at 3.0 T using the eight-channel phased array abdominal coil as the receiver coil. Consecutive stacks of breath-hold spectra were acquired using the point resolved spectroscopy technique at a short echo time of 30 ms and a repetition time of 1500 ms. The spectra were processed with the SAGE software package. Areas and heights for metabolite resonance were obtained. Student's *t* test for unpaired data was used for comparisons of shimming, Cho/Lip2, and lipid content.

RESULTS: There were significant negative correlations between the Cho/Lip2 peak height ratios and BMI ($r = -0.615$) and age ($r = -0.398$) (all $P < 0.01$). Compared with the high-BMI group, the low-BMI group was younger (39.1 ± 13.0 years vs 47.6 ± 8.5 years, $t = -2.954$, $P = 0.005$); had better water suppression ($93.4\% \pm 1.4\%$ vs $85.6\% \pm 11.6\%$, $t = 2.741$, $P = 0.014$); had higher Cho/Lip2 peak heights ratio (0.2 ± 0.14 vs 0.05 ± 0.04 , $t = 6.033$, $P < 0.000$); and had lower lipid content (0.03 ± 0.08 vs 0.29 ± 0.31 , $t = -3.309$, $P = 0.004$). Compared with the older group, the younger group had better shimming effects (17.1 ± 3.6 Hz vs 22.0 ± 6.8 Hz, $t = -2.919$, $P = 0.008$); higher Cho/Lip2 peak heights ratios (0.03 ± 0.05 vs 0.09 ± 0.12 , $t = 2.4$, $P = 0.020$); and lower lipid content (0.05 ± 0.11 vs 0.23 ± 0.32 , $t = -2.337$, $P = 0.031$). Compared with the low-choline peak group, the high-choline peak group had lower lipid content (0.005 ± 0.002 vs 0.13 ± 0.23 , $t = -3.796$, $P < 0.000$); lower BMI (19.6 ± 2.4 vs 23.9 ± 3.0 , $t = -4.410$, $P < 0.000$); and younger age (34.7 ± 10.0 years vs 43.2 ± 12.5 years, $t = -2.088$, $P = 0.041$).

CONCLUSION: Lipid accumulation could result from the increased fat in the body depending on age and BMI. Lipid can mask the resonance signal of choline.

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Key words: Magnetic resonance spectroscopy; High-field imaging; Choline

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INTRODUCTION

Magnetic resonance spectroscopy (MRS) is a non-invasive technique that is being increasingly applied to describe biochemical changes in the liver^[1-5]. The recent installation of higher field strength (3 T) clinical magnets with multicoil arrays offers new opportunities for performing whole-body MRS. The improved signal-to-noise ratio (SNR) can reduce acquisition times, and the higher field strength also provides better separation of resonances^[6,7].

Choline is a precursor of acetylcholine and a component of the phospholipid metabolism of cell membranes. It is known that MRS may be used to diagnose malignancy; usually by measuring the choline peak. Absolute quantification of hepatic metabolite concentrations offers several advantages for the evaluation of *in vivo* MRS data. Unfortunately, absolute quantification of choline-containing compounds is impractical for most clinical applications. A few studies of *in vivo* MRS have reported an increase in choline-containing compounds relative to lipids within tumors such as hepatocellular carcinoma, and a reduction in the lipid-to-choline ratio after transarterial embolization for hepatocellular carcinoma. However, the ability to distinguish reliably benign and malignant tumors from normal liver parenchyma has yet to be established. A major limitation is the observation that relatively large amounts of choline-containing compounds may occur even in normal liver^[8-13].

Knowledge of the normal findings associated with age and body mass index (BMI) is valuable. Therefore, the central questions of our study were: (1) does choline/lipid2 (Cho/Lip2) have a relationship with BMI? and (2) does Cho/Lip2 have a relationship with age? Finally, we try to explain why no observable choline peak was detected on liver MRS in obese and elderly individuals.

MATERIALS AND METHODS

Patients

The study was approved by our institutional review board, and written informed consent was obtained from all patients. We evaluated non-hepatic disease and fatty liver in 58 patients (29 men, 29 women; age range, 19-65 years; median age, 42 years) with no history of liver disease and with normal liver function test results. The mean height of the cohort was 164.4 ± 7.4 cm (range, 150-178 cm), mean body weight was 62.7 ± 11.3 kg (range, 39-93 kg), and mean BMI was 23.1 ± 3.3 (range, 16.9-30.4).

MRS protocol

MRS was performed using a GE Signa 3.0 T whole-body

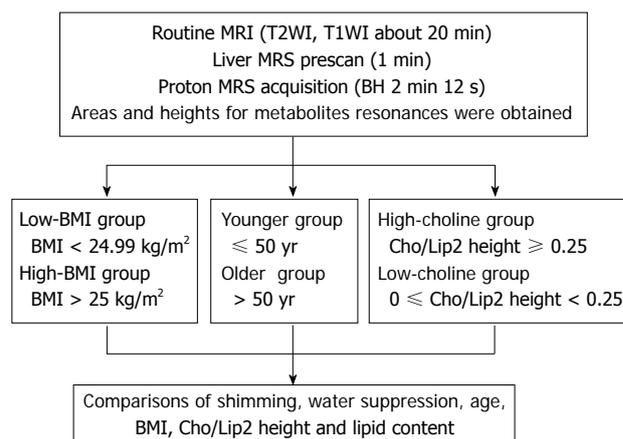


Figure 1 Detailed scanning protocols and statistical analysis. BMI: Body mass index; MRS: Magnetic resonance spectroscopy; Cho/Lip2: Choline/lipid2; MRI: Magnetic resonance imaging.

system (GE Signa Excite HD; GE Medical Systems, Milwaukee, WI, United States) with the standard proton MRS acquisition software provided by the manufacturer. The body coil was used as the transmitter, and a torso phased-array coil (eight coils, four anterior and four posterior coils; Waukesha, WI, United States) was used as the receiver. Single-volume spin-echo point-resolved spectroscopy was used with parameters of 1500/30/64 [repetition time (TR)/echo time (TE)/excitations] in all patients. The patients entered the magnetic field in the supine position with their feet first. The total acquisition time (2 min 12 s per scan) was split into consecutive blocks to match the length of a breath-hold period of about 15-40 s, while data acquisition was performed at end expiration. Volume of interest of 2 cm × 2 cm × 2 cm was localized in the middle portion of the right hepatic lobe, on the basis of T2-weighted single-shot fast spin-echo pictures in the transverse planes with a TR of 935 ms, TE of 83 ms, a matrix of 288 × 192, field of view of 40 cm, section thickness of 8 mm, an intersection gap of 1.5 mm, and a number of excitations of 0.62. The voxel was localized in such a way as to avoid large vessels, bile ducts, and fatty tissue (Figure 1).

After acquisition, data were processed by using the MR spectroscopic analysis package provided by the manufacturer (SAGE 7.1; GE Medical Systems). The raw data were zero-filled once, apodized with a 5-Hz Gaussian filter, Fourier transformed, and phase and baseline corrected. Marquardt curve fitting was performed by using a Gaussian line shape to calculate the area under the peak. MR spectroscopic data were analyzed by a single medical physicist (Xu L) with > 7 years experience in MRS analysis. For each MRS measurement of unsuppressed water, we normalized the amplitude of the lipid signal to the sum of the lipid plus water signals to obtain the percentage lipid within the liver.

For all data acquisition, water suppression was performed using a series of three chemical-shift-selective pulses with predefined flip angles to leave a significant amount of residual water in the spectrum, and high-order

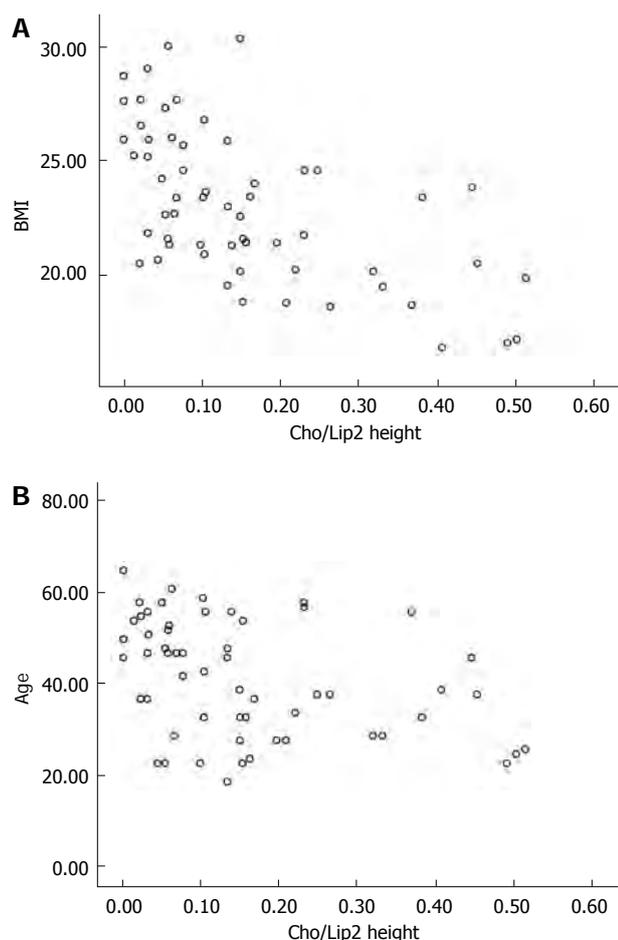


Figure 2 Scatter plots of relationships. A: Between choline/lipid2 (Cho/Lip2) and body mass index (BMI), a good inverse correlation was observed; B: Between Cho/Lip2 and age, a moderate inverse correlation was observed.

shimming followed by automatic local shimming adjustment was used. Line widths (full-width half-maximum) and water suppression were obtained.

Statistical analysis

The study group was divided into two subgroups: low BMI (< 24.99 kg/m²) and high BMI group (> 25 kg/m²). The participants were divided into two subgroups according to age: younger (≤ 50 years) and older (> 50 years). The participants were divided into two subgroups based on the Cho/Lip2 ratio: high choline (Cho/Lip2 ≥ 0.25) and low/non-choline (0 ≤ Cho/Lip2 < 0.25) (Figure 1).

For all tests, *P* < 0.05 was considered to indicate statistically significant differences. Statistical analysis was performed using SPSS version 10.0.1 (SPSS, Chicago, IL, United States). Using Spearman's correlation, we determined the relationship between Cho/Lip2 and BMI, and between Cho/Lip2 and age.

Student's *t* test for unpaired data was used for comparison of shimming, Cho/Lip2 and lipid content between the low-BMI and high-BMI groups, and between the younger and older groups. Student's *t* test for unpaired data was also used for comparison of shimming,

BMI and age between the high-choline and non-choline groups.

RESULTS

Scatter plots and Spearman's correlations

The scatter plots were used to reveal the relationships between the variables (Figure 2). There were significant negative correlations between the Cho/Lip2 peak heights ratios and BMI (*r* = -0.615, *P* < 0.000), and age (*r* = -0.398, *P* = 0.002).

Comparison between low-BMI and high-BMI group

Compared with the high-BMI group, the low-BMI group was younger (39.1 ± 13.0 years *vs* 47.6 ± 8.5 years, *t* = -2.954, *P* = 0.005); had better water suppression (93.4% ± 1.4% *vs* 85.6% ± 11.6%, *t* = 2.741, *P* = 0.014); had higher Cho/Lip2 peak heights ratios (0.20 ± 0.14 *vs* 0.05 ± 0.04, *t* = 6.033, *P* < 0.000); and had lower lipid content (0.03 ± 0.08 *vs* 0.29 ± 0.31, *t* = -3.309, *P* = 0.004) (Figure 3).

Comparison between younger and older groups

Compared with the older group, the younger group had better shimming effects (17.1 ± 3.6 Hz *vs* 22.0 ± 6.8 Hz, *t* = -2.919, *P* = 0.008); higher Cho/Lip2 peak heights ratios (0.03 ± 0.05 *vs* 0.09 ± 0.12, *t* = 2.4, *P* = 0.020); and lower lipid content (0.05 ± 0.11 *vs* 0.23 ± 0.32, *t* = -2.337, *P* = 0.031) (Figure 3).

Comparison between high-choline and low-choline groups

Compared with the low-choline group, the high-choline group had lower lipid content (0.005 ± 0.002 *vs* 0.13 ± 0.23, *t* = -3.796, *P* < 0.000); lower BMI (19.6 ± 2.4 *vs* 23.9 ± 3.0, *t* = -4.410, *P* < 0.000); and younger age (34.7 ± 10.0 years *vs* 43.2 ± 12.5 years, *t* = -2.088, *P* = 0.041) (Figure 4).

DISCUSSION

MRS at 3.0 T provides improved SNR and spectral resolution compared with 1.5 T MRI scanners, therefore, it is expected to yield more reliable measurements of metabolite concentrations^[6,8,14]. The principal metabolite that has been targeted in focal liver disease is choline. In general, choline is elevated in tumors, because choline is a cell membrane component and increased cell turnover is associated with malignancy. *In vivo* ¹H MRS has proved valuable in the diagnosis of tumors in the brain, prostate, breast, and uterine cervix. ¹H MRS is also useful for evaluation of treatment responses in malignant tumors of the head and neck, as well as in breast cancer^[1,13,15-17].

However, the ability to distinguish reliably benign and malignant tumors from normal liver parenchyma has yet to be established. According to the data from our study, the lipid content in liver parenchyma increased with both age and BMI; there were significant negative correlations between Cho/Lip2 and age; and there was no observ-

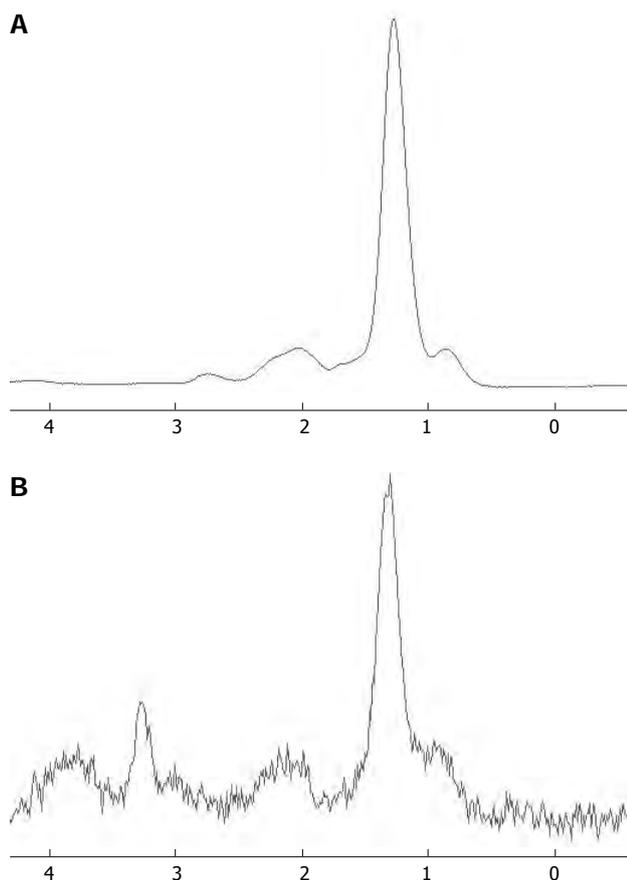


Figure 3 Point resolved spectroscopy-localized single voxel ¹H magnetic resonance spectrum. A: Originating from liver parenchyma of an obese and elderly (body mass index = 30.04, 52 years) volunteer. No observable choline peak was detected at 3.2 ppm; B: Originating from liver parenchyma of a lean and young volunteer (body mass index = 18.87, 23 years). High-choline peak was detected at 3.2 ppm.

able choline peak in obese and elderly individuals. Possible reasons for the above conclusions include: (1) the lipid content in liver parenchyma increased with both age and BMI; and (2) the choline level in liver parenchyma changed with both age and BMI.

The signals of the corresponding lipid groups can be observed at 1.3 ppm for (-CH₂)_n and 0.9 ppm for (-CH₃), and between 2.0 and 2.3 ppm for (-CH=CH-CH₂-), with signals of markedly lower intensity. In this study, the analysis of the spectra only focused on the concentrations of (-CH₂)_n. The lipid content in liver parenchyma increased with both age and BMI. This conclusion has been confirmed by Müller *et al.*^[1] and Fischbach *et al.*^[18]. Assuming that all of the metabolites that contribute to the choline peak remain constant, when the lipid volume fraction is high, the choline fraction should decrease in signal intensity. In other words, the hydrogen found in lipid can produce very strong resonance signals that can mask the resonance signal of lower concentration compounds of interest, usually metabolites such as choline^[18-23].

Choline is a nutrient essential for normal function of all cells. It is a precursor not only for acetylcholine but also for phospholipids that are found in intracellular

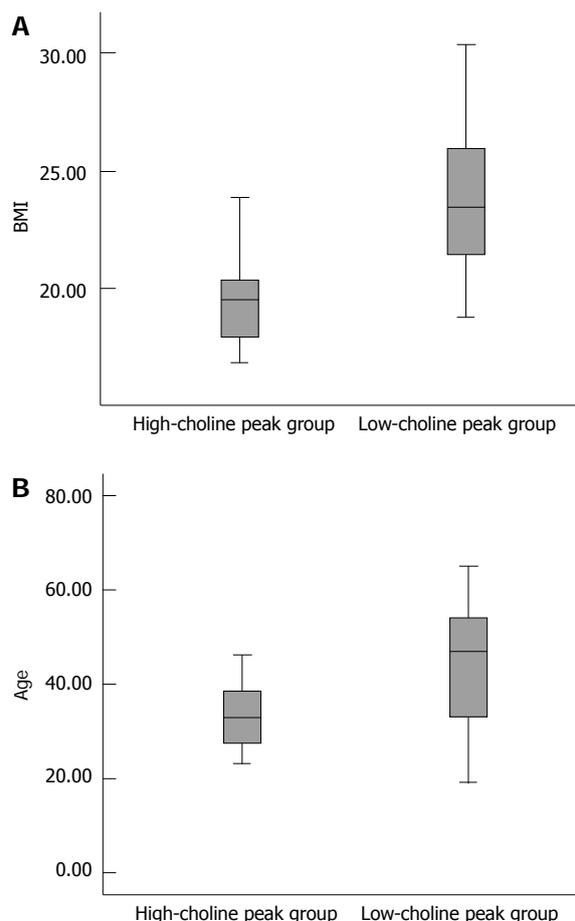


Figure 4 High-choline peak group. A: Had lower body mass index than the low-choline group; B: Was younger than the low-choline group. BMI: Body mass index.

membranes and in the cell membrane. The liver parenchyma already contains large choline metabolite pools. Although catabolic and anabolic reactions are predominant in the liver, leading to elevated choline-containing compounds (CCC) levels. Aging and high BMI may decrease hepatic metabolism, resulting in lower choline concentration. Unfortunately, this conclusion has not been confirmed by previous studies. Fischbach *et al.*^[18] have acquired 113 spectra in normal-appearing parenchyma. The mean \pm SD of the normalized measurements of CCC for the younger group (≤ 40 years) and the older group (> 40 years) was 8.14 ± 6.0 and 7.20 ± 4.32 , respectively. The researchers found that no significant differences were observed.

The presence of a large lipid peak should in principle not influence the fitting of the choline peak. It is known that the concentration of lipid in human liver is 10-1000 times greater than that of most tissue metabolites such as choline. Consequently, the signal of lipid is dominant in ¹H-MRS in some cases. Experimental evidence suggests that spectroscopic data, or metabolic measurements, may be affected by a dominant lipid peak, which makes visualization of the metabolites of interest difficult. This is because the large lipid peak overlaps with adjacent small peaks, and scaling the signal intensity is difficult. This is

the reason why no observable choline peak was detected on liver MRS in obese and elderly individuals. To compensate, fat-suppression techniques are recommended for hepatic MRS to distinguish benign and malignant tumors from normal liver parenchyma^[23-26].

This technique has its limitations in methodology. Although we used a 3.0 T MR imager and shorter TE to increase SNR, spectra containing only noise without any identifiable choline metabolite peaks still existed in a few cases. High-field MRI equipment and/or advanced techniques, such as nuclear Overhauser effect enhancement and proton decoupling, may demonstrate improved SNRs and spectral resolution between MRS peaks. The application of those new techniques may be necessary to answer this question.

In conclusion, Lipid accumulation in the liver could result from increased fat in the body, depending on age and BMI. Hydrogen found in subjects with lipid accumulation can produce very strong resonance signals that can mask the resonance signal of lower concentration compounds such as choline. This is the reason why no observable choline peak was detected on liver MRS in obese and elderly individuals. Fat suppression techniques are recommended for hepatic MRS to distinguish benign and malignant tumors from normal liver parenchyma.

COMMENTS

Background

It is known that magnetic resonance spectroscopy (MRS) can be used to diagnose malignancy; usually by measuring the choline peak. A major limitation is the observation that relatively large amounts of choline-containing compounds may occur even in normal liver. Knowledge of the normal findings associated with age and body mass index (BMI) is valuable.

Research frontiers

A few studies of *in vivo* MRS have reported an increase in choline-containing compounds relative to lipids within tumors such as hepatocellular carcinoma, and a reduction in the lipid-to-choline ratio after transarterial embolization for hepatocellular carcinoma. However, the ability to distinguish reliably benign and malignant tumors from normal liver parenchyma has yet to be established.

Innovations and breakthroughs

The ability to distinguish reliably benign and malignant tumors from normal liver parenchyma has yet to be established. According to the data from this study, the lipid content in liver parenchyma increased with both age and BMI, there was a significant negative correlation between choline/lipid2 (Cho/Lip2) and age, and there was no observable choline peak in obese and elderly individuals.

Applications

Fat suppression techniques are recommended for hepatic MRS to distinguish benign and malignant tumors from normal liver parenchyma.

Terminology

Breathholding either eliminated or markedly reduced phase and frequency shifts and outer voxel contamination that were associated with the motion of the abdomen during breathing.

Peer review

The authors investigated the normal hepatic MRS findings (Cho/Lip2) associated with age and BMI. The study was well designed, and this paper is important and interesting.

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E- Editor Li JY



Clinical outcome of pediatric collagenous gastritis: Case series and review of literature

Nadia Mazen Hijaz, Seth Steven Septer, James Degaetano, Thomas Mario Attard

Nadia Mazen Hijaz, Seth Steven Septer, Thomas Mario Attard, Section of Pediatric Gastroenterology, Children's Mercy Hospital, Kansas, MO 64108, United States

James Degaetano, Department of Pathology, Mater Dei Hospital, Malta Mater Dei Hospital, MSD 2090 Msida, Malta

Thomas Mario Attard, Department of Pediatrics, Mater Dei Hospital, Malta Mater Dei Hospital, MSD 2090 Msida, Malta

Author contributions: Hijaz NM, Septer SS, Degaetano J and Attard TM jointly conceptualised the paper; Hijaz NM and Attard TM performed data acquisition and literatures search; Hijaz NM performed the analyses and the interpretation in collaboration with Septer SS and Attard TM; Hijaz NM wrote the manuscript and all coauthors contributed actively; Attard TM provided guidance on the analysis of the results, conclusions and final critique of the paper; and all authors read and approved the final manuscript.

Correspondence to: Nadia Mazen Hijaz, MD, Section of pediatric Gastroenterology, Children Mercy Hospital, Clinics 2401 Gillham Road, Kansas, MO 64108, United States. nmhijaz@cmh.edu

Telephone: +1-816-2343016 Fax: +1-816-8021465

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Abstract

Collagenous gastritis (CG) is characterized by patchy subepithelial collagen bands. Effective treatment and the clinical and histological outcome of CG in children are poorly defined. The aim of this study is to summarize the published literature on the clinical outcome and response to therapy of pediatric CG including two new cases. We performed a search in Pubmed, OVID for related terms; articles including management and clinical and/or endo-histologic follow up information were included and abstracted. Reported findings were pooled in a dedicated database including the corresponding data extracted from chart review in our patients with CG. Twenty-four patients were included (17 females) with a mean age of 11.7 years. The clinical presentation included iron deficiency anemia and

dyspepsia. The reported duration of follow up (in 18 patients) ranged between 0.2-14 years. Despite most subjects presenting with anemia including one requiring blood transfusion, oral iron therapy was only documented in 12 patients. Other treatment modalities were antisecretory measures in 13 patients; proton pump inhibitors (12), or histamine-2 blockers (3), sucralfate (5), prednisolone (6), oral budesonide in 3 patients where one received it in fish oil and triple therapy (3). Three (13%) patients showed no clinical improvement despite therapy; conversely 19 out of 22 were reported with improved symptoms including 8 with complete symptom resolution. Spontaneous clinical resolution without antisecretory, anti-inflammatory or gastroprotective agents was noted in 5 patients (4 received only supplemental iron). Follow up endo-histopathologic data (17 patients) included persistent collagen band and stable Mononuclear cell infiltrate in 12 patients with histopathologic improvement in 5 patients. Neither collagen band thickness nor mononuclear cell infiltrate correlated with clinical course. Intestinal metaplasia and endocrine cell hyperplasia were reported (1) raising the concern of long term malignant transformation. In summary, CG in children is a chronic disease, typically with a variable clinical response and an indolent course that is distinct from the adult phenotype. Long term therapy usually included iron supplementation but cannot be standardized, given the chronicity of the disease, variability of response and potential for adverse events.

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Key words: Collagenous gastritis; Pediatric; Gastritis; Collagenous colitis; Lymphocytic gastritis

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INTRODUCTION

Collagenous gastritis (CG) is a rare idiopathic disorder characterized by the distinctive endoscopic-histopathologic finding of a thickened ($> 10 \mu\text{m}$) gastric subepithelial collagen layer in association with inflammatory cell infiltrate in the lamina propria^[1]. Collagenous gastritis was first described in a 15 years old by Colletti *et al*^[2] subsequent reports helped define the two distinct phenotypes of the disease in children and adult patients^[1,3]. The classic pattern in children centers around upper gastrointestinal symptoms including abdominal pain and severe anemia^[1,4-6]. In contrast to the adult phenotype, inflammatory changes and collagenous band deposition is usually limited to stomach and CG has a variable clinical and histopathological response to therapy^[6,7]. Adult type CG is associated with several autoimmune processes and celiac disease^[8] which is unusual in the childhood form. Concomitant collagenous colitis may be present and gastrointestinal symptoms include abdominal pain, malabsorptive symptoms and, protein losing enteropathy^[3,9]. Adult CG is characterized by the simultaneous occurrence of collagenous colitis and duodenitis, and absence of nodularity in the mucosa of the colon and duodenum. The histological appearance of adult onset CG is also characterized by extensive inflammation of the entire gastrointestinal tract.

The pathophysiology of collagenous gastritis is poorly understood; one hypothesis includes a primary vascular abnormality with increased vascular permeability resulting in deposition of extruded protein and collagen deposition. Alternatively a primary inflammatory process results in a secondary fibrotic scarring process in susceptible individuals. This is supported by the earlier observation of intraepithelial lymphocytic infiltrate and over expression of human leukocyte antigen DR and CD25^[2] suggesting an immune mediated inflammatory process.

Effective treatment of CG in either age group remains elusive and poorly defined; treatment strategies have revolved around the etiopathologic observations suggesting an inflammatory process and the association with celiac disease. Both anti-inflammatory and anti-secretory measures as well as gluten free diet have been tried but there has been, to date, no comprehensive review of treatment strategies and outcomes in this population.

The purpose of this study is to synthesize the published experience for the clinical course and outcome following different treatment modalities in children with CG including two additional cases with one patient responsive to a combination of oral budesonide in fish oil.

CASE REPORT

We performed a comprehensive chart review of the two cases of pediatric Collagenous Gastritis presenting to our institution from 2009 to 2012, and accrued through query of our institutional dedicated pediatric endoscopic database. Relevant historic, clinical, endoscopic-histologic findings and treatment modalities were summarized and

pooled with the cases reported in the literature.

Pooled literature analysis

We performed a literature search of Pubmed and Ovid for terms: collagenous gastritis, lymphocytic gastritis and Collagenous colitis. Accrued publications were filtered for inclusion of pediatric subjects. Related articles for relevant publications were also reviewed. Articles including pediatric subjects were accrued. We identified sixty peer reviewed publications from Pubmed and an additional 30 publications from Ovid. We excluded duplicate patient reports when identifiable and series where individual therapy and outcome pairs could not be determined.

We abstracted relevant demographic, clinical, endoscopic-histopathologic findings at presentation and upon follow up whenever available and all therapeutic interventions whenever reported. Reports defining therapeutic modalities and clinical or endoscopic-histopathologic outcome were included with our two cases above.

Case 1

An 11 year old boy presented with profound, symptomatic iron deficiency anemia (hemoglobin 4.7 g/dL, mean corpuscular volume 52, serum iron 10 mg/dL, serum ferritin $< 1.5 \text{ ng/dL}$), thrombocytosis (platelets were $540\,000/\mu\text{L}$). Celiac serology was negative. Upper endoscopy and colonoscopy with biopsy showed marked inflammatory changes and nodularity in the gastric body mucosa but otherwise normal findings. The corresponding gastric biopsies showed a mixed mucosal lymphocytic - neutrophilic infiltration and a prominent subepithelial collagen band ($> 10 \mu\text{m}$) that stained positive with Masson Trichrome. Wireless capsule endoscopy was also negative. The patient was treated with oral iron supplements, then standard dose omeprazole with continued periodic treatment with oral iron. Upon repeat esophago-gastroduodenoscopy at 16 years of age biopsies showed marked interval improvement in gastritis, the development of multiple gastric (fundic gland) polyps and resolution of the collagenous band. He is asymptomatic and has been weaned off omeprazole and is being monitored for iron deficiency.

Case 2

A 7 years old girl presented with chronic, mild, diffuse abdominal pain and pallor. Laboratory investigations confirmed iron deficiency anemia (hemoglobin 4.6 g/dL, mean cell volume 59.7, serum ferritin $< 1.5 \text{ ng/dL}$) with platelets count of $435\,000/\mu\text{L}$ normal inflammatory indices (erythrocyte sedimentation rate and C-reactive protein), and negative celiac serology. Upon upper endoscopy and colonoscopy with biopsy moderate to severe hemorrhagic gastropathy was noted and the corresponding biopsies were reported showing a thick Masson Trichrome staining subepithelial hyaline band partly enveloping some of the superficial glands and moderate superficial chronic inflammatory infiltrate (Figure 1). The rest of the upper intestinal biopsies as well biopsies and

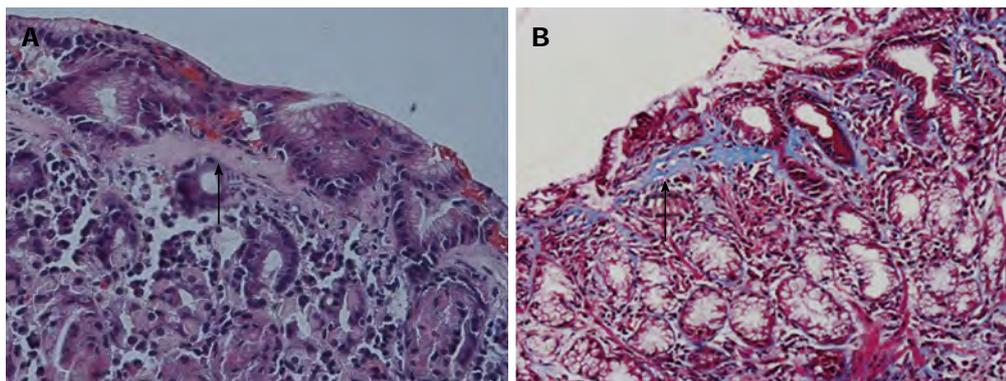


Figure 1 Gastric body surface epithelium showing marked thickening of subepithelial collagen in hematoxylin and eosin stain $\times 200$ (arrow in A) or in masson trichrome $\times 200$ stain (arrow in B) with many intraepithelial lymphocytes.

endoscopic findings on complete simultaneous colonoscopy were normal.

The patient received standard dose esomeprazole approximately 1 mg/kg per day and oral iron supplementation (sodium ferredetate) for 6 mo but upon follow up was reporting episodic, postprandial abdominal pain with corresponding epigastric tenderness on exam. Repeat endoscopy showed unchanged gross and histopathologic findings.

The treatment was escalated in view of refractory disease to include budesonide capsules (6 mg) crushed and suspended in 5 mL of fish oil given orally, daily and continuing esomeprazole and iron supplementation. Upon follow up, in 6 mo she was noted to have improved symptoms, mild constipation and was weaned off supplemental iron. Repeat endoscopy showed grossly improved gastritis (minimal diffuse antral nodularity), interval decreased inflammatory infiltrate but persistent collagen band on most biopsies. The patient was continued on the same medical treatment regimen and on follow up after a further 6 mo was noted to be asymptomatic, with stable hematologic indices.

Our search identified 16 publications and 22 pediatric cases with CG; their clinical presentation showed that anemia was predominantly observed in 17 cases (71%), Abdominal pain in 10 (41%), vomiting in 4 (17%), diarrhea in 4 (17%) and other presentations in 4 (17%) such as failure to thrive and hemocult blood, hememesis and chest pain. their demographic data, therapeutic modalities duration of follow up and outcomes were pooled with our two cases and summarized in Table 1^[1,2,4,6,9-20]. The majority of patients (17) were female, the mean age at presentation was 11.7 years (SD 4.5 years) and similar for both genders.

Gross endoscopic findings were abnormal in all reported cases, several showed gastric mucosal nodularity (11/24). Other reported finding were erythema, erosions, ulceration and hemorrhages in the gastric body, fundus and to a lesser extent in antrum.

Simultaneous colonoscopic findings were reported in 12 patients including 8 patients with gross and histological normal findings; in 3 patients there was collagenous

colitis. In addition, an infantile case showed diffuse atrophic mucosa and increased subcutaneous collagen throughout gastrointestinal (GI) tract sections^[10,11].

When reported (18 patients), the duration of follow up ranged (mean) between 0.2-14 (4) years. Duration of follow up did not significantly correlate with a consistent change in either outcome measure or disease phenotype. This is reflected by the reports by Winslow *et al.*^[13] and Kamimura *et al.*^[15], wherein follow up of 12 and 14 years respectively, and therefore into adulthood did not report features of adult onset type CG.

Despite most subjects presenting with anemia, with one patient requiring blood transfusion, oral iron therapy was only documented in 12 patients. Other treatment modalities were antisecretory measures in 13 patients; Proton pump inhibitors (12), or histamine-2 blockers (3). This includes 3 patients who received triple therapy for *Helicobacter pylori* (*H. pylori*) gastropathy in relation to the findings of CG. Sucralfate was used either alone or combined with other therapy in 5 patients.

Oral steroids were trialed in 9 patients either as systemic steroids (6) or oral budesonide (3) or were not specified (1). Steroids were used after other therapeutic modalities were ineffective. There were no cases reported in which steroids were able to eradicate the symptoms alone; in 6 cases intermittent response was noted; this included improved hematological indices or normalization of growth while recurrence of symptoms occurred in 3 cases after discontinuation of therapy^[1,10,17]. The use of topical steroids produced variable success in 3 cases^[10,12] and case 2. Our case used budesonide in fish oil and showed clinical and hematological improvement that led to eventual weaning of steroids.

In 6 individuals with CG treated with steroids and who underwent repeat endoscopy, histopathology was reported showing unchanged mononuclear (MN) cell infiltrate and collagen band in 3 patients increase in MN cells infiltrate in 2 patients and resolution of MN cells with decreased thickness of collagen in one case.

Additional measures included isolated patients treated with misoprostol, furazolidone, metronidazole and bismuth subsalicylate, hypoallergenic diet, gluten free

Table 1 Demographic outline, treatment modalities, clinical course and outcome pooled cases of collagenous gastritis in children

| Study | Age/gender | Treatment modalities | F/U | Histologic outcome | Clinical outcome |
|--|------------|--|------|---|---|
| Colletti <i>et al</i> ^[2] | 15/F | Ranitidine sucralfate and furazolidane | N/R | No pathological improvement | No clinical improvement |
| Côté <i>et al</i> ^[1] | 9/F | Oral iron, sucralfate, omeprazole prednisolone | 2.5 | Unchanged subepithelial collagen bands Unchanged MN infiltrate Unchanged chronic active gastritis | Intermittent epigastric pain |
| Meunier <i>et al</i> ^[4] | 11/M | Oral iron | 6.25 | Chronic atrophic gastritis Unchanged subepithelial collagen bands Increasing severity MN infiltrate | N/R |
| Winslow <i>et al</i> ^[13] | 12/F | Oral iron | 0.6 | Unchanged histologic findings | Asymptomatic |
| | 14/F | Sucralfate, ranitidine Misoprostol, furazolidone Clarithromycin Metronidazole Omeprazole, prednisone | 12 | Progressive chronic active gastritis Increasing severity MN infiltrate Development of intestinal metaplasia Linear endocrine cell hyperplasia Stable subepithelial collagen bands | Intermittent abdominal pain |
| Mahadevan <i>et al</i> ^[19] | 15/F | Blood transfusion | 0.5 | Unchanged collagen band | N/R |
| Kori <i>et al</i> ^[6] | 12/F | Oral iron Omeprazole Clarithromycin-based triple therapy | 1 | Severe erosive gastritis histologically and macroscopically | Intermittent nausea and vomiting Normalized weight gain |
| | 12/F | Oral, intravenous iron Omeprazole Clarithromycin-based triple therapy, predinsone | 6 | Unchanged subepithelial collagen bands Stable MN infiltrate | Marked clinical improvement, normalized weight gain, anemia Resolved, intermittent epigastric pain |
| | 12/F | Omeprazole | 0.2 | N/R | Improved abdominal pain and heartburn Asymptomatic |
| Kamimura <i>et al</i> ^[15] | 17/M | No therapy | 14 | Increased collagen band thickness, moderate MN cell infiltrate | Asymptomatic |
| Dray <i>et al</i> ^[4] | 15/F | Oral iron, triple therapy Prolong PPI, predinsone | 0.83 | N/R | Clinical remission |
| Ravikumara <i>et al</i> ^[20] | 9/F | Oral iron | 4.1 | Unchanged subepithelial collagen bands Stable MN infiltrate Decreased chronic gastritis | Asymptomatic |
| Leiby <i>et al</i> ^[17] | 0.16/M | Steroids, PPI mesalamine Bismuth subsalicylate | 6 | N/R | CG clinical improvement relapse after finishing steroids Asymptomatic |
| Brain <i>et al</i> ^[9] | 16/F | Ranitidine Omeprazole Oral iron, exclusion diet | N/R | Unchanged histologic findings | Asymptomatic |
| Billiémaz <i>et al</i> ^[10] | 0.75/M | Prednisolone Budesonide Parenteral nutrition Gluten free diet | 14 | Showed a diffuse atrophic mucosa and an increase in the subcutaneous collagen in the gastrointestinal tract | Complete clinical improvement with TPN |
| Suskind <i>et al</i> ^[11] | 9/F | Oral iron | 0.33 | N/R | Asymptomatic |
| | 15/M | Prednisone Lansoprazole Mesalamine | 1 | N/R | Asymptomatic |
| Leung <i>et al</i> ^[12] | 15/F | Budesonide | 3.4 | Moderate gastric collagen deposition that decreased in thickness no IEL | Symptomatic improvement after therapy |
| | 14/F | Pantoprazole Sucralfate | N/R | N/R | No improvement |
| | 19/M | Sucralfate | 0.25 | Moderate gastric collagen deposition in body/fundus, no IEL | Symptomatic improvement |
| Wilson <i>et al</i> ^[16] | 12/F | Oral iron | N/R | N/R | Aymptomatic Normalized weight gain |
| Camarero Salces <i>et al</i> ^[18] | 9/F | Meslazine | N/R | Unchanged | Unchanged |
| This series | 11/M | Oral iron Omeprazole | 5 | Decreased/resolved collagen bands Decreased chronic gastritis Decreased MN infiltrate (fundic gland polyps) | Improved abdominal pain |
| | 7/F | Oral iron Esomeprazole Budesonide/fish oil | 1.5 | Unchanged subepithelial collagen band | Improved abdominal pain |

F/U: Duration of follow up (yr); IEL: Intraepithelial lymphocytosis; MN: Mononuclear; N/R: Not reported; TPN: Total parenteral nutrition; F: Female; M: Male; CG: Collagenous gastritis; PPI: Proton-pump inhibitor.

diet and parenteral nutrition. Mesalamine was prescribed in 3 cases that were reported with concomitant collagenous colitis.

The clinical outcome was reported in 22 patients, repeat endoscopy and therefore histologic follow up was reported only in 17 patients. Three out of 22 patients showed no clinical improvement despite therapy; conversely, 19 out of 24 were reported with improved symptoms including 8 patients with complete symptom resolution. Spontaneous clinical resolution without anti-secretory, anti-inflammatory or gastroprotective agents was noted in 5 patients (4 received only supplemental oral iron).

Complete resolution of symptoms was also reported after the use of oral prednisone in a patient with concomitant CC whereas another three patients with concomitant CC treated with mesalamine were reported with variable response.

In the patients with repeat endoscopy-histopathology reported; persistent histopathologic abnormalities were reported in 12 cases including persistent collagen band and stable mononuclear cell infiltrate. Histopathologic improvement was shown in 5 cases, 3 of them with decreased mononuclear cell infiltrate and decreased/resolved collagen bands in 2 patients on repeated endoscopy. In contrast, mononuclear cell infiltrate was increased in 2 patients^[3,13]. Neither MN cell infiltration nor the collagen band changes correlated with the clinical course. Intestinal metaplasia and endocrine cell hyperplasia were reported in one case raising the concern of long term malignant transformation^[13].

DISCUSSION

Collagenous gastritis is a rare diagnosis in children; its pathophysiology is unknown and is complicated by overlap with lymphocytic gastritis and celiac disease^[8,12]. Pediatric CG is more common in girls and tends to present with severe anemia. In some cases pediatric CG coexists with collagenous sprue and collagenous colitis. The adult phenotype of collagenous gastritis is, in turn, associated with autoimmune disorders. The natural history, treatment modalities and long term outcome of pediatric CG are unknown. Herein, in addition to reporting two new cases of CG and a novel approach to treatment, we have reported on the most comprehensive review to date on the treatment modalities, response and outcome in children with CG described in the peer-reviewed literature.

The demographic outline and clinical presentation of our filtered dataset of treated children with CG and with described clinical and, or endoscopic-histopathologic outcomes reported appears representative of the whole population of pediatric cases reported in the literature. Our subset of cases includes a predominance of females, the mean age at diagnosis is 11.7 years and the duration of reported follow up (4 years) attests to the chronicity of the disease.

The presenting symptoms in our study; abdominal

pain and iron deficiency anemia are identical to those reported elsewhere^[9]. Although the pathophysiology of the iron deficiency remains unclear, most reports include the use of enteral iron supplementation and we have not come across any reference to parenteral iron supplementation alone, suggesting that blood loss rather than malabsorption, is thought to be the cause of iron deficiency.

Half the patients included in this series had undergone colonoscopy; four (16%) had collagenous colitis and three of them were treated with anti-inflammatory measures including mesalamine^[11,17,18]. It was unclear whether lower gastrointestinal symptoms were present in the patients undergoing colonoscopy; it is likely that, as in our patients, this was mandated by suspicion of GI hemorrhage at the time of presentation. The collagen deposition of the entire gastrointestinal tract described in these cases was similar to those described in adult phenotype in their presentation. Although the reported subepithelial deposit in collagenous colitis tends to spread diffusely and continuously, collagen deposition and inflammatory infiltrate in collagenous gastritis tend to vary and are often irregular. The theory behind involvement of colon in some of these patients is that collagen bands arise from panenteric insult through the same pathological process in susceptible individuals, generally more severe in the colon in adulthood compared to children^[9]. Anti-inflammatory drugs including corticosteroids have variable effect on collagenous colitis although relapses can occur, and diarrhea may resolve with or without treatment.

In our pooled analysis, coexistent collagenous colitis did not correlate with a worse prognosis. It is reasonable however to recommend colonoscopy in all patients with CG at the time of initial diagnosis in view of a potential response to therapy if coexistent CC is diagnosed.

A proven therapeutic paradigm does not exist for CG and our review failed to define a consistent response to the therapeutic strategies that were reported in the literature. It appears that the consensus approach to the treatment of isolated CG includes oral supplementation to address iron deficiency as discussed above; anti-secretory strategies, especially proton-pump inhibitors, and anti-inflammatory measures including systemic and, as in our case topically active steroids. In 3 of the patients, concomitant *H. pylori* infections were noted and warranted triple therapy^[6,14]. Measures to detect *H. pylori* on endoscopy are therefore indicated when CG is diagnosed or suspected. This also raises interesting speculation of the possible association of *H. pylori* in the causation of CG.

A therapeutic strategy that includes long term anti-secretory and anti-inflammatory measures has its limitations, central to which, given the chronicity of the disease, is adverse events from long-term treatment. This is especially true given that our analysis is the first to suggest that pediatric CG is, in the most part an indolent, benign process. Adverse effects include systemic steroid side effects are exhaustively described elsewhere^[21]. There are other potential adverse events, for example, the association of proton-pump inhibitor (PPI) use with neu-

roendocrine tumors as suggested by Winslow *et al*^[13] in one patient in our pooled reports. PPI are also reported to interfere with iron absorption^[22]. It therefore follows that, in addition to a clear explanation of our limited understanding of the efficacy of our treatment modalities, aside from oral iron supplementation, parents should also be offered treatments with the least adverse effects and for the shortest possible duration. Histamine-2-receptor Antagonists may be as suitable as PPI but entail less concern with long term use. Steroids may be considered a bridging therapy to achieve clinical and hematological response although there is a significant risk of recurrence of symptoms with discontinuation of therapy. Budesonide may be as efficacious as, and entail less potential for toxicity as systemic steroids.

There seems to be very limited evidence of restriction diet or gluten free diet improving CG. One patient was reported to respond to total parenteral nutrition but this seems an isolated case^[10]. Several other treatment modalities (*e.g.*, furazolidone) were also reported but with limited success.

This report highlights the disconnect between the endoscopic-histopathologic abnormalities that, in the large part, persisted in those patients with follow up endoscopy and the clinical outcome that was typically benign. This is especially true of the reported thickness of the pathognomonic sup epithelial collagen band that remained unchanged in most of the patients. This observation contrasts with the conclusions drawn by Leung *et al*^[12] who suggested that collagen thickness may correlate with severity of disease. In addition, mononuclear cell infiltrate did not correlate with collagen band thickness or clinical outcome.

In two reported cases, long term follow up over 12 years and into adulthood did not; however show a reversion to the adult-phenotype of the disease. This includes a 35-year-old woman presenting with a nodular pattern on Barium X-ray screening study 14 years after her initial presentation^[15]. In this patient the endoscopic and histological findings of this disease were shown to progress gradually in absence of therapy. Endoscopically, the nodular appearance became more conspicuous, in addition to a thicker collagen band evident on biopsy in the context, however, of resolved clinical symptoms. In contrast, Winslow *et al*^[13] reported on collagenous gastritis in a single patient who received multiple therapies including anti-inflammatory treatment during a 12-year period. The patient's biopsy specimens showed a significant corpus endocrine cell hyperplasia, leading to speculation on an increased risk of endocrine neoplasia with gradual progression in disease severity, and unchanged collagen band over the 12-year period. In both cases no colonic involvement as described in adult phenotype reported after this long period. These reports suggest that the pediatric and adult disease phenotypes are different and that the pediatric form does not evolve in the adult form.

This study has several limitations; it is an attempt at a review of case reports and case series that were not, in

the large part focused on therapy, response or long term outcome. There is potentially significant heterogeneity in the accuracy of reporting follow up duration, therapeutic modalities, histopathological and clinical response. Our sample size is a further significant limitation that impacts on the conclusions that can be drawn. Further studies with a more consistent method of qualifying endoscopic-histologic abnormalities are needed.

In conclusion, collagenous gastritis in children tends to be an isolated process that follows a generally benign course with limited long-term morbidity and no increased mortality reported to date. Initial investigation in children with CG needs to include investigation for *H. pylori* and colonoscopy to rule out collagenous colitis. Routine extensive investigation for autoimmune disorders appears unjustified. Response to medication is variable and needs to be individualized with the proviso that anecdotal evidence synthesized in this paper supports the use of oral iron supplementation and the judicious use of anti-secretory and anti-inflammatory agents for the shortest time feasible.

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Shoichiro Kawai, Tsutomu Nishida, Yoshito Hayashi, Hisao Ezaki, Takuya Yamada, Shinichiro Shinzaki, Masanori Miyazaki, Takayuki Yakushijin, Kenji Watabe, Hideki Iijima, Masahiko Tsujii, Tetsuo Takehara, Department of Gastroenterology and Hepatology, Osaka University Graduate School of Medicine, Osaka 565-0871, Japan

Kei Nakai, Kohji Nishida, Department of Ophthalmology, Osaka University Graduate School of Medicine, Osaka 565-0871, Japan
Author contributions: Kawai S and Nishida T contributed equally to this work and wrote the paper; Hayashi Y, Ezaki H, Yamada T, Shinzaki S, Miyazaki M, Nakai K, Yakushijin T, Watabe K, Iijima H, Tsujii M, Nishida K and Takehara T revised the paper.

Correspondence to: Tetsuo Takehara, MD, PhD, Department of Gastroenterology and Hepatology, Osaka University Graduate School of Medicine, 2-2 Yamadaoka, Suita, Osaka 565-0871, Japan. takehara@gh.med.osaka-u.ac.jp

Telephone: +81-6-68793621 Fax: +81-6-68793629

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Abstract

Choroidal or cutaneous metastasis of gastric cancer is rare. Gastrointestinal cancer was found in only 4% in patients with uveal metastasis. Choroidal metastasis from gastric cancer was reported in two cases in earlier literature. The frequency of gastric cancer as a primary lesion was 6% in cutaneous metastasis of men, and cutaneous metastasis occurs in 0.8% of all gastric cancers. We report a patient with gastric adenocarcinoma who presented with visual disorder in his left eye and skin pain on his head as his initial symptoms. These symptoms were diagnosed to be caused by choroidal and cutaneous metastasis of gastric adenocarcinoma. Two cycles of chemotherapy consisted of oral S-1 and intravenous cisplatin (SPIRITS regimen); this was markedly effective to reduce the primary gastric

lesion and almost all the metastatic lesions.

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Key words: Stomach neoplasms; Neoplasm metastasis; Choroid neoplasms; Skin neoplasms

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INTRODUCTION

Gastric adenocarcinoma is one of the most common causes of death from cancer worldwide and more than half of the world's gastric adenocarcinoma cases arise in Asia^[1]. Gastric adenocarcinoma usually develops slowly but eventually grows to show multiple sites of metastasis. Choroidal metastasis of gastric cancer is, however, extremely rare. The uvea consists of the iris, ciliary body, and choroid. Uveal metastasis was reported in 4% of the patients who had a primary cancer in the gastrointestinal tract. Metastasis was found in the iris in 90 (9%), ciliary body in 22 (2%), and choroid in 838 (88%) of the 950 metastatic foci in the uvea^[2]. Cutaneous metastasis of gastric cancer is also relatively rare^[3,4]. We report a patient with gastric adenocarcinoma who presented with a visual disorder in his left eye and skin pain on his head as his initial symptoms.

CASE REPORT

A 75-year-old man visited our hospital in February 2011



Figure 1 Cutaneous metastasis lesion in the head. A: One of the two cutaneous nodules on his head at first visit; B: The size of the cutaneous tumor was rapidly enlarged at 1 mo after the first visit.

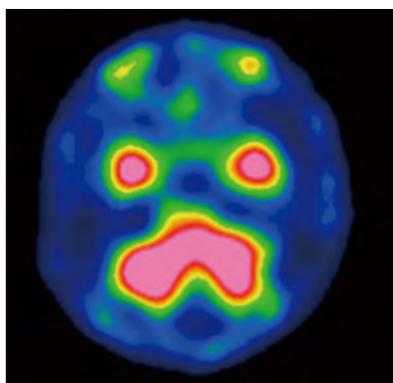


Figure 3 N-isopropyl-p-(123I) iodoamphetamine single photon emission computed tomography findings. 123I iodoamphetamine single photon emission computed tomography showed no accumulation in bilateral eyes.

because of a visual disorder in his left eye and skin pain on his head. He had two cutaneous nodules on his head. One of the cutaneous nodules grew rapidly one month after the first visit (Figure 1A and B). To evaluate the cause of the visual disorder, fundoscopic examination (Figure 2A), optical coherence tomographic examination (Figure 2B), and magnetic resonance imaging (MRI) (Figure 2C and D) were performed and an elevated choroidal neoplasm was detected. Uveal melanoma is known as the most common primary intraocular malignant tumor^[5]. The diagnostic efficacy of N-isopropyl-*p*-(¹²³I) iodoamphetamine single photon emission computed tomog-

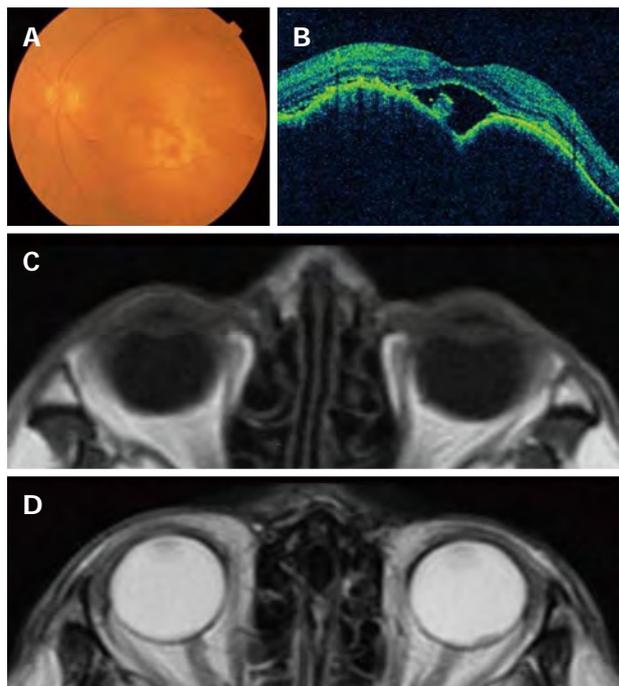


Figure 2 An elevated choroidal neoplasm. A: Fundoscopic examination; B: Optical coherence tomographic examination; C: Magnetic resonance imaging (MRI) T1-weighted image; D: MRI T2-weighted image.

raphy (¹²³I-IMP SPECT) for uveal melanoma has been established^[6]. We performed ¹²³I-IMP SPECT to evaluate the elevated choroidal neoplasm. It, however, showed no accumulation in bilateral eyes that is not specific for melanoma (Figure 3). We diagnosed his choroidal tumor as metastatic tumor, not melanoma.

To detect the primary malignancy, we performed 18F-fluorodeoxyglucose-positron emission tomography/computed tomography (18-FDG-PET/CT) and it showed multiple uptake in the stomach, the bilateral adrenal glands, the abdominal lymph nodes, the right pubis, the femur, and one of the head nodules, but there was no abnormal uptake in his left eye. He underwent esophagogastroduodenoscopy because of the gastric hot spot in the 18-FDG-PET/CT. Endoscopic finding revealed an advanced gastric cancer on the lesser curvature of the stomach (Figure 4A). Gastric biopsy specimens showed moderately differentiated adenocarcinoma (Figure 4B). Pathologic examination of the biopsy specimens of the cutaneous nodule also revealed poorly differentiated adenocarcinoma. Carcinoembryonic antigen (CEA) and alpha fetoprotein (AFP) were elevated to 82 ng/mL and 21 ng/mL, respectively. Abdominal contrast-enhanced CT showed multiple liver metastases, in addition to the known lesions. Thus, we made a final diagnosis of stage IV gastric cancer with multiple metastases; cutaneous, choroid, bilateral adrenal glands, lymph nodes, and liver, right pubis, femur (T4N2M1).

We started chemotherapy with oral S-1 (40 mg/m², twice a day, on days 1-21) and intravenous cisplatin (60 mg/m², on day 8), every 5 wk (SPIRITS regimen)^[7]. Chemotherapy was effective at shrinking a primary gastric

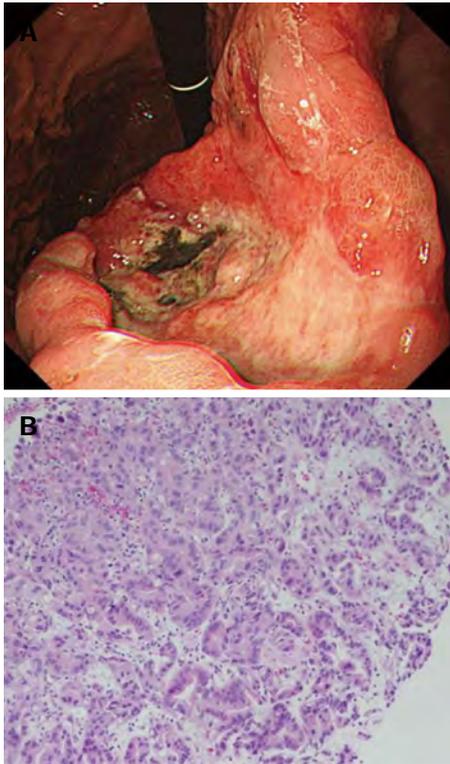


Figure 4 Esophagogastroduodenoscopy and pathological findings of gastric cancer. A: Esophagogastroduodenoscopy was performed and revealed an advanced gastric cancer; B: The gastric biopsy specimens revealed moderately differentiated adenocarcinoma (hematoxylin and eosin stain, $\times 400$).

lesion (Figure 5A), an elevated choroidal lesion (Figure 5B and C), and other metastatic tumors, and improving skin pain relief on his head after 2 cycles, but was not effective in treating the larger cutaneous lesion of the head. The larger cutaneous nodule of the head increased from 25 mm to 32 mm (Figure 5D). Serum levels of CEA and AFP decreased to 8 ng/mL and 8 ng/mL, respectively.

DISCUSSION

Gastric adenocarcinoma shows multiple sites of metastasis. The most common metastatic sites from gastric cancer are liver, peritoneum, and non-regional or distant lymph nodes. Choroidal metastasis is extremely rare, and cutaneous metastasis is also relatively rare. In our case, the initial symptoms related to an advanced gastric cancer showed a visual disorder in his left eye and skin pain on his head. Therefore we reported this case as a remarkably rare case because of both metastases occurring concurrently.

Choroidal metastasis has been increasing due to the improvement of ophthalmic diagnosis techniques. A previous report showed choroidal metastasis mostly comes from breast and lung cancers. In a survey, gastrointestinal cancer was found in only 4% of 420 patients with uveal metastasis^[2]. Choroidal metastasis from gastric cancer was reported in two cases in earlier literature^[8,9], and these cases were found to be recurrent cases of gastric cancer. To the best of our knowledge, this is the first case in which a

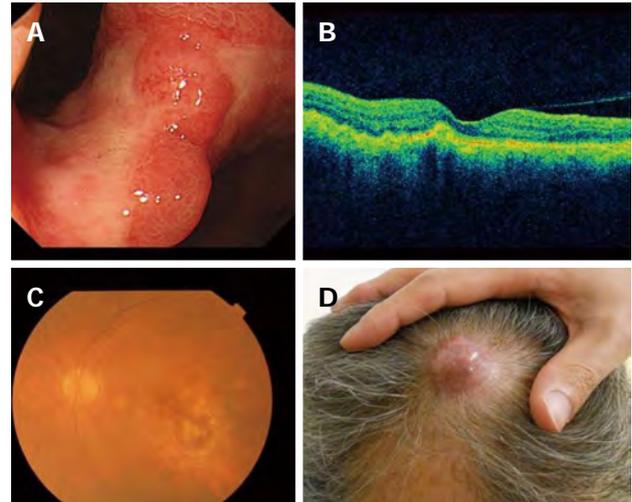


Figure 5 Response to chemotherapy. A: The primary gastric lesion was markedly reduced after 2 cycles of chemotherapy; B, C: The choroidal metastatic tumor was also markedly reduced; D: The larger cutaneous nodule of head enlarged from 25 mm to 32 mm, and headache vanished.

choroidal tumor was found because of a visual disorder as the first symptom before gastric cancer was diagnosed.

In this case, we could not pathologically diagnose the choroidal tumor of this patient as a metastatic tumor. Generally, melanoma and metastatic tumors are the most common malignant choroid tumors^[5]. Therefore we needed to differentiate melanoma and metastatic tumor. Normally, ^{123}I -IMP SPECT is clinically useful for the differential diagnosis of these conditions^[6]. In this case ^{123}I -IMP SPECT showed no accumulation in bilateral eyes. In addition, MRI of the orbits demonstrated a well-circumscribed subretinal mass, where the T1-weighted image was isointense and the T2-weighted image was hypointense. MRI findings were also compatible with a metastatic tumor, not a melanoma^[10]. Chemotherapy for the gastric adenocarcinoma was effective in treating the choroidal tumor. In general, malignant melanoma is resistant to chemotherapy and its treatment regimen is quite different from gastric adenocarcinoma^[11,12]. In our case, the choroidal tumor was markedly reduced in size after chemotherapy for gastric adenocarcinoma (consisting of S-1 and cisplatin). Based on these reasons, it would be appropriate to diagnose this choroidal tumor as a metastatic tumor, not a melanoma.

The frequency of cutaneous metastasis is relatively low, and varies with gender according to the type of underlying malignancies. The most common origins of cutaneous metastasis were reported in lung cancer, colon cancer, melanoma, squamous cell carcinoma of the oral cavity, and renal cell carcinoma in men^[3]. On the other hand, the most common origins in women were breast cancer, colon cancer, melanoma, ovarian cancer, and lung cancer^[3]. The frequency of gastric cancer as a primary lesion was 6% in cutaneous metastasis of men^[3], and cutaneous metastasis occurs in 0.8% of all gastric cancers^[13]. In addition, the cutaneous metastasis was reported to

be close to the site of the primary tumor, therefore the abdomen was the most frequent site in gastric cancer^[14]. Our patient, however, had cutaneous metastasis only on his head and we could make a pathological diagnosis based on pathological findings.

In summary, choroidal or cutaneous metastasis of gastric cancer is rare. We report a patient with gastric adenocarcinoma who presented with both choroidal and cutaneous metastasis of the gastric cancer as initial symptoms.

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Single port laparoscopic right hemicolectomy for ileocolic intussusception

Jia-Hui Chen, Jhe-Syun Wu

Jia-Hui Chen, Jhe-Syun Wu, Department of Surgery, Menno-
nite Christian Hospital, Hualien 970, Taiwan

Jia-Hui Chen, Division of General Surgery, Department of Sur-
gery, Tri-Service General Hospital, National Defense Medical
Center, Taipei 100, Taiwan

Jia-Hui Chen, Graduate Institute of Medical Sciences, Tzu Chi
University, Hualien 97064, Taiwan

Author contributions: Chen JH and Wu JS contributed to the
manuscript writing and revision.

Correspondence to: Jhe-Syun Wu, MD, Department of Sur-
gery, Mennonite Christian Hospital, 44 Min-chuan Road, Hualien
970, Taiwan. gib6234@yahoo.com.tw

Telephone: +886-3-8241241 Fax: +886-3-8236499

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tomy for ileocolic intussusception. *World J Gastroenterol* 2013;
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Abstract

A 36-year-old male was admitted with right lower abdominal pain and diarrhea for more than 3 mo. Colonoscopy and a barium enema study revealed a submucosal tumor over the cecum, but computed tomography showed an ileal lipoma. There was no definitive diagnosis preoperatively, but ileocolic intussusception was noted during surgery. Single port laparoscopic radical right hemicolectomy was performed because intra-operative reduction failed. The histological diagnosis of the resected tumor was lipoma. Single port laparoscopic surgery has recently been proven to be safe and feasible. There are advantages compared with conventional laparoscopic surgery, such as smaller incision wounds, fewer port site complications, and easier conversion. However, there are some drawbacks which need to be overcome, such as difficulties in triangulation and instrument clashing. If there are no contraindications to laparoscopy, single port laparoscopic surgery can be performed safely and should be considered for diagnosis and treatment of intussusception in adults. Here, we report the first case of ileocolic intussusception successfully treated by single port

INTRODUCTION

Intussusception is primarily a childhood disease. It is uncommon in adults and about 70%-90% of adult intussusception cases have a leading cause^[1,2]. Lipoma, which frequently arises in the terminal ileum, is the second most common benign tumor of the small intestine, and it tends to cause intussusception^[3]. As adult patients are at high risk of malignancy, surgical intervention is recommended in cases of intussusception. In recent years, laparoscopic surgery has been able to confirm the diagnosis and to resect the tumor causing intussusception of the small intestine in adults^[4]. Furthermore, single port laparoscopic surgery has been used for various abdominal procedures with safety and feasibility^[5]. We report the first case of ileocolic intussusception successfully treated by single port laparoscopic radical right hemicolectomy.

CASE REPORT

A 36-year-old male was admitted because of right lower abdominal pain and diarrhea for more than 3 mo. Colonoscopy disclosed a submucosal tumor over the cecum. The mass was a ball-like form with an eroded surface

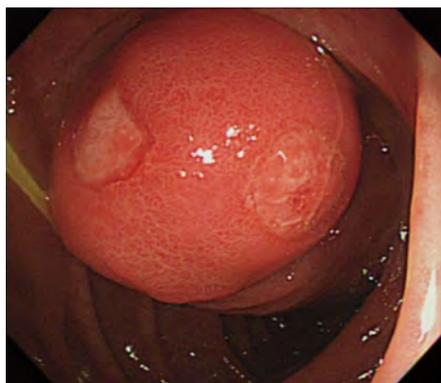


Figure 1 Colonoscopy disclosed a submucosal tumor over the cecum. The mass was a ball-like form with eroded surface.



Figure 3 Barium enema study showed a bulging lesion over the cecum.

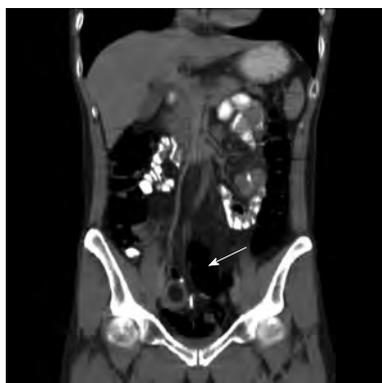


Figure 2 Computed tomography scan showing a round and low density mass about 2.5 cm in the right pelvic region.



Figure 4 The terminal ileum had invaginated through the ileocecal valve and ileocolic intussusception was observed.

(Figure 1). The tumor was initially diagnosed as an ulcer and confirmed by biopsy.

On physical examination, no palpable mass was noted in the abdomen. There was no swelling of superficial lymph nodes. He had no specific past history or family history. Laboratory studies on admission yielded normal blood hematology and chemistry results. The levels of carcinoembryonic antigen and carbohydrate antigen 19-9 were within normal limits. An X-ray of the abdomen was normal. An abdominal computed tomography scan showed a round and low density mass about 2.5 cm in the right pelvic region (Figure 2). A barium enema study showed a bulging lesion over the cecum (Figure 3).

The diagnosis was a cecal submucosal tumor or ileal lipoma. His symptoms were not remarkable. Thus, elective single port laparoscopic right hemicolectomy or single port laparoscopic enterotomy with lipoma resection was scheduled. Through inspection of the abdomen *via* the single port, the terminal ileum was found to be invaginated through the ileocecal valve, and ileocolic intussusception was observed (Figure 4). Initially, we tried manipulation and reduction of the intussusception, but failed. Then we performed single port radical right hemicolectomy because of the high risk of a malignant cause.

For the single-port laparoscopic approach (Figure 5), a vertical incision was created through the umbilicus ap-

proximately 3 cm in length to accommodate the single-port access device. The SILS Port from Covidien Inc. (Mansfield, MA, United States) was used as the single-port access device. This included an insufflation attachment and 3 access ports with associated minitrochars. A rigid 10-mm, 30° laparoscope was used for viewing and 5-mm instruments were used for manipulation of tissues and dissection. In general, a lateral to medial approach was used. The hepatic flexure and lateral peritoneal reflection was mobilized from superior to inferior. The ileocolic pedicle was elevated to allow dissection beneath the ileocolic vessels with identification of the origin of the right colic artery and then the duodenum. The right colon and proximal transverse colon were then lifted off the retroperitoneum. The ileocolic vascular pedicle underwent ligation with the use of a high energy device. The portion of the omentum attached to the specimen was then divided proximally. The right branch of the middle colon was divided. After placement of a wound protector, the tumor was exteriorized *via* the umbilical incision. After resection of the tumor, ileocolic end-to-end anastomosis was performed. The bowel was returned to the abdomen, and then reexamined *in situ*. The fascial incision was closed with a Vicryl suture. Care was taken to maintain oncologic principles, with tight vascular ligation and minimal tumor manipulation. The total surgical time was 3 h and blood loss was about 30 mL.

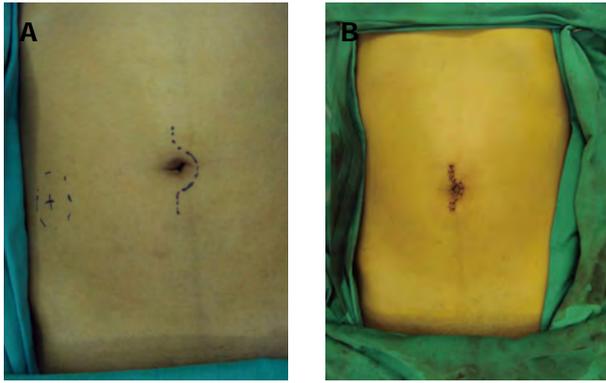


Figure 5 Single-port laparoscopic approach. A: A vertical incision was created through the umbilicus approximately 3 cm in length which accommodated the single-port access device; B: Postoperative suture wound.

The diagnosis was ileocolic intussusception caused by lipoma in the ileal region. Macroscopically, the tumor measured 2.5 cm × 2.5 cm × 2.0 cm, and was located 15 cm from the ileocecal valve (Figure 6). The tumor was ball-like with an irregular surface and soft consistency. The cut surface was yellowish-white, and histopathologic examination revealed fat cells proliferating in the submucosal layer. These characteristics confirmed a diagnosis of lipoma of the ileum.

Postoperative recovery was good and the patient was discharged on the 7th postoperative day. At present, 3 mo after surgery, he is free of symptoms and continues an uneventful course.

DISCUSSION

Single port laparoscopic surgery has been performed for the resection of benign and malignant gastrointestinal tumors in recent years; however, its safety and feasibility is still controversial. Waters *et al*^[6] reported that single port laparoscopic right hemicolectomy is a safe and effective technique for cases of colonic malignant and benign lesions. There are also several reports of benign ileocolic intussusception successfully treated by laparoscopy^[7]. Here, we successfully resected an ileal lipoma causing intussusception using single port laparoscopic radical right hemicolectomy, and to date, no report has described an ileocolic intussusception caused by ileal lipoma which was resected *via* single port laparoscopy.

In 1956, Dean *et al*^[8] classified adult intussusception as enteric; colocolic; ileocecal, with the ileocecal valve as lead point; and ileocolic, with the ileum through the ileocecal valve. The most common type is enteric, occurring in 43% of patients^[9] and our patient had the ileocolic type with ileal lipoma as the leading cause.

Adult intussusception is an uncommon clinical entity encountered by surgeons. The exact mechanism is unknown. However, it is believed that any lesion in the bowel wall or irritant within the lumen that alters normal peristaltic activity is able to initiate an invagination^[2,10]. Ingested food and subsequent peristaltic activity of the



Figure 6 The round tumor measured 2.5 cm × 2.5 cm × 2.0 cm in size and was 15 cm from the ileocecal valve.

bowel produce an area of constriction above the stimulus and relaxation below, thus telescoping the lead point (intussusceptum) through the distal bowel lumen (intussusciptiens)^[1,2,10]. The most common locations are at the junctions between freely moving segments and retroperitoneally or adhesively fixed segments^[11].

The signs and symptoms of pediatric intussusception include a classic triad of a palpable mass, current jelly stools, and pain, and occur infrequently in adults. Adult intussusception commonly presents with nonspecific signs and symptoms similar to a bowel obstruction, such as colicky pain or cramps, nausea and vomiting, palpable abdominal mass, fever, hematochezia, and diarrhea^[9]. The most common symptom is abdominal pain and the less common one is diarrhea. Our patient presented with chronic symptoms of abdominal pain and diarrhea. Because the symptoms often are nonspecific, correct preoperative diagnosis of intussusception is difficult. Eisen *et al*^[12] reported a preoperative diagnosis rate of 40.7%. Similarly, our patient was not diagnosed with intussusception preoperatively. However, laparoscopy is able to evaluate the entire small and large bowel and is another diagnostic tool, too. Although we could not reduce the intussusception *via* single port laparoscopic surgery in our patient, we think that this new technique may provide a better way than conventional laparoscopic surgery to manipulate and reduce intussusceptions.

Symptoms could be acute or chronic. The duration was reported as less than 7 d in 34% of patients and between 7 d and 3 mo in 48%^[13-15]. Gupta *et al*^[13] and Stubenbord *et al*^[14] reported that 22% experienced symptoms of more than 3 mo' duration, while some patients have reported symptoms lasting up to 1 year^[1]. Our patient presented with chronic symptoms for more than 3 mo. We found that the intussusception in our patient was dynamic, changing preoperatively from the colonoscopy and imaging studies. Spontaneous reduction of the intussusception may present in symptomatic or asymptomatic children and occurs more commonly than previously reported. These intussusceptions are usually short-segment, small-bowel intussusceptions with no recognizable leading cause^[16]. Only a few case reports presented a spontaneous

reduction of the intussusception in adults^[17,18]. We postulated that spontaneous reduction of the intussusception in our patient led to obstruction and chronic symptoms, and made preoperative diagnosis more difficult.

A precipitating lesion is found in 90% of adult intussusception cases, but in only 10% of pediatric patients. In most infants and young children, reduction of the intussusception may be tried using barium enema or surgery. However, in adults, definitive surgical resection remains the recommended treatment in nearly all cases because of its nonspecific nature, varying duration of symptoms, the large proportion of structural anomalies, and the relatively high incidence of malignancy^[2,19,20]. Although most authors agree that laparotomy is mandatory, the optimal surgical management of intussusception remains controversial. A reduction at surgery before resection may theoretically permit a more limited resection; however, the risks include intraluminal tumor seeding, a reduction in the externally viable bowel despite mucosal necrosis, venous embolization of malignant cells, spillage of fecal matter through inadvertent perforation, and anastomotic complications in cases of an edematous and weakened bowel^[19]. The main problem is to distinguish benign from malignant lesions before reduction. The cause of intussusception in adults varied by location. Large bowel causative lesions were more frequently malignant than small bowel lesions. The incidence of either primary or metastatic malignancy in the small bowel was 31% compared with 70% in colon lesions^[9]. Benign lesions, including lipomas that cause intussusception were predominantly found in the cecum and terminal ileum^[1,21]. The most common benign lesions of the colon causing intussusception were lipomas^[21].

In our patient, a cecal submucosal tumor or ileal lipoma was suspected preoperatively, but ileocolic intussusception was diagnosed during surgery. Because a benign lesion was highly suspected as the cause, we intended to reduce the intussusception initially, but in vain. Then we decided to perform radical right hemicolectomy.

Laparoscopic surgery has been a standard strategy for a variety of gastrointestinal diseases. The use of laparoscopic surgery for benign bowel tumors and ileocolic intussusception is increasing^[4,7,9]. Single port laparoscopic surgery is a development in the field of minimally invasive surgery. Potential advantages of single port laparoscopic surgery over conventional laparoscopic surgery are thought to be related to improvement in cosmesis and incisional pain and avoidance of port site-related complications. Other aspects, namely operative time, patient selection, patient outcomes, and surgeon efficiency, showed no difference between the two procedures in the existing literature. The conversion rate between the two procedures showed no significant difference, but it is easier for single port laparoscopic surgery to convert to open surgery. Furthermore, we think that a single port can provide access for intussusception diagnosis, manipulation, and reduction.

Although single port laparoscopic surgery can be performed with a conventional rigid laparoscope and

straight instruments, the crowding over the access port usually leads to clashing of instruments. In addition, the handling of both straight instruments in parallel with the laparoscope through a small single incision decreases the freedom of motion for the surgeon and hinders handling of a laparoscope for the assistant. Furthermore, lack of tissue triangulation significantly increases difficulties in exposure and dissection of the lesion. Some of the steps must be performed using the cross-hand maneuver, which is generally avoided in conventional laparoscopy surgery. In attempts to improve surgical exposure, most surgeons use 30° laparoscopes and some used articulating or curved instruments. Some investigators recommended using longer laparoscopes to avoid cluttering of instruments.

In conclusion, we think that single port laparoscopic surgery can be performed safely for ileocolic intussusception caused by ileal lipoma in adults, and should be considered if contraindications are not present.

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Diffuse hemolymphangioma of the rectum: A report of a rare case

Gang Chen, Wei Cui, Xi-Qing Ji, Jun-Feng Du

Gang Chen, Wei Cui, Xi-Qing Ji, Jun-Feng Du, Department of General Surgery, General Hospital of PLA Beijing Military Command, Beijing 100700, China

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Correspondence to: Jun-Feng Du, PhD, Department of General Surgery, General Hospital of PLA Beijing Military Command, 5th South Gate Position, Beijing 100700, China. yacindy@gmail.com

Telephone: +86-10-84008099 Fax: +86-10-84008099

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Abstract

Intestinal hemolymphangioma is a rare vascular and lymphatic malformation and is manifested as anaemia and recurrent alimentary tract hemorrhage. Few cases of hemolymphangioma occurring in small intestine, spleen, esophagus and other organs have been reported. We herein report a case of a 37-year-old man with severe rectal bleeding. Digital examination revealed nodular mucosa. No rectal mass was palpated, but bleeding in the ampulla was detected. Colonoscopy revealed an extensive hypervascular submucosal lesion arising from the rectosigmoid junction colon to the distal edge of the anus. Endoscopic ultrasonography demonstrated an extensive anechoic mass with clear edge. Magnetic resonance imaging (MRI) showed a significant thickness of the rectal wall, extending to the distal edge of the anus, with a narrowing lumen. A sphincter-saving rectal surgery was performed. Due to a lack of knowledge of the clinical, endoscopic and radiological features, preoperative recognition of hemolymphangioma is not easy. Computed tomography and MRI are helpful in confirming the diagnosis, and defining the

extent and invasion of the lesion. For the low malignant potential tumors, a sphincter-saving rectal surgery is recommended after a full evaluation of the tumor.

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Key words: Rectum; Hemolymphangioma; Rectal bleeding

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INTRODUCTION

Hemolymphangioma is very rare, and few cases occurring in the small intestine, spleen, esophagus, chest wall, mediastinum, adrenal gland have been reported^[1-6]. Hemolymphangioma occurs predominately in young patients, especially in newborns and infants, and often arises from congenital malformation of vascular and lymphatic system. It is believed that hemolymphangioma is a benign disease without invasive ability. This report describes the first case of a diffuse hemolymphangioma of the rectum.

CASE REPORT

A 37-year-old man was admitted to a local hospital because of massive rectal bleeding in November 2010. He was managed with emergency care, such as blood transfusion. Upon admission, the patient's hemoglobin was 69 g/L. He had no medical history of abdominal trauma or operation. His major symptoms were rectal bleeding and tenesmus. Because it was hard to distinguish the rectal lesions from haemorrhoids by digital examination, he started on conservative treatments such as local hemostasis. Due to recurrent bleeding, colonoscopy was



Figure 1 Colonoscopy and endoscopic ultrasonography results. A, B: Colonoscopy showed an extensive hypervascular submucosal lesion, with tortuous submucosal veins and nodular mucosa; C: Endoscopic ultrasonography revealed an extensive anechoic mass with clear demarcation.

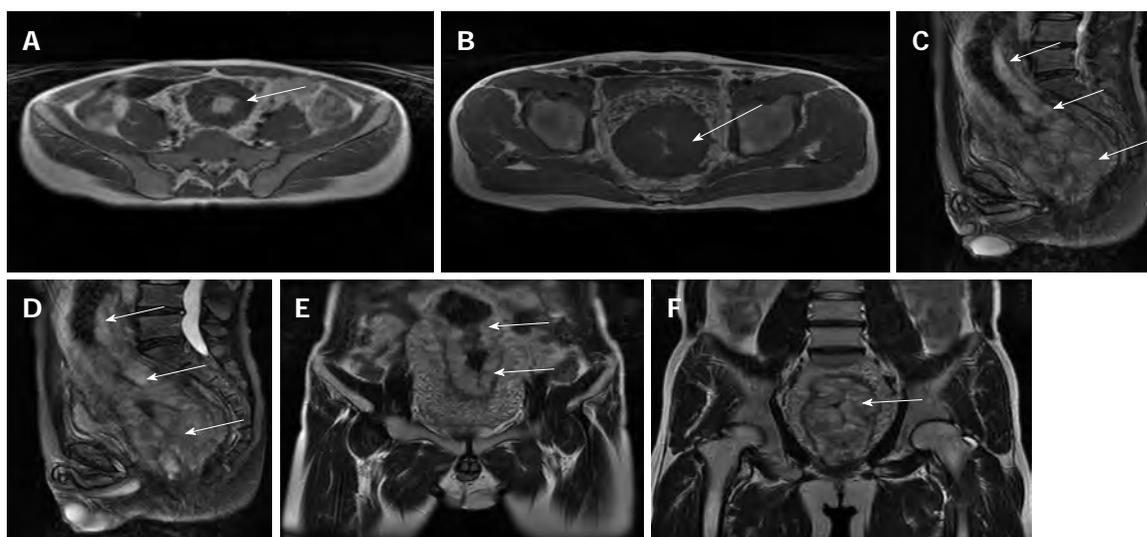


Figure 2 Magnetic resonance imaging showed a significant thickness of the rectal wall, extending to the distal edge of the anus, with a narrow lumen (arrows). A, B: Horizontal plane imaging; C, D: Sagittal plane imaging; E, F: Coronal plane imaging.

performed, which showed an extensive hypervascular submucosal lesion arising from the rectosigmoid junction colon to the distal edge of the anus, and exhibiting tortuous submucosal veins and nodular mucosa. Biopsy was not performed to avoid the secondary severe hemorrhage. The endoscopic ultrasonography indicated an extensive anechoic mass with clear edge (Figure 1). He was transferred to our hospital with the diagnosis of a tumor at the rectum by computed tomography (CT) scan in December 2010. Physical examination revealed mild tenderness on the left lower quadrant. Rectal examination revealed nodular mucosa and red blood in the ampulla, but no obvious mass was found. No other abnormalities were found except for anemia shown in laboratory data including tumor markers such as carcino-embryonic antigen. Magnetic resonance imaging (MRI) showed significant a thickness of the rectal wall, extending to the distal edge of the anus, with a narrow lumen (Figure 2).

Based on the clinical and imaging evidence, preoperative diagnosis of the lesions was cavernous rectal haemangioma. Laparotomy showed diffuse colorectal proliferative lesions arising from sigmoid colon to anus with no ascites or peritoneal dissemination. A frozen-section

examination of the rectal mass indicated a vascular tumor. Subsequently, a sphincter-saving procedure, *i.e.*, low anterior resection of the rectosigmoid colon with hand-sewn transanal colo-anal anastomosis, was performed. The rectal mass weighed 840 g and measured 20 cm × 8 cm × 8 cm (Figures 3 and 4). Gross examination showed a cavernous, soft and compressible tumor. Microscopic examination revealed a tumor which was composed of blood and lymphatic vessels mainly located in submucosa, occupied the entire intestinal wall, and extended into the surrounding fatty tissues (Figure 5). The definitive histological diagnosis was hemolymphangioma of the rectum. No evidence of malignancy was found. The postoperative course was uneventful. The patient was discharged 12 d after surgery. After one year of follow-up, there were no complaints or signs of recurrence.

DISCUSSION

Hemolymphangioma is a very rare and benign tumor. Its incidence varies from 1.2 to 2.8 per 1000 newborns, and both genders are equally affected^[1]. In a literature review until January 2013 (PubMed), no report of hemolymph-

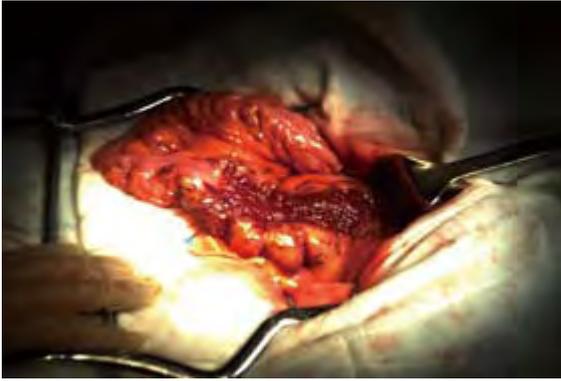


Figure 3 Diffuse colorectal proliferative lesions with no ascites or peritoneal dissemination were found during the operation.



Figure 4 The rectal mass weighed 840 g and measured 20 cm × 8 cm × 8 cm, presenting as a cavernous, soft and compressible tumor.

angioma in the rectum has been published. The primary hemolymphangioma is considered a congenital malformation of the vascular system. The formation of this tumor may be explained by obstruction of the veno-lymphatic communication between dysembryoplastic vascular tissue and the systemic circulation^[1]. Secondary hemolymphangioma is usually caused by the injury of lymphatic vessels in trauma or operation, which induces inadequate lymph fluid drainage. Hemolymphangioma often presents as a cystic or cavernous lesion. Microscopically, the tumor consists of abnormal blood and lymphatic vessels with polycystic spaces. These cysts have connective septa covered by endothelium^[7,8]. When hemolymphangioma of the intestine exists, the submucosal vascular and lymphatic network is affected, with many dilated, thin-walled and irregular blood and lymph fluid filled spaces, mainly located within the mucosa and submucosa^[1]. This tumor could also invade the adjacent structures^[8].

Hemolymphangioma of intestine is manifested clinically as recurrent, acute or chronic painless alimentary tract hemorrhage^[1]. Differential diagnoses include adenomatous polyps, tumors and hemorrhoids. Clinical examination might show mild tenderness, and nodular mucosa, however, no obvious mass could be detected. Complications like massive rectal bleeding, severe anemia, vessel compression or even pelvic organ infiltration could

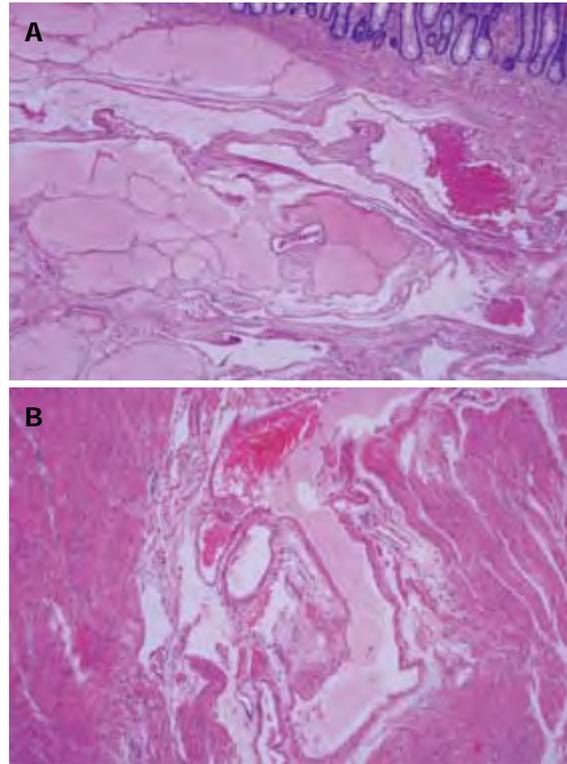


Figure 5 The tumor was composed of lymphatic and blood vessels mainly at the submucosa, occupied the entire wall (A), and extending into the surrounding fatty tissues (B).

sometimes occur.

Colonoscopy should be performed to rule out a malignancy or other problems. Elevated blue nodular lesions or dilated vessels are characteristic findings in the colon wall. Biopsy should not be performed because of the high risk of massive bleeding. The endoscopic ultrasonography is recommended because of its characteristic findings and anechoic mass with clear edge. CT and MRI are useful in defining the extent and the invasion of the mass, and planning the surgical strategy^[9]. It is very important and crucial for radiologists to recognize these lesions and establish an accurate diagnosis so as to avoid a biopsy which could cause severe hemorrhage. However, accurate diagnosis could not usually be established preoperatively.

Elective treatment should be performed and complete surgical resection of the hemolymphangioma is considered the most effective treatment. A thorough exploration of the abdominal cavity should be performed because of the potential invasion to the surrounding organs. Other options of treatment include sclerotherapy, electrocautery, radium implantation, cryosurgery and laser therapy, but these are non-surgical techniques and may only result in temporary clinical outcomes^[10,11]. Angiography and embolization can be applied in cases of acute bleeding, but rebleeding may occur. For the low malignant potential tumors, a sphincter-saving surgery is recommended after a full evaluation of the tumor, followed by sufficient follow-up^[12].

In summary, hemolymphangioma of the rectum is an uncommon vascular and lymphatic lesion, presenting

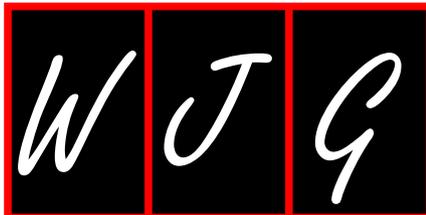
with recurrent and painless rectal bleeding. Preoperative imaging examinations such as endoscopic ultrasonography, CT and MRI are helpful in confirming the diagnosis, and in planning the surgical strategy, and a sphincter-saving surgery is recommended after a full evaluation of the tumor.

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Quadruple primary malignancy patient with survival time more than 20 years

Feng Jiao, Hai Hu, Li-Wei Wang

Feng Jiao, Hai Hu, Li-Wei Wang, Department of Oncology, Shanghai Jiaotong University Affiliated Frist People's Hospital, Shanghai 201620, China

Author contributions: Jiao F and Hu H contributed equally to this work; Jiao F and Hu H collected and interpreted the clinical data; Wang LW wrote the manuscript.

Correspondence to: Li-Wei Wang, MD, PhD, Department of Oncology, Shanghai Jiaotong University Affiliated Frist People's Hospital, 650 New Songjiang Road, Shanghai 201620, China. yzwlv@yahoo.com

Telephone: +86-21-37798322 Fax: +86-21-37798322

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Abstract

Multiple primary carcinoma (MPC) is defined as two or more carcinomas without subordinate relationship detected in the same or other organs of an individual patient. The diagnosis of MPC must comply with the following standards: each of the tumors must present a definite picture of malignancy, each tumor must be histologically distinct, and the probability of one being a metastasis of the other must be excluded. MPC often occurs in the digestive system, but its pathogenesis remains unclear involving genetic susceptibility, tumor immunity and iatrogenic factors, including radiotherapy and chemotherapy. Most MPC patients are double primary malignancy; the occurrence of quadruple primary malignancy is below 0.1%. Here we present a rare case of quadruple primary malignancy involving the small intestine, descending colon, renal pelvis and pancreas. Due to its rarity, the relevant literature is also reviewed. In general, the incidence of MPC is rising, so prevention, early diagnosis and treatment will become necessary and important. Therefore, further research should focus on the etiology and mechanism of MPC.

Key words: Multiple primary carcinoma; Quadruple primary malignancy; Pathology; Surgery; Digestive system

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INTRODUCTION

Multiple primary carcinoma (MPC) is an infrequently occurring disease that is defined as two or more carcinomas without subordinate relationship detected in the same or other organs of an individual patient. It is also called multiple primary malignant neoplasms or multiplicity cancer. The current criteria for the diagnosis of MPC were first presented by Warren *et al*^[1] in 1932: Each of the tumors must present a definite picture of malignancy, each tumor must be histologically distinct and the probability of one being a metastasis of the other must be excluded. MPC could be classified into two categories depending on the diagnosis time of each malignancy. Synchronous MPC refers to the malignancies occurring at the same time or within an interval of six months, while metachronous MPC refers to malignancies following in sequence and more than six months apart^[2]. MPC often occurs in the digestive system. Of all the MPC patients, most are double primary malignancy; the occurrence of quadruple primary malignancy has been reported to be below 0.1%^[3]. Here we present a rare case of quadruple primary malignancy involving the small intestine, descending colon, renal pelvis and pancreas. Considering its rarity, we also reviewed relevant literature.

CASE REPORT

Our patient, a 64-year-old man from Shanghai, China, is

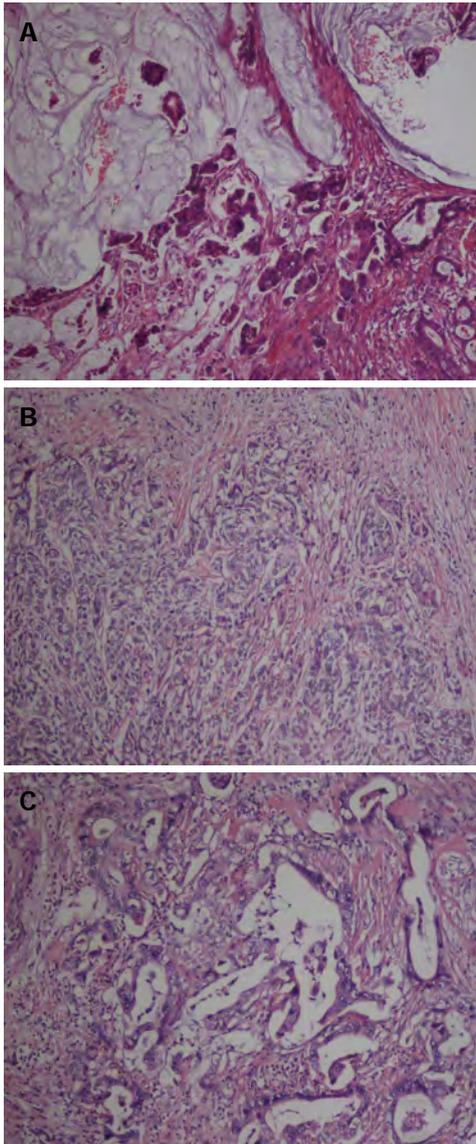


Figure 1 Pathological findings. A: Mucinous adenocarcinoma in the descending colon; B: High-grade invasive urothelial carcinoma of the pelvis; C: Grade II-III adenocarcinoma in the body and tail of the pancreas (hematoxylin and eosin staining, $\times 100$).

still alive and healthy now. He has undergone five surgeries, four for primary malignancies, and one for chronic cholecystitis and cholelithiasis. His mother died of hepatic carcinoma at the age of 81 years, and other members (his wife, daughter and younger brother) in the family have no history of malignancy. He has been suffering from hypertension for 30 years and diabetes for 20 years, and being treated with telmisartan and insulin for each disease, respectively. In addition, he is allergic to penicillin and sulfa drugs. He smokes one pack of cigarettes per day and drinks beer temperately. Other history of trauma and surgery are denied. The following are the detailed records of his medical history.

The first primary malignancy was small intestine adenocarcinoma, which was detected in 1992. According to the description of the patient, the initial symptom was mild pain in the upper abdomen. Considering his good

physical condition, he did not pay much attention to it. The pains worsened over time, and he eventually visited Shanghai Wujing Hospital. Strangely, all of the related examination results were negative, so an exploratory laparotomy was performed immediately. Exposure of the surgical site showed that the small intestine was infected and its contents poured into the peritoneal cavity. A small intestine resection surgery was necessary. The postoperative pathology result revealed small intestine adenocarcinoma. Unfortunately, we no longer have access to the surgery records or the record of the adenocarcinoma because too much time has passed. Owing to anemia caused by long-term absorption disorder after the operation, the patient underwent a blood transfusion that led to hepatitis C infection. Aside from the anemia and transfusion complications, the postoperative recovery of the patient was near perfect and he was scheduled for regular follow-up appointments.

In March 2000, the patient was sent to the Shanghai Liqun Hospital because of a six-month abdominal dull pain and a two-week mucus bloody stool. Colonoscopy performed in the outpatient department showed a tumor in the descending colon. He then underwent a surgical resection after hospitalization. The postoperative histological examination report showed an ulcerated mucinous adenocarcinoma (Figure 1A). All the surgical margins and peripheral lymph nodes were negative. The patient's postoperative recovery was excellent. He was treated with ten cycles of adjuvant chemotherapy after the operation, but the detailed regimen is not available now. All these medical treatment procedure were uneventful and he was scheduled for regular follow-up appointments.

In 2010, the patient was admitted to the department of urology of Ruijing Hospital due to observing gross hematuria symptoms for 20 d. No sign of urinary frequency, urgency, or dysuria were observed, and all physical examinations were normal except the left lumbar. B ultrasonic examination revealed the left renal pelvis was occupied by a solid mass and the ureter was expanded with hydronephrosis. In addition, an ensured abdominal computed tomography (CT) scan revealed left renal carcinoma with hydronephrosis and multiple cysts in both kidneys. Taken together, he was diagnosed as renal pelvic malignancies. Then a radical resection of the left kidney was performed. The postoperative histological examination results revealed high-level invasive urothelial carcinoma of the pelvis (Figure 1B) and vascular tumor thrombus. All surgical margins, perirenal adipose tissue and the left ureter were negative. After the operation, he underwent urinary bladder irrigation, but the detailed regimen is not available now. The patient was again scheduled for regular follow-up appointments.

At the end of 2011, he again complained of upper abdominal pain. A laboratory investigation showed that his CA 19-9 level was 1175.7 IU/mL, much higher than normal. Subsequently, an abdominal CT scan revealed a 2.86 cm \times 3.81 cm mass in the tail of pancreas. As a result of comprehensive examinations above, he was diagnosed with pancreatic cancer. Subsequently, he was im-

mediately transferred to Ruijin Hospital, where he underwent distal pancreatectomy, splenectomy and enterolysis. During the operation, a 4 cm cystic mass that infringed the retroperitoneal space was found. The postoperative histological examination reported grade II-III adenocarcinoma in the body and tail of the pancreas (Figure 1C), and positive peripancreatic lymph nodes (1/1). No tumor cells were found in the resection margins and the spleen. The postoperative recovery was uneventful and the patient was given postoperative adjuvant chemotherapy by using Xeloda. The patient is currently attending regular follow-up appointments and periodically goes to the department of general surgery for his chemotherapy.

DISCUSSION

MPC was proposed in 1889 by Billroth *et al*^[4] in the form of case report. Though there have been an increasing amount of reports on this subject since then, cases of MPC were still considered to be medical curiosities until 1932 when Warren *et al*^[1] gave an extremely detailed compilation study of 1259 cases. More importantly, they established the diagnostic criteria for MPC in their report, which is still used today. Despite its low incidence, the association of two malignancies in a single patient has been widely reported in the literature^[5-7], while only a few cases of quadruple malignancies have been described^[8]. Our patient presents a case of quadruple primary malignancy involving the small intestine, descending colon, pelvis and pancreas. All the malignancies were confirmed by surgery and postoperative pathology results, fulfilling the diagnostic criteria of MPC. Cases of quadruple primary malignancies are very rare, but what is even more astounding is the fact that this patient is still alive today, 20 years after his initial diagnosis. Further, his state of mind and physical condition are excellent, so much so that he cares for himself. Therefore we do think that this is a useful case worthy of our report.

MPC is still quite rare, but the occurrence is on the rise and differs by region and time. Age, diagnostic technique improvements, and longer life spans are all contributing factors. As is widely known and broadly accepted, age is a risk factor for many diseases, and MPC is a no exception. Spratt *et al*^[9] concluded that persons living to an extreme age were expected to have MPC with greater frequency. Another study showed that tumor survivors had a 14% higher risk of developing a new malignancy than would have been expected in the general population^[10]. Based on these findings, we speculate that countries with aging populations will begin to see more and more patients with MPC. Individual genetic susceptibility and other factors, such as hormonal stimulation, iatrogenic factors, environmental degradation and immunologic defects may be involved in the carcinogenesis and progression of MPC. However, nearly all the studies on MPC are descriptive, and detailed studies exploring the molecular mechanisms of MPC are urgently needed. Many hypotheses have been proposed to explain the

pathogenesis of MPC. Iioka *et al*^[11] observed that patients with MPC had a high frequency of microsatellite instability (MSI), arguing that MSI affected the pathogenesis of some MPC cases. Field cancerization is another possible mechanism, which suggests that when an organ is exposed to carcinogens, other nearby organs are also exposed to the carcinogens and carry a high risk of cancer^[12]. Lauchlan *et al*^[13] suggested that cancers developing in other sites originate from histologically similar epithelium. Travis *et al*^[14] discovered that radiotherapy, a kind of iatrogenic factor, following the treatment of a primary cancer lead to a second malignancy. The detailed etiology and mechanism for MPC formation still need further clarification.

Our research group has two hypotheses relating to the prevalence of MPC. The first is genetic susceptibility. All the members of a family share the similar living environment, why one does not get cancer, another may develop single primary cancer (SPC), while others may have multiple primary cancer? Maybe genetic susceptibility is a very important factor. The second is tumor immunity. The survival time of metachronous MPC patients is longer than synchronous MPC patients and metastatic SPC patients on average. A most interesting phenomenon is that the longer the interval between the malignancies, the better the prognosis of the patients (our unpublished data). Immunity abnormality is thought to exist in these patients. We speculate that the immunity of an organism is first impaired, but then is triggered and becomes strengthened by cytokines and growth factors secreted by tumor and other cells, and finally exhausted. This triggered and strengthened immunity is likely to contribute to the longer survival time of the metachronous MPC patients. Although our group has been doing relevant work and has yielded important information, both of these hypotheses still need further study and verification. In conclusion, MPC occurs rarely but is increasing in prevalence. Prevention, early diagnosis, and treatment are important factors in treating MPC. The etiology and its mechanism remain controversial, and thus further research should focus on this distinctive group - MPC patients.

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Subcapsular hepatic haematoma after endoscopic retrograde cholangiopancrea-tography: An unusual case

Bao-Ying Fei, Cai-Hong Li

Bao-Ying Fei, Cai-Hong Li, Department of Gastroenterology, Zhejiang Provincial People's Hospital, Hangzhou 310014, Zhejiang Province, China

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Correspondence to: Bao-Ying Fei, PhD, Professor, Department of Gastroenterology, Zhejiang Provincial People's Hospital, Hangzhou 310014, Zhejiang Province, China. feibaoying@hotmail.com

Telephone: +86-571-85893430 Fax: +86-571-85131448

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Abstract

Subcapsular hepatic haematoma is a rare complication of endoscopic retrograde cholangiopancrea-tography (ERCP), and there are few reports about this unusual complication worldwide. The primary symptom of most cases reported in the literature is abdominal pain. We report an unusual case with the primary symptom of fever. A 56-year-old man who had a six-month history of recurrent episodes of upper abdominal pain was diagnosed with a common bile duct stone by magnetic resonance cholangiopancrea-tography. Endoscopic biliary sphincterotomy was performed, and stones from the common bile duct were successfully extracted with a basket. The patient had a persistent fever after ERCP, and treatment with intravenous antibiotics was unsuccessful. Computed tomography showed a 13 cm × 6 cm subcapsular hepatic haematoma filled with air and liquid on the surface of the right hepatic lobe. The patient was successfully treated with peritoneal drainage under B-ultra guidance. Subcapsular liver haematoma should be considered when hard-to- explain symptoms persist in the early period after ERCP. Percutaneous drainage is an effective treatment.

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Key words: Endoscopic retrograde cholangiopancrea-tography; Hepatic; Hematoma; Complication; Treatment

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INTRODUCTION

Endoscopic retrograde cholangiopancrea-tography (ERCP) is a minimally invasive procedure for the diagnosis and treatment of biliary and pancreatic disease. Even for an expert, serious complications from therapeutic ERCP occur in 2.5%-8% of cases, with mortality ranging from 0.5%-1.0%^[1]. Pancreatitis, cholangitis, perforation, and bleeding as a result of papillotomy are the most frequently described complications^[2-6]. Subcapsular hepatic haematoma is a rare complication of ERCP, and there are few reports about this unusual complication worldwide^[7-9]. We report an unusual case of subcapsular hepatic haematoma post-ERCP with fever as the primary symptom. By contrast, the primary symptom in most cases reported in the literature is abdominal pain.

CASE REPORT

A 56-year-old man with a six-month history of recurrent episodes of upper abdominal pain was diagnosed with a common bile duct stone by magnetic resonance cholangiopancrea-tography. He was admitted for ERCP. A physical examination was unremarkable, and his laboratory tests were normal. Endoscopic biliary sphincterotomy was performed over a 0.035-inch diameter guide wire. Stones from the common bile duct were successfully extracted with a basket.

Two hours post-procedure, the patient developed a

fever (approximately 38.9 °C) without any chills. No other symptoms or findings were observed during physical examination. Laboratory tests revealed a white blood cell count of $13.3 \times 10^9/L$ [normal limit $(4-10) \times 10^9/L$] and serum C-reactive protein of 91 mg/L (normal limit < 5 mg/L). Serum amylase was elevated to 240 U/L (normal limit 30-110 U/L). We considered the cause to be cholangitis after ERCP and administered intravenous antibiotics. On the third day after ERCP, the patient complained of sudden-onset abdominal pain that disappeared after 20 min. At this time, serum amylase was normal. On the following day, the patient felt mild pain in the right upper quadrant of the abdomen without tenderness or signs of peritonism. On the 6 d post-ERCP, his laboratory data demonstrated white blood cell count of $12.3 \times 10^9/L$, neutrophils of 88.4%, and haemoglobin of 9.6 g/dL (normal 12-16 g/dL). His serum biochemistry was within normal limits. The patient had a persistent fever, and his highest body temperature was 39.4 °C. Computed tomography (CT) was performed, and demonstrated a 13 cm \times 6 cm subcapsular hepatic haematoma filled with air and liquid on the surface of the right hepatic lobe (Figure 1).

The patient was haemodynamically stable and treated with peritoneal drainage under B-ultra guidance. His body temperature returned to normal after 1 wk of drainage, and the patient gradually recovered. The drainage catheter was withdrawn after four weeks. During the following week, a follow-up CT scan showed resolution of the haematoma.

DISCUSSION

Subcapsular hepatic haematoma after ERCP is a rare complication, with few cases reported in the literature. It may be explained by an accidental puncture of the intrahepatic biliary tree by the guide wire and rupture of a small calibre intrahepatic vessel^[10].

The occurrence of persistent abdominal pain or hypotension after ERCP should raise the suspicion of subcapsular hepatic haematoma. In this case, the patient only presented with a fever within 2 d after ERCP and had no abdominal pain or hypotension. His body temperature was not controlled after the administration with intravenous antibiotics. The existence of air and liquid inside the haematoma revealed local infection, which explained of persistent fever. Therefore, persistent fever after ERCP suggests that certain precautions should be taken. Laboratory tests did not provide major indicators of the development of a subcapsular hepatic haematoma, except for a decrease in the haemoglobin level. Imaging modalities (ultrasound, CT, and magnetic resonance imaging) are the methods of choice for the diagnosis and surveillance of this complication^[11,12].

Most patients are managed conservatively. Surgical management should be considered when the general condition deteriorates, haemodynamic instability and signs of peritoneal irritation develop, and abdominal CT demonstrates free fluid. In the previously reported cases,



Figure 1 Computed tomography scan of the upper abdomen showing a 13 cm \times 6 cm subcapsular haematoma on the surface of the right lobe of the liver.

two cases^[11,13] were treated surgically and three cases^[14-16] were treated with percutaneous drainage. In this case, the patient's condition didn't improve after the administration of broad-spectrum antibiotics. Adequate percutaneous drainage was effective, suggesting that removing the liquid from within the haematoma is important.

In conclusion, subcapsular liver haematoma is a rare complication and should be kept in mind considered when hard-to-explain symptoms persist in the early period after ERCP. Conservative treatment will be sufficient in most cases. Percutaneous drainage is an effective treatment.

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Ferruccio Bonino, MD, PhD, Professor of Gastroenterology, Director of Liver and Digestive Disease Division, Department of Internal Medicine, University of Pisa, Director of General Medicine 2 Unit University Hospital of Pisa, Via Roma 67, 56124 Pisa, Italy

Myung-Hwan Kim, MD, PhD, Professor, Head, Department of Gastroenterology, Director, Center for Biliary Diseases, University of Ulsan College of Medicine, Asan Medical Center, 388-1 Pungnap-2dong, Songpa-gu, Seoul 138-736, South Korea

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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
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Beijing 100025, China
Telephone: +86-10-85381892
Fax: +86-10-85381893

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- 3 **Tian D**, Araki H, Stahl E, Bergelson J, Kreitman M. Signature of balancing selection in Arabidopsis. *Proc Natl Acad Sci USA* 2006; In press

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- 4 **Diabetes Prevention Program Research Group**. Hypertension, insulin, and proinsulin in participants with impaired glucose tolerance. *Hypertension* 2002; **40**: 679-686 [PMID: 12411462 PMID:2516377 DOI:10.1161/01.HYP.0000035706.28494.09]

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- 5 **Vallancien G**, Emberton M, Harving N, van Moorseelaar 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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- 9 Outreach: Bringing HIV-positive individuals into care. *HRSA Careaction* 2002; 1-6 [PMID: 12154804]

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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. Wiczorek RR, editor. White Plains (NY): March of Dimes Education Services, 2001: 20-34

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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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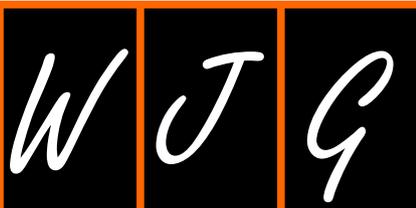
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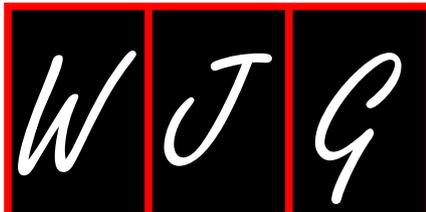
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Epidemiology and inflammatory bowel diseases

Ahmed Mahmoud El-Tawil

Ahmed Mahmoud El-Tawil, Department of Surgery, University Hospital Birmingham, East Corridor, Ground Floor, Birmingham B15 2TH, United Kingdom

Author contributions: El-Tawil AM solely contributed to this paper.

Correspondence to: Ahmed Mahmoud El-Tawil, Department of Surgery, University Hospital of Birmingham, East Corridor, Ground Floor, Birmingham B15 2TH,

United Kingdom. atawil20052003@yahoo.co.uk

Telephone: +44-121-6978231 Fax: +44-121-4466220

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Abstract

The role of alcohol in causing or aggravating the pathogenesis of inflammatory bowel disease is unclear. For finding a conclusive answer for this valuable question we conducted this review. Only two studies were identified that successfully fulfilled our inclusive criteria. Usual consumption of alcohol reduced the risk compared with less frequent use (odds ratio = 0.57, 95%CI: 0.37-0.86). Light alcoholic drinking has protective effects against development of ulcerative colitis. But this inverse association disappeared when smoking was included.

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Key words: Epidemiology; Inflammatory bowel diseases; Crohn's diseases; Ulcerative colitis; Alcohol

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INTRODUCTION

Alcohol is widely consumed in the Western part of the globe where inflammatory bowel diseases are prevalent.

However, the role of alcohol in the pathogenesis of inflammatory bowel diseases is not clear. The question is "what the role of alcohol in the pathogenesis of inflammatory bowel disease is?"

METHODOLOGY

For finding this (these) answer (s), we conducted search throughout Medline, EMBASE, Current content, CINAHL, DARE, the Cochrane Central Register, the Cochrane Database of Systematic Reviews, Joanna Briggs Institute's website, the Campbell Collaboration, the Centre for Evidence-Based Medicine, the NHS Centre for Reviews and Dissemination, ISI web of knowledge, TRIP database, INTUTE and Bandolier were searched from inception to July 2009. The search terms: alcohol (use, drinking, utilize, consume, consumption, ingestion and intake), and beverages were matched against Crohn's disease, ulcerative colitis and inflammatory bowel disease for identifying all published articles. Also, the search included reviewing the published abstracts in online journals for unpublished reports. References from relevant articles were checked as well.

Our criteria for selection were: (1) the study should be of good quality: A. the hypothesis/aim/objective of the study should be clearly described; B. a case-definition and the sources for patients and controls should be clearly described and both cases and controls were representative of their entire population; C. the selected cases should fulfill Leonard-Jones criteria (the diagnosis was confirmed by at least two investigations of these three: Barium studies, colonoscopy and/or biopsy); D. the selected controls should have no family background of inflammatory bowel diseases (Crohn's disease and ulcerative colitis). This is the minimum safest way to ensure that the selected controls had not conducted inflammatory bowel disease by excluding a hereditary genetic factor; E. clear definitions of different "alcohol drinking" categories should be plainly mentioned in the article; F. the main findings of the study should be clearly described; G. the published studies in non-English language would be excluded; (2) the study should be unbiased; (3) patients and controls should be

randomly selected and were recruited from the same population and over the same period of time; (4) the studies should not be based on long term historical recalls; (5) the statistical tests used for assessing the main outcomes should be appropriate; (6) the outcome measures should be clearly described; and (7) the outcome measures should have sufficient statistical power.

FINDINGS

Only nine studies examining the association between alcohol intake and inflammatory bowel diseases were identified. The nine studies were published in six peer review journals^[1-9] (*Z Gastroenterology*, *American Journal of Gastroenterology*, *American Journal of Epidemiology*, *Journal of Clinical Gastroenterology*, *Nippon Eisegaku Zasshi* and *Gut*). Three of them were excluded because they were written in non-English language (Brandes *et al*^[1], Katschinski *et al*^[3] and Higashi *et al*^[9]). Another two were excluded because of lack of controls (Jowett *et al*^[6] and Zutshi *et al*^[8]). A further study was excluded because the authors did not examine the association before the onset of the disease (Samuelsson *et al*^[10]). The study of Boyko *et al*^[2] was also excluded because of the lack of addressing the family background of the selected controls and the types of their diseases were not explained in the article. Therefore, only two of the nine articles fulfilled our inclusion criteria. The validated studies came from Asia with none from Europe and United States. An association between Crohn's disease and alcohol intake was not examined in either of the two studies (Table 1).

Study design

Nakamura *et al*^[4] study: A. It was a case-control study; B. Patients selection (inclusion criteria): patients were residents of areas covered by 93 selected public health centers. Patients who had begun to receive financial aid for treatment of ulcerative colitis during the period of the study were asked to participate in this survey. Three hundreds and eighty-four out of the 490 randomly selected patients with ulcerative colitis (78.4%) agreed to participate in the study; and C. Controls were healthy fit volunteers who were pair matched by age and sex to patients. They were selected randomly among those included in the schedules of health checkup programs.

Jiang *et al*^[7] study: Patients selection (inclusion criteria): 177 inpatients with ulcerative colitis were recruited prospectively from 5 major hospitals in a specified city in central China. Controls were healthy volunteers who were randomly selected among those who were neighbors and colleagues to patients. Patients and controls were matched by sex and age.

Instrument for data collection

Two studies used questionnaires for data collection. It was a self-administered questionnaire in the study by Nakamura *et al*^[4]; and the participants were interviewed to complete a detailed questionnaire in the study by Jiang *et al*^[7].

Details of alcohol consumption

(1) No indication of the level of alcohol consumption among participants in their samples was provided in either of the two studies; (2) In the study of Nakamura *et al*^[4], alcohol consumption was categorized by three levels of drinking frequency: daily alcohol drinkers: "who drank alcoholic beverages 5 d or more a week"; moderate alcohol drinkers: "who drank alcoholic beverages 1-4 d a week"; and non-drinkers: "who had drunk alcoholic beverages less than a day per week". This classification was also used in the former drinkers; and (3) But in the study by Jiang *et al*^[7], 4 categories were determined. Frequent drinking was defined as "alcoholic drinking 3 d or more per week for continuous 6 mo before the diagnosis of ulcerative colitis"; light drinking was defined as "drinking alcoholic beverages less than 3 d a week"; non drinking was defined as "never or rarely drinking"; and former drinking was defined as "patients who had quit drinking for more than 6 mo before the diagnosis of ulcerative colitis". No information was provided about either the duration of alcohol drinking or the type of alcoholic beverage.

Outcome measures

Alcohol consumption and time to onset of the disease:

Onset of the disease was defined as "the time when the related symptoms first appeared". This timing was used to discriminate between pre- and post-illness in the two acknowledged studies. No specified period before the first appearance of symptoms was determined in either.

Alcohol consumption and probability of ulcerative colitis:

Nakamura *et al*^[4] demonstrated that usual consumption of alcohol reduced the risk of developing the disease compared with less frequent use (odds ratio = 0.57, 95%CI: 0.37-0.86). But there was no significant association between ulcerative colitis and alcohol use was found by Jiang *et al*^[7].

Identification and adjustment for confounding factors:

Cigarette smoking was considered as a confounding factor by the two studies. Jiang *et al*^[7] revealed that light alcoholic drinking had protective effect against development of ulcerative colitis. But this inverse association disappeared when smoking was included.

DISCUSSION

Thousands of reports examining possible role of environmental factors in the development of inflammatory bowel disease were published. However, the outcome was trivial. It was just smoking was beneficial in patients with ulcerative colitis but was aggressive in subjects with Crohn's disease. But no reason was given. It is likely due to the use of the time of diagnosis for differentiating between pre- and post illness. But in the case of inflammatory bowel diseases as in other chronic-non-infectious diseases, the time interval between onset of the disease (conduction of the disease without symptomatic presentation) and time of diagnosis (symptomatic presentation) may be ten years

Table 1 The outcome of the study

| The study | The outcome |
|--------------------------------------|--|
| Nakamura <i>et al</i> ^[4] | Regular consumption of alcohol reduced the risk of ulcerative colitis compared with less frequent use (odds ratio = 0.57, 95%CI: 0.37-0.86) |
| Jiang <i>et al</i> ^[7] | Light alcoholic drinking had protective effect against ulcerative colitis and this effect disappeared when smoking associated with the light drinking of alcohol |

or more. Therefore, it is likely that the majority of these studies compared between two conditions of post-illness. Yet it is still possible by following this policy to assess the effect of different environmental factors on the progress of the disease.

As it can be seen from the above results, a correlation between the progress of ulcerative colitis and consumption of alcohol was investigated in only two studies. However, it was not possible to mix the results of the two studies up due to the existence of heterogeneity (different definitions on the consumption of alcohol). It is also noticeable that Nakamura *et al*^[4] failed to include light (mild) drinking in their categorization. This error was avoided by Jiang *et al*^[7]. This is likely the reason for achieving this constructive conclusion about the protective effect of light drinking in subjects with ulcerative colitis.

The reason for this beneficial effect is likely due the existence of different phenols (rich in antioxidants) in alcoholic beverages^[11]. Antioxidants have inhibitory effect against the production and function of pro-inflammatory cytokines^[12].

Yet, this beneficial action is contradicted by the activity of other contents in alcohol. Ethanol and its metabolite, "the acetaldehyde", both stimulate the production of reactive species^[13,14]. This destructive activity is dose and strength related. This may explain the reason for discovering the useful activity in only light alcohol consumers. However, it is interesting to observe that despite of the fact that both of smoking^[15] and light drinking could solely have protective effects in subjects with ulcerative colitis this advantage disappeared when both combined together. This evidently proves that the pathogenesis of inflammatory bowel disease is quite complex and the conduction of experimental studies on animal models and linking the outcome to the pathogenesis of inflammatory bowel diseases do not do any useful help but it might hurt.

OUTCOME AND IMPLICATIONS OF THE REVIEW FOR PRACTICE AND RESEARCH

People who drink regularly were at less risk for developing ulcerative colitis. Light drinking had protective effect but this effect could be abolished by smoking. Further

research studies are needed for confirming these conclusions and to assess the existence of possible correlation between different concentrations of alcohol and severity of the disease.

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Aflatoxins, hepatocellular carcinoma and public health

Arvin Magnussen, Mansour A Parsi

Arvin Magnussen, Sandefjord High School, 3228 Sandefjord, Norway

Mansour A Parsi, Section for Therapeutic and Pancreatobiliary Endoscopy, Digestive Disease Institute, Cleveland Clinic, Cleveland, OH 44195, United States

Author contributions: Magnussen A searched pubmed for relevant articles, gathered and interpreted the data, and wrote the draft manuscript; Parsi MA co-authored and critically revised the manuscript.

Correspondence to: Mansour A Parsi, MD, MPH, Section Head, Section for Therapeutic and Pancreatobiliary Endoscopy, Digestive Disease Institute, Cleveland Clinic, 9500 Euclid Avenue, Cleveland, OH 44195, United States. parsim@ccf.org
Telephone: +1-216-4454880 Fax: +1-216-4446284

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regulations and testing are not in place in many countries. The purpose of this editorial is to summarize the current knowledge on association of aflatoxin and HCC, encourage future research and draw attention to this global public health issue.

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Key words: Aflatoxins; Hepatocellular carcinoma; Environmental health; Food safety; Public health

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Abstract

Hepatocellular carcinoma (HCC) is one of the leading causes of cancer deaths worldwide, primarily affecting populations in the developing countries. Aflatoxin, a food contaminant produced by the fungi *Aspergillus flavus* and *Aspergillus parasiticus*, is a known human carcinogen that has been shown to be a causative agent in the pathogenesis of HCC. Aflatoxin can affect a wide range of food commodities including corns, oilseeds, spices, and tree nuts as well as milk, meat, and dried fruit. Many factors affect the growth of *Aspergillus* fungi and the level of aflatoxin contamination in food. Drought stress is one of the factors that increase susceptibility of plants to *Aspergillus* and thus aflatoxin contamination. A recent drought is thought to be responsible for finding of trace amounts of aflatoxin in some of the corn harvested in the United States. Although it's too soon to know whether aflatoxin will be a significant problem, since United States is the world's largest corn producer and exporter, this has raised alarm bells. Strict regulations and testing of finished foods and feeds in the United States should prevent a major health scare, and prevent human exposure to deleterious levels of aflatoxin. Unfortunately, such

INTRODUCTION

On August 30, 2012, Reuters reported high alert in United States grain sector because of finding of trace amounts of aflatoxin in some of the corn harvested in the United States^[1]. Since strict regulations in the United States prohibit sale of aflatoxin contaminated crops, the alert was mainly because of potential economic consequences for United States farmers. Aflatoxins are one of the most important of the environmental toxins especially in the regions of the world where dietary foodstuffs are highly contaminated. Aflatoxins are primarily produced by fungal species *Aspergillus flavus*, *Aspergillus parasiticus*, and *Aspergillus nomius*, which colonize a wide variety of food commodities including maize, oilseeds, spices, groundnuts, tree nuts, milk and dried fruit^[2]. There are several different types of aflatoxins and nearly all of them can cause illness in human beings and animals. Aflatoxin B1 is the most abundant and toxic member of the family. It is also the most potent hepatocarcinogen known. Aflatoxin B1 has been classified by the World Health Organization as a "group A" carcinogen because of its proven contribution to the pathogenesis of hepatocellular carcinoma (HCC)^[3,4].

HCC is a leading cause of cancer related death and a major public health problem worldwide^[5,6]. Annual mortality associated with HCC is virtually identical to its incidence throughout the world, underscoring the high case fatality rate of this cancer type^[7].

The incidence of HCC varies greatly according to geographic region. The distribution of HCC also differs among different ethnic groups and regions within the same country. In parts of Asia and Africa, HCC accounts for nearly 70% of cancer deaths^[8]. In China, HCC is the third leading cause of cancer mortality accounting for at least 250 000 deaths per year and with an incidence rate in some counties approaching 100 cases per 100 000 individuals per year^[9].

The differences in distribution of HCC are thought to be because of different levels of exposure to HCC risk factors. In developing countries the major risk factors for HCC are chronic infection with hepatitis B virus and exposure to aflatoxins, while in developed countries the major risk factor is cirrhosis of the liver due to hepatitis C virus infection and alcohol abuse. In developed countries the majority of HCCs occur in older patients with long-standing chronic liver disease. In regions with high frequency of hepatitis B virus carriers and aflatoxin exposure, like sub-Saharan Africa, the mean age of presentation of HCC is decades earlier than in Western countries, often decreased to as low as 33 years^[10,11].

TOXICOLOGY

Aflatoxins are associated with both toxicity and carcinogenicity in human and animal populations^[12]. Acute aflatoxicosis results in death, whereas chronic aflatoxicosis results in more prolonged pathologic changes, including cancer and immunosuppression^[12]. The liver is the primary target organ, and liver damage has been documented in rodents, poultry, and nonhuman primates following ingestion of aflatoxin B1. Acute aflatoxicosis has been manifested in humans as an acute hepatitis^[12]. In India in 1974, an outbreak of hepatitis occurred in which 100 people died following consumption of maize that was heavily contaminated with aflatoxin. Aflatoxin B1 was detected in high concentration in the livers of those individuals who died^[13,14].

It has been hypothesized that kwashiorkor, a severe malnutrition disease, and Reye syndrome, marked by encephalopathy and fatty degeneration of the viscera, represent forms of pediatric aflatoxicosis. Although aflatoxins have been found in the livers of children with kwashiorkor and in Reye syndrome patients, a strong cause-and-effect relationship between aflatoxin exposure and these disease states has not been established^[12].

Chronic low-level exposure to aflatoxins in the diet is a risk factor for the development of HCC. Such exposure has been shown experimentally to produce cancer in many animal species and several epidemiologic investigations have shown that increased aflatoxin ingestion correlates with increased risk of HCC in humans^[12].

The mechanism of aflatoxin-induced carcinogenesis is thought to involve tumor promotion or progression. There is evidence that aflatoxin is involved in the activation of proto-oncogenes and mutations in the tumor suppressor gene p53. Aflatoxin exposure and p53 mutations have been tightly linked in epidemiologic studies in Africa and China. Specifically, aflatoxin has been linked to a p53 mutation whereby there occurs a G-to-T transversion at codon 249^[15,16]. This biomarker has been used in epidemiologic studies to establish the link between aflatoxins and hepatic cancer and also to show that co-factors such as infection with hepatitis B virus increase the risk of HCC substantially. It has also been suggested that aflatoxin induces various chromosomal aberrations, unscheduled DNA synthesis and chromosomal strand breaks in human cells. Another suggested mechanism for aflatoxin-mediated carcinogenesis is production of mutagenic substances as a result of metabolism of Aflatoxin by hepatic cytochrome p450.

ENVIRONMENTAL RISKS

Aflatoxins can affect a wide range of commodities including cereals, oilseeds, spices, and tree nuts as well as milk, meat, and dried fruit. The major sources of exposure are maize and groundnuts as these are the foods that are most susceptible to contamination and consumed in the greatest amounts. Developing countries located in the tropical regions, are at greatest risk given their reliance on these commodities as their staple food source^[2]. Food insufficiency and lack of food diversity substantially increases the risk of exposure to aflatoxins among individuals who live in these regions.

Many factors affect the growth of *Aspergillus fungi* and the level of aflatoxin contamination in food. Contamination can occur at any stage of food production from pre-harvest to storage^[2,17]. Factors that affect aflatoxin contamination include the climate of the region, the genotype of the crop planted, soil type, minimum and maximum daily temperatures, and daily net evaporation^[2]. Aflatoxin contamination is also promoted by stress or damage to the crop due to drought prior to harvest, insect activity, poor timing of harvest, heavy rains at harvest and post-harvest, and inadequate drying of the crop before storage. Humidity, temperature, and aeration during drying and storage are also important factors^[2].

VULNERABLE POPULATIONS

Children

Children differ from adults in many ways that have relevance to environmental health^[18,19]. Due to smaller body weights, doses that might not affect adults may induce illness in children. They have more immature neurologic and immune systems and so are more prone to develop complications. Children's developmental state may have a disproportionate impact on their reaction to different environmental toxins.

It is well established that dietary aflatoxins reduce the rate of growth and other measures of productivity in animals. To assess the effects of aflatoxin exposure on growth in humans, Gong *et al.*^[20,21] conducted two separate epidemiologic studies in West Africa. Those studies revealed a striking association between exposure to aflatoxin in children and both stunting (a reflection of chronic malnutrition) and being underweight (an indicator of acute malnutrition). Aflatoxin exposure has also been shown to be a factor in modulating the rate of recovery from kwashiorkor in children^[22,23]. The exact mechanisms underlying these effects of aflatoxins have not been elucidated.

Individuals with viral hepatitis infection

The risk of liver cancer in individuals exposed to chronic hepatitis B virus infection and aflatoxin is up to 30 times greater than the risk in individuals exposed to aflatoxin alone^[24,25]. These two HCC risk factors, aflatoxin and hepatitis B virus, are prevalent in poor nations worldwide. Within these nations, there is often a significant urban-rural difference in aflatoxin exposure and hepatitis B virus prevalence, with both these risk factors typically affecting rural populations more strongly^[25,26].

Aflatoxin also appears to have a synergistic effect on hepatitis C virus-induced liver cancer, although the quantitative relationship is not as well established as that for aflatoxin and hepatitis B virus in inducing HCC^[25,27-29]. Studies have also shown that the genetic characteristics of the virus, and the age and sex of the infected person may play a role in increasing the risk of aflatoxin induced HCC^[27].

PREVENTIVE MEASURES AND INTERVENTION STRATEGIES

Role of politics

The political will at a national level to address the issue of aflatoxin exposure is probably the most important factor in reducing the health hazards associated with aflatoxins in poor countries. As signatories to Codex Alimentarius (World Health Organization and Food and Agricultural Organization documents that deal with food quality) aflatoxin regulatory programs are already in place in most countries^[30]. On the export side these regulatory programs are strictly enforced to protect the export market of agricultural commodities, otherwise the importing countries would reject the commodities resulting in a loss of valuable foreign exchange earnings. On the other hand, domestic regulatory measures on aflatoxins have received very little attention and are rarely enforced, with no incentives given for the aflatoxin free produce and no heavy penalty on the violators of aflatoxin regulations.

Information dissemination

Considerable information has been gathered concerning the health hazards of aflatoxin exposure and conditions that lead to mold growth and aflatoxin contamination

during growing, harvesting and storage of crops. Steps that can be followed to avoid or minimize contamination have been developed. Information is also available on safe storage, handling and transportation practices of agricultural commodities. However, this information is rarely communicated to farmers, traders and all those who need to be informed. Much could be done if the value of different interventions is communicated and the information is disseminated in an appropriate and accessible manner^[31]. As an example, during an outbreak in Kenya in 2005, individuals who received information on maize drying and storage had lower serum aflatoxin levels than those who did not receive this information^[2].

Agricultural strategies

Agricultural interventions are methods or technologies that can be applied either in the field (“pre-harvest”) or in drying, storage, and transportation (“post harvest”) to reduce aflatoxin levels in food^[32].

The presence and growth of *Aspergillus* on pre-harvested crops is dependent on the environment. Agricultural practices including proper irrigation and pest management can reduce aflatoxin contamination^[2]. Pre-harvest interventions include choosing crops with resistance to drought, disease, and pests and choosing strains of that crop which are genetically more resistant to the growth of the fungus and the production of aflatoxins^[2]. Elimination of inoculum sources such as infected debris from the previous harvest may prevent infection of the crop^[2].

Before storage, crops should be properly dried to prevent the development of aflatoxins. Sorting and disposing of visibly moldy or damaged kernels before storage has proven to be an effective method for reducing the development of aflatoxins^[2,33,34]. During storage, moisture, insect, and rodent control can prevent damage to the crop and reduce aflatoxin development. Aflatoxin contamination of maize is influenced by the facilities used for storage, storage time, and the form of maize stored^[2,35]. A community-based intervention study in Africa showed that simple and inexpensive measures such as thorough drying and proper storage of groundnuts can have a significant impact on aflatoxin levels^[34].

Vaccination against hepatitis B virus

Hepatitis B virus infection increases the risk of HCC in individuals exposed to aflatoxins exponentially. Vaccination against hepatitis B virus in infancy is an effective approach to prevent HCC, particularly in developing countries where both incidence of hepatitis B virus and exposure to aflatoxins are high^[26,36,37]. Although the vaccine itself has no impact on actual aflatoxin levels in diets, it reduces aflatoxin-induced HCC by lowering hepatitis B virus risk, thereby preventing the synergistic impact of hepatitis B virus and aflatoxin in inducing liver cancer^[38]. Those who already have chronic hepatitis B virus infection would not benefit from the vaccine, which is why vaccination should be offered in infancy^[38]. Hepatitis B vaccination in infancy has been shown to be safe and effective^[26,39].

RESEARCH PROBLEMS AND NEEDS

Better funding

Aflatoxin research programs need adequate resources in terms of qualified personnel, capital investment, and analytical and technical facilities. Improved funding for aflatoxin research is in dire need, especially in countries with high rate of aflatoxin food contamination. Currently, on a global basis, there is an imbalance between the extent of aflatoxin problem and the funds that have been allocated to its research.

Promotion of a multidisciplinary approach

Aflatoxin contamination of food and feed is a problem that affects multiple disciplines such as agriculture, toxicology, medicine, biology, microbiology, veterinary medicine and other related fields. Hence, a multidisciplinary research approach to the problem should be encouraged and promoted.

Better sampling methods

Determination of aflatoxin levels in food or feed is done via sampling. Sampling of lots of food or feed for aflatoxin analysis is an important step in exposure prevention. Current sampling methods for aflatoxin are not considered totally reliable. Furthermore, sampling techniques have not been standardized and various trade bodies have their own sampling procedure and technique^[40]. Research is needed to identify better and cheaper sampling methods which can in turn lead to standardization of sampling procedures.

Development of resistant cultivars

One of the possible ways to reduce aflatoxin contamination of food commodities is the use of cultivars resistant to seed invasion by aflatoxin-producing fungi^[41]. Breeding for resistance to aflatoxin producing *Aspergillus* species can play a significant role in preventing aflatoxin contamination of food.

Development of competitive and antagonistic microorganisms

Theoretically, competitive and antagonistic native microorganisms that can reduce the populations of aflatoxin producing fungal strains present in the soil have the potential to reduce infection of the plants^[42]. Research is needed to develop such microorganisms and also to make sure that they do not pose any dangers to humans, animals or environment by their own.

Identification of modulators of aflatoxin toxicity

Whereas it is highly desirable that food is not contaminated, the reality is that in parts of the world food contamination with aflatoxins is unavoidable. Identification of substances that can be incorporated into the human diet to reduce or prevent aflatoxin toxicity would have a great potential in reducing the incidence of aflatoxin induced HCC in endemic areas.

Determination of in utero and early childhood exposure effects

In endemic areas, pregnant women are often exposed to aflatoxin contaminated food. There is lack of knowledge about the effects of aflatoxin exposure in utero and early childhood. Research is needed to better understand these effects. This knowledge is fundamental in identification and design of preventive strategies.

CONCLUSION

The purpose of this editorial is to summarize the current knowledge on association of aflatoxin and HCC, encourage future research and draw attention to this global public health issue.

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Microbial manipulation as primary therapy for Crohn's disease

Randy S Longman, Arun Swaminath

Randy S Longman, Arun Swaminath, Division of Digestive and Liver Disease, Department of Medicine, Columbia University Medical Center, New York, NY 10032, United States
Author contributions: Longman RS and Swaminath A wrote the manuscript.

Correspondence to: Arun Swaminath, Assistant Professor, Division of Digestive and Liver Diseases, Department of Medicine, Columbia University Medical Center, 622 West 168th Str VC5, New York, NY 10032,

United States. as3576@mail.cumc.columbia.edu

Telephone: +1-212-3051021 Fax: +1-212-3055576

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Abstract

While antimicrobials are clinically effective in preventing post-operative recurrence, the role for antibiotics in primary therapy for Crohn's disease (CD) remains unclear. The recent multicenter phase 2 trial by Prantera *et al* received wide attention because it demonstrated an increase in the week 12 remission rate in patients with moderately active CD treated with rifaximin and renewed interest in microbial manipulation as primary therapy for CD. In this commentary, we discuss aspects of durability, immune cell polarization, and safety of microbial manipulation as primary therapy for CD.

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Key words: Inflammatory bowel disease; Rifaximin; Microbiome

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COMMENTARY ON HOT TOPICS

Inflammatory bowel disease (IBD) results from a dysregulated immune response to environmental and microbial antigens in a genetically susceptible host. Although we cannot readily manipulate the host genotypes of our patients, numerous clinical studies have attempted to modulate the inflammatory immune response with prebiotic, probiotic, and antimicrobial therapy. The results of randomized controlled trials of antibiotics, however, have been mixed (Table 1). While antimicrobials have gained traction in preventing post-operative recurrence, the role for antibiotics in primary therapy for Crohn's disease (CD) remains unclear. *Post-hoc* analysis of these studies has suggested that antibiotics may be more appropriate in patients with large bowel involvement, but this remains unproven in a randomized controlled trial. Furthermore, despite statistically significant differences in reduction in Crohn's disease activity index (CDAI) in several studies, the lack of effect on true disease remission (CDAI < 150), the concern for medication side effects, and the increasing rate of antibiotic associated *Clostridium difficile* (*C. difficile*) in the IBD population has limited its use in practice.

Given these mixed results, the recent multicenter phase 2 trial by Prantera *et al*^[1] received wide attention because it demonstrated an increase in the week 12 remission rate in patients with moderately active CD treated with 800 mg rifaximin extended release (ER) twice per day (*bid*) (62% vs 43%, $P = 0.005$) and renewed interest in microbial manipulation as primary therapy for CD. As we evaluate the implications of this work, several questions arise: What is the durability of this effect both clinically and microbially? How do we select the patients who will derive the most benefit from antimicrobial therapy? Are these therapies safe?

Durability of response is crucial in coordinating medical therapy and prognosticating clinical course. Evidence of mucosal healing in addition to clinical indicators of disease activity represented in the CDAI define a "deep

Table 1 Randomized controlled trials of antibiotic therapy in inflammatory bowel disease

| Ref. | Antibiotic therapy | Patients (n) | Primary outcome | Results |
|---|--|--------------|---------------------|--------------------------------|
| Afdhal <i>et al</i> ^[12] | Clofazimine | 49 | DAS < 5 | 64% (vs 50% placebo, NS) |
| Sutherland <i>et al</i> ^[13] | Metronidazole | 105 | ↓CDAI (16 wk) | 81 (vs -1 placebo, P = 0.001) |
| Prantera <i>et al</i> ^[14] | Ethambutol, rifampicin, clofazimine, dapsone | 45 | Relapse (9 mo) | Likelihood ratio: 4.6 |
| Steinhart <i>et al</i> ^[15] | Ciprofloxacin, metronidazole | 130 | Remission (8 wk) | 33% (vs 38% placebo, NS) |
| Arnold <i>et al</i> ^[16] | Ciprofloxacin | 47 | ↓CDAI (6 mo) | 75 (vs 25 placebo, P < 0.001) |
| Prantera <i>et al</i> ^[17] | Rifaximin | 83 | ↓CDAI < 150 (12 wk) | 52% (vs 33% placebo, NS) |
| Selby <i>et al</i> ^[18] | Clarithromycin, rifabutin, clofazimine | 213 | Relapse (2 yr) | 66% (vs 50% placebo, P = 0.02) |
| Leiper <i>et al</i> ^[19] | Clarithromycin | 41 | ↓CDAI < 150 (3 mo) | 26% (vs 27% placebo, NS) |

DAS: Disease Activity Score; CDAI: Crohn's disease activity index; NS: Not significant.

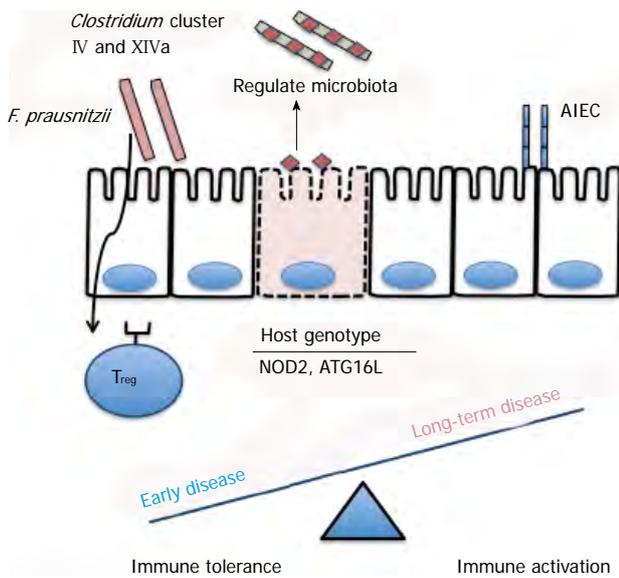


Figure 1 Microbiota regulate immune tolerance and activation in inflammatory bowel disease. *Clostridium* cluster IV and XIVa can induce regulatory T cell (T_{reg}) polarization in the lamina propria^[7]. One member of this cluster, *Fecalibacterium prausnitzii* (*F. prausnitzii*), correlates with reduced post-operative recurrence in Crohn's disease (CD)^[6]. Adherent invasive *Escherichia coli* (AIEC) are found with greater frequency in ileal CD^[11]. Interaction with microbial species may differentially modulate the immune response in early inflammatory disease compared to long-term fibrotic disease^[8]. Host genotype may regulate luminal microbial species. NOD2: Nucleotide-binding ligomerization domain-containing protein 2; ATG16L: Autophagy-related protein 16-1^[9].

remission” associated with a durable response. While the CDAI data generated by Prantera *et al*^[1] are encouraging, remission and response depended solely on clinical indicators, some of which are subjective and not necessarily reflective of systemic inflammation. Objective endoscopic and serologic endpoints to define local and systemic control of inflammation will be crucial in follow up studies of antibiotics as primary therapy.

The second aspect of durability is the effect of rifaximin on the intestinal microbiome. The intestinal “microbiome” refers to the totality of intestinal bacteria and the collection of genetic data that it encodes is called a metagenome. Advances in sequencing technology over the last decade have enabled broader analysis of the types of bacteria that are present in the intestine. Pioneering work defining the full spectrum of intestinal microbiota

in patients with IBD by 16S ribosomal RNA sequence (instead of conventional culture methods)^[2] led to the characterization of an IBD microbiome, reflecting a general reduction in bacterial diversity, a decrease in the clostridial family *Lachnospiraceae*, and an expansion of proteobacteria. More specific characterization of clinical phenotype [ileal Crohn's disease (ICD), colonic CD, ulcerative colitis] in a cohort of Swedish twins revealed the particular prevalence of *Escherichia coli* (*E. coli*) species in ICD with a unique contribution of *Ruminococcus gnavus*^[3]. One interesting finding by Scarpignato *et al*^[4] is that clinical remission was maintained in 63% of the patients in the treatment group up to 12 wk after finishing rifaximin therapy. Prior studies have shown return of pretreatment levels of microbiota within 1-2 wk after discontinuing rifaximin, so it remains unclear whether the durability of this treatment is secondary to a permanent change in the intestinal microbiota or a suppression of a specific pathogenic species. While this analysis is beyond the scope of the study offered by Prantera *et al*^[1], future studies will need to incorporate microbial analysis as well as metatranscriptomic analysis (*e.g.*, what the bacteria are doing) in order to recognize the full diagnostic and therapeutic potential of antimicrobial therapy.

Given rifaximin's broad spectrum of activity against anaerobic and aerobic gram-negative and gram-positive organisms, it is possible that rifaximin treatment eliminates a particular pathogenic or group of pathogenic bacteria that was unaffected by the antibiotics used in previous investigations. If so, does this explain the lack of a dose response in the study? In contrast to previous studies, Prantera *et al*^[1] show no benefit to colonic location of disease [odds ratio (OR) 0.5, P = 0.004]. Does this mean that a suspected pathogen isn't restricted to the colon or that colonic localization is not required? Given the distribution of CD throughout the gastrointestinal tract, this may be a reasonable conclusion.

Microbial analysis suggests several candidate bacteria may be involved in the pathophysiology of CD. Notably, adherent-invasive *E. coli* (AIEC) have been described to be attached to the ileal mucosa of patients with ICD^[5]. These invasive bacteria may sustain inflammation in genetically susceptible individuals and generate systemic immune responses (reflected by serologies) (Figure 1). While *E. coli* are sensitive to rifaximin *in vitro*, the effect

of rifaximin on AIEC populations *in vivo* has not been clearly defined, but could be studied in this cohort. In addition to AIEC, analysis of a post-operative ICD cohort revealed the correlation of the clostridial species, *Faecalibacterium prausnitzii* (*F. prausnitzii*), with a decreased incidence of post-operative recurrence^[6]. *Clostridium* species IV and XIVa (which include *F. prausnitzii*) have been shown in mouse models to induce the accumulation of regulatory T cells in the colon^[7] (Figure 1). Further microbial analysis of primary antimicrobial therapy for CD may offer deeper insight into the mechanism of rifaximin therapy.

If the efficacy of rifaximin depends on microbial mediated disease, perhaps there are clinical or diagnostic parameters that may highlight patients that will derive maximal benefit from antimicrobial therapy? Subgroup analysis by Prantera *et al*^[1] revealed maximal benefit in patients with “early disease”, defined as < 3 years at time of entry into the study (OR 1.7, *P* = 0.02). Recent data in mice showed that the timing of introduction of microbiota into “germ-free” animals regulates the influx of immune cells into intestinal tissue by modulating the expression of genes involved in recruiting these cells^[8]. Perhaps “early disease” maintains immunologic plasticity whereas long-standing disease has already been programmed to support inflammation. Further characterization may reveal distinct microbial components of their cohort. Finally, it would be interesting to know if disease susceptibility alleles correlate with antimicrobial response. A recent study of microbiota in patients with IBD revealed that genetic susceptibility alleles for nucleotide-binding oligomerization domain-containing protein 2 and autophagy-related protein 16-1 correlate with alterations in the microbiome^[9]. These clinical and genotypic parameters may improve the targeted use of antibiotic therapy for CD.

The safety of widespread and long-term antibiotics also remains an issue of concern. Rifaximin has minimal systemic absorption. As such, rifaximin does not have notable systemic side effects or interactions associated with imidazole or fluoroquinolone antibiotics. *C. difficile* remains a major problem in the clinical management of IBD with rising rates of CDI associated with morbidity, mortality and need for colectomy^[10]. Although rifaximin may help treat *C. difficile*, one case of *C. difficile* was seen in the 800 mg ER *bid* group. Further studies will be needed to determine the strength of this association. Rifaximin resistance has evolved in AIEC and should be evaluated before widespread use is adopted^[11].

In summary, this study by Prantera *et al*^[1] offers important results and safety data for the use of rifaximin and supports the role for this anti-microbial in improving remission rates in mild to moderate CD. Hard endpoints including mucosal healing and measurements of systemic inflammation will enable crucial evaluation of the efficacy of rifaximin in phase 3 trials. Further analysis of the microbial alterations during rifaximin therapy are important in not only understanding the biology of the microbiome in IBD, but also in designing rational therapeutic strate-

gies for microbial manipulation. Disease location, systemic inflammatory markers, host genotype, and intestinal microbial signatures will ultimately guide a personalized medical approach to the clinical use of directed antimicrobial and/or bacteriotherapy. Although many questions of mechanism and durability remain, Prantera *et al*^[1] offer an important step forward in the role for microbial manipulation in the clinical management of IBD.

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Quality colonoscopy: A matter of time, technique or technology?

Robert H Lee

Robert H Lee, Veterans Affairs Long Beach Health Care System, University of California Irvine, Long Beach, CA 93109, United States

Author contributions: Lee RH solely contributed to this work.
Correspondence to: Robert H Lee, MD, Clinical Assistant Professor of Medicine, Veterans Affairs Long Beach Health Care System, University of California Irvine, 5901 E. Seventh St., Long Beach, CA 93109, United States. rlee8@uci.edu
Telephone: +1-562-8265752 Fax: +1-562-8265569

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Abstract

Quality colonoscopy is defined by the detection of adenomatous polyps at least 25% of the time in men and 15% of the time in women. Recent studies highlight the importance of key aspects of high quality colonoscopy. These include the amount of time spent examining the mucosa or withdrawal time, the quality of withdrawal technique and new technologies which seek to maximize the detection of colonic neoplasia. This review summarizes the latest evidence regarding the role of time, technique and technology in shaping the quality of colonoscopy.

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Key words: Adenoma; Colorectal cancer; Quality colonoscopy; Colorectal cancer screening

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COMMENTARY ON HOT TOPICS

Colonoscopy is widely considered to be the most effective

tool for colorectal cancer (CRC) screening. However, recent studies suggest that discrepancies in the quality of colonoscopy are the cause of uneven outcomes in CRC detection and prevention. As a result, clinical researchers, professional societies, and governmental policy-makers have sought to identify benchmarks for quality colonoscopy. The recent article by Filip *et al*^[1] represents an important contribution to this ongoing effort to delineate the key aspects of high quality colonoscopic examination. Furthermore, it brings into focus the salient questions which define the current debate about quality improvement in screening colonoscopy.

What is quality colonoscopy?

To address this question, the American College of Gastroenterology and the American Society for Gastrointestinal Endoscopy in 2006 developed guidelines establishing quality indicators for colonoscopy. Outlining intra-procedural standards for colonoscopy, these guidelines establish a withdrawal time (WT) ≥ 6 min, and a cecal intubation rate of $\geq 95\%$ as quality indicators^[2]. However, the most important benchmark is an adenoma detection rate (ADR) of $\geq 25\%$ in men and $\geq 15\%$ in women for average risk screening colonoscopy^[3]. European guidelines concur with this observation and outline a goal ADR of 20% for average-risk colorectal screening in patients over the age of 50^[4]. ADR was chosen as the primary quality indicator because the main benefit of colonoscopy, the detection and removal of neoplastic lesions has been estimated to prevent 76%-90% of CRCs^[5-7]. While more recent studies suggest that the polyp detection rate (PDR) which includes the detection of non-adenomatous polyps (hyperplastic polyps) can be used as a surrogate for ADR, ADR remains the principal quality indicator for colonoscopy^[4,8,9].

Do we perform quality colonoscopy?

Over the last decade, colonoscopy has been increasingly utilized as the primary modality for CRC screening in the United States, with a 14% increase in use among Medicare recipients from 2000-2003^[10]. However, recent evi-

dence suggests that the increase in colonoscopy utilization has not uniformly resulted in a concomitant reduction in CRC-related morbidity and mortality. In a case control study, Baxter *et al*^[11] demonstrated that screening colonoscopy decreased overall CRC-related mortality [odds ratio (OR) 0.69, 95%CI: 0.63-0.74] and left-sided CRC-related mortality (OR 0.69, 95%CI: 0.28-0.39). However, alarmingly, the study found that colonoscopy did not significantly decrease the risk of death from right-sided CRC (OR 0.99, 95%CI: 0.86-1.14)^[11]. This finding was remarkable given that it questioned the long-standing presumption that colonoscopy was superior to other CRC screening modalities primarily through its ability to detect right-sided neoplasms.

The relationship between the use of colonoscopy and its variable impact on CRC prevention is further elucidated by data on missed CRCs from the Manitoba cancer registry^[12]. Defining a missed cancer as a CRC occurring within 6-36 mo of colonoscopy, the investigators found that nearly 1 in 13 CRCs were likely missed on initial colonoscopic examination^[12]. Furthermore, risk factors for missed CRCs included colonoscopy with polypectomy, and proximal location, thus potentially implicating failed cecal intubation and the incomplete resection of polyps as potential causes^[12].

The importance of missed proximal colonic polyps is highlighted by the emerging recognition of sessile serrated adenomas (SSA) as distinct colonic neoplasia with malignant potential. Histologically marked by disorganized and distorted crypt patterns, SSA tend to be proximal in location and to appear as flat or depressed lesions that are easily missed without careful examination^[13]. The potential association between missed CRCs and these lesions is significant in that SSA have been found to carry an increased risk of proximal CRC (OR 4.79, 95%CI: 2.16-5.03)^[14].

The most compelling evidence linking the quality of colonoscopy to CRC prevention outcomes comes from a study by Kaminski *et al*^[15] which examined endoscopists' ADR and the risk for interval CRC after colonoscopy. In comparing endoscopists with mean ADR of < 11% *vs* those with ADR of > 20%, the investigators found a cumulative hazard rate for the development of interval CRC of 10.94 (95%CI: 1.37-87.01)^[15]. In a Cox proportional hazards regression model, endoscopists' ADR along with the patients' age were the only independent predictors of interval CRC^[15]. This study, along with the others previously discussed, strongly suggests that quality colonoscopy is not uniformly performed. Furthermore, it highlights the potential adverse impact of poor quality colonoscopy when it comes to CRC prevention.

Are endoscopists to blame for poor quality colonoscopy?

While factors such as poor bowel preparation, and patients' genetic predisposition for colorectal neoplasia have been implicated in missed neoplasia and the development of CRC between colonoscopies, the preponderance of evidence points to the role of the endoscopist

in determining the quality of colonoscopy^[16]. In a study of over 10 000 colonoscopies, Chen *et al*^[17] found a high degree of variability in mean ADR ranging from 14% to 34.6% among 9 endoscopists. In a multi-variable analysis, the identity of the endoscopist was found to have a similar impact on ADR as patient age and gender^[17]. In a separate study involving missed polyps found on tandem colonoscopy (back-to-back colonoscopies performed to assess for missed lesions), Rex *et al*^[18] found similar variability among participating endoscopists with adenoma miss rates ranging from 17% to 48%. Other factors related to the identity of the endoscopist such as medical specialty (gastroenterologist *vs* non-gastroenterologist), and training level have also been implicated as having an impact on ADR^[12,19,20]. Consequently, it is clear that factors related to the individual endoscopist have a large impact on the quality of colonoscopy.

Is quality colonoscopy a matter of time?

The debate over quality colonoscopy has largely centered on the issue of colonoscopy WT or the amount of time inspecting the colonic mucosa for neoplastic lesions. This is largely due to the landmark paper by Barclay *et al*^[21] which compared ADR among endoscopists with varying WT. Defining WT as the time from cecal identification to withdrawal of the scope from the anus, the investigators found that endoscopists with WT \geq 6 min had higher ADR compared to those with WT < 6 min (28.3% *vs* 11.8%, $P < 0.001$)^[21]. In a similar retrospective study of over 10 000 colonoscopies, Simmons *et al*^[22] found that prolonged WT was associated with higher PDRs ($r = 0.76$, $P < 0.001$) and that overall median polyp detection corresponded to a WT of > 6.7 min.

However, since the publishing of these initial studies, efforts at quality improvement by simply mandating a minimal WT have largely proven to be unsuccessful in significantly improving ADR. In a study by Sawhney *et al*^[23] the establishment of a mandatory WT of \geq 7 min produced a significant increase in the compliance rate for WT from 65% to 100%. However, in spite of this, there was no concomitant increase in the PDR (slope 0.0006, $P = 0.45$)^[23]. Similar studies involving continuous feedback regarding mean WT to endoscopists have also been disappointing in producing significant increases in ADR^[24].

One potential explanation for these findings is the possibility that there may be a ceiling to the degree of improvement in ADR that can be achieved by simply prolonging WT. This was well illustrated by retrospective data from the VA cooperative study where the mean WT was well above 12 min^[25]. While mean WT was associated with initial adenoma detection, it did not correlate with the probability of finding interval neoplasia on surveillance colonoscopy ($P = 0.61$)^[25]. A similar finding was found in a German study where WT did not correlate with variability in ADR when the mean WT ranged from 6-11 min^[26]. Given these observations, there is clear cut evidence that while WT is certainly an important perfor-

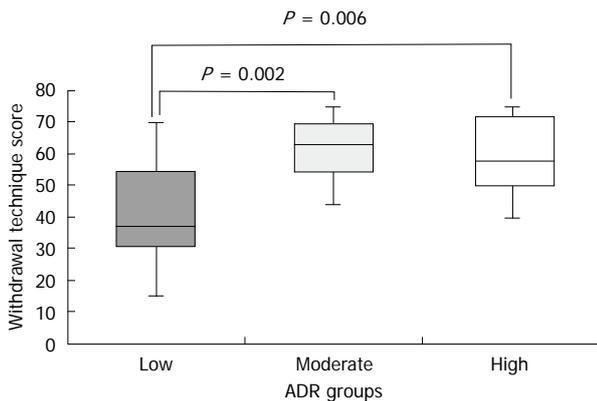


Figure 1 Withdrawal technique scores among endoscopists with low, moderate and high adenoma detection rates. ADR: Adenoma detection rate.

mance parameter, it may not necessarily be the deciding factor in determining the overall quality of colonoscopy.

Is colonoscopy a matter of technique?

Along with the speed of withdrawal, recent attention has focused upon the technique that is used to examine the colonic mucosa for neoplasia. The first study to examine this by Rex *et al*^[27] compared two endoscopists with markedly different adenoma miss rates found in a separate tandem colonoscopy study. Using video-recordings of colonoscopy withdrawals and a 5 point scale to grade the quality of withdrawal technique, the investigators found that the endoscopist with the lower adenoma miss rate (17%) had higher scores for all aspects of withdrawal (distension, cleansing, time spent viewing, examination of proximal aspects of folds) compared to the endoscopist with the highest adenoma miss rate (48%)^[27].

Our research team recently further elucidated the potential relationship between WT and withdrawal technique among a broader set of endoscopists from varying institutions (11 endoscopists from 2 Veterans Affairs Hospitals and 3 University Hospitals)^[28]. A video-recording protocol and grading system was utilized to characterize withdrawal technique and WT of endoscopists with low (11.8% ± 3.4%), moderate (34.1% ± 2.6%) and high ADR (49.0% ± 3.7%)^[28]. Withdrawal technique was assessed using a scale adapted from Rex *et al*^[27] that assigned points (0-5) for three specific dimensions: (1) fold examination (0 = not looking behind folds, 5 = look behind all folds); (2) distension (0 = not cleaning pools, 5 = cleaning all pools); and (3) cleansing (0 = not distended or in spasm, 5 = good distension). Scores for each dimension were assigned for 5 areas of the colon (cecum, ascending, transverse, descending, sigmoid). Only colonoscopies performed for average-risk CRC screening in which cecal intubation was achieved were evaluated. Using this scoring system, we found that High and Moderate ADR endoscopists had higher withdrawal technique scores compared to low ADR endoscopists (Figure 1)^[28]. Furthermore, when the highest and lowest ADR endoscopists were compared, we did not find a significant difference in WT (6.6 ± 1.7 min *vs* 7.4 ± 1.7 min) ($P =$

0.36), but did find a nearly 2-fold difference in technique score (36.2 ± 9 *vs* 61 ± 9.9, $P = 0.0001$)^[28]. One potential explanation for this was the possibility that low ADR endoscopists purposely slowed down the speed of withdrawal to meet the 6 min goal but nonetheless failed to perform a high level of quality withdrawal technique.

The importance of withdrawal technique was also recently highlighted by a quality improvement study by Barclay *et al*^[29]. Unlike the Sawhney study^[23] which solely focused upon a minimal WT, the quality improvement protocol utilized by Barclay *et al*^[29] included both a WT mandate and an institution-wide meeting among endoscopists that established guidelines on optimal withdrawal technique. Following this two-pronged approach, the investigators demonstrated an improvement in ADR (37.8% post-intervention *vs* 23.5% pre-intervention, $P < 0.0001$) and a higher number of advanced neoplasia per patient screened^[29].

The development of newer techniques for mucosal inspection also holds great promise for efforts to enhance the quality of colonoscopy. East *et al*^[30] recently showed that the use of dynamic changes in patient position during withdrawal resulted in a mean ADR of 52% compared with an ADR of 34% ($P < 0.001$) in cases where withdrawal was only performed while the patient was in the left lateral decubitus position. The use of large volume water immersion during colonoscopy along with water exchange to remove residual stool may improve mucosal visualization, with a recent meta-analysis showing an increased detection of right-sided adenomas when using this technique^[31]. Finally, utilizing the concept of the Hawthorne effect, which describes the phenomenon in which individuals often will perform better when they know that they are being monitored, Rex *et al*^[32] have demonstrated that the simple act of video-recording the procedure results in improved WT and withdrawal technique.

Is quality colonoscopy a matter of technology?

Innovations in endoscope development and imaging have shifted the focus towards finding a technological solution to the task of ensuring quality colonoscopy. One of the earliest methods to be applied to the goal of maximizing adenoma detection is chromoendoscopy. Using a spray catheter to coat the lining of the colonic mucosa with either methylene blue or indigo carmine dyes, this approach enhances colonic pit patterns and demarcates the border between normal and abnormal mucosa. Because of its ability to differentiate flat adenomas, a recent meta-analysis has demonstrated that chromoendoscopy is associated with a higher ADR (OR 1.67, 95%CI: 1.29-2.15) and a higher detection rate for ≥ 3 neoplastic lesions (OR 2.55, 95%CI: 1.49-4.36) compared to white-light endoscopy (WLE)^[33]. Furthermore, Stoffel *et al*^[34] conducted a study where patients underwent either chromoendoscopy or WLE as the second part of a tandem colonoscopy study. Here, they found that chromoendoscopy detected a higher percentage of missed adenomas (44% *vs* 17%,

$P = 0.04$) even when controlled for WT^[34]. Given these results, the investigators conclude that the higher ADR seen with chromoendoscopy is due to the method itself rather than as a consequence of the endoscopist having to take a longer time in inspecting the colon^[34].

While current evidence suggests that chromoendoscopy does result in higher ADR, the method is time-consuming and requires additional equipment. Consequently, modalities that rely upon imaging that is built into the processor of the colonoscope have been examined as a means of maximizing ADR. Narrow band imaging (NBI) is the most widely available technology utilizing short wave-length light that is primarily absorbed by hemoglobin in the superficial mucosa^[35,36]. Highlighting mucosal pit patterns and vascularity, NBI offers the ability to potentially differentiate abnormal from normal mucosa with the simple press of a button on the colonoscope. However, a systematic review of both observational and clinical trials recently demonstrated that NBI did not result in higher ADR compared with WLE (OR 1.19, 95%CI: 0.86-1.64). Furthermore, NBI did not yield a higher number of adenomas per patient (relative ratio of means 1.23, 95%CI: 0.93-1.61)^[37]. While other evidence suggests that NBI has sufficient sensitivity and specificity in differentiating adenomatous from non-adenomatous tissue to potentially give rise to a resect and discard strategy for colonic polyps, current data does not support its use as a means of enhancing ADR^[37].

Another potential imaging modality that has been proposed to increase ADR is auto-fluorescence imaging (AFI). AFI relies upon the observation that the colonic mucosa emits auto fluorescent light in response to illumination by ultraviolet light^[38]. Furthermore, the wavelength of the auto fluorescent light is dependent on architecture, light-absorptive properties and the metabolic status of the tissue that is being illuminated^[38]. Exploiting this capability, AFI has been characterized as a potential “red-flag” technology that would warn the endoscopist to carefully inspect an area where a flat neoplastic lesion is located.

Preliminary studies which have examined the relationship between AFI and adenoma detection have thus far proven to be disappointing. In a head to head study of AFI *vs* high resolution endoscopy (HRE), van den Broek *et al*^[39] found no significant differences in adenoma miss rates (29% *vs* 20%, $P = 0.35$). In a study examining the use of tri-modal imaging (AFI plus NBI plus HRE), Kuiper *et al*^[40] found an ADR that was virtually the same as that seen with standard WLE (34% *vs* 37%, $P = 0.61$).

Other technologies on the horizon which hold promise include the third-eye retroscope (Avantis Medical Systems, Sunnyvale, California) which allows for the retrograde visualization of neoplastic lesions behind mucosal folds. In a tandem colonoscopy study, Siersema *et al*^[41] recently showed that the Third Eye system resulted in a lower adenoma miss rate when compared with WLE. While these results are promising, the broad preponderance of the evidence regarding new technologies sug-

gests that technology by itself cannot guarantee quality in colonoscopy.

The way forward for quality colonoscopy

Given the world-wide economic challenges surrounding health care delivery, governments and third party payers are placing a renewed focus on policies that provide the most cost-effective approach towards disease prevention. As part of this trend, quality benchmarks for colonoscopy stand as obvious targets for Pay-For-Performance measures that seek to reward patient-oriented outcomes in CRC screening. Faced with this imperative, the endoscopic community will need to find innovative approaches to measuring and improving the quality of colonoscopy.

The review conducted in this paper illustrates that there probably is not just one solution to the dilemma of ensuring quality in colonoscopy. While recent studies have clearly demonstrated that the amount of time spent examining the mucosa is a vital component, evidence from quality improvement programs suggest that there is likely a ceiling effect to WT. Furthermore, while withdrawal technique does have a definitive impact on ADR, measuring the quality of technique is time-consuming, burdensome and not easily performed. Finally, given the mixed results of technological solutions for enhancing ADR, it is clear that further work must be done to integrate these advances into everyday practice.

The quest to preserve colonoscopy as the primary tool for quality CRC screening demands a multi-pronged approach using research and innovation to enhance all three aspects of colonoscopy. By providing a potential means for easily quantifying the quality of withdrawal technique, the paper by Filip *et al*^[1] stands as an important contribution to this process. Similar research endeavors are clearly required to achieve the goal of ensuring quality in colonoscopy.

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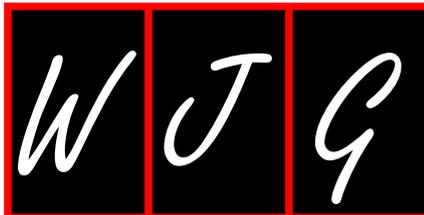
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Intestinal metaplasia surveillance: Searching for the road-map

Angelo Zullo, Cesare Hassan, Alessandro Repici, Bruno Annibale

Angelo Zullo, Cesare Hassan, Gastroenterology and Digestive Endoscopy Unit, Nuovo Regina Margherita Hospital, 00153 Rome, Italy

Alessandro Repici, Department of Gastroenterology, IRCCS Istituto Clinico Humanitas, 20089 Milan, Italy

Bruno Annibale, Department of Digestive and Liver Disease, Sant'Andrea Hospital, II School of Medicine University Sapienza of Rome, 00153 Rome, Italy

Author contributions: Zullo A and Hassan C wrote the manuscript; Repici A and Annibale B supervised the manuscript.

Correspondence to: Angelo Zullo, MD, Gastroenterology and Digestive Endoscopy Unit, Nuovo Regina Margherita Hospital, Via E. Morosini, 30, 00153 Rome, Italy. zullo66@yahoo.it

Telephone: +39-6-58446608 Fax: +39-6-58446533

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tailoring the endoscopic follow-up. Finally, some data would suggest that a 3-year follow-up in patients with extensive gastric precancerous conditions could result in an inadequate secondary prevention.

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Key words: Intestinal metaplasia; Guidelines; Atrophic gastritis; Gastric cancer; Follow-up

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Abstract

Atrophic gastritis and intestinal metaplasia (IM) of the stomach are common and are associated with an increased risk for gastric cancer. In the absence of guidelines, a pragmatic management has been performed in Western countries in patients with these premalignant conditions. Recently, formal European guidelines have been delivered on this topic. Basically, it has been recommended that patients with extensive atrophic gastritis (AG) and/or extensive IM should be offered endoscopic surveillance every 3 years. On the contrary, no scheduled endoscopic/histological control has been advised for those patients with precancerous conditions confined to the antrum. In this commentary, we highlighted some potential weaknesses in the management formally recommended by the new guidelines. In detail, we discussed that AG and IM patients do not share the same gastric cancer risk, at least in Western countries, deserving a different approach. Some factors significantly associated with gastric cancer risk, such as IM type, first-degree family history of gastric cancer, and smoking habit have not been considered in

COMMENTARY ON HOT TOPICS

We read with great interest the recent article by Dinis-Ribeiro *et al*^[1] reporting the first European Guidelines on management of precancerous conditions and lesions in the stomach (MAPS), and strongly recommend it to readers. Despite gastric cancer incidence is decreasing, such a neoplasia remains the fourth most prevalent tumor and second most frequent cause of cancer-related mortality in the world^[2]. The endoscopic-based screening programs performed in a few Asian countries^[3], where the gastric cancer incidence is extremely high, are not feasible in other countries due to a distinctly lower frequency of such a neoplasia. Therefore, in the Western countries, surveillance of precancerous conditions [gastric atrophy and intestinal metaplasia (IM)] and lesions (dysplasia) is the only reliable procedure able either to reduce gastric cancer onset - *i.e.*, by removing dysplasia areas at endoscopy or to diagnose an already-developed cancer in an early stage, so that patient survival is distinctly improved^[4,5]. However, the American Society for Gastrointestinal Endoscopy Guideline recommended against the surveillance

for patients with IM^[6]. Until few months ago, no European guidelines were available on the management of these precancerous lesions, leaving both gastroenterologists and general practitioners (GP) to empirically manage these patients without any actual reference standard. For this reason, Dinis-Ribeiro *et al*^[1] should be commended for having organized a workshop involving a vast panel of experts, in order to deliver the first European guideline on such a topic.

According to these new guidelines, it has been recommended that patients with extensive atrophy gastritis (AG) and/or extensive IM - *i.e.*, involving both antral and gastric body mucosa - should be offered endoscopic surveillance (evidence level 2++, recommendation grade B) every 3 years (evidence level 4, recommendation grade D). On the contrary, no scheduled endoscopic/histological control has been advised for those patients with precancerous conditions confined to the antrum. Therefore, both gastroenterologists and GPs have now a “road map” to systematically schedule the surveillance in patients with either AG or IM.

Although any guideline is better than no guideline, we would further discuss some potential flaws entailed in the management recently recommended. First, it might appear questionable that the same endoscopic follow-up has been advised for both AG and IM patients. In a nationwide study^[7], the 10-year gastric cancer incidence was estimated to be 0.8% and 1.8% in 22 365 AG and 61 707 IM patients, respectively, corresponding to an adjusted yearly incidence of 0.055% and 0.1%^[8]. Given the two-fold different gastric cancer risk, perhaps a differently scheduled endoscopic surveillance should be proposed for AG and IM patients. Differently from IM^[9], a strict follow-up in AG patients may be not cost-effective in Western countries^[10].

Secondly, appropriateness of the suggested interval for endoscopic follow-up is not well documented. The 3-year interval selected for patients with extensive AG or IM does not appear to be corroborated by any prospective study. Indeed, the panel of experts downgraded this statement as level 4, grade D. However, at least two studies demonstrated that only 36% and 38% of detected gastric cancers were in an early stage (*i.e.*, stage I disease), when scheduling the endoscopic surveillance interval at 1- or 2-years, respectively^[11,12]. Therefore, despite patients underwent a more intensive follow-up than the 3-year interval now officially recommended^[1], gastric cancer was diagnosed in an advanced stage in as many as 62%-64% of the cases. Based on these observations, an even worse scenario cannot be excluded when a 3-year interval follow-up is to be implemented in clinical practice. The dismal prognosis of gastric cancer diagnosed in an advanced stage poses ethical concerns about recommending a 3-year surveillance interval for IM patients. On the other hand, it is also unquestionable that an appropriate use of endoscopic procedures is essential to the rational use of finite resources. To dissipate economic resources in

performing yearly endoscopic controls in all IM patients - most of which would never develop gastric cancer - would also be unethical. A possible solution could be represented by a patient-tailored approach. Similarly to the extensive spreading of AG or IM in the stomach - the only risk factor considered in the MAPS guidelines^[1] - several studies demonstrated that other factors increased gastric cancer risk, including IM type, first-degree family history of gastric cancer, and smoking habit. Presence of incomplete type IM significantly increased the hazard ratio of gastric cancer as compared to complete IM (hazard ratio: 11.3, 95%CI: 3.8-33.9)^[13]. A first-degree family history of gastric cancer also increases such risk by 2.6-3.5 times, with a calculated attributable risk of 8%^[14,15]. Indeed, the gastric carcinogenetic cascade in these subjects seems to start earlier than in controls^[16]. A meta-analysis, considering 14 442 cases and 73 918 controls, found that smoking significantly increased gastric cancer risk, with an overall odds ratio (OR) of 1.48 (95%CI: 1.28-1.71), and an OR of 1.69 (95%CI: 1.35-2.11) for current smoker status in comparison to never smokers^[17].

Therefore, IM patients with at least 1 of these risk factors (incomplete IM, family history, smoking habit) - information easily available in clinical practice - would appear at a further increased risk of gastric cancer and may probably benefit of a more intensive endoscopic surveillance, rather than the 3-year follow-up uniformly suggested for all patients^[1]. We recently proposed a patient individualized follow-up with an yearly endoscopic control in those patients with adjunctive risk factors, and a less intensive (2-3 years) follow-up in the remaining IM patients^[18]. For instance, a patient with incomplete IM confined to the antral mucosa would not appear to be at lower gastric cancer risk as compared to a patient with extensive, complete IM. Similarly, a smoker patient with complete IM in the antrum or with a family history of gastric cancer likely deserves endoscopic surveillance, contrary to what recommended by the MAPS. However, further studies on this topic are needed.

Finally, the gastric biopsy sampling proposed in the MAPS includes ≥ 2 biopsies in the antrum and ≥ 2 biopsies in the gastric body. However, in the updated Sydney System, 5 biopsies were recommended, including 1 additional specimen on the *incisura angularis*^[19]. The need of this additional biopsy was based on the evidence that IM prevalence is higher in this gastric site as compared to any other part of the stomach^[20]. Indeed, IM generally initiates in the *incisura angularis*, subsequently spreading in both antrum and gastric body^[21]. Despite it could be argued that IM only located in the angulus does not increase gastric cancer risk, it is also true that IM would presumably spread to both antrum and body in the majority of patients^[21]. Therefore, by simply taking 1 biopsy on the angulus it is possible to early detect IM - that is a generally irreversible, precancerous lesion^[22]. Taking 1 further biopsy on the angulus is a simple and rapid procedure, without any additional patient discomfort and cost.

Indeed, as suggested in the Operative Link for Gastritis Assessment (OLGA) system, the additional biopsy specimens taken on the angulus should be pushed in the same vial of antral biopsies^[23]. The MAPS guideline did not include angulus biopsy among the recommendations^[1]. However, the guideline suggested that biopsies should be histologically assessed according to the OLGA/OLGIM system^[23,24], for which 5 biopsies (1 on the angulus) are required. Therefore, it would appear reasonable to include *incisura angularis* in the biopsy sampling.

In conclusion, while waiting for large prospective, randomized, multicenter studies comparing different follow-up strategies, it would appear reasonable to take into account some additional risk factors for gastric cancer in the follow-up strategy for management of gastric precancerous lesions. A simple, patient-tailored surveillance may be probably more appropriate than a single schedule proposed for all patients. Despite MAPS represents a good start, a more patient-orientated road-map(s) could be also considered.

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Actual concept of "probiotics": Is it more functional to science or business?

Michele Caselli, Francesca Cassol, Girolamo Calò, John Holton, Giovanni Zuliani, Antonio Gasbarrini

Michele Caselli, School of Gastroenterology, University of Ferrara, I-44121 Ferrara, Italy

Francesca Cassol, Department of Gastroenterology, S Anna Hospital, I-44121 Ferrara, Italy

Girolamo Calò, Department of Experimental and Clinical Medicine, Section of Pharmacology, University of Ferrara, I-44121 Ferrara, Italy

John Holton, Department of Health and Social Science, University of Middlesex, London, NW4 4BT, United Kingdom

Giovanni Zuliani, Department of Clinical and Experimental Medicine, Section of Internal Medicine, Gerontology and Geriatrics, University of Ferrara, I-44121 Ferrara, Italy

Antonio Gasbarrini, Division of Internal Medicine and Gastroenterology, Catholic University of Rome, I-00168 Rome, Italy

Author contributions: Caselli M and Cassol F conceived the study and drafted the article; Holton J revised the article and revised the microbiological aspects; Calò G contributed to acquisition and analysis of data; Zuliani G and Gasbarrini A revised the article and critically revised the clinical aspects.

Correspondence to: Francesca Cassol, MD, Department of Gastroenterology, S Anna Hospital, 44121 Ferrara, Italy. cassol.francesca@libero.it

Telephone: +39-348-4121382 Fax: +39-532-762096

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Abstract

It is our contention that the concept of a probiotic as a living bacterium providing unspecified health benefits is inhibiting the development and establishment of an evidence base for the growing field of pharmacobiotics. We believe this is due in part to the current regulatory framework, lack of a clear definition of a probiotic, the ease with which currently defined probiotics can be positioned in the market place, and the enormous profits earned for minimum investment in research. To avoid this, we believe the following two actions are mandatory: international guidelines by a forum of stakeholders made available to scientists and clinicians, patient organizations, and governments; public

research funds made available to the scientific community for performing independent rigorous studies both at the preclinical and clinical levels.

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Key words: Probiotics; Market; Regulations; Guidelines; Metanalysis

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INTRODUCTION

Probiotics are generally defined as live microorganisms, preferentially of human origin, that upon ingestion in specific and sufficient numbers confer unspecified health benefits to the host. During the last twenty years the therapeutic potential of probiotic bacteria has been evaluated in a large number of basic, experimental and clinical studies^[1-3] and their use in different clinical conditions has received considerable scientific and commercial attention.

Today probiotics represent a very big business. The global functional food market has been recently estimated at up to \$50 billion annual share^[4], while the world probiotic market is estimated at \$15 billion. Today, this market is growing at a pace of 5%-30% depending on the country and product type^[5]. The marketing agency Frost and Sullivan believes that the possibility to use salutistic indications on the label of the products containing probiotics, as permitted according to CE 1924/2006 rules, can further increase the consumer interest. Proper communication paired with effective marketing strategies will prove to be useful to this aim. Consumer acceptance varies greatly across Europe, with the most developed

market in Northern European and Scandinavian countries, having a long traditional consumption of probiotic products^[5]. The existing consumer confusion over the different probiotic strains as well as skepticism about their efficacy do not seem sufficient to counteract the salutistic propaganda of the media advertisements.

The regulatory status for probiotic products is not well established at international level yet. The United States Food and Drug Administration apply a conceptual distinction among “medical foods”, “dietary supplements”, “drugs” and “biological products” to probiotic products. The regulatory consequences that accompany a probiotic product that is categorized as a dietary supplement obviously dramatically differ from those that accompany a probiotic product categorized as a drug. If the probiotic product meets the definition of dietary supplement, the manufacturers may place the probiotic product on the market without any pre-market approval and may market the product with claims concerning the effect that the product has on the structure or function of the body. The European Commission has recognized probiotic bacteria as having the status of nutrients; in addition probiotics in powder, capsule or tablet form are in most European countries regarded as “food supplements” but with important differences: according to Bianca Herr of the Leatherhead Food International, in Italy and in Hungary probiotic products are widely accepted as food supplements, in Germany these products are accepted as food supplements in some cases but their acceptance as drugs depends on their concentration, while in Spain there is no specific legislation or guidance for probiotic products. Thus, in most cases, these products reach the market without being tested in the expensive three phases required for approval of a new drug. For these reasons not only big pharma and manufacturers of probiotics but also national pharmaceutical industries and even family farms are involved in this market. Also, the work of the European Food Safety Authority regarding claims made on food labeling and advertising concerning nutrition and health provides an important but very partial solution to the problem.

One would expect that the available scientific evidence is comparable to the size of this market; however, this is certainly not the case. Food And Agriculture Organization and World Health Organization defined the following characteristics of probiotic microorganisms: (1) probiotics should be taxonomically classified and deposited in an internationally recognized culture collection; (2) they have to remain viable and stable after culture, manipulation, and storage before consumption; (3) they have to survive to gastric acid and biliary and pancreatic digestion; (4) they have to induce a host response once ingested by adhering to gut epithelium or by other mechanisms; (5) they have to yield a functional or clinical benefit to the host when consumed; and (6) finally they have to be safe, not only regarding the assessment of side effects, but also in relation to antibiotic resistance patterns. In fact beneficial bacterial populations may play

a role in the transfer of antibiotic resistance to pathogenic and opportunistic bacteria. These general rules are certainly meaningful but not sufficient as guidelines for this field. Although there are few international organizations that purport to be independent of industry, such as International Scientific Association for Probiotics and Prebiotics (ISAPP), whose mission is to engender and disseminate information on high quality, multidisciplinary, scientific investigation in the field of probiotics, in actual fact there is no organization, agency or scientific network able to (1) reduce the incredible confusion related to every aspect of probiotics; (2) direct the rudder of basic and experimental research on probiotics and, in the future, on pharmacobiotics (a fundamental goal is to move away from the restrictive and perhaps outdated term “probiotics” and over to the more inclusive term “pharmacobiotic or pharmabiotic”); and (3) propose well accepted guidelines for evaluating these products in controlled clinical trials. To date variability is the keyword and includes every aspect of probiotics: strain, dose, route of administration, trial methodology, endpoints and outcomes. A very large number of probiotic strains have been used in clinical studies for the treatment of the same clinical condition, and the same strain of probiotics has been used to treat very different disease states. In addition an incredible large range of doses [from 4.5×10^2 colony-forming units (CFUs) to 3.6×10^{12} CFUs] of probiotics has been assayed in clinical trials. Furthermore, in different studies probiotics were administered in a great variety of ways: capsules, powders, tablets, drops or yogurts. An equally great variability exists in methodology, endpoints and outcomes of clinical trials carried out so far, even limiting the analysis to a single clinical condition. As an example we summarized in Tables 1-3 the number of patients, duration of treatment, probiotic strains used, dose used and outcomes of clinical trials carried out on three adult clinical conditions in which probiotics have widely tested: irritable bowel syndrome, ulcerative colitis and Crohn's disease. The tables end with the indication of published meta-analyses. Despite the existence of guidance^[6] and recommendations^[7] for probiotic use in these intestinal diseases, it seems clear from the tables that the lack of homogeneity of the published studies does not allow to draw final conclusions and to generate, through an evidence-based approach, true guidelines useful for adult patients. This is corroborated by meta-analysis studies that recognize the variety of species, strains and doses of probiotic used associated to an evident heterogeneity of study methodologies as main limitations in the field.

This would not be accepted in clinical pharmacology. No drug can be approved for the market with a defined clinical indication without sufficient knowledge of its mode of action, pharmacokinetic parameters, toxicological features, tolerability and effectiveness. In addition this knowledge will be substantially increased by post-marketing surveillance. By contrast, probiotics are commonly commercialized and prescribed for spe-

Table 1 Results of clinical trials with probiotics in irritable bowel syndrome

| Ref. | Patients (n) | Duration of therapy | Probiotic strains | Dose (CFU/d) | Outcomes |
|---|--|---------------------|--|---------------------------|--|
| Maupas <i>et al</i> ^[88] | 34 | 1 mo | <i>Saccharomyces boulardii</i> | 9 × 10 ⁹ | Improved stool number and consistency |
| Gade <i>et al</i> ^[89] | 54 | 1 mo | Paraghurt (<i>Streptococcus faecium</i>) | 1 × 10 ¹² | Improved symptoms |
| Halpern <i>et al</i> ^[90] | 18 | 4 mo | <i>Lactobacillus acidophilus</i> | 2 × 10 ¹⁰ | Improved symptoms |
| O'Sullivan <i>et al</i> ^[91] | 25 | 1 mo | <i>Lactobacillus</i> GG | 1 × 10 ¹⁰ | No benefit |
| Nobaek <i>et al</i> ^[92] | 60 | 1 mo | <i>Lactobacillus plantarum</i> 299V Pro-Viva® | 5 × 10 ⁷ | Improved global symptoms |
| Niedzielin <i>et al</i> ^[93] | 40 | 1 mo | <i>Lactobacillus plantarum</i> 299V Pro-Viva® | 2 × 10 ¹⁰ | Improved global symptoms |
| Kim <i>et al</i> ^[94] | 25 | 2 d-IBS | VSL3® | 9 × 10 ¹¹ | Reduced bloating |
| Tsuchiya <i>et al</i> ^[95] | 68 | 3 mo | <i>Lactobacillus acidophilus</i> | 1.5 × 10 ⁶ | Improved symptoms |
| | | | <i>Lactobacillus helveticus</i> | 1.3 × 10 ⁹ | |
| | | | <i>Bifidobacterium</i> | 4.95 × 10 ⁹ | |
| O'Mahony <i>et al</i> ^[96] | 80 | 2 mo | <i>Bifidobacterium longum</i> subspecies <i>infantis</i> vs <i>Lactobacillus salivarius</i> | 1 × 10 ¹⁰ | <i>B. infantis</i> : improved global symptoms and anti-inflammatory cytokine profile <i>Lactobacillus salivarius</i> : no benefit |
| Kajander <i>et al</i> ^[97] | 103 | 6 mo | Mixture (2 strains of <i>Lactobacillus rhamnosus</i> , <i>Bifidobacterium breve</i> , <i>Propionibacterium freudenreichii</i>) | 8-9 × 10 ⁹ | Improved global symptoms |
| Bittner <i>et al</i> ^[98] | 25 | 0.5 mo | 29 bacteria + prebiotic Prescript-Assist® | 2.6 × 10 ⁸ | Improved wellbeing |
| Sen <i>et al</i> ^[99] | 12 | 1 mo | <i>Lactobacillus plantarum</i> 299V Pro-Viva® | 5 × 10 ⁷ | No benefit; Study design flawed |
| Bausserman <i>et al</i> ^[100] | 50 | 1.5 mo | <i>Lactobacillus</i> GG | 2 × 10 ¹⁰ | No benefit |
| Niv <i>et al</i> ^[101] | 39 | 6 mo | <i>Lactobacillus</i> GG | 2 × 10 ⁸ | No benefit Francis severity IBS score |
| Kim <i>et al</i> ^[102] | 48 | 1 or 2 mo | VSL3® | 8 × 10 ⁹ | Reduced flatulence, retarded colonic transit |
| Whorwell <i>et al</i> ^[103] | 362 | 1 mo | <i>Bifidobacterium longum</i> subspecies <i>infantis</i> | 1 × 10 ⁶ | Improved global symptoms |
| | | | 35 624 in 3 doses | 1 × 10 ¹⁰ | |
| Long <i>et al</i> ^[104] | 60 | 0.5 mo | <i>Bifidobacterium</i> | 2 × 10 ⁸ | Symptoms resolved |
| Gawrońska <i>et al</i> ^[105] | 104 | 1 mo | <i>Lactobacillus</i> GG | 6 × 10 ⁹ | Reduced frequency of pain |
| Moon <i>et al</i> ^[106] | 34 | 1 mo | <i>Bifidobacterium subtilis</i> , <i>Streptococcus faecium</i> | 750 mL/d, CFU/d not given | Reduced frequency pain |
| Marteau <i>et al</i> ^[107] | 116 | 1 mo | Lactibiane® (4 strains of <i>Bifidobacterium longum</i> , <i>Lactobacillus acidophilus</i> , <i>Lactobacillus lactis</i> , <i>Streptococcus thermophilus</i>) | 1 × 10 ¹⁰ | Reduced pain Increased colonic transit in those with constipation |
| Simrén <i>et al</i> ^[108] | 76 | 1.5 mo | <i>Lactobacillus plantarum</i> 299V | 2 × 10 ⁹ | No benefit |
| Simrén <i>et al</i> ^[109] | 118 | 2 mo | <i>Lactobacillus paracasei</i> ssp <i>paracasei</i> | 2 × 10 ¹⁰ | No benefit |
| Guyonnet <i>et al</i> ^[110] | 274 | 1.5 mo | <i>Bifidobacterium animalis</i> , <i>Streptococcus thermophilus</i> and <i>Lactobacillus bulgaricus</i> | 1.25 × 10 ¹⁰ | Improved bloating and constipation |
| Drouault-Holowacz <i>et al</i> ^[111] | 116 | 1 mo | <i>Bifidobacterium longum</i> , <i>Lactobacillus acidophilus</i> , <i>Lactobacillus lactis</i> , <i>Streptococcus thermophilus</i> | 1 × 10 ¹⁰ | Not significant in relieving symptoms |
| Sinn <i>et al</i> ^[112] | 40 | 1 mo | <i>Lactobacillus acidophilus</i> | 2 × 10 ⁸ | Improved abdominal pain and discomfort |
| Enck <i>et al</i> ^[113] | 297 | 1 mo | <i>Escherichia coli</i> , <i>Enterococcus faecalis</i> | 4.5 × 10 ⁷ | Improvement in pain |
| Hun <i>et al</i> ^[114] | 44 | 2 mo | <i>Bacillus coagulans</i> | 8 × 10 ⁸ | Improvement abdominal pain and bloating |
| Dolin <i>et al</i> ^[115] | 61 | 2 mo | <i>Bacillus coagulans</i> | 2 × 10 ⁹ | Diminution of diarrhea |
| Ligaarden <i>et al</i> ^[116] | 16 | 1 mo | <i>Lactobacillus plantarum</i> | 10 ¹⁰ /L | Worsening of symptoms |
| Moayyedi <i>et al</i> ^[117] | 19 randomised controlled trials in 1650 patients | | | | Probiotics appear to be efficacious but the magnitude of benefit and the most effective strains are uncertain |

CFU: Colony-forming unit; IBS: Irritable bowel syndrome.

cific clinical indications in the absence of any conclusive proof concerning their putative pharmacological properties. Finally, it should be remembered that the safety of probiotics should not be considered a foregone datum: in abdominal surgery, translocation of bacteria from the gastrointestinal tract through the mucosa could occur^[8], and probiotic treatment has been associated with increased mortality in patients with acute pancreatitis^[9].

Only a few cases based on studies regarding pediatric population formal meta-analyses have been used to generate clinical guidelines. These studies demonstrated ben-

eficial effects of probiotics in acute diarrhea of children. These effects are strain- and dose-dependent, being generally greater with doses > 10¹⁰ CFUs, highly significant for watery diarrhea and viral gastroenteritis but less so for invasive bacterial diarrhea, more evident when the treatment is started early in the course of disease, and more evident in children living in developed than in developing countries^[10]. In May 2008, probiotics were for the first time included in a guideline document named “the guidelines for the management of acute gastroenteritis” and produced by a Committee of the European

Table 2 Results of clinical trials with probiotics in ulcerative colitis

| Ref. | Patients (n) | Duration of therapy | Probiotic strains | Dose (CFU/d) | Outcomes |
|---|--------------|---------------------|--|----------------------------|---|
| Kruis <i>et al</i> ^[118] | 120 | 12 wk | <i>Escherichia coli</i> Nissle 1917 | 50 × 10 ¹⁰ | Maintaining the remission (similar to 5-ASA) |
| Rembacken <i>et al</i> ^[119] | 116 | 1 yr | <i>Escherichia coli</i> Nissle 1917 | 5 × 10 ¹⁰ | Induction of remission (similar to 5-ASA); maintaining of relapses (similar to 5-ASA) |
| Venturi <i>et al</i> ^[120] | 20 | 1 yr | VSL3® | 5 × 10 ¹¹ | Maintaining the remission |
| Ishikawa <i>et al</i> ^[121] | 21 | 1 yr | Milk with bifidobacteria | 10 × 10 ⁸ | Maintaining the remission |
| Guslandi <i>et al</i> ^[122] | 25 | 4 wk | <i>Saccharomyces boulardii</i> | 250 mg × 3 | Induction of remission |
| Kruis <i>et al</i> ^[123] | 327 | 1 yr | <i>Escherichia coli</i> Nissle 1917 | 2.5-25 × 10 ⁹ | Induction of remission (5-ASA better than probiotic) |
| Tursi <i>et al</i> ^[124] | 90 | 8 wk | Balsalazide/VSL3® | 900 × 10 ⁸ | Induction of remission |
| Cui <i>et al</i> ^[125] | 30 | 8 wk | Bifidobacteria | 1.26 g/d | Maintaining of remission |
| Kato <i>et al</i> ^[126] | 20 | 12 wk | <i>Bifidobacterium</i> -fermented milk <i>vs</i> placebo | 10 ⁹ | CDAI lower in <i>Bifidobacterium</i> fermented milk than in placebo |
| Furrie <i>et al</i> ^[127] | 18 | 4 wk | <i>Bifidobacterium longum</i> + prebiotic (Synergy 1) | 4 × 10 ¹¹ | Induction of remission |
| Bibiloni <i>et al</i> ^[128] | 32 | 6 wk | VSL3® | 1800 billion × 2 | Induction of remission |
| Zocco <i>et al</i> ^[129] | 187 | 12 mo | <i>Lactobacillus GG</i> <i>vs</i> mesalazina | 18 × 10 ⁹ | No difference between the treatment groups |
| Henker <i>et al</i> ^[130] | 34 | 12 mo | <i>Escherichia coli</i> Nissle 1917 | 5 × 10 ¹⁰ | Maintenance of remission |
| Miele <i>et al</i> ^[131] | 29 | 12 mo | VSL3® | 450-1800 × 10 ⁹ | Induction of remission (92.8% in treated with VSL3® and 36.4% in the placebo group) |
| Sood <i>et al</i> ^[132] | 147 | 12 wk | VSL3® | 3.6 × 10 ¹² | Induction of remission (42.9% against 15.7% in the placebo group) |
| Matthes <i>et al</i> ^[133] | 57 | 4 wk | <i>Escherichia coli</i> Nissle 1917 | 10-40 × 10 ⁸ | Induction of remission |
| Sang <i>et al</i> ^[134] | 13 RCTs | | | | Heterogeneity between the studies in their methodology and results |

5-ASA: 5-aminosalicylic acid; CDAI: Crohn's disease activity index; CFU: Colony-forming unit; RCTs: Randomised controlled trials.

Table 3 Results of clinical trials with probiotics in patients with Crohn's disease

| Ref. | Patients (n) | Duration of therapy | Probiotic strains | Dose (CFU/d) | Outcomes |
|--|--------------|---------------------|---|------------------------|--|
| Malchow <i>et al</i> ^[135] | 24 | 3 mo | <i>Escherichia coli</i> Nissle 1917 | 2.5 × 10 ¹⁰ | Maintaining the remission |
| Guslandi <i>et al</i> ^[136] | 32 | 6 mo | <i>Saccharomyces boulardii</i> | 1 g | Postsurgical prevention of CD recurrence (relapse rate probiotic+ 5-ASA <i>vs</i> 5-ASA alone) |
| Prantera <i>et al</i> ^[137] | 45 | 1 yr | <i>Lactobacillus GG</i> | 12 × 10 ⁹ | Postsurgical prevention of CD recurrence (no effects) |
| Schultz <i>et al</i> ^[138] | 11 | 6 mo | <i>Lactobacillus GG</i> | 2 × 10 ⁹ | Probiotics are not superior to placebo in maintaining remission |
| Bousvaros <i>et al</i> ^[139] | 75 | 1 yr | <i>Lactobacillus GG</i> | 2 × 10 ¹⁰ | Probiotics are not superior to placebo in maintaining remission |
| Marteau <i>et al</i> ^[140] | 98 | 6 mo | <i>Lactobacillus johnsonii</i> | 4 × 10 ⁹ | Postsurgical prevention of CD recurrence (recurrence rate decreased <i>vs</i> placebo) |
| Chermesh <i>et al</i> ^[141] | 30 | 24 mo | Synbiotic 2000 (<i>Pediococcus pentoseceus</i> , <i>Lactobacillus raffinolactis</i> , <i>Lactobacillus paracasi</i> susp <i>paracasi</i> , <i>Lactobacillus plantarum</i> 2362) and 4 fermentable fibers <i>vs</i> placebo | 10 ¹¹ | Postsurgical prevention of CD recurrence (NS) |
| Van Gossum <i>et al</i> ^[142] | 70 | 12 wk | <i>Lactobacillus johnsonii</i> or placebo | 10 ¹⁰ | Postsurgical prevention of CD recurrence (NS) |
| Rolfe <i>et al</i> ^[143] | 7 RCTs | | | | No benefit of probiotics in the maintenance of remission of CD |
| Rahimi <i>et al</i> ^[144] | 8 RCTs | | | | None benefit for probiotic treatment in the maintenance of clinical remission of CD |

RCTs: Randomised controlled trials; CD: Crohn's disease; 5-ASA: 5-aminosalicylic acid; CFU: Colony-forming unit; NS: Not significant.

Society for Pediatric Infectious Diseases^[11]; this guideline document was developed through an evidence based systematic review approach that incorporates tables of evidence with their grading. The guidelines state that only the use of probiotic strains with proven efficacy and in appropriate doses is suggested for the management of acute diarrhea in European children as an adjunct to rehydration therapy. The evidence of efficacy regards only two strains: *Lactobacillus rhamnosus GG* was rated as 1A and *Saccharomyces boulardii* was rated as 2B,

corresponding to the level of evidence based respectively on meta-analysis of randomised controlled trials (RCTs) and properly designed RCTs of appropriate size. This evidence is actually confined only to the prevention/treatment of childhood acute gastroenteritis and of antibiotic-associated diarrhea. In the few conditions in which selected probiotic bacteria have shown a proven efficacy competitive mechanisms or mechanisms of restoration of bacteria flora seem to be involved. No final evidence is available in other conditions or diseases in

which probiotic agents are largely used. It appears evident that the tremendous dichotomy between the huge market of probiotic products and the insufficient knowledge of probiotic-based therapies. This would be unacceptable for any other pharmacological treatment.

We believe that some important fields of research exist that should be encouraged due to the possibility of getting information of incommensurate value in the near future. These fields of investigation will possibly permit development of a new concept of “probiotic agents”^[12,13], and should be adequately investigated.

A NEW CONCEPT OF “PROBIOTICS”

The relationship between probiotic agents and innate immune system

In recent years there have been tremendous advances in our understanding of the structure and function of signal receptors, and the pivotal role of pattern recognition receptors (PRRs) and cells of the innate immune system in processing bacterial and food components is now well established^[14-17]. PRRs include trans-membrane Toll-like receptors (TLRs) and Dectin- I; endosomal PRRs (TLR 3, 7/8 and 9); and cytosolic nucleotide oligomerization domain (NOD)-like receptors: (NOD1 and NOD2), Rig-1-like RNA helicase receptor (retinoic acid-inducible gene-1 and iron-regulated surface determinant sensors). The cells involved are dendritic cells (DC), intraepithelial lymphocytes, macrophages, neutrophils and enterocytes. At this level microorganisms are recognized only as microorganism-associated molecular patterns (MAMPs). MAMPs are first recognized by a PRR, and activation of the receptor by binding of the MAMP sequentially activates intracellular molecules such as the cytoplasmic adapter molecule MyD88, leading to the activation of transcription factors including nuclear factor- κ B (NF κ B) and activator protein-1 (AP-1), which are required for gene transcription and cytokine synthesis. The different receptors of the innate immune system are obviously only able to process specific molecular components of microorganisms and foods, whereas the recognition of a whole bacterium or food does not appear possible although simultaneous activation of several PRR's may be characteristic of a specific organism or food and lead to a different outcome than activation by single PRR. For example, studies on host mucosal gene expression following exposure to different whole bacteria have demonstrated up-regulation of different gene networks for each organism. Networks stimulated by these probiotic bacteria included cell proliferation, Th-1/Th2 balance, control of blood pressure, tissue development, water and ion regulation and wound healing. Major host differences were noted in the stimulated transcriptome. The pathways stimulated by the whole organism corresponded to pathways stimulated by known pharmacological preparations. However, the specific molecules of the bacteria that caused these effects are currently unknown^[18]. Further, whether the bacterium is alive or

dead does not seem relevant for the recognition of a molecular pattern by specific PRRs. The accessibility of MAMPs for PRRs and the presence of other microbial effector molecules, such as toxins produced by pathogens, have a pivotal role in the modulation of host immune response. Other important factors determining the host response are host-derived direct or indirect negative regulators of PRR signaling.

To date pathogenic, probiotic and commensal bacteria are considered to induce different levels of immune response: a strong host response stimulated by pathogens, an intermediate response induced by probiotics and finally a homeostatic control of the response is triggered by commensal bacteria. An important exception to this concerns a restricted number of commensal bacteria, the prototype of which is the segmented *Filamentous Bacteria* (SFB), which could largely recapitulate and orchestrate a broad spectrum of B and T cell responses^[19,20]. SFB colonized mice had low levels of ATP, suggesting that host sensing of SFB does not involve TLR or NOD receptors^[21]. We have recently showed that the progressive penetration of the holdfast segments of these bacteria within the specialized epithelial cells of the terminal ileum could permit an impressive presentation of bacterial antigens directly to the lymphocytes contained in the lymphoid packets characteristic of the M cells and to antigen presenting cells^[22].

It should also to be remembered that interactions between PRR and ligands are not as specific as those between antigens and antibodies, and ligands for PRR such as TLRs are generally present in repetitive structures to increase avidity.

Therefore, some very important and specific questions concerning immune-mediated probiotic activity are: (1) Are whole live bacteria essential to promote biological effects on the immune system? (2) Can the concept of probiotics be extended to include bacterial-derived molecular bioactive components? (3) Moreover, can probiotic molecules be also produced by non-probiotic bacteria? and (4) Finally, can probiotics be genetically manipulated to synthesize specific bioactive molecules?

Probiotic molecules

Bacterial DNA: Bacterial genomic DNA of probiotics in VSL-3TM induced a remarkable strain-specific immune response in humans as evaluated by the release of interleukin (IL)-1 β , IL-6 and IL-10. Total bacterial DNA from faeces increased the Th-1 cytokine IL-1 β more than IL-10 compared to DNA from the probiotic bacteria which had the reverse effect. However, total DNA from faeces, after being given a course of the probiotic bacteria, produced a greater stimulation of IL-10 compared to IL-1^[23]. Notably, the respective role of IL-1 β and IL-6 in the beginning and maintenance of a Th17 response is well known^[24,25]. An important and provocative study^[26] showed that in a mouse irritable bowel disease model the protective effects of probiotics contained in VSL-3 are mediated by their DNA rather than by their ability

Table 4 Reference studies concerning the probiotic role of bacterial DNA

| Ref. | Outcomes |
|---|--|
| Lammers <i>et al</i> ^[23] | Bacterial DNA from faeces collected after VSL-3 administration modulated a decrease of IL-1 β and an increase of IL-10 |
| Rachmilewitz <i>et al</i> ^[26] | Study in a mouse IBD model: protective effects of probiotics contained in VSL-3 are mediated by their DNA and TLR9 signaling mediates anti-inflammatory effect |
| Iliev <i>et al</i> ^[27] | <i>Lactobacillus rhamnosus</i> GG DNA induces B-cell proliferation and activate DC |
| Ghadimi <i>et al</i> ^[28] | Bacterial DNA inhibited IL-4 and IL-5 secretion in different <i>Lactobacilli</i> |
| Ménard <i>et al</i> ^[30] | Study from 5 bifidobacterial strains: unmethylated CpG motifs are specific to bacterial DNA by activating TLR9 |

IL: Interleukin; IBD: Irritable bowel disease; TLR: Toll-like receptor.

to colonize the gut mucosa. TLR 9 signaling is essential in mediating the anti-inflammatory effects of probiotics. TLR-9 is an endosomal TLR which is known to interact with bacterial DNA upon bacterial lysis. The authors suggested that DNA-TLR9 signaling resulted in the differentiation of naive cluster of differentiation-4 (CD4) T lymphocytes into regulatory T cells, mediating the protective action. Another example of the immunomodulatory capacity of probiotic DNA is represented by DNA of *Lactobacillus rhamnosus* GG that induces B-cell proliferation and activates DCs^[27]. More recently, the effects on the Th1/Th2 balance by genomic DNA of different probiotic bacteria (*Lactobacillus rhamnosus* GG, *Lactobacillus gasseri*, *Bifidobacterium bifidum*, *Bifidobacterium longum*) were compared with the effects of live bacteria by using peripheral blood mononuclear cells from healthy subjects and from patients with an allergy against the house dust mite^[28]. Compared with live *Lactobacilli*, bacterial DNA inhibited IL-4 and IL-5 secretion in a similar way, and based on the maximal effects achieved with *Lactobacilli* and their DNA, more than 50% of these effects seem to be due to their DNA (Table 4).

The immunomodulatory activity of DNA is characterized by unmethylated CpG motifs which can activate innate immune responses through binding to TLR9 and triggers the translocation of NF κ B and AP-1 from the cytoplasm to the nucleus thereby up-regulating gene expression pathways. Stimulatory oligodeoxynucleosides contain the CpG within a flanking region to give a motif of Pur-p-Pur-p-CpG-p-Pyr-p-Pyr. Typically more than one CpG is present in the immunostimulatory oligodeoxynucleoside and maximal effect occurs if they are separated by 1-2 base pairs. A 5' TpC and pyrimidine rich 3' ends also increases the immunostimulatory effects. In terms of a potential therapeutic, the *in-vivo* degradation can be decreased by synthesizing a phosphorothioate backbone which increases the stimulatory activity of the motif^[29]. A very recent study based on entire genome sequences from five bifidobacterial strains^[30] showed that *Bifidobacterium* genomes contained several CpG motifs and biologically active sequences previously identified in

Table 5 Reference studies concerning the probiotic role of molecules presented at the bacterial surface

| Ref. | Outcomes |
|--|---|
| Mazmanian <i>et al</i> ^[33] | Bacterial capsular PSA elaborated by <i>Bacteroides fragilis</i> activates CD4+ and elicits cytokine production |
| Mazmanian <i>et al</i> ^[35] | Purified PSA suppress pro-inflammatory IL-17 production and protects from inflammatory disease by induction of IL-10 |
| Ryu <i>et al</i> ^[36] | Purified LTA from Gram-positive bacteria has lower potency in the stimulation of Toll-like receptor-2 pathway to induce pro-inflammatory molecules. |
| Grangette <i>et al</i> ^[37] | Modified LTA is able to induce secretion of anti-inflammatory IL-10 |
| Benz <i>et al</i> ^[39] | Lipoproteins and glycoproteins at the cell surface are attractive candidates as probiotic molecules |
| Schlee <i>et al</i> ^[40] | Flagellins of the <i>Escherichia coli</i> Nissle 1917 induces expression of human β -defensin 2 |
| Matsumoto <i>et al</i> ^[33] | Purified PSPG- 1 from <i>Lactobacillus casei</i> Shirota has anti-inflammatory actions in chronic intestinal inflammatory disorders |

PSA: Polysaccharide A; IL: Interleukin; LTA: Lipoteichoic acid; PSPG: Polysaccharide-peptidoglycan.

Lactobacilli. These bioactive sequences induced the production of monocyte chemotactic protein-1 and tumor necrosis factor- α (TNF- α) through a pattern of TLR-9 stimulation of macrophages. An inter- and intra-species investigation of 71 strains of *Bifidobacteria* of various origins showed that these bioactive DNA sequences were highly conserved in the genus. The results of these studies clearly suggest the necessity of further investigation.

MOLECULAR PRESENT AT THE BACTERIAL SURFACE

Bacterial cells wall molecules are potential probiotic ligands that can interact with PRRs and induce signaling pathways resulting in probiotic effects (Table 5).

The immune system is able of recognizing any biological polymer constituting the bacterial cell wall and presenting it to T cells. Most probiotics are typically *Gram-positive bacteria*, in which the cell wall is composed of a thick peptidoglycan layer with proteins, teichoic acids and polysaccharides^[31]. However few *Gram-negative probiotics* exist, such as *Escherichia coli* strain Nissle 1917; in this case the cell wall is composed of a thin peptidoglycan layer and an outer membrane which contains lipopolysaccharides (LPS) that is further decorated with the proteins and polysaccharides^[32].

Although adaptive immune responses have been considered the territory of antigenic proteins or glycoproteins, whereas carbohydrates were considered as not recognized by the adaptive immune system, recent studies have revolutionized this assumption. Bacterial wall polysaccharides and glycolipids are now considered perhaps the more attractive targets in the research for immunomodulatory molecules. Interestingly, the bacterial capsular polysaccharide A (PSA), the most immunodominant among the zwitterionic polysaccharides elaborated by *Bacteroides*

fragilis, a commensal *Gram-negative anaerobe* that colonizes the mammalian lower gastrointestinal tract, has been demonstrated to be the archetypal bacterial molecule capable of mediating development of the host immune system^[33]. PSA presented by intestinal DCs activates CD4+ T cells and elicits appropriate cytokine production. *Bacteroides* species are among the earliest colonizing and most represented microorganisms of the gut microbiota^[34], and they are not considered probiotic species. More recently Mazmanian *et al*^[35] showed that the *Bacteroides fragilis*-produced PSA protects mice from experimental colitis induced by *Helicobacter hepaticus*: purified PSA is required to suppress pro-inflammatory IL-17 production by intestinal T cells, and it also protects from inflammatory disease by induction of IL-10-producing CD4+ T cells. Therefore, although bacteria may have developed polysaccharide capsules known to be not recognized by the immune system, it may be that the host not only tolerates but also has evolved to require cooperation by commensal bacteria for its health. Strikingly, the finding that PSA from *Bacteroides fragilis* is a natural anti-inflammatory molecule that promotes health, so clearly performing important probiotic activities, is not produced by a probiotic bacteria, provides a fundamental platform for the discovery of new biomolecules having important probiotic effects independently from their bacterial derivation.

Polysaccharides synthesized extracellularly (exopolysaccharides, EPSO) also represent attractive candidates as probiotic effector molecules interacting with PRRs. EPSO are produced by both probiotic and symbiotic bacteria, and also potentially pathogenic bacteria, but they have not yet been studied in detail.

On the other hand, lipoteichoic acid (LTA) is considered the major immunostimulating component of the cell wall of *Gram-positive bacteria* via TLR 2 (most of the known probiotics, *Lactobacilli* and *Bifidobacteria*, are *Gram-positive bacteria*), in the same way as LPS is the major immunostimulating component in the cell wall of *Gram-negative bacteria* via TLR 4. Two important concepts concerning LTA have emerged in recent years: the first concerns the much lower potency in the stimulation of TLR 2 pathway to induce pro-inflammatory molecules by using purified LTA from a probiotic strains of *Lactobacillus plantarum* in comparison with a pathogenic strain of *Staphylococcus aureus*^[36]; the second very important concept is related to the possible modification of LTA molecules to induce a substantial reduction in *D*-alanine content with a marked increase in glucose substitutions^[37]. These modified LTA may be candidates as probiotic effector molecules able to induce secretion of anti-inflammatory IL-10.

On the other hand, LPS synthesized by *Gram-negative bacteria* of the gut microbiota have been recently involved in the development of inflammation, obesity and type 2 diabetes induced by a high fat diet^[38]. If confirmed, these findings open up a new possible role in this field not only for a direct bacterial competition by live probi-

otics, but also for research into non-immunostimulating molecules competing with LPS for the TLR 4 pathway.

Finally, both lipoproteins and glycoproteins present at the cell surface are also attractive candidates as probiotic molecules for their interactions with TLR 2 receptors, but to date their role is unexplored even in pathogenic bacteria^[39]. However flagellins of the *Escherichia coli* Nissle 1917 induce the expression of human β -defensin 2, an inducible antimicrobial peptide^[40].

Recombinant probiotics: Colonizing (*e.g.*, *Streptococcus gondii*, *Lactobacillus plantarum*, *Lactobacillus paracasei*, *Lactobacillus casei*, *Lactobacillus acidophilus*) as well as non-colonizing (*e.g.*, *Lactobacillus lactis*) bacterial species have been investigated both as live vaccine vehicles (acting as carriers for protective antigens) and as active producers of molecules with known pharmacological properties.

In respect to the use of these microorganisms as carriers for antigens, the most complete studies have been carried out with the 50 kDa carboxy-terminal fragment of tetanus toxin^[41]; this approach has now been extended to additional antigens eg the B subunit of cholera toxin^[1-3].

Transfected bacteria have also been used to deliver cytokines, but this technique was recently used to investigate other biological properties. Steidler *et al*^[42] chose to construct recombinant *Lactobacillus lactis* strains secreting murine IL-10. These authors demonstrated that these recombinant strains were able to prevent and treat inflammation in two murine models of colitis. Significantly, the same effects were obtained with much lower doses of IL-10 than those required when IL-10 itself was used as a drug. The same authors further constructed a safe (no antibiotic resistance markers and a chromosomally integrated transgene) strain of *Lactobacillus lactis* secreting IL-10 of human origin^[43]. Authorization by a local ethical committee to carry out a phase 1 clinical study on voluntary patients has been obtained in the Netherlands^[44]. In this study, Crohn's disease patients were treated with recombinant *Lactobacillus lactis* (LL-THY 12) in which the thymidylate synthase gene was replaced with a synthetic sequence encoding mature human IL-10. The oral administration of this strain was safe and a decrease in disease activity was observed. The authors concluded that the use of genetically modified bacteria for mucosal delivery of therapeutic proteins is a feasible strategy in human beings. This strategy avoids systemic side effects and appears suitable as maintenance treatment for chronic intestinal diseases. Novel therapeutic strategies for acute and chronic colitis based on recombinant probiotics were also assessed by the generation and *in vivo* evaluation of *Lactobacillus lactis* strains secreting bioactive murine trefoil factors (TFF)^[45]. The authors demonstrated that intragastric administration of this bacterial strain, but not of purified TFF, led to prevention and healing in the acute dextran sodium sulfate (DSS)-induced murine model of colitis, and was similarly effective in reducing established chronic DSS colitis. It has also to be remem-

bered that production and mucosal delivery of different bioactive molecules such as allergens, digestive enzymes and single-chain Fw antibodies have been achieved using lactic acid bacteria^[3]. Targeted diseases included vaginal candidiasis^[46], dental caries^[47], allergies^[48-50], autoimmune diseases^[51,52], human papillomaviruses-induced tumors^[53] and pancreatic insufficiency^[54]. More recently, Rosberg-Cody *et al.*^[55] investigated whether a recombinant strain of *Lactobacillus paracasei*, previously isolated from the human gastrointestinal tract, expressing conjugated linoleic acid (CLA) isomerase from *Propionibacterium acnes*, could influence the fatty acid composition of different tissues in the mouse. Ingestion of the *Lactobacillus paracasei* strain expressing CLA isomerase was associated with a 4-fold increase ($P > 0.001$) in t10c12 CLA in adipose tissues of the mice when compared with animals that received the non-CLA producing isogenic strain. These data show that a single gene encoding CLA isomerase expressed by an intestinal bacterium can influence the fatty acid composition of the host adipose tissue. This t10c12 CLA isomer is also associated with decreased body fat and increased lean body mass in various animal species^[56-60] and, to some extents, human beings^[61-65]. It is also well known that t10c12 CLA isomer is the most potent isomer in terms of potential to prevent cell proliferation and induce apoptosis in cancer cells^[66-69]; notably, when the microbially derived t10c12 CLA was incubated with SW480 colon cancer cells for 5 d, cell viability was decreased by 92%^[70], and it is possible that a CLA-producing probiotic will be able to keep colon cancer cells in check. Although commensal *Bifidobacterium* and *Lactobacillus* species from the gastrointestinal tract have been shown to produce CLA *in vitro*^[71-73], the majority of these studies have demonstrated the production of c9t11 CLA from linoleic acid, while only a few bacteria such as *Propionibacterium acnes*^[74], the rumen bacterium *Megasphaera elsdenii*^[75], and the human derived *Lactobacillus rhamnosus* PL60 and *Lactobacillus plantarum* PL62^[76,77] have been reported to produce t10c12 CLA. Modulation of fatty acid production by bacteria may represent very important probiotic activity and recombinant probiotics may become useful for this in the near future.

Recombinant probiotics may be linked not only to the addition of one or more genes but also to the deletion of one or more genes. In fact, to study the molecular mechanisms involved in the induction and repression of intestinal inflammation, Mohamadzadeh *et al.*^[78] have recently deleted the phosphoglycerol transferase gene that plays a key role in LTA biosynthesis in *Lactobacillus acidophilus* NCK 56.

The results of these authors show that the *Lactobacillus acidophilus* LTA⁻ not only down regulated IL-12 and TNF- α , which are known pro-inflammatory cytokines, but also significantly enhanced IL-10 production by DC and controlled the regulation of co-stimulatory DC functions, resulting in their inability to induce CD 4+ T cell activation. The treatment of mice with *Lactobacillus acidophilus* LTA⁻, compared with *Lactobacillus acidophilus* LTA⁺, signifi-

cantly counteracted DSS-induced colitis. These authors concluded that directed alteration of cell-surface components of *Lactobacillus acidophilus* represents a potential new strategy for the treatment of inflammatory intestinal disorders.

Moreover, efforts have been devoted to improve the efficacy of probiotic bacteria as delivery systems; in this context cell wall mutants of *Lactobacillus plantarum* and *Lactobacillus lactis* defective in alanine racemase (*alr* gene) were constructed^[79,80]: each of these mutants behaved as a substantially improved antigen delivery system compared with its wild-type counterpart^[81]. The potency of the *Lactobacillus plantarum* Alr⁻ mutant was further confirmed using a weak immunogen, such as *Helicobacter pylori* urease B, as a protective antigen; a significant reduction of the *Helicobacter pylori* load in the mouse stomach was achieved after immunization with the recombinant mutant *Lactobacillus plantarum* strain in contrast to results obtained with its wild-type counterpart^[82].

Any gene coding for an active molecule, potentially useful for human health, may be used to generate recombinant probiotic bacteria; in this context, an impressive number of options are available to be investigated in *in vitro* and *in vivo* studies. It is worthy of note, however, that several gene products need glycosylation, phosphorylation or other more complex chemical changes; these may require the enzymatic machinery of eukaryotic cells. Thus, although current available genomic information should greatly facilitate the generation of useful recombinant probiotics, several technical issues and biologically limiting factors have to be taken attentively in consideration. In any case, the use of rapidly evolving genomic technology will surely help to evolve this intriguing and fascinating field and we can expect that from the present pioneering status we will soon progress to the generation of innovative therapeutic tools.

CONCLUSION

We are convinced that, even if as mentioned above there is a very large amount of work to be performed in this field, the available evidence is already enough to move from the actual concept of probiotics to novel and very promising pharmacobiotic strategies. In fact, probiotic molecules and recombinant probiotics may represent an unlimited resource for innovative therapeutics. The following questions arise from the present analysis of available knowledge: (1) Why the therapeutic potential of probiotic molecules and recombinant probiotics has been neglected so far? (2) Why important studies showing that whole live bacteria are not needed for probiotic activity have not received adequate attention by the scientific community? (3) Why molecules such as polysaccharide-peptidoglycan (PSPG)- I from *Lactobacillus shirota*, which have demonstrated to be able to suppress inflammation in chronic intestinal inflammatory disorders *via* inhibition of IL-6, have not been extensively investigated yet? IL-6 plays a pivotal role both in activation and sustainment

of Th 17 response as well as a crucial role as a proinflammatory IL in Th 17 and Th1 cell responses. Thus the dose dependent pharmacological inhibition of IL-6 levels could have a crucial clinical impact, as suggested by animal studies^[83]. Based on these considerations, (4) Why, after identification of adequate drug delivery strategies (in fact, there may be major challenges with formulation and delivery in single cases), the clinical effectiveness of PSPG- I has not been assessed yet? and (5) Why has only a phase I study has been performed with recombinant probiotics? These are crucial questions with important implications. Thus these questions should be discussed at international level by a forum involving different players including, basic researchers, clinicians (gastroenterologists, pediatricians, allergologists, pneumologists, *etc.*), lawgivers and regulatory agencies, and probiotics pharma. Although there are already international organizations that declare to be independent of the industry, such as the aforementioned ISAPP, which tackle these issues, these have within them industry advisory committee members and have not been able until now to pull the current outdated concept of probiotics to the more inclusive concept of pharmabiotics.

Although guidelines and recommendations substantiating the evidence for beneficial effects of probiotics in different clinical conditions of adult patients have been published^[6,7,84-87], the only clinical conditions in which strains of whole live probiotics have been shown to be effective thus far are acute gastroenteritis and antibiotic-associated diarrhoea. It seems therefore that live probiotics can exert a competitive action and can have a role in restoration of intestinal flora. However, a specific role in the cure of chronic and/or autoimmune diseases has not been conclusively demonstrated. Despite this, an entire world involving probiotic molecules and recombinant probiotics is ready to be investigated. In any case, if specific live probiotic strains have been or will be found effective in specific concentrations for specific disease conditions should they still categorized as food supplements? To what extent does the market influence the national regulatory laws in this area? We think that gut microbiota and probiotic bacteria represent an inexhaustible mine from which countless molecules of potential value for human health can be obtained and investigated. If this does not happen, we risk going on discussing whether a live strain is better than another without ever reaching any definitive conclusion for many years. Even if single RCTs demonstrate a level of evidence 1a, but the findings are not confirmed by other authors in order to remove any doubt about the therapeutic role of that strain in the given clinical condition at that specific doses and route of administration, it does not solve the problem and continues to maintain doubts about the role of probiotic therapy. In addition, it should be underlined that clinical studies are almost always sponsored by companies and results of rigorous RCTs are restricted to the strains of company interest. Who needs to maintain the “status quo” without moving the research to a plot of

real pharmacobiotic strategies? Is the huge market based on “easy” trade of live microorganisms involved? We do not want to be unpleasant to anybody, but we think that opening an international forum on this important issue would be of great benefit to both physicians and patients. If to tell the story of salutistic products through well-made advertisements in the media induces big gains without big expenses, we fear that hardly anyone will decide to invest in this area. This way the birth of the pharmacobiotic era will turn away more and more. The resources that are available to us are often sacrificed by humans on the altar of interests and market strategies: among the chief concerns of the scientific community is the need to denounce all those situations in which scientific rigor is sacrificed to commercial interests. To avoid this, the following two actions are mandatory: (1) international guidelines by the forum of players made available to scientists and clinicians, patient organizations, and lawgivers; and (2) public research funds made available to the scientific community for performing independent rigorous studies both at preclinical and clinical levels.

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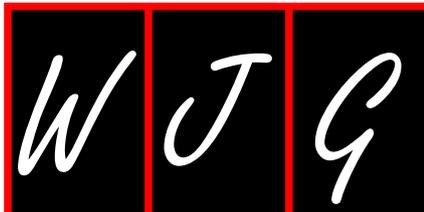
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Upper gastrointestinal microbiota and digestive diseases

Zi-Kai Wang, Yun-Sheng Yang

Zi-Kai Wang, Yun-Sheng Yang, Department of Gastroenterology and Hepatology, the Chinese PLA General Hospital, the Chinese PLA Medical Academy, Beijing 100853, China

Author contributions: Wang ZK wrote the manuscript; Yang YS revised the manuscript; and both authors have read and approved the final version.

Correspondence to: Yun-Sheng Yang, MD, PhD, Department of Gastroenterology and Hepatology, the Chinese PLA General Hospital, the Chinese PLA Medical Academy, Beijing 100853, China. sunny301ddc@126.com

Telephone: +86-10-55499005 Fax: +86-10-55499005

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Abstract

Metagenomics which combines the power of genomics, bioinformatics, and systems biology, provide new access to the microbial world. Metagenomics permit the genetic analysis of complex microbial populations without requiring prior cultivation. Through the conceptual innovations in metagenomics and the improvements in DNA high-throughput sequencing and bioinformatics analysis technology, gastrointestinal microbiology has entered the metagenomics era and become a hot topic worldwide. Human microbiome research is underway, however, most studies in this area have focused on the composition and function of the intestinal microbiota and the relationship between intestinal microbiota and metabolic diseases (obesity, diabetes, metabolic syndrome, *etc.*) and intestinal disorders [inflammatory bowel disease, colorectal cancer, irritable bowel syndrome (IBS), *etc.*]. Few investigations on microbiota have been conducted within the upper gastrointestinal tract (esophagus, stomach and duodenum). The upper gastrointestinal microbiota is essential for several gastrointestinal illnesses, including esophagitis, Barrett's esophagus, and esophageal carcinoma, gastritis and gastric cancer, small intestinal bacterial overgrowth, IBS and celiac disease. However, the constitution and diversity of the microbiota in different sections of the upper gastrointestinal tract under health and various

disease states, as well as the function of microbiota in the pathogenesis of various digestive diseases are still undefined. The current article provides an overview of the recent findings regarding the relationship between upper gastrointestinal microbiota and gastrointestinal diseases; and discusses the study limitations and future directions of upper gastrointestinal microbiota research.

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Key words: Microbiota; Upper gastrointestinal tract; Digestive diseases; 16S rDNA; Metagenomics

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INTRODUCTION

Microbes associated with the human body include bacteria, archaea, fungi, and viruses. The vast majority of studies on microbiota have focused on bacteria. A large number of micro-organisms colonize on the surface of and within the human body and can reach counts of 10^{12} - 10^{14} , 10 times that of the cells of the human body, while the number of genes in these micro-organisms, approximately 3.30 million, is 150 times higher than that in the human body^[1]. Humans are a type of super organism composed of the human body and symbiotic micro-organisms^[2], therefore, researching human diseases from only the human body point of view reveals only a partial view of a condition; thus, the role of commensal microbiota in human health and disease must be considered. Micro-organisms and their metabolites play important roles in human energy metabolism^[3], the absorption of nutrients^[4], immune function^[5] and other important physiological activities^[6]. In fact, a variety of human diseases will be induced when commensalism between the

host and the microorganisms is disrupted.

Microbiota composition has classically been analyzed using cost-effective and reproducible culture techniques that use differential media to select for specific bacterial populations. Early microbiological studies relied mainly on traditional culture-based methods. However, the overall microbial community structure, spatial distribution and dynamics could not be fully demonstrated using traditional culture-based methods due to the fact that 99% of such micro-organisms are uncultured^[1]. As the gene sequence of 16S rRNA has a combination of highly conserved and variable sequences in a relatively short portion of the bacterial chromosome^[7], it is increasingly used to characterize the diversity of the complex microbial communities that can be sampled from different sites of the body in both healthy individuals and patients with diverse pathological conditions^[8-12]. Many bacterial 16S rRNA gene-dependent methods, including terminal restriction fragment length polymorphism (TRFLP)^[13], denaturing and temperature gradient gel electrophoresis (DGGE and TGGE)^[14], ribosomal intergenic spacer analysis (RISA)^[15], DNA microarray^[16] and fluorescence *in situ* hybridization (FISH)^[17], have emerged as molecular biology techniques; however, the information obtained by the above methods has been very limited, thus, ongoing studies cover only a small portion of this field.

Recent microbiological studies are firmly supported technically along with the introduction of the concept of metagenomics^[18], the development of high-throughput DNA sequencing and bioinformatics technology. Metagenomics mainly include two strategies: first, the high-throughput sequencing of 16S rDNA hypervariable regions, which can provide diversity and abundance information on microbial communities, while not supplying the functional genes of microbiota and that is mainly used in the classification, identification and comparison of microbiota^[8,9]; second, metagenomic sequencing of whole community DNA, not only provides information on microbiota structure and abundance, but can also be used in the functional annotation and building of metabolic networks, and favors the in-depth investigation and screening of functional genes^[11,19,20]. Over the past 3-5 years, sequencing cost and time have been greatly reduced; thus, the feasibility of comprehensive and detailed studies of the microbiota has increased significantly.

The United States National Institutes of Health launched the Human Microbiome Project (HMP) in 2007^[20]. The European Commission also initiated the metagenomics of the human intestinal tract funded by the 7th Framework Program of the European Union in 2008. Scholars from various countries established the International Human Microbiome Consortium in 2009, an international cooperative effort aiming to explore the relationship between microbiota and human health and disease. The editors of *Science* had predicted that human microbe research may become a new hot topic worldwide^[2]; in fact, studies of microbiota in human health and disease based on high-throughput sequencing and bioinformatics

technology have already been initiated. Not long ago, the HMP Consortium investigated the human microbiome based on healthy adults sampled from five body regions including the feces, oral cavity, airway, skin and vagina, which generated 5177 microbial taxonomic profiles from 16S ribosomal RNA genes and > 3.5 terabases of metagenomic sequence to date^[19]. However, the mucosa-associated microbiota of the gastrointestinal tract was not evaluated in this study. Most researches today have focused on intestinal microbiota and the relationship between intestinal microbiota and metabolic diseases (obesity, diabetes, metabolic syndrome, *etc.*)^[11,21-23] and intestinal disorders [inflammatory bowel disease (IBD), colorectal cancer (CRC), irritable bowel syndrome (IBS), *etc.*]^[1,24-26], and rarely examined upper gastrointestinal tract microbiota. Here we review the evidence for a microbiota concept in upper gastrointestinal diseases and highlight recent studies that have enhanced our appreciation of the relationship between microbiota and human health and disease.

MICROBIOTA AND THE ESOPHAGUS

The esophagus, unlike the oral cavity, stomach and colon, does not retain food contents. Studies using culturing methods have suggested that the esophagus is either sterile or contains only a few transient microbes originating from the oropharynx by swallowing or from the stomach by gastroesophageal reflux^[27]. Moreover, under certain disease conditions, several pathogenic microorganisms, such as *Candida albicans*, *Cryptococcus* or *Herpesvirus*, can infect the esophagus^[28-31]. Whether an imbalance of esophageal microbiota is responsible for esophageal disorders remains unclear; in fact, investigations of esophageal microbiota remain limited^[27,32-38].

Culture-based studies mainly used luminal washes of esophageal contents^[27,36] and their results were not convincing. Gagliardi *et al.*^[27] demonstrated that *Streptococcus viridans* (*S. viridians*) may be the most numerous microorganism in both the normal esophagus and the oropharynx. Norder Grusell *et al.*^[36] also reported that *S. viridians* was the most common bacterium using both brush samples and biopsies; and this study confirmed that the human esophagus could be colonized with a resident flora of its own, although it was similar to the flora present in the oral cavity.

Culture-independent methods have recently been used more frequently to characterize the diversity of the microbiota in the esophagus^[33,37,38]. Pei *et al.*^[33] investigated the composition of microbiota in the normal distal esophagus using broad-range 16S rDNA polymerase chain reaction (PCR). They confirmed that the majority of esophageal microbiota were known and cultivable; and found that *Streptococcus*, *Prevotella* and *Veillonellaceae* were the most prevalent genera in esophageal biopsies. Yang *et al.*^[37] characterized the diversity of the microbiota of the distal esophagus in individuals with normal esophagus and in patients with esophagitis and Barrett's esophagus using

16S rDNA sequencing. They classified the esophageal microbiota into two types: *Streptococcus*-dominated in the normal esophagus and *Gram-negative anaerobes* in Barrett's esophagus and esophagitis^[37]. However, this study could not answer the question of how the microbiota participates in the pathogenesis of esophageal inflammation.

Gastroesophageal reflux impairs the mucosal barrier in the distal esophagus, allowing chronic exposure of the epithelial cells to diverse microbiota and inducing chronic inflammation. Chronic inflammation may play a critical role in the progression from reflux-related intestinal metaplasia or Barrett's esophagus to esophageal carcinoma^[39]. Until now, there has been no research on the diversity of esophageal microbiota in patients suffering from esophageal squamous or adenocarcinoma.

Interestingly, Fillon *et al.*^[40] sampled the microbiome in normal histological esophageal mucosa using a novel device, the Enterotest™ capsule, and found that the microbiota phylum-level diversity was similar in samples from the esophageal mucosa biopsy, esophageal string test, oral string and nasal swab as identified using 454 pyrosequencing; moreover, at the genera level, the most common three genera, *Streptococcus*, *Prevotella*, and *Veillonella*, were similar in the esophageal string test and mucosal biopsy samples, a finding that was consistent with the findings of Pei *et al.*^[33]. In fact, this novel instrument could be used for future research on the microbiota within the human esophagus. However, this study did not eliminate the effect of proton pump inhibitors (PPI)^[41,42], steroids^[43] or restricted diet on esophageal microbiota. It is also evident that PPI could have an effect on gastrointestinal microbiota^[41], and long-term PPI treatment is very common in reflux esophagitis, therefore, it may be meaningful to estimate the effect of long-term PPI treatment on esophageal microbiota.

In addition, Chagas' megaesophageal disease caused by *Trypanosoma americanum* infection is usually associated with esophageal bacterial overgrowth, recurrent pulmonary infections and esophageal neoplasia^[32]. Pajecski *et al.*^[32] showed that patients with megaesophageal disease could present with a wide variety of microbiomes, mainly aerobic *Gram-positive* and *anaerobic bacteria*. The imbalance of esophageal microbiota could play a causal role, and high-throughput sequencing technology could be used to understand esophageal bacterial overgrowth in Chagas' megaesophageal disease.

MICROBIOTA AND THE STOMACH

The stomach is a special area in the gastrointestinal microecosystem. Its unique ecological environment and characteristic microbial community are due to gastric acid secretion. Because the stomach connects the esophagus and oral cavity on the upper side and the duodenum on the lower side, bacteria from the mouth, pharynx, nose, respiratory tract, esophagus and small intestine can enter the stomach. It was once believed that gastric acid could kill the bacteria entering the stomach and that the

stomach environment was not suitable for bacterial colonization; however, some studies using traditional culture methods confirmed that large numbers of acid-resistant bacterial strains exist in the stomach and are mainly derived from the transient flora in the mouth and food, including *Streptococcus*, *Neisseria* and *Lactobacillus*, while the content was generally $< 10^3$ colony-forming unit/mL (CFU/mL)^[44].

In 1984, Marshall *et al.*^[45] isolated *Helicobacter pylori* (*H. pylori*) from the stomach, thus starting a new era of *H. pylori* and digestive diseases research, and won the Nobel Prize in medicine. With the development of molecular biology and bacterial 16S rDNA identification techniques, the composition of the stomach flora was gradually investigated using new molecular biological methods. Using bacterial 16S rDNA PCR and TGGE, Monstein *et al.*^[46] demonstrated that some other microbes, including *Enterococcus*, *Pseudomonas*, *Staphylococcus* and *Stomatococcus*, exist within the gastric mucosa.

The identification of stomach flora has increased dramatically with the development of metagenomics and high-throughput sequencing technology. Bik *et al.*^[47] performed a 16S rDNA sequencing analysis of the stomach flora of 23 patients with gastric diseases, identified 128 kinds of phylotypes belonging to eight classes, and obtained 1056 non-*H. pylori* clones. Li *et al.*^[48] performed a 16S rDNA high-throughput sequencing analysis of the gastric mucosa-associated flora in *H. pylori*-negative patients with gastritis who had never used non-steroidal anti-inflammatory drugs and obtained a total of 1223 non-*H. pylori* clones that could be classified into 133 kinds of phylotypes belonging to eight bacterial classes. Although the above two studies were conducted in populations within different regions and ethnic groups, the stomach flora composition was very similar among those populations. The two studies identified approximately 130 phylotypes belonging to 7-8 classes, and 77.4% and 79.8% of clone fragments separately for each study were homogeneous. The two species with the highest abundance, *Streptococcus* and *Prevotella*, were the same. Anderson *et al.*^[49] conducted pyrosequencing analyses of gastric mucosa-associated flora in six healthy subjects and obtained 262 phylotypes belonging to 13 classes, including strains that had not been confirmed by other studies such as *Chlamydia* and *Cyanobacteria*. Stearns *et al.*^[50] performed high-throughput 16S rDNA sequencing analysis of the oral, stomach, duodena and colon mucosa-associated flora as well as feces-related flora in four healthy subjects. The obtained stomach flora constitution was more detailed than that obtained by Bik *et al.*^[47] due to the higher sequencing depth. The high-throughput 16S rDNA sequencing techniques based on the metagenomics strategy were adopted in the above studies; thus, the obtained stomach flora information was detailed and showed that huge numbers of bacteria other than *H. pylori* exist in the stomach.

Gastrointestinal microbiota distribution is spatially specific. The gastric juice-associated microbiota is easily

affected by diet and other factors; thus, they are variable. In contrast, the gastric mucosa-associated microbiota is relatively stable and less affected by interference factors. Furthermore, gastric mucosa-associated flora can affect the host more directly and are more closely related to the pathogenesis of gastric disease. Li *et al.*^[48] thoroughly washed gastric mucosal biopsy samples, but found no change in the constitution of gastric mucosa-associated flora; thus, the gastric flora proved to be closely associated with gastric mucosa and could not be readily washed away. Although some microbes such as *Streptococcus* genus have a high abundance in both the oral cavity and stomach^[47,48,51], the study by Li *et al.*^[48] indicated that many non-*H. pylori* microbes could be resident flora in the human stomach, and not just transient flora from the oral cavity.

The use of feces has been widely adopted in the study of the relationship between intestinal microbiota and diseases as it is easily sampled and reflects the overall constitution of the intestinal flora. It is worth noting that feces-associated flora are less interfered with by host DNA; thus, they can be sequenced for whole communities of DNA or for 16S rDNA hypervariable regions. Gastrointestinal mucosa-associated flora are usually interfered with by host DNA; thus, they are commonly investigated using 16S rDNA sequencing.

Stomach flora and *H. pylori*

H. pylori is a Gram-negative bacillus that colonizes the stomachs of approximately 50% of individuals worldwide. *H. pylori* has been investigated more deeply than any other stomach pathogen. Although *H. pylori* is the major pathogenic factor of chronic gastritis and peptic ulcer and is one of the risk factors for gastric adenocarcinoma and mucosa-associated lymphoid tissue lymphoma^[52,53], the relationship between *H. pylori* infection and gastric diseases remains unclear. Stomach colonization was negative even after several weeks of oral implantation of *H. pylori* in specific pathogen-free animal models; however, it was usually positive in germ-free animal models^[54], suggesting that other stomach flora affected the intragastric colonization of *H. pylori* and its activity in gastric diseases.

In vivo and *in vitro* studies based on animal models have found that some probiotics including *Lactobacillus*, *Bifidobacterium* and *Saccharomyces* can prevent the adhesion, colonization and growth of *H. pylori* in gastric mucosa. Zaman *et al.*^[55] confirmed that some *Lactobacillus* species prevented the colonization of *H. pylori* in the stomachs of Mongolian gerbils, while *Eubacterium limosum* promoted the colonization of *H. pylori*. Yin *et al.*^[56] found that long-term intragastric colonization of *H. pylori* could affect the distribution and number of flora in the stomach and duodenum. For example, the propagation of *Lactobacilli* was inhibited, while *Enterococcus*, *Staphylococcus aureus*, *Bifidobacterium* and *Bacteroides* were rarely affected. Sun *et al.*^[57] believed that some *Lactobacillus* species were dominant strains in the stomachs of Mongolian gerbils that were not affected by *H. pylori* infection; *Lactobacillus gasseri* and

Lactobacillus reuteri existed in the gerbils' stomachs and suppressed the colonization and growth of *H. pylori*.

A small number of studies based on the human body also confirmed that *H. pylori* interacted with other gastric flora. Using traditional culture methods, Garcia *et al.*^[58] analyzed the gastric mucosa-associated flora and showed that some *Lactobacillus* species competed with *H. pylori* for colonization resources. Hu *et al.*^[59] conducted bacterial cultures using gastric mucosal biopsy samples and found that the dominant species, including *Streptococcus*, *Neisseria*, *Rothia* and *Staphylococcus*, were primarily sourced from the upper respiratory tract. They believed that *H. pylori* infection was usually accompanied by the colonization of non-*H. pylori* bacteria; they noted that these non-*H. pylori* bacteria played certain roles in gastric diseases. Sanduleanu *et al.*^[60] thought that non-*H. pylori* bacteria and their products could persist in the stomach as antigenic stimulators that enhance the immune response caused by *H. pylori* infection and that their co-infection could promote the development of atrophic gastritis. Clinical trials have initially confirmed that probiotics treatment can reduce the gastric colonization density of *H. pylori*^[61]. Probiotics can increase the eradication rate, decrease the relapse rate and reduce antibiotic side effects; thus, they may be used as an effective supplement in *H. pylori* eradication therapy.

The above results suggested that the *in vivo* colonization of *H. pylori* was closely related with other stomach flora; however, most of the above studies were based on animal models and limited by their methods. As such, composition of the human stomach flora and which stomach flora can inhibit or promote the intragastric colonization of *H. pylori* have not been or cannot be comprehensively understood.

Stomach flora and gastric cancer

Gastric cancer is one of the most common malignancies worldwide. The pathogenesis of gastric cancer is a process of multiple stages and steps affected by multiple factors that involve a huge number of molecules and complex regulation networks. The cause of gastric cancer is still not clear despite deep decades-long studies. It is generally believed that environmental, dietary, *H. pylori* infection and genetic factors participate in the pathogenesis of gastric cancer. Furthermore, *H. pylori* is closely related with gastric cancer, although whether other intragastric flora facilitate or inhibit the effect of *H. pylori* in gastric cancer development remains unknown and very few studies have examined this issue.

The pathogenesis of CRC may be the result of interactions among the intestinal flora, intestinal mucosal immunity and host genetic susceptibility^[62]. Intestinal flora interfere with the signal mechanisms of pro-inflammatory reactions in the intestinal mucosa and results in excessive repair of mucosal injury and ultimately induces tumorigenesis and canceration^[63]. Some intestinal microbes and their metabolites have direct or indirect cytotoxic effects on intestinal mucosal epithelial cells, and incomplete repair of damaged intestinal mucosal epithelium may result

in neoplastic transformation^[63]. Animal studies have also confirmed that permanent and normal intestinal flora are necessary for intestinal tumorigenesis^[64]. Studies on the relationship between intestinal flora and CRC indicated that the permanent and normal intragastric flora and their composition might participate in the pathogenesis of gastric cancer. However, few studies have focused on the issue of whether new ways of enabling early warning and early diagnoses of gastric cancer can be established using in-depth analysis and studies of gastric flora composition. The primary reason for this is that due to the large and complex flora network, comprehensive and detailed information on flora constitution could not be obtained by studies using culture-based methods; thus, the microecological study of the relationship between other stomach flora and gastric cancer pathogenesis could not be conducted. Dicksved *et al.*^[65] recently analyzed the stomach flora constitution of patients with gastric cancer and found no significant differences in the stomach flora of patients with gastric cancer and those of patients with dyspepsia and normal gastric mucosa. However, there were many limitations in that research, including the small size of the included samples and the fact that a new generation of high-throughput sequencing technology was not used; thus, the results remain to be confirmed since the stomach flora were not comprehensively and deeply studied. To date, no study on the relationship between stomach flora and gastric cancer using high-throughput sequencing technology based on the metagenomics strategy has been performed.

Stomach flora and gastric polyp

Gastric polyps are focal elevated lesions within the gastric epithelium mucosa. The current limited systematic studies of gastric polyps focus mainly on the relationship between gastric polyps and cancer as well as the role of *H. pylori* in the pathogenesis. The possible pathogenesis related to the development of gastric polyps include hereditary factors, bile reflux, *H. pylori* infection, etc., while none have been directly proved. As such, the etiology and biological characteristics of gastric polyps and its long-term effects on the human body are not yet clear. Studies of gastric polyps are far less detailed than those of colonic polyps. The intestinal flora are involved in the pathogenesis of colonic polyps^[24,25], while no study focusing on the relationship between stomach flora constitution and gastric polyps pathogenesis from the perspective of gastric microbiota using bacterial 16S rDNA sequencing has been reported.

Overall, the constitution and diversity of stomach flora under various disease states, the interactions between *H. pylori* and other stomach flora and their underlying mechanisms as well as the effect of stomach flora in the pathogenesis of various stomach diseases are expected to be uncovered more deeply from the perspective of intestinal microbiota using high-throughput bacterial 16S rDNA sequencing technology based on the metagenomics strategy.

MICROBIOTA AND THE DUODENUM

Much less is known about the microbes that are present within the duodenum, particularly because collecting samples for such microbial ecology studies is much more challenging. However, continued efforts in this regard are needed, especially in light of the growing recognition of the composition of the duodenal microbiota and the association with health and gastrointestinal disorders as revealed in recent studies. Duodenal microbiota studies have focused predominantly on small intestinal bacterial overgrowth (SIBO), IBS, and celiac disease (CD).

Duodenal flora and SIBO

Some risk factors, such as demographics (older age), anatomic abnormalities (*e.g.*, small intestinal diverticula and gastric resection), motility disorders (*e.g.*, CD, diabetic neuropathy and scleroderma), organ system dysfunction (*e.g.*, cirrhosis, chronic pancreatitis and end-stage renal disease), and medications (*e.g.*, recurrent antibiotics and gastric acid inhibitors), are closely associated with SIBO^[66].

SIBO has been traditionally defined according to the number and type of culturable bacteria within duodenal or jejunal aspirates: 10^5 CFU/mL of colonic-type bacteria has been commonly used^[67]. Although some studies have diagnosed SIBO using a direct test—that is, bacterial cultures of aspirate from the small bowel^[68], the majority of gastrointestinal microbiota could not be cultured, the culture-based method can not reveal the real changes in microbiota in the small intestine in various disease conditions. The lactulose/glucose breath tests have also recently been used for the diagnosis of SIBO^[69,70], however, these are indirect tests with poor sensitivity and specificity^[71,72]. In the future, high-throughput bacterial 16S rDNA sequencing may be used to determine the composition of microbiota in the small intestine in some disorders which could lead to SIBO, and contribute to new diagnostic criteria for SIBO.

Duodenal flora and IBS

IBS is a common disorder characterized by abdominal pain or discomfort associated with disturbed bowel function such as constipation and/or diarrhea^[73]. Epidemiological and clinical data support the new bacterial concept of IBS^[74]. Altered intestinal microbiota composition^[75-79] and gut flora metabolites (*e.g.*, short-chain fatty acids butyrate, acetate, and propionate; CH₄ and H₂ gases)^[79,80] were observed in patients with IBS. An etiological role of gastrointestinal infection in the development of IBS has been confirmed^[81]. The final and most promising area is that of alterations in small intestinal microbiota in a subset of patients with IBS^[74,82]. Several probiotics^[83,84] and antibiotics^[85] might play a potential therapeutic role in IBS.

Most studies in this area have investigated the changes in fecal microbiota in patients with IBS^[86,87], however, corresponding investigations of the microbial composi-

tion of the small intestine, the duodenum in particular, in patients with IBS are rare^[88,89]. A recent specific real-time PCR-based investigation using duodenal mucosa brush samples noted that the percentage of *Bifidobacterium* corresponding to the species *Bifidobacterium catenulatum* was significantly lower in patients with IBS than in healthy subjects^[88]. The same group showed higher levels of *Pseudomonas aeruginosa* in the upper small intestine of patients with IBS than in healthy subjects^[88]. Although further investigation is required, these findings suggest that therapies involving modulation of the small intestinal microbiota merit consideration. The relationship between SIBO and IBS is highly inconsistent among studies, and there is no evidence of SIBO being absent before IBS symptoms are evident and present after IBS emerges^[56].

Duodenal flora and CD

CD typically presents in early childhood with chronic inflammation of the small intestinal mucosa and permanent intolerance to dietary gluten. Several studies have confirmed that other factors such as abnormalities in the small intestinal microbiota might be associated with this disorder^[90-93]. Nadal *et al*^[91] conducted bacteriological analyses of duodenal biopsy specimens based on pediatric patients with CD. Their results showed that patients with active CD had significantly higher numbers of total bacteria, especially Gram-negative bacteria, compared with asymptomatic patients and healthy subjects^[91]. The ratio of *Lactobacillus-Bifidobacterium* to *Bacteroides-Escherichia coli* was lower in patients with CD. Nistal *et al*^[92] analyzed the bacterial *16S rRNA* gene sequencing of DNA extracted from duodenal biopsies and showed that the diversity of duodenal microbiota was significantly different between treated and untreated adults with CD due to treatment with a gluten-free diet. Furthermore, Di *et al*^[93] found that a gluten-free diet lasting two or more years could not completely restore the microbiota. Undoubtedly, the fecal-associated microbiota composition and related metabolites could also be disturbed in patients with CD^[94,95]. Disruption of the duodenal microbiota in patients with CD was linked overall to the symptomatic presentation and could favor the pathogenesis of CD.

The composition of the microbiota within the small intestine has not been analyzed comprehensively using a high-throughput *16S rRNA* gene or metagenomic sequencing method, either in healthy individuals or in patients with gastrointestinal conditions. The study of specimens from the small intestine (especially the distal duodenum, jejunum, and proximal ileum) collected from organ donations and transplantation could be a good way of understanding the abundance and variety of normal microbiota within the small intestine.

STUDY LIMITATIONS AND FUTURE OF HUMAN MICROBIOTA RESEARCH

It is evident that gastrointestinal microbiota contribute to human health and disease. The composition and function

of microbiota within the human gastrointestinal tract have been sought for decades, but efforts have been hampered by the following factors: the complexity of gastrointestinal microbiota, especially with regard to the abundance and diversity of commensal fungi and viruses within the human gastrointestinal tract^[96,97]; the heterogeneity and multifactorial pathophysiology of gastrointestinal diseases; the impact of the variability of host genotype, diet^[98,99], age^[100,101], race^[98], geographic location^[98], drug treatment^[102], and medical intervention^[103] on gastrointestinal microbiota; inherent limitations in the methodologies used to assess the composition and function of gastrointestinal microbiota; and a lack of suitable animal models (similar to human microecology) for studying the pathogenesis of various disorders. High-throughput sequencing and bioinformatics analyses are evolving rapidly and providing us with fascinating insight into the microbiota present within the human gastrointestinal tract. We are in the midst of a revolutionary period with respect to investigation of the gastrointestinal microbiota. There have been remarkable advances with respect to establishing which microbes are altered in healthy subjects^[19] vs those in individuals suffering from IBD^[1], obesity^[1], and type 2 diabetes^[23]. However, these studies mainly focused on the fecal-associated microbiota, because the gut microbiota has a major impact on human health and disease and is the best-studied ecosystem; and fecal samples are easy to collect and suitable for the metagenomic sequencing of whole community DNA.

At present, there are more study limitations for the microbiota in the upper gastrointestinal tract, especially the choice of representative human specimens and the application of a reliable analytical method. Endoscopic biopsy specimens, aspirate samples, mucosa brush samples, and surgical specimens from the esophagus, stomach and upper duodenum could be used for microbiota analysis. However, sample collection from the distal duodenum, jejunum, and proximal ileum is still difficult; the surgical and aspirate samples, especially the specimens from organ donations and transplantation may be suitable for analysis. In addition, contamination by the oral microflora and the microbiota from other sections of the upper gastrointestinal tract, and contamination with human host DNA could represent major and permanent methodical problems. Microbiota studies are subject to the restriction of missing distinction between transient and resident microflora in the esophagus, stomach and small intestine, thus, the collection and handling of specimens are of great importance.

A number of culture-based techniques and PCR-based molecular approaches including TRFLP, DGGE and TGGE, RISA, DNA microarray and FISH, have been applied to analyze the human microbiota. Furthermore, the next-generation high-throughput DNA sequencing techniques based on 454 pyrosequencing or Illumina (Solexa) sequencing platforms are the most powerful to investigate the composition, abundance and function of the gastrointestinal microbiota. The analytical method

selected for the assessment of upper gastrointestinal microbiota will depend on the expected target as well as the time and cost-effectiveness associated with the research. Currently, the high-throughput sequencing of 16S rDNA, but not the metagenomics sequencing of the whole microbial community DNA, may be the best molecular method for upper gastrointestinal microbiota. However, it is difficult to distinguish DNA coming from dead or live microbes, when using extracted DNA in the PCR-based molecular analysis. At present, both molecular and culture-based methods should be used to investigate the microbiota composition in the human gastrointestinal tract. Although it could be an arduous task, it is essential for scientific researchers to sequence and characterize the microbiota within the upper gastrointestinal tract. In the future, the use of metagenomics combined with human genome-wide association studies, as well as metabonomics and metaproteomics, may be an ideal approach to understand the microbiota-host interaction and unravel the significance of specific microbiota to determine which microbiota are causative and which are present merely as a consequence of disease. Perhaps one day specific microbes and microbiota-based biomarkers will be developed for diagnostic and therapeutic purposes.

CONCLUSION

In summary, the upper gastrointestinal microbiota is implicated in several gastrointestinal illnesses. There are many study limitations for the upper gastrointestinal microbiota, which could be prevented or mitigated. Through the conceptual innovations in metagenomics and the improvements in DNA high-throughput sequencing and bioinformatics analysis technology, it is now possible to explore the genetic nature of the microbiome in the esophagus, stomach, and small intestine, and the interactions between the host and the residing microbial community.

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Scolopendra subspinipes mutilans protected the cerulein-induced acute pancreatitis by inhibiting high-mobility group box protein-1

Il-Joo Jo, Gi-Sang Bae, Kyoung-Chel Park, Sun Bok Choi, Won-Seok Jung, Su-Young Jung, Jung-Hee Cho, Mee-Ok Choi, Ho-Joon Song, Sung-Joo Park

Il-Joo Jo, Kyoung-Chel Park, Sun Bok Choi, Ho-Joon Song, Sung-Joo Park, Department of Herbology, School of Oriental Medicine, Wonkwang University, Iksan, Jeonbuk 540-749, South Korea

Il-Joo Jo, Mee-Ok Choi, Department of Beauty Science, Kwangju women's University, Kwangju 506-713, South Korea
Gi-Sang Bae, Sung-Joo Park, Hanbang Body-fluid Research Center, Wonkwang University, Iksan, Jeonbuk 540-749, South Korea

Won-Seok Jung, Su-Young Jung, Jung-Hee Cho, Jeollanamdo Development Institute for Korean Traditional Medicine, Jangheung, Jeollanamdo 529-851, South Korea

Author contributions: Jo IJ and Park SJ designed the research; Jo IJ, Bae GS, Park KC, Choi SB, Jung WS, Jung SY, Cho JH, Choi MO and Song HJ performed the research; Jo IJ and Park SJ analyzed the data; Jo IJ and Park SJ wrote the paper.

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Correspondence to: Sung-Joo Park, MD, PhD, Department of Herbology, School of Oriental Medicine, Wonkwang University, Iksan, Jeonbuk 540-749, South Korea. parksj08@wku.ac.kr
Telephone: +82-63-850-6450 Fax: +82-63-856-2283

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Abstract

AIM: To evaluate the inhibitory effects of *Scolopendra subspinipes mutilans* (SSM) on cerulein-induced acute pancreatitis (AP) in a mouse model.

METHODS: SSM water extract (0.1, 0.5, or 1 g/kg) was administrated intraperitoneally 1 h prior to the first injection of cerulein. Once AP developed, the stable cholecystokinin analogue, cerulein was injected hourly, over a 6 h period. Blood samples were taken 6 h later to determine serum amylase, lipase, and cytokine levels. The pancreas and lungs were rapidly removed for

morphological examination, myeloperoxidase assay, and real-time reverse transcription polymerase chain reaction. To specify the role of SSM in pancreatitis, the pancreatic acinar cells were isolated using collagenase method. Then the cells were pre-treated with SSM, then stimulated with cerulein. The cell viability, cytokine productions and high-mobility group box protein-1 (HMGB-1) were measured. Furthermore, the regulating mechanisms of SSM action were evaluated.

RESULTS: The administration of SSM significantly attenuated the severity of pancreatitis and pancreatitis associated lung injury, as was shown by the reduction in pancreatic edema, neutrophil infiltration, vacuolization and necrosis. SSM treatment also reduced pancreatic weight/body weight ratio, serum amylase, lipase and cytokine levels, and mRNA expression of multiple inflammatory mediators such as tumor necrosis factor- α and interleukin-1 β . In addition, treatment with SSM inhibited HMGB-1 expression in the pancreas during AP. In accordance with *in vivo* data, SSM inhibited the cerulein-induced acinar cell death, cytokine, and HMGB-1 release. SSM also inhibited the activation of c-Jun NH2-terminal kinase, p38 and nuclear factor (NF)- κ B.

CONCLUSION: These results suggest that SSM plays a protective role during the development of AP and pancreatitis associated lung injury *via* deactivating c-Jun NH2-terminal kinase, p38 and NF- κ B.

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Key words: *Scolopendra subspinipes mutilans*; Cytokines; Acute pancreatitis; High-mobility group box protein-1

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INTRODUCTION

The annual incidence of acute pancreatitis (AP) is in the range of 300 or more patients per million^[1,2]. The mortality rate for severe AP is approximately 30%, whereas that for moderate pancreatitis is about 3%. The main causes of death are circulatory shock renal, respiratory, and hepatic failure. Thus, many patients with AP develop multiple organ failure (MOF)^[3]. Generally, AP is characterized by activation of pancreatic digestive enzyme production, widespread inflammatory cell infiltration, leukocyte activation, and release of various pro-inflammatory mediators such as tumor necrosis factor (TNF)- α and interleukin (IL)^[4-6]. Although numerous approaches have attempted to identify the pathogenesis of AP^[7-9], the detailed mechanism remains unclear.

Recent studies have shown that high-mobility group box protein-1 (HMGB-1) is a late activator in the inflammatory cascade, when released into the extracellular space^[10]. Neutralization of HMGB-1 has been shown to protect against systemic inflammatory responses such as in sepsis and MOF as HMGB-1 acts as a downstream cytokine of early inflammatory factors such as TNF and ILs^[11-13]. In addition, HMGB-1 has been speculated to be a target for treating AP^[14].

Scolopendra subspinipes mutilans (SSM) is a venomous arthropod, which can be found throughout the world. SSM and its venom have been reported to exhibit many biochemical and physiological effects^[15,16]. The water soluble fractions from SSM have antimicrobial and anti-inflammatory activity and hemolytic action of the toxins^[17,18]. In addition, SSM has been prescribed for the treatment of cardiovascular diseases in South Korea, China, and other Far Eastern Asian countries for several hundred years^[16]. However, the protective activities of SSM in cerulein-induced AP have not been examined to date. Our study was designed to assess the protective effect of SSM in cerulein-induced AP.

Here, we investigated the *in vivo* and *in vitro* activities of SSM using a murine model of experimental pancreatitis. To examine the role of SSM in AP, we examined pancreatic and lung histology, myeloperoxidase (MPO) activity, pancreatic weight (PW)/body weight (BW) ratio, levels of serum amylase, lipase, and cytokines such as TNF- α and IL-1 β as well as expression levels of HMGB-1. Furthermore, we examined mitogen activated protein kinases (MAPKs) and nuclear factor (NF)- κ B to find out the inhibitory mechanisms of SSM in AP.

MATERIALS AND METHODS

Chemicals and reagents

Avidin-peroxidase, cerulein, hexadecyltrimethylammo-

nium bromide, Triton X-100, and tetramethylbenzidine were purchased from Sigma-Aldrich (St. Louis, MO, United States). Anti-mouse TNF- α and IL-1 β antibodies, and recombinant TNF- α and IL-1 were purchased from R-D Systems (Minneapolis, MN, United States).

Preparation of SSM

SSM was purchased from a standard commercial source (Omni Herb, Seoul, South Korea). The identity of the SSM was confirmed by Professor Seung-Heon Hong from Wonkwang University. SSM was prepared by decocting the dried prescription of SSM (100 g) with boiling distilled water (1 L). The decoction time was about 2 h. The water extract was frozen at -80 °C and then freeze-dried to produce a powder form (20.4 g). The yield of extract was 20.4%. The powder was extracted with distilled water and filtered. The filtrates were stored at 4 °C until use.

Animal model

All experiments were performed according to protocols approved by the Animal Care Committee of Wonkwang University. C57BL/6 mice (age 6-8 wk; weight 15-20 g) were purchased from Orient Bio (Sungnam, KyungKiDo, South Korea). All animals were bred and housed in standard shoebox cages in a climate-controlled environment with an ambient temperature of 23 \pm 2 °C and a 12-h light-dark cycle for 7 d. The animals were fed standard laboratory chow, given water, and were randomly assigned to the control or experimental groups. The mice were fasted for 18 h before the induction of AP. Six mice were included in each experimental group.

Experimental design

AP was induced by intraperitoneal injection of supra-maximal concentrations of the stable cholecystokinin analog cerulein (50 μ g/kg) or saline; injections were performed hourly for 6 h. To verify the prophylactic effects of SSM, SSM (0.1, 0.5, or 1 g/kg) was injected 1 h before the first cerulein injection. Mice were sacrificed 6 h after the last cerulein injection. Blood samples were taken to determine serum amylase, lipase, and cytokine levels. For histological examination and scoring, the entire pancreas and lungs were rapidly removed from each mouse and fixed in formalin. To measure tissue MPO activity, as an indicator of neutrophil sequestration, and to perform real-time reverse transcriptase-polymerase chain reaction (RT-PCR) examinations, 3 portions of both pancreas and lungs were stored at -80 °C.

Histological analysis

The entire pancreas of at least 6 mice from each treatment group were examined and semi-quantitatively assessed for levels of necrosis, vacuolization, inflammation, and edema. The entire section, representing a minimum of 100 fields, was examined for each sample and scored on a scale of 0-3 (0 being normal and 3 being severe) on the basis of the number of necrotic acinar cells and the presence of vacuolization, interstitial edema, and inflam-

matory cells infiltration. These characteristics include the presence of acinar-cell ghosts, vacuolization and swelling of the acinar cells, and/or the destruction of the histo-architecture of whole or parts of the acini. For scoring the lungs, the sections were examined for the presence of interstitial inflammation and edema.

Measurement of serum amylase and lipase levels

Blood samples, for the determination of serum amylase and lipase levels, were obtained 6 h after induction of pancreatitis. Mice were anesthetized with an intraperitoneal injection of ketamine (80 mg/kg) and xylazine (4 mg/kg). After anesthetization, blood was withdrawn from the heart of each mouse into a syringe. The levels of serum amylase and lipase were measured using an assay kit (BioAssay Systems, Hayward, CA, United States).

RT-PCR

RT-PCR was performed to measure mRNA transcript levels in the mouse pancreatic tissues and pancreatic acinar cells. Total RNA was isolated from the mouse pancreas using TRIzol (Invitrogen, Carlsbad, CA, United States) and was subjected to reverse transcription using SuperScript II RT (Invitrogen, Carlsbad, CA, United States). TaqMan quantitative RT-PCR using the Light-Cycler 2.0 detection system was performed according to the instructions of the manufacturer (Roche, Basel, Switzerland). For each sample, triplicate test reactions and a control reaction without reverse transcription were analyzed for expression of the gene of interest, and the results were normalized to those of the “housekeeping” hypoxanthine-guanine phosphoribosyl transferase (HPRT) mRNA. Arbitrary expression units were calculated by dividing the expression level for the gene of interest by the ribosomal protein HPRT mRNA expression level. The sequences of forward, reverse, and probe oligonucleotide primers for multiplex real-time TaqMan PCR were as follows: for mouse TNF- α (forward, 5'-TCTCTTCAAGGGACAAGGCTG-3'; reverse, 5'-ATAGCAAATCGGCTGACGGT-3'; probe, 5'-CCC-GACTACGTGCTCCTCACCCA-3'), for mouse IL-1 β (forward, 5'-TTGACGGACCCAAAAGAT-3'; reverse, 5'-GAAGCTGGATGCTCTCATCTG-3'; universal probe, M15131.1-Roche Applied Science).

Enzyme-linked immunosorbent assay

Enzyme-linked immunosorbent assays (ELISAs) for TNF- α and IL-1 β were carried out in duplicate in 96-well plates (Nunc, Roskilde, Denmark), which had been incubated with 100 L aliquots of either anti-mouse TNF- α or anti-mouse IL-1 β monoclonal antibodies (1.0 μ g/mL in phosphate-buffered saline (PBS) at pH 7.4) overnight. The plates were washed in PBS containing 0.05% Tween-20 and blocked with PBS containing 10% fetal bovine serum for 2 h. After additional washes, the standards and the serum, pancreatic homogenates and pancreatic acinar cell supernatants were added to the plates and incubated at room temperature for 3 h. To obtain pancreatic homog-

enates, the pancreas were thawed and then homogenized in PBS. After washing the wells, 0.2 μ g/mL of biotinylated anti-mouse TNF- α or IL-1 β were added to each well. Incubation was continued at room temperature for 1 h. The wells were washed, avidin-peroxidase was added, and plates were incubated for 30 min at room temperature. Wells were washed again, and 3, 3', 5, 5'-tetramethylbenzidine substrate was added. Color development was measured at 450 nm using an automated microplate ELISA reader. Standard curves were obtained for each sample by using serial dilutions of recombinant TNF- α and IL-1 β .

MPO activity estimation

Neutrophil sequestration in the pancreas was quantified by measuring the tissue MPO activity. Tissue samples were thawed, homogenized in 20 mmol/L phosphate buffer (pH 7.4), and centrifuged (15 000 revolution/min, 10 min), and the resulting pellet was resuspended in 50 mmol/L phosphate buffer (pH 6.0) containing 0.5% hexadecyltrimethylammonium bromide. The sample was then centrifuged (15 000 revolution/min, 5 min), and the supernatant used for the MPO assay. The reaction mixture consisted of the supernatant, 1.6 mmol/L tetramethylbenzidine, 80 mmol/L sodium phosphate buffer (pH 5.4), and 0.3 mmol/L hydrogen peroxide. The mixture was incubated at 37 °C for 110 s, the reaction was terminated with 2 mol/L of H₂SO₄, and the absorbance was measured at 450 nm. This absorbance was then corrected for the DNA content of the tissue sample.

Histological and immunohistochemical analysis

Fixed pancreatic tissues were embedded in paraffin, cut into 4-mm sections, and stained with hematoxylin-eosin for standard histological examination. Immunohistochemical (IHC) staining for HMGB-1 was performed using a DAB IHC kit (DAKO, Cytomation, Denmark). The relative intensity was measured using the Leica microscopy software (Wetzlar, Germany).

Acinar cell isolation

Pancreatic acini were isolated from C57BL/6 mice using collagenase digestion. All experiments were performed according to protocols approved by the Animal Care Committee of Wonkwang University. Briefly, pancreatic tissue was minced with scissors and digested for 15 min in solution Q (120 mmol NaCl, 20 mmol HEPES, 5 mmol KCl, 1 mmol MgCl₂, 1 mmol CaCl₂, 10 mmol sodium pyruvate, 10 mmol ascorbate, 10 mmol glucose, 0.1% bovine serum albumin, 0.01% soybean trypsinogen inhibitor, and 150 units of collagenase/mL). Cells were continuously shaken and gassed with 100% O₂ in a 37 °C water bath and subsequently washed in fresh isolation medium. After collagenase digestion, the tissue was gently pipetted. Dispersed acini were filtered through a 150- μ m nylon mesh, centrifuged 3 times (each for 90 s at 720 rpm), resuspended in Waymouth medium (Invitrogen, Gibco, CA) and incubated with 95% O₂ and 5% CO₂ for 4 h.

Table 1 Effect of *Scolopendra subspinipes mutilans* water extract on pancreatic histological scoring during acute pancreatitis (mean \pm SE, $n = 6$)

| Group | Edema | Inflammation | Vacuolization | Necrosis |
|--------------|-------------------------------|-------------------------------|-------------------------------|-------------------------------|
| Saline | 0.1 \pm 0.02 | 0.3 \pm 0.05 | 0.1 \pm 0.03 | 0.2 \pm 0.02 |
| AP | 2.6 \pm 0.03 ^a | 2.8 \pm 0.04 ^a | 2.4 \pm 0.02 ^a | 2.5 \pm 0.05 ^a |
| SSM 0.1 + AP | 2.3 \pm 0.05 ^{a,c} | 2.0 \pm 0.02 ^{a,c} | 1.6 \pm 0.04 ^{a,c} | 1.8 \pm 0.01 ^{a,c} |
| SSM 0.5 + AP | 1.0 \pm 0.02 ^{a,c} | 1.5 \pm 0.05 ^{a,c} | 1.2 \pm 0.02 ^{a,c} | 1.5 \pm 0.04 ^{a,c} |
| SSM 1 + AP | 0.6 \pm 0.01 ^{a,c} | 1.0 \pm 0.03 ^{a,c} | 0.7 \pm 0.05 ^{a,c} | 0.9 \pm 0.03 ^{a,c} |

The results were similar in 3 further experiments. ^a $P < 0.05$ vs control group; ^c $P < 0.05$ vs cerulein treatment alone. SSM: *Scolopendra subspinipes mutilans*; AP: Acute pancreatitis.

Cell viability assay

Cell viability was assayed using a modified colorimetric technique that is based on the ability of live cells to convert the tetrazolium compound 3-(4,5-dimethylthiazol)-2,5-diphenyltetrazolium bromide (MTT) into purple formazan crystals. MTT (5 mg/mL) was dissolved in Krebs-Henseleit buffer (115 mmol NaCl, 3.6 mmol KCl, 1.3 mmol KH₂PO₄, 25 mmol NaHCO₃, 1 mol CaCl₂, and 1 mol MgCl₂), and 50 μ L was added to each well. After incubating for 30 min at 37 $^{\circ}$ C, the suspension was removed, and the formazan crystals formed were dissolved in 200 μ L dimethyl sulfoxide. Aliquots from each well were seeded in the wells of a 96-well plate in duplicate and assayed at 540 nm using a microplate ELISA reader. The number of viable cells was expressed as a percentage of the control.

Western blotting

Pancreatic tissues and pancreatic acini were homogenized, following which the lysates were boiled in a sample buffer [62.5 mmol Tris-HCl, pH 6.8, 2% sodium dodecyl sulfate (SDS), 20% glycerol, and 10% 2-mercaptoethanol]. Proteins in the cell lysates were then separated using 10% SDS-polyacrylamide gel electrophoresis and transferred to a nitrocellulose membrane. Then, the membrane was blocked with 5% skim milk in PBS-Tween-20 for 2 h at RT and then incubated with primary antibodies overnight. After washing 3 times, each blot was incubated with peroxidase-conjugated secondary antibody for 1 h, and antibody-specific proteins were visualized using an enhanced chemiluminescence detection system (Amersham, Piscataway, NJ) according to the manufacturer's recommended protocol.

High-performance liquid chromatography sample preparation and conditions

An aliquot of 5.0 mg extract powder was dissolved with 1.0 mL of methanol and then filtered through a 0.45 μ m filter membrane before use. A volume of 20 μ L was injected into the high-performance liquid chromatography (HPLC) sample injector system. Chromatographic experiments were performed on a SYKAM series HPLC instrument equipped with sample injector and diode-array UV/Vis detector. For all experiments a SHISEIDO

CAPCELL PACK C-18 column (4.6 mm \times 250 mm; 5 μ m) was used as stationary phase and injection volume were set 20 μ L, respectively. The mobile phase composed of water (A) and acetonitrile (B), applying gradient program starting from 10 %B to 40 %B in 40 min. The column cleaned with 10 %B for 20 min, and then the system was equilibrated for 20 min with the starting conditions. Flow rate was 0.7 mL/min, and the detection wavelength adjusted to 210 nm. The quantifications of peak are 91% (1st), 4% (2nd), 0.5% (3rd), 4.5% (4th) to total.

Statistical analysis

The results were expressed as mean \pm SE. The significance of change was evaluated using the one-way analysis of variance (ANOVA). Differences between the experimental groups were evaluated by performing ANOVA. P values < 0.05 were considered statistically significant.

RESULTS

Effect of SSM on pancreatic histology during cerulein-induced AP

In saline-treated mice, the histological features of the pancreas showed typically normal architecture. Mice treated with intraperitoneal injections of cerulein developed AP. Histological examination of the pancreas (6 h after the final injection of cerulein) revealed tissue damage characterized by mild interstitial edema, inflammatory cell infiltration, vacuolization, and acinar cell necrosis. Compared to saline pre-treatment, SSM pre-treatment resulted in a significant reduction in pancreatic injury as shown by reduced edema, inflammation, vacuolization, and necrosis, in a dose-dependent manner (Figure 1A, B and Table 1).

Effect of on the MPO activity in cerulein-induced AP

As an additional quantitative assessment of the severity of the inflammatory response, we measured MPO activity as an indicator of neutrophil sequestration in the pancreas, following the induction of AP. MPO activity in the pancreas of the SSM pre-treated AP mice was lesser than that in the pancreas of the saline pre-treated AP mice (Figure 1C).

Effect of SSM on PW/BW and serum amylase and lipase levels in cerulein-induced AP

In order to assess the effect of SSM on pancreatic edema, the PW/BW was measured. As shown in Figure 2A, the PW/BW was increased in saline-treated mice with AP. SSM treatment, however, inhibited the AP-induced PW/BW ratio increase compared with the saline treated group (Figure 2A). Serum amylase and lipase levels are most commonly used biochemical markers of pancreatic disease, particularly in AP^[19-21]. Therefore, we examined serum amylase and lipase levels during cerulein-induced AP. The administration of SSM significantly reduced the serum amylase and lipase levels (Figure 2B and C).

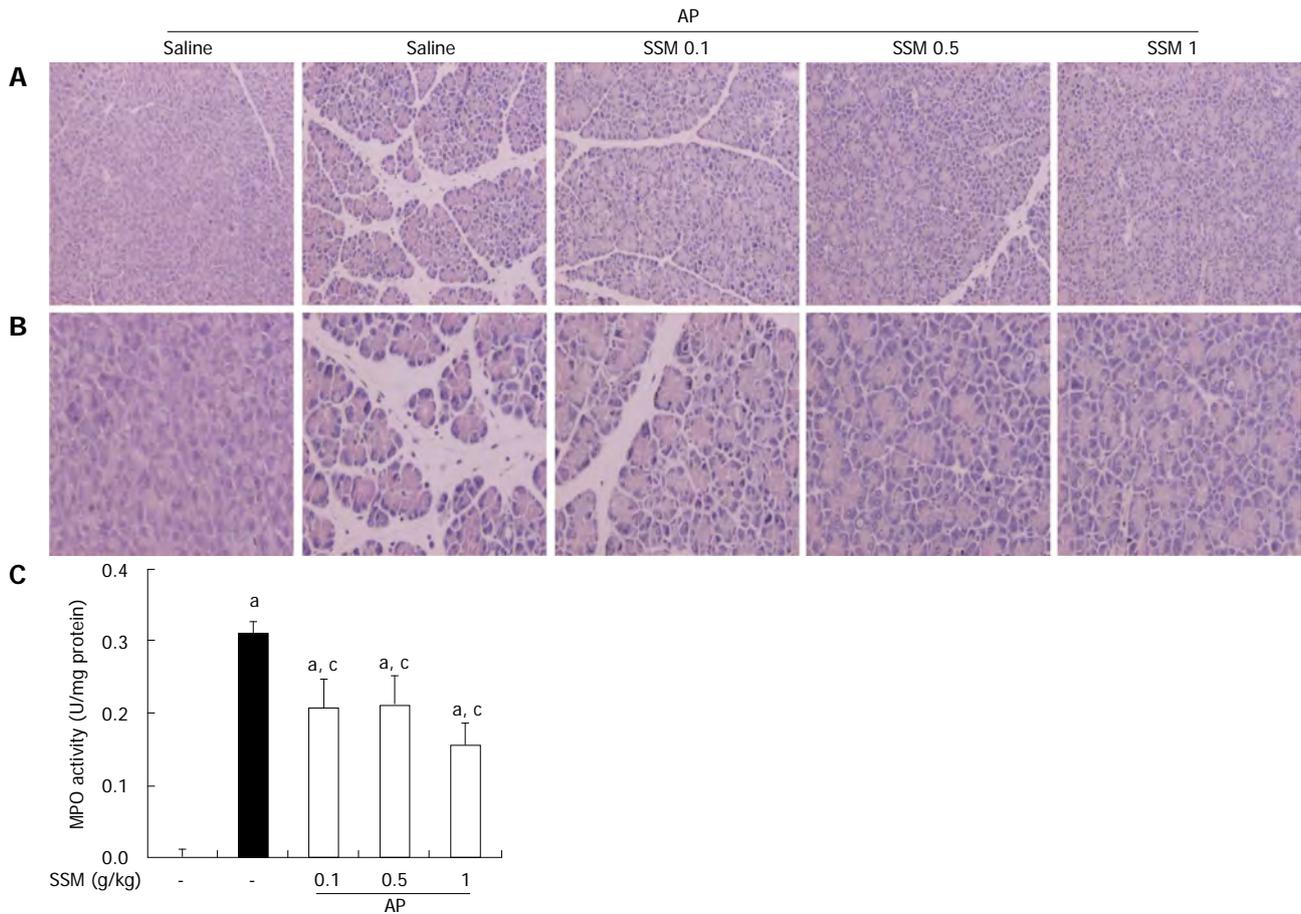


Figure 1 Effects of *Scolopendra subspinipes mutilans* on inflammation in the pancreas following pancreatitis. A, B: 200 × (A) and 400 × (B) magnification of representative hematoxylin and eosin stained pancreatic sections of control mice and mice pretreated with *Scolopendra subspinipes mutilans* (SSM) (0.1, 0.5 or 1 g/kg) 1 h before the cerulein (50 μg/kg)-mediated induction of acute pancreatitis (AP); C: Myeloperoxidase (MPO) activity was measured in the pancreas 6 h after completion of the cerulein injections. Data are expressed as U/mg protein. Data are represented as mean ± SE (n = 6 in each group). The results were similar in 3 further experiments. ^aP < 0.05 vs control group, ^cP < 0.05 vs cerulein treatment alone.

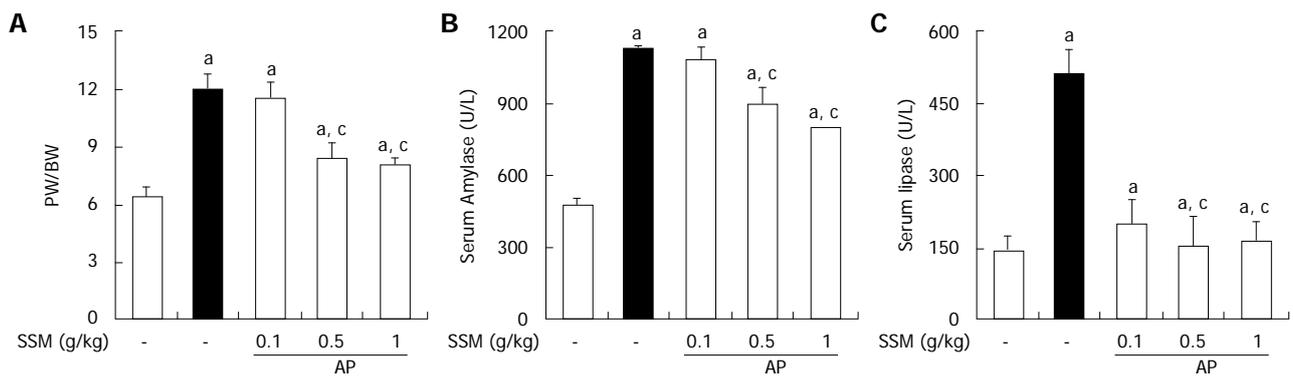


Figure 2 Effects of *Scolopendra subspinipes mutilans* pretreatment on the pancreatic weight/body weight ratio and the production of digestive enzymes such as serum amylase and serum lipase during cerulein-induced acute pancreatitis. Mice pretreated with *Scolopendra subspinipes mutilans* (SSM) (0.1, 0.5 or 1 g/kg) were challenged with intraperitoneal injections of cerulein (50 μg/kg). Mice were sacrificed 6 h after the last cerulein injection. Serum and pancreas were harvested and the pancreatic weight (PW)/body weight (BW) (A) and levels of digestive enzymes such as amylase (B) and lipase (C) were measured as indicated in the experimental protocol. Data are represented as mean ± SE (n = 6 in each group). The results were similar in 3 further experiments. ^aP < 0.05 vs control group, ^cP < 0.05 vs cerulein treatment alone. AP: Acute pancreatitis.

Effect of SSM on TNF-α and IL-1β production in cerulein-induced AP

Several inflammatory mediators have been shown to increase during AP^[22]. Therefore, to examine the effect of SSM on the occurrence of a systemic inflammatory

response during cerulein-induced AP, we measured the level of TNF-α and IL-1β induction. Compared to control mice, mice with AP showed a significant increase in the levels of these inflammatory mediators in the pancreatic tissue and serum (Figure 3). However, SSM pre-

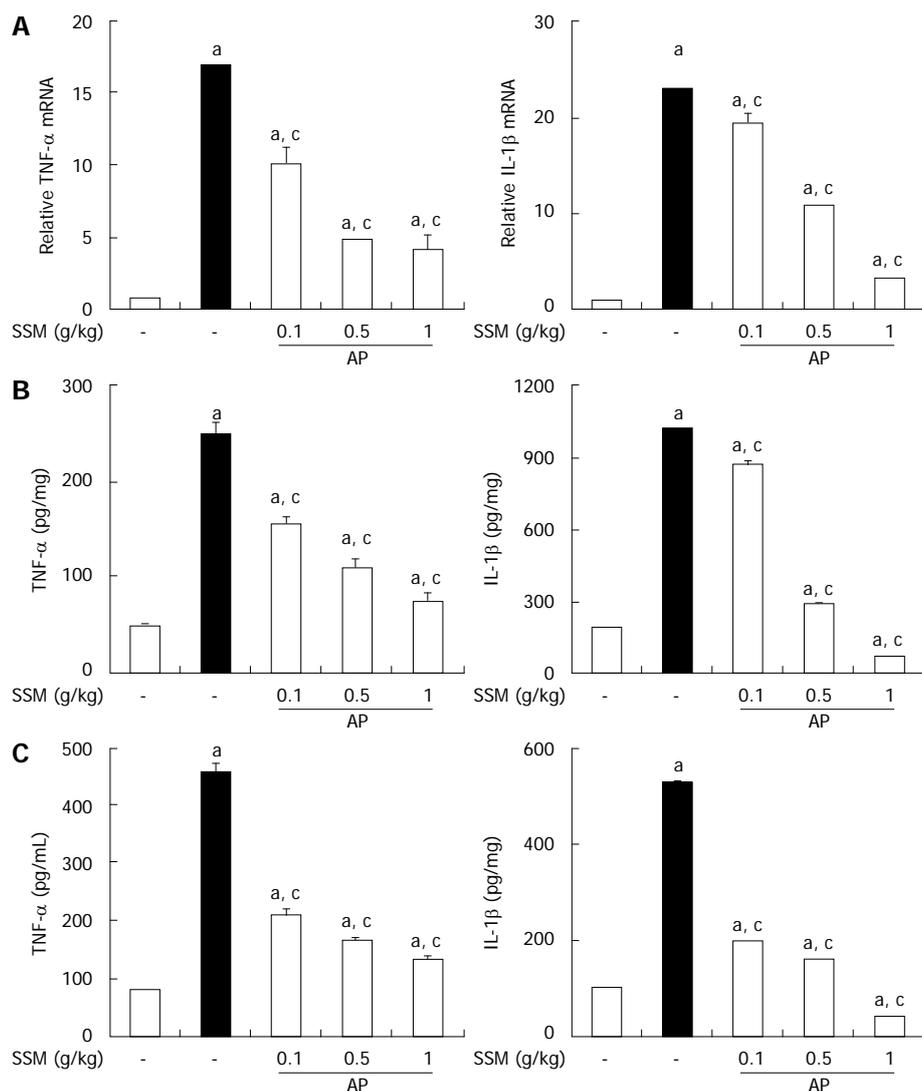


Figure 3 Effect of *Scolopendra subspinipes mutilans* on tumor necrosis factor- α and interleukins-1 β during cerulein-induced acute pancreatitis. Mice pretreated with *Scolopendra subspinipes mutilans* (SSM) (0.1, 0.5, or 1 g/kg) were challenged with intraperitoneal injections of cerulein at a supramaximal dose (50 μ g/kg). Mice were sacrificed 6 h after the last cerulein injection. A-C: Levels of pancreatic tumor necrosis factor (TNF)- α and interleukin (IL)-1 β mRNA were quantified by real-time reverse transcriptase-polymerase chain reaction (A) and the corresponding protein levels were measured in the pancreatic tissue (B) and serum by enzyme-linked immunosorbent assay (C). Data are represented as mean \pm SE ($n = 6$ in each group). The results were similar in 3 further experiments. ^a $P < 0.05$ vs control group, ^c $P < 0.05$ vs cerulein treatment alone. AP: Acute pancreatitis.

treatment reduced the cytokine levels in both pancreatic tissue (Figure 3A and B) and serum (Figure 3C).

Effect of SSM on lung histological changes during cerulein-induced AP

The lung is typically affected in cases of pancreatitis^[23-25]. Lung injury, characterized by edema and inflammation, commonly develops early in AP^[26]. Lungs from cerulein-induced AP show alveolar thickening and inflammatory cell infiltration^[26]. However, these changes were significantly reduced in lungs from the SSM pre-treated group, and this effect was dose-dependent (Figure 4A, B and Table 2).

Effect of SSM on HMGB-1 expression in cerulein-induced AP

To measure the HMGB-1 expression, an IHC method

was used. IHC analysis showed that HMGB-1 expression was detected in the pancreas by the presence of a brown color. As shown in Figure 5, HMGB-1 was slightly expressed in control mice, but strongly expressed in AP mice. However, compared to the saline pre-treated AP mice, SSM pre-treated AP mice showed a significant reduction in HMGB-1 expression in the pancreatic tissue (Figure 5).

Effect of SSM on inflammatory responses in the isolated pancreatic acinar cells

The local inflammation caused in pancreatic acinar cells results in acinar cells death and organ destruction^[8]. Thus, acinar cells death can be a hallmark of AP. To assess whether SSM water extract inhibits acinar cells death, we evaluated cell viability by using the MTT assay. At 1 h after SSM pretreatment, cerulein was added for 6 h into

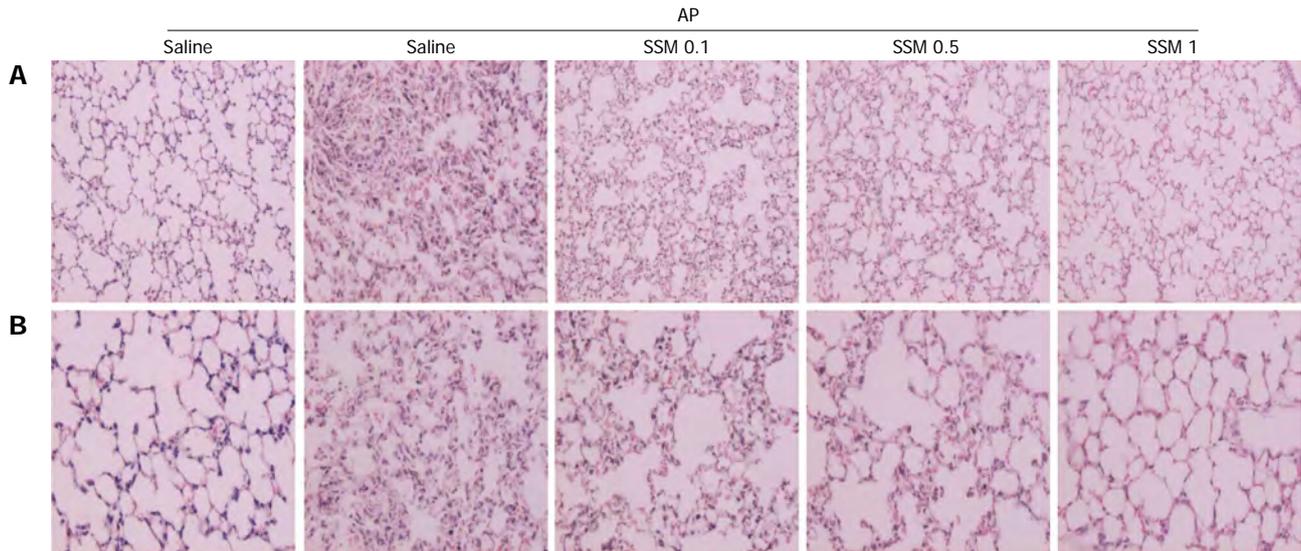


Figure 4 Effects of *Scolopendra subspinipes mutilans* on acute pancreatitis-associated lung injury. A, B: 200 × (A) and 400 × (B) magnification of representative hematoxylin and eosin-stained lung sections of control mice and mice pretreated with *Scolopendra subspinipes mutilans* (0.1, 0.5, or 1 g/kg) 1 h before the cerulein (50 μg/kg)-mediated induction of acute pancreatitis. Data are represented as mean ± SE (n = 6 in each group). The results were similar in 3 additional experiments.

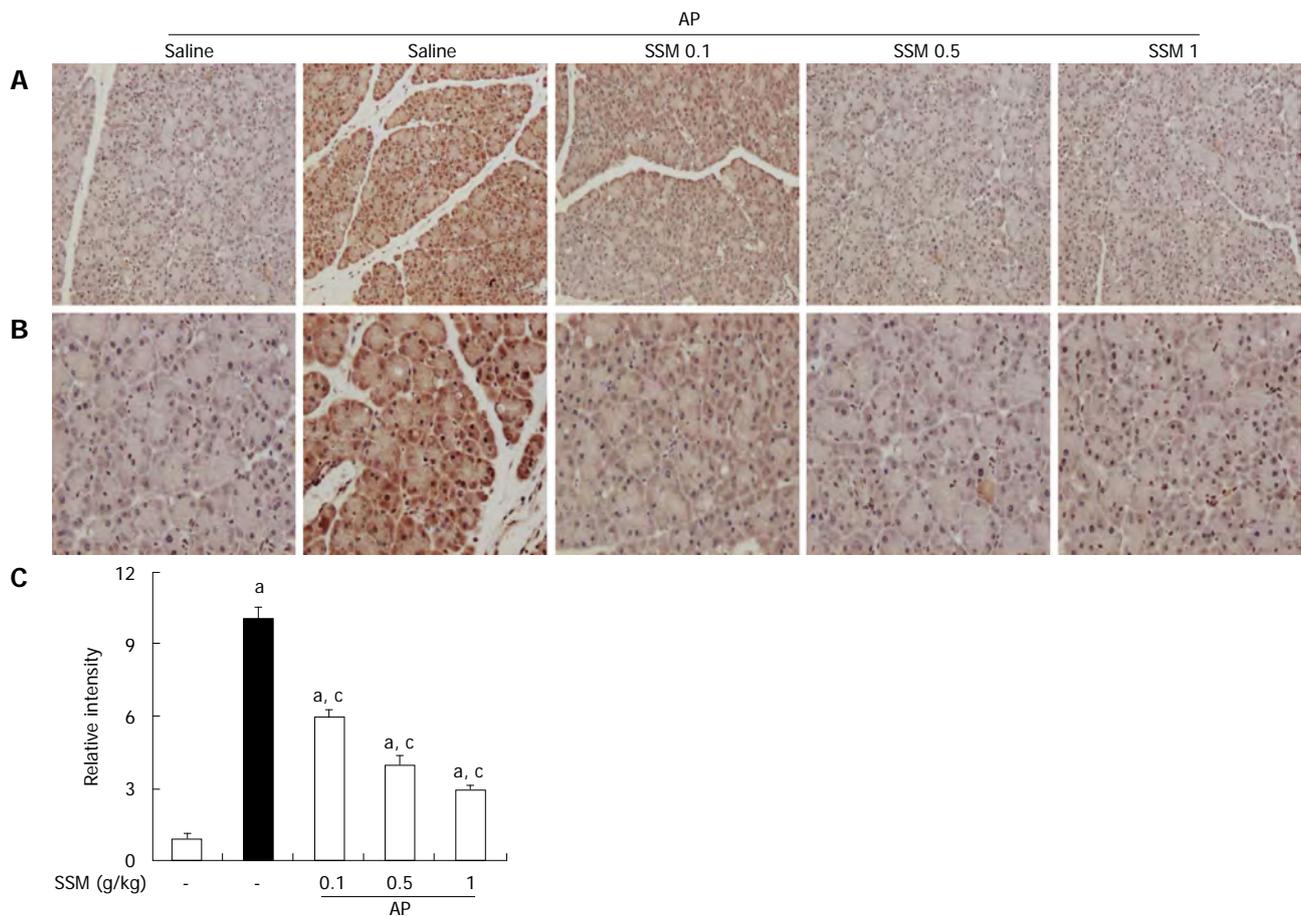


Figure 5 Effects of *Scolopendra subspinipes mutilans* on pancreatic high-mobility group box protein-1 expression in cerulein-induced acute pancreatitis. A, B: 200 × (A) and 400 × (B) magnification of representative immunohistochemical data, detecting high-mobility group box protein-1 (HMGB-1) expression in pancreatic tissue sections of control mice and mice pretreated with *Scolopendra subspinipes mutilans* (SSM) (0.1, 0.5, or 1 g/kg) 1 h before the cerulein (50 μg/kg)-mediated induction of acute pancreatitis (AP). Mice were sacrificed 6 h after the last cerulein injection; C: Relative intensity of HMGB-1 staining was scored as described in Materials and Methods. Data are represented as mean ± SE (n = 6 in each group). The results were similar in 3 further experiments. ^aP < 0.05 vs control group, ^cP < 0.05 vs cerulein treatment alone.

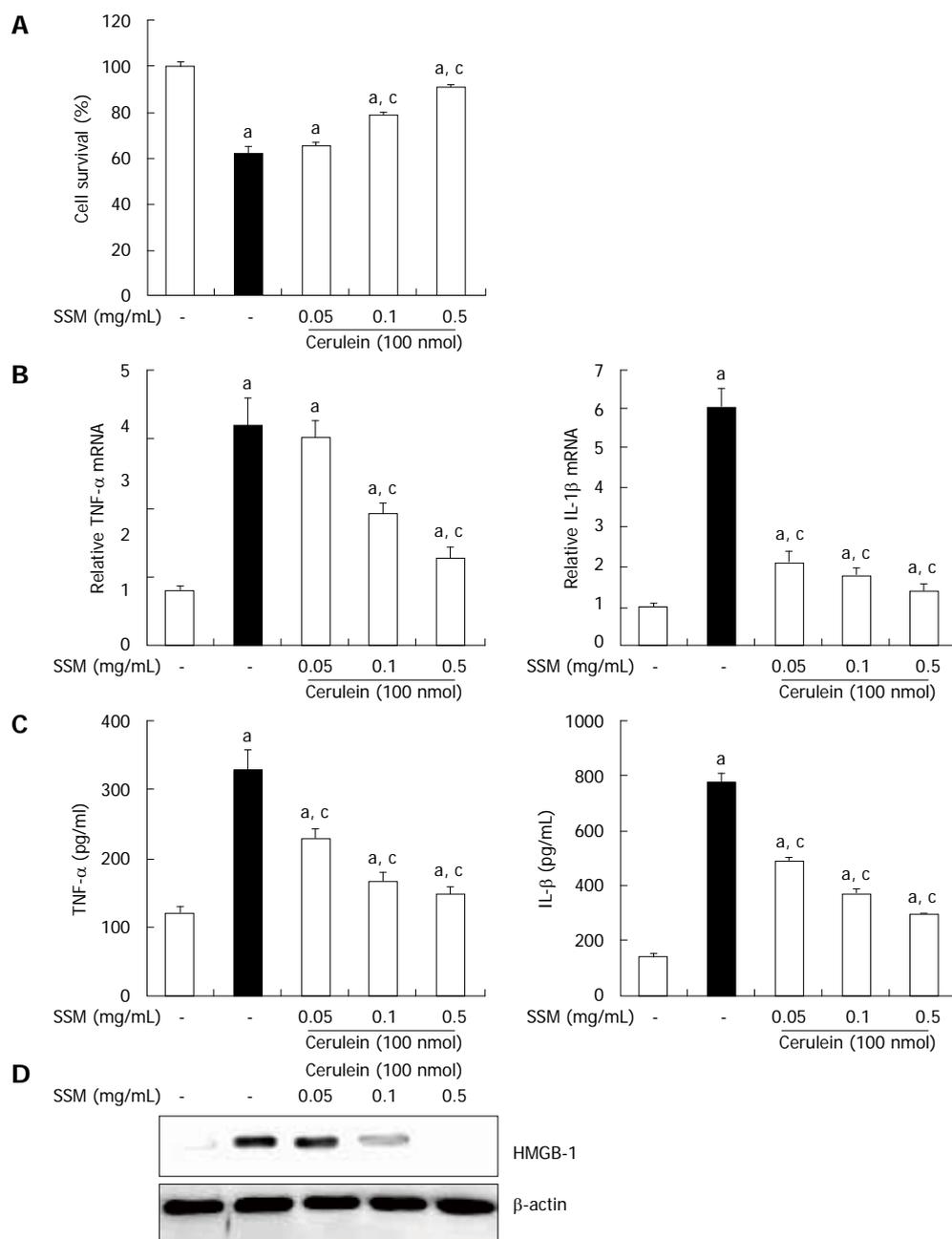


Figure 6 Effect of *Scolopendra subspinipes mutilans* on cerulein-induced acinar cell death and the production of inflammatory mediators. The acinar cells were pretreated with *Scolopendra subspinipes mutilans* (SSM) for 1 h at indicated doses. A: 6 h after cerulein stimulation, the cell viability was measured; B, C: After 24 h of cerulein stimulation, cytokine levels in isolated pancreatic acinar cells were examined using enzyme-linked immunosorbent assay (B) and real-time reverse transcription polymerase chain reaction (C); D: And also the high-mobility group box protein-1 (HMGB-1) levels were measured by western blot. Data are represented as mean \pm SE ($n = 6$ in each group). The results were similar in 3 further experiments. ^a $P < 0.05$ vs control group, ^c $P < 0.05$ vs cerulein treatment alone. TNF: Tumor necrosis factor; IL: Interleukins.

cultured acinar cells. As shown in Figure 6A, the number of cerulein-induced acinar cells death was significantly reduced by SSM (Figure 6A). Next, we also examined cytokine production in isolated pancreatic acinar cells. Pretreatment with SSM inhibited the production of cytokines, such as TNF- α and IL-1 β in a dose dependant (Figure 6C and D). In addition, SSM inhibited the cerulein-induced HMGB-1 expression, which means SSM protected the acinar cells necrosis (Figure 6E).

Further, to examine the inhibitory mechanism(s) against cerulein-induced responses in acinar cells, the activa-

tion of MAPKs and NF- κ B were examined. We assessed the activation of MAPKs and NF- κ B *via* phosphorylation and I κ -B α degradation, respectively. Cerulein treatment resulted in the phosphorylation of MAPKs and degradation of I κ -B α . However, SSM treatment inhibited the activation of c-Jun NH₂-terminal kinase (JNK), p38, and the degradation of I κ -B α but not ERK1/2 (Figure 7A). To clarify whether down-regulation of the molecules in JNK, p38 and NF- κ B by SSM is responsible for the reduced inflammatory responses, JNK inhibitor (SP600125), p38 inhibitor (SB239063) and NF- κ B inhibitor (n-acetyl

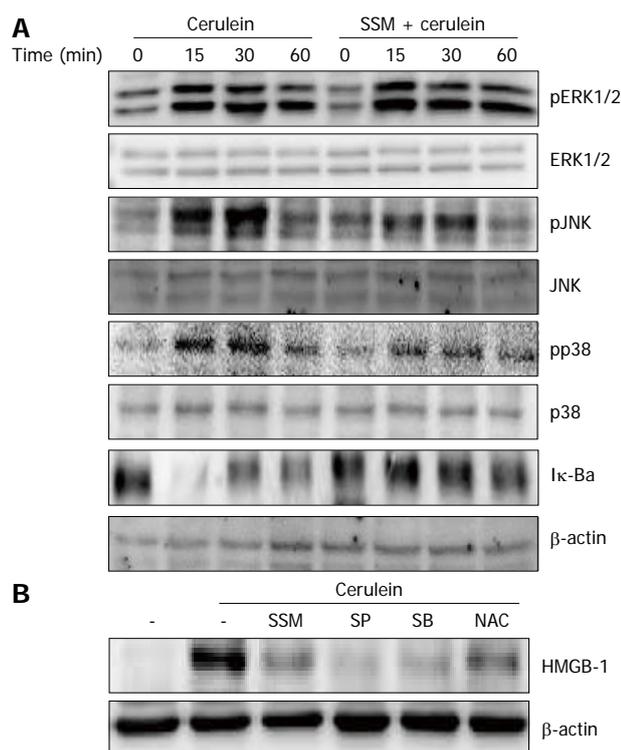


Figure 7 Effect of *Scolopendra subspinipes mutilans* on activation of mitogen activated protein kinases and nuclear factor- κ B. A: Isolated pancreatic acinar cells were pretreated with *Scolopendra subspinipes mutilans* (SSM) for 1 h and then stimulated with cerulein for indicated times. The expression of phosphospecific mitogen activated protein kinases and degradation of I κ -B α in pancreatic acini was examined *via* western blot; B: The acinar cells were pretreated with c-Jun NH $_2$ -terminal kinase (JNK) inhibitor (SP600125 50 μ mol), p38 inhibitor (SB239063 20 μ mol), nuclear factor- κ B inhibitor (N-acetyl cystein, NAC, 10 mmol) or SSM (0.5 mg/mL) for 1 h. Then cerulein was added into isolated pancreatic acinar cells and incubated for 24 h. High-mobility group box protein-1 (HMGB-1) expression was measured *via* western blot. The results were similar in 3 further experiments. ERK: Extracellular signal-related kinase.

cystein; NAC) were used. The inhibition of JNK, p38 and NF- κ B resulted in the reduction of HMGB-1 expression (Figure 7B).

Characterization of the principal component of SSM

SSM was analyzed by HPLC to characterize its main component. A chromatogram of SSM is shown in Figure 8. The peaks of the principal components of SSM have not yet been identified. Further studies to evaluate the principal components of SSM would be needed.

DISCUSSION

In this study, we have provided evidence that SSM water extract attenuated the development of cerulein-induced AP and AP-associated lung injury. Pre-treatment of mice with SSM significantly inhibited serum amylase and lipase production, TNF- α and IL-1 β expression, and MPO activity. In addition, SSM pre-treatment inhibited HMGB-1 expression in the pancreas. In accordance with *in vivo* experiments, SSM inhibited the acinar cell death, cytokine productions, and HMGB-1 production. Furthermore, SSM inhibited the activation of JNK, p38 and NF- κ B.

Table 2 Effect of *Scolopendra subspinipes mutilans* on lung histological scoring during acute pancreatitis (mean \pm SE, $n = 6$)

| Group | Edema | Inflammation |
|--------------|-------------------------------|-------------------------------|
| Saline | 0.2 \pm 0.05 | 0.3 \pm 0.03 |
| AP | 2.7 \pm 0.02 ^a | 2.8 \pm 0.05 ^a |
| SSM 0.1 + AP | 2.2 \pm 0.04 ^{a,c} | 2.3 \pm 0.02 ^{a,c} |
| SSM 0.5 + AP | 1.6 \pm 0.03 ^{a,c} | 1.5 \pm 0.04 ^{a,c} |
| SSM 1 + AP | 1.0 \pm 0.05 ^{a,c} | 0.8 \pm 0.03 ^{a,c} |

The results were similar in 3 further experiments. ^a $P < 0.05$ vs control group, ^c $P < 0.05$ vs cerulein treatment alone. SSM: *Scolopendra subspinipes mutilans*; AP: Acute pancreatitis.

These findings suggested that SSM protected the AP *via* JNK, p38 and NF- κ B deactivation.

Recently, many studies have reported the anti-inflammatory activity of SSM. Wang *et al.*^[27] showed the protective effects of SSM on acute renal failure and multiple focal neuropathy, and Ren *et al.*^[28] reported the anti-inflammatory effects of SSM in Alzheimer's disease. Therefore, to further investigate the anti-inflammatory activities of SSM, we selected to examine the effects of SSM in a cerulein-induced AP model, which has not previously been assessed. As we expected, SSM water extract significantly inhibited pancreatic and lung inflammation in a dose-dependent manner (Figures 1 and 4). We supposed that the anti-inflammatory effects of SSM on AP would be due to anti-microbial effects of SSM. Ren *et al.*^[17] reported that water soluble fraction of SSM could remove the all type of bacteria such as gram-positive, gram-negative bacteria and fungi. Because one of the main causes of AP would be bacterial infection^[29,30], the removal ability of SSM would be helpful to protect AP. Thus, the anti-microbial ability of SSM might contribute to inhibition of pancreatic inflammation.

Amylase and lipase levels are used alone, or in combination, to diagnose patients with AP^[31]. An increased level of serum amylase, at least 3 times over the normal limit, indicates AP. Amylase activity rises quickly during the early phase after the onset of symptoms and returns to normal quickly^[31]. Serum amylase activities could reflect the exocrine pancreatic insufficiency, thus resulting in mal-digestion^[32]. In comparison with serum amylase activity, serum lipase activity remains increased (up to 16-28 fold) for longer, thereby giving greater opportunity in patients with a delayed presentation. Pancreatic lipase activities are less likely to be affected by other environmental factors^[33]. Thus, the serum amylase and lipase activities play a key role in determining the severity of AP. In this experiment, cerulein stimulation resulted in significant elevation in serum amylase and lipase levels. This increase was inhibited by SSM pre-treatment, suggesting that SSM is effective against the induction of AP (Figure 2).

The activation of inflammatory cells that release cytokines such as TNF- α and IL-1 β is an important cascade in the pathogenesis of AP^[34-36]. TNF- α and IL-1 β are derived predominantly from activated macrophages and

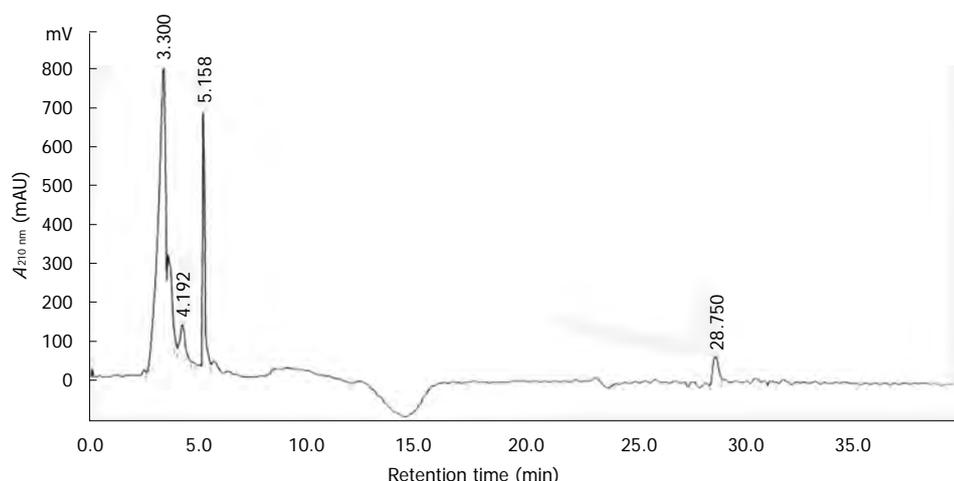


Figure 8 High-performance liquid chromatography chromatogram of the *Scolopendra subspinipes mutilans* at the length of 210 nm.

act *via* specific cell membrane-bound receptors. Levels of both these pro-inflammatory mediators are elevated on initiation of and during AP^[37,38]. Intrapancreatic TNF- α and IL-1 β can be detected 1 h after induction of AP, and the levels of these cytokines increase rapidly over the next 6 h^[37,38]. Recently, many studies have reported that both TNF- α and IL-1 β play an important role in AP^[7,39]. In our experimental model of pancreatitis, the serum levels of TNF- α and IL-1 β were elevated during AP. However, when mice were pre-treated with SSM water extract, this elevation of TNF- α and IL-1 β was inhibited (Figures 3 and 6).

In this study, we examined the role of HMGB-1 as a late inflammatory mediator in AP. Generally, HMGB-1, a DNA-binding intranuclear protein, is known to be a late activator in the inflammatory cascade^[10]. HMGB-1 has the capacity to induce cytokines and activate inflammatory cells when applied extracellularly^[10]. This implicates that HMGB-1 is a pro-inflammatory mediator. Recent investigations reported that serum HMGB-1 levels increase in patients with sepsis/endotoxemia^[40], hemorrhagic shock^[41], acute lung injury^[42], rheumatoid arthritis^[43], and disseminated intravascular coagulation^[44]. Similarly, many studies have shown the pivotal role of HMGB-1 plays in the development of pancreatic inflammation in AP^[45-48]. In this study, compared to saline pre-treatment, SSM pre-treatment significantly inhibited AP-induced HMGB-1 expression (Figures 5A and 6).

Oxidative stress and pro-inflammatory cytokines trigger common signal transduction pathways involved in the inflammatory cascade, particularly through activation of MAPK^[49]. We previously reported that the inhibition of MAPKs could inhibit the cytokine productions^[7,8]. In the present study, acinar cells with cerulein showed increased TNF- α and IL-1 β release *via* MAPKs and NF- κ B activation. However, SSM treatment inhibited activation of JNK, p38 and NF- κ B but not ERK1/2, consequently inhibiting cytokine release and HMGB-1 (Figures 6 and 7). In addition, we have shown here that cerulein-induced HMGB-1 expression was inhibited in pancreatic acinar cells by inhibition of JNK, p38 and NF- κ B activation

(Figure 7). These data suggest that SSM inhibits expression of HMGB-1 *via* inhibition of JNK, p38 and NF- κ B activation.

In conclusion, this study shows that SSM attenuates the severity of cerulein-induced AP and pancreatitis-associated lung injury through the inhibition of tissue injury, pro-inflammatory cytokine production, and HMGB-1 expression. Therefore, SSM exerts potent anti-inflammatory effects in AP and could be a beneficial agent in the AP and its pulmonary complications.

COMMENTS

Background

Acute pancreatitis (AP) is a serious, unpredictable clinical problem, whose pathophysiology remains poorly understood. Therefore, drugs and therapies need to be developed.

Research frontiers

Scolopendra subspinipes mutilans (SSM) is a venomous arthropod, which can be found throughout the world. SSM and its venom have been reported to exhibit many biochemical and physiological effects. In addition, SSM has been prescribed for the treatment of cardiovascular diseases in South Korea, China, and other Far Eastern Asian countries for several hundred years. However, the protective activities of SSM in cerulein-induced AP have not been examined to date. This study aimed to assess the protective effect of SSM in cerulein-induced AP.

Innovations and breakthroughs

Many studies have been tried to explore the possible candidate for treatment of acute pancreatitis (AP), but failed to find out. Nowad, the drug of AP is limited in protease inhibitors, and also the pathogenesis is not well-studied. In this paper, the authors studied the possible candidate to develop drug for AP, in line with their previous report. Also the authors provided the regulating mechanisms in AP. This finding could strengthen up the further studies of AP. Furthermore, this *in vivo* and *in vitro* studies would suggest that SSM could protect cerulein-induced AP *via* inhibiting high mobility group box chromosomal protein-1 release.

Applications

By understanding how SSM is effective in AP, these results could provide the clinical basis for development of drug or compound to treat AP and/or other inflammatory diseases.

Terminology

AP is a sudden inflammation of the pancreas. It can have severe complications and high mortality despite treatment. While mild cases are often successfully treated with conservative measures and aggressive intravenous fluid rehydration, severe cases may require admission to the intensive care unit or even surgery to deal with complications of the disease process.

Peer review

The role of SSM is mainly deleterious and also anti-inflammatory and antimicrobial, what in literature has support about the protective effect of SSM in AP. The study is designed reasonably and the methods and the results seem mostly proper to show interesting protective effect of SSM on AP.

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MDCT angiography to evaluate the therapeutic effect of PTVE for esophageal varices

Aitao Sun, Yong-Jun Shi, Zhuo-Dong Xu, Xiang-Guo Tian, Jin-Hua Hu, Guang-Chuan Wang, Chun-Qing Zhang

Aitao Sun, Yong-Jun Shi, Zhuo-Dong Xu, Xiang-Guo Tian, Jin-Hua Hu, Guang-Chuan Wang, Chun-Qing Zhang, Department of Gastroenterology, Provincial Hospital Affiliated with Shandong University, Jinan 250021, Shandong Province, China

Zhuo-Dong Xu, Department of Radiology, Provincial Hospital Affiliated with Shandong University, Jinan 250021, Shandong Province, China

Author contributions: Sun A and Shi YJ wrote the paper and contributed equally to this work; Zhang CQ designed the research; Zhang CQ, Xu ZD and Hu JH performed the procedures; Tian XG was responsible for the statistical work; Wang GH performed the clinical follow up.

Correspondence to: Chun-Qing Zhang, MD, Department of Gastroenterology, Provincial Hospital Affiliated to Shandong University, 324 Jingwu Weiqi Road, Jinan 250021, Shandong Province, China. zhchqing@medmail.com.cn

Telephone: +86-531-66953227 Fax: +86-531-87906348

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Abstract

AIM: To evaluate the role of multi-detector row computed tomography (MDCT) angiography for assessing the therapeutic effects of percutaneous transhepatic variceal embolization (PTVE) for esophageal varices (EVs).

METHODS: The subjects of this prospective study were 156 patients who underwent PTVE with cyanoacrylate for EVs. Patients were divided into three groups according to the filling range of cyanoacrylate in EVs and their feeding vessels: (1) group A, complete obliteration, with at least 3 cm of the lower EVs and peri-/EVs, as well as the adventitial plexus of the gastric cardia and fundus filled with cyanoacrylate; (2) group B, partial obliteration of varices surrounding the gastric cardia and fundus, with their feeding vessels being obliterated with cyanoacrylate, but without reaching lower EVs; and (3) group C, trunk obliteration, with

the main branch of the left gastric vein being filled with cyanoacrylate, but without reaching varices surrounding the gastric cardia or fundus. We performed chart reviews and a prospective follow-up using MDCT images, angiography, and gastrointestinal endoscopy.

RESULTS: The median follow-up period was 34 mo. The rate of eradication of varices for all patients was 56.4% (88/156) and the rate of relapse was 31.3% (41/131). The rates of variceal eradication at 1, 3, and 5 years after PTVE were 90.2%, 84.1% and 81.7%, respectively, for the complete group; 61.2%, 49% and 42.9%, respectively, for the partial group; with no varices disappearing in the trunk group. The relapse-free rates at 1, 3 and 5 years after PTVE were 91.5%, 86.6% and 81.7%, respectively, for the complete group; 71.1%, 55.6% and 51.1%, respectively, for the partial group; and all EVs recurred in the trunk group. Kaplan-Meier analysis showed *P* values of 0.000 and 0.000, and odds ratios of 3.824 and 3.603 for the rates of variceal eradication and relapse free rates, respectively. Cyanoacrylate in EVs disappeared with time, but those in the EVs and other feeding vessels remained permanently in the vessels without a decrease with time, which is important for the continued obliteration of the feeding vessels and prevention of EV relapse.

CONCLUSION: MDCT provides excellent visualization of cyanoacrylate obliteration in EV and their feeding veins after PTVE. It confirms that PTVE is effective for treating EVs.

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Key words: Multi-detector row computed tomography; Percutaneous transhepatic variceal embolization; Cyanoacrylate; Esophageal varices; Therapeutic effect

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INTRODUCTION

Percutaneous transhepatic variceal embolization (PTVE) was introduced in the 1970s to manage esophageal varices (EVs)^[1,2], but this approach has not attained widespread clinical acceptance because of the associated high rebleeding rate^[3-5]. PTVE with cyanoacrylate is a modified procedure for conventional percutaneous transhepatic obliteration^[6-12]. Since both the varices and their feeding vessels are sufficiently and permanently obliterated by cyanoacrylate, this modified PTVE technique has been confirmed as an effective and safe method for preventing EV rebleeding^[11,12]. Multi-detector row computed tomography (MDCT) angiography has significantly improved our understanding of the complex vascular structural changes that occur during portal hypertension^[13-18], along with their clinical and prognostic significance^[19-24]. This technique enables the noninvasive study of the hemodynamic changes in the portal venous system^[14] and plays an important role in the treatment and follow-up of esophageal and gastric varices^[19,24].

The purpose of this study was to assess the relationship between hemodynamic changes in portosystemic collaterals and the long-term results of modified PTVE with cyanoacrylate for patients with EVs using multiplanar reformatted image MDCT imaging.

MATERIALS AND METHODS

Patients

This was a retrospective-prospective follow-up study to evaluate the long-term results of modified PTVE with cyanoacrylate for treating variceal bleeding. Patients with cirrhosis who had a history of EVs bleeding within 6 mo between January 2005 and December 2011 and who underwent PTVE were selected. A chart review of all patients was performed. We recorded demographic and clinical information, including type of underlying liver disease, liver function tests, and renal function prior to and after PTVE. Liver function was classified according to the Child-Pugh classification. PTVE was performed as a rescue maneuver in all patients, including patients who had uncontrolled severe bleeding during endoscopic therapy or those who had recurrent bleeding episodes from EVs during or after band ligation. All patients underwent diagnostic endoscopy before the intervention to confirm the presence of EV as the bleeding source. This study was approved by the local ethics committee and informed written consent was obtained from each patient.

The inclusion criteria were as follows: (1) cirrhosis confirmed by laboratory and imaging examinations; (2) preoperative gastroscopy showing mild to severe EVs and gastric fundal varices; (3) acute bleeding because of ruptured varices or a history of rupture and bleeding

within the last 3 mo; (4) surgery not indicated or preferred; and (5) the patient and their family agreeing to complete preoperative examinations and postoperative follow-ups.

The exclusion criteria were as follows: (1) concomitant liver cancer or other cancers; (2) concomitant widespread portal vein embolism or severe portal vein cavernous transformation; (3) phase II or higher hepatic encephalopathy; (4) obvious jaundice with total bilirubin levels three times higher than normal; (5) obvious bleeding tendency with a prothrombin time > 25 s; (6) severe hypertension, coronary heart disease, or cardiopulmonary insufficiency; and (7) concomitant hepatorenal syndrome or chronic renal insufficiency.

Computed tomography scanning and three-dimensional computed tomography portogram

MDCT images were used to evaluate EVs and their feeding vessels before and after the procedures. Computed tomography (CT) was performed *via* a 64-MDCT scanner (Sensation, Siemens Healthcare, Erlangen, Germany) during the unenhanced, arterial, and portal venous phases. A bolus-tracking technique was applied with a trigger threshold at the upper abdominal aorta of 150 Hounsfield units, with a delay time of 10 s for the arterial phase and an additional 30 s after the first acquisition for the portal venous phase. Iohexol 350 (100-150 mL) was administered at a dose calculated according to patient weight at a rate of 3 mL/s. MDCT was performed with Virtual Place Advance (AZE Ltd., Irvine CA, United States). Three reformatting techniques were used for image reconstruction in this study.

Image analysis evaluation of EVs and portosystemic collaterals

Imaging studies were independently reviewed by two abdominal imaging specialists with fellowship-level training and 5 years of experience as attending radiologists. Said reviewers were blinded to all other clinical and imaging information. EVs and peri-/para-EVs, as well as the adventitial plexus of the gastric cardia and fundus, could be detected by cross-sectional CT scanning during the unenhanced and portal venous phases. After PTVE, the varices adequately embolized with cyanoacrylate to show hyperdensity without enhancement on post-procedural images, whereas isodensity was observed on pre-contrast images in patients with inadequate eradication of the vessels, and with no enhancement being shown on post-contrast images. Portosystemic collaterals were independently assessed on MDCT images before and after PTVE with cyanoacrylate. The supplying vessels, including the left gastric vein (LGV), posterior gastric vein (PGV), and paraesophageal vein were identified on the three-dimensional CT portogram.

According to the findings of CT images, patients were divided into three groups on the basis of the filling range of cyanoacrylate in EVs and their feeding vessels: (1) group A, complete obliteration, with at least 3 cm of

Table 1 Clinical characteristics of patients included in the study

| Characteristics | Value (n = 156) |
|--|-----------------------|
| Gender: Male/female | 91/65 |
| Age range, yr (mean \pm SD) | 35-74 (53 \pm 13.6) |
| Cause: Hepatitis B/hepatitis C/ alcoholic liver disease/other | 109/6/25/16 |
| Child-Pugh classification: A/B/C | 41/79/36 |
| Variceal size: F2/F3 | 31/125 |
| Portal hypertensive gastropathy (yes/no) | 45/111 |
| Encephalopathy: (II stage/ I stage/no) | 23/56/77 |
| Ascites (yes/no) | 62/94 |
| Laboratory findings | |
| Leukocytes ($\times 10^9/L$) | 4.6 \pm 2.1 |
| Hemoglobin (g/L) | 83.7 \pm 18.8 |
| Platelets ($\times 10^9/L$) | 94.9 \pm 53.2 |
| ALT (U/L) | 88.2 \pm 34.5 |
| Albumin (g/L) | 32.1 \pm 12.4 |
| Bilirubin ($\mu\text{mol/L}$) | 29.3 \pm 18.1 |
| Prothrombin time (s) | 16.4 \pm 3.8 |
| Creatinine ($\mu\text{mol/L}$) | 72.7 \pm 23.8 |

ALT: Alanine aminotransferase.

the lower EVs and peri-/para-EVs, as well as the adventitial plexus of the gastric cardia and fundus filled with cyanoacrylate; (2) group B, partial obliteration, with the varices surrounding the gastric cardia, fundus, and their feeding vessels being obliterated with cyanoacrylate, but without reaching the lower EVs; and (3) group C, trunk obliteration, with the main branch of the LGV being filled with cyanoacrylate, but without reaching the varices surrounding the gastric cardia or fundus.

Follow-up study

All subjects included in this study were followed up at regular intervals of 1, 3 and 6 mo after the procedures, and then every 6-12 mo in a specialized clinic under coordination by a research nurse. Patients underwent a brief recording of medical history at each visit, including possible gastrointestinal bleeding, alcohol intake, concurrent medications, and patient complaints. A brief physical examination, including estimation of ascites and HE by routine neurological examination and laboratory profiling, was performed. Gastrointestinal endoscopy and CT portal venography were performed every 6-12 mo to evaluate EVs and their feeding veins.

Relapse after PTVE was assessed by endoscopy. Follow-up endoscopy was performed at 6 mo intervals after treatment. EVs were evaluated independently during endoscopy by two experienced endoscopists. The endoscopic EVs findings were evaluated according to the classification system of the Japanese Society for Portal Hypertension and EVs^[25]. A final decision regarding the endoscopic findings was reached by consensus. The appearance of the localized red color sign F1 or bleeding on follow-up endoscopy was regarded as a relapse of EVs. In this prospective study, we defined relapse of EVs as the primary endpoint, and survival as the secondary endpoint. The relationship between the cyanoac-

rylate filling range in the vessels and prognosis of PTVE for EVs was determined by the MDCT imaging results.

Modified PTVE procedure

The PTVE procedure was performed under radiological guidance as described previously. In brief, after transhepatic puncture of the intrahepatic portal vein branch under ultrasonographic or fluoroscopic guidance, a 5F Cobra catheter was introduced into the splenic vein, and splenoportography was performed to evaluate the index varices as well as the feeding vessels and draining veins. The main feeding vessel (*e.g.*, left, short or PGV) was selected with the 5F cobra catheter, and cyanoacrylate was injected into all varices in the lower esophagus and gastric fundus, as well as into all feeding vessels. Next, splenoportography was repeated to assess the extent of varix obliteration. If other feeding vessels were detected, the procedure was repeated until blood flow in the varices completely ceased. The lower EVs, peri-EVs, and/or the gastric cardiac submucosal and perforating vessels were sufficiently obliterated with cyanoacrylate. Finally, the 5F sheath system was withdrawn, and the puncture tract was embolized with microcoils.

Statistical analysis

All data are expressed as mean \pm SD or median values. The cumulative relapse-free rate and cumulative survival rate among the groups based on the reduction rate after treatment were determined using the Kaplan-Meier method and JMP version 5 statistical software (SAS Institute, Tokyo, Japan). Significance was tested using the generalized Wilcoxon signed-rank test and Student's *t*-test. A *P* value < 0.05 was regarded as significant.

RESULTS

Demographics

A total of 181 patients underwent PTVE during the study period. Fifteen patients with hepatocellular carcinoma and five cases of technical failures were excluded. Five cases were lost in follow-up. Thus, the final study population consisted of 156 patients (Figure 1). The clinical characteristics of the patients in the groups, including age, gender, etiology of cirrhosis, severity of liver disease, and size of EVs, are shown in Table 1.

CT detection of EVs and their feeding vessels

(1) All EVs were detected in all patients by MDCT angiography during the portal venous phase, and ninety-seven of the 156 patients had paraesophageal varices. According to the MDCT angiography findings before PTVE, 86 of the patients had a LGV alone (Figure 2A), 46 had a LGV plus a PGV (Figure 2B), and 37 patients had a LGV plus a PGV and a short gastric vein (SGV) (Figure 2C). The supplying portosystemic collaterals were further confirmed according to the findings on DSA following the PTVE procedures; and (2) MDCT scanning and angiography during the portal venous phase were

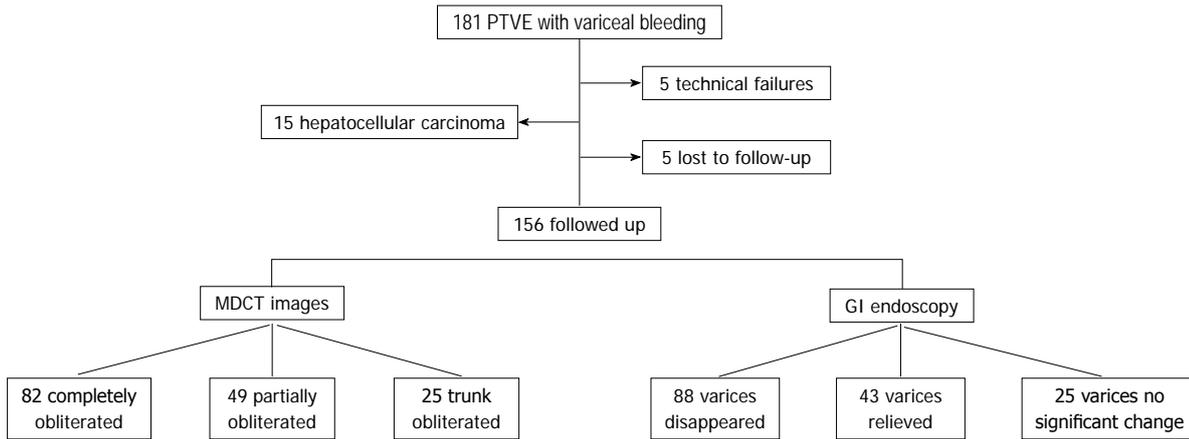


Figure 1 Study flow chart. PTVE: Percutaneous transhepatic variceal embolization; MDCT: Multi-detector row computed tomography; GI: Gastrointestinal.

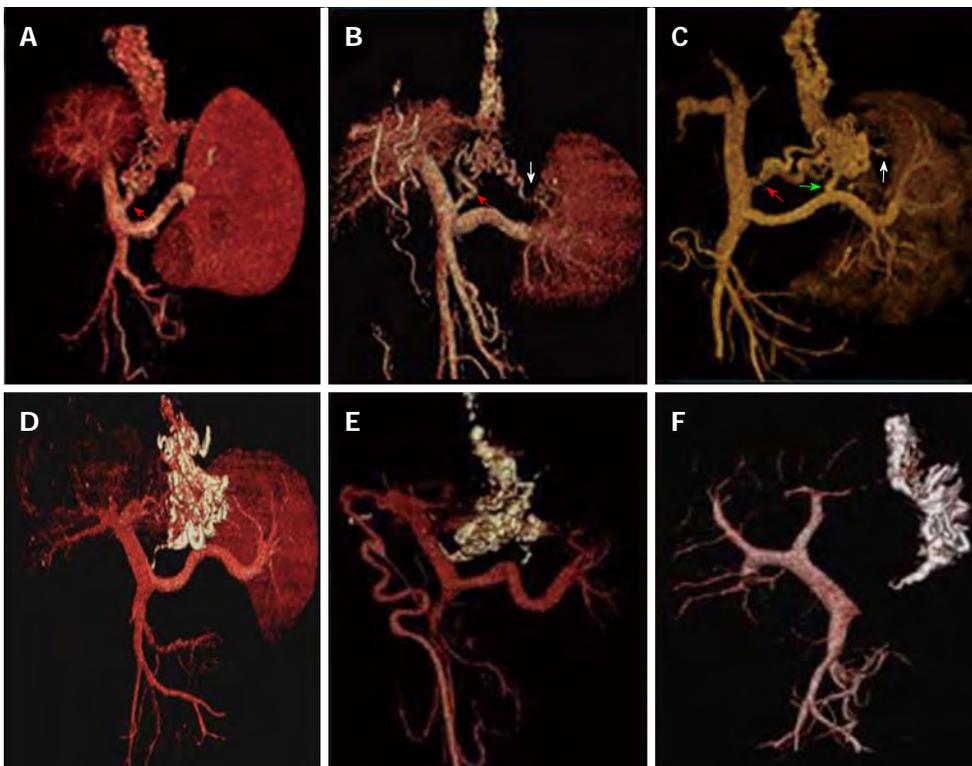


Figure 2 Volume rendering of the multi-detector row computed tomography angiography data set during the portal venous phase demonstrated the esophageal-gastric varices and their afferent vessels before and after percutaneous transhepatic variceal embolization. A-C: Volume rendering of multi-detector row computed tomography angiography data set during the portal venous phase demonstrates the esophageal-gastric varices and their afferent vessels (left gastric vein (LGV) only for A, LGV and short gastric vein (SGV) for B, LGV, posterior gastric vein and SGV for C); D-F: All varices and their feeding vessels are completely filled with cyanoacrylate (arrows) after percutaneous transhepatic variceal embolization; no flow signals were revealed in any of the cases.

used to evaluate EVs and their collaterals after the procedures. Figures 2 and 3 show the three typical groups of obliteration with cyanoacrylate according to coronal thin slab maximum intensity projection of MDCT angiography follow-up. Group A: complete obliteration, the lower EVs and peri-/para-EVs, as well as the adventitial plexus of the gastric cardia and fundus, were completely filled with cyanoacrylate; Group B: partial obliteration, the varices surrounding the gastric cardia and fundus and their feeding vessels were obliterated with cyanoacrylate, but cyanoacrylate did not obliterate the lower EVs; Group

C: trunk obliteration, the main branch of the LGV was filled with cyanoacrylate, but it did not reach the varices surrounding the gastric cardia or fundus. Volume rendering of the MDCT angiography data set during the portal venous phase demonstrated that the esophageal-gastric varices and afferent vessels were completely filled with cyanoacrylate, but without flow signal in the varices (Figure 2D-F). Of the 156 patients, 82 achieved complete obliteration, 49 achieved partial obliteration, and 25 achieved trunk obliteration.

During the follow-up, CT images revealed dynamic

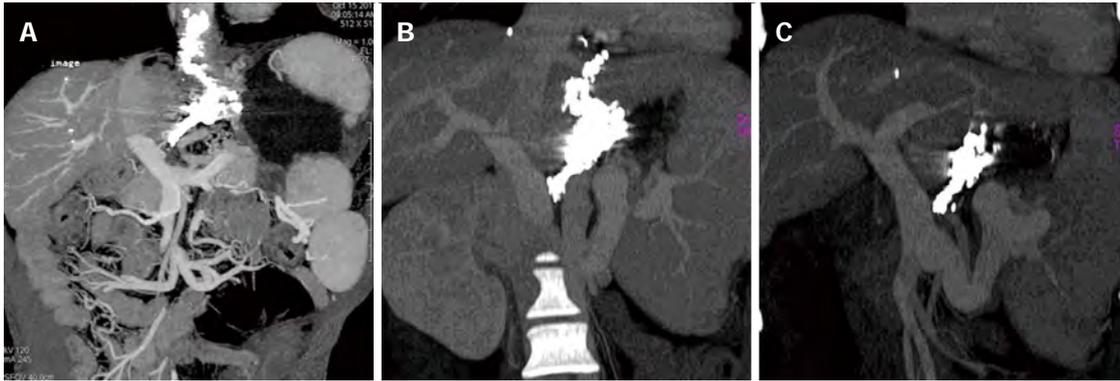


Figure 3 Coronal thin-slab maximum intensity projection of multi-detector row computed tomography angiography during the portal venous phase demonstrates the three different obliteration types of cyanoacrylate. A: Complete obliteration: with the lower esophageal varices (EVs) and peri-/para-EVs, as well as the adventitial plexus of the gastric cardia and fundus, filled with cyanoacrylate; B: Partial obliteration: along with the left gastric vein (LGV) and its main branches being obliterated with cyanoacrylate, the varices surrounding the gastric cardia and fundus are also obliterated, but without reaching the lower EVs; C: Trunk obliteration: cyanoacrylate mainly obliterates the LGV and its main branches outside of the gastric wall.

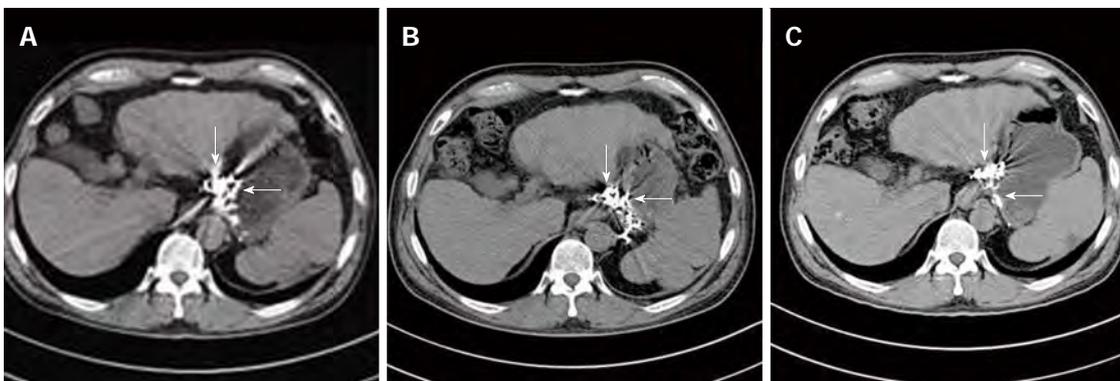


Figure 4 Computed tomography image follow-up revealed the different outcome of cyanoacrylate in the different veins: cyanoacrylate in submucosal varices (within the wall of the fundus, large arrow) disappeared with time, but those in the para- and peri-gastric varices (outside the wall of the fundus, small arrow) remained permanently in the vessels without a time-dependent decrease. A: The cyanoacrylate in both the gastric varices and peri- and para-gastric varices stayed full at 3 mo after percutaneous transhepatic variceal embolization (PTVE); B: The cyanoacrylate in the gastric varices was reduced at 6 mo after PTVE; C: The cyanoacrylate in the gastric varices nearly disappeared at 12 mo after PTVE, but the cyanoacrylate in the peri- and para-gastric vessels retained the same as before.

changes and outcome of cyanoacrylate injection in different veins. Cyanoacrylate in esophageal and gastric varices gradually reduced with time, but that in the para- and peri-varices was retained permanently in the vessels without a time-dependent decrease, which is important to continue the obliteration of the feeding vessels and prevent the relapse of esophageal-gastric varices (Figure 4).

Cumulative relapse-free rates

The median follow-up period was 34 mo, ranged from 6 mo to 71 mo. The rate of eradication of varices for all patients was 56.4% (88/156), and the rate of relapse was 31.3% (41/131). The rates of variceal eradication at 1, 3, and 5 years after PTVE were 90.2%, 84.1% and 81.7%, respectively, for the complete group; 61.2%, 49% and 42.9%, respectively, for the partial group; and with no varices disappearing in the trunk group (Figure 5A). The relapse free rates at 1, 3 and 5 years after PTVE were 91.5%, 86.6% and 81.7%, respectively, for the complete group; 71.1%, 55.6% and 51.1%, respectively, for the par-

tial group; and all EVs recurring in the trunk group (Figure 5B). Kaplan-Meier analysis showed a $P = 0.000$ and 0.000 and odds ratios (OR) = 3.824 and 3.603 for rates of variceal eradication and relapse free rates, respectively (Tables 2 and 3).

Rebleeding

Thirty-six of the 156 patients experienced upper gastrointestinal rebleeding during the follow-up period. The overall bleeding rate was 23.1% (36/156). Seven patients (8.5%, 7/82) in the complete obliteration group, 11 patients (22.4%, 11/49) in the partial obliteration group, and 18 patients (72%, 18/25) in the trunk obliteration group experienced rebleeding. The different rebleeding rates among the groups was statistically significant (Kaplan-Meier values: $P = 0.000$; OR, 3.560; Figure 5A). Portal hypertensive gastropathy was the main cause of bleeding in the complete obliteration group (six cases, the remaining one was bleeding from a peptic ulcer). Esophagogastric variceal rupture was the main cause of bleeding in the trunk obliteration group (12 cases,

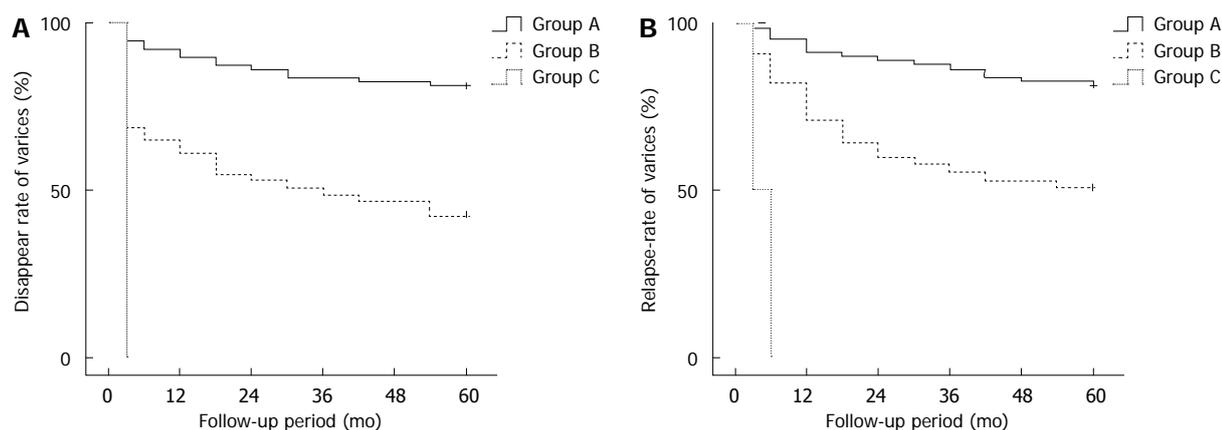


Figure 5 Kaplan-Meier analysis of the disappearance rate (A) and non-relapse rate (B) of varices shown at gastroscopy during the follow-up. Group A: Complete obliteration; Group B: Partial obliteration; Group C: Trunk obliteration.

Table 2 Single- and multiple-factor analysis of relative re-bleeding rate factors after modified percutaneous transhepatic variceal embolization

| Variable | Univariate | Multivariate | |
|--|------------|-----------------------|---------|
| | P value | Hazard ratio (95%CI) | P value |
| Gender (male vs female) | 0.591 | - | NS |
| Age, yr (≥ 53 vs < 53) | 0.966 | - | NS |
| Child-Pugh classification C vs (A + B) | 0.031 | 1.730 (1.1394-2.6279) | 0.010 |
| Cause: Hepatitis B + hepatitis C vs alcoholic liver disease vs other | 0.557 | - | NS |
| Obliteration type | 0.000 | 3.279 (2.132-5.042) | 0.000 |
| Variceal grade, severe | 0.909 | - | NS |
| Portal hypertensive gastropathy (yes vs no) | 0.036 | 2.525 (0.898-7.097) | 0.075 |
| Encephalopathy (yes vs no) | 0.065 | - | NS |
| Ascites (yes vs no) | 0.024 | 0.702 (0.252-1.958) | 0.217 |
| Laboratory findings | | | |
| Leukocytes ($\times 10^9/L$) (≥ 4.6 vs < 4.6) | 0.726 | - | NS |
| Hemoglobin (g/L) (< 83.7 vs ≥ 83.7) | 0.103 | - | NS |
| Platelets ($\times 10^9/L$) (< 94.9 vs ≥ 94.9) | 0.437 | - | NS |
| ALT (U/L) (< 88.2 vs ≥ 88.2) | 0.649 | - | NS |
| Albumin (g/L) (< 32.1 vs ≥ 32.1) | 0.138 | - | NS |
| Bilirubin ($\mu\text{mol/L}$) (< 29.3 vs ≥ 29.3) | 0.240 | - | NS |
| Prothrombin time(s) (≥ 16.4 vs < 16.4) | 0.176 | - | NS |
| Creatinine ($\mu\text{mol/L}$) (≥ 72.7 vs < 72.7) | 0.385 | - | NS |

ALT: Alanine transaminase; NS: Not significant.

the others were five cases of peptic ulcer bleeding and one case of portal hypertensive gastropathy); four cases of variceal rupture occurred in the partial obliteration group (two due to portal hypertensive gastropathy, and three from a peptic ulcer).

Survival rates

Thirty-eight patients died during the follow-up period; 14 patients (17%, 14/82) in the complete obliteration group died. Of these, three died of gastrointestinal tract bleeding, eight of hepatic failure, and three of primary liver cancer. Fifteen patients (30.6%, 15/49) in the par-

Table 3 Single- and multiple-factor analysis of relative factors of survival rate after modified percutaneous transhepatic variceal embolization

| Variable | Univariate | Multivariate | |
|--|------------|----------------------|---------|
| | P value | Hazard ratio (95%CI) | P value |
| Gender (male vs female) | 0.256 | - | NS |
| Age, yr (≥ 53 vs < 53) | 0.715 | - | NS |
| Child-Pugh classification C vs (A + B) | 0.015 | 1.827 (1.184-2.819) | 0.006 |
| Cause: Hepatitis B + hepatitis C vs alcoholic liver disease vs other | 0.654 | - | NS |
| Obliteration type | 0.020 | 2.535 (1.779-3.613) | 0.000 |
| Variceal grade, severe | 0.721 | - | NS |
| Portal hypertensive gastropathy (yes vs no) | 0.249 | - | NS |
| Encephalopathy (yes vs no) | 0.041 | 3.240 (0.725-6.874) | 0.082 |
| Ascites (yes vs no) | 0.087 | - | NS |
| Laboratory findings | | | |
| Leukocytes ($\times 10^9/L$) (≥ 4.6 vs < 4.6) | 0.759 | - | NS |
| Hemoglobin (g/L) (< 83.7 vs ≥ 83.7) | 0.073 | - | NS |
| Platelets ($\times 10^9/L$) (< 94.9 vs ≥ 94.9) | 0.641 | - | NS |
| ALT (U/L) (< 88.2 vs ≥ 88.2) | 0.528 | - | NS |
| Albumin (g/L) (< 32.1 vs ≥ 32.1) | 0.193 | - | NS |
| Bilirubin ($\mu\text{mol/L}$) (< 29.3 vs ≥ 29.3) | 0.282 | - | NS |
| Prothrombin time (s) (≥ 16.4 vs < 16.4) | 0.149 | - | NS |
| Creatinine ($\mu\text{mol/L}$) (≥ 72.7 vs < 72.7) | 0.591 | - | NS |

ALT: Alanine transaminase; NS: Not significant.

tial obliteration group died. Of these, four died of gastrointestinal tract bleeding, six from hepatic failure, and two from primary liver cancer. Nineteen patients (76%, 19/25) in the trunk obliteration group died. Of these, 10 died of gastrointestinal tract bleeding, five from hepatic failure, and four from primary liver cancer. The overall 5-year mortality rate after the operation was 30.8% (48/156). The differences in the mortality rates among the groups were statistically significant (Kaplan-Meier values, $P = 0.02$; OR, 2.822; Figure 6B). A Cox regression analysis was performed for factors that might influence the rebleeding and survival rates after the operation

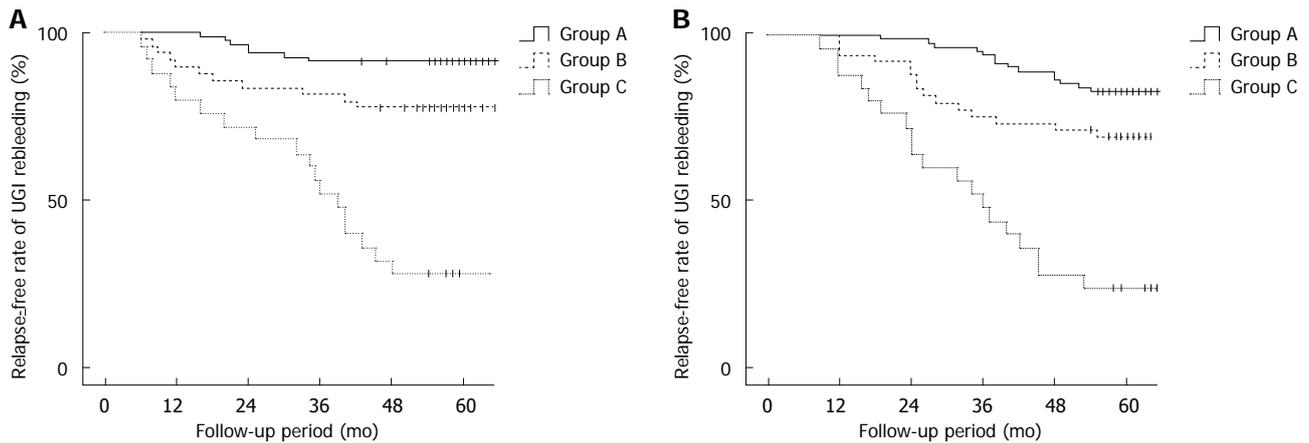


Figure 6 Kaplan-Meier analysis of non-bleeding (A) and survival rates (B) among the different obliteration types after modified percutaneous transhepatic variceal embolization. Group A: Complete obliteration; Group B: Partial obliteration; Group C: trunk obliteration.

(type of obliteration, gender, age, Child-Pugh classification, cause of cirrhosis, severity of varices, hepatic encephalopathy, ascites, and laboratory data) and showed that the range of obliteration and Child-Pugh grade were independent predictors of rebleeding and survival rates after the embolism operation (Tables 2 and 3).

DISCUSSION

MDCT has recently become more useful for examining EVs and the entire portosystemic shunt^[14,26]. MDCT allows for visualization of abnormal vessels of the intrinsic circulation, such as peri-EVs that are attached to the muscularis externa of the esophagus and para-EVs localized to the surrounding tissue^[14,27]. MDCT also allows for the detection of perigastric and paragastric varices^[23,28].

EVs are located within the wall of the lower esophagus, whereas para-esophageal veins are situated outside the wall of the lower esophagus. These vessels are supplied primarily by the LGV, which divides into anterior and posterior branches. The anterior branch supplies EVs, and the posterior branch forms the paraesophageal veins. EVs are usually connected with para-EVs in the distal esophagus^[29-31]. Previous studies have shown that large paraesophageal collateral veins (> 5 mm) are associated with high rates of variceal recurrence and rebleeding after banding^[32-34], and the increased risk of bleeding also seems to be associated with large perforating veins^[29,35].

With regard to the vascular anatomy of the lower esophagus and upper stomach in patients with EVs, it is important to achieve not only obliteration of EVs, but also embolization of the feeding vessels supplied by the portal venous system. We have a long-lasting interest in applying PTVE with cyanoacrylate to sufficiently obliterate the entire lower esophageal and peri-/para-EVs, and the adventitial plexus of the gastric cardia and fundus, to achieve this purpose^[11,12].

In this study we used multi-slice helical CT and three-dimensional portography to evaluate the hemodynamic

changes in EVs and their collaterals after PTVE with cyanoacrylate. Patients were divided into three groups on the basis of the filling range of cyanoacrylate filling in EVs and their feeding vessels: (1) Group A, complete obliteration: at least 3 cm lower than EVs and peri-/para-EVs, with the adventitial plexus of the gastric cardia and fundus being filled with cyanoacrylate; (2) Group B, partial obliteration: the varices surrounding the gastric cardia and fundus and their feeding vessels being obliterated with cyanoacrylate, but not reaching the lower EVs; and (3) Group C, trunk obliteration: the tissue adhesive filling the main branch of the LGV, but not reaching the varices surrounding the gastric cardia or fundus.

Our results demonstrated the relationship between MDCT image-based embolization range and the prognosis of patients with EVs after PTVE. Of the 156 patients who underwent PTVE with cyanoacrylate for EVs, 82 had their varices completely obliterated; relapse and rebleeding of varices were very low in this group [13.4% (11/82) and 8.5% (7/82), respectively]. However, the relapse rate and rebleeding rate of varices was significantly higher in patients with inadequate embolization of the varices and the feeding vessels than in those with adequate embolization. Multivariate analyses showed that the cyanoacrylate embolization range was an independent risk factor for rebleeding and the relapse of EVs. Thus, a close relationship was observed between the range of EV obstruction, their feeding veins, and the recurrence of EVs. These results demonstrate that it is important to achieve not only obliteration of the feeding vessels, but also embolization of EVs. This result may also explain why conventional PTVE does not achieve long-term prevention of variceal rebleeding, as it focuses mainly on the embolization of feeding veins using a coil or gelatin sponge.

In this study, we first reported the different changes and regression of cyanoacrylate in different veins. Cyanoacrylate in esophageal and gastric varices (vessels within the wall) gradually reduced with time, but the cyanoacrylate in the para- and peri- varices (vessels outside the wall) was permanently retained in the vessels without

a time-dependent decrease. This is important in order to continue obliterating the feeding vessels and prevent the relapse of esophageal-gastric varices, as the para- and peri- varices (vessels outside the wall) are the feeding vessels for EVs.

In summary, standard liver MDCT has significantly improved our understanding of the complex vascular structural changes that occur during portal hypertension, and MDCT provides excellent visualization of glue obliteration in EVs and their feeding veins. The results of our study emphasize the importance of sufficient eradication of EVs and their feeding vessels.

ACKNOWLEDGMENTS

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COMMENTS

Background

Bleeding from esophageal varices (EVs) is a serious complication of portal hypertension and a leading cause of death in patients with liver cirrhosis. Recurrent variceal bleeding is a challenging problem in patients with advanced cirrhosis.

Research frontiers

Percutaneous transhepatic variceal embolization (PTVE) with cyanoacrylate, a modified procedure for conventional percutaneous transhepatic obliteration, has been confirmed as an effective and safe method for preventing EVs rebleeding. Multi-detector row computed tomography (MDCT) has recently become more useful for examining EVs and the entire portosystemic shunt.

Innovations and breakthroughs

PTVE is effective for treating EVs, and the sufficient eradication of EVs and their feeding vessels is important in treating varices. MDCT has significantly improved our understanding of the complex vascular structural changes that occur during portal hypertension, and provides excellent visualization of cyanoacrylate obliteration in EVs and their feeding veins.

Applications

With the extensive and permanent obliteration of both EVs and their feeding veins, PTVE with cyanoacrylate is a prospective modality for the treatment of esophageal varices. MDCT is effective in evaluating the range of cyanoacrylate obliteration in EV and their feeding veins after PTVE.

Peer review

In the present study, instead of using EV embolization, PTVE for the eradication of varices feeding veins was instead attempted. The outcomes are encouraging: with clear data showing that PTVE could be an effective method. The MDCT images verified the importance of sufficient eradication of the feeding vessels for the long-term effect of PTVE with cyanoacrylate. The application of this modified technique in the presented cohort with EVs provides sufficient evidence for drawing conclusions. These findings were interesting, and contributed new data to the various effective EV treatment modalities.

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Mitofusin-2 ameliorates high-fat diet-induced insulin resistance in liver of rats

Ke-Xin Gan, Chao Wang, Jin-Hu Chen, Chun-Jing Zhu, Guang-Yao Song

Ke-Xin Gan, Chun-Jing Zhu, Department of Internal Medicine, Hebei Medical University, Shijiazhuang 050017, Hebei Province, China

Chao Wang, Jin-Hu Chen, Department of Endocrinology, the General Hospital of Hebei Province, Shijiazhuang 050051, Hebei Province, China

Guang-Yao Song, Department of Internal Medicine, Hebei Medical University, Shijiazhuang 050017, Hebei Province, China
Author contributions: Song GY, Wang C, Chen JH, Gan KX and Zhu CJ designed the research; Gan KX and Zhu CJ performed the research and analyzed the data; Gan KX wrote the paper; all authors have read and approved the final manuscript.

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Correspondence to: Guang-Yao Song, Professor, Department of Internal Medicine, Hebei Medical University, 361 East Zhongshan Road, Shijiazhuang 050017, Hebei Province, China. sguangyao@sohu.com

Telephone: +86-311-85988267 Fax: +86-311-85988318

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Abstract

AIM: To investigate the effects of mitofusin-2 (MFN2) on insulin sensitivity and its potential targets in the liver of rats fed with a high-fat diet (HFD).

METHODS: Rats were fed with a control or HFD for 4 or 8 wk, and were then infected with a control or an MFN2 expressing adenovirus once a week for 3 wk starting from the 9th wk. Blood glucose (BG), plasma insulin and insulin sensitivity of rats were determined at end of the 4th and 8th wk, and after treatment with different amounts of MFN2 expressing adenovirus (10^8 , 10^9 or 10^{10} vp/kg body weight). BG levels were measured by Accu-chek Active Meter. Plasma insulin levels were analyzed by using a Rat insulin enzyme-

linked immunosorbent assay kit. Insulin resistance was evaluated by measuring the glucose infusion rate (GIR) using a hyperinsulinemic euglycemic clamp technique. The expression or phosphorylation levels of MFN2 and essential molecules in the insulin signaling pathway, such as insulin receptor (INSR), insulin receptor substrate 2 (IRS2), phosphoinositide-3-kinase (PI3K), protein kinase beta (AKT2) and glucose transporter type 2 (GLUT2) was assayed by quantitative real-time polymerase chain reaction and Western-blotting.

RESULTS: After the end of 8 wk, the body weight of rats receiving the normal control diet (ND) and the HFD was not significantly different ($P > 0.05$). Compared with the ND group, GIR in the HFD group was significantly decreased ($P < 0.01$), while the levels of BG, triglycerides (TG), total cholesterol (TC) and insulin in the HFD group were significantly higher than those in the ND group ($P < 0.05$). Expression of MFN2 mRNA and protein in liver of rats was significantly down-regulated in the HFD group ($P < 0.01$) after 8 wk of HFD feeding. The expression of INSR, IRS2 and GLUT2 were down-regulated markedly ($P < 0.01$). Although there were no changes in PI3K-P85 and AKT2 expression, their phosphorylation levels were decreased significantly ($P < 0.01$). After intervention with MFN2 expressing adenovirus for 3 wk, the expression of MFN2 mRNA and protein levels were up-regulated ($P < 0.01$). There was no difference in body weight of rats between the groups. The levels of BG, TG, TC and insulin in rats were lower than those in the Ad group ($P < 0.05$), but GIR in rats infected with Ad-MFN2 was significantly increased ($P < 0.01$), compared with the Ad group. The expression of INSR, IRS2 and GLUT2 was increased, while phosphorylation levels of PI3K-P85 and AKT2 were increased ($P < 0.01$), compared with the Ad group.

CONCLUSION: HFDs induce insulin resistance, and this can be reversed by MFN2 over-expression targeting the insulin signaling pathway.

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Key words: Mitofusin-2; High-fat diet; Insulin resistance; Insulin pathway; Liver

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INTRODUCTION

With obesity and diabetes reaching epidemic proportions worldwide, the role of insulin resistance and its consequences are attracting much attention. Insulin resistance is a critical mechanism of type 2 diabetes mellitus (T2DM) and of predisposing conditions for T2DM such as obesity and metabolic syndrome^[1]. Chronic excess energy consumption has been shown to contribute to hyperinsulinemia and insulin resistance. The liver plays a critical role in energy metabolism and is a major insulin target organ responsible for glucose homeostasis^[2]. In the liver, insulin acts through a complex signaling network and functions as an important regulator of carbohydrate and lipid homeostasis. Deficiency in insulin signaling may cause insulin resistance and subsequently lead to systemic insulin resistance and T2DM^[3,4]. Specific members of the suppressor of cytokine signaling (SOCS) family of proteins are now thought to play a role in the development of insulin resistance owing to their ability to inhibit insulin signaling pathways. Work with hepatocyte-specific suppressor of cytokine signaling 3 (SOCS3)-deficient (L-SOCS3 cKO) mice, reveals that hepatic SOCS3 is a mediator of insulin resistance in the liver^[5]. Therefore, it is of significance to identify the mechanism of insulin resistance and improve the function of the insulin signaling network for the cure of insulin resistance.

Mitochondria generate energy and play central roles in cell energy metabolism^[6]. Studies have shown that insulin resistance states such as T2DM or obesity were correlated with a decrease in mitochondria number and function^[7,8]. Fusion of mitochondria constitutes an important step in the regulation of mitochondrial morphology and function^[9,10]. Mitofusin-2 (MFN2) encodes a mitochondrial membrane GTPase which participates in mitochondrial fusion and contributes to the maintenance and operation of the mitochondrial network^[11]. MFN2 plays a central role in mitochondrial metabolism and may be associated with metabolic diseases such as obesity and T2DM^[12-14].

Previous studies revealed that a high-fat diet (HFD) induced insulin resistance^[15] and MFN2 could play a important role in development of insulin resistance^[12,16,17]. However, the role of MFN2, a key factor for mitochondrial function and energy metabolism, in liver insulin resistance and the insulin signaling pathway should be further elucidated. In this study, we established an HFD-in-

duced insulin resistance model in rats and used an MFN2 expressing adenovirus to investigate the mechanism by which MFN2 ameliorates HFD-induced insulin resistance.

MATERIALS AND METHODS

Animal care and grouping

Male Wistar rats about 60-80 g (4 wk old) were housed in wire bottom cages to prevent coprophagia. The environment was controlled in terms of light (12:12-h light-dark cycle starting at 6:00 AM), humidity, and room temperature (20-23 °C). Except for pretest overnight fasting and the immediate postoperative period, animals had free access to water and chow. The experimental protocols were approved by the Institutional Animal Care and Use Committee of the Centre for Gerontology and Geriatrics of Hebei Province in China.

Seven days after their arrival, rats were randomly divided into 9 groups ($n = 6$), as is schematically represented in Figure 1. In brief, rats were fed with a normal control diet (ND) or an HFD for 4 or 8 wk, and then some groups were infected with Ad-MFN2 or empty Ad adenovirus or PBS control once a week, for 3 wk. The HFD consisted of 59.8% fat, 20.1% protein, and 20.1% carbohydrate (kcal). The normal rodent chow diet contained 10.3% fat, 24.2% protein, and 65.5% carbohydrate (kcal). The MFN2 expressing adenovirus Ad-MFN2 and the empty control adenovirus Ad were obtained from Dr. Zhang, Hebei Medical University^[18].

Blood samples were collected from the abdominal aorta. Blood glucose (BG) levels were measured by Accu-chek Active Meter (ACCU-CHEK® Active; Roche, Germany) and insulin levels were analyzed using a rat insulin enzyme-linked immunosorbent assay kit (Crystal Chem. Inc, United States). The liver tissue samples of rats were taken immediately and kept at -80 °C after being quickly frozen in liquid nitrogen.

Euglycemic hyperinsulinemic clamp

Hyperinsulinemic clamp studies were performed as previously described^[19]. Rats were under general anesthesia (3% pelltobarbitalum natricum, 60 mg/kg, intraperitoneally), and catheters were inserted into the right jugular vein and the thoracic aorta of rats and exteriorized from the back of the neck subcutaneously. At the end, the catheters were flushed with isotonic saline containing heparin (50 U/mL). Rats were allowed to fully recover for a minimum of 3 d and only those that had lost less than 5% of their preoperative weights were used. Euglycemic-hyperinsulinemic clamp tests were performed on fasted, awake, and unrestrained animals. Insulin was infused at 4 mU/(kg min) through the jugular vein catheter for 0-90 min. Glucose concentrations were clamped at euglycemic levels by a variable rate infusion of 30% glucose. BG levels were monitored with a glucometer (ACCU-CHEK® Active; Roche), and glucose infusion rates (GIR) were adjusted every 5-10 min as needed. A stable GIR was obtained within about 60 min after in-

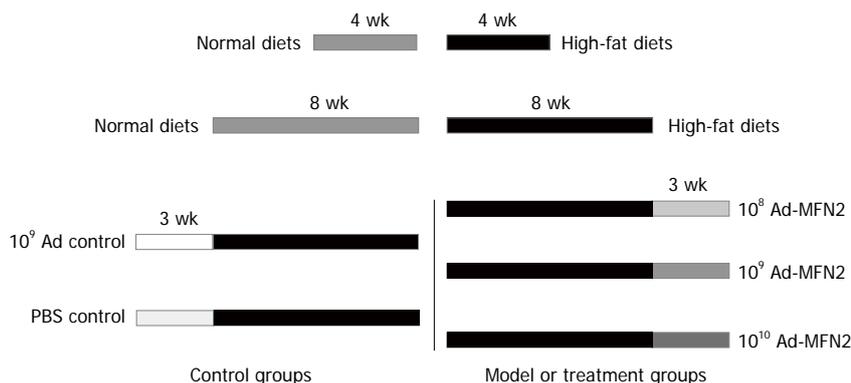


Figure 1 Schematic presentation of rat groups. Rats were fed with control or high-fat diets for 4 or 8 wk, and then some groups were infected with Ad-mitofusin 2 (MFN2) or empty Ad adenovirus or phosphate-buffered saline (PBS) control once a week for 3 wk.

Table 1 Primer sequences for quantitative polymerase chain reaction

| Gene | bp | Forward primer (5'-3') | Reverse primer (5'-3') | GenBank No. |
|-------|-----|------------------------|-------------------------|--------------|
| GAPDH | 120 | TGAACGGGAAGCTCACITGG | GCTTACCACCTTCTGTGATGTC | NM_017008 |
| MFN2 | 160 | AGCGTCTCTCCCTCTGACA | TTCCACACCCTCTCCGAC | NM_130894 |
| INSR | 135 | TTTGCCCAACCATCTGTAAG | GACCATCCAGGTAGAAGTTTCG | NM_017071.1 |
| IRS2 | 81 | TCTCTGGCAGTTCAGGTCCG | AGTCCTCTGGGTAAGGGTTG | NM_001168633 |
| PI3K | 135 | GCCTGCTCTGTAGTGGTAGATG | GGAGGTGTGTTGGTAATGTAGC | NM_013005.1 |
| AKT2 | 79 | CTGAGATGATGGAGGTAGCG | CCGAGGAGTTTGTAGATAATCG | NM_017093.1 |
| GLUT2 | 80 | AGCACATACGACACCAGACG | CAGACAGAGACCAGAGCATAGTG | NM_012879 |
| SOCS3 | 148 | TCACCCACAGCAAGTTTCC | ACGGCACTCCAGTAGAATCC | NM_053565.1 |

GAPDH: Glyceraldehyde-3-phosphate dehydrogenase; MFN2: Mitofusin-2; INSR: Insulin receptor; IRS2: Insulin receptor substrate 2; PI3K: Phosphoinositide-3-kinase; AKT2: Protein kinase beta; GLUT2: Glucose transporter type 2; SOCS3: Suppressor of cytokine signaling 3.

sulin infusion and maintained thereafter. At steady state, mean GIR was normalized to body weight.

Real-time polymerase chain reaction analysis

Total RNA was isolated from liver tissue using TRIzol reagent (Invitrogen, United States) according to the manufacturer’s instructions. Equal amounts of RNA were used to synthesize first strand cDNA (Promega, United States), and quantitative real-time polymerase chain reaction (RT-PCR) was performed on an ABI PRISM 7300 PCR System (Applied Biosystems, United States) using Syber Green I GoTaq[®] qPCR Master Mix (Promega, United States). PCR was performed as: one cycle at 95 °C for 5 min, followed by 40 cycles of 95 °C for 15 s, 58 °C for 20 s and 72 °C for 30 s. Then PCR products were analyzed by melting curve to confirm the specificity of amplification. The PCR primer sequences are shown in Table 1. mRNA expression of target genes was normalized to the internal reference gene glyceraldehyde 3-phosphate dehydrogenase. The relative expression of target genes was obtained using SDS v1.3.2 software linked with the PCR machine.

Western blotting

Protein samples were prepared with lysis buffer (10 mL/L Triton X-100, 150 mmol/L NaCl, 10 mmol/L Tris-HCl, pH 7.4, 1 mmol/L EDTA, 1 mmol/L EGTA, pH 8.0, 0.2 mmol/L Na₃VO₄, 0.2 mmol/L phenylmethyl-

sulfonyl fluoride, and 5 mL/L NP-40). Equal amounts of protein were separated by 10% SDS-PAGE (sodium dodecyl sulfate polyacrylamide gel electrophoresis), and electrotransferred to polyvinylidene difluoride membranes, and were then blocked with 5 g/L bovine serum albumin for 2 h at room temperature. Membranes were incubated with appropriate diluted primary antibodies of MFN2, insulin receptor (INSR), insulin receptor substrate 2 (IRS2), phosphoinositide-3-kinase (PI3K-P85), p-PI3K-P85, AKT2, p-AKT2, glucose transporter type 2 (GLUT2) or β-actin (all from Santa Cruz or Cell Signaling Technology, United states) respectively overnight at 4 °C, and then with the respective secondary antibody for 2 h. Proteins were detected with the enhanced chemiluminescence detection system. β-actin served as an internal control protein. The experiments were replicated three times.

Statistical analysis

Data were shown as mean ± SD. One-way analysis of variance was used to determine statistically significant differences between groups. P < 0.05 was considered statistically significant.

RESULTS

HFD decreased insulin sensitivity in rats

As shown in Figure 2, fasting BG and plasma insulin lev-

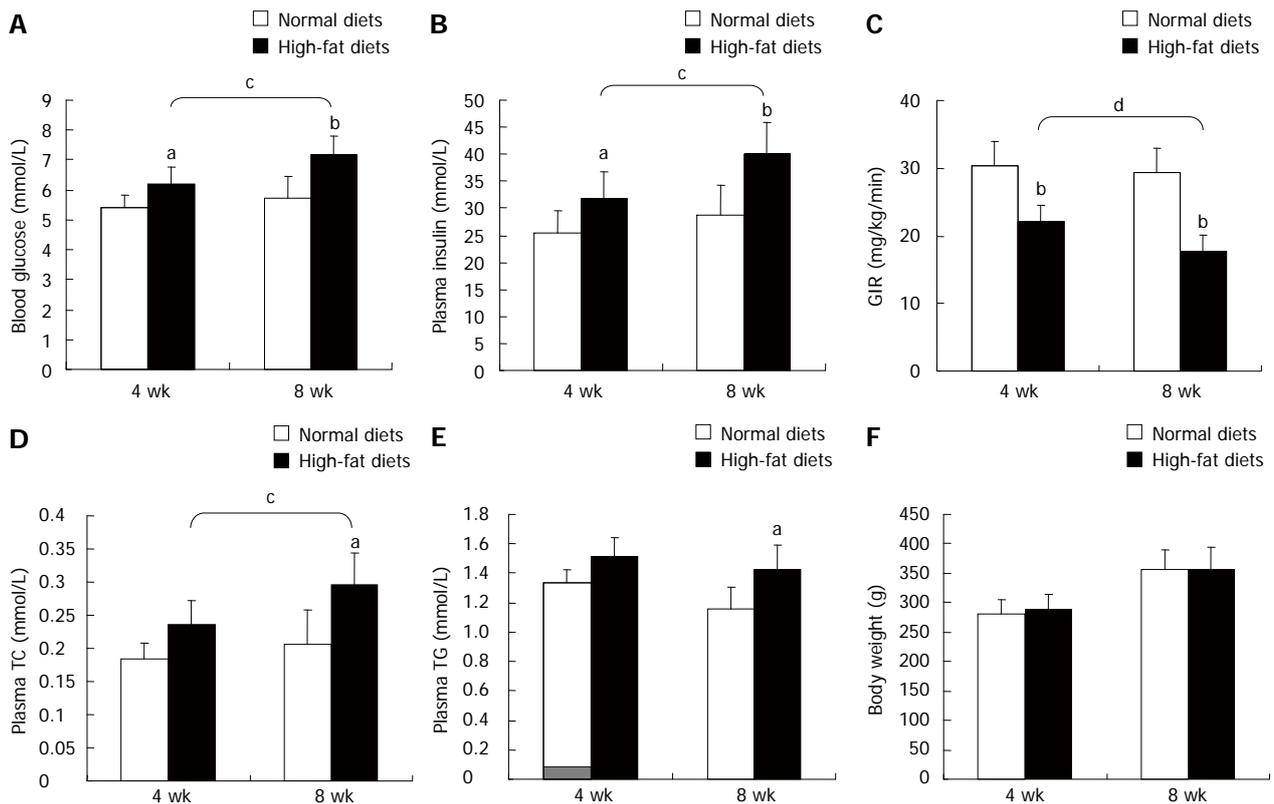


Figure 2 High-fat diets resulted in insulin resistance in rats ($n = 6$). Rats were fed with high-fat diets or normal control diets for 4 wk or 8 wk. A: The level of blood glucose; B: The level of plasma insulin. C: The values of glucose infusion rate (GIR) were used to assess insulin sensitivity of rats assayed by hyperinsulinemic euglycemic clamping; D: The level of plasma total cholesterol (TC); E: The level of plasma triglyceride (TG); F: The body weight. ^a $P < 0.05$, ^b $P < 0.01$ vs normal diets; ^c $P < 0.05$, ^d $P < 0.01$ vs 4 wk.

els of rats increased after 4 or 8 wk HFD feeding, while GIR, a more sensitive indicator for insulin sensitivity, decreased markedly. The levels of plasma total cholesterol (TC) and triglycerides (TG) were higher than those in the ND group at 4 and 8 wk. Greater changes in these values were seen at 8 wk than 4 wk, while the body weights of rats in each group were not significantly different at 4 or 8 wk.

HFD inhibited the expression of MFN2 and insulin signaling pathway factors and their phosphorylation levels

MFN2 is a key factor for energy metabolism, while the IRS2/PI3K cascade is the main insulin signaling pathway in hepatocytes, so we detected the expression of MFN2 and the IRS2/PI3K cascade pathway. As shown in Figure 3, the expression of MFN2, INSR, IRS2 and GLUT2 was down-regulated markedly by HFD both at 4 and 8 wk. While the mRNA and total protein expression of PI3K-P85 and AKT2 were not significantly changed (Figure 3A and B), their protein phosphorylation levels decreased markedly (Figure 3B). However, HFD seemed to have no effects on the expression of IRS1, PI3K-P110 and AKT1 or their phosphorylation (data not shown).

Over-expression of MFN2 ameliorated HFD induced insulin resistance in rats

In order to know the effect of MFN2 on insulin sensitiv-

ity, rats were fed with an HFD for 8 wk, and then were infected with different amounts of Ad-MFN2 (10^8 , 10^9 or 10^{10} vp/kg body weight) or empty Ad adenovirus or PBS control for 3 wk. The results showed that MFN2 expression in the liver of rats increased dramatically after Ad-MFN2 infection (Figure 4A-C). At the same time, fasting BG, plasma insulin, TC and TG levels decreased (Figure 4D-G), while GIR increased (Figure 4H) markedly after infection with different amounts of Ad-MFN2. The body weight of rats in the two groups showed no significant difference (Figure 4I). The results indicated that MFN2 over-expression could neutralize the effects of HFD on insulin sensitivity.

Over-expression of MFN2 neutralized the inhibition of the insulin pathway by HFD in rats

Based on MFN2 over-expression in liver of rats, we detected changes in the insulin pathway by quantitative RT-PCR and Western-blot assays. The results showed that both mRNA and protein levels of INSR, IRS2 and GLUT2 were up-regulated markedly (Figure 5A and B). Though there were no changes in PI3K-P85 and AKT2 expression, their phosphorylation levels increased significantly (Figure 5B). After treated with MFN2 expressing adenovirus, the expression of SOCS3 was decreased (Figure 5C and D). The results suggested that MFN2-induced improvement in insulin sensitivity may be correlated with the promotion of the insulin signaling pathway.

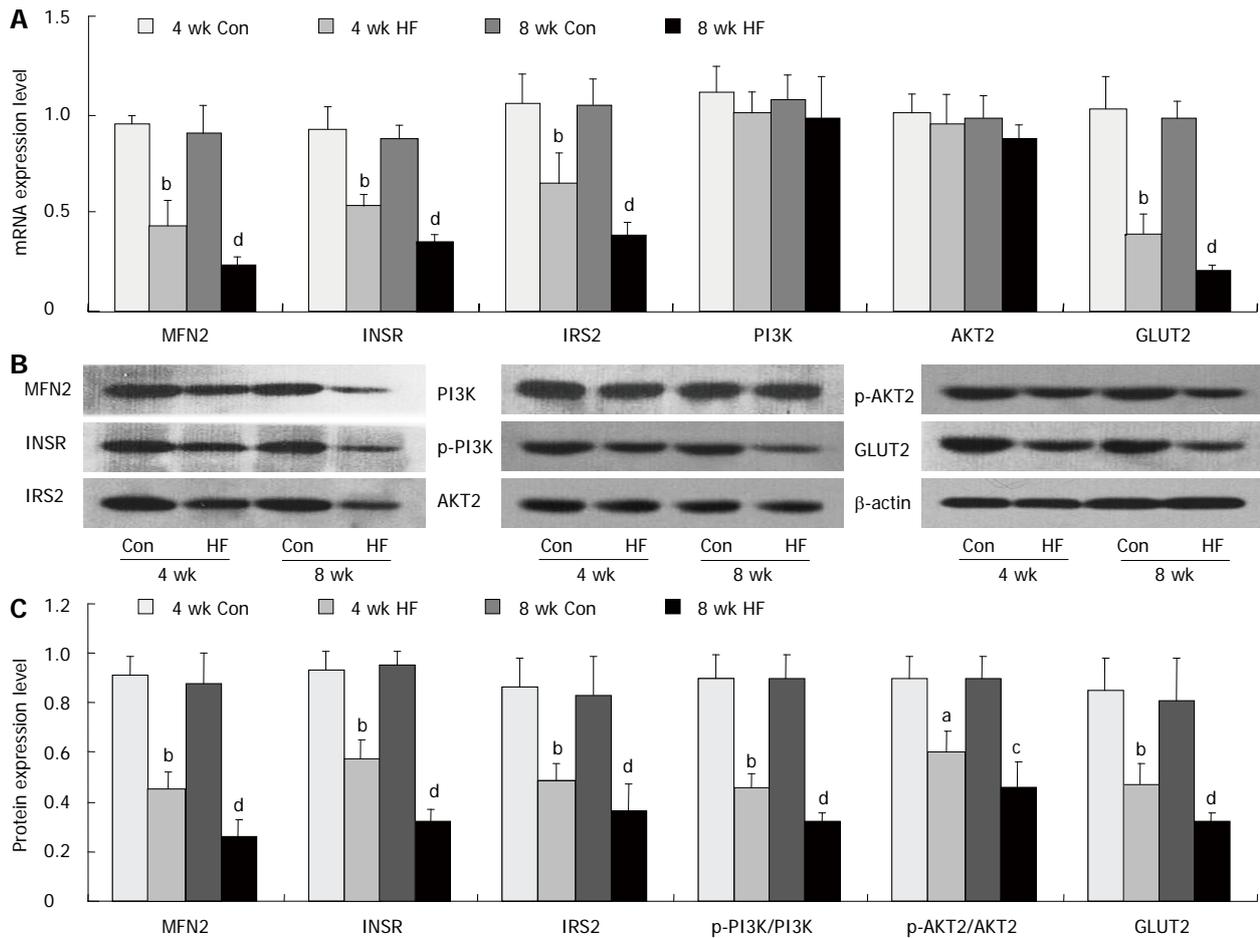


Figure 3 High-fat diets inhibited insulin pathway in liver of rats. Rats were fed with high-fat diets (HF) or normal control diets (Con) for 4 or 8 wk. A: The mRNA expression levels of mitofusin 2 (MFN2), insulin receptor (INSR), insulin receptor substrate 2 (IRS2), phosphoinositide-3-kinase (PI3K), AKT2 and glucose transporter type 2 (GLUT2) were examined by quantitative real-time-polymerase chain reaction; B and C: The protein levels were detected by Western-blotting. ^b*P* < 0.01 vs normal diets for 4 wk; ^d*P* < 0.01 vs normal diets for 8 wk.

Over-expression of MFN2 alleviated hepatic steatosis

All of the tissue sections in the control and Ad groups exhibited diffuse hepatic steatosis under a light microscope. Hepatic steatosis was most obvious around the portal area and was accompanied by inflammatory cell infiltration. The liver HE staining of rats infected with Ad-MFN2 showed a lower cell volume and fat droplet accumulation (Figure 6).

DISCUSSION

HFDs induce dysfunction of energy metabolism and impaired insulin sensitivity^[20]. Insulin resistance is not only the most important pathophysiological feature in many pre-diabetic states, but is also a key component of the metabolic syndrome, in which target cells fail to respond to normal levels of circulating insulin^[21]. The liver is a vital organ for lipid metabolism and glycometabolism, and therefore one of the main organs in which insulin resistance occurs. Mitochondria are the power centres, and also the energy metabolism centres in cells, so mitochondrial dysfunction is the main reason for insulin resistance and is involved in the pathogenesis of T2DM^[22]. The

MFN2 gene plays a central role in mitochondrial metabolism^[23]; however, the role of MFN2 in insulin resistance and the insulin signaling pathway remains uncertain. Our study suggested that MFN2 expression and insulin sensitivity were inhibited by an HFD, while recovery of MFN2 expression could recover impaired insulin sensitivity, and may be associated with improvements of the insulin signaling pathway in liver.

An HFD enriched with lard, a significant contributor to the development of obesity and insulin resistance, has been widely used to induce an animal model of obesity with insulin resistance^[24]. Previous studies have reported a long-term HFD could cause a marked increase in body weight. In this study, we confirmed that an HFD, for only 4 wk, could result in insulin resistance, which progressed further after 8 wk in the absence of major changes in total body weight. It may be because an HFD produces altered fat distribution, leading to the accumulation of visceral fat^[25,26]. At the same time, MFN2 expression was down-regulated dramatically, and the insulin signaling pathway was inhibited markedly in the liver of rats.

MFN proteins have been shown to regulate the biogenesis and maintenance of the mitochondrial network

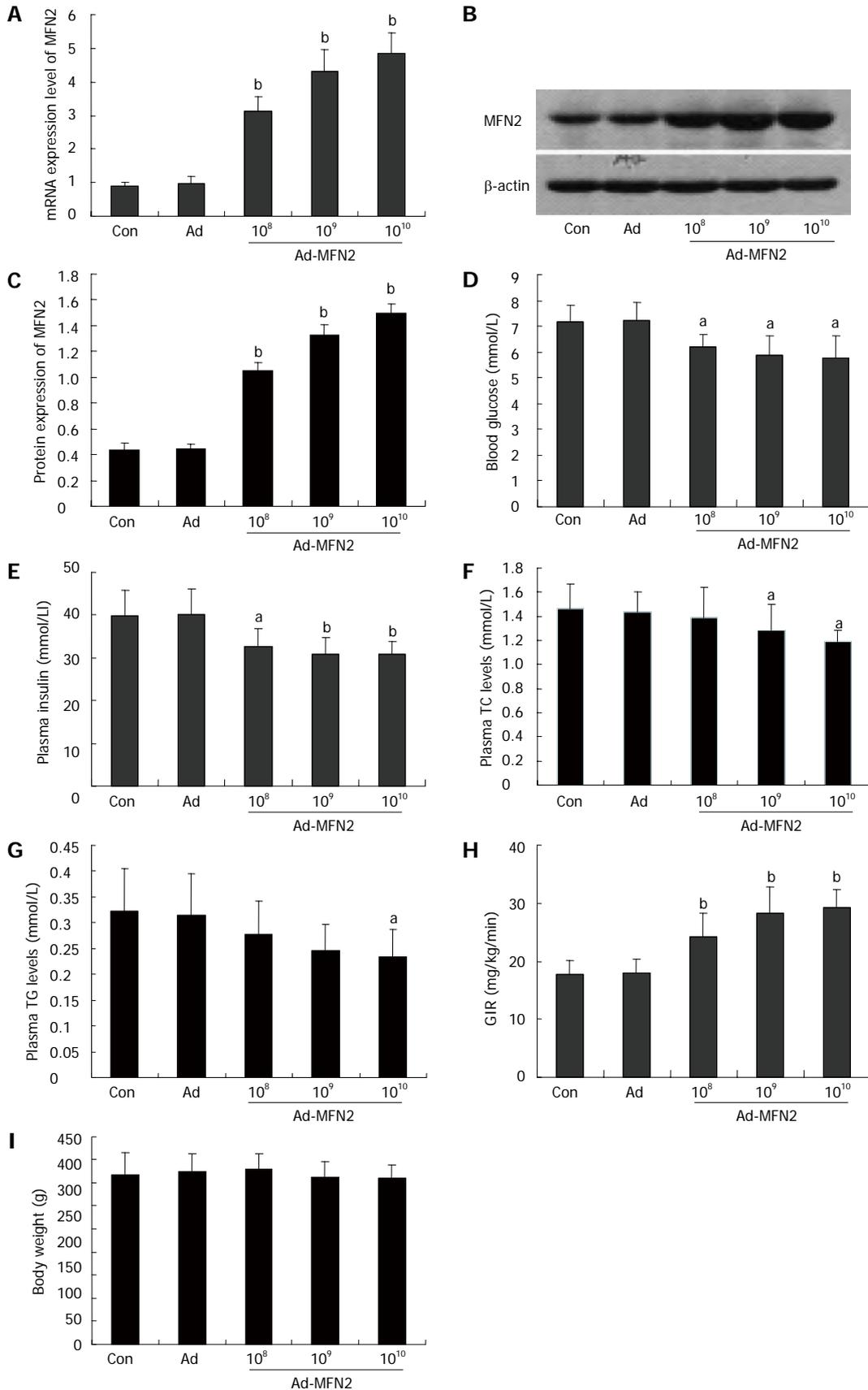


Figure 4 Mitofusin-2 over-expression improved insulin sensitivity of rats ($n = 6$). Rats were fed with high-fat diets for 8 wk, and then were infected with different amount of Ad-mitofusin-2 (MFN2) (10^8 , 10^9 or 10^{10} vp/kg body weight) or empty Ad adenovirus or phosphate-buffered saline (PBS) control for 3 wk. MFN2 over-expression in liver of rats was confirmed by quantitative real-time-polymerase chain reaction (A) and Western-blotting (B and C). The levels of blood glucose (D), plasma insulin (E), triglycerides (TG) (G) plasma total cholesterol (TC) (F) and insulin sensitivity (H) levels were examined, respectively. The body weight of rats was measured (I). ^a $P < 0.05$, ^b $P < 0.01$ vs Ad. GIR: Glucose infusion rate.

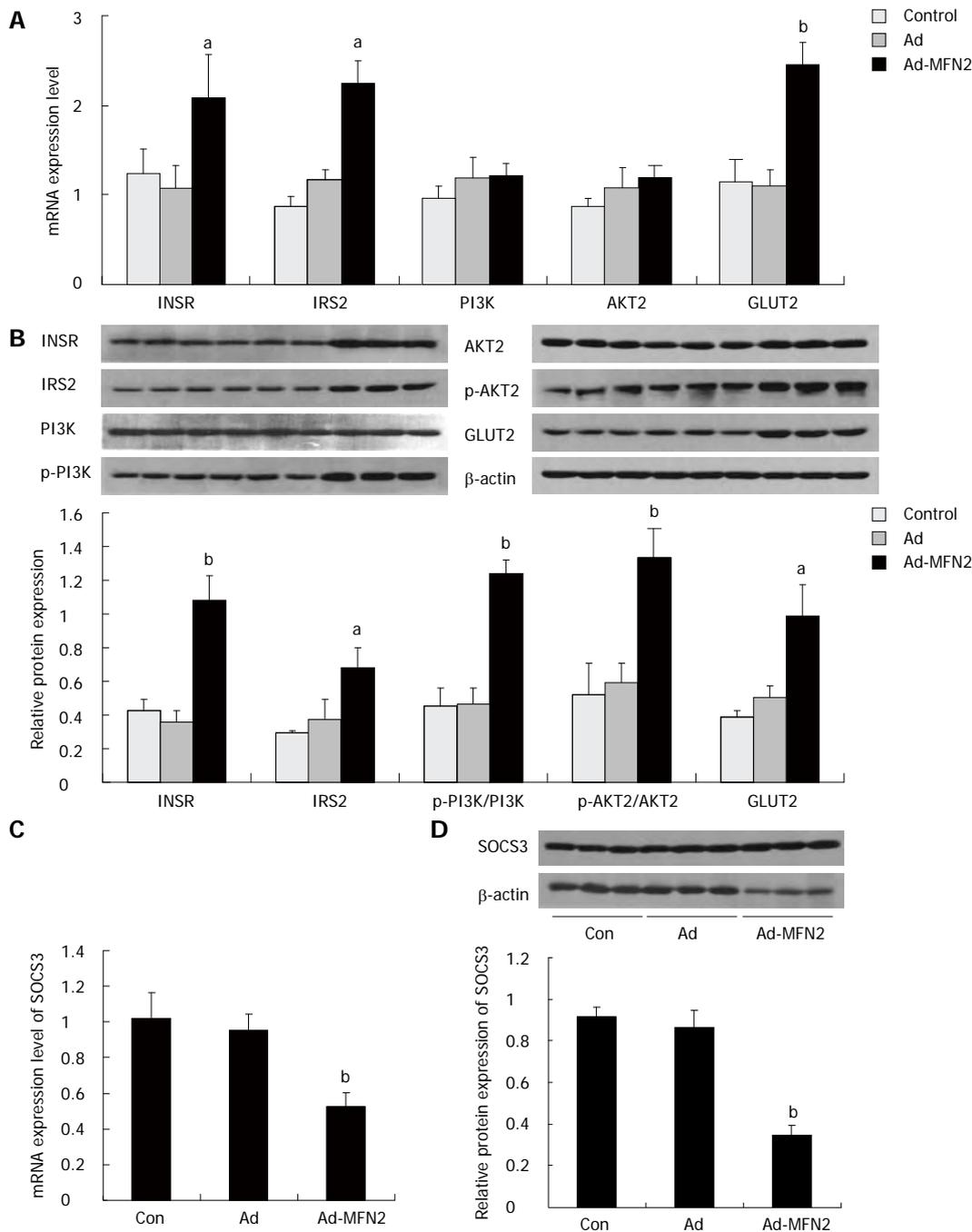


Figure 5 Mitofusin-2 over-expression up-regulated the expression of insulin pathway related genes in liver of rats. Rats were fed with high-fat diets for 8 wk, and then were infected with Ad-mitofusin-2 (MFN2) or empty Ad adenovirus or phosphate-buffered saline (PBS) control for 3 wk. The expression levels of insulin receptor (INSR), insulin receptor substrate 2 (IRS2), phosphoinositide-3-kinase (PI3K), AKT2 and glucose transporter type 2 (GLUT2) were determined by quantitative real-time-polymerase chain reaction (RT-PCR) (A) and Western-blot (B). The phosphorylation levels of PI3K and AKT2 were assayed by Western-blotting (B). The expression level of suppressor of cytokine signaling 3 (SOCS3) was determined by quantitative RT-PCR (C) and Western-blot (D). ^a*P* < 0.05, ^b*P* < 0.01 vs Ad.

rk^[27]. MFN protein deficiency could cause a failure in the mitochondrial architecture and decreases in oxidative capacity and glucose oxidation. MFN2 is a proliferation-inhibiting gene encoding a mitochondrial fusion protein that participates in the maintenance of the mitochondrial morphology and regulates mitochondrial metabolism and intracellular signaling^[6]. A recent study found that liver MFN2 protein was significantly decreased, and fasting BG concentrations were increased in mice after

interference with MFN2 protein expression^[28]. Our data demonstrate that over-expression of MFN2 significantly restored insulin sensitivity and reduced the levels of BG and plasma insulin in rats, suggesting MFN2 as a potential therapeutic target in insulin resistance.

Most metabolic processes are regulated by insulin in muscle, adipocytes, and liver. A recent study indicated that mitochondrial protein down-regulation contributes to defects in insulin signaling in insulin resistance^[29]. Insu-

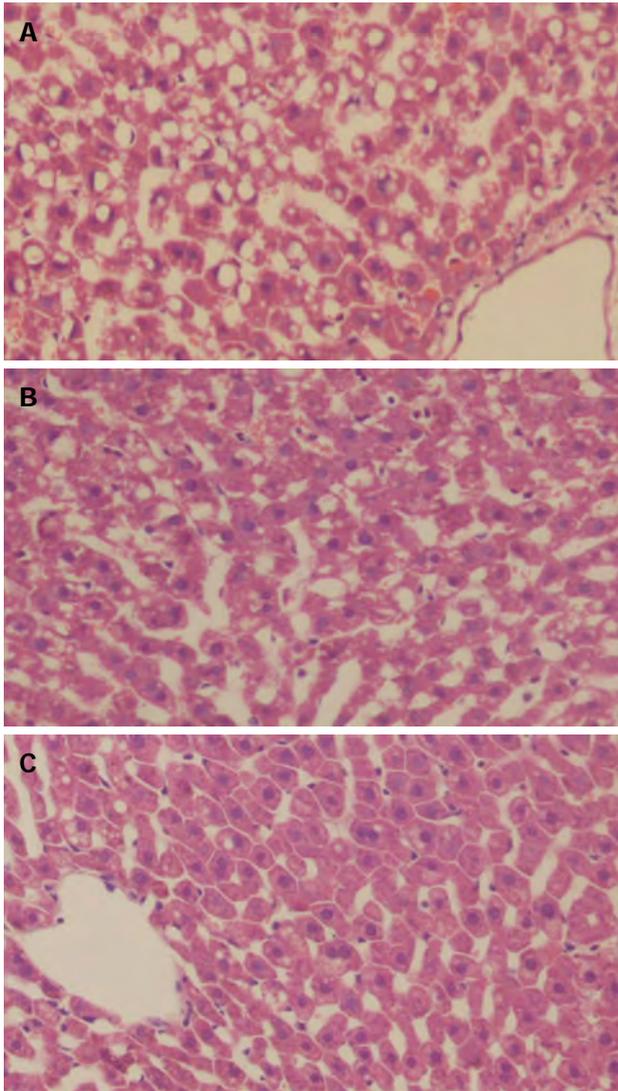


Figure 6 Over-expression of mitofusin-2 improves high-fat diets induced hepatic steatosis. Histological analysis of liver sections from rats fed with high-fat diets infected with phosphate-buffered saline control (A), empty Ad adenovirus (B) or Ad-mitofusin 2 (C) for 3 wk (HE, $\times 200$).

lin acts through a complex signaling network including alternative or complementary pathways, with multiple molecular systems involved^[30]. Abnormalities in the early stages of insulin signaling have been considered as an important component of many insulin-resistant states^[31,32]. Our results showed that the expression of INRS, IRS2 and GLUT2 decreased; the phosphorylation of PI3K-P85 and AKT2 was also inhibited by HFD, but was restored markedly by recovery of MFN2 expression.

Hepatic expression of SOCS3 has been reported to be elevated in rodent models of obesity and insulin resistance^[33,34]. SOCS3 was found to bind to phosphotyrosine 960 of the insulin receptor and prevent STAT5b activation by insulin^[35]. In COS-7 cells, SOCS3 reduced IRS-2 phosphorylation and its subsequent association with p85, the regulatory subunit of PI3K^[36]. In multiple cell lines, SOCS3 has been shown to bind IRS-2 and promote its ubiquitination and subsequent degradation^[34,37]. The inhi-

bition of SOCS3 expression restores IRS-1 and IRS-2 tyrosine phosphorylation, and IRS-1 and IRS-2 association with p85-PI3K and [Ser473] phosphorylation of Akt^[38]. In our study, expression of SOCS3 in the liver of rats treated with MFN2 expressing adenovirus was decreased. MFN2 expression may improve insulin resistance by regulating the expression of SOCS3 in the liver of rats.

In conclusion, MFN2 could ameliorate insulin resistance induced by HFD by improvement of the insulin signaling pathway, and this may be a potential target for the treatment of insulin resistance and metabolic syndrome.

COMMENTS

Background

Insulin resistance is associated with numerous modern health problems, such as obesity, metabolic syndrome and type 2 diabetes mellitus. Mitofusin 2 (MFN2) regulates mitochondrial morphology and signaling, and is involved in the pathogenesis of insulin resistance and development of diabetes, but the exact mechanism is unclear.

Research frontiers

MFN2, a large transmembrane GTPase located in the outer mitochondrial membrane, promotes membrane fusion and is involved in the maintenance of the morphology of mitochondria. Recent studies show that *MFN2* gene expression is positively correlated with insulin resistance. Some metabolic diseases, such as type 2 diabetes show impaired MFN2 expression. MFN2 deficiency impairs insulin signaling in the liver and muscle. The research hotspot is the mechanism of MFN2 in improving insulin sensitivity.

Innovations and breakthroughs

MFN2, a large transmembrane GTPase located in the outer mitochondrial membrane, promotes membrane fusion and is involved in the maintenance of the morphology of mitochondria. Recent studies show that *MFN2* gene expression is positively correlated with insulin resistance and that MFN2 deficiency impairs insulin signaling in the liver and muscle.

Applications

The study results suggest that MFN2 over-expression improves insulin sensitivity, and may be used for preventing the development of diabetes in future.

Terminology

MFN2 encodes a mitochondrial membrane protein that participates in mitochondrial fusion and contributes to the maintenance and operation of the mitochondrial network. This protein is involved in the regulation of vascular smooth muscle cell proliferation, and it may play a role in the pathophysiology of obesity. Mutations in this gene cause Charcot-Marie-Tooth disease type 2A2, and hereditary motor and sensory neuropathy VI, which are both disorders of the peripheral nervous system. Defects in this gene have also been associated with early-onset stroke.

Peer review

The authors examined the expression of insulin signaling pathways and suppressor of cytokine signaling 3 (SOCS3) after infection with an MFN2 expressing adenovirus. It revealed that expression of insulin signaling pathways were increased and SOCS3 was inhibited in the liver of insulin resistant rats. Expression of MFN2 improves the insulin signaling pathway through the inhibition of SOCS3 expression. The results are interesting and may represent a molecular mechanism of MFN2 amelioration of insulin resistance.

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Effects of ginsenoside Rh2 on growth and migration of pancreatic cancer cells

Xi-Ping Tang, Guo-Du Tang, Chun-Yun Fang, Zhi-Hai Liang, Lu-Yi Zhang

Xi-Ping Tang, Guo-Du Tang, Chun-Yun Fang, Zhi-Hai Liang, Lu-Yi Zhang, Department of Gastroenterology, First Affiliated Hospital, Guangxi Medical University, Nanning 530021, Guangxi Zhuang Autonomous Region, China

Author contributions: Tang XP and Tang GD designed the study; Tang XP, Fang CY, Liang ZH and Zhang LY performed the majority of experiments; Tang XP analyzed the data and wrote the manuscript; Tang GD reviewed the manuscript.

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Correspondence to: Dr. Guo-Du Tang, Department of Gastroenterology, First Affiliated Hospital, Guangxi Medical University, Nanning 530021, Guangxi Zhuang Autonomous Region, China. tguodu02@yahoo.com.cn

Telephone: +86-771-5356501 Fax: +86-771-5356501

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Abstract

AIM: To investigate the effects of ginsenoside Rh2 on the human pancreatic cancer cell line Bxpc-3.

METHODS: The human pancreatic cancer cell line Bxpc-3 was cultured *in vitro* and treated with or without ginsenoside Rh2. Growth rates for Bxpc-3 cells were assessed by methyl thiazolyl tetrazolium (MTT) and colony formation assays. Cell cycle changes were analyzed by flow cytometry. Apoptosis was measured by flow cytometry and Hoechst 33258 fluorescence staining. A scratch assay and a Matrigel invasion assay were used to detect cell migration and invasion. Expression of Bax, Bcl-2, survivin, cyclin D1, matrix metalloproteinase (MMP)-2, MMP-9, cleaved caspase-3, caspase-8, and caspase-9 mRNA were determined by reverse transcriptase-polymerase chain reaction (RT-PCR). Bax, Bcl-2, survivin, cyclin D1, cleaved caspase-3, caspase-8 and caspase-9 protein levels were examined by western blotting. Expression of MMP-2 and MMP-9 proteins

in culture supernatants were determined by enzyme-linked immunosorbent assay (ELISA).

RESULTS: Rh2 significantly inhibited Bxpc-3 cell proliferation in a dose- and time-dependent manner, as evaluated by the MTT ($P < 0.05$) and colony formation assays ($P < 0.05$). Compared to the control group, Rh2 significantly increased the percentage of Bxpc-3 cells in the G₀/G₁ phase from 43.32% \pm 2.17% to 71.32% \pm 1.16%, which was accompanied by a decrease in S phase (from 50.86% \pm 1.29% to 28.48% \pm 1.18%) and G₂/M phase (from 5.81% \pm 1.19% to 0.20% \pm 0.05%) in a dose-dependent manner ($P < 0.05$), suggesting that Rh2 arrested cell cycle progression at the G₀/G₁ phase, as measured by flow cytometry. Compared to the control group, cells treated with Rh2 showed significantly higher apoptosis ratios in a dose-dependent manner (percentage of early apoptotic cells: from 5.29% \pm 2.28% to 38.90% \pm 3.42% ($F = 56.20$, $P < 0.05$); percentage of late apoptotic cells: from 4.58% \pm 1.42% to 36.32% \pm 2.73% ($F = 86.70$, $P < 0.05$). Rh2 inhibited Bxpc-3 cell migration and invasion, as detected by scratch wound healing assay and Matrigel invasion assay [percentages of scratch wound healing for 12 h, 24 h and 48 h (control *vs* experimental group): 37.3% \pm 4.8% *vs* 18.30% \pm 1.65%, 58.7% \pm 3.5% *vs* 38.00% \pm 4.09% and 93.83% \pm 4.65% *vs* 65.50% \pm 4.09%, respectively; $t = 6.489$, $t = 6.656$ and $t = 7.926$, respectively, $P < 0.05$; the number of cells invading at various concentrations (0 μ mol/L, 35 μ mol/L, 45 μ mol/L and 55 μ mol/L): 81.10 \pm 9.55, 46.40 \pm 6.95, 24.70 \pm 6.88 and 8.70 \pm 3.34, respectively ($F = 502.713$, $P < 0.05$)]. RT-PCR, western blotting or ELISA showed that mRNA and protein expression of Bax, cleaved caspase-3 and caspase-9 were upregulated ($P < 0.05$), while mRNA and protein expression of Bcl-2, survivin, cyclin D1, MMP-2 and MMP-9 were downregulated ($P < 0.05$).

CONCLUSION: Ginsenoside Rh2 inhibits proliferation, migration and invasion and induces apoptosis of the human pancreatic cancer cell line Bxpc-3.

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Key words: Ginsenoside Rh2; Human pancreatic cancer Bxpc-3 cell; Proliferation; Apoptosis; Migration

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INTRODUCTION

Pancreatic cancer (PC) is a disease with a high mortality rate and the 1-year survival rate is < 10%^[1]. The successful surgical resection rate for PC is not high and current chemotherapy is not effective. Thus, there is an urgent need to develop novel treatment modalities. Ginseng is a traditional herbal medicine that is well known for its wide spectrum of pharmacological effects^[2]. Ginsenoside is the main effective component of ginseng and has been widely used in oriental countries for thousands of years^[3,4]. Recently, some experiments have demonstrated that ginsenoside has a wide variety of biological activities including immunomodulatory effects and anti-inflammatory and antitumor activity^[5-7]. Ginsenoside Rh2 is a pure compound extracted from ginsenosides. Recently, researchers have found that ginsenoside Rh2 could inhibit growth of many kinds of cancer cells, including breast cancer, prostate cancer, hepatoma, gastric cancer and colon carcinoma^[8-13]. Rh2 may play an antitumor role through the following mechanisms: (1) regulating tumor cells through the signaling pathway system, including the signaling pathway of protein kinase C, insulin-like growth factors, caspase family and Bcl-2 family; (2) affecting the activity of cell telomerase; (3) blocking anabolism and metabolism of important components in tumor cells; and (4) reversing abnormal differentiation and resistance of tumor cells.

However, to date, little is known about the role of ginsenoside Rh2 in PC. The purpose of this study was to investigate the effects of ginsenoside Rh2 on proliferation, apoptosis and migration of the human pancreatic cancer cell line Bxpc-3, and to explore the potential mechanisms of the effects.

MATERIALS AND METHODS

Materials

The human pancreatic cancer cell line Bxpc-3 was obtained from the Cell Resource Center, Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences. RPMI 1640 medium and fetal bovine serum were purchased from Gibco BRL (Gaithersburg, MD, United States). Ginsenoside Rh2 was obtained from the National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). The drug was dissolved

in dimethyl sulfoxide (DMSO) with a stock concentration of 40 mmol/L. The following materials were used: monoclonal antibodies to Bcl-2, Bax, survivin, cyclin D1, cleaved caspase-3, caspase-8 and caspase-9 (Santa Cruz Biotechnology, Santa Cruz, CA, United States), enzyme-linked immunosorbent assay (ELISA) kits for matrix metalloproteinase (MMP)-2 and MMP-9 (Boster Bioengineering Co., Wuhan, China), Annexin V-FITC Apoptosis Detection Kit and Cell Cycle Detection Kit (KeyGEN Biotech Co., Nanjin, Jiangsu, China), Hoechst 33258 staining kit (Beyotime Biotechnology Co., Jiangsu, China), Matrigel (BD Biosciences, United States) and 24-well invasion chambers (Corning-Costar, New York, United States).

Cell culture

Bxpc-3 cells were maintained in RPMI 1640 medium supplemented with 10% heat-inactivated fetal bovine serum, 100 U/mL penicillin and 100 µg/mL streptomycin at 37 °C in a 5% CO₂ atmosphere.

Methyl thiazolyl tetrazolium assay

Cells were seeded on 96-well plates in triplicate. Following 24 h culture at 37 °C, the medium was replaced with fresh medium containing increasing concentrations of ginsenoside Rh2 (0 µmol/L, 10 µmol/L, 20 µmol/L, 35 µmol/L, 45 µmol/L, 55 µmol/L and 60 µmol/L) in a final volume of 200 µL. Cells were incubated at 37 °C for 24 h, 48 h and 72 h. Methyl thiazolyl tetrazolium (MTT) [20 µL, 2 mg/mL in phosphate-buffered saline (PBS)] was added and cells were incubated for a further 4 h. The medium was removed and 150 µL DMSO was added to each well. It was shaken mechanically for 10 min and the optical density was measured at 570 nm. The experiment was repeated three times.

Colony formation assay

Cells were plated in six-well plates at a density of 100 cells/well for 48 h, prior to the addition of various concentrations of Rh2 (0 µmol/L, 35 µmol/L, 45 µmol/L and 55 µmol/L). After 48 h treatment, the drug-containing medium was removed and replaced with complete growth medium. The medium was changed every 3 d for 14 d until visible colonies formed. Colonies were simultaneously fixed and stained with 0.5% crystal violet in methanol, and were manually counted. Individually stained colonies in each well were counted and the colony formation fraction was calculated as following: colony number/(number of cells seeded × plating efficiency), where plating efficiency was equivalent to the colony number divided by the number of cells seeded in the drug-free medium. The experiment was repeated three times.

Flow cytometry

Bxpc-3 cells seeded in six-well plates were treated with different concentration (0 µmol/L, 35 µmol/L, 45 µmol/L and 55 µmol/L) Rh2 for 48 h at a cell density of 1.5

$\times 10^5$ cells/mL. Cells were processed using the following assay. (1) Cells were resuspended by adding 500 μ L binding buffer, followed by adding 5 μ L annexin V-FITC and 5 μ L propidium iodide (PI) dye. After mixing at room temperature in the dark for 5-15 min, flow cytometry analysis was performed. Annexin V-FITC-positive and PI-negative cells were considered as apoptotic cells; (2) Cells were resuspended by adding 100 μ L RNase A H₂O and incubated in water at 37 °C for 30 min. After adding 400 μ L PI and mixing at 4 °C for 30 min in the dark, flow cytometry analysis was performed. The G₀/G₁, S and G₂/M stages were compared. The experiment was repeated three times.

Reverse transcriptase-polymerase chain reaction

Bxpc-3 cells (1.5×10^4 /mL) were seeded in six-well plates. After 48 h culture, cells were treated with Rh2 (0 μ mol/L and 45 μ mol/L) for 48 h. Total RNA was extracted using TRIzol reagent. cDNA synthesis was performed using an RNA PCR kit. Samples were separated on 20 g/L agarose gels. The band intensity was determined by a gel image analysis system (Bio-Rad, Hercules, CA, United States) and normalized with β -actin. The PCR primers and regimen were as follows: 5'-AGGATCGAGCAGGGC-GAATG-3', 5'-GCTCCCGGAGGAAGTCCAAT-3' for Bax (345 bp); 5'-GATGGCAAATGACCAGCAGA-3', 5'-GCAGGATAGCAGCACAGGAT- 3' for Bcl-2 (346 bp); 5'-TGCCAGGATGATAAGTTCTT-3', 5'-ATCAAAGGCAGAAGGTTTGTGT-3' for cyclin D1 (316 bp); 5'-CCTCCTCAGCATCTTATCCG-3', 5'-CA-CAAACACCCACCTCAAA-3' for survivin (206 bp); 5'-CCGTGCCCCATCATCAAGTTCC-3', 5'-GCACGA GCAAAGGCATCATCCA-3' for MMP-2 (350 bp); 5'-CTTCCCTGGAGACCTGAGAAC-3', 5'-CCAAACT-GGATGACGATGTCT-3' for MMP-9 (423 bp); and 5'-TgacgTGGACATCCGCAAAG-3', 5'-CTGGAAGGT-GGACAGCGAGG-3' for β -actin (205 bp). The PCR conditions were denaturation at 94 °C for 5 min, annealing at 54 °C (Bax), 58 °C (Bcl-2) and 60 °C (cyclin D1, survivin, MMP-2, MMP-9) for 30 s \times 35 cycles, and extension at 72 °C for 7 min.

Western blotting

Bxpc-3 cells were treated with 0 μ mol/L, 35 μ mol/L, 45 μ mol/L and 55 μ mol/L Rh2 for 48 h, and collected by centrifugation, washed twice with cold PBS, and resuspended in 200 μ L protein lysate. Cells were centrifuged at 4 °C at 12 000 *g* for 5 min and the supernatant was stored at -20 °C. The protein concentration was determined by the bicinchoninic acid method (Beyotime Biotechnology Co., Jiangsu, China), and 40 μ g protein was loaded onto 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis gels. After electrophoresis, proteins were transferred onto polyvinylidene difluoride membranes at 4 °C for 4 h. Membranes were blocked in 5% non-fat milk for 1 h followed by incubation with primary antibodies of Bax, Bcl-2, survivin, cyclin D1, cleaved caspase-3, caspase-8 and caspase-9, overnight at 4 °C with shaking. Af-

ter washing in Tris-buffered saline with Tween 20 (TBST), membranes were incubated with peroxidase-linked IgG conjugates for 2 h at room temperature, washed again in TBST, and detected by an enhanced chemiluminescence reagent kit (Beyotime Biotechnology). The band intensity was determined by a gel image analysis system (Bio-Rad) and normalized with β -actin.

Hoechst 33258 assay for apoptosis

Bxpc-3 cells were treated with 0 μ mol/L, 35 μ mol/L, 45 μ mol/L and 55 μ mol/L Rh2 for 48 h and rinsed with PBS twice, followed by incubation with 10 μ g/mL Hoechst 33258 reagent at 37 °C in the dark for 15 min. Cells were fixed in 0.5 mL 4% paraform for 10 min and rinsed with PBS twice. The stained cells were examined and immediately photographed under a fluorescence microscope (Olympus, Shinjuku-ku, Tokyo, Japan) at an excitation wavelength of 330-380 nm. Apoptotic cells were identified on the basis of morphological changes in their nuclear assembly by observing chromatin condensation and fragment staining with Hoechst 33258. In each group, 10 microscopic fields were randomly selected and counted.

Invasion assay

Transwell chambers (Corning-Costar) were used to examine the ability of cells to invade through a Matrigel-coated filter following the manufacturer's instructions. RPMI 1640 medium was added to the upper chambers and allowed to hydrate for 2 h at 37 °C with 5% CO₂. Next, 5×10^4 Bxpc-3 cells treated with various concentrations of Rh2 (0 μ mol/L, 35 μ mol/L, 45 μ mol/L and 55 μ mol/L) were added to the upper chamber and grown in medium containing 2% fetal bovine serum on 8.0 μ m porous polycarbonate membranes, which were coated with diluted Matrigel basement membrane matrix. The lower chambers were filled with RPMI 1640 medium containing 10% fetal bovine serum. After 24 h incubation, the cells remaining on the upper surface of the filter were removed using cotton tips, and the cells that invaded to the underside of the membrane were fixed with 4% paraform and stained with crystal violet. Cells in 10 random fields of view at 400 \times magnification were counted and expressed as the average number of cells/field of view.

Migration assay

We scribed five paralleled lines on the bottom of six-well plates using a marker pen and seeded cells at a density of 4.0×10^5 cells per well in triplicate for 48 h. A perpendicular scratch wound was generated by scratching with a pipette tip. After rinsing with PBS to remove the detached cells, medium containing different concentrations of Rh2 (0 μ mol/L, 35 μ mol/L, 45 μ mol/L and 55 μ mol/L) was added. Photographic images were taken from each well at 0 h, 12 h, 24 h and 48 h. The distance that cells migrated through the area created by scratching was determined by measuring the wound width at the above times and subtracting it from the wound width at the start. The values

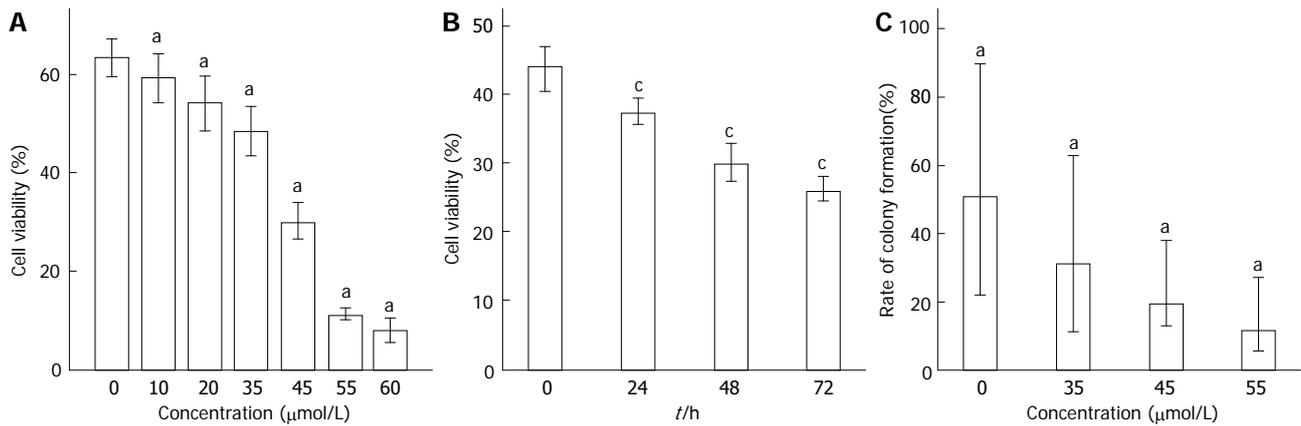


Figure 1 Effect of Rh2 on viability of Bxpc-3 cells. A and B: Methyl thiazolyl tetrazolium assay showed that the inhibitory effects of Rh2 on the viability of BxPc-3 cells were observed in both a dose- and time-dependent manner; C: A colony formation assay was used to examine the growth of BxPc-3 cells, ^a $P < 0.05$ vs 0 µmol/L group; ^a $P < 0.05$ vs 0 h group.

obtained were then expressed as the rate of wound healing. The experiment was repeated three times.

ELISA

Cells were cultured in six-well plates with RPMI 1640 containing different concentrations of Rh2 (0 µmol/L, 35 µmol/L, 45 µmol/L and 55 µmol/L) for 48 h. Supernatants were collected and stored at -80 °C. The protein concentrations of MMP-2 and MMP-9 in culture supernatants were determined using an ELISA kit according to the manufacturer's instructions.

Statistical analysis

The data were analyzed with single factor analysis of variance and a Student's *t* test using SPSS 13.0 software. Data were represented as mean ± SD. $P < 0.05$ was considered statistically significant.

RESULTS

Rh2 inhibiting Bxpc-3 cell viability

The inhibitory effect of Rh2 on the growth of Bxpc-3 cells was assessed by MTT and colony formation assays. The MTT assay showed that the various concentrations of Rh2 inhibited the viability of Bxpc-3 cells in a dose- and a time-dependent manner. The viable Bxpc-3 cells consistently decreased with higher concentrations of Rh2 for 48 h, as shown in Figure 1A (0 µmol/L, 10 µmol/L, 20 µmol/L, 35 µmol/L, 45 µmol/L, 55 µmol/L and 60 µmol/L: 63.867% ± 2.675%, 59.883% ± 3.16%, 54.917% ± 3.553%, 48.850% ± 3.316%, 29.900% ± 2.134%, 10.917% ± 0.671% and 8.267% ± 1.191%, respectively, $F = 477.542$, $P < 0.05$, Figure 1A). When cells were treated with 45 µmol/L Rh2 for 24 h, 48 h or 72 h, the cell viability declined significantly compared to 0 h (24 h, 48 h and 72 h: 37.417% ± 1.710%, 29.900% ± 2.134% and 25.917% ± 1.447%, respectively, $F = 81.633$, $P < 0.05$; Figure 1B). The clonogenic assay showed that Rh2 treatment resulted in significant inhibition of colony formation of Bxpc-3 cells compared with controls in

a dose-dependent manner (0 µmol/L, 35 µmol/L, 45 µmol/L and 55 µmol/L): 50.667% ± 13.204%, 31.000% ± 10.149%, 19.333% ± 5.132% and 10.667% ± 4.041%, respectively, $F = 11.229$, $P < 0.05$; Figure 1C).

Rh2 altering Bxpc-3 cell cycle distribution

The cell cycle distribution of Bxpc-3 cells treated with various concentrations of Rh2 (0 µmol/L, 35 µmol/L, 45 µmol/L and 55 µmol/L) for 48 h is shown in Figure 2A and 2B. The various concentrations of Rh2 altered cell cycle progression. Rh2 significantly increased the percentage of Bxpc-3 cells in the G₀/G₁ phase in a dose-dependent manner (0 µmol/L, 35 µmol/L, 45 µmol/L and 55 µmol/L: 43.32% ± 2.17%, 56.76% ± 1.33%, 67.40% ± 1.12% and 71.32% ± 1.16%, respectively, $F = 208.37$, $P < 0.001$), accompanied by a decrease in the percentage in S phase (50.86% ± 1.29%, 38.29% ± 1.40%, 32.08% ± 0.96% and 28.48% ± 1.18%, respectively, $F = 32.45$, $P < 0.001$) and G₂/M phase (5.81% ± 1.19%, 4.95% ± 0.81%, 1.32% ± 0.83% and 0.20% ± 0.05%, respectively, $F = 214.80$, $P < 0.001$). This suggested that the cell cycle was arrested at the G₀/G₁ phase by Rh2. Western blotting and reverse transcriptase-polymerase chain reaction (RT-PCR) analysis of molecular marker, cyclin D1, which was related to G₁ phase arrest, showed significant downregulation [Figure 2C and D, ratio of cyclin D1/glyceraldehyde-3-phosphate dehydrogenase (GADPH): 1.896 ± 0.104, 1.443 ± 0.074, 1.084 ± 0.162 and 0.225 ± 0.074, respectively, $F = 251.18$, $P < 0.05$; Figure 2E and F, ratio of cyclin D1/β-actin: 0.885 ± 0.083 and 0.687 ± 0.096, respectively, $F = 3.818$, $P < 0.05$]. The western blotting and RT-PCR data were consistent with the G₁ arrest phenomenon observed in flow cytometry analysis.

Rh2 inducing Bxpc-3 cell apoptosis

Rh2-induced apoptotic cell death was found using Annexin V-FITC/PI double stained flow cytometry. Annexin V-FITC-positive and PI-negative cells, which were considered as apoptotic cells, increased in a dose-dependent manner compared to the control group (Figure

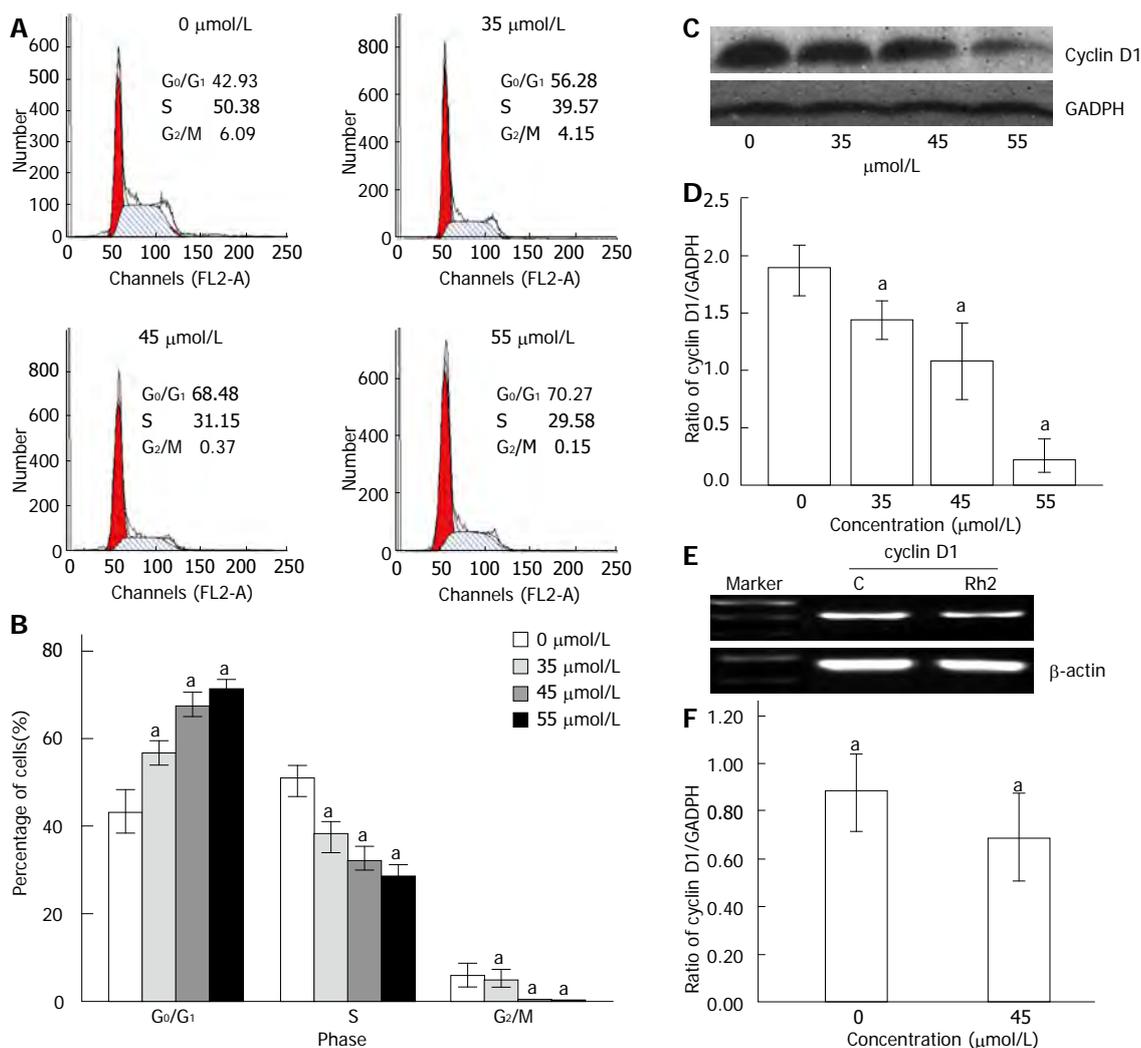


Figure 2 Rh2 induces G₀/G₁ arrest in Bxpc-3 cells treated for 48 h. **A, B:** Cell cycle distribution was assessed by flow cytometry. The results showed that the cell cycle was arrested at the G₀/G₁ phase when treated with Rh2; **C, D:** Western blotting was used to examine protein expression of cyclin D1; **E, F:** Reverse transcriptase-polymerase chain reaction was used to examine the expression of cyclin D1 mRNA. The results indicated that Rh2 downregulated protein and mRNA expression of cyclin D1 in Bxpc-3 cells. ^a*P* < 0.05 vs 0 μmol/L group. GADPH: Glyceraldehyde-3-phosphate dehydrogenase.

3A and B). The percentages of early apoptotic cells (0 μmol/L, 35 μmol/L, 45 μmol/L and 55 μmol/L) were 5.29% ± 2.28%, 12.15% ± 3.58%, 23.88% ± 4.07% and 38.9% ± 3.42%, respectively (*F* = 56.20, *P* < 0.05). The percentages of late apoptotic cells (0 μmol/L, 35 μmol/L, 45 μmol/L and 55 μmol/L) were 4.58% ± 1.42%, 9.9% ± 2.2%, 25.67% ± 3.87% and 36.32% ± 2.73%, respectively (*F* = 86.70, *P* < 0.05).

We also detected morphological changes in apoptotic cells by Hoechst 33258 staining (Figure 3C and D). In the untreated Bxpc-3 cells, the nuclei were stained weak homogeneous blue, while in the group treated with Rh2, bright chromatin condensation and nuclear fragmentation were observed. Furthermore, the rates of bright chromatin condensation and nuclear fragmentation increased in a dose-dependent manner. The percentages of apoptotic cells (0 μmol/L, 35 μmol/L, 45 μmol/L and 55 μmol/L) were 0.40% ± 0.05%, 16.4% ± 2.7%, 39.20% ± 2.28% and 50.4% ± 2.7%, respectively (*F* = 502.71, *P* < 0.05).

In order to investigate the mechanisms for Rh2 induc-

ing Bxpc-3 cell apoptosis, western blotting and RT-PCR analysis of related apoptotic proteins and mRNA were used, including Bax, Bcl-2, survivin, cleaved caspase-3, caspase-8 and caspase-9. The results revealed significant downregulation of Bcl-2 and survivin, and upregulation of Bax, cleaved caspase-3 and caspase-9 (*P* < 0.05), but no change in cleaved caspase-8 (*P* > 0.05; Figure 4). The ratios of Bax/GADPH (0 μmol/L, 35 μmol/L, 45 μmol/L and 55 μmol/L) were the following: 0.815 ± 0.147, 1.169 ± 0.127, 2.226 ± 0.398 and 12.580 ± 2.592, respectively (*F* = 109.651, *P* < 0.05). The ratios of Bcl-2/GADPH were 1.964 ± 0.221, 1.407 ± 0.163, 1.020 ± 0.141 and 0.726 ± 0.136, respectively (*F* = 60.424, *P* < 0.05). The ratios of survivin/GADPH were 2.959 ± 0.296, 1.406 ± 0.118, 1.004 ± 0.169 and 0.473 ± 0.129, respectively (*F* = 187.58, *P* < 0.05). The ratios of cleaved caspase-3/GADPH were 0.257 ± 0.095, 0.460 ± 0.097, 1.439 ± 0.111 and 1.805 ± 0.076, respectively (*F* = 367.81, *P* < 0.05). The ratios of cleaved caspase-9/GADPH were 1.096 ± 0.105, 1.457 ± 0.079, 1.900 ± 0.097 and 2.420 ±

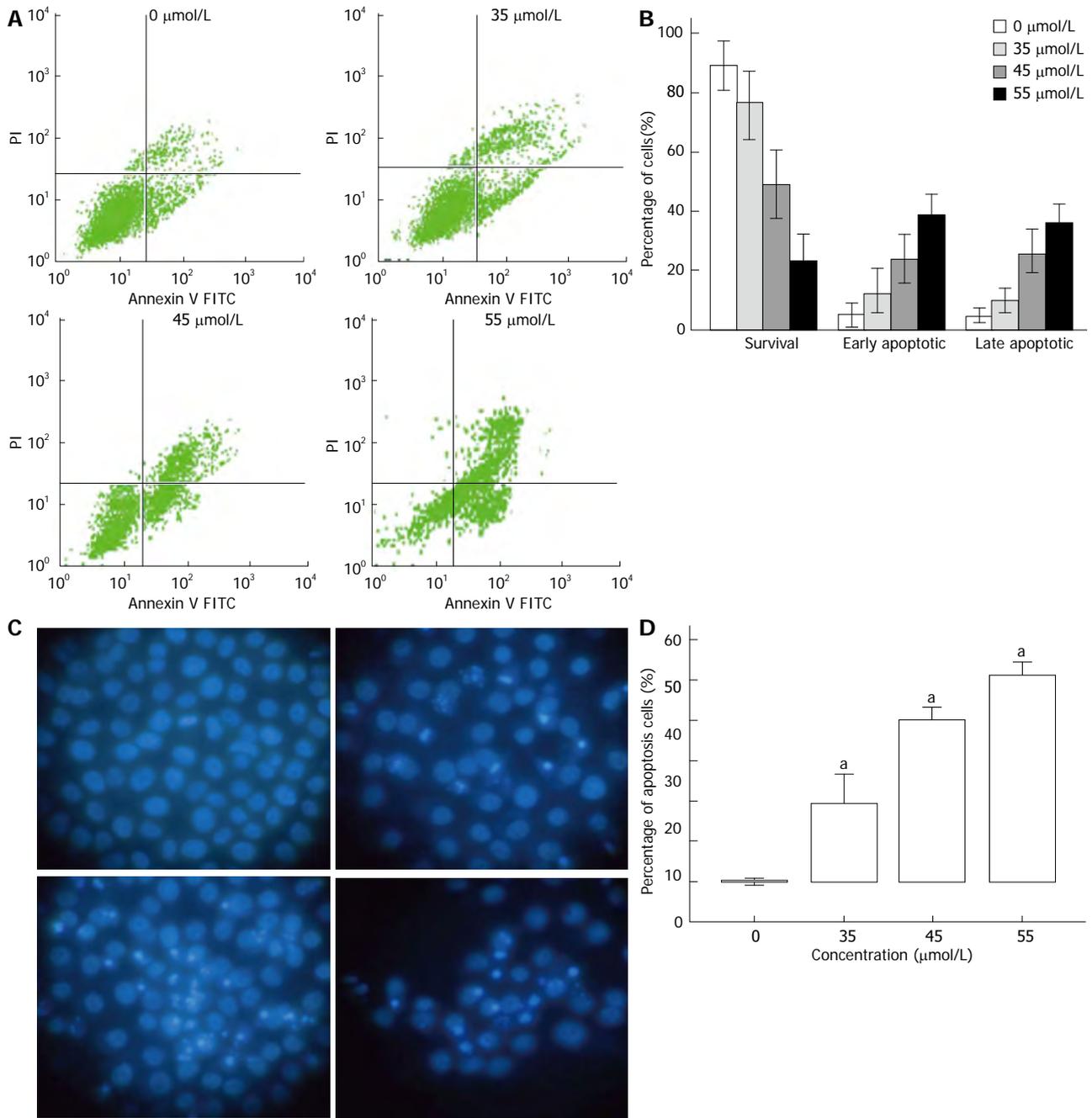


Figure 3 Rh2 induces Bxpc-3 cells apoptosis. A, B: An apoptosis assay was carried out using flow cytometry after Annexin V-FITC/PI staining. Viable cells are in the lower left quadrant, early apoptotic cells are in the lower right quadrant, late apoptotic or necrotic cells are in the upper right quadrant, and nonviable necrotic cells are in the upper left quadrant. The data showed that Rh2 increased the percentages of early and late apoptotic cells; C, D: An apoptosis assay was also carried out using Hoechst 33 258 staining. Nuclei were stained weak homogeneous blue in the normal cells, and bright chromatin condensation and nuclear fragmentation were found in the apoptosis cells. The percentages of apoptosis cells treated with Rh2 were increased, ^a*P* < 0.05 vs 0 μmol/L group.

0.238, respectively (*F* = 93.925, *P* < 0.05). The ratios of cleaved caspase-8/GADPH were 0.464 ± 0.095, 0.469 ± 0.106, 0.507 ± 0.112 and 0.468 ± 0.066, respectively (*F* = 0.255, *P* = 0.857). The ratios of Bax, Bcl-2, and survivin/β-actin (0 μmol/L and 45 μmol/L) were 1.148 ± 0.007 vs 1.361 ± 0.098 (*t* = -4.34, *P* < 0.05), 1.482 ± 0.120 vs 1.149 ± 0.143 (*t* = 4.358, *P* < 0.05) and 1.053 ± 0.144 vs 0.654 ± 0.120, respectively (*t* = 5.235, *P* < 0.05).

Rh2 inhibiting Bxpc-3 cells invasion and migration

We first examined the effect of Rh2 on the migration of

Bxpc-3 cells. For the migration assay, a scratch wound healing assay was used. In the scratch wound healing assay, treatment with Rh2 of 45 μmol/L for 12 h, 24 h and 48 h significantly inhibited the migration of Bxpc-3 cells compared to the control group (Figure 5A and B). Compared to the control group, the rates of scratch wound healing for 12 h, 24 h and 48 h were the following (control vs trial group): 37.3% ± 4.8% vs 18.3% ± 1.65% (*t* = 6.489, *P* < 0.05), 58.7% ± 3.5% vs 38.00% ± 4.09% (*t* = 6.656, *P* < 0.05) and 93.83% ± 4.65% vs 65.50% ± 4.09% (*t* = 7.926, *P* < 0.05), respectively.

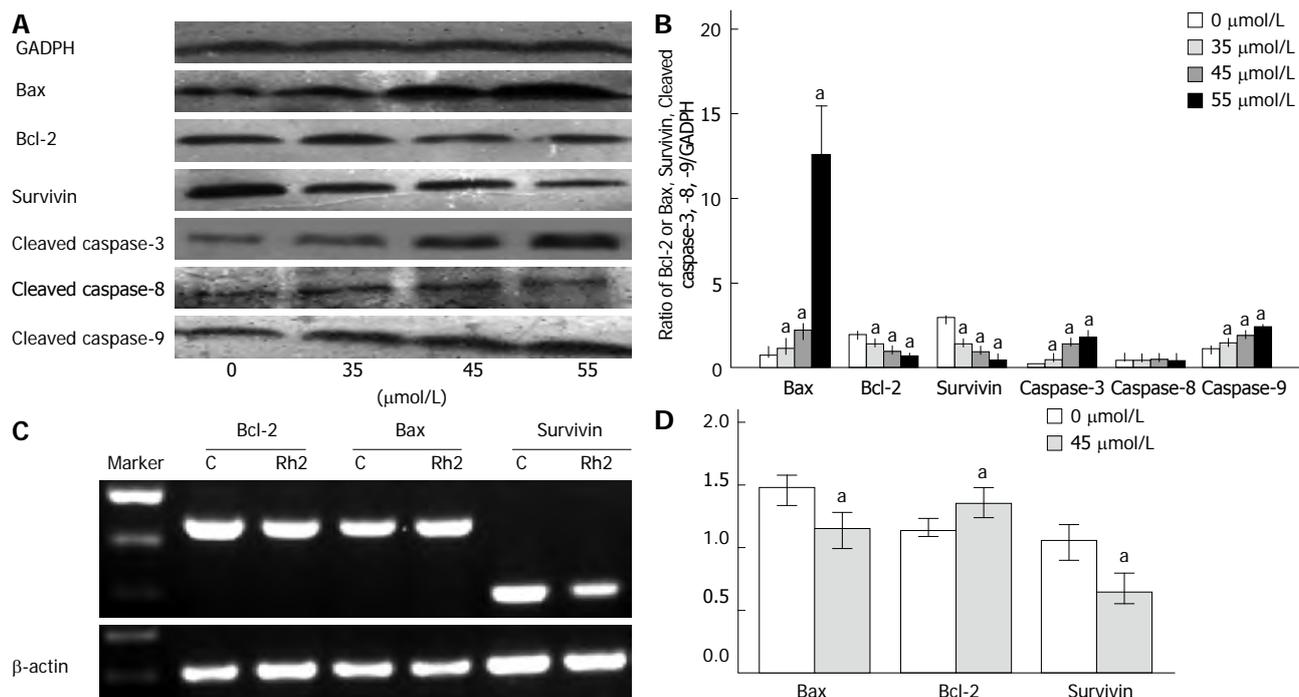


Figure 4 Effects of Rh2 on proteins and mRNA expression of Bax, Bcl-2, survivin, cleaved caspase-3, caspase-8, caspase-9 in Bxpc-3 cells. A, B: Western blotting was used to examine protein expression of Bax, Bcl-2, survivin, cleaved caspase-3, caspase-8 and caspase-9. Rh2 upregulated protein expression of Bax, cleaved caspase-3 and caspase-9 in a dose-dependent manner and downregulated protein expression of Bcl-2 and survivin in a dose-dependent manner. However, Rh2 had no effect on protein expression of cleaved caspase-8; C, D: Reverse transcriptase-polymerase chain reaction was used to examine the expression of Bax, Bcl-2 and survivin mRNA. Rh2 (45 $\mu\text{mol/L}$) upregulated mRNA expression of Bax, and downregulated mRNA expression of Bcl-2 and survivin, $^aP < 0.05$ vs 0 $\mu\text{mol/L}$ group.

We next examined the effect of Rh2 on Bxpc-3 cell invasion using the Matrigel invasion assay. Compared to the control group, Rh2 inhibited cell invasion in a concentration-dependent manner. Even the lowest concentration of Rh2 (35 $\mu\text{mol/L}$) significantly inhibited cell invasion (Figure 5C and D). The numbers of cells invading through Matrigel and filters into the lower surface in the control group and the group treated with Rh2 in various concentrations were as follows (0 $\mu\text{mol/L}$, 35 $\mu\text{mol/L}$, 45 $\mu\text{mol/L}$ and 55 $\mu\text{mol/L}$): 81.10 ± 9.55 , 46.40 ± 6.95 , 24.70 ± 6.88 and 8.70 ± 3.34 , respectively ($F = 502.713$, $P < 0.05$).

We also used RT-PCR and ELISA to detect the expression of factors related to migration: that is, MMP-2 and MMP-9. Compared to the control group, Rh2 downregulated expression of MMP-2 and MMP-9 mRNA and protein in a dose-dependent manner (Figure 6). The ratios of MMP-2/ β -actin (0 $\mu\text{mol/L}$ and 45 $\mu\text{mol/L}$) were 0.644 ± 0.074 vs 0.424 ± 0.063 ($t = 5.543$, $P < 0.05$). The ratios of MMP-9/ β -actin (0 $\mu\text{mol/L}$ and 45 $\mu\text{mol/L}$) were 0.995 ± 0.105 vs 0.408 ± 0.105 ($t = 9.679$, $P < 0.05$). Protein expression levels of MMP-2 (0 $\mu\text{mol/L}$, 35 $\mu\text{mol/L}$, 45 $\mu\text{mol/L}$ and 55 $\mu\text{mol/L}$) were 126.128 ± 9.132 , 86.681 ± 8.134 , 62.033 ± 6.979 and 37.672 ± 6.671 , respectively ($F = 140.802$, $P < 0.05$). Protein expression levels of MMP-9 (0 $\mu\text{mol/L}$, 35 $\mu\text{mol/L}$, 45 $\mu\text{mol/L}$ and 55 $\mu\text{mol/L}$) were 127.652 ± 6.792 , 94.235 ± 7.427 , 67.704 ± 6.731 and 44.195 ± 6.705 , respectively ($F = 161.173$, $P < 0.05$).

DISCUSSION

Ginsenosides are the major pharmacologically active components of ginseng and they exhibit various biological effects such as anti-inflammatory and anticancer effects^[14]. Ginsenoside Rh2 is one of the main bioactive components in ginseng extracts and has been reported in both *in vitro* and *in vivo* studies to possess potent antitumor activity, including inhibition of cell growth and induction of apoptosis in various tumor cells. However, there are no relevant reports about the effects of ginsenoside Rh2 on pancreatic cancer. In this study, we investigated the effects of Rh2 on apoptosis, proliferation, invasion and migration of the human pancreatic cancer cell line Bxpc-3.

The typical characteristics of the abnormal proliferation of tumors are out-of-control cell reproduction and strong growth. Therefore, inhibiting tumor cell proliferation is the key to controlling tumor development. Studies have shown that Rh2 can inhibit cell viability by inducing cell cycle arrest in human breast cancer cells^[15]. In the cell cycle, cyclin D1 regulates cell proliferation by encoding a key regulator of the cell cycle transition from the G₁ to S phase. We demonstrated that the expression of cyclin D1 was depressed in Bxpc-3 cells treated with Rh2. This result is consistent with MTT, clonogenic assay and flow cytometry results. Flow cytometry showed that Rh2 arrested Bxpc-3 cells in G₁ phase. The data suggested that Rh2 may downregulate cyclin D1 and regulate cell cycle transition, thereby suppressing cancer cell proliferation.

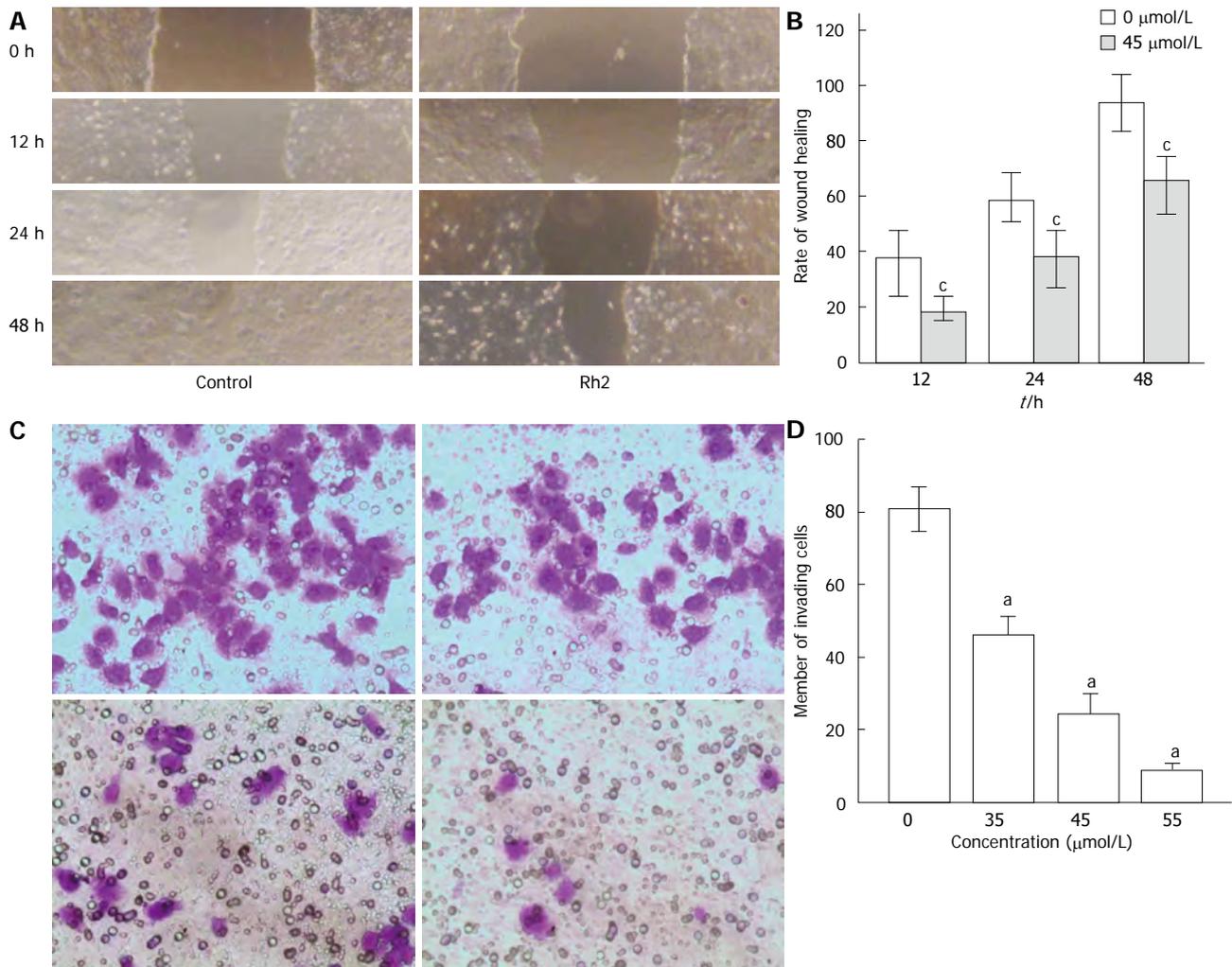


Figure 5 Rh2 inhibits Bxpc-3 cell invasion and migration. A, B: Viability of cell migration was assessed with a scratch wound healing assay and expressed by a wound healing area. Rh2 decreased the rate of scratch wound healing; C, D: Viability of cell invasion was assessed by Matrigel invasion assay and expressed by the number of invading cells. Rh2 decreased the number of invading cells, ^a $P < 0.05$ vs 0 $\mu\text{mol/L}$ group; ^c $P < 0.05$ vs control group.

Except for cell proliferation, we investigated apoptosis in Bxpc-3 cells treated with Rh2. We examined cell apoptosis rates through flow cytometry and Hoechst 33258 fluorescence staining. Our experiments showed that Rh2 could induce Bxpc-3 cell apoptosis in a concentration-dependent manner. What is the mechanism of apoptosis induced by Rh2? Recent reports have shown that Rh2 induces two types of cell death (caspase-dependent apoptosis and caspase-independent paraptosis-like cell death) in colorectal cancer cells through the activation of p53^[16], and Rh2 inhibits cell viability by inducing Bcl-2 family protein-mediated apoptosis in human breast cancer cells^[17]. Therefore, we investigated several apoptosis-related proteins, including Bcl-2, Bax, survivin, cleaved caspase-3, caspase-8 and caspase-9. The Bcl-2 and caspase families are considered to be the most important proteins regulating apoptosis, which can be divided into two types: antiapoptotic and proapoptotic proteins^[18]. Dominant in the two families, Bcl-2 is an antiapoptotic protein, however, Bax, cleaved caspase-3, caspase-8 and caspase-9 are proapoptotic proteins. The activation pathway of

apoptotic protein is divided into the endogenous and exogenous pathways. In the endogenous pathway, caspase-3 is activated, which initiates the apoptotic program^[19]. The other above-mentioned apoptotic proteins, except for caspase-8, belong to the endogenous pathway. Activation of endogenous and exogenous apoptosis-related proteins regulates the expression of cleaved caspase-3, which plays a different apoptotic role. Another important apoptosis inhibiting factor, survivin, is an important member of the inhibitors of apoptosis family and promotes many tumor cells including pancreatic cancer cell survival by regulating the G₁ checkpoint and G₂/M phase of the cell cycle and directly inhibiting caspase-3 and caspase-7 activation^[20-22]. Survivin has attracted growing attention as a potential target for cancer treatment because its expression has been found in primary and cultured tumor cells and its overexpression is associated with poor prognosis^[23]. Our results showed that Rh2 downregulated Bcl-2 and survivin, and upregulated Bax, cleaved caspase-3 and caspase-9. However, it had no effect on cleaved caspase-8. This indicated that Rh2 could induce apoptosis of Bxpc-3 cells mainly

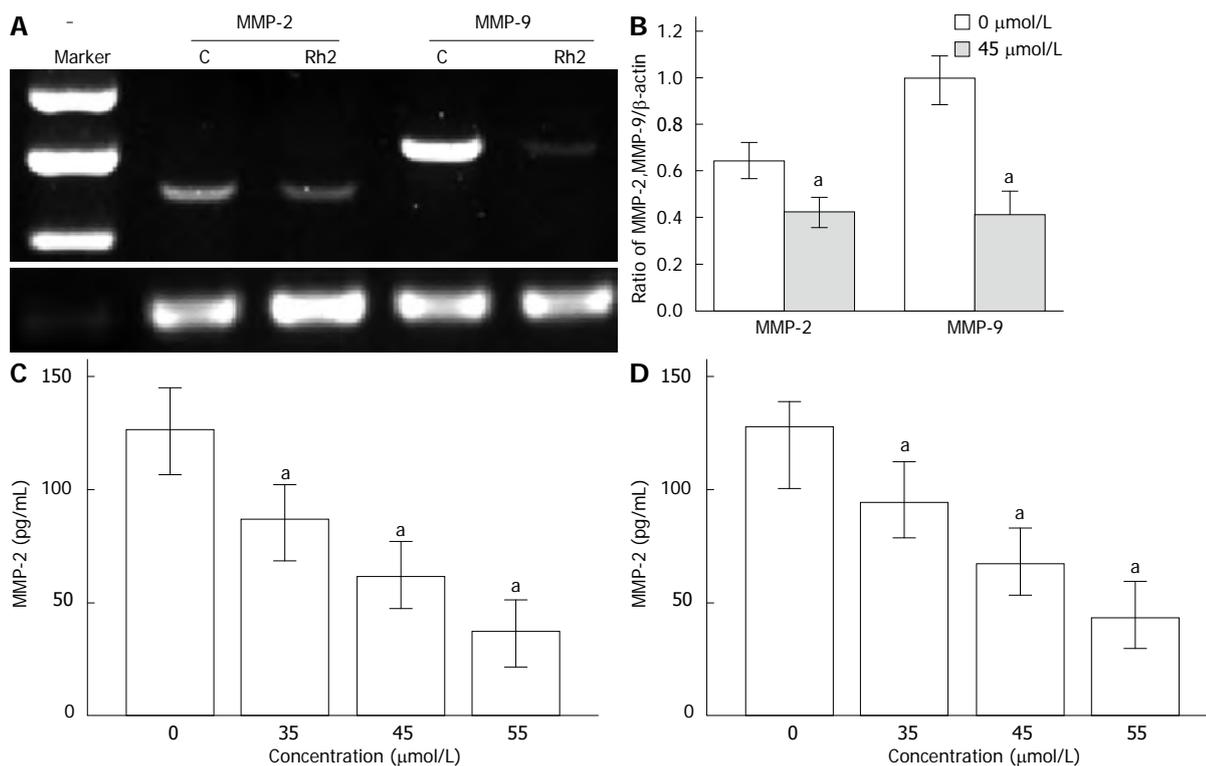


Figure 6 Effects of Rh2 on protein and mRNA expression of matrix metalloproteinase-2 and matrix metalloproteinase-9 in Bxpc-3 cells. A, B: Reverse transcriptase-polymerase chain reaction was used to examine the expression of matrix metalloproteinase (MMP)-2 and MMP-9 mRNA. Rh2 (45 $\mu\text{mol/L}$) downregulated expression of MMP-2 and MMP-9 mRNA; C, D: Enzyme-linked immunosorbent assay was used to examine protein expression of MMP-2 and MMP-9. Rh2 downregulated protein expression of MMP-2 and MMP-9 in supernatants of Bxpc-3 cells in a dose-dependent manner, ^a $P < 0.05$ vs 0 $\mu\text{mol/L}$ group.

through initiating the endogenous apoptotic pathway. However, it remains unclear how Rh2 induced apoptosis of Bxpc-3 cells by initiating the endogenous apoptotic pathway instead of the exogenous pathway. This mechanism merits further investigation in the future.

Another typical characteristic of pancreatic cancer is aggressive metastasis^[24], including local invasion of adjacent structures and metastasis to lymph nodes and liver in the very early stages. Therefore, efforts must be focused on not only targeting the primary tumor but also controlling metastases of pancreatic cancer. Therefore, we examined the effect of Rh2 on invasion and migration of Bxpc-3 cells by using a Matrigel invasion assay and a scratch wound healing assay. Our results showed that Rh2 significantly inhibited cell invasion and migration in a concentration-dependent manner. What is the mechanism of Rh2 in inhibiting Bxpc-3 cell invasion and migration? In invasion and metastasis of tumors, cataplasia of the extracellular matrix is an important component. In such processes, MMPs, as a family of endopeptidases, play a major role and can induce extracellular matrix degradation related to cancer cell invasion and metastasis. Among all members of the MMP gene family, MMP-2 and MMP-9 are considered to be especially important in the degradation of the extracellular matrix that is associated with malignant behavior in a variety of tumor cells^[25-30], including pancreatic cancer^[31]. We therefore examined the expression of MMP-2 and MMP-9, and found that Rh2 significantly downregulated MMP-2 and MMP-9. This

suggested that Rh2 prevented Bxpc-3 cells from invasion and migrating through downregulating MMP-2 and MMP-9.

In summary, we demonstrated the effects of Rh2 on Bxpc-3 pancreatic cancer cells for the first time: (1) Rh2 inhibits cell proliferation by downregulating cyclin D1 and arresting cells in G₁ phases; (2) Rh2 induces cell apoptosis mainly through initiating the endogenous apoptotic pathway; and (3) Rh2 prevents cells from invading and migrating through downregulating MMP-2 and MMP-9. Our study shows that Rh2 can inhibit the proliferation and invasion and induce apoptosis of Bxpc-3 pancreatic cancer cells. As a natural and safe medicine, Rh2 may have future utility in clinical applications for treating pancreatic cancer. Our study will provide a new experimental basis for the clinical application of Rh2 in pancreatic cancer treatments.

COMMENTS

Background

Several studies have revealed that ginsenoside Rh2 could inhibit the growth of many kinds of cancer cells, including breast cancer, prostate cancer, hepatoma, gastric cancer and colon carcinoma. However, little is known about the role of ginsenoside Rh2 in pancreatic cancer. In this study, the authors investigated the effects of ginsenoside Rh2 on the proliferation, apoptosis and migration of cells in the human pancreatic cancer cell line Bxpc-3, and the potential mechanisms were explored.

Research frontiers

This is the first report on ginsenoside Rh2 relevant to pancreatic cancer cells.

Innovations and breakthroughs

By studying the growth-inhibiting, apoptosis-inducing and migration-inhibiting effects of ginsenoside Rh2 on the human pancreatic cancer cell line Bxpc-3, the authors concluded that Rh2 could induce apoptosis and inhibit the growth and migration of cells in the human pancreatic cancer cell line Bxpc-3.

Applications

As a natural and safe medicine, Rh2 may have future utility in clinical applications for treating pancreatic cancer. This study will provide a new experimental basis for the clinical application of Rh2 in pancreatic cancer treatments.

Terminology

Ginsenoside is the main effective component of ginseng, which has been widely used in oriental countries for thousands of years. Ginsenoside Rh2 is a pure compound extracted from ginsenosides.

Peer review

The authors present new findings that constitute the first evidence that this compound may have potential in pancreatic cancer treatment. The data are consistent and provide a solid foundation for future work. The data presented by the authors are interesting. The experiments were well controlled and executed.

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Heme status affects human hepatic messenger RNA and microRNA expression

Herbert L Bonkovsky, Weihong Hou, Nury Steuerwald, Qing Tian, Ting Li, Judy Parsons, Alicia Hamilton, Sunil Hwang, Laura Schrum

Herbert L Bonkovsky, Weihong Hou, Nury Steuerwald, Qing Tian, Ting Li, Sunil Hwang, Laura Schrum, the Liver, Digestive and Metabolic Disorders Laboratory, Cannon Research Center, Carolinas Medical Center, 1542 Garden Terrace, Charlotte, NC 28203, United States

Nury Steuerwald, Judy Parsons, Alicia Hamilton, the Molecular Core Laboratory, Cannon Research Center, Carolinas Medical Center, Charlotte, NC 28203, United States

Author contributions: Bonkovsky HL, Hou W, Steuerwald N, Li T, Hwang S and Schrum L designed the research, analyzed the data, and wrote the paper; Tian Q, Parsons J and Hamilton A performed studies and analyses.

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Correspondence to: Herbert L Bonkovsky, MD, Professor of Medicine, Director of Research, the Liver, Digestive and Metabolic Disorders Laboratory, Cannon Research Center, Carolinas Medical Center, 1542 Garden Terrace, Charlotte, NC 28203, United States. herbert.bonkovsky@carolinas.org

Telephone: +1-704-3557516 Fax: +1-704-3553793

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Abstract

AIM: To assess effects of heme on messenger RNA (mRNA) and microRNA (miRNA) profiles of liver cells derived from humans.

METHODS: We exposed human hepatoma cell line Huh-7 cells to excess iron protoporphyrin (heme) (10 $\mu\text{mol/L}$) or induced heme deficiency by addition of 4, 6-dioxoheptanoic acid (500 $\mu\text{mol/L}$), a potent inhibitor of aminolevulinic acid dehydratase, for 6 h or 24 h. We harvested total RNA from the cells and performed both mRNA and miRNA array analyses, with use of Affymetrix chips, reagents, and instruments (human genome U133 plus 2.0 and miRNA 2.0 arrays). We assessed

changes and their significance and interrelationships with Target Scan, Pathway Studios, and Ingenuity software.

RESULTS: Changes in mRNA levels were most numerous and striking at 6 h after heme treatment but were similar and still numerous at 24 h. After 6 h of heme exposure, the increase in heme oxygenase 1 gene expression was 60-fold by mRNA and 88-fold by quantitative reverse transcription-polymerase chain reaction. We found striking changes, especially up-regulation by heme of nuclear erythroid-2 related factor-mediated oxidative stress responses, protein ubiquitination, glucocorticoid signaling, P53 signaling, and changes in RNAs that regulate intermediary metabolism. Fewer mRNAs were down-regulated by heme, and the fold decreases were less exuberant than were the increases. Notable decreases after 24 h of heme exposure were patatin-like phospholipase domain-containing protein 3 (-6.5-fold), neuronal PAS domain protein 2 (-1.93-fold), and protoporphyrinogen oxidase (-1.7-fold).

CONCLUSION: Heme excess exhibits several toxic effects on liver and kidney, which deserve study in humans and in animal models of the human porphyrias or other disorders.

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Key words: Delta-aminolevulinic acid synthase; Heme; Heat shock proteins; Hepatotoxicity; Messenger RNA; MicroRNA

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INTRODUCTION

Iron protoporphyrin (heme) is a primordial macrocycle upon which nearly all life on earth depends. It has manifold known functions and diverse properties, but there remains much about its roles and functions that is unknown. The importance of a normal pathway and regulation of heme metabolism is underscored by the seriousness of diseases in which there are defects in heme homeostasis. For example, the porphyrias are a group of diseases in which there are defects in normal heme synthesis, due mainly to inborn errors of metabolism that produce deficient activities of the enzymes of normal porphyrin and heme synthesis^[1-4]. Genes and their products of particular importance in heme metabolism are delta-aminolevulinic acid (ALA) synthase 1 (ALAS1) and heme oxygenase 1 (HMOX1), respectively, the rate-controlling enzymes of heme synthesis and catabolism. Regulation of expression of these genes and proteins is complex, but we and others have shown that heme is a major regulator of both, albeit exerting opposite effects. Specifically, heme is a negative regulator of ALAS1 by virtue of decreasing gene transcription, decreasing stability of the messenger RNA (mRNA), decreasing its uptake into mitochondria, where it carries out the synthesis of ALA, and by decreasing the half-life of the mature mitochondrial protein^[2,5-7]. In contrast, heme produces rapid and profound up-regulation of the gene for HMOX1, acting to increase gene transcription by virtue of destabilizing repressive dimers of basic leucine zipper transcription factor 1 (BACH1) and small musculo-aponeurotic factor (maf) proteins and increasing nuclear erythroid-2 related factor (Nrf2)-maf dimers^[8-11].

HMOX1 has emerged as an important anti-inflammatory and cytoprotective principle, and its up-regulation has been associated with several beneficial effects in diverse systems^[2,12]. It is believed to function chiefly by increasing levels of carbon monoxide and biliverdin, which are important signaling and anti-oxidant molecules, respectively. In most mammals, biliverdin is rapidly reduced to the more lipophilic bilirubin, which also has potent anti-oxidant properties and which more readily passes through and dissolves in biological membranes, where it can exert its anti-oxidant and protective functions^[13,14]. In addition, HMOX1 will decrease high levels of heme, which itself is a potential strong pro-oxidant, leading to formation of carbon monoxide CO, biliverdin, and iron. The importance of HMOX1 is underlined by the severe pathology of mice or humans with severe HMOX1 deficiencies^[15-18].

Intravenous (IV) heme was first used as a therapeutic agent more than 40 years ago, for treatment of acute porphyrias in relapse^[19], based upon the understanding that uncontrolled up-regulation of hepatic ALAS1 was a hallmark of acute porphyric attacks and that this enzyme could be dramatically and rapidly down-regulated by administration of heme intravenously. This treatment has stood the test of time, and IV heme continues to be the treatment of choice for all but mild, self-limited attacks

of acute porphyria^[1,3,4,20,21]. Recently, IV heme, in the form of hematin, was reported to also be of benefit in the treatment of acute pancreatitis in mice^[22].

Small, non-coding RNAs, such as microRNAs (miRNAs), have emerged as important modulators of gene expression. They bind to complementary sequences (called “seed sequences”) of mRNAs, especially in 3'-untranslated regions, and alter their stability and their translation into proteins. Recently, we reported important novel effects of miRNAs-122, -196, and -let 7 on expression of HMOX1 and its key repressor BACH1^[17,23], and we recently discovered new and heretofore unexpected roles of proteasomal and other protease pathways that regulate levels of ALAS1, BACH1, and HMOX1^[17,24].

Because of the several effects of heme on hepatic pathways and because of its role as a therapeutic agent, in this work we set out to characterize more nearly completely the comparative effects of heme excess *vs* heme deficiency in human hepatocytes. We performed detailed studies of mRNA and miRNA profiles under these conditions, and we have found evidence for increased oxidative stress and several other changes in metabolic and signaling pathways by heme.

MATERIALS AND METHODS

Chemicals and reagents

Fe protoporphyrin (heme) was purchased from Frontier Scientific (Logan, UT). 4,6-dioxoheptanoic acid (DHA) was from Sigma-Aldrich (St. Louis, MO). Dimethyl sulfoxide (DMSO) was purchased from Fisher Biotech (Fair Lawn, NJ). Fetal bovine serum (FBS), Dulbecco's modified Eagle's medium (DMEM), trypsin and TRIzol reagent were from Invitrogen Inc. (Carlsbad, CA).

Cell culture and treatments

Human hepatoma cell line, Huh-7 (Japan Health Research Resources Bank, Osaka, Japan) was cultured with DMEM supplemented with 100 units/mL penicillin, 100 mg/L streptomycin, and 10% (v/v) FBS. All cells were maintained in a humidified atmosphere of 95% room air and 50 mL/L CO₂ at 37 °C. Freshly prepared heme (dissolved in DMSO) or DHA (dissolved in water) was added to final concentrations of 10 μmol/L or 500 μmol/L, respectively. After 6 h or 24 h at 37 °C in 50 mL/L CO₂/950 mL/L room air, cells were harvested and washed with ice cold phosphate buffered saline once, and lysed directly with TRIzol reagent (Invitrogen, Carlsbad, CA). Total RNA was extracted according to the manufacturer's instructions and stored at -80 °C until mRNA and miRNA microarrays were performed.

cDNA microarray profiling

Total RNA samples were reverse transcribed, amplified and labeled using GeneChip® 3' IVT Express Kit (Affymetrix Inc., Santa Clara, CA). The resultant labeled cRNA (complementary RNA) was then purified and fragmented as per the manufacturer's instructions. The cRNA samples together with probe array controls were

Table 1 Confirmation and comparison of selected messenger RNA array results with those of quantitative real time polymerase chain reaction for selected genes

| Gene | Array | | qRT-PCR | |
|-----------------------------|-------------|-----------------------|-------------|-----------------------|
| | Fold change | P value | Fold change | P value |
| Heme vs DHA-6 h time point | | | | |
| <i>HMOX1</i> | 59.9 | 9.97×10^{-9} | 87.9 | 10^{-35} |
| <i>IL-8</i> | 20.50 | 3.92×10^{-5} | 13.7 | 0.016 |
| <i>ANXA1</i> | 7.18 | 1.87×10^{-5} | 32.3 | 0.015 |
| <i>BACH1</i> | 3.04 | 3.26×10^{-5} | 3.1 | 0.0019 |
| Heme vs DHA-24 h time point | | | | |
| <i>HSPB8</i> | 77.1 | 2.66×10^{-6} | 92 | 0.010 |
| <i>ANXA1</i> | 45.7 | 5.98×10^{-6} | 138 | 0.015 |
| <i>IL-8</i> | 33.2 | 4.19×10^{-5} | 31.7 | 0.016 |
| <i>HMOX1</i> | 25.6 | 2.50×10^{-5} | 27.6 | 1.33×10^{-7} |
| <i>BACH2</i> | 4.50 | 7.24×10^{-5} | 22.6 | 0.011 |
| <i>PNPLA3</i> | -6.48 | 1.74×10^{-4} | -6.65 | 4.59×10^{-7} |

P values are for comparison of results following treatment with iron protoporphyrin (heme) vs 4, 6-dioxoheptanoic acid (DHA). Listing of all messenger RNAs with ≥ 1.5 fold differences in expression at 6 h, 24 h after treatment of Huh-7 cells with heme (10 $\mu\text{mol/L}$) vs DHA (500 $\mu\text{mol/L}$). Cells were cultured, treated, harvested and total RNA prepared and assays as described in Materials and Methods. qRT-PCR: Quantitative real time polymerase chain reaction; *HMOX1*: Heme oxygenase 1; *IL-8*: Interleukin-8; *ANXA1*: Annexin A1; *BACH*: Basic leucine zipper transcription factor; *HSPB8*: Heat shock 22kDa protein 8; *PNPLA3*: Patatin-like phospholipase domain-containing protein 3.

hybridized onto Affymetrix GeneChip[®] Human Genome U133 Plus 2.0 arrays. Hybridization controls were spiked into the cRNA samples in order to monitor and troubleshoot the hybridization process. Probes for housekeeping genes were used to assess sample integrity. Hybridization, washing, staining and scanning were performed using Affymetrix GeneChip[®] system instruments and protocols.

miRNA microarray profiling

The total RNA was Poly (A) tailed and ligated to biotinylated signal molecules using the FlashTag[™] Biotin RNA labeling Kit (Genisphere, Llc in Hatfield, PA, United States). An enzyme linked oligosorbent assay quantitative-competitive assay was performed to verify labeling prior to array hybridization to GeneChip[®] miRNA 2.0 microarrays (Affymetrix, Santa Clara, CA, United States). Hybridization, washing, staining and scanning were performed using Affymetrix GeneChip[®] system instruments and protocols.

Real-time fluorescent reverse transcription-polymerase chain reaction for quantification of mRNAs

First-strand complementary DNA was synthesized using iScript[™] cDNA synthesis kit (Bio-Rad, Hercules, CA, United States). The reverse transcription reaction was incubated at 42 °C for 30 min and stopped by heating to 85 °C for 5 min. 50 ng of final product was used as template for polymerase chain reaction (PCR). Quantitative reverse transcriptase (qRT)-PCR was performed using TaqMan[®] Probe-Based Detection (Applied Biosystems,

Foster City, CA, United States) per manufacturer's instructions with an ABI Prism 7500 Fast Real-Time PCR System, using Taqman[®] gene expression assays and Taqman[®] Gene expression master mix (Applied Biosystems). Template was amplified by 40 cycles of denaturation at 95 °C for 15 s, annealing of primers and probe together with extension at 60 °C for 1 min in triplicate reactions. Fluorescence data were acquired during a combined anneal/extension step. RT negative reactions were run on each plate to confirm the absence of DNA contamination. Fold change values were calculated using comparative Ct analysis and normalized to those of glyceraldehyde phosphate dehydrogenase, which was an invariant control^[25].

Statistical analysis

Affymetrix GeneChip[®] Command Console Software version 3.0.1 was used to analyze microarray image data and to compute intensity values. Affymetrix files containing raw, probe-level signal intensities were analyzed using Partek Genomics Suite (Partek, St. Louis, MO, United States). Robust multichip averaging was used for background correction, quantile normalization, and probe set summarization with median polish^[26]. Statistical differences were assessed by two-way analysis of variance analysis with false discovery rate. Partek miRNA workflow was used to access TargetScan^[27] target prediction databases to perform miRNA-mRNA target integration. Pathway analysis was performed using Ariadne Pathway Studios (Ariadne Genomics, Rockville, MD, United States). The core analysis function in Ingenuity Pathway Analysis (Ingenuity Systems, Redwood City, CA, United States) was used for canonical and toxicity pathway analyses. Cluster and Treeview software were used to perform miRNA hierarchical cluster analysis and visualization.

RESULTS

Heme excess, compared to control or to heme deficiency, produced by DHA, produced a number of striking changes, particularly up-regulation of mRNAs in Huh-7 cells, as shown in Figure 1A and B. The changes were most numerous and striking at 6 h after heme treatment (Figure 1A), but also were similar and numerous at 24 h (Figure 1C). After 6 h of heme exposure, the increase in *HMOX1* gene expression was 60-fold ($P = 9.97 \times 10^{-9}$) by mRNA array and 88-fold by qRT-PCR (Table 1). *HMOX1* is also known as heat shock protein (HSP)-32. Several other stress-response/heat shock-responsive genes also were markedly up-regulated, including interleukin-8 (*IL-8*), sestrin 2 (*SESN2*), heat shock 22kDa protein 8 (*HSPB8*), stanniocalcin 2, *MAFF*, and annexin A1 (*ANXA1*). mRNAs for lipoprotein receptor and *BACH1*, the latter a major repressor of *HMOX1*, were increased 3.9 and 3.0-fold, respectively Table 1 provides a summary list of some of the more striking changes in mRNA levels at 6 h. It also shows results for the more quantitative method of qRT-PCR. Note that, for the genes studied, the fold

Table 2 Summary of data analysis with use of ingenuity pathway

| Name | 6 h | | 24 h | |
|---|------------------------|----------------|------------------------|----------------|
| | P value | Ratio | P value | Ratio |
| Top canonical pathways | | | | |
| Nrf2-mediated oxidative stress response | 2.64×10^{-11} | 28/193 (0.145) | 1.58×10^{-9} | 61/193 (0.316) |
| Protein ubiquitination pathway | 1.44×10^{-9} | 31/274 (0.133) | 3.26×10^{-8} | 75/274 (0.274) |
| Aldosterone signaling in epithelial cells | 8.31×10^{-8} | 21/170 (0.124) | - | - |
| Glucocorticoid receptor signaling | 1.6×10^{-5} | 24/295 (0.081) | - | - |
| P53 signaling | 1.75×10^{-3} | 10/96 (0.104) | - | - |
| Biosynthesis of steroids | - | - | 3.22×10^{-7} | 14/121 (0.116) |
| Propanoate metabolism | - | - | 1.45×10^{-6} | 24/121 (0.198) |
| Urea cycle and metabolism of amino groups | - | - | 5.8×10^{-6} | 16/78 (0.205) |
| Top toxicity lists | | | | |
| Nrf2-mediated oxidative stress response | 3.03×10^{-11} | 30/237 (0.127) | 5.41×10^{-9} | 66/237 (0.278) |
| Renal necrosis/cell death | 1.05×10^{-4} | 25/314 (0.08) | 1.06×10^{-5} | 79/314 (0.252) |
| Liver necrosis/cell death | 1.79×10^{-3} | 14/166 (0.084) | - | - |
| P53 signaling | 2.06×10^{-3} | 10/95 (0.105) | 1.54×10^{-4} | 30/95 (0.316) |
| Liver proliferation | 2.54×10^{-3} | 12/133 (0.09) | - | - |
| Cholesterol biosynthesis | - | - | 2.26×10^{-13} | 16/16 (1) |
| FXR/RXR activation | - | - | 5.84×10^{-4} | 26/86 (0.302) |

P values are for comparison of results following treatment with iron protoporphyrin (heme) vs 4, 6-dioxoheptanoic acid (DHA). Listing of all microRNAs with ≥ 1.5 fold differences in expression at 6 h after treatment of Huh-7 cells with heme (10 $\mu\text{mol/L}$) vs DHA (500 $\mu\text{mol/L}$). Cells were cultured, treated, harvested and total RNA prepared and assays as described in Materials and Methods. FXR/RXR: Farnesoid X receptor/retinoic X receptor; Nrf2: Nuclear erythroid-2 related factor.

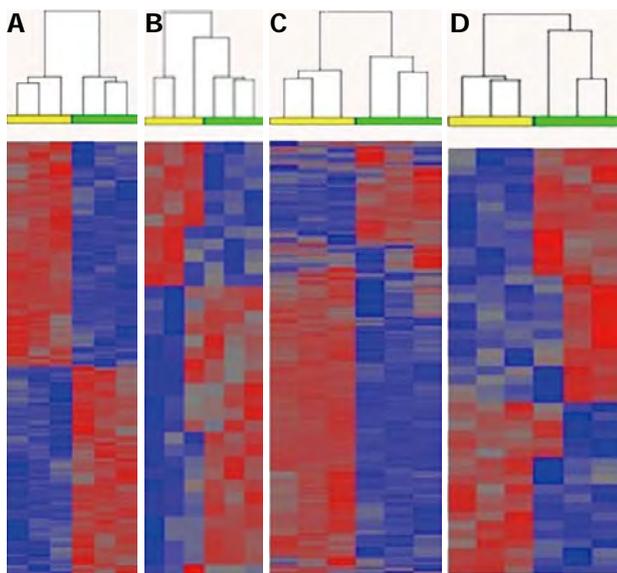


Figure 1 Heat maps with hierarchical clustering of mRNAs (A, C) and microRNAs (B, D) in Huh-7 cells exposed to heme, 10 $\mu\text{mol/L}$ (yellow bars, left side of each panel) or 4, 6-dioxoheptanoic acid, 500 $\mu\text{mol/L}$ (green bars, right side of each panel). A and B: The duration of exposure to heme or DHA was 6 h; C and D: The duration of exposure was 24 h. Cells were cultured, treated, harvested, and RNA arrays performed and analyzed as described in Materials and Methods. Blue color indicates decreased expression; red color indicates increased expression.

differences in expression of mRNAs, comparing heme to DHA treatment, were generally similar by the two methods. The directions of the differences (+ or -) produced by heme vs DHA were always the same (because of limited funds and limited amounts of RNA we were able to directly compare results of mRNA arrays and qRT-PCR for only 4-5 genes).

Fewer mRNA levels were lower with heme excess, and the fold decreases were much less exuberant than were the fold increases. After 24 h of exposure, the greatest fold increase by mRNA array was that of *HSPB8* (77-fold), followed by *ANSA1*, *IL-8*, *HMOX1*, *HSPA1A*, and *SESN2* (46 to 11-fold). At 24 h, there was a notable increase in expression of the *BACH1* gene both by mRNA array (4.5-fold increase by heme) and by qRT-PCT (22.6-fold increase by heme). Notable decreases after 24 h of heme exposure were observed for patatin-like phospholipase domain-containing protein 3 (-6.5-fold), neuronal PAS domain protein 2 (-1.93-fold), and protoporphyrinogen oxidase, the penultimate enzyme of the heme biosynthetic pathway (-1.7-fold). Results of some of the striking differences after 24 h of heme excess vs heme deficiency are summarized in Table 1, which also again shows generally good agreement between results by mRNA array vs qRT-PCR. There also were several changes in miRNA profiles, as shown in Figure 1B and D.

Next, we subjected the mRNA array profiles to analysis by Ingenuity Pathway algorithms. We found striking and highly statistically significant effects at 6 h in Nrf2-mediated oxidative stress response genes, in protein ubiquitination pathway genes, in aldosterone signaling genes in epithelial cells, and in glucocorticoid receptor signaling genes (Table 1). At 24 h, the NRF2-mediated oxidative stress response and the protein ubiquitin pathways continued to show highly significant differences ($P = 1.6 \times 10^{-9}$ and 3.3×10^{-8} , respectively), and pathways involved in biosynthesis of steroids and in propanoate and urea and amino acid metabolism were also significantly affected. Other notable and major effects of heme excess occurred in pathways involved in toxicity, including those that mediate renal necrosis, hepatic necrosis, and

Table 3 Summary of analysis with ingenuity pathway analytical tools

| Name | 6 h | | 24 h | |
|---|---|---------------|---|---------------|
| | P value | No. molecules | P value | No. molecules |
| Hepatotoxicity | | | | |
| Liver proliferation | 6.71×10^{-4} - 6.26×10^{-1} | 12 | 4.24×10^{-3} - 1.63×10^{-1} | 36 |
| Liver necrosis/cell death | 2.08×10^{-3} - 3.77×10^{-1} | 15 | - | - |
| Liver damage | 3.31×10^{-3} - 1.25×10^{-1} | 12 | - | - |
| Liver hemorrhaging | 7.28×10^{-3} - 7.28×10^{-3} | 2 | - | - |
| Hepatocellular peroxisome proliferation | 3.57×10^{-2} - 3.57×10^{-2} | 1 | - | - |
| Liver cholestasis | - | - | 1.11×10^{-5} - 6.55×10^{-1} | 32 |
| Hepatocellular carcinoma | - | - | 2.46×10^{-1} - 2.46×10^{-4} | 54 |
| Liver steatosis | - | - | 2.59×10^{-3} - 1.00×10^{-0} | 34 |
| Liver fibrosis | - | - | 1.51×10^{-2} - 4.43×10^{-1} | 19 |
| Nephrotoxicity | | | | |
| Renal necrosis/cell death | 7.22×10^{-5} - 4.21×10^{-1} | 25 | 7.44×10^{-7} - 1.00×10^{-0} | 79 |
| Kidney failure | 1.27×10^{-3} - 2.24×10^{-1} | 10 | 2.64×10^{-2} - 6.55×10^{-1} | 26 |
| Renal enlargement | 1.27×10^{-3} - 1.03×10^{-1} | 2 | 2.64×10^{-2} - 4.13×10^{-1} | 2 |
| Renal damage | 3.09×10^{-2} - 5.83×10^{-1} | 4 | | |
| Glomerular | 3.57×10^{-2} - 5.17×10^{-1} | 2 | | |
| Renal tubule injury | | | 4.27×10^{-5} - 1.63×10^{-1} | 30 |
| Renal proliferation | | | 2.17×10^{-3} - 4.83×10^{-1} | 24 |

P values for the comparison of iron protoporphyrin (heme) *vs* 4, 6-dioxoheptanoic acid (DHA) are shown. Listing of all microRNAs with ≥ 1.5 fold differences in expression at 24 h after treatment of Huh-7 cells with heme (10 $\mu\text{mol/L}$) *vs* DHA (500 $\mu\text{mol/L}$). Cells were cultured, treated, harvested and total RNA prepared and assays as described in Materials and Methods.

those involved in P53 signaling, liver proliferation, cholesterol biosynthesis, and nuclear receptor (farnesoid X receptor, retinoic X receptor) dependent activation (Table 2).

We examined the pathways of hepatotoxicity and nephrotoxicity in greater detail (Table 3). At 6 h after heme or DHA treatment, 12 molecules involved in liver proliferation, 15 in liver cell damage/necrosis, and 12 in liver damage pathways were markedly affected, whereas, at 24 h, 32 involved in cholestasis, 54 in hepatocellular carcinoma, 34 in hepatic steatosis, and 19 in hepatic fibrosis were increased by heme (Table 3). With respect to molecules involved in pathways of renal damage, many involved in renal cell damage, renal failure, tubular injury and proliferation were up-regulated following heme exposure (Table 2).

Changes in miRNA levels are shown as heat maps in Figure 1B and D. Note that miR-181c, -296-5p, -513a-5p, and -637, were significantly affected by heme, compared with DHA. All were up-regulated by heme, with the exception of miR-513a-5p, which was down-regulated -3.84 fold ($P = 0.034$) at 24 h post-treatment.

Figure 1C and D provides a summary of the major pathways affected by heme excess (*vs* heme deficiency, induced by DHA) in human Huh-7 cells. Note the striking effects on HSPs, on *HMOX1*, on *BACH1* and *BACH2*, and on *FOXP3*, *CEBP*, *JUN*, *MYC*, *ATF3*, *GCLO*, *GDF*, *IRS2*, *EGR1*, and lesser but wide-spread up-regulation of expression of *CYP* genes, including *CYP2E1*, *CY1*, *3A4*, *CYP2B6*, *CYP2C9*, *CYP1A2*.

DISCUSSION

The major findings of this work are as follows: (1) heme

excess *vs* heme deficiency, produced by exposure of cells to DHA for 6 or 24 h, leads to marked changes in global gene expression in the Huh-7 line of cells derived from a human hepatocellular carcinoma; (2) a large number of genes that are activated by stressful conditions (stress response genes), including several HSPs, Nrf2 and NQO1 are markedly up-regulated by heme; (3) several important transcription factors, including FOS, JUN, MYC, ATF, BACH1, BACH2, SMADs, CEBP, are up-regulated by heme excess; (4) marked up-regulation of NRF2-mediated, protein ubiquitination, steroid signaling, P53 signaling, and renal and liver cell necrosis pathways is produced by heme excess; and (5) heme excess also produces changes in miRNA profiles, which likely contribute to the modulation of mRNA expressions observed (Figure 1).

A deficiency of hepatic heme is well-known to be associated with disease phenotypes, especially the acute porphyrias in relapse, in which there are partial defects in genes and enzymes of heme synthesis, which, with other genetic and environmental factors, lead to a critical deficiency in the regulatory heme pool of hepatocytes and hence to uncontrolled and marked up-regulation of hepatic ALS synthase-1, which normally is the rate-controlling enzyme of heme synthesis^[1-4]. Acute attacks of porphyria cause much morbidity loss of productivity, hospitalization, and occasionally acute mortality.

Realization of this cascade of effects led one of us (Bonkovsky HL) to develop heme as a potential therapy for acute porphyria in relapse^[19]. This therapy has withstood the test of time and still today. The prompt administration of heme intravenously is the treatment of choice for acute porphyria attacks^[1,20,21]. It also has benefits in other diseases in which there are defects in normal heme synthesis or excesses of ALA production,

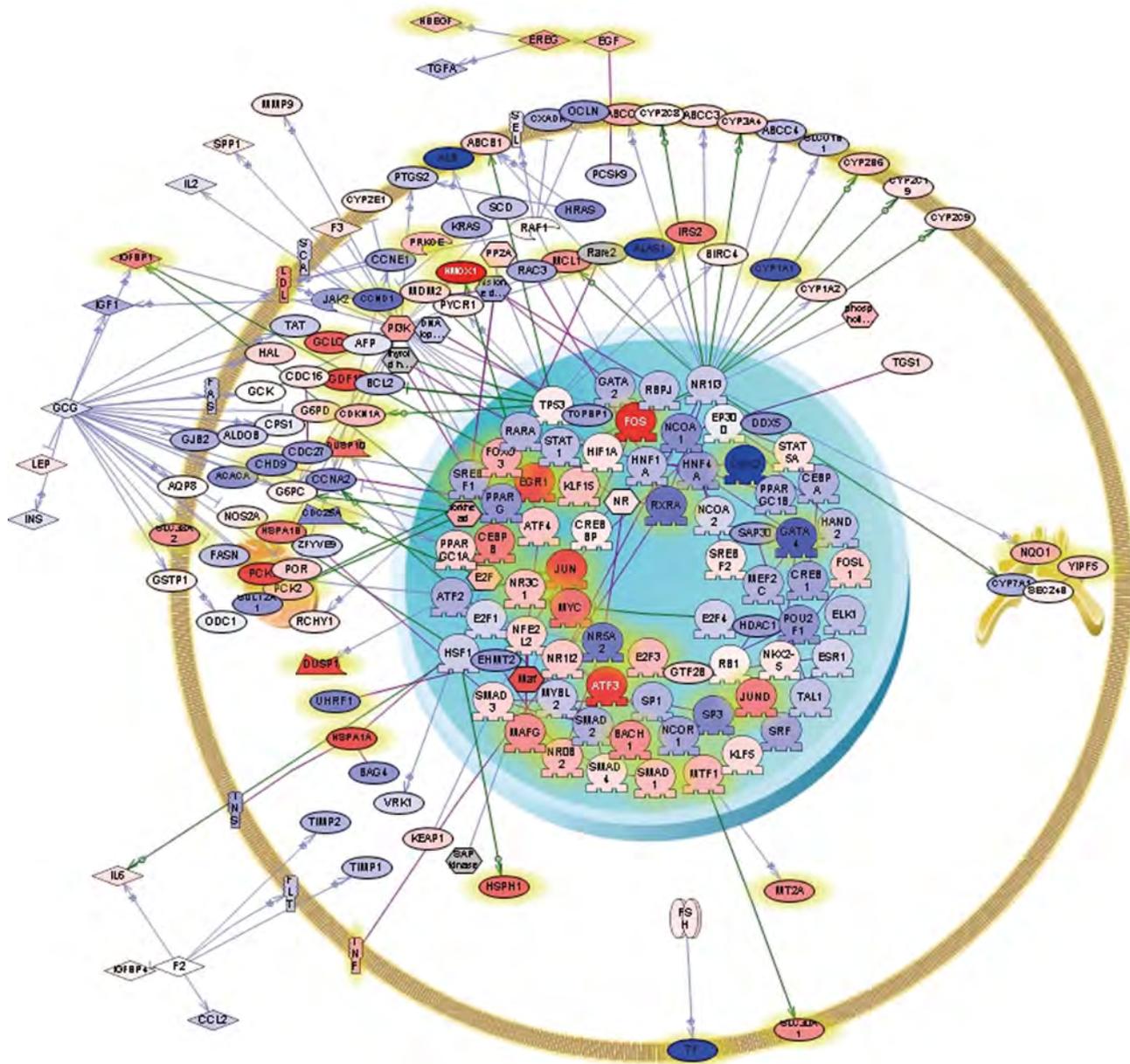


Figure 2 Pathway analysis illustrating differential gene expression in iron protoporphyrin- vs 4, 6-dioxioheptanoic acid- treated Huh-7 cells. Cells were cultured and exposed to iron protoporphyrin (10 μmol/L) or 4, 6-dioxioheptanoic acid (500 μmol/L) for 6 h, after which they were harvested and total RNA isolated using TRIzol. Pathway analysis was performed using Ariadne Pathway Studio by defining sub-networks of genes selected using gene set enrichment analysis ($P \leq 0.01$). The resulting sub-networks were combined. Entities satisfying the filtering criteria are depicted. The intensity of red or blue of the entities themselves indicates the corresponding degree of up- or down-regulation, respectively. Differing shapes are used to represent the entity types and the relationships among them.

which likely is neurotoxic^[28]. In addition, heme has been of benefit in managing other forms of porphyria, especially congenital erythropoietic (uro)porphyria^[29-31] and erythropoietic protoporphyria^[32-34].

In addition, as shown previously by us^[5,9,12,35,36] and others^[37], and as confirmed in this work (Figure 1A and B; Table 1), heme is a potent inducer of the *HMOX1* gene. *HMOX1* is a key cytoprotective and anti-oxidant gene, exerting a myriad of beneficial effects on diverse tissues and in diverse conditions, especially oxidative stress (for reviews see^[2,12,38-40]). Indeed, heme has recently been shown to ameliorate experimental pancreatitis in mice^[22,41], and similar effects seem likely for other inflammatory diseases, based upon the emerging anti-inflammatory and immuno-

suppressive effects of carbon monoxide, biliverdin, and bilirubin, the products of the HMOX-catalyzed breakdown of heme^[21,42]. Heme has also shown promise for blocking the replication of the hepatitis C virus^[23,24,36], at least in part by virtue of its effects BACH1 and HMOX1. The results of this work introduce a note of caution into the therapeutic uses of heme. It may be that heme itself is acting as a pro-oxidant and is increasing oxidative stress and reactive oxygen species, leading to up-regulation of several cytoprotective genes. Thus, heme itself may also be toxic, especially if the doses are too high or if HMOX is deficient^[15-17].

The current findings also lend greater weight to the possible usefulness of other means of inducing HMOX1,

such as by cobalt protoporphyrin, which we^[9,35] and others^[43-45] have shown is a potent and long-acting inducer and which, unlike heme, does not increase oxidative stress nor undergo catabolism by HMOX. Thus, its effects may be achieved with lower doses and for longer times than for heme.

We recognize that our results have limitations: We have performed arrays only of mRNA's and miRNAs, and we have not yet confirmed all major changes and results with qRT-PCR. However, it is reassuring that the qRT-PCR data generally are well correlated with the array data (Table 1). In addition, we have not yet performed detailed proteomic analyses or heme-excess *vs* heme-deficient hepatocytes. However, in a preliminary proteomics study, we have found several changes consistent with our findings. Specifically, at 18 h of heme treatment (10 μ mol/L) *vs* the vehicle, DMSO, we found sixty-six proteins differentially expressed to a highly significant degree. Among these, a total of 24 were decreased, whereas 42 were increased in expression, in keeping with the greater up-regulation of mRNAs by heme, noted above. Among those most strongly increased in expression were HMOX1, ubiquitin and ribosome protein S27a precursor, retinal dehydrogenase, HSP70, ferritin light chain, and ferritin heavy chain. In a similar comparison of DHA (500 μ mol/L *vs* DMSO), 5 proteins were significantly decreased in expression, namely, ATIC, CCT8, GANAB, ENO1, and XP07. Thus, there are similarities in the mRNA and protein results, notably HMOX1 and other HSPs. The increases in ferritin light and heavy chain peptides, but not mRNAs, are in keeping with the known post-transcriptional up-regulatory effects of iron on ferritin chain synthesis^[46,47]. One expects that heme treatment will lead to increases in free iron in hepatocytes, as a result of the action of HMOX^[11,12]. This will be the subject of a later manuscript in this series (Figure 2).

In summary, heme excess in a human hepatocyte line produces manifold changes in mRNA and miRNA profiling, including especially effects on oxidative/stress response, protein ubiquitination, steroid signaling (aldosterone, glucocorticoids), cholesterol, propanoate, urea, and amino acid metabolism. These findings emphasize that additional and more in-depth studies of effects of heme on the transcriptome and proteome are needed and that heme should be used with due caution as a treatment of human disease.

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COMMENTS

Background

Heme is used as therapy for acute porphyria in relapse and for other conditions of iron protoporphyrin (heme) deficiency. Because it is a potent inducer of heme oxygenase-1, a major cytoprotective enzyme, heme is proposed as a drug that

may benefit a variety of acute or chronic inflammatory conditions. Heme has also been found recently to bind to and influence levels and activities of several proteins involved in circadian rhythms and intermediary metabolism of carbohydrates and lipids.

Research frontiers

Although heme is a primordial molecule upon which aerobic life as the authors know it depends, the myriad effects of heme on gene and protein expression and metabolic and circadian pathways remain imperfectly understood.

Innovations and breakthroughs

In this work, authors have carried out detailed analyses of alterations caused by heme excess *vs* heme deficiency on messenger RNA and microRNA profiles in the Huh-7 liver cell line derived from a human subject. They also have performed exploratory measures of effects of heme on the proteome of these cells.

Applications

Their results have important implications especially for cautions regarding the use of heme as a therapeutic agent in humans.

Terminology

Heme is one of the class of metalloporphyrins, which include cobalt protoporphyrin, tin- and zinc porphyrins, an others. The circadian rhythm proteins are proteins that influence the normal daily sleep-wake and other 24 h cycles upon which life on earth depends.

Peer review

The data highlight significant heme-dependent alterations in biochemical pathways, which could contribute in understanding aspects of pharmacological heme toxicity. The findings are appropriately discussed and potential limitations are acknowledged. The study is relatively well-done and the results of the microarray approach are convincing.

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Rates and impact of hepatitis on human immunodeficiency virus infection in a large African cohort

Nimzing Gwamzhi Ladep, Patricia Aladi Agaba, Oche Agbaji, Auwal Muazu, Placid Ugoagwu, Godwin Imade, Graham Cooke, Sheena McCormack, Simon David Taylor-Robinson, John Idoko, Phyllis Kanki

Nimzing Gwamzhi Ladep, Graham Cooke, Simon David Taylor-Robinson, Section of Hepatology, Department of Medicine, Imperial College London, St Mary's Hospital Campus, London W2 1NY, United Kingdom

Patricia Aladi Agaba, Oche Agbaji, Auwal Muazu, Placid Ugoagwu, Godwin Imade, John Idoko, AIDS Prevention Initiative in Nigeria, Jos University Teaching Hospital, Jos 930001, Nigeria

Sheena McCormack, MRC Clinical Trials Unit, London, London NW1 2DA, United Kingdom

John Idoko, National Agency for the Control of AIDS, Central Business District, Abuja 905001, Nigeria

Phyllis Kanki, Immunology and Infectious Diseases, Harvard School of Public Health, Boston, MA 02115-5810, United States
Author contributions: Ladep NG, Muazu A, Cooke G and Idoko J designed, carried out part of clinical duties, analysed data; Agaba PA, Agbaji O and Ugoagwu P collated data, checked statistical correctness of the analysis helped with ethical processes and approved the use of data at study site; Ladep NG, Agaba PA, Imade G, Cooke G, McCormack S, Taylor-Robinson SD, Idoko J and Kanki P wrote up and approved final version of manuscript.

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Correspondence to: Nimzing Gwamzhi Ladep, MD, Liver Unit, Imperial College London, St Mary's Hospital Campus, 10th Floor, QEOM Building, South Wharf Road, London W2 1NY, United Kingdom. n.ladep@imperial.ac.uk

Telephone: +44-203-3121909 Fax: +44-203-3121909

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Abstract

AIM: To determine the rates and impact of hepatitis B virus (HBV) and hepatitis C virus (HCV) infections on response to long-term highly active antiretroviral

therapy (HAART) in a large human immunodeficiency virus (HIV) population in Nigeria.

METHODS: HBV and HCV as well as HIV infections are endemic in sub Saharan Africa. This was a retrospective cohort study of 19 408 adults who were recruited between June 2004 and December 2010 in the AIDS Prevention Initiative in Nigeria in Nigeria programme at Jos University Teaching Hospital. Serological assays, including HBV surface antigen (HBsAg) and hepatitis C antibody were used to categorise hepatitis status of the patients. HBsAg was determined using enzyme immunoassay (EIA) (Monolisa HBsAg Ultra3; Bio-Rad). HCV antibody was tested using third generation EIA (DIA.PRO Diagnostic, Bioprobes srl, Milan, Italy). HIV RNA levels were measured using Roche COBAS AmpliCor HIV-1 monitor test version 1.5 (Roche Diagnostics, GmbH, Mannheim, Germany) with a detection limit of 400 copies/mL. Flow cytometry was used to determine CD4+ cell count (Partec, GmbH Munster, Germany). Comparison of categorical and continuous variables were achieved using Pearson's χ^2 and Kruskal Wallis tests respectively, on MedCalc for Windows, version 9.5.0.0 (MedCalc Software, Mariakerke, Belgium).

RESULTS: With an overall hepatitis screening rate of over 90% for each virus; HBV, HCV and HBV/HCV were detected in 3162 (17.8%), 1983 (11.3%) and 453 (2.5%) HIV infected adults respectively. The rate of liver disease was low, but highest among HIV mono-infected patients (29, 0.11%), followed by HBV co-infected patients (15, 0.08%). Patients with HBV co-infection and triple infection had higher \log_{10} HIV RNA loads (HBV: 4.6 copies/mL vs HIV only: 4.5 copies/mL, $P < 0.0001$) and more severe immune suppression (HBV: 645, 55.4%; HBV/HCV: 97, 56.7%) prior to initiation of HAART compared to HIV mono-infected patients (1852, 48.6%) ($P < 0.0001$). Of 3025 patients who were 4.4 years on HAART and whose CD4 cell counts results at baseline and end of follow up were

available for analyses, CD4 increase was significantly lower in those with HBV co-infection (HBV: 144 cells/mm³; HBV/HCV: 105 cells/mm³) than in those with HCV co-infection (165 cells/mm³) and HIV mono-infection (150 cells/mm³) ($P = 0.0008$).

CONCLUSION: High rates of HBV and HCV infections were found in this HIV cohort. CD4 recovery was significantly diminished in patients with HBV co-infection.

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Key words: Human immunodeficiency virus; Hepatitis B; Hepatitis C; Africa; Liver disease

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INTRODUCTION

Studies of the prevalence of hepatitis in human immunodeficiency virus (HIV) infected individuals confirm that the rates of hepatitis B virus (HBV) in HIV-infected patients vary widely with tendency towards higher values compared to HBV prevalence in the general population^[1,2]. In the north central region of Nigeria, the prevalence of HBV and hepatitis C virus (HCV) in HIV infected people were 27.8% and 18.3% respectively, and triple infection (HBV/HCV/HIV) was found in 7.2% of 180 HIV infected patients^[3]. HBV prevalence in the Nigeria general population ranges between 10% and 20%^[4].

Although it is widely recommended that HIV infected patients be screened for hepatitis before antiretroviral therapy (ART), no data are accessible to ascertain adherence to this guideline in Nigeria. Reports from Thailand confirm that compliance to hepatitis screening in HIV patients prior to initiating antiretrovirals (ARVs) was poor (55%-69%)^[5,6]. Inadequate epidemiological information on hepatitis in HIV patients may underpin one reason for national health schemes in many developing countries not offering integrated hepatitis services in HIV infected persons. As a consequence, patients co-infected with HBV and HCV are being ignored in regards to timing of antiretroviral therapy, screening for cirrhosis of the liver and hepatocellular carcinoma (HCC), as well as in the choice of ARV regimens that have the potential to optimise their care.

Rising trends in the prevalence of HBV and HCV among HIV-infected individuals during the last decade have been reported in a United States study. That study, involving about 30 000 HIV-infected patients recorded a low, but significantly increasing proportion of patients

being screened for hepatitis; from 20% in 1998 to 60% in 2004^[7]. The researchers found that the rate of HBV and HCV increased from 7% to 8.5% and 9% to 24% respectively. To date, no report of trends in the rate of hepatitis in patients infected with HIV from sub Saharan countries has been published.

The choice of ART regimen can be critical in achieving good treatment outcomes; and knowledge of hepatitis co-infection is vital in this regard. Lamivudine resistance in HIV/HBV co-infected patients on ART has been described in some studies within the West African sub region^[8,9]. A French study has recently demonstrated the advantage of treating HBV-HIV co-infected patients with Tenofovir-containing ARV regimen, particularly for wild type precore mutant and lamivudine-resistant HBV^[10]. Guidelines for the choice of ART regimens generally recommend screening for hepatitis, but this is not routinely undertaken and/or largely depends on availability of resources. Even where screening for hepatitis takes place, a large number of HIV physicians base the choice of ART on available drugs rather than on informed co-morbid conditions. However, changes in treatment guidelines have advocated administration of HBV active ART to co-infected patients in Nigeria.

The importance of well-designed research to answer these questions cannot be overemphasised in order to inform adequate provision of resources for the optimisation of care for HIV/hepatitis co-infected individuals in Africa. We thus aimed to determine the rate of hepatitis screening in HIV infected patients, magnitude of hepatitis co-infection in this large cohort, impact of hepatitis co-infection on baseline HIV parameters, HIV suppression and CD4+ cell increase following HAART.

MATERIALS AND METHODS

Study population

The AIDS Prevention Initiative in Nigeria (APIN) and Harvard School of Public Health HIV program, supported by a grant from the United States President's Emergency Plan for AIDS Relief have been providing ART, at no cost to patients in Nigeria from 2004 till date. This programme is run on a community-based model (although the major sites in Nigeria are located within tertiary health centres), in which integrated community prevention outreaches, on-site HIV screening, counselling, provision of medications, follow up, monitoring and evaluation of all activities are embarked upon. Jos University Teaching Hospital (JUTH) site is one of several centres in Nigeria, with latest HIV prevalence of 4.4%^[11]. JUTH has a specialised centre of care for HIV infection where patients are seen at the outpatient facility at planned intervals of 4-12 wk.

The initial first line ARVs in this population included Stavudine/Zidovudine, Lamivudine and Efavirenz/Nevirapine. However, from 2006, Truvada® (Tenofovir plus Emtricitabine) started to be administered to HIV patients initiating ART in the programme. From June 2004 to December 2010, approximately 19 408 HIV-infected

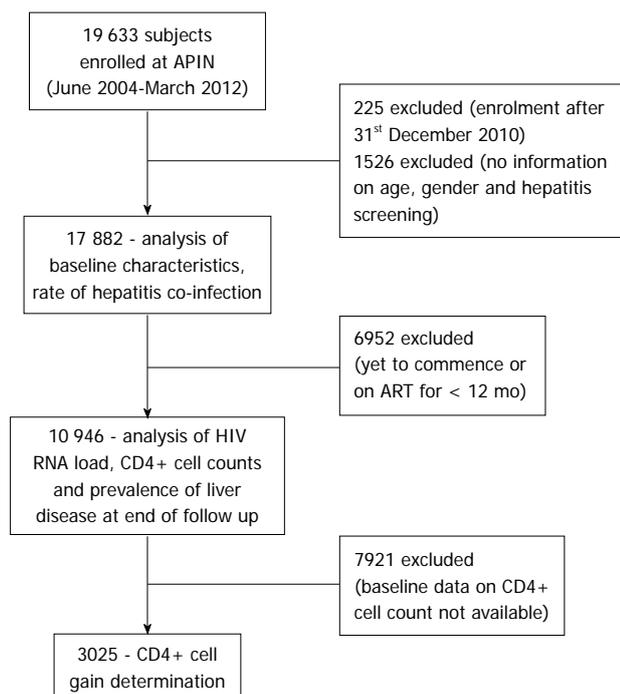


Figure 1 Schematic representation of analyses carried out on recruited human immunodeficiency virus infected individuals at Jos University Teaching Hospital, Nigeria. HIV: Human immunodeficiency virus; ART: Antiretroviral therapies; APIN: Acquired immunodeficiency syndrome prevention initiative in Nigeria.

individuals had been recruited in the JUTH/APIN/Harvard programme and were initiated on ARVs and anti-tuberculosis drugs if indicated.

For the first part of the study, we included all the subjects whose HIV status was confirmed by Western blot assay and enrolled in the programme between 2004 and 2010. Information on age, gender, educational attainment, status of HBV and HCV were obtained. HIV RNA levels and CD4+ cell counts at baseline and most recent assays were also included as were information on the last day of follow up, death or discontinuation of therapy.

Subjects were defined as having HBV and HCV infection if they tested positive for HBV surface antigen (HBsAg) and hepatitis C antibody (HCV Ab) respectively on baseline blood samples. HIV RNA levels and CD4+ cell count were determined at baseline for patients and at 3 monthly intervals until the end point of the study. Hepatotoxicity was defined as alanine aminotransferase (ALT) values ≥ 5 folds over upper limit of normal (ULN) (41 IU/mL for JUTH) or if ≥ 3.5 folds over ULN if baseline ALT was above ULN.

Recruited patients gave written informed consents approved by the ethical committee at JUTH and the institutional review board at the Harvard School of Public Health. For the present work, we obtained a further approval for secondary use of data to study liver-related morbidities in this cohort.

Study design

This was a retrospective cohort study. The pro-forma

utilised in the analyses is summarised in Figure 1. The number of HIV infected individuals that were screened for HBV and HCV were divided by the total number of patients recruited to ascertain the proportion of hepatitis screening. We then calculated the prevalence of HBV and HCV from the numbers that underwent serological testing. We categorised all patients who had hepatotoxicity, liver cirrhosis and hepatocellular carcinoma to a single group (liver disease). As there was overlap of the morbidities, we categorised the cumulative rates of liver related morbidities.

Case-controlled studies of the impact of hepatitis co-infections on baseline HIV viral load and CD4+ cell counts were also embarked upon. HBV, identified by HBsAg, HCV (anti-HCV) and both infections (triple infection) were categorised as cases; which were compared to HIV-only (controls).

Laboratory testing

Before recruitment into the APIN programme, subjects were screened for HIV, using enzyme linked immunoassay and subsequently confirmed by Western blot assay. HBsAg was determined using enzyme immunoassay (EIA) (Monolisa HBsAg Ultra3; Bio-Rad). HCV antibody was tested using third generation EIA (DIA.PRO Diagnostic, Bioprobes srl, Milan, Italy). HIV RNA levels were measured using Roche COBAS Amplicor HIV-1 monitor test version 1.5 (Roche Diagnostics, GmbH, Mannheim, Germany) with a detection limit of 400 copies/mL (Figure 1). Flow cytometry was used to determine CD4+ cell count (Partec, GmbH Munster, Germany).

Statistical analysis

As the diagnoses of HBV, HCV and liver diseases were likely to overlap, we calculated the cumulative prevalence of liver morbidities by overlapping diagnoses; categorised into HBV only, HCV only, HBV/HCV, liver disease only and liver disease with any of HBV, HCV and HBV/HCV. We determined relationships in the demographics of the patients and liver morbidities as well as baseline HIV parameters. The obtained characteristics of HBV, HCV, HBV/HCV and HIV only subjects were compared against each other at baseline using Spearman's chi square and Kruskal Wallis tests for categorical and continuous variables respectively. Analyses were accomplished using MedCalc for Windows, version 9.5.0.0 (MedCalc Software, Mariakerke, Belgium). *P* values of < 0.05 were considered statistically significant.

RESULTS

Between June 2004 and December 2010, 19 408 HIV individuals were enrolled and followed for a median of 53 mo (interquartile range: 31-72 mo). Table 1 presents a summary of the main characteristics of the cohort at baseline. Subjects diagnosed with HBV were more likely to be young (median age: 32 years; $P < 0.001$), male and to have had high HIV RNA loads and CD4+ cell count below 200/mm³. HCV co-infected individuals were more

Table 1 Baseline characteristics by hepatitis status of patients at Jos University Teaching Hospital, 2004-2010 *n* (%)

| Characteristic | Total | HBV | HCV | Triple infection | HIV only |
|------------------------------|---------------|--------------------------|--------------------------|-------------------------|--------------|
| Gender | | | | | |
| Male | 6222 (34.8) | 1214 (38.1) ^b | 764 (37.9) ^b | 178 (39.3) ^b | 4066 (33.2) |
| Female | 11 660 (65.2) | 1971 (61.9) ^b | 1250 (62.1) ^b | 275 (60.7) ^b | 8164 (66.8) |
| Age group (yr) | | | | | |
| 15-29 | 5870 (32.8) | 1097 (34.4) ^b | 463 (23.0) ^b | 120 (26.5) | 4190 (34.3) |
| 30-39 | 7106 (39.7) | 1309 (41.1) ^b | 786 (39.0) ^b | 206 (45.5) | 4805 (39.4) |
| 40-49 | 3619 (20.2) | 615 (19.3) ^b | 533 (26.5) ^b | 94 (20.8) | 2377 (19.5) |
| ≥ 50 | 1287 (7.2) | 164 (5.1) ^b | 232 (11.5) ^b | 33 (7.3) | 828 (6.8) |
| Tuberculosis diagnosis | | | | | |
| Present | 2552 (14.3) | 470 (14.8) | 300 (14.9) | 68 (15.0) | 1714 (14.0) |
| Absent | 15 330 (85.7) | 2715 (85.2) | 1714 (85.0) | 385 (85.0) | 10516 (86.0) |
| Education status | | | | | |
| None | 3230 (18.8) | 527 (17.2) | 471 (24.3) | 87 (19.8) | 2145 (18.3) |
| Primary | 3487 (20.3) | 623 (20.3) | 440 (22.7) | 96 (21.8) | 2328 (19.9) |
| Secondary | 5208 (30.4) | 962 (31.4) | 536 (27.6) | 146 (33.2) | 3564 (30.5) |
| Tertiary | 5219 (30.4) | 954 (31.1) | 494 (25.4) | 111 (25.2) | 3660 (31.3) |
| CD4 (cells/mm ³) | | | | | |
| < 200 | 2937 (50.7) | 645 (55.4) ^b | 343 (52.5) | 97 (56.7) ^b | 1852 (48.6) |
| 200-499 | 2214 (38.2) | 410 (35.3) ^b | 249 (38.1) | 57 (33.3) ^b | 1498 (39.3) |
| ≥ 500 | 646 (11.1) | 108 (9.3) ^b | 61 (9.3) | 17 (9.9) ^b | 460 (12.1) |
| HIV (copies/mL) | | | | | |
| Undetect (< 400) | 2194 (12.4) | 351 (11.2) ^b | 221 (11.1) ^b | 29 (6.7) ^b | 1583 (13.1) |
| Low (400-9999) | 3581 (20.3) | 562 (17.9) ^b | 364 (18.2) ^b | 99 (22.8) ^b | 2556 (21.1) |
| Interm (10 000-29 999) | 2774 (15.7) | 523 (16.7) ^b | 321 (16.1) ^b | 72 (16.6) ^b | 1858 (15.4) |
| High (≥ 30 000) | 9115 (51.6) | 1700 (54.2) ^b | 1090 (53.9) ^b | 234 (53.9) ^b | 6091 (50.4) |

^b*P* < 0.01 *vs* human immunodeficiency virus (HIV) only; Undetect: Undetectable; Interm: Intermediate; HBV: Hepatitis B virus; HCV: Hepatitis C virus.

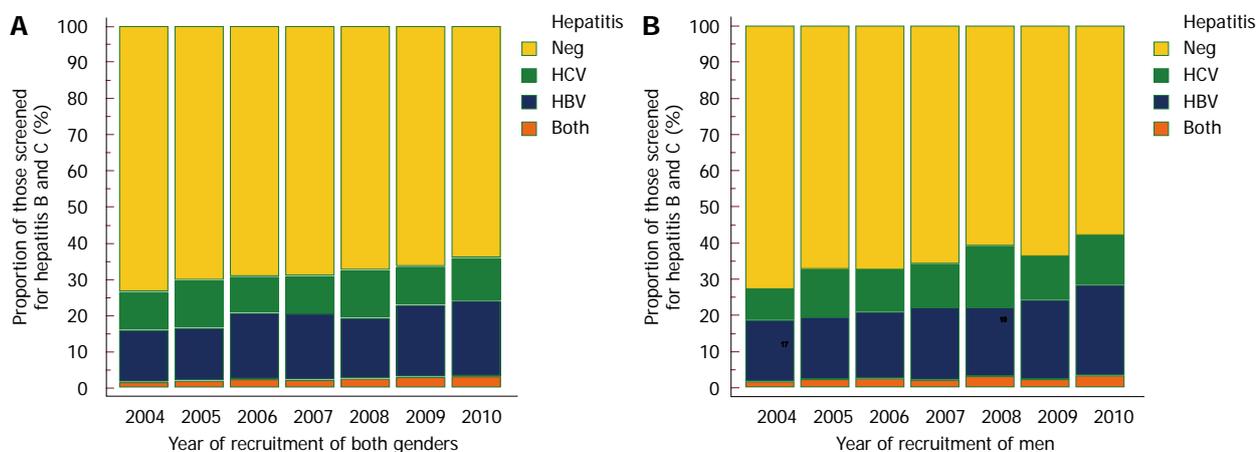


Figure 2 Rates of hepatitis co-infection of human immunodeficiency virus infected individuals in both genders (A) and men (B) among acquired immunodeficiency syndrome prevention initiative in Nigeria cohort, Jos University Teaching Hospital; 2004-2010. HBV: Hepatitis B virus; HCV: Hepatitis C virus.

likely to be males, older (median age: 36 years), have had low level of education and high HIV RNA in their plasma.

Hepatitis screening and prevalence of co-infections

At the beginning of the study period, 99.3% and 99.5% of recruited HIV infected individuals underwent HBV and HCV screening respectively. There has been a significant decline in the rates of screening for HBV and HCV during approximately 7 year study period to 73.0% and 87.6% respectively in 2010 ($P < 0.001$, Figure 2). Overall, the prevalence of HBsAg was 20.7%. A significant increase in the rate of HBV from 14.4% in 2004 to

21.0% in 2010 was observed ($P < 0.001$) and although, fluctuating rates of HCV Ab was recorded among those that were screened, an increasing pattern was noted. The prevalence of HCV Ab was 10.6% in 2004, increasing to 11.7% in 2010. Higher rates of HBV infection was found in men than women rising from 17% in 2004 to 25% in 2010.

Overlapping diagnosis

Cumulatively, 3185 (17.8%) patients were positive to HBsAg and 2014 (11.3%) patients had HCV Ab. 453 (2.5%) patients had evidence of combined HBV and HCV infections (Figure 3). Liver disease was diagnosed

Table 2 Outcome on antiretroviral therapy for at least 12 mo of hepatitis status of human immunodeficiency virus infected individuals at Jos University Teaching Hospital, 2004-2010 *n* (%)

| Variable | Total | HBV | HCV | Both | HIV only |
|------------------------------|---------------|-------------------------|-------------|-------------------------|-------------|
| CD4 (cells/mm ³) | | | | † | |
| < 200 | 2028 (18.5) | 396 (21.3) ^b | 218 (17.5) | 50 (20.2) ^b | 1364 (17.9) |
| 200-499 | 5549 (50.7) | 953 (51.3) ^b | 625 (50.3) | 129 (52.2) ^b | 3842 (50.6) |
| ≥ 500 | 3369 (30.8) | 508 (27.4) ^b | 400 (32.2) | 68 (27.5) ^b | 2393 (31.4) |
| HIV RNA (copies/mL) | | | | | |
| Undetectable (< 400) | 7155 (65.4) | 1186 (63.9) | 809 (65.1) | 155 (62.8) | 5005 (65.9) |
| Detectable (≥ 400) | 3792 (34.6) | 671 (36.1) | 434 (34.9) | 92 (37.2) | 2595 (34.1) |
| Liver disease | | | | | |
| Present | 31 (2.8) | 8 (4.3) | 4 (3.2) | 0 (0.0) | 19 (2.5) |
| None diagnosed | 10 916 (97.2) | 1849 (95.7) | 1239 (96.8) | 247 (100) | 7581 (97.5) |

^b*P* < 0.01 vs human immunodeficiency virus (HIV) only. HBV: Hepatitis B virus; HCV: Hepatitis C virus.

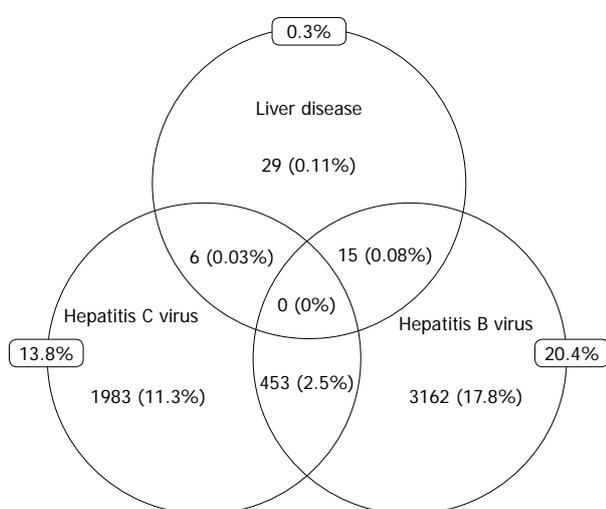


Figure 3 Prevalence of hepatitis B, hepatitis C, hepatitis B and C and liver disease diagnoses among human immunodeficiency virus infected individuals at Jos University Teaching Hospital, 2004-2010.

in 50 (0.3%) patients. Of these, 15 had HBV, 6 had HCV and 29 had no evidence of hepatitis co-infection. None of those with triple infection had a diagnosis of liver disease. Diagnoses of liver disease were achieved via conventional means, including: assessment of sequential liver enzymes, liver ultrasound, and alpha fetoprotein.

Impact of hepatitis co-infection on outcome of ARTs

Higher proportion of HBV (645, 55.4%) and HBV/HCV (97, 56.7%) co-infected patients had CD4+ cell counts below 200 cells/mm³ at baseline compared with HCV (343, 52.5%) and HIV (1852, 48.6%) patients (*P* < 0.0001) (Table 2). The median HIV RNA at baseline was Log₁₀ 4.6 copies/mL each for HBV and HCV patients; and 4.5 copies/mL for HIV mono-infected patients (*P* < 0.0001) (Figure 4). At the end of follow up on ART [median duration: 4.4 years (interquartile range: 2.6-6 years)], no significant difference in HIV RNA load suppression was observed in all study groups. However, there was a significantly lower CD4+ cell increase among those individuals co-infected by HBV/HCV (105 cells/mm³) and HBV (144 cells/mm³) than in HCV (165 cells/mm³)

and HIV-only (150 cells/mm³) patient groups (*P* = 0.008, Figure 5).

DISCUSSION

In this large cohort of HIV infected sub-Saharan African patients, we found that whereas chronic HBV and HCV were frequent diagnoses, liver disease was not common; although investigated only when there were overt clinical symptoms. Overall, one out of every five patients had HBV and more than a tenth had HCV. Almost every patient that was recruited at the beginning of study was screened for HBV and HCV. This is rather remarkable for a resource limited setting and much higher than obtainable in some cohorts in Thailand^[5,6]. The fact that our study site benefited from grants for research and involved the services of specialists may explain the high hepatitis screening rate. However, this initial enthusiasm was not sustained, as there has been a significant decline in the rates of screening for hepatitis during the study period. Interestingly, diagnoses of HBV and HCV showed significant rising trends. We note that there may be a selection bias in this regard as people tend to go to tertiary care centres to seek treatment.

Our findings corroborate the reports of other researchers who had observed higher prevalence of HBV and HCV infection among HIV-infected patients than in the general population^[12]. Studies of prevalence of HBV in the general population of people living within the study area during the period between 2002 and 2007 had found rates of between 10.3% and 15.1%^[13-16]. These confirm that the rates of hepatitis are higher in the HIV patients than in the general population of Nigeria. The fact that HIV and hepatitis viruses share the same routes of transmission supports this explanation. However, it remains unknown whether hepatitis occurs at the same time as HIV infection or predates it.

The precise modes of transmission of HBV and HCV in our cohort are not known. However, it has been reported that transmission of HBV most commonly occurs in early childhood among African populations^[17], compared to high transmission rates among adults in industrialised countries^[18]. Whereas intravenous drug use

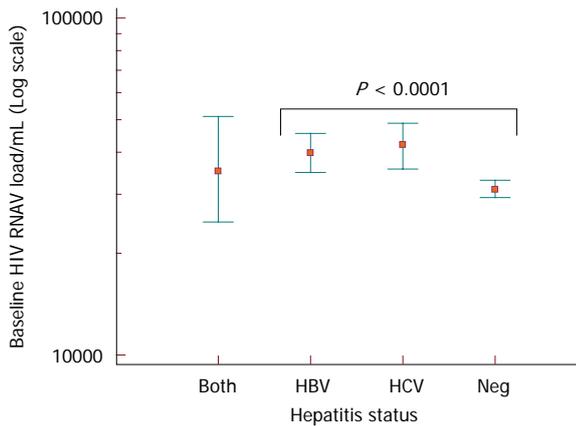


Figure 4 Baseline HIV viral load by hepatitis status among HIV infected patients at AIDS Prevention Initiative in Nigeria, Jos University Teaching Hospital, 2004-2010. HBV: Hepatitis B virus; HCV: hepatitis C virus.

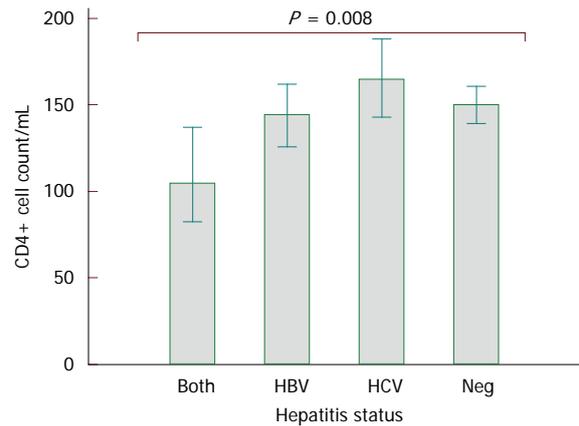


Figure 5 Median CD4 increase by hepatitis status following antiretroviral therapies at Jos University Teaching Hospital, 2004-2010. HBV: Hepatitis B virus; HCV: hepatitis C virus.

is the major route of transmission of HIV and indeed hepatitis viruses in western industrialised countries, heterosexual and horizontal routes, as well as indiscriminate injections (unsterile needles) are thought to be the prevalent modes of transmission of these viral infections in African communities^[19]. Many patients may have iatrogenic transmission from poor sterilisation during routine medical, obstetric, dental and surgical procedures. Most HIV/hepatitis co-infected patients in Nigeria are postulated to have become infected by hepatitis viruses before HIV^[17]. Longitudinal studies will be required to appropriately determine patients that may have acquired hepatitis before, at the same time or after HIV infection. Such a study has the advantage of providing additional information for reinforcing prevention methods, for example HBV vaccination not only for the present cohort but for HIV infected patients in HBV endemic regions.

The incidence of liver disease in the present study was not assiduously documented, although observed to be common. Only 11 patients were documented to have had primary liver cancer in the present study. Eight of these patients were screened for hepatitis. While four were HBsAg positive, 4 were negative to both HBV and HCV. As population-based cancer registries are not routinely available and/or reliable in Nigeria owing to poor registration of diseases and deaths, we did not compare the incidence of primary liver cancer in the present cohort with those from the general population. However, studies in United States have confirmed that primary liver cancer occurs about 6 times more commonly in HIV infected individuals than in the general population^[20,21]. With such a high rate of HBV and HCV infection in this African cohort, there is a chance that a large number of primary liver cancer cases were missed or will yet manifest. It should be noted that prior to free provision of ART to HIV patients in Nigeria from 2004, the cost of these medications was prohibitive and the incidence of HIV mirrored its mortality^[22]. It is thus likely that most patients would have died earlier than they could present with HCC. Furthermore, with prolonged ART, many

of these patients will survive longer and HCC could become more frequently diagnosed.

Unfortunately, the study design did not allow for prospective evaluation of hepatitis status. In addition, the patients were treated with ARVs (*e.g.*, Truvada) that in some cases may have been both active on hepatitis and HIV infection. The baseline evaluation and hepatitis status seems to indicate that other non-infectious causes of hepatic disease may need to be considered. It will be anticipated that HIV hepatitis co-infected patients would have higher incidence of liver disease. This would have been the case if we restricted the definition of liver disease to end stage liver disease (fibrosis, cirrhosis and liver cancer). However, we included hepatotoxicity of ARVs in the definition. This would explain, in part the higher incidence of liver disease in HIV mono-infected patients. Chronic liver disease was small in the cohort, perhaps due to under reporting, or perhaps a high threshold for recording cases in the database. Reasons for the apparent rarity of liver disease in HIV/hepatitis co-infected patients and higher cases of liver disease in HIV mono-infected than hepatitis co-infected patients require further studies.

At baseline, patients with hepatitis co-infection had higher HIV RNA than patients with HIV mono-infection. Correspondingly, a higher proportion of patients co-infected with HBV had CD4+ cell counts below 200/mL compared to HIV mono-infected individuals. The finding of higher HIV RNA at baseline corroborates earlier findings in a study of a small number of patients (1564) from the same study site^[23], as well as another study from China^[24]. Following HAART, the gain in CD4+ cell count was significantly diminished in those patients who had HBV co-infection compared to those with HIV mono-infection. In contrast to our findings, two studies that assessed the impact of HBV on response to HAART found no difference in CD4+ cell gain^[25,26]. Reasons for the differential outcomes are not obvious. Differences in environment are unlikely, as our findings contrast the observation of a study of South African patients^[27]. We

note however, that whereas the South Africa study had a shorter duration of follow up (1.5 years), the present cohort was followed for a longer duration on ART (4.4 years). Nevertheless, results of HBV and long-term HIV outcomes (7 years) in the US found no difference in the HIV load suppression and CD4+ cell gain. Be that as it may, as the natural history of HBV is likely to differ between US and African populations, owing to differential age at acquisition of hepatitis infections, studies comparing African patients on long-term ART would provide a better assessment.

Our findings suggest that CD4+ cell loss by HIV is accentuated by HBV, despite on-going HIV treatment. A few studies have highlighted that active HBV infection is associated with T-lymphocyte exhaustion^[28,29]. This has been further strengthened by the fact that inhibition of HBV DNA replication using anti-HBV drugs resulted in immune restoration^[30,31]. One would expect such an effect to be universal. However, variable outcomes of HAART in regards to HIV load suppression and/or increases in CD4+ cell count in HBV co-infected versus HIV mono-infected patients have been reported. While some studies found no differences between HBV and HIV mono-infected groups^[27,32], others found non-sustained differences in CD4+ cell increases^[25,33]. Studies of HIV treatment outcomes of HBV co-infected and HIV mono-infected African patients comparing HBV suppressive agents versus regimens that are non HBV suppressing will be required to adequately characterise the importance of HBV in the era of HAART.

Our study was not without limitations. First, the use of HBsAg positivity as the sole indicator of chronic HBV infection may be misleading. Definition of chronic HBV would require a positive HBsAg assay consecutively carried out at least 6 mo apart. As single HBsAg was utilised to define cases of HBV in this current study, cases of misclassification might have occurred. Also, delineating HBV cases by their HBV DNA loads and HBeAg status would have provided more meaningful analyses. However, as it is generally known that HBV infection in Africans occur more commonly in childhood, the chance of falsely misclassifying HBV is low. For want of resources, we could not perform HBeAg and HBV DNA. Another issue that could have led to misclassification to hepatitis status is reliance on HCV Ab result to define active HCV infection. Earlier data (unpublished) from a sub group of the current cohort had found HCV viraemia of 33% in those that were HCV Ab positive. It is thus possible that of the patients that were classified “HCV”; only a third may actually be HCV viraemic. More studies with better characterisation of HCV status in this cohort would be required. Missing data was another issue we encountered. In some of the patients, HBsAg and HCV Ab and baseline CD4+ data results were unavailable. Also, we relied on diagnosis of liver diseases (hepatotoxicity, cirrhosis and primary liver cancer) on clinical notes of the patients, where available. As a result of these, we only analysed the rates of hepatitis among those that had hepatitis results. Also, CD4

cell gain was analysed for 3012 patients for whom there were baseline and follow up results and who were on HAART.

In conclusion, high and increasing rates of HBV, HCV and HBV/HCV co-infections were found in this large HIV infected cohort of Africans. The prevalence of liver disease, particularly liver cancer was low; mostly reported among HBV/HIV and HIV-only individuals. HBV co-infection was associated with high HIV RNA load and decreased CD4+ cell counts at baseline and attenuated immunological recovery after a median follow up duration on HAART of 4.4 years. Our findings underscore the urgent need to maintain a strict hepatitis screening policy among HIV infected patients undergoing HAART as well as inclusion of ART regimens with potent anti-HBV activities in HBV endemic regions of the world. Longitudinal studies in African patients to ascertain super-infection of HIV by hepatitis, assessments of impact of HBV-suppressive versus HBV non-suppressive HAART regimens and predictive value of hepatitis on the mortality of the present cohort will form a significant contribution to future research.

ACKNOWLEDGMENTS

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COMMENTS

Background

Owing to shared routes of transmission, co-infection of human immunodeficiency virus (HIV) by hepatitis B (HBV) and hepatitis C viruses (HCV) is common. National guidelines recommend that commencement of antiretroviral therapy be preceded by hepatitis screening. Until the writing of this paper, no data is accessible from Africa to ascertain adherence to this guideline. Perhaps, more interesting is the fact that the impact of HBV (most prevalent hepatitis infection in Africa) on HIV therapy is yet to be determined in a large HIV infected population.

Research frontiers

Studies from Asia have reported a modest hepatitis screening rate among HIV patients. A landmark study in France led to the recommendation of Tenofovir-based antiretroviral regimen for HBV/HIV co-infected patients going on treatment. It will be helpful to lay a foundational study in African patients to ascertain whether the effects of such treatment will be the same; especially as the mode and natural history of chronic viral hepatitis vary between developed and developing countries.

Innovations and breakthroughs

In the present study, the authors characterised the patients based on hepatitis status, comparing them with HIV mono-infected cohort and have shown that HBV in particular was associated with decreased CD4 cell increase following long-term highly active antiretroviral therapy. Additionally, the fact that hepatitis screening rate was about 90% in this Nigeria cohort suggests that the recommendation of pre-highly active antiretroviral therapy (HAART) screening for hepatitis is achievable in resource-limited healthcare settings.

Applications

This study suggests that HBV/HCV infection among HIV population is higher than in the general population of Nigeria and portends considerations for HBV preventive programmes, including vaccination. As HBV co-infection negatively impacted CD4 increase during HAART, individualised treatment algorithms,

which had been advocated by international specialists needs to be followed assiduously in order to reduce morbidity in HIV patients.

Peer review

This article delves into a topic that we have few data, coming from sub-Saharan Africa grappling the scourge of hepatitis and HIV and looks on the impact of the former on HIV care. However, it fails to address the impact of the latter on the course of hepatitis. The surprising revelation is the rarity of liver disease in those with co-infection; this is deserving of further studies.

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Cognitive functioning and depressive symptoms in adolescents with inflammatory bowel disease

Anu E Castaneda, Annamari Tuulio-Henriksson, Eeva T Aronen, Mauri Marttunen, Kaija-Leena Kolho

Anu E Castaneda, Department of Mental Health and Substance Abuse Services, National Institute for Health and Welfare, 00271 Helsinki, Finland

Annamari Tuulio-Henriksson, Research Department, Social Insurance Institute, 00250 Helsinki, Finland

Eeva T Aronen, Child Psychiatry, Children's Hospital, University of Helsinki and Helsinki University Central Hospital, 00250 Helsinki, Finland

Mauri Marttunen, Department of Adolescent Psychiatry, University of Helsinki and Helsinki University Central Hospital, 00271 Helsinki, Finland

Mauri Marttunen, Department of Mental Health and Substance Use Services, National Institute for Health and Welfare, 00271 Helsinki, Finland

Kaija-Leena Kolho, Hospital for Children and Adolescents, Department of Pediatric Gastroenterology, University of Helsinki, 00250 Helsinki, Finland

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Correspondence to: Anu E Castaneda, PhD, Department of Mental Health and Substance Abuse Services, National Institute for Health and Welfare, Mannerheimintie 166, Helsinki PO Box 30, 00271 Helsinki, Finland. anu.castaneda@thl.fi

Telephone: +358-29-5248597 Fax: +358-29-5248478

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sive symptoms in adolescents with inflammatory bowel disease (IBD).

METHODS: A neuropsychological test battery, including subtests of the Wechsler Adult Intelligence Scale-Revised and III, Wechsler Memory Scale-Revised, California Verbal Learning Test (CVLT), Stroop Color-Word Test, and Trail Making Test, which assessed verbal and visual short- and long-term memory, processing speed, logical reasoning, verbal intelligence, attention, and executive functioning, was administered to 13- to 19-year-old patients with IBD ($n = 34$; active disease $n = 20$). Depressive symptoms were measured with the Beck Depression Inventory. The findings were compared with peers with non-acute juvenile idiopathic arthritis (JIA; $n = 23$). Patients with coexisting psychiatric disorders were excluded.

RESULTS: The IBD group, especially patients in the acute phase, made more perseverative errors in the CVLT test that assessed verbal memory than the JIA group (6.0 ± 4.3 vs 3.3 ± 2.9 , $P < 0.01$), but no other differences between the IBD and JIA groups were observed in the neuropsychological tests. The difference was close to statistical significance, even when glucocorticoid medication was controlled for ($P < 0.052$). The IBD group had more depressive symptoms than the JIA group (7.9 ± 7.6 vs 4.0 ± 4.0 , $P < 0.05$). Approximately one third of the IBD group had at least mild depressive symptoms, and those with acute illness had the highest scores. However, depressive symptoms were not related to the difference in the verbal memory test (perseverative errors in the CVLT) between the IBD and JIA groups.

CONCLUSION: Adolescents with acute IBD may have mild verbal memory problems but no major cognitive deficits compared to peers with JIA.

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Abstract

AIM: To investigate cognitive functioning and depres-

Key words: Cognitive impairment; Inflammatory bowel

disease; Crohn's disease; Depressive symptoms; Ulcerative colitis; Adolescents

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INTRODUCTION

Inflammatory bowel disease (IBD) is a chronic life-long disease comprising Crohn's disease, ulcerative colitis and unclassified colitis that may appear at any age. The incidence of IBD among adolescents is increasing^[1-3], and thus, it is important to study the possible burden of the disease on the everyday life of the patients. According to parent reports, adolescents with IBD have more emotional, social, and thought problems and lower competence than their healthy peers^[4]. The disease affects the quality of life of adolescents^[5-7], may have negative consequences on education and school functioning^[8,9], and may cause higher unemployment later in life^[10-13]. Furthermore, adolescents with severe IBD have disturbed sleep and are overtired more often than their healthy peers^[14]. However, research on cognitive dysfunction, which refers to deficits in cognitive information processing, is scarce in patients suffering from IBD.

Two prior studies^[15,16] on adults found some cognitive difficulties among patients with IBD. Attree and co-authors^[15] reported poorer performance in a test measuring verbal functioning in adults with IBD compared to healthy controls. There were, however, no differences in tests assessing attention and mental speed. The other study^[16] also showed that adults with IBD had a decrease in verbal functioning compared with healthy controls and that this was unlikely to be due to premorbid levels of intellectual functioning. Thus, like many other chronic illnesses, IBD seems to be accompanied by some cognitive deficits among adults. However, there are no studies on cognitive functioning among adolescent patients with IBD. In addition, it is not known whether the phase of the illness is associated with cognitive functioning. Given that this disorder often begins in adolescence, it is important to examine whether the illness has an impact on cognitive development and, consequently, the performance of young patients at school.

Depressive symptoms are common among adults^[17] and adolescents with IBD^[18-20]. Depression may also be associated with cognitive impairments^[21,22]. In particular, early-onset depression may be related to deficits in attention, memory, and executive functioning^[23,24]. Depressive symptoms in adolescence might be associated with serious and long-lasting psychosocial difficulties, such as problems in education and work^[25], and cognitive impairments in adolescence and early adulthood may also

complicate school performance and affect successful psychosocial development. Therefore, adolescence is a key period for both the recognition and treatment of mental health and cognitive problems associated with somatic illnesses to avoid long-term sequelae in several areas of psychosocial functioning. Taken together, more studies are needed on illness-related psychosocial and cognitive correlates to improve the care and assessment of young IBD patients.

The main aim of the present study was to examine cognitive functioning among adolescent patients with IBD and investigate whether disease activity or depressive symptoms were associated with cognitive functioning. As a clinical comparison group, an adolescent patient group with minor symptoms of a chronic disease, juvenile idiopathic arthritis (JIA) in the non-acute phase, was included. It was hypothesized that IBD would be related to difficulties in cognitive functioning, especially in verbal functions and that these difficulties would be more pronounced in the acute phase of the disorder.

MATERIALS AND METHODS

Participants

The study groups were enrolled during May 2008-March 2009 at the Hospital for Children and Adolescents, Helsinki, Finland. Consecutive IBD patients aged 13 years or older were invited to participate in the study along with their routine outpatient visits. Likewise, consecutive non-acute JIA patients of the same age group, followed-up at the same outpatient facilities, were invited to participate to represent a control group with non-acute chronic illness (identical recruitment procedure). Patients with coexisting psychiatric, neurological, or developmental disorders (based on hospital case records and information from the patients and their parents) were excluded. All patients were native Finnish speakers and attended regular school, and none had particularly poor school performance (based on self-reports). There were 17 decliners (IBD $n = 8$, JIA $n = 9$). The decliners did not differ from those who participated in age, gender, or severity of the disorder, and the reason for refusal was mainly lack of time. One participant underwent the procedure but was excluded later because of previously diagnosed neuropsychological difficulties and special education at school. None of the included patients had major learning disabilities, but one patient with IBD self-reported as being diagnosed with minor dyslexia in secondary school, with the problems overcome. The final study sample consisted of 34 IBD patients aged 13 to 19 years (ulcerative colitis $n = 16$, Crohn's disease $n = 17$, and unclassified colitis $n = 1$) and 23 JIA patients aged 14-19 years (Table 1). Most patients were full-time students, and only two persons were employees. Current medication included 5-aminosalicylic acid ($n = 26$), azathioprine ($n = 11$), glucocorticoids ($n = 17$), and antibiotics ($n = 3$) in the IBD group and methotrexate ($n = 11$), anti-tumor necrosis factor- α agent ($n = 4$), chloroquine ($n = 4$), and hydroxychloroquine ($n = 4$) in the JIA group.

Table 1 Background data of the study groups

| | IBD (<i>n</i> = 34) | | JIA (<i>n</i> = 23) | |
|-------------------------------------|----------------------|-----------|----------------------|-----------|
| Age (yr) | 16.3 ± 1.7 | 13.6-19.7 | 15.5 ± 1.2 | 14.0-18.6 |
| Age at receiving the diagnosis (yr) | 12.7 ± 3.5 | 2.0-16.7 | 9.0 ± 4.6 | 1.3-15.0 |
| Gender (female) | 15 (44) | | 14 (61) | |
| Socioeconomic status ¹ | | | | |
| Class I | 10 (32) | | 11 (50) | |
| Class II | 9 (29) | | 3 (14) | |
| Class III | 12 (39) | | 7 (32) | |
| Class IV | 0 (0) | | 1 (5) | |
| State (acute) | 20 (59) | | 0 (0) | |
| Disease duration | | | | |
| Less than one year | 6 (18) | | 2 (9) | |
| One to two years | 10 (29) | | 1 (4) | |
| More than two years | 18 (53) | | 20 (87) | |

¹Data missing for three inflammatory bowel disease (IBD) and one juvenile idiopathic arthritis (JIA) patients. Data are expressed as absolute *n* (%) or mean ± SD.

= 1), leflunomide (*n* = 1), and sulfasalazine (*n* = 1) in the JIA group. Three patients with Crohn's disease had undergone surgery more than six months prior to the neuropsychological examination. None of the patients had current psychotropic medication or ongoing psychiatric treatments. The study was approved by the Ethics Committee of the Hospital District of Helsinki and Uusimaa, and it conformed to the provisions of the Declaration of Helsinki and its amendments. Written informed consent was obtained after a complete description of the study from each examined patient or a parent when the examinee was under 18 years of age.

Socioeconomic status

The socioeconomic status of the adolescent's family was classified according to Helsinki City socioeconomic statistics: class I included persons with an academic degree, business managers, and professionals (*e.g.*, engineers); class II included administrative personnel, owners of small businesses, and minor professionals (*e.g.*, graduate nurse); class III included skilled manual employees (*e.g.*, laboratory assistant); and class IV included unskilled employees (*e.g.*, domestic maid). This grading is adapted nationally and comparable to international classification^[26]. The higher socioeconomic class of the parents was considered to be the socioeconomic class of the family (Table 1).

Clinical evaluation

The activity of IBD was based on the clinical evaluation of experienced clinicians (Physician's Global Assessment) and inflammatory markers [erythrocyte sedimentation rate (ESR), C-reactive protein, and fecal calprotectin, with values < 100 µg/g of stool considered normal and values >1000 µg/g considered exceedingly high]^[27]. The disease activity in JIA was based on the child health assessment questionnaire and clinical evaluation by a pediatric rheumatologist.

On the day of the neuropsychological assessment,

patients also completed a questionnaire in the Finnish language gathering information on depressive symptoms and background factors. Depressive symptoms were measured with the Beck Depression Inventory (BDI)^[28]. The BDI is a 21-item questionnaire in which the items are answered using a 4-point rating scale ranging from 0 to 3. High scores indicate a high level of depressive symptoms. The total score was calculated and used in the statistical analyses. Additionally, the BDI total score was categorized into four classes by classifying scores from 0 to 9 as no symptoms, 10 to 18 as mild symptoms, 19 to 29 as moderate symptoms, and 30 to 63 as severe symptoms. Five patients had one missing value in the BDI, which was replaced by their individual item mean scores of the BDI. The patients also completed an in-house questionnaire with questions on school performance, subjective view of learning difficulties, sick leave, employment, and parental professions.

Neuropsychological examination

The neuropsychological examination was conducted individually by a psychologist blinded to the presence of diagnosis or disease activity prior to the examination. The examination took place in one session of approximately one hour. The neuropsychological test battery, including Finnish versions of internationally used, validated test methods administered in a fixed order, was selected to allow for the comparison of verbal and visual short-term memory, verbal long-term memory and learning, attention, logical reasoning and social insight, psychomotor processing speed, and executive functioning between the study samples. Because the cognitive functioning of adolescents with IBD has not been previously investigated, the tests were chosen to evaluate a wide range of neuropsychological functions instead of focusing on only some specific functions. Tests were scored according to standardized procedures by the examiner.

Auditory attention and verbal working memory were assessed with the Digit Span Forward and Backward subtests, respectively, of the Wechsler Memory Scale, Revised^[29] (WMS-R). Visual attention and working memory were measured with the Visual Span Forward and Backward subtests, respectively, of the WMS-R^[29]. Visuo-motor performance and processing speed were assessed with the Digit Symbol subtest of the Wechsler Adult Intelligence Scale, Revised^[30] (WAIS-R). General verbal intelligence was estimated with the Vocabulary subtest of the WAIS-R^[30], and logical reasoning and social insight were assessed with the Picture Arrangement subtest of the WAIS-III^[31]. The Stroop Color-Word Test^[32] (Golden), given in three parts, was administered to evaluate executive functioning, and the interference score was calculated and used in the analysis. The Trail Making Test^[33] (TMT), given in two parts, was administered to evaluate attentive and executive functioning. Part A measures visuo-spatial attention and performance speed, whereas Part B requires mental flexibility, ability to shift attention,

and strategy. Possible errors made by the examinee were not corrected by the examiner. The time to complete Parts A and B and the difference in score between B and A (the executive aspect of the task when the speed component is removed) were used in the statistical analysis. The California Verbal Learning Test^[34] (CVLT), in which the examinee is required to learn a 16-item word list over five trials and recall and/or recognize it after short and long delays, was used to measure various aspects of verbal learning and memory. The following variables of the CVLT were included in the statistical analyses: Total Recall from trials 1-5 (learning performance), Short-Delay Free Recall (short delay memory performance), Long-Delay Free Recall (long delay memory performance), Discriminability (recognition memory taking into account both hits and false positives), Perseverative Repetition Errors, Intrusion Errors, Semantic Clustering (the use of an active learning strategy of reorganizing target words into categorical groups), and Learning Slope (the increase in recalled words per trial over trials 1-5). Higher scores indicate better performance in all tests, except in the Stroop test, TMT and Perseverative and Intrusive Errors of the CVLT.

Three IBD patients had missing values in neuropsychological tests: the Stroop test performance of two patients was excluded due to red-green color blindness. One patient had distractions during the testing situation, and therefore, the results of the Picture Arrangement and the Stroop test were considered invalid in this case.

Statistical analysis

Pearson's χ^2 test was used to compare differences in gender, a one-way analysis of variance (ANOVA) was used to compare differences in age, and a two-tailed Mann-Whitney test was used to compare differences in the socioeconomic status of the adolescent's family between the study groups.

Neuropsychological test scores were compared between the IBD and JIA groups with a univariate ANOVA. The main group comparisons were performed separately for each test score as a dependent variable, with group membership as an independent variable and gender as an additional independent variable to adjust for gender effects. Neuropsychological test performance was also compared between subgroups of active and non-active IBD groups and the JIA group with Bonferroni's *post hoc* test. To adjust for the effects of glucocorticoids, group comparisons of neuropsychological functioning were conducted when this medication (yes *vs* no) was treated as an additional independent variable.

Depressive symptoms were compared between the groups with ANOVA. ANOVAs with *post hoc* tests, corrected for multiple testing with the Bonferroni correction, were also used to compare depressive symptoms between the subgroups of active and non-active IBD patients and the JIA group. Linear regression models were used to predict depression score by age or gender. In addition, to adjust for the effects of depressive symptoms

on neuropsychological test performance, these group comparisons were also conducted with the continuous BDI score as an additional covariate factor.

All analyses were conducted with SPSS 16.0 software, and a *P* value < 0.05 was defined as indicating a statistically significant result throughout the study. Raw test scores were used. Scores that were not normally distributed were log (TMT: A, B, B-A; CVLT: Perseverations, Intrusions) or cube (CVLT: Discriminability) transformed.

RESULTS

The IBD and JIA groups did not differ in age ($F = 3.210$, $\nu = 1, 55$, $P = 0.079$), gender ($\chi^2 = 1.540$, $\nu = 1$, $P = 0.215$), or socioeconomic status of the family ($U = 304.500$, $P = 0.483$). Among IBD patients with the acute state ($n = 20$), the median ESR was 19 mm/h (range from 3 to 53 mm/h), fecal calprotectin was 870 $\mu\text{g/g}$ (range from 101 to 3130 $\mu\text{g/g}$), and 14 of the 20 patients were on glucocorticoids. The respective figures for IBD patients with quiescent disease ($n = 14$) were 4 mm/h for ESR (from 1 to 28 mm/h), 183 $\mu\text{g/g}$ for fecal calprotectin (from 150 to 505 $\mu\text{g/g}$) and three on low-dose glucocorticoids.

The only statistically significant difference in the neuropsychological test performance between the groups appeared in Perseverative Repetition Errors of the CVLT, with the IBD group performing poorer than the JIA group (Table 2). When the subgroups of active and non-active IBD patients were compared with each other and the JIA group, the only difference was again in Perseveration Errors ($F = 5.150$, $\nu = 2, 51$, $P = 0.009$), with the acute IBD group performing poorer than the JIA group ($P = 0.027$; other data not shown). When glucocorticoid medication was treated as an additional independent variable, the difference in Perseveration Errors was close to the level of statistical significance ($F = 3.948$, $\nu = 1, 51$, $P = 0.052$; other data not shown).

The IBD group had scores suggesting more depressive symptoms than the JIA group (mean \pm SD, IBD: 7.9 ± 7.6 , JIA: 4.0 ± 4.0 ; $F = 5.046$, $\nu = 1, 55$, $P = 0.029$), especially IBD patients in the active disease phase ($F = 3.966$, $\nu = 2, 54$, $P = 0.025$; active IBD *vs* JIA $P = 0.022$). In the IBD group, 24% had scores suggesting mild depressive symptoms (half of the patients were in the active and half in the non-active state), and 9% had scores suggesting moderate or severe depressive symptoms (all in the active state). In the JIA group, 9% had scores for mild depressive symptoms, while none had moderate or severe symptoms. None of the patients reported suicidal ideation or suicidal behavior in the BDI. The depression score was not predicted by age ($\beta = 0.247$, $\nu = 1, 55$, $P = 0.064$) or gender ($F = 2.766$, $\nu = 1, 55$, $P = 0.102$).

When the BDI score was set as a covariate in the group comparisons of the neuropsychological data, the difference in Perseveration Errors remained significant between the study groups ($F = 4.591$, $\nu = 1, 52$, $P = 0.004$), and no other differences between the groups emerged (data not shown).

Table 2 Neuropsychological test results (row scores) in the study groups

| | IBD (<i>n</i> = 34) | JIA (<i>n</i> = 23) | IBD vs JIA | | |
|---|----------------------|----------------------|-----------------------|----------|----------------|
| | | | <i>F</i> ¹ | <i>ν</i> | <i>P</i> value |
| Attention | | | | | |
| WMS-R: Digit span forward | 7.3 ± 1.4 | 7.0 ± 1.8 | 0.866 | 1, 53 | 0.356 |
| WMS-R: Visual span forward | 8.3 ± 1.6 | 8.5 ± 2.0 | 0.054 | 1, 53 | 0.817 |
| TMT: A (time) | 31.2 ± 8.6 | 31.0 ± 8.6 | 0.000 | 1, 53 | 0.989 |
| Executive functioning | | | | | |
| TMT: B (time) | 74.7 ± 25.3 | 66.4 ± 15.0 | 0.812 | 1, 53 | 0.372 |
| TMT: B-A (time) | 43.6 ± 22.1 | 35.3 ± 14.0 | 0.986 | 1, 53 | 0.325 |
| Stroop: Interference score (time) | 56.5 ± 13.9 | 57.9 ± 19.5 | 0.190 | 1, 50 | 0.665 |
| Working memory | | | | | |
| WMS-R: Digit span backward | 6.3 ± 1.7 | 6.1 ± 1.5 | 0.596 | 1, 53 | 0.444 |
| WMS-R: Visual span backward | 8.7 ± 1.3 | 8.3 ± 1.3 | 1.218 | 1, 53 | 0.275 |
| Processing speed | | | | | |
| WAIS-R: Digit symbol | 55.3 ± 10.0 | 56.7 ± 9.7 | 0.001 | 1, 53 | 0.970 |
| Basic ability | | | | | |
| WAIS-R: Vocabulary | 39.7 ± 9.7 | 40.6 ± 10.4 | 0.049 ² | 1, 53 | 0.826 |
| Logical reasoning/social insight | | | | | |
| WAIS-III: Picture arrangement | 13.4 ± 4.0 | 12.9 ± 4.2 | 0.277 | 1, 52 | 0.601 |
| Verbal learning and memory | | | | | |
| CVLT: Total recall of trials 1-5 | 55.3 ± 8.7 | 56.8 ± 8.3 | 0.009 | 1, 53 | 0.925 |
| CVLT: Short-delay free recall | 11.7 ± 2.9 | 11.4 ± 3.1 | 0.250 ² | 1, 53 | 0.619 |
| CVLT: Long-delay free recall | 12.3 ± 2.5 | 12.5 ± 2.3 | 0.010 | 1, 53 | 0.920 |
| CVLT: Discriminability | 1.0 ± 0.0 | 1.0 ± 0.0 | 0.280 | 1, 53 | 0.599 |
| CVLT: Perseverative errors | 6.0 ± 4.3 | 3.3 ± 2.9 | 8.249 | 1, 53 | 0.006 |
| CVLT: Intrusion errors | 2.8 ± 3.4 | 2.0 ± 2.8 | 0.527 | 1, 53 | 0.471 |
| CVLT: Semantic clustering | 1.8 ± 0.7 | 1.7 ± 0.7 | 0.168 | 1, 53 | 0.683 |
| CVLT: Learning slope | 1.3 ± 0.3 | 1.2 ± 0.5 | 0.346 ² | 1, 53 | 0.559 |

¹A one-way analysis of variance (group and gender as independent factors); ²Levene's test of equality of error variances $P < 0.05$. IBD: Inflammatory bowel disease; JIA: Juvenile idiopathic arthritis; WMS-R: Wechsler Memory Scale-Revised; WAIS-III: Wechsler Adult Intelligence Scale-Third Edition; WAIS-R: Wechsler Adult Intelligence Scale-Revised; TMT: Trail Making Test; CVLT: California Verbal Learning Test.

DISCUSSION

To our knowledge, this is the first study that describes cognitive functioning in adolescent patients with IBD. We found only minor impairments in the verbal memory test, in which IBD patients, particularly in the acute phase, produced more perseverative errors than patients with non-acute JIA. Perseveration in the CVLT test may be related to a momentary loss of alertness in the tiresome and long verbal memory test. However, no other differences in cognitive functioning between the study groups were detected. These findings indicate that adolescents with active IBD may have some mild problems in verbal memory but no major cognitive deficits. The two prior studies in adults with IBD found deficits, particularly in verbal functioning^[15,16], suggesting that in the clinical evaluation of young patients with IBD, it may be relevant to pay attention to even minor cognitive problems that may be aggravated in the process of growing up.

Depressive symptoms were measured using the BDI on the same day as the neuropsychological examination. Adolescents with IBD, especially those in the acute phase of the illness, may more commonly have depressive symptoms compared with peers with mild disease or non-acute JIA. Approximately one third of the IBD patients had scores indicative of at least mild depres-

sive symptoms, and some of those with acute illness scored for moderate or severe symptoms. This finding is noteworthy, as in non-acute JIA, no patients reported moderate or severe depressive symptoms, and less than one out of ten reported mild symptoms. In line with our results, previous studies have described depressive symptoms among young patients with IBD^[18-20]. Although the minor impairment in the verbal memory test in IBD patients was not explained by the severity of depressive symptoms, depressed mood may have other severe and long-lasting consequences for the psychosocial functioning of these patients.

One limitation of the present study is the lack of a healthy control group, which potentially weakens the generalization of the findings. The patient control group was chosen to represent adolescents with similar life surroundings (chronic disorder with inflammatory pathology) but with less severe health problems that have not been found to be related to cognitive deficits^[35]. The strength of the present study is that the JIA patients underwent exactly the same procedure as the IBD group in the outpatient clinic of the same hospital during the same time period and with the same examiners, who were blinded to patient data. Furthermore, the patient groups did not differ in age or gender. There was no major attrition of participants that could limit the interpretation of the results; however, the sample

sizes were still relatively small. Another limitation is that there may be some potential confounders, such as sleep disturbances or acute stressful life events, *e.g.*, divorce of parents, that should be taken into account. In the IBD group, the majority (70%) of the patients in the acute state were on glucocorticoids, which may affect cognitive functioning^[36]. Unfortunately, the total exposure time of glucocorticoids was not available. However, it is unlikely that the only reason for the minor problem in the verbal memory test was glucocorticoids because the group difference between the IBD and JIA patients remained close to the level of statistical significance when glucocorticoid medication was adjusted for in the statistical analyses. Furthermore, in active IBD, patients need either glucocorticoids or other immunosuppressive therapy, and it would be unethical to decline these medications for study purposes. Thus, studying treatment-naïve patients with active IBD is challenging.

Taken together, this is the first study that investigates cognitive functioning in adolescents with IBD. The results indicate that young patients with IBD, especially in the acute state of the disease, may have some problems in verbal memory, particularly when assessed with a test requiring alertness in a tiresome context. However, no major cognitive deficits among young patients with IBD were found compared to peers with JIA. The clinical significance of this finding and its possible impact on school performance, is unclear, warranting further studies. Likewise, the relation of cognitive functioning to medication and disease-related factors, such as sleep disturbances or depression, needs to be assessed in larger study samples. In particular, longitudinal studies on disease activity and cognitive functioning related to psychosocial functioning are warranted in adolescents with IBD.

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COMMENTS

Background

The incidence of inflammatory bowel disease (IBD) among adolescents is increasing, and therefore, it is important to study its possible burden on patients' everyday life. However, research on cognitive functioning has been scarce in IBD, especially among adolescents.

Research frontiers

It is important to examine whether IBD has an impact on cognitive development and possibly consequently on the performance of young patients at school and everyday life.

Innovations and breakthroughs

Cognitive functioning in IBD has previously been investigated in only two studies, which both found some cognitive deficits relating to IBD, but both studies were conducted in adults. There are no studies investigating cognitive functioning in adolescents with IBD. This study shows that adolescents with acute IBD may have mild verbal memory problems but no major cognitive deficits.

Applications

In the clinical evaluation of young patients with IBD, it may be relevant to pay attention to even minor cognitive problems that may be aggravated in the process of growing up and affect school performance.

Terminology

Cognitive functioning refers to functions of cognitive information processing, such as memory, attention, concentration, processing speed, and executive functions.

Peer review

The present study includes an interesting research question with an original design. The authors utilize numerous neuropsychological tests to assess cognition from various perspectives.

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Influence of percutaneous local therapy for hepatocellular carcinoma on gastric function

Mitsuyoshi Kobayashi, Fumihiko Kinekawa, Kazuya Matsuda, Shintaro Fujihara, Noriko Nishiyama, Takako Nomura, Joji Tani, Hisaaki Miyoshi, Hideki Kobara, Akihiro Deguchi, Hirohito Yoneyama, Hirohito Mori, Tsutomu Masaki

Mitsuyoshi Kobayashi, Shintaro Fujihara, Noriko Nishiyama, Takako Nomura, Joji Tani, Hisaaki Miyoshi, Hideki Kobara, Akihiro Deguchi, Hirohito Yoneyama, Hirohito Mori, Tsutomu Masaki, Department of Gastroenterology and Neurology, Faculty of Medicine, Kagawa University, Miki-cho, Kita-gun, Kagawa 761-0793, Japan

Fumihiko Kinekawa, Department of Internal Medicine, Sanuki Municipal Hospital, Sanuki-city, Kagawa 769-2393, Japan

Kazuya Matsuda, Department of Internal Medicine, Matsuda Clinic, Takamatsu-city, Kagawa 760-0071, Japan

Author contributions: Kobayashi M, Kinekawa F and Matsuda K designed the research; Kobayashi M, Fujihara S, Nishiyama N, Nomura T, Tani J and Miyoshi H analyzed and interpreted the data; Kobayashi M and Kinekawa F drafted the article; and Kobara H, Deguchi A, Yoneyama H, Mori H and Masaki T revised the article for important intellectual content.

Correspondence to: Mitsuyoshi Kobayashi, MD, Department of Gastroenterology and Neurology, Faculty of Medicine, Kagawa University, 1750-1 Iknobe, Miki-cho, Kita-gun, Kagawa 761-0793, Japan. koba@med.kagawa-u.ac.jp

Telephone: +81-87-8912156 Fax: +81-87-8912158

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Abstract

AIM: To investigate the influence of percutaneous local therapy on gastric myoelectrical activity in patients with hepatocellular carcinomas.

METHODS: Forty-four patients with hepatocellular carcinoma (HCC) [27 males and 17 females, ranging in age from 49 to 81 years old (69.7 ± 8.01 years)] who were admitted for percutaneous local therapy were enrolled in this study. We examined clinical abdominal symptoms using the Gastrointestinal Symptom Rating Scale (GSRS) before and 3 d after percutaneous local therapy. We also measured cutaneous fasting and

postprandial electrogastrography (EGG) recordings before and 3 d after percutaneous local therapy.

RESULTS: We found that the percentage of normogastria in the fasting period was lower in the Child B group than in the Child A group ($66.8\% \pm 8.6\%$ vs $84.0\% \pm 3.8\%$). After percutaneous local therapy for HCC, the percentages of normogastria in the fasting period were significantly decreased ($81.6\% \pm 3.5\%$ vs $75.2\% \pm 4.5\%$). None of the postprandial EGG parameters changed significantly after percutaneous local therapy for HCC. Percutaneous local therapy for HCC reduced the power ratio (PR). In particular, the PR of tachygastria was significantly decreased after therapy ($P < 0.01$). However, no significant differences were found in the postprandial EGG parameters. Likewise, no significant differences were found in the calculated GSRS scores obtained from the questionnaire before and after therapy.

CONCLUSION: Gastric slow-wave dysrhythmias were induced by percutaneous local therapy in HCC patients, even though the GSRS scores obtained from the questionnaire did not change significantly.

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Key words: Gastric myoelectrical activity; Electrogastrography; Hepatocellular carcinoma; Percutaneous ethanol injection; Radiofrequency ablation

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INTRODUCTION

In treating hepatocellular carcinoma (HCC), various percutaneous local therapies, such as percutaneous ethanol injection (PEI), microwave coagulation, and radio frequency ablation (RFA), are frequently used because of their demonstrated effectiveness. Although these treatment modalities can induce coagulated tumor necrosis effectively, they are also known to cause adverse effects on extrahepatic abdominal organs^[1]. There are, however, few published reports on the influence of percutaneous local therapy on gastric myenteric activity. Therefore, it is unclear whether gastric function is affected by percutaneous local therapy.

Gastric myoelectrical activity is regulated by electrical pacemaker activity known as slow waves. Gastric slow waves originating from pacemaker cells on the major curvature of the stomach can be measured noninvasively by using a cutaneous electrogastrography (EGG) recorder and placing electrodes on the abdominal skin. In this study, to clarify the influence of percutaneous local therapy for HCC on gastric function, we continuously recorded gastric myoelectric activity by EGG and estimated the influence of percutaneous local therapy on HCC gastric function. In addition, we investigated whether gastric slow-wave dysrhythmias were associated with clinical abdominal symptoms occurring after therapy.

MATERIALS AND METHODS

Patient population

Forty-four patients with HCC [27 males and 17 females, ranging in age from 49 to 81 years old (69.7 ± 8.01 years)] were enrolled in the present study. Patients with diseases known to affect gastric myoelectrical activity, such as diabetes mellitus, who had received a partial or total gastrectomy or who were taking medication known to alter gastrointestinal electrical activity were excluded from this study. After being provided with a careful explanation regarding the goals of the investigation, all patients provided informed consent. The study was conducted in accordance with the local ethical guidelines and the recommendations of the Declaration of Helsinki. HCC was diagnosed by its characteristic appearance on ultrasonography, computed tomographic scan, angiography, serum α -fetoprotein assays, and serum protein induced by vitamin K absence or antagonist-2 assays.

We have developed several novel percutaneous local therapies for HCC. The first of these therapies is the combination of PEI and RFA (PEI-RFA). In this treatment modality, RFA is performed immediately after PEI. The second therapy is percutaneous ethanol-lipiodol injection (PELI). In this modality, a 10:1 mixture of pure ethanol and lipiodol, a lipid-based contrast medium, is injected percutaneously into the HCC. The third therapy is the combination of PELI and RFA (PELI-RFA). The final therapy is RFA alone. The relative usefulness of each of these new treatment modalities has been reported elsewhere^[2-6]. In the present study, 18 patients with

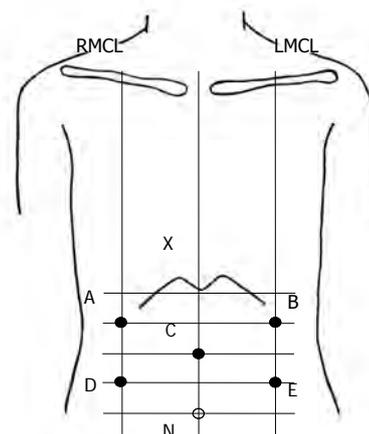


Figure 1 Positions of electrodes for electrogastrography recording. X: Xyphoid process; N: Navel; A: Channel 1 placed on the intersecting point between the right mid-clavicular line (RMCL) and the vertical bisectrix of the line XC; B: Channel 2 placed on the intersecting point between left mid-clavicular line (LMCL) the avertical bisectrix of the line XC; C: Central terminal electrode placed on the patient's ventral midline approximately halfway between the umbilicus and the xyphoid process; D: Channel 3 placed on the intersecting point between RMCL and the avertical bisectrix of the line NC; E: Channel 4 placed on the intersecting point between LMCL and a vertical bisectrix of the line NC.

HCC underwent PEI-RFA, 7 patients underwent PELI, 6 patients underwent PELI-RFA, and 13 patients underwent RFA.

Assessment of abdominal symptoms after therapy

We examined clinical abdominal symptoms using the questionnaire developed by Svedlund *et al.*^[9] (Gastrointestinal Symptom Rating Scale, GSRS), which was translated into Japanese. The GSRS consists of 15 items that assess symptoms of the digestive tract on an interview-based rating scale. Items on the GSRS are scored using a seven-point scale, where a score of 1 represents the absence of any troublesome symptoms and a score of 7 represents very troublesome symptoms. The 15 items on the GSRS evaluate the following 5 symptom categories: reflux (heartburn and regurgitation), abdominal pain (abdominal pain, hunger pains, and nausea), diarrhea (diarrhea, loose stools, and an urgent need to defecate), indigestion (borborygmus, abdominal distension, eructation, and increased flatus), and constipation (constipation, hard stools, and a feeling of incomplete evacuation).

Electrogastrography

We recorded an EGG before therapy began and on the third day after therapy and compared the results. The EGGs were recorded with a portable electrogastrographic recorder (NIPRO, EGG, A and D, Tokyo, Japan). The central terminal electrode for the EGG recorder was placed at the midpoint between the processus xiphoideus and the navel, and 4 other electrodes were placed above, below, and to the left and right of the stomach (Figure 1). The recordings of the EGGs were stable because of the tenth filter sampling at 1-s cycles. Data recording was performed at 13 bits and at a frequency between 2.1 and 6.0 cycles/min. Electrogastrography was performed

Table 1 Clinical characteristics of the subjects

| | |
|----------------------------|----------------|
| Sex (male/female) | 27/17 |
| Age (yr), means \pm SD | 69.7 \pm 8.0 |
| Cause of liver dysfunction | |
| HBV | 5 |
| HCV | 30 |
| Alcoholic | 3 |
| PBC | 1 |
| NBNC | 5 |
| Child-Pugh A/B | 33/11 |
| Therapy | |
| PELI | 7 |
| PELI-RFA | 6 |
| PEI-RFA | 18 |
| RFA | 13 |

PELI-RFA: Combination of percutaneous ethanol-lipiodol injection and radio frequency ablation (RFA); PEI-RFA: Combination of percutaneous ethanol injection (PEI) and RFA; PBC: Primary biliary cirrhosis; NBNC: Negative for both hepatitis C virus (HCV) and hepatitis B virus (HBV).

with the patients in a dorsal position. Patients were asked to remain as still as possible to reduce motion artifacts. Electrogastrograms were recorded for 30 min during a fasting period and again during a postprandial period.

EKG analysis

The EKG data were downloaded to a personal computer via an RS-232C port, and a frequency analysis (fast Fourier transformation; FFT analysis) was performed on 512 points and analyzed using EGS2 Ver.1.31 software (Gram, Japan). Using an FFT analysis, we evaluated the percentages of bradycardia (< 2.4 cycles/min), normogastria (2.4-3.6 cycles/min), and tachycardia (> 3.6 cycles/min), as well as the dominant frequency and the postprandial-to-fasting power ratio (PR).

Statistical analysis

Measured values are expressed as the mean \pm SE. Comparisons before and after therapy were performed by the paired Student *t* test, and $P < 0.05$ was accepted as indicating a significant difference. A regression analysis was performed to examine the relationship between decreases in the percentage of normogastria (%normogastria) after treatment and clinical factors. A P value < 0.05 was considered statistically significant. The software package used for the statistical analysis was SPSS (SPSS statistics 20 IBM Corporation, New York, United States).

RESULTS

According to the Child-Pugh classification of liver disease severity, there were 33 patients in stage A and 11 patients in stage B (Table 1), and the conditions of their Child-Pugh classifications remained the same during the administration of percutaneous local therapy. The causes of liver dysfunction in the HCC patients were as follows: chronic hepatitis B virus infection in 5 cases, chronic hepatitis C virus infection in 30 cases, negative for both hepatitis B surface antigen and hepatitis C antibody in 5

Table 2 Clinical characteristics of Child A and B patients

| | Child A group | Child B group |
|----------------------------|----------------|----------------|
| Sex (male/female) | 23/10 | 4/7 |
| Age (yr) | 69.4 \pm 8.0 | 70.6 \pm 8.8 |
| Cause of liver dysfunction | | |
| HBV | 4 | 1 |
| HCV | 23 | 7 |
| Alcoholic | 2 | 1 |
| PBC | 0 | 1 |
| NBNC | 4 | 1 |

PBC: Primary biliary cirrhosis; NBNC: Negative for both hepatitis C virus (HCV) and hepatitis B virus (HBV).

cases, alcoholism in 3 cases, and primary biliary cirrhosis in 1 case.

No major complications occurred during or after treatment. All the subjects tolerated the electrogastrographic examination well, and no gastrointestinal complaints were reported by the patients during the EGG recording. First, we examined the gastric myenteric activity among the liver cirrhosis subgroups before the treatment. The clinical characteristics of the patients in the Child A and Child B group are shown in Table 2, and the pretreatment EGG data are shown in Table 3.

We found that the percentage of normogastria in the fasting period was lower in the Child B group than in the Child A group. However, no significant differences were found in the calculated GSRS scores obtained from the questionnaire. Furthermore, no differences in symptom categories were significant (Table 4).

Next, we examined the change in EGG associated with treatment. The EGG results are summarized in Table 3. Examples of the raw EGG signals before and after therapy are shown in Figure 2.

After percutaneous local therapy for HCC, the percentages of normogastria in the fasting period were significantly decreased ($P < 0.05$). Although the ratio of bradycardia increased, the change was not significant ($P = 0.967$). None of the postprandial EGG parameters changed significantly after percutaneous local therapy for HCC. Therapy reduced the PR of bradycardia, normogastria, and tachycardia. In particular, the PR of tachycardia was significantly decreased after therapy ($P < 0.01$).

Conversely, no significant differences were found in the calculated GSRS scores changes within any symptom category (Table 4). A single regression analysis was performed to examine the relationship between decreases in the percentage of normogastria (%normogastria) after treatment and the following factors: patient sex, patient age, ethanol injection, location of the treated hepatoma (right or left lobe), tumor size (diameter), use of RFA, and the Child-Pugh score. The results revealed that the decreases in %normogastria were positively correlated with patient age ($P = 0.037$). Moreover, the decreases in %normogastria were significantly larger in the group with ethanol consumption than in the group without ethanol consumption ($P = 0.018$). A multiple regression

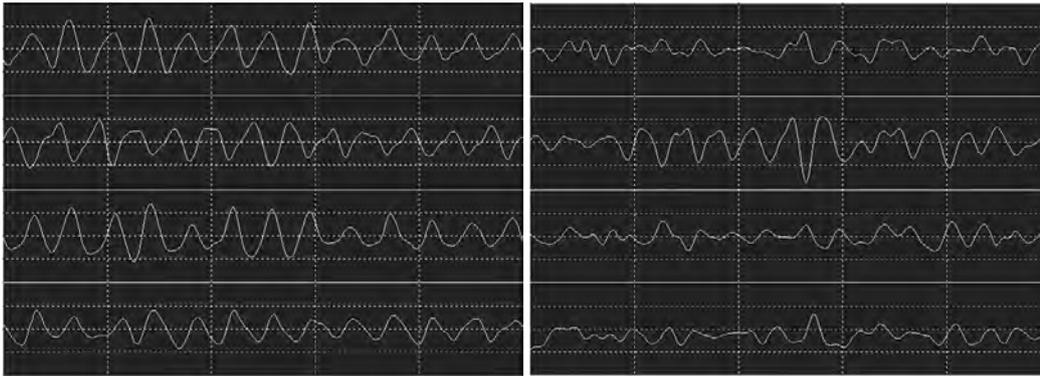


Figure 2 Example of the raw electrogastrography signals before percutaneous local therapy for hepatocellular carcinoma (left) and after therapy (right).

Table 3 Relationship between the liver function classification of cirrhotic patients electrogastrography results and the comparisons between before and after therapy (mean ± SE)

| | Child A (n = 33) | Child B (n = 11) | P value | Before | After | P value |
|--------------------|------------------|------------------|---------|--------------|--------------|---------|
| Fasting EGG | | | | | | |
| DF (circles/min) | 2.89 ± 0.08 | 2.73 ± 0.18 | NS | 2.89 ± 0.07 | 2.84 ± 0.08 | NS |
| Normogastria | 84.0% ± 3.8% | 66.8% ± 8.6% | < 0.05 | 81.6% ± 3.5% | 75.2% ± 4.5% | < 0.05 |
| Bradygastria | 14.8% ± 3.8% | 30.1% ± 8.8% | NS | 16.6% ± 3.5% | 23.2% ± 4.3% | NS |
| Tachygastria | 1.7% ± 5.5% | 1.6% ± 6.1% | NS | 1.7% ± 0.9% | 1.6% ± 0.9% | NS |
| Post-meal EGG | | | | | | |
| DF (circles/min) | 2.53 ± 0.09 | 2.56 ± 0.14 | NS | 2.55 ± 0.08 | 2.67 ± 0.08 | NS |
| Normogastria | 54.9% ± 4.8% | 51.7% ± 8.3% | NS | 54.9% ± 4.3% | 53.8% ± 4.3% | NS |
| Bradygastria | 43.2% ± 4.8% | 40.5% ± 8.2% | NS | 41.5% ± 4.3% | 37.5% ± 4.0% | NS |
| Tachygastria | 1.9% ± 1.0% | 7.8% ± 4.8% | NS | 3.6% ± 1.5% | 8.7% ± 3.0% | NS |
| PR of bradygastria | 2.3 ± 0.4 | 2.2 ± 0.8 | NS | 2.3 ± 0.4 | 1.9 ± 0.2 | NS |
| PR of normogastria | 1.4 ± 0.2 | 1.4 ± 0.3 | NS | 1.4 ± 0.2 | 1.4 ± 0.1 | NS |
| PR of tachygastria | 2.2 ± 0.3 | 2.3 ± 0.7 | NS | 2.2 ± 0.3 | 0.7 ± 0.1 | < 0.01 |

DF: Dominant frequency; PR: Power ratio; NS: Not significant; EGG: Electrogastrography.

Table 4 Gastrointestinal Symptom Rating Scale before therapy in Child A and Child B groups and before and after therapy (mean ± SE)

| | Child A | Child B | P value | Before | After | P value |
|----------------|-----------|-----------|---------|-----------|-----------|---------|
| GSRS score | 1.5 ± 0.1 | 1.7 ± 0.3 | NS | 1.5 ± 0.1 | 1.6 ± 0.1 | NS |
| Reflux | 1.4 ± 0.1 | 1.4 ± 0.3 | NS | 1.4 ± 0.1 | 1.4 ± 0.2 | NS |
| Abdominal pain | 1.3 ± 0.1 | 1.6 ± 0.2 | NS | 1.4 ± 0.1 | 1.6 ± 1.2 | NS |
| Indigestion | 1.4 ± 0.1 | 1.9 ± 0.3 | NS | 1.6 ± 0.1 | 1.6 ± 0.1 | NS |
| Diarrhea | 1.3 ± 0.1 | 1.5 ± 0.2 | NS | 1.4 ± 0.1 | 1.6 ± 0.1 | NS |
| Constipation | 1.8 ± 0.2 | 2.2 ± 0.4 | NS | 1.9 ± 0.2 | 1.9 ± 0.2 | NS |

NS: Not significant; GSRS: Gastrointestinal Symptom Rating Scale.

analysis revealed, however, that none of the independent variables were significantly related to the decreases in %normogastria. In assessing the effect of the independent variables based on P value, tumor size appeared to have the strongest influence on the changes in %normogastria (Table 5).

DISCUSSION

We previously reported the influence of percutaneous local therapy for HCC on gastric myenteric activity^[10].

This is the first formal report with additional cases.

The first electrogastrogram measurement in humans was performed by Alvarez, who found that ECG waves recorded on the body surface of a middle-aged woman occurred at a rate of three cycles per minute.

This method can be used to noninvasively assess the electrical activity generated by gastric smooth muscles^[11].

Although the role of EGG in clinical gastroenterology has not yet been clearly defined and the cause of gastric dysrhythmias at the cellular level is unknown, EGGs are believed to reflect the electrical control activity and gastric motility regulated by pacemakers. In humans, these EGG waves originate from the pacemaker area along the major curvature of the stomach and propagate aborally with increasing velocity at intervals of approximately 20 s. EGGs have been shown to provide useful information for clinical diagnoses.

Abnormal gastric slow-wave frequencies have been described in disorders of gastric emptying, nausea and vomiting in pregnancy^[12], motion sickness^[13], anorexia nervosa^[14], functional dyspepsia^[15-18], and diabetic gastroparesis^[19-21].

Patients with cirrhosis of the liver frequently present with many gastrointestinal complaints that are most likely due to abnormal gastrointestinal motility.

Table 5 Clinical factors associated with decreases in the %normogastric after treatment using regression analysis

| | Regression coefficient | 95%CI | | P value | Partial regression coefficient | 95%CI | | P value |
|----------------------------|------------------------|--------|-------|---------|--------------------------------|--------|-------|---------|
| Sex (M) | -10.77 | -27.34 | 5.79 | 0.196 | -11.58 | -29.4 | 6.25 | 0.196 |
| Age(yr) | 1.05 | 0.07 | 2.04 | 0.037 | 0.73 | -0.45 | 1.91 | 0.22 |
| Ethanol (use) | 20.6 | 3.75 | 37.46 | 0.018 | 13.99 | -3.88 | 31.87 | 0.121 |
| Tumor location (left lobe) | -12.26 | -32.3 | 7.78 | 0.224 | -11.83 | -32.05 | 8.39 | 0.243 |
| Tumor size (cm) | -5.78 | -14.32 | 2.77 | 0.18 | -6.6 | -14.89 | 1.68 | 0.115 |
| RFA (use) | -0.54 | -23.04 | 21.96 | 0.962 | -5.74 | -27.61 | 16.13 | 0.598 |
| Child-Pugh (score) | -4.52 | -11.96 | 2.92 | 0.227 | -8.05 | -15.3 | -0.8 | 0.031 |

Regression analysis was performed to examine the relationship between decreases in the percentage of normogastric after treatment and the following factors: patient sex, patient age, ethanol injection, location of the treated hepatoma (right or left lobe), tumor size (diameter), use of radio frequency ablation (RFA), and the Child-Pugh score.

However, reports on the gastric myoelectrical activity in liver diseases are surprisingly scarce, with only a few papers. Caras *et al*^[22] demonstrated an abnormal electro-gastrogram in 8 of 14 (57%) patients with end-stage liver disease awaiting liver transplants. Usami *et al*^[23] found a decreased share of the power within the normogastric range relative to the total power of the entire frequency band considered in a study group comprising 32 patients with liver cirrhosis of various etiologies and stages.

Their study also confirmed that the results of EGG became less favorable as the severity of liver damage increased.

Gastrointestinal hormones may increase in liver cirrhosis owing to reduced hepatic metabolism and portosystemic shunting. Metabolic abnormalities of these peptides have been reported in patients with liver cirrhosis.

In some studies, the levels of plasma vasoactive peptides with liver cirrhosis have been shown to be higher compared with the normal population^[24,25].

It has been shown that transcatheter arterial chemo-embolization affects gastric myenteric activity and that overproduction of endogenous prostaglandin is related to dysrhythmia of the gastric myenteric activity, suggesting that prostaglandin is related to the activation of the inflammatory response, production of pain, and fever^[26].

Pain and stress may affect the stomach's electrical and mechanical activities. Therefore, it is useful to know whether the abdominal pain induced by percutaneous local therapy produces changes in gastric myoelectric activity.

In this study, multiple regression analysis revealed that tumor size was the strongest and has strong adverse effects on gastric motility.

The pathophysiology of gastric dysrhythmias induced by percutaneous local therapy in HCC patients is poorly understood.

In this study, we demonstrated that gastric myenteric activity was affected by percutaneous local therapy for HCC, even though abdominal symptoms were not apparent and the GSRS scores obtained from the questionnaire did not change significantly after therapy. Delayed gastric transit is a significant clinical matter that may

occur after percutaneous therapy for HCC. Because patients with HCC have reportedly tended to have gastrointestinal dysfunction^[27], we must monitor gastrointestinal dysfunction after percutaneous local therapy for HCC even when there no clinical symptoms are present.

We found that the percentage of normogastric during fasting was decreased after therapy, suggesting that therapy may have an adverse effect on the interdigestive migrating contractions (IMCs) of the fasting period.

IMCs occur approximately every two hours in humans. The physiological role of IMCs is thought to be the housekeeping of the gastrointestinal tract by transferring food residues and detached epithelium toward the rectum through intense contractions that migrate from the stomach to the end of the ileum^[28]. IMCs have four phases, as described by Szurszewski^[29], and there are two types of IMCs. IMCs originating in the stomach are called gastrointestinal IMCs (GI-IMCs), and those originating in the duodenum or lower tract are called intestinal IMCs (I-IMCs). Motilin, which is secreted by the duodenum and upper intestinal mucosa, triggers GI-IMC but is not involved in I-IMCs^[30]. In recent years, ghrelin and serotonin in the digestive tract have been of interest as mediators of IMCs^[31-33].

Normal gastrointestinal movement is also involved in immunity. The secretion of immunoglobulin A peaks during IMCs in the intestinal tract^[34], and a lack of housekeeping by IMCs induces bacterial overgrowth (BO)^[35]. Patients with hepatic cirrhosis are known to have prolonged oro-cecal transit times and are likely to suffer from small intestine BO^[36]. As shown in animal models, aggravated BO may allow bacterial translocation and the development of sepsis. Thus, maintaining normal gastrointestinal movement is of clinical significance in patients with hepatic cirrhosis who are susceptible to infection.

In conclusion, gastric slow-wave dysrhythmias were induced by percutaneous local therapy in HCC patients, but no significant differences were found in the calculated GSRS scores obtained from patient questionnaires collected before and after therapy. The mechanisms underlying the effect of percutaneous local therapy for HCC on extrahepatic abdominal organs require further exploration.

COMMENTS

Background

In the treatment of hepatocellular carcinoma (HCC), various percutaneous local therapies are frequently used, but they are also known to cause adverse effects on extrahepatic abdominal organs. However, the influence of percutaneous local therapy on gastric myenteric activity has not been well investigated.

Research frontiers

Because patients with HCC tend to have gastrointestinal dysfunction, we must monitor gastrointestinal dysfunction after percutaneous local therapy for HCC. In this study, the authors continuously recorded the gastric myoelectric activity by electrogastrography (EGG) and estimated the influence of percutaneous local therapy on HCC gastric function to clarify the influence of percutaneous local therapy for HCC on gastric function.

Innovations and breakthroughs

There are few published reports on the influence of percutaneous local therapy on gastric myenteric activity. To the best of the authors' knowledge, the present study was the most detailed and systematic study investigating the influence of percutaneous local therapy on gastric myoelectrical activity in patients with HCC.

Applications

Gastric slow-wave dysrhythmias were induced by percutaneous local therapy in HCC patients, even though abdominal symptoms were not apparent and the Gastrointestinal Symptom Rating Scale scores obtained from the questionnaire did not change significantly after therapy.

Terminology

EGG is a method that enables gastric electrical activity to be recorded using abdominal surface electrodes. The method has the advantages of being noninvasive, convenient, and providing considerable functional information.

Peer review

This is a very well organized and well conducted study. The results are clearly described, and the discussion and conclusions do not overstate the results.

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Effect of autotransfusion system on tumor recurrence and survival in hepatocellular carcinoma patients

Sami Akbulut, Cuneyt Kayaalp, Mehmet Yilmaz, Volkan Ince, Dincer Ozgor, Koray Karabulut, Cengiz Eris, Huseyin Ilksen Toprak, Cemalettin Aydin, Sezai Yilmaz

Sami Akbulut, Cuneyt Kayaalp, Mehmet Yilmaz, Volkan Ince, Dincer Ozgor, Koray Karabulut, Cengiz Eris, Cemalettin Aydin, Sezai Yilmaz, Division of Liver Transplantation Institute, Department of Surgery, Inonu University Faculty of Medicine, 44280 Malatya, Turkey

Huseyin Ilksen Toprak, Department of Anesthesiology, Inonu University Faculty of Medicine, 44280 Malatya, Turkey

Author contributions: Yilmaz S, Kayaalp C and Aydin C performed the surgical procedure; Akbulut S, Ozgor D, Ince V and Yilmaz M wrote the article and performed literature review, including a comprehensive literature search; Akbulut S Eris C, Kayaalp C and Toprak HI designed the study and wrote the article; Toprak HI provided the CATS usage information; Karabulut K performed the statistical analysis.

Correspondence to: Sami Akbulut, MD, Division of Liver Transplantation Institute, Department of Surgery, Inonu University Faculty of Medicine, 44280 Malatya, Turkey. akbulutsami@gmail.com

Telephone: +90-422-3410660 Fax: +90-422-3410036

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Abstract

AIM: To investigate the therapeutic efficacy and safety of continuous autotransfusion system (CATS) during liver transplantation of hepatocellular carcinoma patients.

METHODS: Eighty-three hepatocellular carcinoma (HCC) patients who underwent liver transplantation with intraoperative CATS ($n = 24$, CATS group) and without ($n = 59$, non-CATS group) between April 2006 and November 2011 at the Liver Transplant Institute of Inonu University were analyzed retrospectively. Post-operative HCC recurrence was monitored by measuring alpha-fetoprotein (AFP) levels at 3-mo intervals and performing imaging analysis by thoracoabdominal multidetector computed tomography at 6-month intervals. Inter-group differences in recurrence and correlations

between demographic, clinical, and pathological data were assessed by ANOVA and χ^2 tests. Overall and disease-free survivals were calculated by the univariate Kaplan-Meier method.

RESULTS: Of the 83 liver transplanted HCC patients, 89.2% were male and the overall mean age was 51.3 ± 8.9 years (range: 18-69 years). The CATS and non-CATS groups showed no statistically significant differences in age, sex ratio, body mass index, underlying disease, donor type, graft-to-recipient weight ratio, Child-Pugh and Model for End-Stage Liver Disease scores, number of tumors, tumor size, AFP level, Milan and University of California San Francisco selection criteria, tumor differentiation, macrovascular invasion, median hospital stay, recurrence rate, recurrence site, or mortality rate. The mean follow-up time of the non-CATS group was 17.9 ± 12.8 mo, during which systemic metastasis and/or locoregional recurrence developed in 25.4% of the patients. The mean follow-up time for the CATS group was 25.8 ± 15.1 mo, during which systemic metastasis and/or locoregional recurrence was detected in 29.2% of the patients. There was no significant difference between the CATS and non-CATS groups in recurrence rate or site. Additionally, no significant differences existed between the groups in overall or disease-free survival.

CONCLUSION: CATS is a safe procedure and may decrease the risk of tumor recurrence in HCC patients.

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Key words: Liver transplantation; Hepatocellular carcinoma; Intraoperative blood salvage autotransfusion; Recurrence; Tumor cell dissemination

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INTRODUCTION

Since the first report of a successful human liver transplantation (LT) in 1963, this procedure has become the gold standard treatment for end-stage liver diseases, including acute liver failure, some metabolic liver diseases, primary liver cancers [such as hepatocellular carcinoma (HCC)], and some metastatic liver tumors^[1-3]. Despite its widespread application, intraoperative hemorrhaging remains a significant limitation of the procedure^[1,4]. The liver is directly connected to three different vascular systems, all of which may be weakened by the underlying disease's pathogenic processes. Serious collateral circulation damage can accompany chronic liver disease and parallel severity of the disease status, increasing the risk of hemorrhage during LT surgery.

Clinical studies have identified the most important factors influencing development of major hemorrhage during surgery as adhesions between cirrhotic liver and surrounding structures, impaired clotting factor synthesis, inherent hemorrhage tendency of some liver diseases, retransplantation, portal vein thrombosis, and the experience level of the involved hepatobiliary surgical team^[3,5-7]. Moreover, the extent of any hemorrhage that develops during surgery has been defined as one of the most significant factors affecting patient morbidity and mortality^[6,8]. Suitable and balanced blood replacement is vital for patients undergoing major surgery. Unfortunately, the exogenous blood transfusion methods remain inadequate for resolving major blood loss that occurs during surgery. Various intraoperative blood salvage autotransfusion systems have been developed to overcome this clinical challenge, and are based upon re-use of the patient's own blood that has accumulated at the surgical site^[4,9].

Intraoperative blood salvage autotransfusion (IBSA) systems have emerged as cost-effective intraoperative tools that reduce the blood requirement by up to 60%^[5]. However, some studies have indicated that the IBSA systems may cause cancer cell dissemination when used during cancer surgery^[5], thereby increasing a patient's risk for metastasis or recurrence. These results have not been upheld by other studies^[4] and the therapeutic efficacy and safety of IBSA systems remain to be definitely established. The IBSA system employed in our health-care institute is the continuous autotransfusion system (CATS) manufactured by Fresenius Kabi AG (Bad Homburg, Germany). To gain a better understanding of the therapeutic efficacy and safety of CATS during LT, this research study was designed to retrospectively analyze groups of HCC patients who underwent LT with and without CATS to determine postoperative rates of tumor recurrence and survival.

MATERIALS AND METHODS

Between April 2006 and November 2011, a total of 702 LTs were performed for 664 patients at the Liver Transplant Institute of Inonu University. Histopathological evaluation of the resected hepatectomy specimens revealed HCC foci of varying sizes and numbers for 17.1% (114/702) of the LT recipients. Of those, 28.1% (32/114) were considered ineligible for this study based upon insufficient follow-up data (< 3 mo; *n* = 2) and other-cause mortality within 3 mo after the LT (*n* = 29). From the remaining cohort of LT HCC patients, only those receiving primary liver transplants were selected for study inclusion. These 83 patients were then divided into two groups according to use of CATS during the LT surgery: CATS group, *n* = 24; non-CATS group, *n* = 59. The study participant selection process is outlined in Figure 1.

The following data were recorded for comparative analysis between the CATS and non-CATS groups: age, sex, body mass index (BMI; in kg/m²), underlying disease [hepatitis B virus (HBV) infection, hepatitis C virus (HCV) infection, or primary HCC], donor type (living/deceased), graft-to-recipient weight ratio (GRWR), Child-Pugh score, model for end-stage liver disease (MELD) score, number of tumors (< 10 or > 10), mean tumor size (in cm), Milan selection criteria (within/beyond), University of California San Francisco (UCSF) selection criteria (within/beyond), preoperative alpha-fetoprotein (AFP) levels (< 200 or > 200 ng/mL)^[10], tumor differentiation (good/average/poor), macrovascular invasion (positive/negative), systemic metastasis and locoregional recurrence (positive/negative), recurrence site (hepatic and/or extrahepatic), current status (alive/died), and duration of follow-up (in mo).

The LT patients had been classified according to the Milan and UCSF criteria, both of which consider the diameter and number of HCC lesions observed by pathological examination to identify individuals likely to benefit from and survive the LT procedure. Specifically, patients with a single lesion of ≤ 5 cm diameter or with three or less lesions for which the largest was ≤ 3 cm were defined as 'within' Milan criteria, whereas patients with a single lesion of ≤ 6.5 cm or with three or less nodules for which the largest was ≤ 4.5 cm and the total tumor diameter was ≤ 8 cm were defined as 'within' UCSF criteria^[2,11]. Donor type (deceased or living) was based on recommendations of the Milan group and the criteria accepted by the United Network of Organ Sharing. HCC cases within the Milan criteria (*n* = 30) were first given the chance of deceased-donor liver transplantation (DDLT; *n* = 3), whereas HCC cases beyond the Milan criteria were given living-donor liver transplantation (LDLT; *n* = 80).

In the LDLT procedure, venous drainage was facilitated by creating a wide-orifice venous drainage model using a saphenous vein graft followed by a wide-aperture anastomosis created between the wide-orifice hepatic vein and inferior vena cava. In the DDLT procedure, a hepato-caval anastomosis was created by using the standard piggyback technique. Anastomoses of other vascular

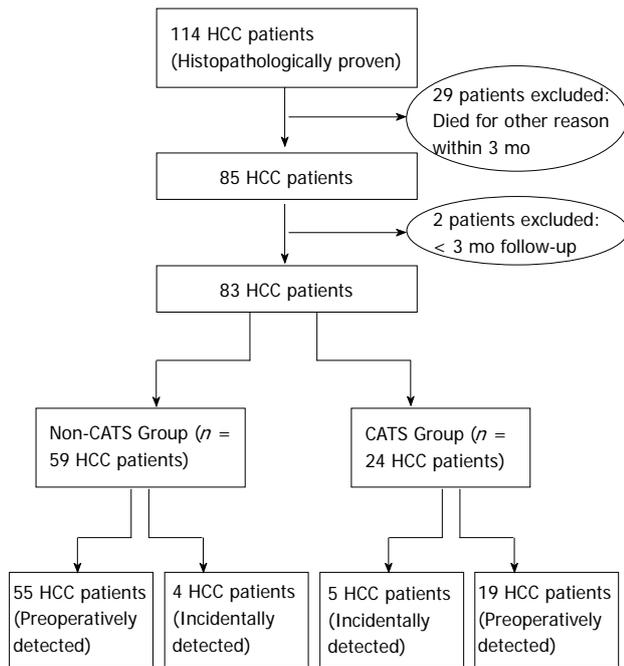


Figure 1 Flow chart showing the study selection methodology for hepatocellular carcinoma patients. HCC: Hepatocellular carcinoma; CATS: Continuous autotransfusion system.

structures and the biliary tract were created by standard techniques.

In the early postoperative period, all known HCC cases were given the immunosuppressive regimen of low-dose sirolimus, steroids, and mycophenolate mofetil. The postoperative pharmaceutical regimen for non-HCC patients who received LT for end-stage liver disease included the calcineurin inhibitor; however, nine of those patients were subsequently diagnosed with HCC by pathology and were immediately switched to sirolimus treatment.

All patients were followed-up with measurement of AFP levels at 3-mo intervals and thoracoabdominal multidetector computed tomography (MDCT) imaging at 6-mo intervals. Suspicion of tumor recurrence led to shorter interval monitoring. Sixty-nine patients with chronic liver disease showed enhanced AFP values during follow-up and were monitored by dynamic MDCT. Five additional cases of primary HCC showed enhanced AFP levels by other tests carried out for symptoms unrelated to clinical liver disease and were then monitored by dynamic MDCT. HCC recurrence was indicated when dynamic MDCT scan images showed heterogeneous contrast with the arterial phase, evidenced by hypodense or isodense regions in the normal liver parenchyma of the portal phase, and contrast enhancement extending to the capsule in the late phase. In cases of suspected HCC, ultrasonography or CT-guided biopsy was performed, depending on mass location. A total of 74 patients were diagnosed with HCC during follow-up.

HCC recurrence was diagnosed by enhanced blood AFP levels, MDCT tumor detection, and/or biopsy-detected cancer of the same cell type as the originally re-

sected HCC. Recurrence was categorized as either systemic metastasis (in a different organ system with no other identifiable cause) or locoregional (within the transplanted liver and/or perihepatic lymph node chain). Overall survival was defined as the time interval from LT to death from any cause, or to the last outpatient clinical follow-up for censored patients. Disease-free survival was defined as the time from LT to HCC recurrence or death from any cause.

Statistical analysis

Statistical analyses were performed by the SPSS software package (version 13.0; SPSS, Chicago, IL, United States). Categorical data were analyzed by the χ^2 test. Continuous data were analyzed by the ANOVA test. Overall and disease-free survivals were calculated by the univariate Kaplan-Meier method. Statistical significance was indicated by $P < 0.05$. Data are expressed as mean \pm SD errors of the mean (SEM).

RESULTS

Eighty-three HCC patients who underwent LT were analyzed. The male:female ratio was strongly biased (74:9). The mean age was 51.3 ± 8.9 years (range: 18-69). The CATS procedure was used in only 28.9% of the LT patients (CATS group, $n = 4$; non-CATS group, $n = 59$). No significant differences were identified between the two groups in terms of age, sex, BMI, underlying disease, donor type, GRWR, Child-Pugh score, MELD score, tumor count, tumor size, AFP levels, Milan criteria, UCSF criteria, tumor differentiation, macrovascular invasion, presence of recurrence, recurrence site, mortality rate, or duration of hospital stay. Clinical and demographic data of both groups are shown in Table 1. The tumor characteristics of all patients are shown in Table 2. There was no difference between the groups for disease-free ($P = 0.9$) or overall ($P = 0.06$) survival (Figure 2).

The mean follow-up time for the CATS group was 25.8 ± 15.1 mo (range: 4-53), during which systemic metastasis and/or locoregional recurrence was detected in 29.2% (7/24) of the patients. Four of these patients experienced locoregional recurrence together with distant organ metastasis. One patient developed distant organ metastasis only, and the remaining two patients developed locoregional recurrence only. The mean follow-up time of the non-CATS group was 17.9 ± 12.8 mo (range: 4-56 mo), during which systemic metastasis and/or locoregional recurrence developed in 25.4% (15/59) of the patients. Ten patients experienced locoregional recurrence together with distant organ metastasis. Two patients developed distant organ metastasis only, and the remaining three patients developed locoregional recurrence only. There was no significant difference between the CATS and non-CATS groups in recurrence rate ($P < 0.7$) or site ($P < 0.8$) (Table 2).

DISCUSSION

IBSA collects a patient's own blood accumulated at a

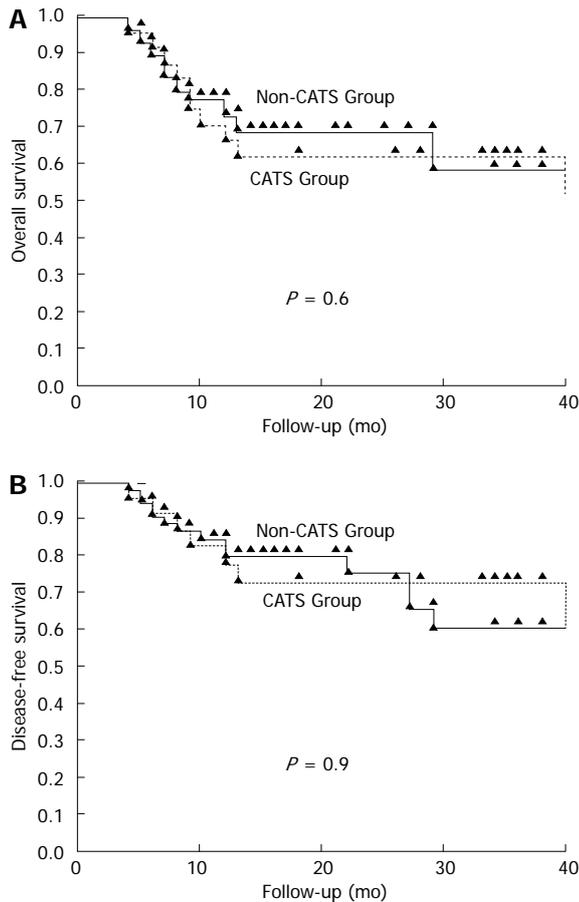


Figure 2 Kaplan-Meier overall survival curves of hepatocellular carcinoma patients undergoing liver transplantation with and without continuous autotransfusion system. A: Overall survival curves; B: Disease-free survival curves.

surgical site for re-use after aspiration, washing, filtration, and reinfusion^[9,12-15]. Following the first description of an IBSA system, the Bentley autotransfusion device, the Haemonetics Co. manufactured an IBSA-based device for washing and concentrating of erythrocytes; known as the Cell Saver (Haemonetics, Braintree, MA, United States), this system became commercially available in 1974. Nearly two decades later, the CATS was produced by the Fresenius Kabi Co. and has since been successfully applied in clinical practice.

Correlations of IBSA with postoperative complications, tumor recurrence, and mortality of patients receiving either homologous (allogenic) or autologous blood transfusion have been extensively studied, but the results have been inconsistent^[16]. Some studies have shown increased infection rates, delayed wound healing, and increased mortality, depending on the amount of blood used following homologous transfusion. Similarly, increased rates of tumor recurrence and mortality were reported for patients who underwent homologous transfusion. Further study has suggested this situation is associated with suppression of natural killer cells and cytotoxic T-cells, and activation of T-suppressor cells^[7,13,16-18]. Some studies have also demonstrated that the infectious and

Table 1 Clinical and demographic characteristics of 83 hepatocellular carcinoma patients undergoing liver transplantation with and without continuous autotransfusion system *n* (%)

| Patient Characteristics | CATS group (<i>n</i> = 24) | Non-CATS group (<i>n</i> = 59) | <i>P</i> |
|--------------------------|-----------------------------|---------------------------------|----------|
| Sex | | | |
| Male | 22 (91.7) | 52 (88.1) | 0.6 |
| Female | 2 (8.3) | 7 (11.9) | |
| Age, yr | | | |
| mean ± SE | 52.0 ± 1.8 | 51.0 ± 1.2 | 0.7 |
| Median (range) | 54 (37-67) | 53 (18-69) | |
| BMI, kg/m ² | 25.5 ± 0.8 | 25.1 ± 0.6 | 0.8 |
| Underlying disease | | | |
| HBV | 18 (75) | 39 (66) | 0.2 |
| HBV + HCV | 0 | 1 (1.7) | |
| HBV + HDV | 5 (20.8) | 9 (15.3) | |
| HCV | 0 | 5 (8.5) | |
| Primary HCC | 0 | 5 (8.5) | |
| Wilson | 1 (4.2) | 0 | |
| Donor type | | | |
| Living | 24 (100) | 56 (95) | 0.1 |
| Deceased | 0 | 3 (5) | |
| GRWR | 1.10 ± 0.06 | 1.20 ± 0.05 | 0.9 |
| Child score | | | |
| A | 6 (25.0) | 18 (30.5) | 0.7 |
| B | 11 (45.8) | 29 (49.2) | |
| C | 7 (29.2) | 12 (20.3) | |
| MELD score | 14.5 ± 0.9 | 13.6 ± 0.8 | |
| Current status | | | |
| Alive | 13 (54.2) | 41 (69.5) | 0.2 |
| Mortality | 11 (45.8) | 18 (30.5) | |
| Hospital stay, d (range) | 35.0 ± 13.8 (14-69) | 33.0 ± 38.2 (7-274) | 0.6 |
| Follow-up, mo (range) | 25.8 ± 15.1 (4-53) | 17.9 ± 12.8 (4-56) | |

HBV: Hepatitis B virus; HCV: Hepatitis C virus; CATS: Continuous autotransfusion system; BMI: Body mass index; HCC: Hepatocellular carcinoma; CATS: Continuous autotransfusion system; GRWR: Graft-to-recipient weight ratio; MELD: Model for End-Stage Liver Disease.

non-infectious complications associated with homologous blood transfusion occur less frequently with autologous blood transfusion^[13,15,17]. The autologous transfusion strategy boasts other advantages as well, including: lower volume requirement for exogenous blood, which increases cost-effectiveness; higher oxygen-carrying capacity, which accelerates wound healing; an immunostimulant effect, which reduces tumor recurrence and increases patient survival rates^[5,7,13-15,18,19].

Although positive results have been obtained with autologous transfusion in patients undergoing tumor surgery, the possibility that tumor dissemination may be caused by the IBSA transfusion method remains a significant concern^[17,20]. This potential risk was first reported by Yaw *et al.*^[15], who demonstrated the ability of tumor cells to pass through the filter of the Bentley autotransfusion device. Subsequently, the American Medical Association designated IBSA systems as unsuitable for use in cancer surgery^[13,17]. However, this restriction was later modified by the National Institute of Clinical Excellence, which indicated that IBSA systems could be used in cancer surgery when combined with leukocyte depletion filters (LDFs)^[13,19]. Since then, the Association of Anaesthetists of Great Britain and Ireland, Obstetric Anaesthetists As-

Table 2 Detailed tumor characteristics of liver-transplanted hepatocellular carcinoma patients in the continuous autotransfusion system and non-continuous autotransfusion system groups *n* (%)

| Tumor characteristics | CATS group (<i>n</i> = 24) | Non-CATS group (<i>n</i> = 59) | <i>P</i> value |
|-------------------------------------|--------------------------------|------------------------------------|-------------------|
| Tumor count | | | |
| < 10 | 17 (70.8) | 47 (79.6) | 0.4 |
| > 10 | 7 (29.2) | 12 (20.3) | |
| Mean tumor size, cm | 5.8 ± 0.9 | 5.2 ± 0.5 | 0.7 |
| Milan criteria | | | |
| Within | 8 (33.3) | 22 (37.3) | 0.7 |
| Beyond | 16 (66.6) | 37 (62.7) | |
| UCSF criteria | | | |
| Within | 9 (37.5) | 29 (49.2) | 0.3 |
| Beyond | 15 (62.5) | 30 (50.8) | |
| AFP levels | | | |
| < 200 | 17 (70.8) | 43 (73) | 0.7 |
| > 200 | 7 (29.2) | 16 (27) | |
| Tumor differentiation | | | |
| Good | 12 (50.0) | 24 (41.0) | 0.6 |
| Average | 9 (37.5) | 23 (39.0) | |
| Poor | 3 (12.5) | 12 (20.0) | |
| Macrovascular invasion | | | |
| Present | 2 (8.4) | 3 (5.0) | 0.6 |
| Absent | 22 (91.6) | 56 (95.0) | |
| HCC recurrence | | | |
| Present | 7 (29.2) | 15 (25.4) | 0.7 |
| Absent | 17 (70.8) | 44 (74.6) | |
| Recurrence and/or metastasis | | | |
| Hepatic | 2 | 3 | 0.8 |
| Extrahepatic | 1 | 2 | |
| Both | 4 | 10 | |
| Recurrence site | | | |
| Liver | 2 | 3 | |
| Lung | 0 | 2 | |
| Liver + bone | 1 | 0 | |
| Liver + lung | 1 | 1 | |
| Liver + stomach | 1 | 0 | |
| Liver + bone + lung | 1 | 0 | |
| Lung + surrenal gland | 1 | 0 | |
| Liver + surrenal gland | 0 | 4 | |
| Liver + surrenal gland + lung | 0 | 2 | 0 |
| Liver + esophagus | 0 | 1 | |
| Liver + lung + brain | 0 | 1 | |
| Liver + surrenal gland + peritoneum | 0 | 1 | |

UCSF: University of California San Francisco; CATS: Continuous autotransfusion system; AFP: Alpha-fetoprotein; HCC: Hepatocellular carcinoma.

sociation, American College of Obstetricians and Gynecologists, and British Confidential Enquiry of Maternal and Child Health, have confirmed the clinical utility and safety of various IBSA systems alone or integrated with LDF in cancer surgery.

The most comprehensive study of IBSA systems in cancer surgery was conducted as a meta-analysis of 10 published studies of 2326 prostate, liver, cervical, and gastrointestinal cancer patients^[12]. The results showed that IBSA did not constitute a risk in terms of tumor recurrence and metastasis. Yet, it has been shown in many studies that 91%-100% of tumor cells passing through the IBSA system filter reach the reinfusion bag^[1,9,13,14,16,17,19,21]; therefore, LDF should be integrated into the IBSA systems.

Catling *et al*^[17] showed that the Cell Saver filter failed to remove 91.2% of tumor cells but that integration of LDF resolved this completely. Another study of patients undergoing LT for HCC showed that the Cell Saver filter system alone removed only 25% of tumor cells, but 93.3% of tumor cells when integrated with an LDF^[1]. However, the IBSA CATS system used in our study was not integrated with LDF and was not associated with increased tumor recurrence. This difference may reflect the pore diameters of the different IBSA systems' filters; for example, the pore diameter range of the Cell Saver's filter is 20-150 μm , whereas the CATS machine's filter is 40-120 μm . The different cancer cells types examined in the different studies may also influence the finding. Cervical cancer cells generally range from 25-65 μm diameter, whereas HCC cells range from 41-55 μm . Thus, certain cancers may require different filters and IBSA systems, integrated or unintegrated, should be applied accordingly.

Most studies of IBSA systems in gastrointestinal surgery have involved the liver and emergency situations^[17]. The complicated vascular structure of the liver and the frequency of severely cirrhotic conditions necessitate highly expert surgeons to lessen the high risk of hemorrhage. Nonetheless, massive blood loss remains a common occurrence in liver tumor surgeries involving major resection, and particularly in LT^[17]. Two studies have investigated the survival rates associated with IBSA systems in patients undergoing liver resection for HCC. Fujimoto *et al*^[22] found equal cumulative overall and disease-free survival rates for patients treated with and without IBSA, and showed that the IBSA system was superior in its requirement for a lower volume of transfused blood. Hirano *et al*^[7] reported better 10-year overall and disease-free survival rates for patients treated with IBSA. These authors also showed that the overall and disease-free survival rates were better for early-stage HCC cases treated with IBSA, but no significant advantage was found when used in advanced-stage HCC cases.

A few studies have reported on IBSA systems in LT. One study by Philips *et al*^[23], in which HCC and sepsis cases had been excluded, use of the Cell Saver during LT reduced the requirement for homologous blood transfusion and was cost-effective. Similarly, Sankarankutty *et al*^[8] showed that use of the Cell Saver during LT reduced the requirement for homologous blood transfusion and lowered the risk of infection. In a study of CATS during LT performed by Massicote *et al*^[24], in which patients with preoperative infection had been excluded, the procedure reduced the requirement for homologous blood transfusion. Finally, Liang *et al*^[3] showed that the Cell Saver integrated with LDF reduced bacterial contamination rates by 90.3%. Thus, IBSA systems exhibit a reduced requirement for homologous blood transfusion, are cost-effective, and have less risk of microbiological contamination.

According to our knowledge, only three studies have investigated the effect on tumor recurrence of IBSA systems used during LC in HCC patients^[1,4,5]. Foltys *et al*^[5] reported use of the Cell Saver in 40 of 136 HCC patients

undergoing LT showed no influence on recurrence rate, overall survival, or disease-free survival during a mean follow-up period of 1015 d. In another study of the Cell Saver autotransfusion system in 31 of 47 HCC patients undergoing LT, Muscari *et al*^[4] reported no influence on recurrence rates during a mean 34-month follow-up period. Liang *et al*^[1] reported that 15 samples of blood collected in the re-infusion bag by the Cell Saver from the surgical sites of 20 HCC patients undergoing LT contained tumor cells; however, when a two-stage LDF system was used with the Cell Saver process, 14 of those samples were tumor-cell negative. In these studies, cases that were beyond the Milan and UCSF criteria and developed tumor perforation due to manipulation during surgery, the Cell Saver system was shown to be insufficient. It is, thus, presumed that the cell-holding capacity of the filters decreases due to the combined tumor load in the surgical area and that resulting from the IBSA device.

Studies in our laboratory have attempted to determine whether the IBSA system-displaced tumor cells enter systemic circulation and, if so, to what extent and whether their presence promotes metastasis. It is well established that the surgical procedure itself can passage tumor cells into the patient's systemic circulation. However, once in circulation, these tumor cells have a 0.000 001%-0.01% chance of causing metastasis^[9,17,19,20]. Therefore, cells passing into the circulation via the IBSA system are expected to have a less-than-absolute potential to cause metastases. In the current study, there was no difference in postoperative metastasis between the CATS group and the non-CATS group. This finding may reflect the fact that > 50% of the CATS group was beyond the Milan and UCSF criteria. Thus, our results indicate that cancer cells passing into the circulation did not cause metastasis.

This study has some important limitations that may have influenced the results. First, some patients' records were missing blood transfusion data, making it impossible to determine how much CATS reduced the need for homologous blood transfusion. Second, the retrospective design of the study restricted the data available for analysis. A future study of prospective design may allow for quantitative detection of tumor cells or AFP using a genetic-based procedure, such as polymerase chain reaction; in this way, blood collected from surgical sites and in the re-infusion bag can be compared to a preoperative peripheral blood sample to more directly determine whether the CATS procedure contributed to recurrence, as opposed to preexisting tumor cells in circulation.

In conclusion, our findings suggest that the use of IBSA systems during LT of HCC patients has no effect on tumor recurrence and survival.

such as liver transplantation. However, tumor cell dissemination may be caused by autotransfusion systems when used during cancer surgery.

Research frontiers

The authors examined whether the use of CATS during liver transplantation to treat hepatocellular carcinoma (HCC) affected tumor recurrence and mortality rates by retrospectively analyzing tumor development and overall and disease-free survival in 83 HCC patients who underwent liver transplantation with ($n = 24$) and without ($n = 59$) CATS.

Applications

Kaplan-Meier analysis was used to compare disease-free and overall survival associated with the CATS procedure during liver transplantation to treat HCC. There was no difference observed between the CATS and non-CATS groups in terms of disease-free or overall survival.

Terminology

Systemic metastasis was defined as metastatic cancer of the same cell type in a different organ system with no other identifiable cause. Locoregional recurrence was defined as recurrent cancer within the transplanted liver or perihepatic lymph node chain, or both. Overall survival was defined as the time interval from liver transplantation to death from any cause or to the last outpatient clinic follow-up visit in censored patients. Disease-free survival was defined as the time from liver transplantation until recurrence of tumor or death from any cause

Peer review

This article has retrogradely investigated the effects of Intraoperative blood salvage autotransfusion (IBSA) on the survival, recurrence and metastasis of hepatocellular carcinoma patients who underwent liver transplantation. Generally, the study is of interest and useful for evaluating IBSA on liver transplantation of HCC patients.

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COMMENTS

Background

Intraoperative continuous autotransfusion system (CATS), a system that involves collecting and washing blood from surgical sites and returning washed blood products to the same patient, has been proven effective for saving blood and reducing hemorrhagic complications during many surgical procedures,

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Long-term outcome in patients with obscure gastrointestinal bleeding after negative capsule endoscopy

Seong-Joon Koh, Jong Pil Im, Ji Won Kim, Byeong Gwan Kim, Kook Lae Lee, Sang Gyun Kim, Joo Sung Kim, Hyun Chae Jung

Seong-Joon Koh, Ji Won Kim, Byeong Gwan Kim, Kook Lae Lee, Department of Internal Medicine, Seoul National University Boramae Hospital, Seoul National University College of Medicine, Seoul 110-744, South Korea

Jong Pil Im, Sang Gyun Kim, Joo Sung Kim, Hyun Chae Jung, Department of Internal Medicine and Liver Research Institute, Seoul National University College of Medicine, Seoul 110-744, South Korea

Author contributions: Koh SJ contributed to data collection and writing the manuscript; Im JP was in charge of this study; Kim JW, Kim BG, Lee KL, Kim SG, Kim JS and Jung HC contributed to data acquisition; all the authors have read and approved the final version of the manuscript.

Correspondence to: Jong Pil Im, MD, Department of Internal Medicine and Liver Research Institute, Seoul National University College of Medicine, 28 Yongon-Dong, Chongno-Gu, Seoul 110-744, South Korea. jp-im@hanmail.net

Telephone: +82-2-7408112 Fax: +82-2-7436701

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Abstract

AIM: To investigate long-term outcome in obscure gastrointestinal bleeding (OGIB) after negative capsule endoscopy (CE) and identify risk factors for rebleeding.

METHODS: A total of 113 consecutive patients underwent CE for OGIB from May 2003 to June 2010 at Seoul National University Hospital. Ninety-five patients (84.1%) with a subsequent follow-up after CE of at least 6 mo were enrolled in this study. Follow-up data were obtained from the patients' medical records. The CE images were reviewed by two board-certified gastroenterologists and consensus diagnosis was used in all cases. The primary outcome measure was the detection of rebleeding after CE, and factors associated with rebleeding were evaluated using multivariate analysis.

RESULTS: Of the 95 enrolled patients (median age 61 years, range 17-85 years), 62 patients (65.3%) were male. The median duration of follow-up was 23.7 mo (range 6.0-89.4 mo). Seventy-three patients (76.8%) underwent CE for obscure-overt bleeding. Complete examination of the small bowel was achieved in 77 cases (81.1%). Significant lesions were found in 38 patients (40.0%). The overall rebleeding rate was 28.4%. The rebleeding rate was higher in patients with positive CE (36.8%) than in those with negative CE (22.8%). However, there was no significant difference in cumulative rebleeding rates between the two groups (log rank test; $P = 0.205$). Anticoagulation after CE examination was an independent risk factor for rebleeding (hazard ratio, 5.019; 95%CI, 1.560-16.145; $P = 0.007$), regardless of CE results.

CONCLUSION: Patients with OGIB and negative CE have a potential risk of rebleeding. Therefore, close observation is required and alternative modalities should be considered in suspicious cases.

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Key words: Capsule endoscopy; Gastrointestinal hemorrhage; Risk factors; Prognosis; Enteroscopy

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INTRODUCTION

Obscure gastrointestinal bleeding (OGIB) represent about 5% of all gastrointestinal (GI) hemorrhage and is defined

as recurrent or persistent bleeding or iron-deficiency anemia originating from the GI tract, with negative results from upper and lower endoscopy^[1,2]. It has been reported that small-bowel hemorrhage is the most common cause for OGIB^[3]. However, the difficulty in establishing a diagnosis in patients suspected of having small-bowel hemorrhage has made assessment of OGIB problematic, and diagnosis is often delayed. Recently, there have been advances in the identification of small-bowel hemorrhage using capsule endoscopy (CE) or balloon-assisted endoscopy.

CE is useful in the detection of the cause of small-bowel hemorrhage, and CE has a higher diagnostic yield than other diagnostic modalities^[4-7]. In addition, CE has advantages over balloon-assisted endoscopy, in that CE allows observation of the whole small bowel and identification of the bleeding foci^[8]. Furthermore, CE allows non-invasive viewing of the whole small-bowel mucosa. Most investigators, therefore, agree that CE should be the initial form of investigation for OGIB^[3].

Several studies have evaluated the clinical implications of negative CE results over the long-term. However, there are contradictory findings regarding long-term outcome in patients with OGIB and negative CE results^[9-11]. In addition, most of these studies comprise small numbers of patients and have short-term follow-up. Furthermore, it has been reported that significant small-bowel pathology may be missed during CE examinations, but can be subsequently diagnosed using alternative diagnostic tools including double-balloon enteroscopy (DBE)^[12,13]. On the basis of these results, establishment of long-term clinical outcomes in patients with OGIB and negative CE remains unknown.

The aim of this study was to investigate the long-term outcomes in patients with OGIB and negative CE results and to identify the risk factors that are associated with rebleeding.

MATERIALS AND METHODS

Patients and study design

Between May 2003 and June 2010, a total of 113 consecutive patients at Seoul National University Hospital that had OGIB underwent CE to identify the cause of bleeding. Among those, long-term follow-up (> 6 mo) data were available for 95 (84.1%) patients. OGIB was defined as either obscure-overt (presented as melena or hematochezia) or obscure-occult (iron-deficiency anemia with or without positive fecal occult blood) GI bleeding. Patient enrollment required one or more non-diagnostic esophagogastroduodenoscopy and colonoscopy examinations prior to CE. Clinical information and follow-up data were obtained from the patients' medical records. The data included age, sex, comorbidity, anticoagulant use, aspirin use, nonsteroidal anti-inflammatory drugs (NSAIDs) use, hemoglobin level, and type of treatment for bleeding. This study was approved by the Institutional Review Board of Seoul National University Hospital.

CE and outcome measurement

CE was performed with the PillCam SB (Given Imaging, Yoqneam, Israel) or MiroCam (IntroMedic, Seoul, South Korea) CE system. After 12 h fasting, the capsule was taken by the patients. According to our unit's protocol, bowel preparation was not performed. Patients were allowed to drink water 2 h after swallowing the capsule and to have a light meal 4 h later. The recorder was stopped at about 8 h or 12 h after swallowing the PillCam SB or MiroCam. Patients were advised to keep away from magnetic exposure until capsule excretion.

The CE images were reviewed by two board-certified gastroenterologists; consensus diagnosis was used in all cases. Inter-observer agreement was > 95%. The videos were read at a speed of 15 frames/s.

According to standard practice guidelines, CE findings were categorized into three types: lesions considered to have a high potential for OGIB (P2); lesions with uncertain bleeding potential (P1); and lesions with no bleeding potential (P0)^[14,15]. Positive studies were defined as examinations that identified one or more P2 lesions, whereas those that identified only P1 or no abnormal lesions were regarded as negative results. Further evaluations such as abdominal computed tomography (CT), small-bowel follow-through, bleeding scan, or conventional angiography were performed in patients with persistent overt GI bleeding. However, because it was only introduced in our institution in September 2009, balloon-assisted endoscopy was only performed in four patients who had positive CE findings. Patients who showed minor OGIB without recurrence were carefully observed without further evaluation.

Each patient's subsequent management was decided according to their CE results and clinical conditions. Specific treatment was performed in patients with identifiable causes on CE or with persistent overt bleeding, which included endoscopic, angiographic or surgical hemostasis; discontinuation of anticoagulants, nonsteroidal anti-inflammatory drugs, aspirin, or other antiplatelet agents; steroids for patients with Crohn's disease; and anti-tuberculosis medication for patients with tuberculosis enterocolitis. Red blood cell (RBC) transfusion, iron supplementation, or watchful waiting, which were classified as non-specific treatments, were performed in patients with negative CE after minor OGIB.

The primary outcome measure was the detection of rebleeding after CE. Rebleeding was defined as evidence of GI bleeding at least 30 d after the initial bleeding. Evidence of GI bleeding was defined as overt bleeding (melena or hematochezia) or a fall in hemoglobin value of ≥ 2 g/dL compared with the baseline value, and in the absence of other causes of decline in hemoglobin level^[10]. Secondary outcome measures were the rate of transfusion and subsequent hospitalization after CE.

Statistical analysis

Univariate analyses were performed using Student's *t* test for continuous variables and the χ^2 or Fisher's exact tests

Table 1 Clinical characteristics of patients with obscure gastrointestinal bleeding *n* (%)

| Characteristics | Total (<i>n</i> = 95) |
|---|------------------------|
| Age (yr), median (range) | 61.0 (17-85) |
| Male | 62 (65.3) |
| Obscure-overt bleeding | 73 (76.8) |
| Complete small-bowel visualization | 77 (81.1) |
| Comorbidity | 43 (45.3) |
| Hemoglobin concentration at the time of the procedure (g/dL), mean \pm SD | 8.3 \pm 2.0 |
| Need for transfusion before capsule endoscopy | 50 (52.6) |
| Diagnostic yield | 38 (40.0) |
| Follow-up duration (mo), median (range) | 23.7 (6.0-89.4) |
| > 12 mo follow-up | 73 (76.8) |
| Aspirin use | 23 (24.2) |
| Other antiplatelet agent | 13 (13.7) |
| Anticoagulation | 8 (8.4) |
| Nonsteroidal anti-inflammatory drugs | 10 (10.5) |

for categorical variables. A Kaplan-Meier curve with a log rank test was used to analyze the cumulative rebleeding rates. Multivariate analysis was done by using the Cox proportional hazards model to identify the risk factors associated with rebleeding. Statistical significance was determined using a *P* value < 0.05. All analyses were carried out using SPSS for Windows version 12.0 (SPSS Inc., Chicago, IL, United States).

RESULTS

Patient characteristics

Ninety-five patients who had undergone CE, with subsequent follow-up of > 6 mo within the defined period were studied. The baseline characteristics of the patients are summarized in Table 1. The median age was 61.0 years (range 17-85 years) and 62 patients (65.3%) were men. Seventy-three (76.8%) underwent CE for obscure-overt GI bleeding. Complete small-bowel visualization was achieved in 77 patients (81.1%). The median follow-up period was 23.7 (range 6.0-89.4) mo after CE. The majority of patients had undergone additional diagnostic workup, which included abdominal CT (61.1%, 58/95), small-bowel follow-through (21.1%, 20/95), RBC scan (14.7%, 14/95), and conventional angiography (9.5%, 9/95).

CE findings

The CE results are summarized in Table 2. Thirty-eight (40.0%) patients had a significant abnormality that showed as one or more P2 lesions. These included erosion or ulcer (21.1%, 8/38), angiodysplasia (26.3%, 10/38), inflammatory bowel disease (IBD) including tuberculosis enteritis (23.7%, 9/38), small-bowel tumors (5.3%, 2/38), and active bleeding of unknown origin (23.7%, 9/38). There was no significant difference in the prevalence of positive findings according to the initial manifestation (*P* = 0.921), with 29 of 73 patients with overt GI bleeding showing a positive CE result (39.7%), compared to 9 of

Table 2 Capsule endoscopy results of patients with obscure gastrointestinal bleeding and rebleeding rates *n* (%)

| Findings | Total (<i>n</i> = 95) | Specific treatment | Re-bleeding |
|--------------------------------------|------------------------|--------------------|-------------|
| P2 lesion | 38 | 24 (63.2) | 14 (36.8) |
| Ulcer or erosion | 8 | 6 (75.0) | 3 (37.5) |
| Tumor | 2 | 2 (100) | 0 (0) |
| Angiodysplasia | 10 | 3 (30.0) | 4 (40.0) |
| Bleeding from unknown focus | 9 | 4 (44.4) | 4 (44.4) |
| IBD including tuberculosis enteritis | 9 | 9 (100) | 3 (33.3) |
| P1 lesion | 6 | 0 (0) | 1 (16.7) |
| Erosion | 2 | 0 (0) | 1 (50.0) |
| Non-bleeding polyp | 2 | 0 (0) | 0 (0) |
| Lymphangiectasia | 2 | 0 (0) | 0 (0) |
| P0 lesion | 51 | 3 (5.9) | 12 (23.5) |
| Total | 95 | 27 (28.4) | 27 (28.4) |

IBD: Inflammatory bowel disease.

22 patients with occult bleeding showing a positive CE result (40.5%).

Clinical course and management after CE

Of the 38 patients with positive CE results, 24 received specific treatments. Conservative management such as iron replacement, watchful waiting, or blood transfusion was performed in 14 patients. Of the 10 patients with angiodysplasia, argon plasma coagulation (APC) was performed in three. The rebleeding rate was higher in patients treated with APC (66.7%, 2/3) than in those undergoing conservative management (28.6%, 2/7). In eight patients with small-bowel ulcer or erosion, one patient with multiple ulcers was considered to have involvement of myeloproliferative disease, which was treated with systemic chemotherapy. Five patients discontinued NSAID use. Two patients with small-bowel tumor underwent surgical resection; their tumors were histologically diagnosed as GI stromal tumor and an inflammatory fibroid polyp, respectively. Of the nine patients classified in the IBD group, including Crohn's disease and tuberculosis enterocolitis, all were treated with steroids or anti-tuberculosis medication. Of the four patients who had active bleeding without identifiable cause, one was treated with explorative laparotomy and diagnosed with radiation enterocolitis. One patient was confirmed with Crohn's disease after performing balloon-assisted endoscopy. One patient was also confirmed with early stage Crohn's disease that involved the terminal ileum and cecum, after performing colonoscopy and biopsy. Finally, one patient was diagnosed with angiodysplasia of the duodenum and treated with APC.

Of the 51 patients with negative CE, rebleeding was identified in 12, and nine of these patients underwent additional evaluation for recurrent overt GI bleeding. The evaluation included abdominal CT, small-bowel follow-through, RBC scan, Meckel's scan, and explorative laparotomy. Despite these examinations, the focus of significant bleeding was not detected in six patients. However,

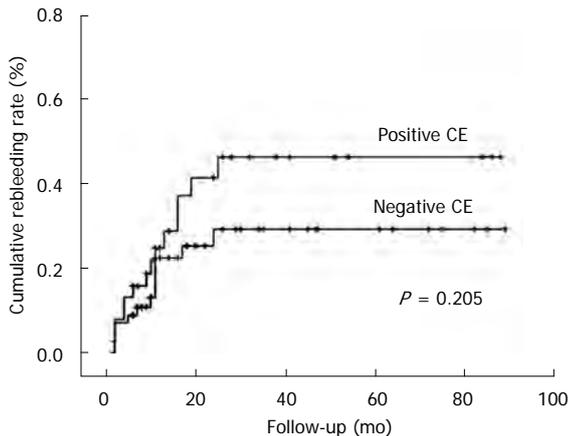


Figure 1 Cumulative rebleeding rates according to the initial capsule endoscopy results. CE: Capsule endoscopy.

significant small-bowel lesions were detected in three patients. In one of these, a bleeding diverticulum arising from distal ileum was identified on CT angiography and treated with angiographic embolization. In another, an ulcer was identified in the distal ileum using small-bowel follow-through. That lesion was confirmed with extranodal marginal zone lymphoma after explorative laparotomy. In the last case, jejunal angiodysplasia was identified as the focus of the recurrent bleeding through explorative laparotomy with intraoperative enteroscopy. The remaining three patients who showed recurrent occult bleeding and had negative CE results received symptomatic treatment including iron replacement.

Subsequent risk for rebleeding and long-term outcome in patients with OGIB

The rebleeding rates in the CE results are summarized in Table 2. Of the 95 patients, 27 (28.4%) showed one or more rebleeding events during follow-up. The median time to rebleeding was 10.0 mo (range 1.0-25.0 mo). The rebleeding rate in patients with positive and negative CE was 36.8% and 22.8%, respectively. There was no significant difference in rebleeding between these two groups ($P = 0.205$, log rank test; Figure 1). In addition, there was no significant difference in the cumulative rebleeding rates between P1 and P0 lesion groups ($P = 0.711$, log rank test). Subsequent hospitalization for bleeding was required in five patients (13.2%) in the CE-positive group compared to seven (12.3%) in the CE-negative group ($P = 1.000$). Subsequent blood transfusions were given in three (7.9%) *vs* six (10.5%) patients, respectively ($P = 1.000$).

On the basis of multivariable analysis *via* the Cox proportional hazards analysis, anticoagulation therapy was independently associated with an increased risk of rebleeding [hazard ratio (HR), 5.019; 95%CI, 1.560-16.145; $P = 0.007$]. However, negative CE and specific treatment were not associated with a decreased risk of rebleeding (Table 3). To identify the risk factors for rebleeding in patients with negative CE, we performed subgroup analysis.

Anticoagulation therapy was identified as an independent risk factor for rebleeding in patients with negative CE (HR, 7.069; 95% CI: 1.942-29.809; $P = 0.004$).

DISCUSSION

CE is a safe and effective tool in evaluating small-bowel disease. It is generally accepted as the first diagnostic choice for patients with OGIB^[3]. It provides a higher diagnostic yield compared to other modalities because of its improved visualization. However, there are several limitations of CE reported in evaluations of small-bowel pathology. These include a limited visual field of the bowel lumen, poor bowel preparation, inadequate luminal distension, rapid passage around the proximal small bowel, and incomplete study of the cecum^[16,17]. Therefore, it is plausible that CE can miss significant lesions; a risk which could translate into poor prognosis in patients with OGIB in the long term. Although there have been many reports determining the clinical impact of negative CE, the long-term risk of recurrent bleeding in patients with OGIB after negative CE remains controversial. According to a prospective analysis comparing CE with intraoperative endoscopy as the standard of reference, the negative predictive value (NPV) for CE was 86%^[18]. Delvaux *et al*^[19], in a 12-mo follow-up study, reported that the NPV was 100% in patients with normal findings on CE. In addition, several studies have reported that patients with OGIB and negative CE results have very low rebleeding rates^[9,10,20,21]. Therefore, it has been generally accepted that a negative CE result predicts a favorable prognosis in patients with OGIB. However, a well-designed prospective study reported that the rebleeding rate during 1-year follow-up was 33% in patients with normal CE findings^[22]. Moreover, a retrospective study demonstrated that there was no significant difference in the cumulative rebleeding rates between patients with positive CE and those with negative CE^[11]. The present study demonstrated that the overall rebleeding rates in patients with positive and negative CE results during the minimum 6 mo follow-up period were 36.8% and 22.8%, respectively. There was no significant difference in the cumulative rebleeding rates between these two groups. In addition, multivariate analysis showed that CE results were not associated with a risk of rebleeding. Therefore, our results indicate that the risk for recurrent bleeding is considerable, even if patients with OGIB have negative CE results.

According to the current recommendations for OGIB from the American Gastroenterological Association, subsequent intervention directed by CE findings is recommended in patients with positive CE results. Moreover, further diagnostic testing can be deferred in patients with negative CE, and balloon-assisted endoscopy is considered only in patients with a high suspicion of small-bowel pathology. However, little information is available on the duration of follow-up. The duration of follow-up in previous studies, which found very low rebleeding rates,

Table 3 Risk factors for rebleeding in patients with obscure gastrointestinal bleeding

| Variables | Hazard ratio | 95%CI | P value |
|------------------------|--------------|--------------|---------|
| Male | 2.082 | 0.882-4.910 | 0.094 |
| Age > 50 yr | 0.980 | 0.328-2.922 | 0.971 |
| Hb < 8 g/dL | 0.861 | 0.365-2.029 | 0.732 |
| Transfusion before CE | 1.719 | 0.674-4.382 | 0.257 |
| Comorbidity | 1.619 | 0.661-3.969 | 0.292 |
| Aspirin use | 1.020 | 0.357-2.914 | 0.970 |
| Anticoagulation use | 5.019 | 1.560-16.145 | 0.007 |
| NSAIDs use | 1.153 | 0.314-4.232 | 0.830 |
| Obscure-overt bleeding | 1.143 | 0.382-3.416 | 0.811 |
| Specific treatment | 1.123 | 0.368-3.422 | 0.839 |
| Positive CE | 1.564 | 0.561-4.355 | 0.392 |

NSAIDs: Nonsteroidal anti-inflammatory drugs; CE: Capsule endoscopy.

was only 12 mo^[19,21]. However, increased rebleeding rates have been reported with longer follow-up periods in recent studies^[9,10]. In the present study, approximately 50% of the patients showed a first rebleeding episode > 1 year after the initial bleeding, while the maximum time to rebleeding was 24 mo after a negative CE result. Moreover, Park *et al*^[11] reported a rebleeding rate of 35.7% during 32 mo follow-up. On the basis of these results, a close follow-up duration of at least 2 years is needed in patients with OGIB, even if patients have negative CE findings (Table 4).

CE can improve the diagnostic yield in patients with OGIB, but it remains uncertain whether CE improves clinical outcomes. A recent, prospective, randomized control trial demonstrated that a substantial improvement in diagnostic yield with the use of CE did not lead to improved outcome in patients with OGIB^[22]. In addition, a recent study showed that positive CE results are not predictive of a favorable outcome in patients with iron-deficiency anemia^[23]. On that basis, treatment directed by CE may not improve long-term outcome in patients with OGIB. In contrast, Park *et al*^[11] demonstrated that specific treatments decrease long-term rebleeding after CE, suggesting that vigorous investigation to detect the bleeding focus could definitely reduce the rebleeding. In addition to this, Delvaux *et al*^[19] also showed that only one among 18 patients who were treated directed by CE relapsed during 1 year follow-up. In the present study, a significant proportion (63.2%) of patients with positive CE results had specific treatment. Higher rebleeding rate was found in patients with angiodysplasia and IBD, while patients with tumors exhibited no rebleeding after surgical intervention. There was no significant difference in the cumulative rebleeding rates regardless of specific treatment. In addition, multivariate analysis showed that specific treatment did not reduce the risk of rebleeding. These results suggest that CE plays a limited role in clinical outcome among patients with OGIB. However, these results should be interpreted with caution because our data were retrospectively obtained from a single tertiary referral hospital. Outcomes in patients with OGIB

Table 4 Follow-up duration and rebleeding rates in patients with obscure gastrointestinal bleeding after negative capsule endoscopy

| Study | No. of enrolled cases | Follow-up duration (mo) | Rebleeding rates after negative capsule endoscopy |
|--|-----------------------|-------------------------|---|
| Lai <i>et al</i> ^[9] | 49 | 12 | 6% |
| Macdonald <i>et al</i> ^[10] | 49 | 17 | 11% |
| Park <i>et al</i> ^[11] | 51 | 32 | 36% |
| Delvaux <i>et al</i> ^[19] | 44 | 12 | 0% |
| Lorenceanu-Savale <i>et al</i> ^[21] | 35 | 12 | 0% |
| Current study | 51 | 23 | 23% |

are likely attributable to various etiologies and to the severity of initial presentation. Moreover, the natural history of etiology such as angiodysplasia remains unclear. Therefore, prospective, well-designed, long-term follow-up studies that include the various etiologies of OGIB are required to determine whether diagnostic testing with CE will translate into a significant improvement in the management and outcome in patients with OGIB.

There are no clear guidelines for evaluating patients with initially negative CE results. The management of these patients with OGIB remains elusive. However, patients with evidence of ongoing or recurrent OGIB need further investigation. The options include repeat upper and lower endoscopy, CE, DBE, radiology or nuclear medicine, and intraoperative enteroscopy^[24]. In a recent study, patients with initial negative CE results benefited from a second-look CE if the bleeding presentation changed from occult to overt, or if hemoglobin decreased by ≥ 4 g/dL^[25]. In addition to this, DBE could be useful in evaluating patients with negative CE results because it has a diagnostic yield similar to that of CE^[26]. Furthermore, it has great advantages in providing histological confirmation and simultaneous treatment^[27]. Therefore, well-designed prospective studies are required to improve the management in OGIB patients with a nondiagnostic CE test.

Our study had a few limitations. First, the data were obtained from a single tertiary referral hospital and the study was of retrospective design. Second, balloon-assisted endoscopy was not performed in most of the patients. Therefore, the possibility exists that a less-invasive approach might have led to higher rebleeding rates in both groups. Finally, it is possible that some lesions may have been missed because our data included incomplete CE results.

In conclusion, patients with OGIB and negative CE have a potential risk of rebleeding. Therefore, close observation is needed even in patients with negative CE and alternative modalities should be considered in clinically suspicious cases.

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COMMENTS

Background

Obscure gastrointestinal bleeding (OGIB) represent about 5% of all gastrointestinal hemorrhage and is defined as recurrent or persistent bleeding or iron-deficiency anemia originating from the gastrointestinal tract, with negative results from upper and lower endoscopy. Capsule endoscopy (CE) is useful for detection of the cause of small-bowel hemorrhage, and has a higher diagnostic yield than other diagnostic modalities.

Research frontiers

Wireless CE is considered a first-line investigation in patients with OGIB. However, a significant proportion of patients with OGIB have nondiagnostic CE results. Although many studies have investigated the diagnostic yield of CE, little information is available about long-term outcome in patients after negative CE. In addition, it remains uncertain whether treatment directed by CE improves long-term outcome in patients with OGIB.

Innovations and breakthroughs

This study showed that the rebleeding rate in patients with OGIB after negative CE results was substantial. Treatment directed by CE did not reduce the risk of rebleeding.

Applications

The study showed that negative CE did not predict a favorable outcome, which suggests that close observation for rebleeding is warranted.

Peer review

The authors have reported that patients with OGIB and negative CE have a potential risk of rebleeding. Treatments directed by CE were not associated with a decreased risk of rebleeding. The data provided in this study contribute to understanding the long-term outcome in patients with OGIB.

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Long-term outcomes and prognostic factors for patients with esophageal cancer following radiotherapy

Chuang-Zhen Chen, Jian-Zhou Chen, De-Rui Li, Zhi-Xiong Lin, Ming-Zhen Zhou, Dong-Sheng Li, Zhi-Jian Chen

Chuang-Zhen Chen, Jian-Zhou Chen, De-Rui Li, Zhi-Xiong Lin, Ming-Zhen Zhou, Dong-Sheng Li, Zhi-Jian Chen, Department of Radiation Oncology, Cancer Hospital of Shantou University Medical College, Shantou 515031, Guangdong Province, China

Author contributions: Chen CZ and Chen JZ contributed equally to this work; Chen ZJ and Li DR designed the research; Chen CZ, Chen JZ, Lin ZX, Zhou MZ and Li DS performed the research; Chen CZ and Chen JZ analyzed the data and wrote the paper.

Correspondence to: Zhi-Jian Chen, MD, Chief, Department of Radiation Oncology, Cancer Hospital of Shantou University Medical College, 7 Raoping Road, Shantou 515031, Guangdong Province, China. zjchenmd@stu.edu.cn

Telephone: +86-754-88555844 Fax: +86-754-88560352

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Abstract

AIM: To evaluate long-term outcomes and prognostic factors for esophageal squamous cell carcinoma (SCC) treated with three dimensional conformal radiotherapy (3D-CRT).

METHODS: Between January 2005 and December 2006, 153 patients (120 males, 33 females) with pathologically confirmed esophageal SCC and treated with 3D-CRT in Cancer Hospital of Shantou University were included in this retrospective analysis. Median age was 60 years (range: 37-84 years). The proportion of tumor location was as follows: upper thorax (including the cervical region), 73 (48%); middle thorax, 73 (48%); lower thorax, 7 (5%), respectively. The median radiation dose was 64 Gy (range: 50-74 Gy). Fifty four cases (35%) received cisplatin-based concurrent chemotherapy. Univariate and multivariate analysis were performed to determine the association between the correlative factors and prognosis.

RESULTS: The five-year overall survival rate was 26.3%, with a median follow-up of 49 mo (range: 3-66 mo) for patients who were still alive. On univariate analysis, lesion location, lesion length by barium esophagogram, computed tomography imaging characteristics including Y diameter (anterior-posterior, AP, extent of tumor), gross tumor volume of primary lesion (GTV-E), volume of positive lymph nodes (GTV-LN), and the total target volume (GTV-T = GTV-E + GTV-LN) were prognostic for overall survival. By multivariate analysis, only the Y diameter [hazard ratio (HR) 2.219, 95%CI 1.141-4.316, $P = 0.019$] and the GTV-T (HR 1.372, 95%CI 1.044-1.803, $P = 0.023$) were independent prognostic factors for survival.

CONCLUSION: The overall survival of esophageal carcinoma patients undergoing 3D-CRT was promising. The best predictors for survival were GTV-T and Y diameter.

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Key words: Esophageal neoplasm; Three dimensional conformal radiotherapy; Multivariate analysis; Prognostic factor

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INTRODUCTION

In the Radiation Therapy and Oncology Group (RTOG) 85-01 trial, patients with locally advanced, unresectable esophageal carcinoma (EC) were randomized to receive either chemoradiation or radiation alone. Those patients

receiving chemoradiotherapy had a 26% 5-year survival rate compared with 0% in those treated with radiotherapy alone^[1]. Since the results of this study were reported, concurrent chemoradiotherapy has become a widely accepted standard treatment for patients with EC who are treated with non-surgical methods. However, not all the patients are good candidates for chemoradiotherapy due to the presence of multiple co-morbid medical conditions. The outcomes and factors that predict overall survival in patients treated with radiotherapy alone should also be considered in the management of esophageal cancer. The development of three-dimensional conformal radiotherapy (3D-CRT) and other advanced radiotherapy techniques has allowed clinicians to treat patients with increased accuracy and normal tissue sparing capabilities. These advantages may allow for the safe delivery of higher radiation doses compared with historic radiotherapy approaches^[2-5].

To our knowledge, this study was the largest single institution experience to report the long-term survival for esophageal squamous cell carcinoma (SCC) in patients treated with 3D-CRT with or without chemotherapy in English.

MATERIALS AND METHODS

Patient selection

Patients with non-metastatic and pathologically confirmed SCC who received definitive 3D-CRT, with or without chemotherapy, in the Department of Radiation Oncology, Cancer Hospital of Shantou University Medical College (SUMC) between Jan 2005 and December 2006 were enrolled in the study. Patients were treated with definitive chemoradiotherapy because the disease was not amenable to resection, patients had multiple medical co-morbidities that would preclude surgery, or the patient declined surgery. Patients were treated with radiotherapy alone if other co-morbid medical conditions precluded them from receiving concurrent chemotherapy or the patient declined chemotherapy.

Treatment

The gross tumor volume (GTV) was identified using both diagnostic and radiotherapy planning computed tomography (CT) images and barium swallow examination. The clinical target volume (CTV) was then expanded from the GTV with a margin of 1.0 cm laterally and a 2 cm margin in the superior and inferior dimensions. An additional 0.5 cm margin expansion around the CTV was included for the planning target volume. For treatment planning purposes, lung V_{20} 30%-35% and spinal cord maximum dose < 45 Gy were used as dose constraints. All 153 patients were treated using 3D-CRT to a median dose 64 Gy (range: 50-74 Gy) in 25 to 37 fractions. Of these, 137 patients received conventionally fractionated radiotherapy at 2 Gy per fraction, five times a week throughout the treatment course, while 16 patients received an accelerated course of treatment in which the

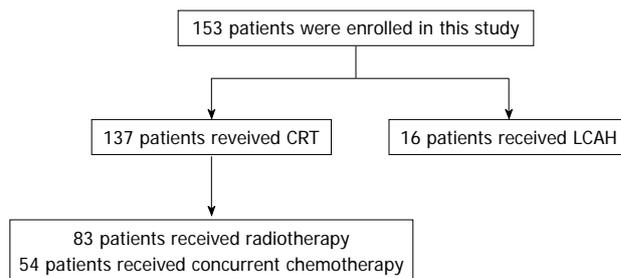


Figure 1 Patient flow chart of treatment allocation. CRT: Conventionally fractionated radiotherapy; LCAH: Late course accelerated hyperfractionated radiotherapy.

first 40 Gy was conventionally fractionated and the latter course was treated with accelerated hyperfractionated radiotherapy consisting of 24 Gy given 1.5 Gy *bid*, to a total dose of 64 Gy. All fractions were delivered at least 6 h apart. Fifty four patients (35.3%) received cisplatin-based concurrent chemotherapy. The treatment allocation was showed in a patient flow chart (Figure 1).

Clinical variables for prognostic analysis

General clinical features included gender, age, lesion location, late course accelerated hyperfractionation, chemotherapy and radiation dose. Imaging parameters included the lesion length, as defined by esophageal barium swallow; and lesion characteristics on CT imaging, such as the largest diameter, X diameter (maximum lateral extent of tumor) and Y diameter (anterior-posterior, AP, extent of tumor); esophageal wall thickness (maximum thickness of esophageal wall if the esophageal lumen is visible or half of the largest diameter with invisible esophageal lumen); lesion length; invasion to adjacent structures (including thoracic aorta, bronchus, trachea, pericardia); supraclavicular lymph nodes metastasis; number of positive lymph nodes; volume of the primary lesion (GTV-E); the volume of positive lymph nodes (GTV-LN); and the total gross tumor volume ($GTV-T = GTV-E + GTV-LN$).

Statistical analysis

Survival curves were generated using the Kaplan-Meier method with mortality data being estimated from first day of radiotherapy until date of death or date of last follow-up. The log-rank test was used to compare the survival curves. A univariate analysis was performed to identify factors for the multivariable model. A multivariate Cox-regression analysis was then performed incorporating significant univariate factors to determine the relevant prognostic factors. Results were reported using hazard ratios (HRs) and 95% CIs. All analyses were performed with SPSS (version 19.0; Chicago, IL). All reported *P* values were 2-sided with a value < 0.05 considered statistically significant.

RESULTS

Between January 2005 and December 2006, there were

Table 1 Radiographic tumor characteristics

| Characteristic | mean \pm SD | Range |
|--|-----------------|-----------|
| Length of lesion in barium esophagogram (cm) | 5.8 \pm 2.3 | 1.6-12.8 |
| Largest diameter (cm) | 3.4 \pm 1.0 | 1.5-6.0 |
| X dimension (cm) | 3.2 \pm 1.0 | 1.2-5.9 |
| Y dimension (cm) | 2.5 \pm 0.6 | 1.2-4.4 |
| Esophageal wall thickness (cm) | 1.5 \pm 0.6 | 0.5-4.2 |
| Length of lesion in CT (cm) | 6.6 \pm 2.2 | 2.4-16.0 |
| GTV-E (cm ³) | 31.8 \pm 20.7 | 3.4-114.3 |
| GTV-LN (cm ³) | 4.3 \pm 10.6 | 0-78.8 |
| GTV-T (cm ³) | 36.1 \pm 23.8 | 3.4-124.0 |

GTV-E: Gross tumor volume of primary lesion; GTV-LN: Volume of positive lymph nodes; GTV-T: Total gross tumor volume.

637 patients with EC that were prospectively registered in the Department of Radiation Oncology, Cancer Hospital of SUMC. One hundred and fifty three of these patients met the enrollment criteria. There were 120 males and 33 females, with a median age of 60 years (range: 37-84 years). The number of patients with primary tumor location in upper (including neck), middle and lower thorax was 73 (48%), 73 (48%) and 7 (5%), respectively. Of these patients, 57.5% had invasion to adjacent structures and 64.1% were found with positive regional lymph nodes metastases. Table 1 summarized the radiographic tumor characteristic of 153 patients.

Survival

For the 153 patients included in this study, the median follow up time was 49 mo (range: 3-66 mo) for the 47 living patients. The 1-, 3- and 5-year overall survival (OS) rates were 72.5%, 34.7% and 26.3%, respectively. The 1-, 3- and 5-year OS rates for the 99 patients that were treated with radiotherapy alone were 69.9%, 34.3% and 26.5%, respectively, and for the 54 patients treated with chemoradiotherapy, the 1-, 3-, and 5-year OS rates were 79.2%, 35.8% and 25.5%, respectively.

Predictors of overall survival

We performed an analysis to identify factors that predicted for OS for patients with EC. The results of univariate analysis and multivariate analysis are shown in Table 2. By univariate analysis, lesion location ($P = 0.011$); lesion length by barium esophagogram ($P = 0.003$); lesion characteristics by CT imaging such as the largest diameter ($P = 0.002$), X diameter ($P = 0.006$) and Y diameter of lesion ($P = 0.001$); thickness of esophageal wall ($P = 0.016$); length of lesion ($P = 0.024$); invasion to adjacent structure ($P = 0.007$); invasion of bronchus ($P = 0.046$); number of metastatic lymph nodes ($P = 0.008$); GTV-LN ($P = 0.010$); GTV-E ($P = 0.007$); and GTV-T ($P = 0.002$) were significant prognostic factors. By multivariate analysis, only the Y diameter (HR 2.219, 95%CI 1.141-4.316, $P = 0.019$) and GTV-T (HR 1.372, 95%CI 1.044-1.803, $P = 0.023$) were independent prognostic factors for survival. The survival curves with different Y diameters and GTV-T are shown in Figure 2.

Table 2 Univariate analysis for survival of patients

| Feature | Cases | Median survival (mo) | 5-yr OS | χ^2 value | P value |
|--|----------------------|----------------------|---------|----------------|--------------|
| Gender | Male | 120 | 19.2 | 24.2% | 0.896 0.344 |
| | Female | 33 | 24.3 | 34.6% | |
| Age (yr) | ≤ 60 | 77 | 22.1 | 29.5% | 1.159 0.282 |
| | > 60 | 76 | 20 | 23.2% | |
| Lesion location | Neck, upper thorax | 73 | 23.4 | 38.8% | 6.497 0.011 |
| | Middle, lower thorax | 80 | 18.5 | 13.5% | |
| | LCAH | | | | |
| LCAH | No | 137 | 19.2 | 26.7% | 0.257 0.612 |
| | Yes | 16 | 30.4 | 25% | |
| RT dose (Gy) | ≤ 64 | 86 | 19.4 | 23.8% | 0.429 0.512 |
| | > 64 | 67 | 22.4 | 29.7% | |
| Chemotherapy | No | 99 | 18.4 | 26.5% | 0.438 0.508 |
| | Yes | 54 | 23.4 | 25.5% | |
| The length of lesion in barium esophagogram (cm) | ≤ 3 | 12 | - | 80.8% | 13.627 0.003 |
| | $> 3, \leq 5$ | 57 | 22.4 | 23.8% | |
| | $> 5, \leq 7$ | 52 | 16.8 | 20.9% | |
| | > 7 | 32 | 17.8 | 21.7% | |
| Largest diameter (cm) | ≤ 2 | 11 | - | 72.7% | 12.729 0.002 |
| | $> 2, \leq 3$ | 43 | 23.4 | 33.7% | |
| | > 3 | 99 | 17.3 | 17.3% | |
| Y diameter (cm) | ≤ 2 | 29 | - | 60.3% | 11.574 0.001 |
| | > 2 | 124 | 18.4 | 18.3% | |
| X diameter (cm) | ≤ 2 | 16 | - | 62.5% | 15.319 0.006 |
| | $> 2, \leq 3$ | 49 | 22.1 | 31.7% | |
| | $> 3, \leq 4$ | 59 | 18.1 | 23% | |
| | > 4 | 29 | 17.8 | 0% | |
| Thickness of esophageal wall (cm) | ≤ 1 | 32 | 34.8 | 46.2% | 8.224 0.016 |
| | $> 1, \leq 2$ | 104 | 18.4 | 22.0% | |
| | > 2 | 17 | 17.2 | 12.8% | |
| Length of lesion (cm) | < 4 | 10 | - | 57.1% | 7.462 0.024 |
| | $\geq 4, \leq 9$ | 126 | 20 | 26% | |
| | > 9 | 17 | 13.9 | 9.2% | |
| GTV-E (cm ³) | < 18 | 40 | 37.8 | 42.9% | 9.934 0.007 |
| | $\geq 18, \leq 48$ | 82 | 18.4 | 24.6% | |
| | > 48 | 31 | 14.5 | 8.6% | |
| Invasion to adjacent structure | No | 65 | 24.3 | 38.1% | 7.289 0.007 |
| | Yes | 88 | 17.7 | 17.8% | |
| Invasion to trachea | No | 96 | 22.5 | 27.5% | 0.865 0.352 |
| | Yes | 57 | 17.8 | 24.5% | |
| Invasion to bronchus | No | 131 | 22.1 | 29.9% | 3.992 0.046 |
| | Yes | 22 | 15.8 | 5.6% | |
| Invasion to aorta | No | 128 | 22.1 | 29% | 1.469 0.226 |
| | Yes | 25 | 17.8 | 13.9% | |
| Invasion to pericardia | No | 145 | 20 | 27.9% | 1.559 0.212 |
| | Yes | 8 | 16.9 | 0% | |
| SLNM ¹ | Yes | 25 | 15.9 | 21.1% | 1.317 0.251 |
| | No | 107 | 22.4 | 23.6% | |
| Number of positive lymph nodes ² | 0 | 55 | 30.4 | 38.4% | 9.709 0.008 |
| | 1-2 | 85 | 18.4 | 21.2% | |
| | > 2 | 13 | 13.8 | 7.7% | |
| GTV-LN (cm ³) | 0 | 55 | 30.4 | 38.4% | 9.229 0.010 |
| | 0-3 | 59 | 19.2 | 22.8% | |
| | > 3 | 39 | 15.9 | 14% | |
| GTV-T (cm ³) | < 20 | 41 | 39.8 | 41.5% | 12.891 0.002 |
| | 20-40 | 64 | 18.1 | 28.6% | |
| | > 40 | 48 | 15.4 | 9.2% | |

¹SLNM: Supraclavicular lymph node metastasis, only the thoracic cases were included; ²N0: No positive lymph node was observed. N+: Periesophageal lymph node with longest diameter of ≥ 5 mm, mediastinal lymph nodes or supraclavicular lymph nodes with shortest diameter of ≥ 10 mm. LCAH: Late course accelerated hyperfractionated radiotherapy; OS: Overall survival; GTV-E: Gross tumor volume of primary lesion; GTV-LN: Volume of positive lymph nodes; GTV-T: Total gross tumor volume.

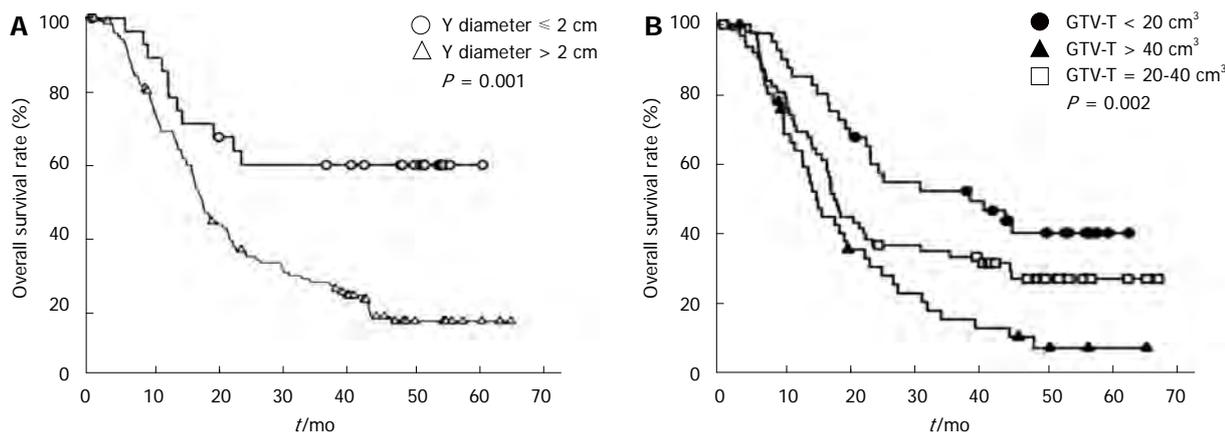


Figure 2 Overall survival of patients. A: With different Y diameter; B: With different total target volume. GTV-T: Total gross tumor volume.

DISCUSSION

EC is an uncommon malignancy in the United States and adenocarcinoma accounts for nearly 60%^[6,7]. In China, EC is endemic and the majority of cases are SCC. We retrospectively analyzed 153 cases of esophageal SCC that were treated with 3D-CRT during 2005 to 2006. The radiation was delivered by 3D-CRT in this patient cohort, which should be contrasted with the 2D conventional radiotherapy technique reported in RTOG 85-01 trial. The 1- 3- and 5-year OS rates were 72.5%, 34.7% and 26.3% respectively, which compares favorably to the results of RTOG 85-01. For the 99 patients who received 3D-CRT alone, the median survival and the 5-year OS rates were 18.4 mo and 26.5%, respectively, which was similar to previous reports from patients receiving chemoradiotherapy^[8-12].

Although the RTOG 85-01 trial reported no 5 year survivors in those patients receiving radiation alone, in this current retrospective review, a 5-year OS rate of 26.5% in those patients receiving 3D-CRT alone was observed. We hypothesize that the improvement in outcome may be a function of increased dose conformity and improved tumor targeting. These advances in 3D-CRT likely lead to decreased treatment related toxicities and reduced the potential for “marginal misses”^[2-4]. Furthermore, 35.3% of patients received chemotherapy in this study, but no significant difference in survival was found between patients with or without chemotherapy ($\chi^2 = 0.438, P = 0.508$). However, due to the limitations of this retrospective study, it is difficult to define the precise role of chemotherapy in EC treated with 3D-CRT.

Tumor length was replaced by depth of esophageal wall invasion as a staging factor in the 1987 version of the American Joint Committee on Cancer (AJCC) tumor node metastasis (TNM) staging system based on the EC registration database of Japan between 1969 and 1980, which demonstrated that depth of tumor invasion was a better predictor for 10-year survival of EC patients than the superficial extent of tumor^[13,14]. In the newly published edition of the AJCC TNM staging system, histological grading and tumor location, as well as depth

of esophageal wall invasion, are regarded as prognostic factors for both adenocarcinoma and SCC^[15,16]. Nevertheless, several previously published results proposed that tumor length was still an independent staging factor for EC other than previously mentioned prognostic factors^[17-20]. However, most, if not all of these results were based on surgery patients, which may be quite different from the outcome of non-surgical cases. Meanwhile, there were few English publications about the prognosis of EC treated with non-surgical approaches.

Many potential prognostic tumor factors achieving significance in univariate analysis were identified in our study. However, only the AP dimension (Y diameter) and GTV-T maintained significance for OS on multivariate analysis. Deep anterior-posterior invasion or large tumor size indicated poor prognosis.

Wang *et al*^[21] reported a Cox model analysis on 100 cases of EC treated with 3D-CRT which also showed similar results as our study, indicating that the GTV-T was an independent predictor for OS. Generally, larger GTV-T means heavier tumor load, increasing numbers of radioresistant hypoxic tumor cells and clonogenic cells and greater limitation of relevant organs at risk that lead to poor survival, which has been regarded as an important predictive factor for cancer in other sites such as lung, breast and head and neck.

Tumor cells tend to invade through the mucosa of the esophagus or spread longitudinally as it is a hollow tube structure. Therefore, deeper invasion through a transverse direction generally means more advanced status. Indeed, the AJCC staging system for EC classifies T staging based upon the depth of tumor invasion^[15,16]. However, transverse diameters are more reasonable predictors of the invasion of tumor especially for non-surgical patients due to the imprecise measurement of thickness of esophageal wall in CT images. Y diameter was the only independent prognostic factor in our study. Concerning the adjacent structures, trachea, bronchus and pericardia are anterior and aorta and vertebrae are posterior to the esophagus. The esophageal tumor tends to grow in other directions than anterior or posterior due to the limitation of these adjacent structures. We found Y diameter was much sma-

ller than the other two diameters (2.5 cm vs 3.2 cm or 3.4 cm). This may explain why the Y diameter becomes the most sensitive predictor to the invasion of esophageal cancer, the bigger Y diameter, the lower OS.

In addition to tumor invasion, the AJCC TNM staging system also incorporates the number of metastatic lymph nodes as an independent staging factor for EC^[15,16]. Several prior studies have confirmed the prognostic significance of lymph nodes^[22-25]. In these studies, lymph node metastases were determined pathologically while in the current study of patients treated by nonsurgical approaches, the lymph node status was determined by CT imaging. Clinical staging is never as accurate as surgical staging which likely explains why lymph node involvement in our study lost significance on multivariate analysis.

In conclusion, 3D-CRT with or without chemotherapy should be considered as a definitive treatment option for patients with inoperable esophageal SCC. Big anterior-posterior tumor dimension (Y diameter) or large tumor size predicted for worse survival which may provide additional prognostic information to the non-surgical staging system and clinical decision making for esophageal SCC.

COMMENTS

Background

Esophageal cancer is the eighth most common cancer worldwide. The incidence of this disease in the United States is relatively low and adenocarcinoma accounts for nearly 60%, while in China, esophageal cancer is endemic and the majority of cases are squamous cell cancer (SCC). Radiation therapy is the most commonly used treatment method for patients with this disease under the following conditions: (1) unresectable disease; (2) resectable disease, but with medical co-morbidities that would preclude surgery; (3) patients declined surgery. Since the report of the results of Radiation Therapy and Oncology Group (RTOG) 85-01 trial in 1999, concurrent chemoradiotherapy has become a widely accepted standard treatment for patients with esophageal carcinoma (EC) who are treated with non-surgical methods.

Research frontiers

In the RTOG 85-01 trial, patients with locally advanced, unresectable EC were randomized to receive either chemoradiation or radiation alone. Those patients receiving chemoradiotherapy had a 26% 5-year survival rate compared with 0% in those treated with radiotherapy alone. However, not all patients are good candidates for chemoradiotherapy due to the presence of multiple co-morbid medical conditions. The outcomes and factors that predict overall survival in patients treated with radiotherapy alone should also be considered in the management of esophageal cancer. Furthermore, the technique used in this report was a conventional method, whereas the development of three-dimensional conformal radiotherapy (3D-CRT) and other advanced radiotherapy techniques has allowed clinicians to treat patients with increased accuracy and normal tissue sparing capabilities. Therefore, it is meaningful to explore the outcomes and predict outcome factors of patients with the advent of modern radiation techniques.

Innovations and breakthroughs

Patients with esophageal cancer in the previous reports were mostly treated with conventional radiation techniques. This study was the largest single institution experience to report the long-term survival for SCC in patients treated with 3D-CRT with or without chemotherapy in English. The tumor node metastasis (TNM) staging system has long been used to predict the outcomes of patients with esophageal cancer after treatment. However, the current TNM staging category of esophageal cancer is wholly based on pathological findings from surgery. Hence it is inapplicable in patients treated with a non-surgical method, especially radiation therapy. Factors other than pathological findings are desperately needed to help predict outcomes or make clinical decisions. In this study, we enrolled general clinical features (including gender, age, lesion location, late course accelerated hyperfractionation, chemotherapy, radiation dose)

and imaging parameters (including the lesion length as defined by esophageal barium swallow and lesion characteristics on computed tomography imaging), (in total 22 factors) in the prediction analysis. These factors could be attained easily through general clinical examinations without surgical approaches.

Applications

The study results suggest that 3D-CRT with or without chemotherapy should be considered as a definitive treatment option for patients with inoperable esophageal SCC. Big anterior-posterior tumor dimension (Y diameter) or large tumor size predict for worse survival which may provide additional prognostic information to the non-surgical staging system and clinical decision making for esophageal SCC.

Terminology

3D-CRT is a type of modern radiation technique, in which the profile of each radiation beam is shaped to fit the profile of the target from a beam's eye view using multileaf collimator (or lead block) and a variable number of beams. This technique has allowed clinicians to treat patients with increased accuracy and normal tissue sparing capabilities and deliver a higher dose of radiation to the tumor than conventional techniques would allow. Since the start of this century, 3D-CRT has been gradually implemented in the radiation treatment of esophageal cancer.

Peer review

This is a good study in which authors evaluate long-term outcomes and prognostic factors for esophageal SCC treated with 3D-CRT. The results are interesting and suggest that the overall survival of EC patients undergoing 3D-CRT is promising.

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Assessment by meta-analysis of interferon-gamma for the diagnosis of tuberculous peritonitis

Si-Biao Su, Shan-Yu Qin, Xiao-Yun Guo, Wei Luo, Hai-Xing Jiang

Si-Biao Su, Shan-Yu Qin, Xiao-Yun Guo, Wei Luo, Hai-Xing Jiang, Department of Gastroenterology, the First Affiliated Hospital of Guangxi Medical University, Nanning 530021, Guangxi Province, China

Author contributions: Jiang HX designed the study, searched the databases, extracted the data, analyzed the results, and wrote the manuscript; Su SB helped with study design, searched the databases, wrote and revised the manuscript; Qin SY formulated the research question, and helped with database searches and analysis; Guo XY and Luo W helped design the data abstraction form and served as second reviewers in extracting the data.

Correspondence to: Dr. Hai-Xing Jiang, Department of Gastroenterology, the First Affiliated Hospital of Guangxi Medical University, No. 22 Shuangyong Road, Nanning 530021, Guangxi Province, China. jihaxi@163.com

Telephone: +86-771-5356725 Fax: +86-771-5356585

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Abstract

AIM: To investigate the performance and diagnostic accuracy of interferon-gamma (IFN- γ) for tuberculous peritonitis (TBP) by meta-analysis.

METHODS: A systematic search of English language studies was performed. We searched the following electronic databases: MEDLINE, EMBASE, Web of Science, BIOSIS, LILACS and the Cochrane Library. The Standards for Reporting Diagnostic Accuracy initiative and Quality Assessment for Studies of Diagnostic Accuracy tool were used to assess the methodological quality of the studies. Sensitivity, specificity, and other measures of the accuracy of IFN- γ concentration in the diagnosis of peritoneal effusion were pooled using random-effects models. Receiver operating characteristic (ROC) curves were applied to summarize overall test performance. Two reviewers independently judged study eligibility while screening the citations.

RESULTS: Six studies met the inclusion criteria. The

average inter-rater agreement between the two reviewers for items in the quality checklist was 0.92. Analysis of IFN- γ level for TBP diagnosis yielded a summary estimate: sensitivity, 0.93 (95%CI, 0.87-0.97); specificity, 0.99 (95%CI, 0.97-1.00); positive likelihood ratio (PLR), 41.49 (95%CI, 18.80-91.55); negative likelihood ratio (NLR), 0.11 (95%CI, 0.06-0.19); and diagnostic odds ratio (DOR), 678.02 (95%CI, 209.91-2190.09). χ^2 values of the sensitivity, specificity, PLR, NLR and DOR were 5.66 ($P = 0.3407$), 6.37 ($P = 0.2715$), 1.38 ($P = 0.9265$), 5.46 ($P = 0.3621$) and 1.42 ($P = 0.9220$), respectively. The summary receiver ROC curve was positioned near the desirable upper left corner and the maximum joint sensitivity and specificity was 0.97. The area under the curve was 0.99. The evaluation of publication bias was not significant ($P = 0.922$).

CONCLUSION: IFN- γ may be a sensitive and specific marker for the accurate diagnosis of TBP. The level of IFN- γ may contribute to the accurate differentiation of tuberculosis (TB) ascites from non-TB ascites.

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Key words: Tuberculosis; Tuberculous peritonitis; Interferon-gamma; Diagnosis; Meta-analysis

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INTRODUCTION

Tuberculous peritonitis (TBP) is a manifestation of tuberculosis (TB), which constitutes about 3% of extrapulmonary tuberculosis (EPTB) cases, and EPTB constitutes

about 15%-20% of all cases of TB in immunocompetent patients and accounts for more than 50% of cases in human immunodeficiency virus-positive individuals^[1]. TBP is one of the most common forms of EPTB and cases of TBP are expected to increase with the increasing incidence of TB worldwide^[2,3]. Early diagnosis of TBP is beneficial for anti-TB treatment, the prevention of complications, and reduction of mortality rate^[4]. However, current clinical diagnostic techniques for TBP are time-consuming and inefficient. The definitive diagnosis requires histological confirmation of caseous granulomas. As ascites is one of the clinical signs of TBP, bacteriologic confirmation can be performed using ascitic fluid-derived acid-fast bacilli smears as well as cultures for *Mycobacterium tuberculosis* (*M. tuberculosis*). However, four weeks are required for the cultivation of *M. tuberculosis*, and acid-fast bacilli smears are too insensitive to meet the current diagnostic demand^[5]. Laparoscopy-guided biopsy is advantageous for rapid TBP diagnosis, but has complications related to anesthesia and potential injury and bleeding^[6]. An evaluation of existing techniques is urgently required as is the development of new methods with high sensitivity and specificity for early and accurate TBP diagnosis.

M. tuberculosis infection initiates an immunologic cascade involving the secretion of various cytokines and recruitment of Th1 lymphocytes. With abundant cell recruitment at the morbid site, the levels of various cytokines are markedly elevated. Interferon-gamma (IFN- γ) is an important cytokine following infection with *M. tuberculosis*^[7,8]. Studies assessing the level of IFN- γ have been reported. Several studies from different parts of the world have demonstrated the efficacy of IFN- γ for the diagnosis of TB pleural and pericardial effusions^[9,10], and its diagnostic efficacy has been compared with that of adenosine deaminase (ADA) in terms of cost-effectiveness^[11]. Some studies have also evaluated the role of IFN- γ in the diagnosis of TB ascites^[12-14]. However, whether IFN- γ detection contributes to accurate TBP diagnosis remains controversial. In the present study, we systematically analyzed and assessed the overall efficacy of IFN- γ in the diagnosis of TBP *via* meta-analysis techniques.

MATERIALS AND METHODS

Search strategy and study selection

We searched the following electronic databases: MEDLINE (1980-2011); EMBASE (1980-2011); Web of Science (1990-2011); BIOSIS (1993-2011) and LILACS (1980-2011). We also reviewed the Cochrane Library to identify relevant studies. Updated searches were carried out in December 2011. The following search terms were used: "tuberculosis" "*Mycobacterium tuberculosis*" "peritonitis" "peritoneal effusion/peritoneal fluid/abdominal effusion/ascitic fluid/ascites" "interferon/IFN" "sensitivity and specificity" and "accuracy". We contacted experts in the specialty and searched the reference lists of primary and review articles. Although no language restrictions

were imposed initially, our resources only permitted the review of articles published in the English language for the full text review and final analysis. Conference abstracts and letters were excluded due to unavailable data.

A study was included when it provided both the sensitivity (true-positive rate) and specificity (false-positive rate) of IFN- γ for TBP diagnosis, or provided IFN- γ values in a dot-plot form that allowed results to be extracted for individual study subjects. Patients of any age diagnosed with TBP underwent smear or culture of *M. tuberculosis* and/or histologic observation of peritoneal tissue, as well as clinical diagnosis, such as response to anti-TB therapy. In addition, we selected studies including at least 10 TBP specimens which were eligible for inclusion in order to reduce selection bias due to a small number of participants. Two reviewers (Su SB and Jiang HX) independently judged study eligibility while screening the citations. Disagreements were resolved by consensus.

Data extraction and quality assessment

Two reviewers (Su SB and Jiang HX) checked and extracted data independently. The reviewers were blinded to publication details, and disagreements were resolved by consensus. Data retrieved from the reports included participant characteristics, assay methods, sensitivity and specificity data, cutoff values, year of publication, and methodological quality. Peritonitis IFN- γ values provided in dot plots were measured by placing scalar grids over the plots, and were analyzed by a receiver operating characteristic (ROC) curve for each study (SPSS; Chicago, IL, United States). A summary of each study, including the numbers of true-positive, false-positive, false-negative and true-negative findings, is displayed in Table 1.

We assessed the methodological quality of studies using guidelines established by the Standards for Reporting Diagnostic Accuracy (STARD)^[15] initiative and the Quality Assessment for Studies of Diagnostic Accuracy (QUADAS) tool^[16]. In addition, the following study design characteristics were retrieved: (1) cross-sectional design *vs* case-control design; (2) consecutive or random sampling of patients; (3) blind (single or double) interpretation of determination and reference standard results; and (4) prospective data collection. If primary studies did not show data that met the above criteria, we requested them from the authors. The "unknown" items were then treated as "no" if the authors did not respond.

Statistical analysis

We used standard methods recommended for meta-analyses of diagnostic test evaluations^[17]. Analyses were performed using a professional statistical software program (Meta-DiSc for Windows; XI Cochrane Colloquium; Barcelona, Spain). The following measures of test accuracy were analyzed for each study: sensitivity, specificity, positive likelihood ratio (PLR), negative likelihood ratio (NLR) and diagnostic odds ratio (DOR).

The analysis was based on a summary ROC (SROC) curve^[17]. Sensitivity and specificity as a single test thresh-

Table 1 Summary of included studies

| Ref. | Patients | Assay method | Cut off | Test results | | | | Quality score | |
|--------------------------------------|----------|--------------|------------------|--------------|----|----|----|---------------|--------|
| | | | | TP | FP | FN | TN | STARD | QUADAS |
| Ribera <i>et al</i> ^[12] | 86 | RIA | 3 U/mL or 9 U/mL | 16 | 0 | 0 | 70 | 11 | 9 |
| Soliman <i>et al</i> ^[13] | 50 | ELISA | 26 pg/mL | 13 | 0 | 3 | 33 | 15 | 12 |
| Sathar <i>et al</i> ^[14] | 92 | RIA | 3.2 U/mL | 25 | 1 | 2 | 54 | 13 | 10 |
| Saleh <i>et al</i> ^[28] | 41 | ELISA | 0.35 IU/mL | 13 | 0 | 1 | 27 | 16 | 11 |
| Sharma <i>et al</i> ^[29] | 119 | ELISA | 112 pg/mL | 30 | 3 | 1 | 85 | 18 | 13 |
| Sathar <i>et al</i> ^[30] | 52 | ELISA | 20 pg/mL | 21 | 0 | 2 | 29 | 14 | 12 |

ELISA: Enzyme-linked immunosorbent assay; RIA: Radioimmunoassay; TP: True-positive; FP: False-positive; FN: False-negative; TN: True-negative; STARD: Standards for Reporting Diagnostic Accuracy, maximum score 25, guidelines that aim to improve the quality of reporting in diagnostic studies; QUADAS: Quality Assessment for Studies of Diagnostic Accuracy, appraisal by use of empirical evidence, maximum score 14, expert opinion and formal consensus to assess the quality of primary studies of diagnostic accuracy.

Table 2 Characteristics of included studies

| Ref. | TB/N-TB patients | Reference standard | Cross-sectional design | Consecutive or random | Blinded design | Prospective |
|--------------------------------------|------------------|--------------------|------------------------|-----------------------|----------------|-------------|
| Ribera <i>et al</i> ^[12] | 16/70 | Bac/His or Clin | No | Yes | No | Yes |
| Soliman <i>et al</i> ^[13] | 17/33 | Bac/His or Clin | No | Yes | Yes | Yes |
| Sathar <i>et al</i> ^[14] | 30/62 | Bac/His | No | Yes | No | Yes |
| Saleh <i>et al</i> ^[28] | 14/27 | Bac/His or Clin | No | Yes | No | Yes |
| Sharma <i>et al</i> ^[29] | 31/88 | Bac/His | Yes | Yes | Yes | Yes |
| Sathar <i>et al</i> ^[30] | 23/29 | Bac/His or Clin | No | Yes | No | Yes |

TB: Tuberculosis; Bac: Bacteriology; His: Histology; Clin: Clinical course.

old identified for each study were used to plot an SROC curve^[18]. A random-effects model was adopted to calculate the average sensitivity, specificity, and other measures across studies^[19,20].

The term heterogeneity refers to the degree of variability in results across studies, which was used in relation to meta-analyses. We detected statistically significant heterogeneity with the χ^2 test (Fisher exact tests). To assess the effects of STARD and QUADAS scores on the diagnostic ability of IFN- γ , we included them as covariates in univariate meta-regression analysis (inverse variance weighted). We also analyzed the effects of other covariates on DOR, such as cross-sectional design, consecutive or random sampling of patients, single or double interpretation of determination, reference standard results, and prospective data collection. The relative DOR (RDOR) was calculated according to standard methods to analyze the change in diagnostic precision in the study per unit increase in the covariate^[21,22]. Since publication bias is of concern for meta-analyses of diagnostic studies, we tested for the potential presence of this bias with funnel plots and the Egger test^[23].

RESULTS

Selection and summary of studies

Eleven out of 25 publications dealing with peritonitis IFN- γ concentration for TBP diagnosis were considered to be eligible for inclusion in the meta-analysis^[10,12-14,24-30]. Among these publications, five studies^[10,24-27] were excluded because IFN- γ was detected only in peritoneal dialysis patients^[27], there was no detailed data^[25] and the

number of participants was 10 or less^[10,24,26] (Figure 1). Finally, 6 studies^[12-14,28-30] including 131 TBP patients and 309 non-TBP patients were available for analysis, and the clinical characteristics of these studies, along with QUADAS scores, are outlined in Table 1.

Quality of reporting and study characteristics

The average inter-rater agreement between the two reviewers for items in the quality checklist was 0.92. All studies (100%) were collected from consecutive patients. The average sample size was 69 (range, 41-119) in the included studies. In four studies^[12,13,28,30], a small proportion of the patients received the diagnosis according to clinical presentation, peritoneal effusion analysis, radiology findings and responsiveness of the patient to anti-TB chemotherapy. However, the diagnosis of peritoneal TB was confirmed in most of the TBP patients based on the conventional "gold standard" which was a smear or a positive *M. tuberculosis* culture which was taken from ascitic fluid and/or histology showing a caseating granuloma. In two studies^[14,29], all patients were diagnosed with TBP based on a smear or culture that was positive for *M. tuberculosis* and had been taken from ascitic fluid and/or histology showing a caseating granuloma. All studies (100%) which reported that the study design was prospective could be identified (Table 2). Two studies (33.3%) reported blinded interpretation of the IFN- γ assay independent of the reference standard.

Diagnostic accuracy

The sensitivity and specificity of 6 IFN- γ assays for the diagnosis of TBP are shown in the forest plot (Figure 2).

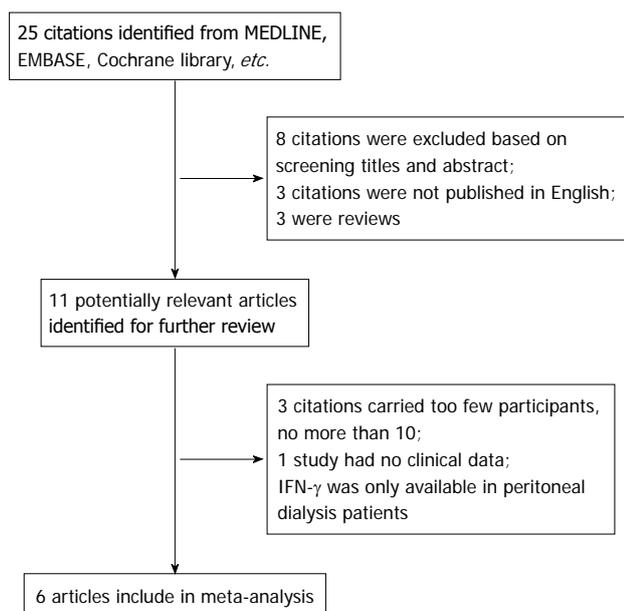


Figure 1 Flowchart of study selection. IFN- γ : Interferon-gamma.

Sensitivity of IFN- γ for TBP diagnosis ranged from 0.54 to 1.00 (mean, 0.93; 95%CI, 0.87-0.97), while specificity ranged from 0.87 to 1.00 (mean, 0.99; 95%CI, 0.97-1.00). We also noted that PLR was 41.49 (95%CI, 18.80-91.55), NLR was 0.11 (95%CI, 0.06-0.19) and DOR was 678.02 (95%CI, 209.91 to 2190.09). χ^2 values of sensitivity, specificity, PLR, NLR and DOR were 5.66 ($P = 0.3407$), 6.37 ($P = 0.2715$), 1.38 ($P = 0.9265$), 5.46 ($P = 0.3621$) and 1.42 ($P = 0.9220$), respectively, indicating no significant heterogeneity for sensitivity, specificity, PLR, NLR and DOR between studies.

The SROC plot is different from the traditional ROC plot and explores the effect of varying thresholds on sensitivity and specificity in a single study. In a SROC plot, any of the data points represent a separate study. The SROC curve represents a global summary of test performance and shows the tradeoff between sensitivity and specificity. A graph of the SROC curve for IFN- γ determination showing true-positive rates and false-positive rates from individual studies is shown in Figure 3. As a global measure of test efficacy, we used the Q -value, the intersection point of the SROC curve with a diagonal line from the left upper corner to the right lower corner of the ROC space, which corresponds to the highest common value of sensitivity and specificity for the test. This point represents an overall measure of the discriminatory power of a test. Our data showed that the SROC curve was positioned near the desirable upper left corner and that the maximum joint sensitivity and specificity was 0.97. The area under the curve (AUC) was 0.99. This indicated a high level of overall accuracy.

Multiple regression analysis and publication bias

By using the STARD guidelines^[15], a quality score for each study was compiled on the basis of title and introduction, methods, results and discussion (Table 1). Qual-

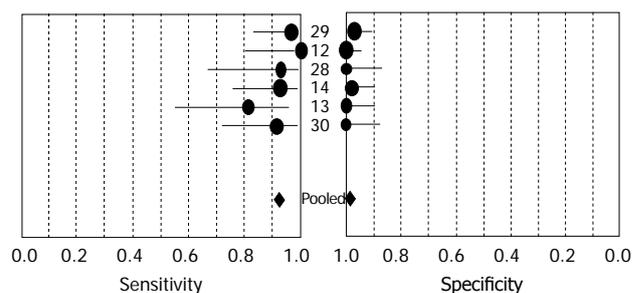


Figure 2 Forest plot showing the sensitivity and specificity of interferon-gamma in the diagnosis of tuberculous peritonitis. Forest plot shows the sensitivity and specificity of interferon-gamma (IFN- γ) for tuberculous peritonitis diagnosis. The point estimates of sensitivity and specificity from each study are shown as solid circles. Error bars indicated 95%CI. Numbers indicate the studies included in the meta-analysis, as cited in the reference list. Pooled estimates for IFN- γ assay were as follows: sensitivity, 0.93 (95%CI, 0.87 to 0.97), specificity, 0.99 (95%CI, 0.97 to 1.00).

ity scoring was also carried out using QUADAS^[16], in which a score of 1 indicated a fulfilled criterion, 0 if an unclear criterion, and -1 if criterion not achieved. These scores were used in the meta-regression analysis to assess the effect of study quality on the RDOR of IFN- γ in the diagnosis of TBP. As shown in Table 3, studies with higher quality (STARD score, ≥ 13 ; QUADAS score, ≥ 10) produced RDOR values that were not significantly higher than those studies with lower quality. We also noted that differences for studies with or without blinded, cross-sectional, consecutive/random and prospective designs did not reach statistical significance, indicating that the study design did not substantially affect the diagnostic accuracy.

The evaluation of publication bias showed that the results from the Egger test were not significant ($P = 0.922$). These results indicated little potential for publication bias.

DISCUSSION

The diagnosis of extrapulmonary mycobacterial infection is often difficult to establish since it has a nonspecific clinical presentation. Conventional diagnostic tests such as microscopic examination of peritonitis fluid by Ziehl-Neelsen staining, culture of mycobacteria from peritoneal effusion and peritonitis pathological examinations, are not always helpful in making the diagnosis because of their limitations. Invasive procedures, such as peritoneoscopy, laparotomy and peritoneal biopsy, which require appropriate and adequate clinical specimens are complex and sometimes risky^[6,14]. A negative smear for acid-fast bacilli, a lack of granulomas on histopathology, and failure to culture *M. tuberculosis* do not exclude the diagnosis^[5,31-33]. Furthermore, the culture of *M. tuberculosis* takes 4 wk, and acid-fast bacilli smears are too insensitive to meet current needs^[5,34].

The most popular biomarkers which have been proposed for TBP diagnosis are ADA and INF- γ . The levels of both were significantly higher in TBP patients than in

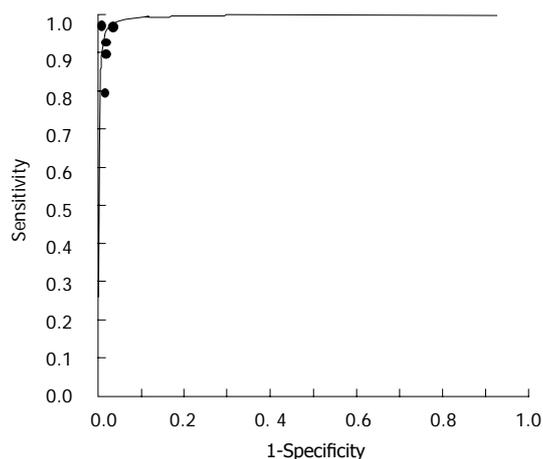


Figure 3 Summary receiver operating characteristic curves for interferon-gamma assays. Solid circles represent each study included in the meta-analysis. The size of each study is indicated by the size of the solid circle. Summary receiver operating characteristic curves summarize the overall diagnostic accuracy.

non-TBP patients. Both showed relatively high sensitivity and specificity in diagnosing TBP^[12,14,24,26,28,29]. However, there are also other methods for TBP diagnosis, such as the molecular rapid amplification-based tests [for example polymerase chain reaction (PCR)] which detect specific DNA or RNA fragments of *M. tuberculosis*. Many reports suggest that various PCR tests have good performance with sensitivity reaching up to 95% in smear-positive patients. However, Ziehl-Neelsen staining in patients with TB peritonitis is positive in only 3% of cases, and PCR sensitivity would be similarly very low^[35]. At present, the ADA assay has been recommended not only in the TB peritonitis diagnostic test^[36], but also as a tool for the differential diagnosis of different forms of EPTB, such as pleuritis, synovitis, and meningitis, infections of the female genital system and peripheral lymph nodes, and uveitis^[37]. Liang *et al.*^[38] and his colleagues have completed a meta-analysis (including 63 studies) to estimate ADA in the diagnosis of tuberculous pleurisy. The meta-analysis showed that the mean sensitivity of the ADA assays was 0.92, while the mean specificity was 0.90, the maximum joint sensitivity and specificity was 0.91, while AUC was 0.96, indicating a relatively high level of overall accuracy. However, the present meta-analysis showed that the mean sensitivity of the IFN- γ assay was 0.93, while the mean specificity was 0.99, and that the maximum joint sensitivity and specificity was 0.97, while the AUC was 0.99, indicating a higher level of overall accuracy.

The DOR is a single indicator of test accuracy^[39] that combines the data from sensitivity and specificity into a single number. The DOR of a test is the ratio of the odds of positive test results in the patient with disease relative to the odds of positive test results in the patient without disease. The value of DOR ranges from 0 to infinity, the higher values indicate better discriminatory test performance (higher accuracy). A DOR of 1.0 indicates that a test does not discriminate between patients with

Table 3 Weighted meta-regression of the effects of study design methods and methodological quality on diagnostic accuracy of interferon-gamma assays

| Covariate | Studies | Coefficient | RDOR (95%CI) | P |
|------------------------|---------|-------------|----------------------|-------|
| Consecutive or random | 6 | - | - | - |
| Prospective | 6 | - | - | - |
| Cross-sectional design | 1 | -1.763 | 0.17 (0.00, 2053.55) | 0.593 |
| Blinded design | 2 | -0.649 | 0.52 (0.01, 31.46) | 0.649 |
| Methods | | | | |
| RIA | 2 | -0.815 | 0.44 (0.01, 26.58) | 0.571 |
| ELISA | 4 | | | |
| QUADAS ≥ 10 | 5 | -2.137 | 0.12 (0.00, 98.54) | 0.387 |
| STARD ≥ 13 | 5 | -2.137 | 0.12 (0.00, 98.55) | 0.387 |

ELISA: Enzyme-linked immunosorbent assay; RIA: Radioimmunoassay; STARD: Standards for Reporting Diagnostic Accuracy, maximum score 25, guidelines that aim to improve the quality of reporting in diagnostic studies; QUADAS: Quality Assessment for Studies of Diagnostic Accuracy, appraisal by use of empirical evidence, maximum score 14, expert opinion and formal consensus to assess the quality of primary studies of diagnostic accuracy; RDOR: Relative diagnostic odds ratio.

and those without disease. In the present meta-analysis, we found that the mean DOR was 678.02, also indicating a high level of overall accuracy.

Since the SROC curve and the DOR are not easy to interpret or use in clinical practice^[40], and likelihood ratios are considered to be more clinically meaningful^[40], we also presented both PLR and NLR as our measures of diagnostic accuracy. Likelihood ratios of > 10 or < 0.1 generate large and often conclusive shifts from pre-test to posttest probability (indicating high accuracy). A PLR value of 41.49 suggests that patients with TBP have an approximately 41-fold higher chance of being IFN- γ assay-positive compared with patients without TBP. This high probability would be considered high enough to begin or to continue anti-TB treatment of TBP patients, especially in the absence of any evidence of malignancy. On the other hand, NLR was found to be 0.11 in the present meta-analysis. If the IFN- γ assay result was negative, the probability that this patient has TBP is approximately 10%, which is not low enough to rule out TBP. These data suggest that a negative IFN- γ assay result should not be used alone as a justification to deny or to discontinue anti-TB therapy. The choice of therapeutic strategy should be based on the results of microscopic examination of smear or culture of *M. tuberculosis* and/or histologic observation of peritoneal tissue, as well as other clinical data, such as response to anti-TB therapy.

An exploration of the reasons for heterogeneity rather than computation of a single summary measure is an important goal of meta-analysis^[41]. In our meta-analysis, both STARD and QUADAS scores were used in the meta-regression analysis to assess the effect of study quality on RDOR. Most of the studies were of high quality (STARD score of ≥ 13 or QUADAS score of ≥ 10), with the exception of one study^[12] which was assessed to be of low quality (STARD score of 11 and QUADAS score of 9). We found that there was no statis-

tical heterogeneity for sensitivity, specificity, PLR, NLR, and DOR among the studies, which indicated that the differences for studies with or without a blinded, cross-sectional, consecutive/random and prospective design did not reach statistical significance, and the study design did not substantially affect diagnostic accuracy.

It should be emphasized that a definite TBP diagnosis is achieved when *M. tuberculosis* is demonstrated in peritonitis specimens, or when caseating granulomas are found in peritonitis biopsy specimens. As mentioned above, *M. tuberculosis* requires 4 wk of culture, and acid-fast bacilli smears are too insensitive to meet current needs^[15,34]. Where diagnostic difficulty exists, measuring the levels of several biomarkers, such as ADA and IFN- γ , in ascitic fluid is useful, and clinicians can embark on empirical anti-TB therapy while awaiting culture results, especially in young patients from areas with a high prevalence of TB. One criticism of the use of biomarkers rather than cultures for TBP diagnosis is that culture results are not available to guide anti-TB therapy. In short, none of the biomarkers, including IFN- γ , provide culture and sensitivity data. Culture results are particularly useful if drug resistant TB is prevalent^[42].

Our meta-analysis had several limitations. Firstly, the exclusion of conference abstracts, letters to the editor, and non-English-language studies might have led to publication bias, which was not found in the present review. However, a review of these abstracts and letters suggested that the overall results were similar to the results in the English language studies included. Secondly, misclassification bias may occur. TBP is not always diagnosed by either histologic or microbiological examination. Actually, some patients were diagnosed with TBP infection based just on the clinical course. This issue regarding accuracy of diagnosis could cause nonrandom misclassification, leading to biased results. Finally, the number of studies that met the inclusion criteria was not large enough. Multi-center and large blinded randomized controlled trials with IFN- γ assays using peritoneal effusion for TBP diagnosis should be conducted.

Based on this study, IFN- γ may play a potential role in accurate TBP diagnosis. This may be helpful in clinical findings and conventional tests including microbiological examination and peritoneal biopsy. Numerous studies are required to further establish the role of IFN- γ for early and accurate TBP diagnosis.

ACKNOWLEDGMENTS

We are grateful to Professor Shi HZ for his great assistance in the statistical analysis and translating foreign language articles.

COMMENTS

Background

Tuberculous peritonitis (TBP) is a manifestation of tuberculosis. Its diagnosis is still challenging and of great importance. Early and accurate diagnosis contributes to effective therapy and good survival rates. However, current clinical

diagnostic techniques for TBP are time-consuming and inefficient. Interferon-gamma (IFN- γ) in peritoneal effusion has been shown to be a marker for the diagnosis of TBP.

Research frontiers

How does IFN- γ in ascitic fluid testing relate to blood interferon- γ release assay (IGRA) tests? The blood IGRA test is an established test for latent tuberculous (TB) infection, however, there is little information on the difference between IFN- γ and IGRA tests in clinical diagnosis. Multi-center and large blinded randomized controlled trials using both these tests for TB diagnosis should be conducted in the future.

Innovations and breakthroughs

Authors assessed IFN- γ for TBP diagnosis by meta-analysis, and the clinical findings may greatly facilitate the diagnosis and differential diagnosis of TBP.

Applications

IFN- γ may play a potential role in the accurate diagnosis of TBP. This may be helpful in clinical findings and conventional tests including microbiological examination and peritoneal biopsy. The level of IFN- γ may contribute to the accurate differentiation of tuberculosis ascites from non-tuberculosis ascites.

Peer review

This systematic review and meta-analysis of IFN- γ testing for TBP was generally of good quality, and adhered to relevant guidelines for systematic review and quality assessment.

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Case report and review of esophageal lichen planus treated with fluticasone

Marie Lourdes Ynson, Faripour Forouhar, Haleh Vaziri

Marie Lourdes Ynson, Department of Medicine, University of Connecticut Health Center, Farmington, CT 06030, United States
Faripour Forouhar, Department of Pathology, University of Connecticut Health Center, Farmington, CT 06030, United States
Haleh Vaziri, Department of Gastroenterology, University of Connecticut Health Center, Farmington, CT 06030, United States
Author contributions: Ynson ML, Forouhar F and Vaziri H contributed to manuscript writing and revisions.
Correspondence to: Marie Lourdes Ynson, MD, Department of Medicine, University of Connecticut Health Center, 263 Farmington Avenue, Farmington, CT 06030, United States. mynson@resident.uhc.edu
Telephone: +1-860-6796524 Fax: +1-860-6793159
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Abstract

Lichen planus is a fairly common chronic idiopathic disorder of the skin, nails and mucosal surfaces. Esophageal involvement of this disease on the other hand is rare and only about 50 cases have been reported in literature. Given its rarity, it can be difficult to diagnose and may be easily misdiagnosed as reflux esophagitis. Currently, there are no clear recommendations on the optimal management of this disease and little is known about the best treatment approach. Systemic steroids are usually the first line treatment and offer a favorable response. In this report, we would like to present a novel approach in the management of esophageal lichen planus in a middle-aged woman treated successfully with swallowed fluticasone propionate 220 mcg twice a day for 6 wk, as evidenced by objective clinical findings. Based on our review of related literature and experience in this patient, we feel that a trial of swallowed fluticasone may be a prudent approach in the management of these patients since it has a more favorable side effect profile than systemic treatment.

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Key words: Lichen planus; Lichen rubra planus; Anti-inflammatory agents; Steroids; Dysphagia; Fluticasone

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INTRODUCTION

Lichen planus is a well-recognized chronic idiopathic disorder involving the skin, nails and mucosal surfaces including the mouth, pharynx and perineum^[1]. It affects less than 1% of the general population^[2]. Mucosal surface involvement is found in about 30%-70% of patients diagnosed with lichen planus^[3]. Esophageal involvement, on the other hand, is considered to be rare with its true prevalence unknown. The study by Dickens *et al*^[4] demonstrated esophageal lichen planus in 26% of patients with mucocutaneous lesions while a larger study by Eisen^[5] found esophageal lesions in only 1% of patients. Here we describe a case of esophageal lichen planus who was treated with swallowed fluticasone with good response and present a review of this topic.

CASE REPORT

A 63-year-old female with history of gastric bypass surgery, coronary artery disease, and hyperlipidemia reported having intermittent solid food dysphagia for the past 3 years. She also had symptoms of gastroesophageal reflux disease that was initially controlled with omeprazole 20 mg daily. Later on, she developed multiple episodes of breakthrough and nocturnal reflux symptoms accompanied by occasional difficulty in swallowing large pills that happened 2-3 times a week. She denied food regurgitation, odynophagia, nausea, vomiting or weight loss. For further evaluation of her dysphagia, a barium swallow

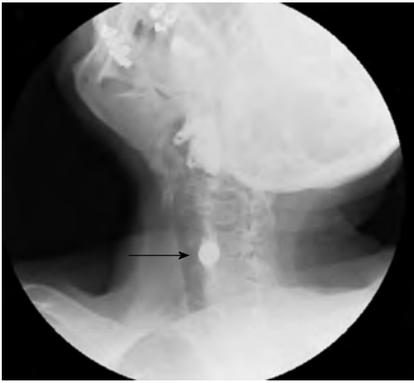


Figure 1 Barium swallow showing barium pill (arrow) trapped in C6-C7.



Figure 2 Endoscopy image showing friable mucosa and dried blood in upper esophagus with a mild to moderate stricture at upper and mid-esophageal junction.



Figure 4 Endoscopy image showing resolution of lesions in the upper esophagus post treatment.

was performed. During the procedure, the barium pill briefly got trapped in the upper esophagus at the level of C6-C7. This location correlated with the reported site of dysphagia (Figure 1). The rest of the exam was uneventful and no mass or stricture was noted except for a small sliding hiatal hernia. She subsequently underwent an upper endoscopy which showed areas of friable mucosa and dried blood in the upper esophagus with a mild to moderate stricture at the upper and mid-esophageal junction (Figure 2). Esophageal biopsies were obtained which showed hyperkeratosis, parakeratosis and spongiosis of the lower third of the epithelium with focal dys-

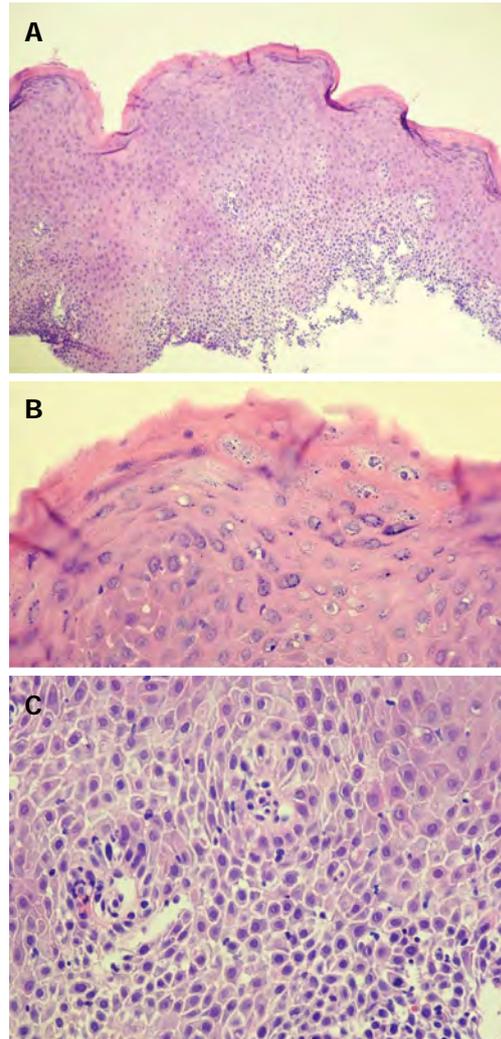


Figure 3 Chronic inflammatory cell infiltration of the epithelium was also present and was mainly composed of lymphocytes. A: Low power view of upper esophagus shows extensive severe keratinization, organizational disarray of cell arrangement and spongiosis of lower layer associated with inflammatory infiltration. (E, $\times 40$); B: High power of parakeratotic cells and accumulation of keratohyaline granules in the cytoplasm of the mature keratinocytes. In this zone keratinization is mild. (HE, $\times 400$); C: High power view of spongiosis of lower layers of squamous epithelium associated with sprinkling of lymphocytes typical of longstanding chronic inflammation. (HE, $\times 400$).

keratosis. Chronic inflammatory cell infiltration of the epithelium was also present and was mainly composed of lymphocytes (Figure 3). Submucosa was not present for evaluation of lichenoid inflammation. Nonetheless, in the presence of the clinical history, the findings were interpreted to be consistent with lichen planus. On further questioning, patient reported having an oral lesion diagnosed to be lichen planus in 2009. Due to her history of previous lichen planus and endoscopy findings, patient was diagnosed with esophageal lichen planus and was started on swallowed fluticasone propionate 220 mcg twice daily for 6 wk. At her 4th wk follow-up she noted resolution of her symptoms. On follow-up endoscopy at 15 wk, only small light pink plaques were noted in the mid-esophagus while the rest of the esophagus showed normal looking mucosa (Figure 4). Pathology

Table 1 Differentiation between types of lichen planus

| | Cutaneous | Oral | Esophageal |
|-----------------------------|---|---|---|
| Age and gender distribution | No gender preference | No gender preference | Generally middle aged females ¹ |
| Clinical findings | Eruptions of violaceous, scaling, pruritic plaques ^[7] | Atrophic lesions, erosions, lace-like reticulated plaques ^[2] | Elevated lacy white papules, esophageal webs, pseudomembranes, desquamation, and superficial pinpoint erosions with and without stenosis ^[4,5,15,22-25] |
| Duration of illness | Self-limited with spontaneous regression in 1-2 yr ^[21] | Propensity for chronicity ^[7] | Propensity for chronicity ^[7] |
| Histologic findings | Hypergranulosis, hyperorthokeratosis, acanthosis and "saw-tooth" elongation of the rete pegs ^[21] Typical band-like inflammatory infiltrate with a predominance of mature T cells and basal layer degeneration including characteristic Civatte bodies (<i>i.e.</i> , apoptotic basal keratinocytes) ^[21] | Oral lesions closely resemble esophageal lesions Typical band-like inflammatory infiltrate with a predominance of mature T cells and basal layer degeneration including characteristic Civatte bodies (<i>i.e.</i> , apoptotic basal keratinocytes) ^[21] | Parakeratosis, atrophic epithelium, lacks hypergranulosis, variable thinning or acanthosis ^[21] Typical band-like inflammatory infiltrate with a predominance of mature T cells and basal layer degeneration including characteristic Civatte bodies (<i>i.e.</i> , apoptotic basal keratinocytes) ^[21] |
| Risk of malignancy | No increased risk of malignant transformation | Increased risk of oral malignancy; Associated with hepatitis C | Some case reports have shown malignancy associated with esophageal lichen planus |

¹One report by Chryssostalis *et al*^[10] showed esophageal lichen planus in a 22-yr-old male patient.

showed normal to atrophic squamous mucosa with mild non-specific inflammation including rare eosinophils and no evidence of interface band-like lymphocytic infiltrate which was compatible with lichen planus post-therapy.

DISCUSSION

Lichen planus is a common disorder of squamous epithelium^[2]. The exact cause of this disease is unknown; however infectious (viral), neurologic, genetic and immunologic causes have been proposed^[6]. Lichen planus appears to be mediated by cytotoxic CD8+ T cells that attack an antigen in the basal epithelium in a manner resembling graft-versus-host disease^[2]. There are no detailed studies published to specifically address the pathogenesis of esophageal lichen planus. Chandan *et al*^[7] suggests that the pathogenesis may be similar to oral lichen planus. It has been suggested that oral lichen planus is due to an immune response to an exogenous or endogenous antigen found in the basal keratinocytes. This activates the Langerhans cells which then present the antigen to the CD4+ T lymphocytes migrating to the oral mucosa lamina propria. Keratinocytes respond to the injury by producing cytokines that promote CD8+ T lymphocyte stimulation. These CD8+ cytotoxic cells will distribute near the epithelium and destroy it^[8]. Table 1 summarizes the differences between esophageal *vs* oral and cutaneous lichen planus.

Esophageal lichen planus is considered to be a rare disease with only about 50 cases reported in English literature. Retrospectively, Eisen^[5] suggests a prevalence of less than 1% among patients with oral lichen planus but because of subtle clinical findings and lack of characteristic histologic features, the true prevalence is hard to determine^[9]. All reported patients are usually middle-aged females except in the study by Chryssostalis *et al*^[10] that

reported a case of a 22-year-old male with esophageal involvement. Esophageal lichen planus is often present in patients with extraesophageal involvement, but it may also be present in patients without dermal or oropharyngeal manifestations^[11]. In a case series of esophageal lichen planus, the initial site of involvement was esophagus in 48% of patients^[12]. Symptoms can range from asymptomatic to odynophagia and dysphagia^[4]. The proximal or mid-esophagus is the most common site of involvement^[7], although the entire esophagus can also be affected with sparing of the gastroesophageal junction^[13]. Different types of lesions have been reported and can range from elevated lacy white papules, esophageal webs, pseudomembranes, desquamation, and superficial pinpoint erosions with and without stenosis. Most case reports mention the characteristic finding of peeling of the mucosa away from the esophagus leaving a friable, inflamed surface that bleeds on contact^[11]. Strictures are also common in these patients and may represent progression from inflammation to ulceration, fibrosis with subsequent stricture formation^[14].

Esophageal lichen planus may be misdiagnosed as reflux esophagitis which further compounds the situation. In our patient, esophageal lichen planus was favored over reflux esophagitis due to the extensive degree of hyperkeratosis and focal parakeratosis with accumulation of keratohyaline granules which are unlikely in reflux related injury. The presence of spongiosis in the lower third of the epithelium was also more consistent with esophageal lichen planus. The spongiosis in reflux diseases tend to occur in the upper third of the epithelium where acid is in contact with the mucosa. The other factor that can help to differentiate these 2 entities is the site of involvement in the esophagus. While reflux disease is usually more severe in the distal esophagus, lichen planus affects the middle and upper esophagus in most cases.

The most characteristic histologic finding in esophageal lichen planus is a bandlike or lichenoid lymphocytic infiltrate involving the superficial lamina propria and basal epithelium^[7]. A predominance of mature T cells is present within this infiltrate. These are associated with basal keratinocyte degeneration which often include Civatte bodies (necrotic keratinocytes with anucleate remnants)^[7]. Lymphocytic infiltration of the mucosa is not pathognomonic of this disease and medications such as gold, thiazide, and anti-malarials can induce lichen planus-like lesions and need to be excluded clinically^[15].

No clear guidelines are present for the treatment of esophageal lichen planus and there is no specific way to treat this entity. Historically, systemic corticosteroids have been used as first-line treatment with a response rate of up to 74% based on multiple reports. However, relapse rate can be as high as 85% with steroid withdrawal^[11]. Treatment response was also reported with adrenocorticotrophic hormone injection, etretinate, topical tacrolimus, intralesional corticosteroids and cyclosporine^[2,16]. Esophageal dilation is commonly used to treat strictures, although intralesional steroid injections and/or oral tacrolimus may decrease the frequency of required dilations. It is important to note that koebner phenomenon which is defined as development of new lesions along the lines of trauma, can occur with dilatation.

Although effective, systemic corticosteroid treatment can be associated with serious side effects and therefore there is a need for a safer alternative. In our review of literature, we have come upon case reports and a recent case series where treatment with swallowed fluticasone propionate has resulted in symptomatic improvement as well as endoscopic improvement in 4 out of 6 treated patients^[14,17]. Because of these promising results, we decided to try this novel approach for our patient which proved to be successful.

Oral lichen planus has a 1%-3% risk of malignant transformation to squamous cell carcinoma^[3]. It is unknown however if the same is true for esophageal lichen planus. To date, 3 case reports have described squamous cell carcinoma and 1 described a verrucous carcinoma arising from these lesions^[10,18,19]. In these reports, squamous cell carcinoma developed more than 20 years after the diagnosis of esophageal lichen planus^[18]. Due to this risk, some advocated surveillance endoscopies in patients with esophageal lichen planus to detect early malignancy. In the study by Quispel *et al.*^[20], high magnification indigo carmine chromoendoscopy was used to establish the prevalence of endoscopic and histopathologic esophageal abnormalities consistent with lichen planus and dysplasia in a cohort of patients with lichen planus. It was found that up to 50% of patients with orocutaneous lichen planus had esophageal involvement but no dysplasia was found. The authors of this study proposed to set a low threshold for performing endoscopy in patients with lichen planus and symptoms suggestive of esophageal involvement but recommended against routine screening^[20]. Based on these data, we recommend that the frequency

of screening by endoscopy be individualized, but the possibility of malignancy be kept in mind while evaluating these patients with a low threshold for further evaluation in patients with symptoms suggestive for esophageal cancer.

In conclusion, esophageal lichen planus should be suspected in middle-aged female patients who present with symptoms of dysphagia and odynophagia and found to have mucosal abnormalities involving the upper third of the esophagus. Once the diagnosis of esophageal lichen planus is made, these patients should be considered for treatment with swallowed fluticasone before other systemic and more toxic therapies. It is important to keep in mind that secondary to the risk for malignant transformation, these patients should be followed closely.

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PEComa of the colon resistant to sirolimus but responsive to doxorubicin/ifosfamide

Wolfgang Scheppach, Nikolaus Reissmann, Thomas Sprinz, Ekkehard Schippers, Bjoern Schoettker, Justus G Mueller

Wolfgang Scheppach, Nikolaus Reissmann, Department of Medicine, Juliussspital Wuerzburg, D-97070 Wuerzburg, Germany
Thomas Sprinz, Ekkehard Schippers, Department of Surgery, Juliussspital Wuerzburg, D-97070 Wuerzburg, Germany
Bjoern Schoettker, Onkologische Schwerpunktpraxis, D-97070 Wuerzburg, Germany

Justus G Mueller, Department of Pathology, University of Wuerzburg, 97070 Würzburg, Germany

Author contributions: Scheppach W and Reissmann N diagnosed and treated the patient in hospital; Sprinz T and Schippers E operated on the patient; Schoettker B treated the outpatient; Mueller JG analysed the tumor specimens histologically; all authors contributed significantly to the acquisition, analysis and interpretation of data; Scheppach W drafted the article; all coauthors revised it critically and finally approved it for publication.

Correspondence to: Wolfgang Scheppach, MD, Department of Medicine, Juliussspital Wuerzburg, Juliuspromenade 19, D-97070 Wuerzburg, Germany. gastroenterologie@juliussspital.de
Telephone: +49-931-3931701 Fax: +49-931-3931702

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Abstract

A 23-year-old male presented with a three-week-history of crampy abdominal pain and melaena. Colonoscopy revealed a friable mass filling the entire lumen of the cecum; histologically, it was classified as perivascular epithelioid cell tumor (PEComa). An magnetic resonance imaging scan showed, in addition to the primary tumor, two large mesenteric lymph node metastases and four metastatic lesions in the liver. The patient underwent right hemicolectomy and left hemihepatectomy combined with wedge resections of metastases in the right lobe of the liver, the resection status was R0. Subsequently, the patient was treated with sirolimus. After 4 mo of adjuvant mammalian target of rapamycin inhibition he developed two new liver metastases and a local pelvic recurrence. The visible tumor formations

were again excised surgically, this time the resection status was R2 with regard to the pelvic recurrence. The patient was treated with 12 cycles of doxorubicin and ifosfamide under which the disease was stable for 9 mo. The clinical course was then determined by rapid tumor growth in the pelvic cavity. Second line chemotherapy with gemcitabine and docetaxel was ineffective, and the patient died 23 mo after the onset of disease. This case report adds evidence that, in malignant PEComa, the mainstay of treatment is curative surgery. If not achievable, the effects of adjuvant or palliative chemotherapy are unpredictable.

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Key words: Perivascular epithelioid cell tumor; Colon; Liver metastases; Mammalian target of rapamycin inhibitor; Sirolimus; Chemotherapy; Doxorubicin; Ifosfamide

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INTRODUCTION

Perivascular epithelioid cell tumor (PEComa) are rare mesenchymal neoplasms for which, according to a World Health Organization classification, histologically and immunohistochemically distinctive perivascular epithelioid cells are diagnostic^[1]. Clinical courses are highly variable from benign behaviour to aggressive local tumor growth and seeding of metastases^[2]. In this case report, a highly malignant type of PEComa in a 23-year-old male and its response to multimodal therapies is described.

CASE REPORT

A 23-year-old male was admitted to the hospital because of crampy abdominal pain and melaena for three weeks. Colonoscopy revealed a 5.5 cm mass lesion in the cecum surrounding the ileocecal valve (Figure 1). At biopsy, the friable tumor tissue was bleeding easily. An magnetic resonance imaging (MRI) scan showed, in addition to the primary tumor, two mesenteric lymph node metastases (each 5 cm in diameter) and 4 metastatic lesions in the liver (1-2 cm in diameter, segments 1, 2, 4a and 6) (Figure 2). Additional staging procedures at the time of primary diagnosis [abdominal and chest computed tomography (CT), positron emission tomography] revealed no further tumor manifestations.

In a two-stage procedure, the patient underwent right hemicolectomy and, after recovery, left hemihepatectomy combined with atypical wedge resections of hepatic segments 1 and 6 (resection status R0). On the basis of biopsy and resection material, a diagnosis of malignant PEComa was made (see below).

Owing to the aggressive nature of the tumor, both clinically and histologically, the patient received adjuvant treatment with the mammalian target of rapamycin (mTOR) inhibitor sirolimus (2 mg/d). However, after 4 mo the drug had to be discontinued due to two new liver metastases in segments 7 and 8 which were removed by atypical wedge resection. Simultaneously, a local pelvic recurrence of 13 cm × 12 cm × 8 cm with bilateral ureteral obstruction and rectal impression was diagnosed. A debulking operation was performed which resulted in Hartmann's situation (resection status R2); additionally, splints were inserted into both ureters.

Palliative chemotherapy with doxorubicin (75 mg/m²) and ifosfamide (5000 mg/m²) every 3 wk was started. This regime was well tolerated until cycle 7 when the dose had to be reduced due to hematotoxicity. Altogether, the patient received 12 cycles of doxorubicin/ifosfamide under which the disease was stable for 9 mo as evaluated by CT scans every 8-12 wk.

Afterwards renewed tumor growth in the pelvic cavity was observed, aggravated by malignant ascites. Three cycles of second line chemotherapy (gemcitabine 900 mg/m² on days 1 and 8 combined with docetaxel 100 mg/m² on day 8 every 21 d) were administered without measurable effect. The patient died 23 mo after the onset of disease.

Pathology

The specimen obtained at hemicolectomy showed a 5.5 cm measuring mass in the cecum with metastases in 2 of 18 regional lymph nodes, each measuring 5 cm in diameter. The tumor was located in the bowel wall, with broad ulceration of the overlying mucosa. Histology (Figure 3) revealed a tumor of low to moderate cellularity, with a vague nodular pattern, an epithelioid and solid arrangement of the tumor cells and a sinusoidal vascular pattern without stromal desmoplasia. The tumor cells had a broad clear to granular eosinophilic cytoplasm,



Figure 1 Endoscopic aspect of a soft and friable perivascular epithelioid cell tumor of the cecum.

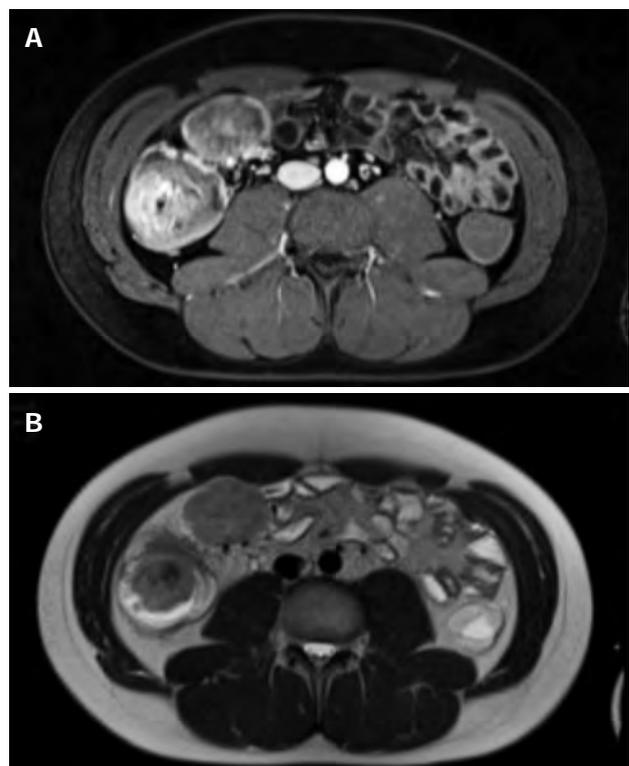


Figure 2 Magnetic resonance imaging of cecal perivascular epithelioid cell tumor and mesenteric lymph node metastasis. A: T2-weighted image; B: T1-weighted image after iv administration of contrast medium.

with moderate PAS positivity. The distinct cellular membranes exhibited some wrinkling. Most tumor nuclei showed moderate nuclear pleomorphism, but there were some highly pleomorphic hyperchromatic tumor cell nuclei. Sixty percent of the tumor area was necrotic. The mitotic rate was 12 per 10 high-power field (HPF). In some areas, the tumor was well demarcated, but there were other areas with a more infiltrative pattern of invasion.

Immunohistochemistry revealed positivity for HMB45 and negativity for melanoma antigen recognized by T cells 1 and microphthalmia-associated transcription factor. There was a weak expression of pankeratin markers (AE1/3, KL1) and CD56 in few tumor cells. Other mark-

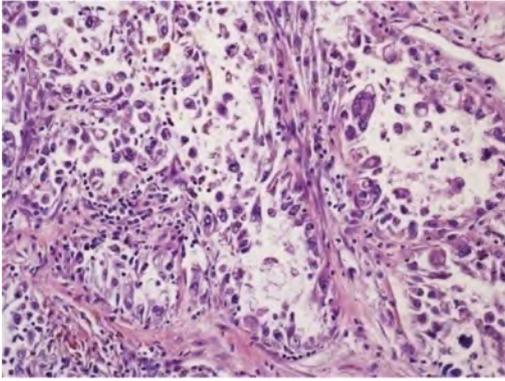


Figure 3 Histologic section of the primary tumor in the cecum. A representative part of the tumor shows epithelioid tumor cells. The cytoplasm exhibits both clear and granular eosinophilic parts. The tumor cells are arranged in large nodules with necrotic debris in the central parts. In the lower left corner, a cytoplasmic brown pigment is seen that upon ultrastructural evaluation turned out to be melanin pigment and melanosomes. There are narrow stalks of collagen-rich stroma with a scant lymphocytic infiltrate (Hematoxylin and eosin, magnification 200 ×).

ers (Synaptophysin, Chromogranin, PanLeu, CD34, CD31, S100, CD117, DOG1, Myogenin, MyoD1, EMA, Actin, Caldesmon, Desmin, CD30) were absent. Ki67 labeled 50%-60% of the tumor cells. PCR analysis of fresh frozen tumor material was negative for translocations suggestive of clear cell sarcoma [t(12;22)], synovial sarcoma [t(X;18)], myxoid liposarcoma [t(12;16)] and alveolar rhabdomyosarcoma [t(2;13)].

The diagnosis of PEComa was suggested in the biopsies obtained at endoscopy. Because of the rarity of this tumor and the missing expression of smooth muscle markers usually found in PEComas, the tissue was sent to a reference pathologist (Fletcher CDM, Boston, MA, United States) who confirmed the diagnosis of PEComa. From the surgical material, a tumor area with brown cytoplasmic pigment (with negativity in the Prussian blue and PAS stains) was selected for electron microscopy; in this sample typical melanosomes could be demonstrated.

The tumor material obtained at the resection of liver metastases did not differ histologically from that of the primary tumor. However, following chemotherapy with doxorubicin/ifosfamide, there was a tremendous increase in nuclear pleomorphism with many extremely large hyperchromatic bizarre tumor nuclei, many tumor cells with nuclear fragmentation and micronuclei, and a decrease in the amount of mitotic figures. These morphologic alterations resemble regressive tumor changes following chemotherapy. However, the area of necrotic tumor cells was 20% at this time point, i.e. most of the tumor contained still viable cells.

DISCUSSION

This report of malignant PEComa has to be seen in the context of other single case descriptions or small case series on an extremely rare tumor entity. Predictors of prognosis in PEComa have been described in a clinicopathologic study on 26 cases by Folpe *et al*³¹. A

significant association between tumor size > 5 cm, infiltrative growth pattern, high nuclear grade and cellularity, mitotic rate $\geq 1/50$ HPF, necrosis, vascular invasion and subsequent aggressive clinical behaviour has been seen. In a more recent review article⁴¹ on the basis of 234 PEComas the only pathologic factors of recurrence after surgical resection were primary tumor size ≥ 5 cm and a high mitotic rate of > 1/50 HPF. All of these “worrisome” pathologic features were present in the 23-year-old patient of the actual case. Additionally, the presence at initial diagnosis of two large metastases in mesenteric lymph nodes (each measuring 5 cm in diameter) and of 4 hepatic metastases had to be considered as clinical indicators of poor prognosis.

PEComas arise from various organs such as uterus and vagina, kidney, digestive tract, retroperitoneum, bone, skin and eye. Intestinal origins include stomach, colon and rectum, peritoneal cavity and falciform ligament. Considering only PEComas of the colon and rectum, there are 4 reports on 7 patients^{5-8]} in whom the clinical course was benign (5 × operation only, 2 × operation and adjuvant chemotherapy, no evidence of disease at the end of follow-up). These findings are in contrast with the actual case when mesenteric and hepatic metastases were present at the time of diagnosis. The organ of origin, therefore, does not seem to be a predictor of prognosis.

Concerning treatment strategies, Bleeker *et al*⁴¹ stated that cytotoxic chemotherapy and radiation had shown little benefit in malignant PEComa. According to the authors, the emerging role of mTOR inhibitors would raise enthusiasm in the therapy of these rare tumors. The clinical course reported herein reflects the opposite impression: After R0 resection of the primary tumor and mesenteric/hepatic metastases, adjuvant mTOR inhibition with sirolimus given for 4 mo at a dose of 2 mg/d (suitable for liver transplant recipients) failed to prevent a local recurrence and new liver metastases. On the contrary, cytotoxic chemotherapy (doxorubicin/ifosfamide) considered first choice in soft tissue sarcomas was associated with stable disease for 9 mo. Thus, the combination of repetitive surgery with conventional chemotherapy may still be a choice in the palliative therapy of malignant PEComa. The benefit of mTOR inhibition (sirolimus, temsirolimus, everolimus), although theoretically attractive, is at present unpredictable^{9,10]}. Many other therapies have been tried to control unresectable PEComa, *e.g.*, dacarbazine, epirubicin, paclitaxel, gemcitabine, oxaliplatin, imatinib, α -interferon, thalidomide, alone or in combinations. However, clinical outcomes have been extremely variable and a standard treatment is not in sight.

Some PEComas are associated with phakomatosis and hamartomatous diseases, *e.g.*, the tuberous sclerosis complex (TSC). In these conditions the mTOR signalling pathway is activated which may thus be targeted by sirolimus and related compounds^{11]}. In the present case of the 23-year-old patient there was no indication of TSC. Due to the paucity of data it is unknown if the presence or absence of TSC can be used as a predictor of susceptibility to sirolimus therapy.

Given the extreme rarity and heterogeneity of PEComas, a comparative study with a focus on optimal treatment will unlikely be performed. Instead, a PEComa registry based at a sarcoma center would be a reasonable option. Well documented clinical courses, histological features and empirical therapies could thus be accumulated and best practice procedures deduced.

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Infliximab induces remission in cryptogenic multifocal ulcerous stenosing enteritis: First case

Heiko De Schepper, Elisabeth Macken, Veerle Van Marck, Maarten Spinhoven, Paul Pelckmans, Tom Moreels

Heiko De Schepper, Elisabeth Macken, Paul Pelckmans, Tom Moreels, Department of Gastroenterology and Hepatology, Antwerp University Hospital, B-2650 Edegem-Antwerp, Belgium

Veerle Van Marck, Department of Pathology, Antwerp University Hospital, B-2650 Edegem-Antwerp, Belgium

Maarten Spinhoven, Department of Radiology, Antwerp University Hospital, B-2650 Edegem-Antwerp, Belgium

Author contributions: All authors contributed equally to this case report; all authors have read and approved the final manuscript.

Correspondence to: Heiko De Schepper, MD, Department of Gastroenterology and Hepatology, Antwerp University Hospital, Wilrijkstraat 10, B-2650 Edegem-Antwerp, Belgium. heiko.de.schepper@uza.be

Telephone: +32-3-8215585 Fax: +32-3-8214478

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Abstract

We present the case of a 29-year-old patient with a history of abdominal pain and vomiting. Based on wireless video capsule findings he was previously diagnosed with ileal Crohn's disease at a different institution, although the clinical and radiological picture was not typical and the response to corticosteroids was poor. We performed a single-balloon enteroscopy showing a short, ulcerous stenosis 50 cm proximal from Bauhin's valve. The endoscopic and clinical histopathological findings were compatible with cryptogenic multifocal ulcerous stenosing enteritis (CMUSE). High dose corticosteroids were again started, without effect. The monoclonal tumor necrosis factor- α (TNF- α) antibody infliximab was added to the medical therapy. After induction therapy, both clinical and endoscopic amelioration was obtained. Larger case studies are needed to confirm the efficacy of TNF- α inhibition in steroid refractory CMUSE.

Key words: Cryptogenic multifocal ulcerous stenosing enteritis; Infliximab; Stenosis; Intestinal ulceration; Inflammatory bowel disease

De Schepper H, Macken E, Van Marck V, Spinhoven M, Pelckmans P, Moreels T. Infliximab induces remission in cryptogenic multifocal ulcerous stenosing enteritis: First case. *World J Gastroenterol* 2013; 19(10): 1661-1664 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v19/i10/1661.htm> DOI: <http://dx.doi.org/10.3748/wjg.v19.i10.1661>

INTRODUCTION

Ulceration of the small intestine poses a rather limited but difficult differential diagnosis. The most common causes are Crohn's disease, nonsteroidal anti-inflammatory drug (NSAID) associated enteritis, lymphoma, tuberculous enteritis and (mainly in immunocompromised patients) cytomegalovirus (CMV) enteritis. Less frequent etiologies should be kept in mind however. A recently described differential diagnosis is cryptogenic multifocal ulcerous stenosing enteritis (CMUSE), which may be difficult to treat.

CASE REPORT

A 29-year-old male patient presented at our department in April 2011 for a second opinion. He suffered from aspecific abdominal complaints since 2003. Initially, a diagnosis of irritable bowel syndrome was put forth. In 2008, an ileocolonoscopy was performed in a different institution which showed several small ulcerations in the terminal ileum in the presence of a normal colon. Gastroduodenoscopy was normal. A wireless video capsule examination was performed showing multiple small ulcerations in the ileum. A tentative diagnosis of Crohn's disease was made. The patient was started on corticosteroids in January 2009 (prednisolone 40 mg/d), azathioprine was associated as a maintenance therapy but not



Figure 1 Computed tomography showing the location of a potential short stricture (arrow) in the preterminal ileum with mild pre- and poststenotic dilatation. This stricture was identified only after second reading of the computed tomography images and based upon the enteroscopic findings. A: Transverse plane; B: Coronal plane; C: Parasagittal plane.

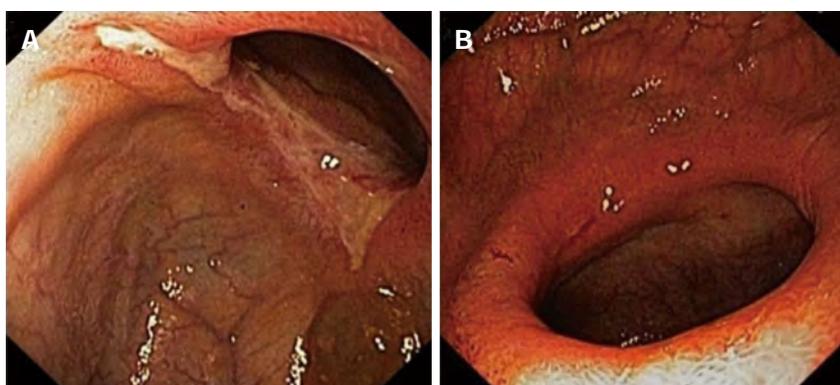


Figure 2 High definition endoscopic image of a circular ulcerative stenosis in the ileum, 50 cm proximal from the ileocecal valve. After infliximab induction therapy, the ulceration almost completely disappeared and only a short hyperemic and less pronounced stenosis remained. A: Before infliximab; B: After infliximab.

tolerated due to intractable nausea. The steroids were slowly tapered over 8 mo, after which the correctness of the diagnosis of Crohn's disease was questioned for unspecified reasons and all treatment stopped. The patient consulted our department for a second opinion.

When we first met this patient in April 2011, diffuse abdominal cramping pain was still the chief complaint. The pain was worse postprandially leading to sitophobia. There was frequent vomiting and a 15 kg weight loss over the past 5 years was noted. Stools tended to be loose with the occasional presence of bloody slimes, the frequency was approximately once daily. There was no fever. His medical history was unremarkable apart from the presumed inflammatory bowel disease. There was no familial history of digestive disease. He smoked 20 cigarettes per week and used marijuana on a daily basis for medicinal purposes. There was no other substance (ab) use mentioned nor detected upon repeated urine toxicology. The use of NSAIDs was systematically denied. Blood work showed normal hemoglobin and red blood cell count and no leucocytosis or C-reactive protein elevation. Antinuclear antibodies and anti-neutrophil cytoplasmic antibodies titers were not elevated.

Since all previous examinations only mentioned small intestinal involvement, a computed tomography (CT) en-

terography was performed to guide subsequent endoscopy but failed to show intestinal inflammation or sequelae of inflammation such as strictures. We subsequently performed a retrograde single-balloon enteroscopy in May 2011 showing the presence of multiple circular but short ileal ulcerations over a distance of 20 cm, starting at the ileocaecal valve. Based upon these findings the CT enterography was consequently reviewed with the radiologist. After a careful second reading, a very short segment in the preterminal ileum was identified which could represent a small intestinal stricture although differentiation with a segmental contraction could not be made (Figure 1). Reconsidering the possibility of a mild form of Crohn's disease, the patient was again started on corticosteroids (budesonide tapered over 3 mo).

In November 2011 a repeat enteroscopy was performed, which showed a small ulceration 20 cm proximally from the ileocaecal valve and a short but circular and stenosing ulceration 30 cm more proximally (Figure 2). The microscopic examination showed superficial ulceration in the ileal mucosa, without associated signs of Crohn's disease in the surrounding, preserved mucosa (Figure 3A).

Based upon the clinical course and the characteristic endoscopic image, CMUSE was diagnosed. Considering the lack of endoscopic or clinical response to treatment

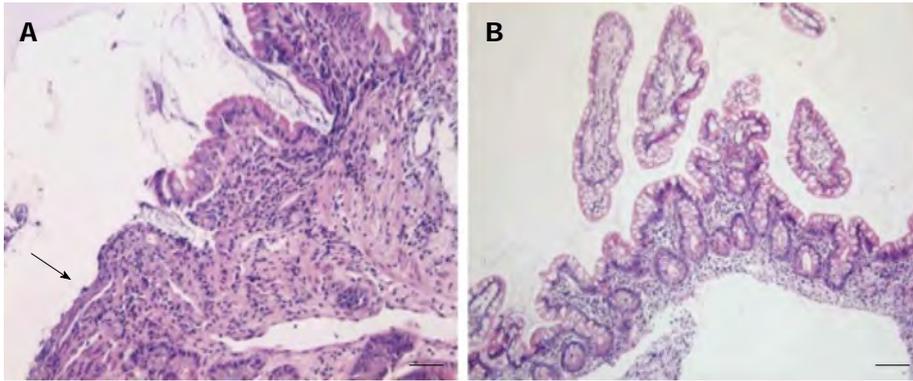


Figure 3 Ileal biopsy before and after infliximab treatment. A: Superficial ulceration of the mucosa (arrow), with an acute inflammatory infiltrate in the lamina propria (HE stained paraffin section, bar: 100 μ m); B: Ileal biopsy after treatment with infliximab, showing restoration of the villus architecture and only slight, non-specific inflammatory changes (bar: 200 μ m).

with budesonide so far, the patient was switched to high dose prednisolone (40 mg daily) in December 2011. After two months, no clinical improvement was noticed, in contrast, the patient almost completely stopped oral food intake because of postprandial cramping and was started on continuous nasogastric tube feeding. In addition, a third enteroscopy confirmed that the endoscopic image was unaltered. To avoid surgery, the ethical committee of our institution was consulted to consider our patient for a compassionate use treatment with the monoclonal tumor necrosis factor- α (TNF- α) antibody infliximab. A standard induction scheme was started in March 2012 (intravenous infliximab at a dose of 5 mg/kg on weeks 0, 2 and 6), followed by maintenance treatment (intravenous infliximab at a dose of 5 mg/kg every 8 wk). After administration of the induction scheme, significant clinical improvement occurred with a reduction in abdominal pain and significant amelioration of sitophobia, leading to cessation of nasogastric tube feeding and prednisolone treatment. A fourth enteroscopy was performed, showing disappearance of the ileal circular ulceration (Figure 2A). A nonsignificant and probably irreversible stenosis remained. Histological examination showed only minor, non-specific inflammatory changes and a restoration of the villus architecture in the ileal biopsies (Figure 3B).

Infliximab treatment is currently still ongoing, clinical remission is maintained up till 6 mo after starting treatment.

DISCUSSION

Small intestinal ulceration is a less frequent cause of abdominal pain. Its differential diagnosis includes lymphoma, Crohn's disease, tuberculous enteritis, CMV enteritis, NSAID related enteritis, oral potassium chloride toxicity, severe celiac disease (ulcerative jejunoileitis), systemic vasculitis and CMUSE. Small intestinal strictures are seen in chronic NSAID, ischemic enteritis, abdominal irradiation, Crohn's disease and CMUSE^[1].

CMUSE is an independent entity showing characteristics of inflammatory bowel disease and ischemic enteritis.

The etiology is unclear but angiography studies suggest that in at least a subset of patients, CMUSE may involve vasculitis and even represent a visceral variant of polyarteritis nodosa. It seems likely that CMUSE incorporates chronic nonspecific ulcers of the small intestine (CNSU), a disease reported solely in the Japanese population^[2]. CNSU does not respond to corticosteroid administration, and can therefore be seen as steroid-refractory CMUSE.

CMUSE patients always report intestinal symptoms such as abdominal pain, diarrhea and vomiting related to intestinal (sub)obstruction. In 70% of cases, extraintestinal complaints are mentioned: 50% of patients lose weight, 20% develop fever and 10% complain of joint aches. The relatively frequent presence of extraintestinal signs such as oral aphthae, Raynaud's phenomenon, sicca syndrome and pulmonary disease suggests that CMUSE may be part of a more systemic disorder.

Diagnostic criteria for CMUSE were proposed by Perlemuter *et al.*^[3,4], who published the 2 largest collections of patients (totaling 28 patients): unexplained small intestinal strictures found in adolescents and in middle-aged subjects, superficial ulceration restricted to the mucosa and submucosa, a chronic or relapsing clinical course (even after surgery), no biological signs of systemic inflammatory reaction and a typically (initially) beneficial effect produced by steroids. These criteria can be distilled from the clinical history and (balloon assisted) enteroscopy, the latter providing the histological material necessary to confirm the diagnosis. Histopathology shows an inflammatory response and ulcerative damage restricted to the mucosa and submucosa, different from the transmural involvement typical from Crohn's disease. Blood analysis is needed to detect anemia, to exclude systemic inflammation and may show hypoproteinemia due to protein-losing enteropathy. Medical imaging has its own role in diagnosing small intestinal ulceration/stenosis. A simple contrast follow-through fluoroscopy can dynamically show the presence of intestinal stenosis, but provides no specificity. As mesenteric vascular changes only occur in a subset of patients, performing a diagnostic angiography will not *per se* guide the diagnosis nor the therapy, and should be reserved for selected cases. Our case shows that

CT enterography may lack the sensitivity to capture the often short and circular stenoses that are characteristic for CMUSE, in contrast to the often longer and more complex strictures seen in Crohn's disease.

Differential diagnosis was mentioned earlier and should at least and most importantly include Crohn's disease and NSAID-related enteropathy^[5].

Current medical therapy consists of corticosteroid induction and maintenance with slow tapering of daily dosage, although no randomized clinical trials or guidelines are available to defend this strategy. Corticosteroids may prevent surgical intervention but often lead to steroid-dependence. Steroid-refractory CMUSE has recently been reported in one patient^[6]. The use of other immune suppressive medication such as azathioprine or anti TNF- α inhibition is not reported in current literature. The mainstay of treatment of CMUSE stenosis is surgery, although recurrence is frequent (symptoms and strictures recur in 50% of patients). Endoscopic balloon dilatation can be used as a bridge to surgery, in order to prevent extensive resections of the small intestine and the threat of a short bowel syndrome.

Our patient fits the Perlemuter criteria for CMUSE (although we have no proof that the inflammation does not extend to the submucosa, as the latter can only be confirmed on a resection specimen). He evidently was refractory to steroid treatment, confirming an earlier anecdotal report^[6]. To avoid surgical resection and the high risk of disease recurrence it implies, we opted to treat our patient with the standard of care treatment in inflammatory bowel disease, *i.e.*, the monoclonal TNF- α antibody infliximab. In a small series of patients, this immunosuppressive drug was able to induce disease remission for symptomatic enteric Crohn's strictures^[7], providing a rationale for our treatment trial. Moreover, infliximab has shown its worth in treating rheumatoid arthritis and several forms of vasculitis (although the data for the latter are less extensive)^[8].

After the infliximab induction regimen, clinical remission was immediately noted. Enteroscopy showed near

complete endoscopic remission as well. To our knowledge, this is the first report to describe the beneficial effect of TNF- α blockade in steroid-refractory CMUSE. Evidently, larger series are necessary to confirm this observation.

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Metachronous colonic metastasis from pancreatic cancer seven years post-pancreatoduodenectomy

Kentaro Inada, Dai Shida, Kazumasa Noda, Satoru Inoue, Masahiro Warabi, Nobutaka Umekita

Kentaro Inada, Dai Shida, Kazumasa Noda, Satoru Inoue, Nobutaka Umekita, Department of Surgery, Tokyo Metropolitan Bokutoh Hospital, Sumida-ku, Tokyo 1308575, Japan
Masahiro Warabi, Department of Pathology, Tokyo Metropolitan Bokutoh Hospital, Sumida-ku, Tokyo 1308575, Japan
Author contributions: Inada K, Shida D, Noda K, Warabi M, Inoue S and Umekita N collected the data and administered the treatment; Inada K prepared the manuscript; Shida D was responsible for writing the paper and its supervision.

Correspondence to: Dr. Kentaro Inada, Department of Surgery, Tokyo Metropolitan Bokutoh Hospital, 4-23-15 Koto-bashi, Sumida-ku, Tokyo 130-8575, Japan. kentaro.inada@hotmail.co.jp
Telephone: +81-3-36336151 Fax: +81-3-36336173
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received systemic chemotherapy, but unfortunately, he died 14 mo after the surgery.

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Key words: Colonic metastasis; Pancreatic cancer; Immunohistochemical staining; Cytokeratin 7; Cytokeratin 20

Inada K, Shida D, Noda K, Inoue S, Warabi M, Umekita N. Metachronous colonic metastasis from pancreatic cancer seven years post-pancreatoduodenectomy. *World J Gastroenterol* 2013; 19(10): 1665-1668 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v19/i10/1665.htm> DOI: <http://dx.doi.org/10.3748/wjg.v19.i10.1665>

Abstract

Colonic metastasis from other organs is very rare. Here we report the case of a 62-year-old man with a history of pancreatoduodenectomy for stage IIB pancreatic head cancer performed seven years back. He presented with abdominal distension and pain. Under the preoperative diagnosis of bowel obstruction, surgical treatment was performed, and a circumferential lesion causing bowel obstruction of the ascending colon was detected. A right hemicolectomy with lymph node dissection was performed. The specimen showed a 5-cm wall thickening with a cobble-stone like appearance of the ascending colon, which morphologically appeared scirrhous. Histological examination revealed cancer nests invading from the subserosa to the muscular and submucosal layers of the colon. Immunohistochemical analysis of the tumor cells demonstrated positive staining for cytokeratin 7, but negative for cytokeratin 20, which was the same as the previous pancreatic cancer specimen. These pathological and immunohistochemical features strongly supported the diagnosis of colonic metastasis from the pancreas. Thereafter, the patient

INTRODUCTION

Primary colon cancer is the most common malignancy in the Western part of the world^[1], but colonic metastasis from other solid organs such as the pancreas is extremely rare. Only two cases of colonic metastasis from the pancreas have been previously reported in the English literature^[2,3]. Here we report a case of metachronous colonic metastasis from pancreatic cancer, seven years post-pancreatoduodenectomy, marked by bowel obstruction.

CASE REPORT

A 62-year-old man underwent a pancreatoduodenectomy in May 2001 for pancreatic head cancer. The pathological diagnosis was a well-differentiated adenocarcinoma with strong fibrosis, which was defined as a scirrhous type. There was invasion into the pancreatic duct and lymph node metastases. The stage of pancreatic cancer was T2N1M0 stage II B, as per the American Joint Committee on Cancer staging system 7th edition^[4]. Follow-

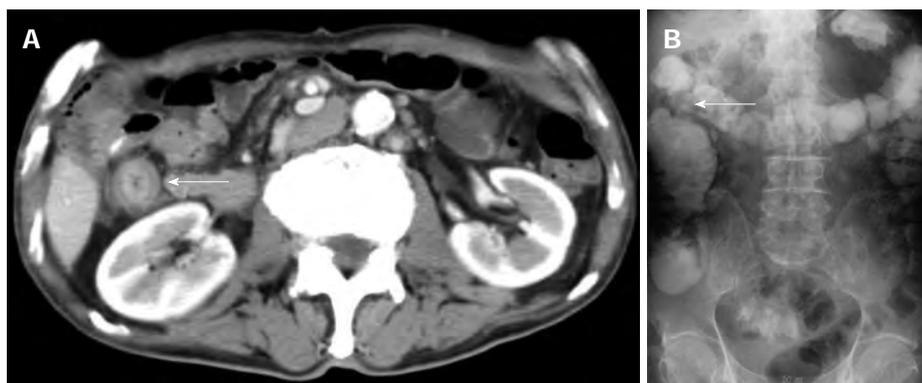


Figure 1 Examinations at seven years after pancreatoduodenectomy. A: An abdominal computed tomography showing thickening of the ascending colon wall with enhancement and luminal narrowing (arrow); B: A contrast examination showing a 5-cm stricture of the ascending colon (arrow).

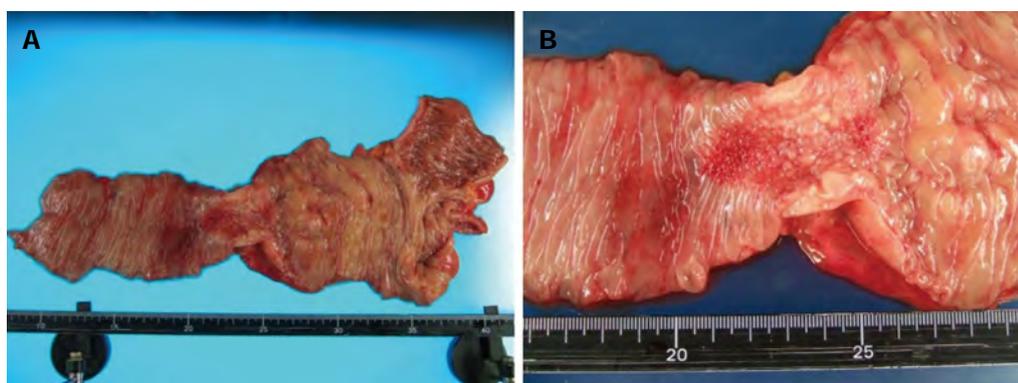


Figure 2 A pathological specimen showing a 5-cm wall thickening and a cobblestone-like appearance of the ascending colon.

up regimen of pancreatic cancer was examinations of computed tomography (CT), ultrasonography and the biological marker every 6 mo. During the seven years of follow-up, he showed no signs of recurrence. In July 2008, he began complaining of abdominal distension and pain. A CT of his abdomen revealed ascending colon wall thickening with luminal narrowing (Figure 1A). There were no other obvious signs of recurrence of pancreatic cancer or ascites. Both chest CT and abdominal ultrasonography also revealed no obvious sign of recurrence. In addition, a contrast examination of the patient's alimentary tract revealed stricture of the ascending colon (Figure 1B). The levels of the biological marker carbohydrate antigen 19-9 (CA19-9) were within the normal range for seven years post-pancreatoduodenectomy; this time, the levels were elevated to 1886.6 U/mL. The carcinoembryonic antigen levels were normal. At this time, a colonoscopy was not performed because of fear of precipitating an overt obstruction. Conservative therapy did not improve the bowel obstruction, thus surgical treatment was performed in August 2008, under the presumptive diagnosis of either an adhesive intestinal obstruction or colon cancer. Intraoperative findings revealed a 5-cm mass in his ascending colon, which was causing the bowel obstruction. Neither peritoneal dissemination nor any other lesions could be observed in the abdomen, including the residual pancreas

or liver. With an intraoperative diagnosis of a tumor in the ascending colon, a right hemicolectomy with adjacent lymphadenectomy was performed. The specimen revealed a 5-cm wall thickening with a cobblestone-like appearance of the ascending colon (Figure 2). The tumor was morphologically scirrhous and infiltrated the colon wall, as seen in gastric linitis plastica. Histological examination revealed cancer nests with glandular structures from the subserosa invading into the muscular and submucosal layers of the colon (Figure 3).

Immunohistochemical staining of the colon tumor as well as the previously resected pancreatic tumor was positive for cytokeratin 7 and negative for cytokeratin 20 (Figure 4). These pathological and immunohistochemical features strongly supported the diagnosis of a tumor in the ascending colon originating from the pancreas and was not a primary adenocarcinoma of the colon. In addition, infiltration of the lymphatic vessels and metastases were observed in two of the 28 dissected lymph nodes. However, the patient had an uneventful postoperative course and was discharged on the 12th post-surgical day. The CA19-9 levels decreased to 840 U/mL after surgery; however, the levels elevated to 8200 U/mL two months later. He was treated with seven cycles of gemcitabine (1000 mg/m²) after pancreatoduodenectomy; nevertheless, his condition deteriorated and he died 14 mo after surgery.

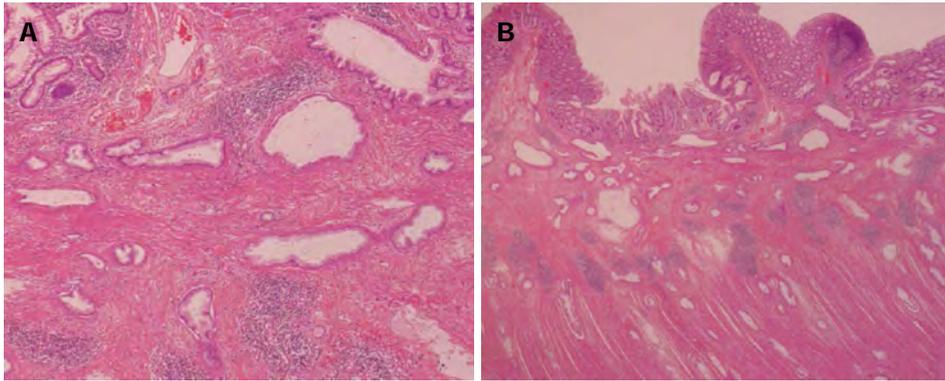


Figure 3 Histological findings revealed the cancer nests invading from the subserosa to the muscular and submucosal layers of the colon. A: $\times 4$; B: $\times 10$.

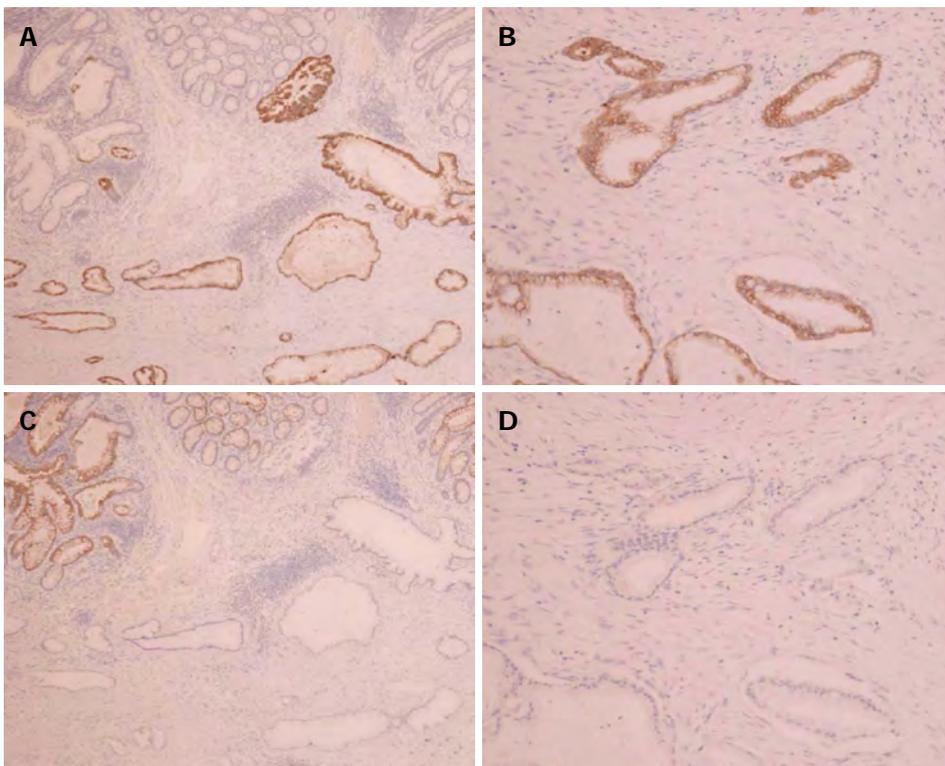


Figure 4 Photomicrographs ($\times 10$). A: Immunostaining showing cytokeratin 7 positivity in colonic metastasis; B: Immunostaining showing cytokeratin 20 negativity in colonic metastasis; C: Immunostaining showing cytokeratin 7 positivity in primary pancreatic cancer; D: Immunostaining showing cytokeratin 20 negativity in primary pancreatic cancer.

DISCUSSION

Pancreatic cancers are very aggressive. Less than 10%-15% of cases present with tumors those are localized to the pancreas at the time of diagnosis. The overall survival rate of advanced pancreatic cancer cases is $< 5\%$ at five years with most patients dying within the first year^[5]. Pancreatic cancer commonly spreads to the liver, lungs, abdomen, regional lymph nodes and peritoneum. Postoperative colonic metastasis from pancreatic cancer is extremely rare, as only two cases have been reported previously in the English literature^[2,3].

Colonic metastases from other organs are rare. However, with regard to treatment and prognosis, a correct diagnosis is very important. Colonic metastases from

other organs typically present scirrhous morphology^[6] and the tumors infiltrate the colon wall in a manner similar to gastric linitis plastica. Specific immunohistochemical staining is useful to diagnose primary tumor sites. Cytokeratin is an intermediate filament protein; cytokeratin 7 is expressed in epithelial cells of the kidney, prostate, pancreas, ovary, lung, and breast, but not in the colon or gastrointestinal tract. However, cytokeratin 20 is expressed in all cases of colorectal carcinomas, 62% of pancreatic carcinoma cases, and 50% of gastric adenocarcinomas^[7]. Thus, a combination of cytokeratins 7 and 20 is very useful to distinguish metastatic colon cancer from ovarian^[8], breast^[9], and primary colon cancers. In two cases, cytokeratin 7 positivity and cytokeratin 20 negativity strongly indicated metastases.

However, in the present case, it was unclear as to how the pancreatic cancer metastasized to the colon. Invasive cancer nests in the muscular layer were conspicuous and the lymph vessels were filled with tumor cells. Only 2 of 28 lymph nodes were positive for metastatic carcinoma. On the basis of these observations, not lymphatic spread but peritoneal (drop-down) metastasis from the pancreas to the colon was assumed as the most likely pathway.

In conclusion, the present report describes a case of colonic metastasis from pancreatic cancer after a pancreatoduodenectomy. Immunohistochemical staining by cytokeratins 7 and 20 were very useful to distinguish metastatic tumors from primary tumors. To our knowledge, this is the third report documenting pancreatic adenocarcinoma metastasizing to the colon by probable lymphatic spread.

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L- Editor A E- Editor Xiong L



Response letter regarding the interpretation of gene expression data

Fusun Ozmen

Fusun Ozmen, Department of Basic Oncology, Cancer Institute, Hacettepe University, 06280 Ankara, Turkey
Author contributions: Ozmen F wrote this letter.
Correspondence to: Fusun Ozmen, MD, PhD, Department of Basic Oncology, Cancer Institute, Hacettepe University, 06280 Ankara, Turkey. fusun.ozmen@hacettepe.edu.tr
Telephone: +90-312-3054322 Fax: +90-312-3242009
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Abstract

This is a response letter to Verna E's comments regarding our previous manuscript published last year in the *World Journal of Gastroenterology* entitled "Relationship between *LYVE-1*, *VEGFR-3* and *CD44* gene expressions and lymphatic metastasis in gastric cancer", which evaluated the relationship between these expression levels and clinicopathological parameters (Ozmen F *et al*, *World J Gastroenterology* 2011; 17: 3220-3228). The mean values for lymphatic vessel endothelial hyaluronan receptor-1, CD44 and vascular endothelial growth factor receptor-3 expression (represented as $2^{-\Delta\Delta Ct}$) were 1.13, 1.24 and 1.17, respectively, suggesting an increase in gene expression in tumor tissue compared to normal tissue. Despite the increase in gene expression in the cancer tissues ($2^{-\Delta\Delta Ct} > 1$), only some of the results reached statistical significance, which was thoroughly discussed in our paper. In the present letter, we report that his comments are flawed and result in confusion. Therefore, we herein provide more explanation regarding gene expression in gastric cancer. We hope that this letter will address Verna E's misunderstandings.

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Key words: Gastric cancer; Lymphatic metastasis; Lymphatic vessel endothelial hyaluronan receptor-1; Vascular endothelial growth factor receptor-3; CD44

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TO THE EDITOR

I recently noticed that a Letter to the Editor written by Verna E, regarding our paper (Ozmen F *et al*, *World J Gastroenterol* 2011; 17: 3220-3228), had been published in the June issue of the Journal this year (Verna E, *World J Gastroenterol* 2012; 18: 3181-3182)^[1,2].

Although I would like to thank Dr. Verna for his interest and for providing us with this opportunity to address his concerns, I have to stress that it is very important to understand the paper in its entirety before coming to certain conclusions: (1) Our study investigated the expression levels of the lymphatic vessel endothelial hyaluronan receptor-1 (*LYVE-1*), vascular endothelial growth factor receptor-3 (*VEGFR-3*), and *CD44* genes in human tissues with or without a tumor using real-time polymerase chain reaction (RT-PCR) and evaluated the relationship in gastric cancer between these expression levels and clinicopathological parameters that included tumor type, stage, differentiation, and the presence of lymph node metastasis, vascular invasion, and neural/perineural invasion^[1]; (2) Relative expression levels were calculated using the PCR cycle threshold (Ct) number for each tissue and control sample using the formula $2^{-\Delta(Ct_{\text{sample}} - \Delta Ct_{\text{control}})}$. ΔCt represents the difference in Ct values between the target and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) transcripts. RT-PCR was performed in duplicate for each sample, and average Ct values were calculated according to the previous papers from Pfaffl^[3] and Livak *et al*^[4], which are references he cited in his letter; (3) It is not clear why he required an explanation of the interpretation of the gene expression data because everyone involved in this type of study is familiar with

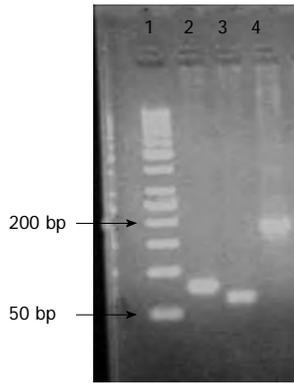


Figure 1 Polymerase chain reaction products of the *CD44*, vascular endothelial growth factor receptor-3, and lymphatic vessel endothelial hyaluronan receptor-1 genes. Lane 1: Ladder (50 bp); Lane 2: *CD44* (80 bp); Lane 3: Vascular endothelial growth factor receptor-3 (63 bp); Lane 4: Lymphatic vessel endothelial hyaluronan receptor-1 (184 bp).

the analysis. As previously explained by Livak *et al*^[4], the choice of the calibrator for the $2^{-\Delta\Delta C_t}$ method depends on the type of gene expression experiment that one has planned. The simplest design is to use the untreated control (in our study, the patient's own normal tissue) as the calibrator and GAPDH as an internal control. Using the $2^{-\Delta\Delta C_t}$ method, the data are presented as the fold change in gene expression normalized to an endogenous reference gene and relative to the normal control. For the control sample, $\Delta\Delta C_t$ equals zero, and 2^0 equals one; as a result, the fold change in gene expression relative to the untreated control equals one, by definition. For the treated samples (tumor tissue), an evaluation of $2^{-\Delta\Delta C_t}$ indicates the fold change in gene expression relative to the untreated control. The gene expression levels in tumor tissues represent the difference from normal controls in our study, and all values were shown in Fig. 2^[1]. There were some values over 1, representing increased expression, and there were also values less than 1, representing decreased expression; (4) The mean values for *LYVE1*, *CD44* and *VEGFR3* expression (represented as $2^{-\Delta\Delta C_t}$ and shown in Fig. 2) were 1.13, 1.24 and 1.17, respectively, suggest-

ing increased gene expression in tumor tissues compared to normal tissue. Therefore, we believe it is natural to conclude from these results that the expression levels were increased. Despite the increase in gene expression in the cancer tissues ($2^{-\Delta\Delta C_t} > 1$), only some of the results reached statistical significance, which was thoroughly discussed in our paper^[1]; (5) In our study, we did not only report the gene expression data, but also presented data obtained using immunohistochemistry, pathology, and other clinical features of the tumors. Although we are commenting on our results, we used all of these data to reach a logical conclusion. As shown in Fig. 3, 4 and 5, gene expression was increased ($2^{-\Delta\Delta C_t} > 1$) with increased T-stage, a PLN/TLN ratio > 0.4 and the presence of perineural invasion^[1]; and (6) In our manuscript, we used Figure 1 to show the PCR products of the genes. Dr. Verna is correct to note that there are some non-specific amplicons of the *VEGFR-3* gene in that Figure. However, we optimized both the conventional PCR and real-time PCR reactions for the *VEGFR-3* gene and also all others, as shown in the lower part of Figure 1. It is easy to note that no nonspecific band is present in this reaction for *VEGFR-3*.

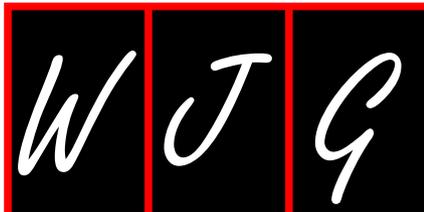
Once again, I would like to thank Dr. Verna for his interest in our study and hope that this letter will resolve any misunderstandings.

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Choice of drugs in the treatment of chronic hepatitis B in pregnancy

Ertugrul Guclu, Oguz Karabay

Ertugrul Guclu, Oguz Karabay, Department of Infectious Diseases and Clinical Microbiology, Sakarya University Faculty of Medicine, 54200 Sakarya, Turkey

Author contributions: Guclu E designed and wrote the paper; Karabay O contributed to the discussion section of article.

Correspondence to: Ertugrul Guclu, Assistant Professor, Department of Infectious Diseases and Clinical Microbiology, Sakarya University Faculty of Medicine, Kemalpaşa Mh 1 Ring Yolu, 54200 Sakarya, Turkey. ertugrulguclu@hotmail.com

Telephone: +90-264-4445400 Fax: +90-264-2759192

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Abstract

The selection of antiviral drugs for chronic hepatitis B (CHB) treatment in pregnancy is very difficult since none of the drugs have been approved for use in pregnancy. Transmission from mother to newborn remains the most frequent route of infection in mothers with high viral load and positive hepatitis B e antigen status, even with the use of appropriate prophylaxis with hepatitis B virus (HBV) immunoglobulin and HBV vaccination. We read from the article written by Yi *et al* that lamivudine treatment in early pregnancy was safe and effective. However, we could not understand why adefovir dipivoxil (ADV) was used in three pregnancy cases, since ADV has been classified as pregnancy category C. In pregnancy, telbivudine or tenofovir should be selected when the treatment of CHB is necessary, since these drugs have been classified as Food and Drug Administration pregnancy risk category B.

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Key words: Pregnancy; Adefovir dipivoxil; Lamivudine; Tenofovir; Entecavir; Chronic hepatitis B; Treatment

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TO THE EDITOR

We read the article entitled “Safety of lamivudine treatment for chronic hepatitis B in early pregnancy”, with great interest. The management of chronic hepatitis B (CHB) in pregnancy is complex. Especially in endemic areas, vertical transmission from mother to newborn remains the most frequent route of infection and this situation often leads to chronic disease. Moreover, even with the use of appropriate prophylaxis with hepatitis B virus (HBV) immunoglobulin and HBV vaccination, a significant risk of vertical transmission remains, particularly in mothers with high viral loads and positive hepatitis B e antigen status^[1].

Without a doubt, the article by Yi *et al*^[2] sheds very important light on CHB treatment in pregnancy. They reported that lamivudine treatment in early pregnancy was safe and effective. In addition they stated that adefovir dipivoxil (ADV) was given in three cases who had HBV DNA above 10⁶ copies/mL, from week 28 of pregnancy. However, we know that ADV is classified as pregnancy category C and there are no adequate and well controlled studies on the use of adefovir during pregnancy^[3]. On the other hand, we have two drugs, tenofovir and telbivudine, which are classified as Food and Drug Administration pregnancy risk category B^[4]. Tenofovir received this classification based on data collected from human exposure, and in addition, Han *et al*^[5] study in pregnant women supports the “B” rating of telbivudine^[1]. In patients treated with ADV or entecavir, these drugs should be switched to safer drugs, if a woman becomes pregnant^[1]. For the sake of clarity for readers, it would be helpful if the authors explained why they selected ADV instead of tenofovir or telbivudine for pregnant women with CHB.

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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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Clinical features of gastroduodenal injury associated with long-term low-dose aspirin therapy

Junichi Iwamoto, Yoshifumi Saito, Akira Honda, Yasushi Matsuzaki

Junichi Iwamoto, Yoshifumi Saito, Akira Honda, Yasushi Matsuzaki, Department of Gastroenterology, Tokyo Medical University, Ibaraki Medical Center, Ibaraki 300-0395, Japan

Author contributions: All the authors contributed equally to this work; all authors read and approved the final manuscript.

Correspondence to: Junichi Iwamoto, MD, Department of Gastroenterology, Tokyo Medical University, Ibaraki Medical Center, 3-20-1 Ami-machi Chuo, Inashiki-gun, Ibaraki 300-0395, Japan. junnki@dg.mbn.or.jp

Telephone: +81-298-871161 Fax: +81-298-883463

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Abstract

Low-dose aspirin (LDA) is clinically used for the prevention of cardiovascular and cerebrovascular events with the advent of an aging society. On the other hand, a very low dose of aspirin (10 mg daily) decreases the gastric mucosal prostaglandin levels and causes significant gastric mucosal damage. The incidence of LDA-induced gastrointestinal mucosal injury and bleeding has increased. It has been noticed that the incidence of LDA-induced gastrointestinal hemorrhage has increased more than that of non-aspirin non-steroidal anti-inflammatory drug (NSAID)-induced lesions. The pathogenesis related to inhibition of cyclooxygenase (COX)-1 includes reduced mucosal flow, reduced mucus and bicarbonate secretion, and impaired platelet aggregation. The pathogenesis related to inhibition of COX-2 involves reduced angiogenesis and increased leukocyte adherence. The pathogenic mechanisms related to direct epithelial damage are acid back diffusion and impaired platelet aggregation. The factors associated with an increased risk of upper gastrointestinal (GI) complications in subjects taking LDA are aspirin dose, history of ulcer or upper GI bleeding, age > 70 years, concomitant use of non-aspirin NSAIDs including COX-2-selective NSAIDs, and *Helicobacter pylori* (*H. pylori*) infection. Moreover, no significant differences have been found

between ulcer and non-ulcer groups in the frequency and severity of symptoms such as nausea, acid regurgitation, heartburn, and bloating. It has been shown that the ratios of ulcers located in the body, fundus and cardia are significantly higher in bleeding patients than the ratio of gastroduodenal ulcers in patients taking LDA. Proton pump inhibitors reduce the risk of developing gastric and duodenal ulcers. In contrast to NSAID-induced gastrointestinal ulcers, a well-tolerated histamine H₂-receptor antagonist is reportedly effective in prevention of LDA-induced gastrointestinal ulcers. The eradication of *H. pylori* is equivalent to treatment with omeprazole in preventing recurrent bleeding. Continuous aspirin therapy for patients with gastrointestinal bleeding may increase the risk of recurrent bleeding but potentially reduces the mortality rates, as stopping aspirin therapy is associated with higher mortality rates. It is very important to prevent LDA-induced gastroduodenal ulcer complications including bleeding, and every effort should be exercised to prevent the bleeding complications.

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Key words: Gastroduodenal ulcer; Upper gastrointestinal bleeding; Low-dose aspirin; Non-steroidal anti-inflammatory drugs

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INTRODUCTION

Low-dose aspirin (LDA) is clinically used for the prevention of cardiovascular and cerebrovascular events with

the advent of an aging society^[1-6]. Worldwide trials of antiplatelet therapy have demonstrated that an antiplatelet regimen (such as aspirin 75-325 mg/d) offers worthwhile protection against myocardial infarction, stroke, and death.

On the other hand, a very low dose of aspirin (10 mg daily) decreases the gastric mucosal prostaglandin levels and causes significant gastric mucosal damage^[7]. The incidence of LDA-induced gastrointestinal (GI) mucosal injury has increased^[8-11]. This review focuses on the clinical characteristics of LDA-induced GI ulcer or erosion and bleeding, including incidence, mechanism, risk of bleeding, clinical manifestations, risk factors, endoscopic features, prevention, and treatment.

CLINICAL FEATURES OF LDA-INDUCED GI INJURY

Incidence of LDA-induced GI ulcer and bleeding

The incidence of upper GI damage in patients taking long-term LDA has been investigated. In a multicenter investigation, Yeomans *et al*^[12] found that the prevalence rates of ulcer and erosion were 10.7% and 63.1%, respectively, in 187 patients taking long-term LDA, and that the incidence rates of ulcer and erosion in 113 patients followed up for 3 mo were 7.1% and 60.2%, respectively, indicating that GI ulcers develop in one in 10 patients taking LDA.

The annual incidence of serious upper GI ulcer bleeding among Japanese patients taking LDA or non-aspirin non-steroidal anti-inflammatory drugs (NSAID) was investigated. The pooled incidence rate of bleeding was 2.65% (range: 2.56%-2.74%) and 1.29% (range: 1.27%-1.31%) per 1000 patient years for LDA and non-aspirin NSAID users, respectively^[13]. Niv *et al*^[14] have investigated, using esophagogastroduodenoscopy, 46 asymptomatic patients taking LDA and they detected ulcer or erosions in 22 patients, erosive gastroduodenitis in 13, gastric ulcer in 14, duodenal ulcer in 2, and gastric and duodenal ulcers in 2, suggesting that esophagogastroduodenoscopy is important for LDA users, even the asymptomatic patients. The incidence and factors influencing the occurrence of upper GI bleeding in 903 consecutive patients taking LDA were analyzed. The results revealed that 4.5% of patients presented with upper GI bleeding requiring hospitalization during follow-up, and the incidence of upper GI bleeding was 1.2 per 100 patient years^[9]. The incidence rates of upper GI bleeding in 27 694 users of LDA were analyzed, and a total of 207 exclusive users of LDA experienced a first episode of upper GI bleeding. The standardized incidence rate of upper GI bleeding among LDA users was 2.6% (range: 2.2%-2.9%), and the standardized incidence rate for combined use of LDA and other NSAIDs was 5.6% (range: 4.4%-7.0%)^[10].

The frequency of gastroduodenal injuries associated with LDA use for prevention of cardiovascular and cerebrovascular events were investigated. The results

showed that mucosal injuries occurred in 61.4% and gastroduodenal ulcers in 18.8% of 101 LDA users with ischemic heart disease who were not receiving antiulcer treatment^[15]. In another investigation, screening upper endoscopic examinations were prospectively performed on 236 patients with ischemic heart disease, and mucosal defects were found in 92 of 190 (48.4%) users of LDA and in 6 of 46 (13.0%) non-users^[16].

Taha *et al*^[17] have investigated the efficacy of famotidine in prevention of peptic ulcers and erosive esophagitis in patients receiving LDA. They showed that gastric ulcers had developed in 3.4% of patients treated with famotidine and in 15.0% of patients on placebo, while duodenal ulcers had developed in 0.5% and 8.5%, respectively. Other previous reports have examined the efficacy of esomeprazole as compared with placebo in prevention of peptic ulcers in patients who were at risk for ulcer development taking low-dose acetylsalicylic acid (ASA). These studies have shown that esomeprazole significantly reduced the cumulative proportion of patients with peptic ulcers, and that 7.4% of placebo recipients developed peptic ulcers^[18]. These studies have revealed not only the efficacy of famotidine or esomeprazole but also the incidence of gastroduodenal ulcer in patients taking long-term LDA without anti-ulcer drugs.

A clinical investigation examined the efficacy of low-dose lansoprazole in the secondary prevention of LDA-associated gastric or duodenal ulcers, and showed that the cumulative incidence of gastric or duodenal ulcers was 3.7% in the lansoprazole group and 31.7% in the placebo group. This investigation indicated that the incidence of gastric or duodenal ulcers was 31.7% in patients with a definite history of gastric or duodenal ulcers who required long-term LDA therapy^[19].

The incidence rates of upper GI events in LDA users are summarized in Table 1.

Mechanism of LDA-induced gastroduodenal mucosal damage

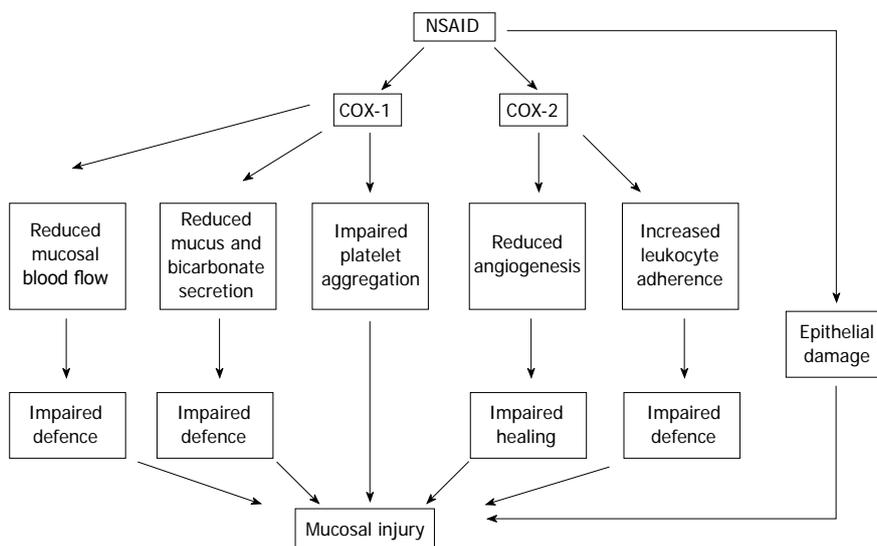
The mechanism of action of NSAIDs or LDA can be subdivided into local action and systemic action, and several mechanisms have been reported in a previous review^[20] (Figure 1). The pathogenesis related to inhibition of cyclooxygenase (COX)-1 includes reduced mucosal flow, reduced mucus and bicarbonate secretion, and impaired platelet aggregation. The pathogenic mechanisms involved in inhibition of COX-2 are reduced angiogenesis and increased leukocyte adherence. The pathogenesis related to direct epithelial damage involves acid back diffusion and impaired platelet aggregation. Aspirin is a more potent inhibitor of COX-1 than of COX-2^[20] (Figure 1).

Both the direct effect of aspirin on the GI mucosa and the systemic effect related to reduction of prostaglandin level are suggested to contribute in the pathogenesis of LDA-induced GI mucosal damage^[7]. Some researchers have suggested that reduction in the ability of the gastric mucosa to synthesize prostaglandin E2 and

Table 1 Incidence of upper gastrointestinal events in low-dose aspirin users

| Ref. | Subjects | GI event | Incidence rate |
|---------------------------------------|-----------------------------|---------------------------------|--|
| Yeomans <i>et al</i> ^[12] | 187 | Gastroduodenal | 10.7% |
| | | Gastroduodenal | 63.1% |
| Ishikawa <i>et al</i> ^[13] | 1657 | Gastroduodenal | 2.65 (95%CI: 2.56-2.74) per 1000 patient years |
| Niv <i>et al</i> ^[14] | 46 asymptomatic | Gastroduodenal ulcer or erosion | 47.83% |
| Taha <i>et al</i> ^[17] | 200 | Gastroduodenal ulcer | 23.5% |
| Scheiman <i>et al</i> ^[18] | 2426 high risk | Gastroduodenal ulcer | 7.4% |
| Sugano <i>et al</i> ^[19] | 235 with a history of ulcer | Gastroduodenal ulcer | 31.7% |
| Serrano <i>et al</i> ^[9] | 903 | Upper GI bleeding | 1.2 per 100 patient years |
| Sorensen <i>et al</i> ^[10] | 27 694 | Upper GI bleeding | 2.6 (95%CI: 2.2-2.9) |
| Nema <i>et al</i> ^[15] | 101 | Gastroduodenal mucosal injury | 61.4% |
| | | Gastroduodenal ulcer | 18.8% |
| Nema <i>et al</i> ^[16] | 190 | Gastroduodenal mucosal defects | 48.4% |

GI: Gastrointestinal.

**Figure 1** Pathogenesis of non-steroidal anti-inflammatory drug-induced gastric injury. Non-steroidal anti-inflammatory (NSAID) drugs induce injury *via* three key pathways: inhibition of cyclooxygenase (COX)-1 activity, inhibition of COX-2 activity, and direct cytotoxic effects on the epithelium. Aspirin is a more potent inhibitor of COX-1 than of COX-2^[20].

the consequent side effects result in injury of the gastric mucosa following aspirin use^[21].

Gastric damage in rats induced by a selective COX-1 inhibitor (SC-560) and a selective COX-2 inhibitor (celecoxib) were investigated. SC-560 alone or celecoxib alone did not cause gastric damage, whereas the combination of SC-560 and celecoxib invariably caused hemorrhagic erosion. This study suggested that inhibition of both COX-1 and COX-2 is required for NSAID-induced gastric injury in the rat^[22].

Another study demonstrated that COX-1-deficient mice survived well and had no gastric pathology, indicating that inhibition of both COX-1 and COX-2 was required for NSAID-induced gastric injury^[23].

A previous review has suggested a mechanism of LDA-induced gastroduodenal mucosal injury, claiming that ion trapping and back diffusion of hydrogen ions lead to gastric erosion and bleeding; this is known as the hypothesis of NSAIDs' dual insult on the stomach^[24].

It has been suggested that NSAID-induced neu-

trophil adherence is associated with NSAID-induced GI mucosal damage. The neutrophil adherence to the vascular endothelium could lead to obstruction in the capillaries with consequent reduction of blood flow in the gastric mucosa. The increased production of oxygen-derived free radicals and the liberation of proteases are also associated with NSAID-induced mucosal damage^[25].

Risk of upper GI injury and bleeding associated with long-term use of LDA

A previous investigation has evaluated the long-term effects of individual doses of aspirin (10 mg, 81 mg, or 325 mg daily for 3 mo) on the GI tract, and revealed that a very low dose of aspirin (10 mg daily) decreased the gastric mucosal prostaglandin levels and caused significant gastric mucosal damage^[7].

Other researchers studied the characteristics of patients with acute upper GI hemorrhage at 3 time points over a 6-year follow-up period, and revealed that the incidence of hemorrhage in patients taking LDA increased

from 15 per 100 000 of the population per annum to 18 and 27, and that the respective incidence rates in patients taking other anti-thrombotic drugs were 4, 8, and 12, respectively. On the other hand, no significant change was found in NSAID users. This study has suggested that the rate of LDA-induced GI hemorrhage is higher than that of non-aspirin NSAID-induced lesions^[26]. A previous case-control study demonstrated that the percentages of regular users of aspirin regimens (300 mg daily or less) among patients with gastric or duodenal ulcer bleeding, hospital and community controls were 12.8%, 9.0%, and 7.8%, respectively, concluding that the regular users of aspirin regimens were at risk for gastric or duodenal ulcer bleeding^[27].

A case-control study in Japan has shown that the odds ratio (OR) of upper GI bleeding was 5.5 for aspirin and 6.1 for non-aspirin NSAIDs, indicating that the risk of upper GI bleeding in the cases taking LDA is almost similar to the risk in the cases taking non-aspirin NSAIDs^[28].

A meta-analysis of 24 randomized controlled trials has evaluated the incidence of GI hemorrhage associated with long-term aspirin therapy to determine the effect of dose reduction and formulation on the incidence of hemorrhage. This meta-analysis revealed that GI hemorrhage occurred in 2.47% of patients taking aspirin as compared with 1.42% taking placebo. This study suggested that long-term therapy with aspirin is associated with a significant increase in the incidence of GI hemorrhage, and no evidence was found supporting the proposal that reducing the dose or using modified release formulations would reduce the incidence of GI hemorrhage^[29]. Another meta-analysis of 6 trials investigated the benefits and GI risk of aspirin use for secondary prevention of cerebrovascular and cardiovascular diseases. It was shown that aspirin reduced all-cause mortality by 18%, the number of strokes by 20%, myocardial infarctions by 30%, and other vascular events by 30%. It was further demonstrated that patients who took aspirin were 2.5 times more likely than those in the placebo group to have GI tract bleeding, concluding that aspirin use for secondary prevention of thromboembolic events has a favorable benefit-to-risk profile^[30].

A previous investigation has demonstrated that following percutaneous coronary intervention, long-term (one year) clopidogrel therapy significantly reduced the risk of adverse ischemic events, indicating the efficacy of clopidogrel therapy in such cases^[31].

The risk of major upper gastrointestinal bleeding associated with various antiplatelet drugs has been examined, and the results revealed that the individual risks of upper GI bleeding were 4.0% (range: 3.2%-4.9%) with acetylsalicylic acid, 2.3% (range: 0.9%-6.0%) with clopidogrel, 0.9% (range: 0.4%-2.0%) with dipyridamole, and 3.1% with ticlopidine (range: 1.8%-5.1%), suggesting that not only acetylsalicylic acid but also the other various antiplatelet drugs have some risk for upper GI bleeding^[32].

The risk of upper GI bleeding following the combined use of LDA and other antithrombotic drugs has

been investigated. Hallas *et al.*^[33] have assessed the risk of serious upper GI bleeding in patients taking LDA alone or in combination with other antithrombotic drugs. This investigation demonstrated that the adjusted odds ratios associating drug use with upper GI bleeding were 1.8 (range: 1.5-2.1) for LDA, 1.1 (range: 0.6-2.1) for clopidogrel, 1.9 (range: 1.3-2.8) for dipyridamole, and 1.8 (range: 1.3-2.4) for vitamin K antagonists. Furthermore, they have shown that the adjusted odds ratios associated with combined use were 7.4 (range: 3.5-15) for clopidogrel and aspirin, 5.3 (range: 2.9-9.5) for vitamin K antagonists and aspirin, and 2.3 (range: 1.7-3.3) for dipyridamole and aspirin^[33]. The higher risk of upper GI bleeding in patients receiving LDA and other antithrombotic drugs was revealed.

Risk factors for LDA-induced GI damage and bleeding

A previous investigation demonstrated that at doses below 163 mg/d, GI hemorrhage occurred in 2.30% of patients taking aspirin as compared with 1.45% taking placebo, and that with modified release formulations of aspirin, the odds ratio was 1.93, indicating that reducing the dose or using modified release formulations would not reduce the incidence of GI hemorrhage^[29].

Another report examined the association between taking LDA and the risk of symptomatic ulcer. The authors demonstrated that the relative risk was 2.9 (range: 2.3-3.6) for aspirin (75 mg) users as compared with non-users, and that the relative risk was similar for doses up to 300 mg daily^[34].

On the other hand, a multivariate analysis examined the risk factors for LDA-related upper GI bleeding, and showed that higher doses of aspirin increased the risk of upper GI bleeding, suggesting a dose-dependent risk for aspirin^[9].

A multicenter case-control study investigated 550 incident cases of upper GI bleeding admitted into hospital with melena or hematemesis and confirmed by endoscopy, in comparison with 1202 controls identified from population census lists about the use of aspirin. This study demonstrated that the relative risks of upper GI bleeding for plain, enteric-coated, and buffered aspirin at average daily doses of 325 mg or less were 2.6, 2.7, and 3.1, respectively, and at doses greater than 325 mg, the relative risk was 5.8 for plain and 7.0 for buffered aspirin^[35]. Another investigation disclosed that the risk of upper GI bleeding was similar among users of non-coated LDA and coated LDA^[10]. It was also suggested in another study that the risk of gastroduodenal ulcer was similarly elevated for both regular and enteric-coated preparations of LDA^[34]. These clinical reports revealed no important differences in risk among the three aspirin forms.

Some studies have been designed to identify patients who are most likely to have adverse events of NSAID therapy. The established risk factors for development of NSAID-associated gastroduodenal ulcers were reportedly advanced age, history of ulcer, concomitant use of corticosteroids, higher dose of NSAID including the use of

more than one NSAID, concomitant administration of anticoagulants, and serious systemic disorders, while possible risk factors were reportedly concomitant infection with *Helicobacter pylori* (*H. pylori*), cigarette smoking, and consumption of alcohol^[36].

The risk factors for upper GI bleeding or peptic ulcer in patients taking LDA have been suggested in a previous investigation. A clinical study evaluated the risk predictors of gastroduodenal ulcers during treatment with vascular protective doses of aspirin, and suggested that older age and *H. pylori* infection increased the risk of gastroduodenal ulcers^[12].

A multivariate analysis examined the risk factors for LDA-related upper GI bleeding, and showed that a history of peptic ulcer or upper GI bleeding correlated with higher risk of upper GI bleeding. On the other hand, antisecretory and nitro-vasodilator drugs correlated with a decreased risk^[9]. It has been suggested that the factors associated with an increased risk of upper GI complications in subjects taking LDA are aspirin dose, history of ulcer or upper GI bleeding, age > 70 years, concomitant use of non-aspirin NSAIDs including COX-2-selective NSAIDs, and *H. pylori* infection^[8].

A previous cohort study investigated the incidence rates of upper GI bleeding in 27 694 users of LDA as compared with the incidence rates in the general population. This study disclosed that the standardized incidence rate ratio was 2.6 (range: 2.2-2.9), and the standardized incidence rate ratio for combined use of LDA and other NSAIDs was 5.6 (range: 4.4-7.0), indicating the higher risk of combined use of LDA and other NSAIDs^[10].

Many investigators have reported the relationship between *H. pylori* infection and use of NSAIDs in the pathogenesis of gastroduodenal ulcer, and this is still controversial. A meta-analysis of 25 studies has shown that both *H. pylori* infection and use of NSAIDs independently and significantly increase the risk of peptic ulcer and ulcer bleeding, concluding that there is synergism for the development of peptic ulcer and ulcer bleeding between *H. pylori* infection and NSAID use^[37]. Another previous study investigated whether *H. pylori* increases the risk of upper GI bleeding in patients taking LDA, evaluating the role of *H. pylori* infection and other clinical factors. The results revealed that *H. pylori* infection was an independent risk factor of upper GI bleeding in this population (OR: 4.7, range: 2.0-10.9)^[38]. On the other hand, another previous report has demonstrated that *H. pylori*-infected patients were less likely to have any gastric erosion than the non-infected, indicating that *H. pylori* infection may partially protect against LDA-induced gastric erosion^[39].

A previous case-control study investigated the risk of peptic ulcer and upper GI bleeding associated with the use of coxibs, traditional NSAIDs, aspirin, or combinations of these drugs. This study demonstrated that the risk associated with coxib use for upper GI bleeding was less than that of non-selective NSAIDs. This report revealed that with combined use of LDA, the lower risk

of coxibs tended to disappear^[40]. The Celecoxib Long-term Arthritis Safety Study has shown that for patients not taking aspirin, the annual incidence rates of upper GI ulcer complications alone and combined with symptomatic ulcers for celecoxib were 0.44% and 1.40%, respectively, and for patients taking aspirin, the corresponding rates were 2.01% and 4.70%, respectively. These results suggested that celecoxib was associated with a lower incidence of GI toxicity, but that the risk increased with concomitant use of LDA^[41].

Although coxibs tend to present a lower risk of upper GI complications than NSAIDs overall, aspirin is a strong effect modifier, abolishing completely the GI safety advantage of coxibs over NSAIDs, suggesting that concomitant use of COX-2 inhibitor and LDA increases the risk of upper GI complications^[42].

Clinical manifestations of LDA-induced gastroduodenal injury

Previous studies have demonstrated a correlation between clinical manifestations and ulcers in patients taking LDA. In a multicenter investigation, Yeomans *et al*^[12] demonstrated that the ulcer prevalence was 10.7% in 187 patients taking long-term LDA, and that only 20% had dyspeptic symptoms, which was not significantly different from the patients without ulcer. This study revealed no significant differences between the ulcer groups and non-ulcer groups in the frequency and severity of symptoms such as nausea, acid regurgitation, heartburn, and bloating^[12]. Another previous report stated that some patients on aspirin complained of symptoms whereas the others remained completely asymptomatic, indicating that the patients remaining free of symptoms seemed to characteristically have a higher gastric sensory threshold^[43]. Niv *et al*^[14] have investigated 46 asymptomatic patients taking mini-dose aspirin for more than 3 mo. Ulcer or erosions developed in 22 of those patients taking mini-dose aspirin, erosive gastroduodenitis in 13, gastric ulcer in 14, duodenal ulcer in 2, and gastric and duodenal ulcers in 2, indicating a high prevalence of ulcerations of the stomach and duodenum in asymptomatic LDA users^[14].

The mechanism of asymptomatic ulceration in aspirin users has been investigated, and it was suggested that subjects on aspirin remaining free of symptoms appear to characteristically have higher gastric sensory thresholds^[43].

Endoscopic features of LDA-induced gastroduodenal mucosal damage

A recent investigation has examined the chronological changes of the GI mucosa with LDA use in 20 healthy *H. pylori*-negative subjects. These patients were divided into those receiving 100 mg aspirin with placebo, and those receiving 100 mg aspirin + 300 mg rebamipide daily for 7 d, and they were examined for mucosal damage at 0, 2, 6, and 24 h on the first day, and then on the third and seventh days. The results revealed that ulcers developed in the duodenum at 24 h and in the antrum at 72 h, and

that erosions mainly developed in the duodenum in the subjects receiving 100 mg aspirin, concluding that damage occurred in the duodenum most frequently and that almost all damage improved gradually in spite of continuous aspirin^[44]. A prospective study on 238 patients with bleeding peptic ulcers demonstrated the endoscopic characteristics of LDA-induced hemorrhagic ulcer. Non-NSAID-induced ulcers were significantly higher in the gastric body than the LDA-induced ulcers, and non-aspirin NSAID-induced ulcers and most of the LDA-induced ulcers were found in the gastric body, angular notch, and duodenum. The number of ulcers was investigated in 18 non-aspirin NSAID-induced ulcers, and the ulcer was single in 44.4% and multiple in 55.6%^[11]. In our previous investigation, the ratios of ulcers located in the antrum of patients taking LDA and non-aspirin NSAIDs were significantly higher than those of patients not taking NSAIDs (the bleeding patients and the whole gastroduodenal ulcer patient population). It also has been shown that the ratios of ulcers located in the body, fundus, and cardia were significantly higher in the bleeding patients than in the whole gastroduodenal ulcer patient population taking LDA^[45]. Shiotani *et al*^[46] investigated 305 patients taking 100 mg aspirin for cardiovascular diseases. They found that 38 patients (12.4%) had ulcer lesions (34 gastric ulcer, 2 duodenal ulcer, and 2 with gastroduodenal ulcer). Of the 34 gastric ulcers, 18 were single and 16 were multiple, and 58.8% of the gastric ulcers were in the gastric body while the maximum ulcer size was 25 mm^[46].

Some investigators analyzed the size of LDA-induced ulcers, and demonstrated that gastric ulcers were more frequent than duodenal ulcers in both *H. pylori*-negative and -positive patients; the gastric ulcers and duodenal ulcers were most commonly 5-10 mm in size^[47]. In a study on 674 upper GI bleeders, erosive esophagitis was detected in 150 cases, suggesting that erosive esophagitis is common in patients with upper GI bleeding taking LDA or antithrombotic agents^[48].

PREVENTION AND TREATMENT OF LDA-INDUCED GASTRODUODENAL MUCOSAL DAMAGE

Prevention of LDA-induced gastroduodenal mucosal damage and bleeding

The primary prevention of LDA-induced gastroduodenal damage has been investigated. It was demonstrated that famotidine is effective in prevention of gastric and duodenal ulcers, as well as erosive esophagitis, in patients taking LDA^[17]. In contrast to NSAID-induced GI ulcers, a well-tolerated histamine H2-receptor antagonist is effective in preventing the development of LDA-induced GI ulcers.

A previous study investigated the efficacy of esomeprazole in reducing the risk of gastric and duodenal ulcers in patients receiving continuous LDA therapy. The results revealed that 5.4% in the placebo group developed

a gastric or duodenal ulcer during the 26-wk treatment as compared with 1.6% in the esomeprazole group, suggesting that esomeprazole (20 mg once daily) reduces the risk of developing gastric and duodenal ulcers^[47]. Another clinical trial revealed that treatment with esomeprazole (40 mg or 20 mg once daily) reduces the occurrence of peptic ulcers in LDA-taking patients who are at risk for ulcer development^[18].

The secondary prevention of LDA-induced gastroduodenal damage has been investigated. A case-control study demonstrated that proton pump inhibitors, H2-receptor antagonists, and nitrates reduced upper GI bleeding risk, suggesting that antisecretory agents or nitrate treatment results in reduced relative risks of upper GI bleeding in patients taking NSAIDs or aspirin^[49]. A clinical trial involving 160 patients with aspirin-related peptic ulcers/erosions compared the efficacy of H2-receptor antagonists (famotidine group) and proton pump inhibitors (pantoprazole group). This trial demonstrated that GI bleeding was significantly more frequent in the famotidine group than in the pantoprazole group^[50]. A clinical investigation in Japan examined the efficacy of low-dose lansoprazole (15 mg once daily) in the secondary prevention of LDA-associated gastric or duodenal ulcers. The patients were randomized to receive lansoprazole 15 mg daily ($n = 226$) or gefarnate 50 mg twice daily ($n = 235$) for 12 mo or longer. The results disclosed that the cumulative incidence of gastric or duodenal ulcers was 3.7% in the lansoprazole group and 31.7% in the placebo group, indicating that lansoprazole was superior to gefarnate for the secondary prevention of LDA-associated gastric or duodenal ulcers^[19]. After the ulcers had healed, the patients who were negative for *H. pylori* were randomly assigned to receive either 75 mg of clopidogrel daily plus placebo or 80 mg of aspirin daily plus 20 mg of esomeprazole twice daily for 12 mo. The results showed that recurrent ulcer bleeding occurred in 13 patients receiving clopidogrel and in 1 receiving aspirin plus esomeprazole. This study concluded that among patients with a history of aspirin-induced ulcer bleeding, aspirin plus esomeprazole was superior to clopidogrel in the prevention of recurrent ulcer bleeding in *H. pylori*-negative patients^[51]. The studied co-treatments for prevention of LDA-induced gastroduodenal events are displayed in Table 2.

Treatment of LDA-induced gastroduodenal injury and bleeding

It has been reported that ranitidine with NSAID discontinuation is effective in the treatment of NSAID-induced gastroduodenal ulcer^[52]. If it is not possible to stop NSAID treatment, rabeprazole should be used as its efficacy for NSAID-induced ulcer under continuous NSAID administration has been confirmed^[53].

Many investigations have studied the efficacy of proton pump inhibitors in the healing of NSAID-induced gastroduodenal ulcer in comparison with the efficacy of H2-receptor antagonists. Most of the reports stated that

Table 2 Prevention of low-dose aspirin-induced gastroduodenal events

| Ref. | Co-treatment | GI event | Incidence rate |
|---------------------------------------|---|---------------------------------------|-------------------------------|
| Taha <i>et al</i> ^[17] | Famotidine 40 mg vs placebo | Gastric ulcers/duodenal ulcers | 3.4% vs 15.0% 0.5% vs 8.5% |
| Scheiman <i>et al</i> ^[18] | Esomeprazole 40 mg vs 20 mg vs placebo | Gastroduodenal ulcers | 1.5% vs 1.1% vs 7.4% |
| Sugano <i>et al</i> ^[19] | Lansoprazole 15 mg vs placebo | Gastroduodenal ulcers | 3.7% vs 31.7% |
| Yeomans <i>et al</i> ^[47] | Esomeprazole 20 mg vs placebo | Gastroduodenal ulcers | 1.6% vs 5.4% |
| Ng <i>et al</i> ^[50] | Famotidine 40 mg vs pantoprazole 20 mg | Dyspeptic or bleeding ulcers/erosions | 20% vs 0% |
| Chan <i>et al</i> ^[67] | Omeprazole 20 mg vs <i>H. pylori</i> eradication | Gastroduodenal bleeding | 0.9% vs 1.9% |
| Lai <i>et al</i> ^[68] | <i>H. pylori</i> eradication + lansoprazole 15 mg vs <i>H. pylori</i> eradication | Gastroduodenal ulcers | 1.6% vs 14.8% |

GI: Gastrointestinal; *H. pylori*: *Helicobacter pylori*.

the proton pump inhibitors healed and prevented ulcers more effectively than H₂-receptor antagonists^[54-56]. An animal experiment revealed that lansoprazole protection against NSAID-induced gastric damage depends on a reduction in mucosal oxidative injury, which is also responsible for an increase in sulfhydryl radical bioavailability^[57].

Another previous report stated that the differences between the efficacy of proton pump inhibitors and H₂-receptor antagonists in the treatment of NSAID-induced ulcer were not statistically significant^[58].

In patients with LDA-induced upper GI bleeding, the risk involved in stopping the LDA was higher than that of non-aspirin NSAID. A previous study compared the therapeutic effects of proton pump inhibitors and H₂-receptor antagonists on the healing rate of gastroduodenal ulcers during continuous use of LDA, and no significant differences were found. The results indicated that not only proton pump inhibitors but also H₂-receptor antagonists are effective in the treatment of gastroduodenal ulcers during continuous use of LDA^[59,60].

Previous research has also revealed that misoprostol is effective for the treatment of gastroduodenal injury in patients taking NSAIDs or LDA^[61-63].

It is a clinical problem whether aspirin therapy should be continued or discontinued in patients who develop peptic ulcer bleeding while receiving LDA. A clinical trial examined whether discontinuation of aspirin therapy is a risk factor for ischemic stroke and compared the frequency of aspirin therapy discontinuation during four weeks before an ischemic cerebral event in patients and four weeks before interview in controls. This trial suggested the importance of continuous aspirin therapy and clarified the risk associated with discontinuation of aspirin therapy in patients at risk for ischemic stroke^[64]. A recent clinical investigation has analyzed the recurrent ulcer bleeding and mortality rates attributable to cardiovas-

cular and cerebrovascular events in 78 patients receiving aspirin and 78 taking placebo for 8 wk immediately after endoscopic therapy. It was demonstrated that recurrent ulcer bleeding rate within 30 d was 10.3% in the aspirin group and 5.4% in the placebo group. The patients who received aspirin had lower all-cause mortality rates than the patients who received placebo (1.3% vs 12.9%), while the patients in the aspirin group had lower mortality rates attributable to cardiovascular, cerebrovascular, or GI complications than the patients in the placebo group (1.3% vs 10.3%). This study suggested that continuous aspirin therapy may increase the risk of recurrent bleeding but potentially reduces the mortality rates, indicating that continuous aspirin therapy had lower mortality rates^[65].

Our previous study has revealed that the ratios of patients taking LDA who required additional endoscopic treatment on the next day following the first procedure were higher than those of non-aspirin NSAID and non-NSAID patients. The duration of hospitalization of the patients taking LDA was significantly longer than that of the patients taking non-aspirin NSAIDs and non-NSAIDs. These results suggested the possibility that the treatment of LDA-induced hemorrhagic gastroduodenal ulcers is more difficult than treatment of those induced by non-aspirin NSAIDs or non-NSAIDs, indicating that every effort should be exercised to prevent bleeding complications in patients receiving LDA^[45]. Endoscopic high-frequency soft coagulation has been recently developed in Japan, and the efficacy of hemostasis with soft coagulation for bleeding gastric ulcer patients (including aspirin users) has been investigated by comparing it with hemoclips. The results demonstrated that 85% of patients in the endoscopic hemostasis with soft coagulation group and 79% patients in the endoscopic hemoclip group were successfully treated^[65]. Another clinical investigation involved 39 cases where hemostasis was attempted with bipolar forceps to deal with non-variceal upper GI bleeding. This study revealed that the technique of bipolar forceps (a new technique of endoscopic hemostasis) is simple, safe and unlikely to induce complications^[66]. Although standard endoscopic hemostasis is reportedly difficult for upper GI bleeding in aspirin users, this new technique of endoscopic hemostasis is potentially effective in such cases^[45,65,66].

Eradication of *H. pylori* in treatment of LDA-induced gastroduodenal damage

A meta-analysis has investigated the prevalence of *H. pylori* infection and NSAID use in patients with peptic ulcer bleeding, and revealed that *H. pylori* infection increased the risk (3.53-fold) of peptic ulcer disease in NSAID takers, in addition to the risk associated with NSAID use, concluding that both *H. pylori* infection and NSAID use independently and significantly increase the risk of peptic ulcer and ulcer bleeding^[37].

A previous report demonstrated that among those taking aspirin, the probability of recurrent bleeding during a 6-mo period was 1.9% for patients who received

eradication therapy and 0.9% for patients who received omeprazole, indicating that eradication of *H. pylori* is equivalent to treatment with omeprazole in preventing recurrent bleeding. On the other hand, omeprazole is superior to eradication of *H. pylori* in preventing recurrent bleeding in patients who are taking non-aspirin NSAIDs^[67].

However, in another study, after the ulcers had healed and the *H. pylori* infection was eradicated, the patients were randomly assigned to treatment with 30 mg of lansoprazole daily or placebo, in addition to 100 mg of aspirin daily, for 12 mo. The results revealed that the treatment with lansoprazole in addition to eradication of *H. pylori* infection significantly reduced the rate of recurrence of ulcer complications^[68].

CONCLUSION

Worldwide trials of antiplatelet therapy have demonstrated that an antiplatelet regimen (75-325 mg/d) offers worthwhile protection against myocardial infarction, stroke, and death. On the other hand, the rate of low-dose aspirin-induced GI hemorrhage has increased more than that of non-aspirin NSAID-induced lesions. Continuous aspirin therapy in the case of GI bleeding may increase the risk for recurrent bleeding but potentially reduces mortality rates. Although the size of gastroduodenal ulcers in patients taking LDA is smaller than that of patients taking non-aspirin NSAIDs, more cases need additional endoscopic treatment on the next day after the first procedure as compared with patients taking non-aspirin NSAIDs. Previous clinical investigation has suggested that co-treatment with proton pump inhibitors is effective in preventing the development of LDA-induced GI ulcer. These results indicate that it is very important to prevent LDA-induced gastroduodenal ulcer complications including bleeding, and that every effort should be exercised to prevent bleeding complications.

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Molecular mechanisms of liver ischemia reperfusion injury: Insights from transgenic knockout models

Gourab Datta, Barry J Fuller, Brian R Davidson

Gourab Datta, Barry J Fuller, Brian R Davidson, Liver Transplantation and Hepatobiliary Unit, Division of Surgery and Interventional Science, Royal Free Campus, University College London, Royal Free Hampstead NHS Trust Hospital, London NW 2QG, United Kingdom

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Correspondence to: Dr. Gourab Datta, MBChir, BA, Liver Transplantation and Hepatobiliary Unit, Division of Surgery and Interventional Science, Royal Free Campus, University College London, Royal Free Hampstead NHS Trust Hospital, Pond Street, London NW 2QG, United Kingdom. gourab.datta@yahoo.co.uk
Telephone: +44-207-7940500 Fax: +44-207-4726226

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Abstract

Ischemia reperfusion injury is a major obstacle in liver resection and liver transplantation surgery. Understanding the mechanisms of liver ischemia reperfusion injury (IRI) and developing strategies to counteract this injury will therefore reduce acute complications in hepatic resection and transplantation, as well as expanding the potential pool of usable donor grafts. The initial liver injury is initiated by reactive oxygen species which cause direct cellular injury and also activate a cascade of molecular mediators leading to microvascular changes, increased apoptosis and acute inflammatory changes with increased hepatocyte necrosis. Some adaptive pathways are activated during reperfusion that reduce the reperfusion injury. IRI involves a complex interplay between neutrophils, natural killer T-cells cells, CD4+ T cell subtypes, cytokines, nitric oxide synthases, haem oxygenase-1, survival kinases such as the signal transducer and activator of transcription, Phosphatidylinositol 3-kinases/Akt and nuclear factor κ B pathways. Transgenic animals, particularly genetic knockout models, have become a powerful tool at elucidating mechanisms of liver ischaemia reperfusion injury and are

complementary to pharmacological studies. Targeted disruption of the protein at the genetic level is more specific and maintained than pharmacological inhibitors or stimulants of the same protein. This article reviews the evidence from knockout models of liver IRI about the cellular and molecular mechanisms underlying liver IRI.

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Key words: Liver; Ischemia/reperfusion; Transgenic; Knockout; Nitric oxide synthase; Haem oxygenase; Mitogen-activated protein kinase; T cell receptor

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INTRODUCTION

Ischemia reperfusion injury is a major cause of morbidity and mortality in liver resection and liver transplantation surgery. Prolonged organ ischemia is characterised reduced tissue oxygenation resulting in tissue adenosine triphosphate (ATP) depletion with a transition to activation of anaerobic metabolic pathways which cannot maintain cellular function for prolonged periods ultimately leading to cell death. Restoration of blood flow is necessary to restore cellular function, but paradoxically reperfusion can initiate a cascade of pathways that cause further cellular injury after prolonged ischaemia. Understanding the mechanisms of liver ischemia reperfusion injury (IRI) and developing strategies to counteract this injury will reduce acute complications in hepatic resection and transplantation, as well as expanding the potential pool of usable donor grafts.

The initial liver injury is initiated by reactive oxygen species (ROS) which cause direct cellular injury and also activate a cascade of mediators leading to microvascular changes, increased apoptosis and acute inflammatory changes with increased necrosis. Not all pathways activated are injurious and some adaptive pathways are activated during reperfusion that dampen the reperfusion injury. Classically two phases of liver injury have been described, an early (< 6 h) and late (> 12 h) phase of injury. In reality, this is a somewhat artificial distinction, as liver injury occurs as a continuum during reperfusion where pathways are activated at various often overlapping time-points.

The extent of liver injury in IRI is normally measured by raised levels of serum liver enzymes, most commonly aspartate transaminase, alanine transaminase (ALT), lactate dehydrogenase and/or serum glutamic-oxaloacetic transaminase, and by histological assessment with the Suzuki classification, with or without modifications, being most widely used in liver IRI^[1]. In this classification sinusoidal congestion, hepatocyte necrosis and ballooning degeneration are graded 0 to 5. No necrosis, congestion/centrilobular ballooning is given a score of 0 whereas severe congestion/ballooning degeneration, as well as > 60% lobular necrosis is given a score of 5 (Table 1).

Transgenic animals, particularly genetic knockout models, have become a powerful tool at elucidating mechanisms of liver ischaemia reperfusion injury and are complementary to pharmacological studies^[2-9]. The mechanistic insights derived from transgenic knockout models of liver ischaemia reperfusion injury will be reviewed. Knockout models provide a very specific targeted disruption of a particular protein at the genetic level which is more informative than the use of “specific” pharmacological inhibitors or stimulants of the same protein are used.

REACTIVE OXYGEN SPECIES

Depletion of intracellular and extracellular ATP during ischaemia results in increased ATP degradation products, including adenosine, hypoxanthine and xanthine and a shift towards anaerobic metabolism. On reperfusion, initially the increase in oxygen delivery exceeds the rate at which cellular metabolism returns to aerobic pathways, which generates damaging free radicals. A wide variety of ROS are generated, the most widely implicated being superoxide, hydrogen peroxide and reactive nitrogen species, such as peroxynitrite.

There are thought to be three main pathways for the generation of ROS: conversion of xanthine dehydrogenase to xanthine oxidase during ischaemia, nicotinamide adenine dinucleotide phosphate (NADPH) oxidase activation and uncoupling of the mitochondrial electron transport chain^[10,11]. Although hepatocytes can directly produce ROS, physiologically Kupffer cells are thought to be the main source of ROS in the early stages of liver IRI with natural killer T-cells (NKT) cells generating ROS

Table 1 Suzuki classification of liver ischaemia reperfusion injury

| Numerical assessment | Sinusoidal congestion | Vacuolisation/ballooning | Necrosis |
|----------------------|-----------------------|--------------------------|-------------|
| 0 | None | None | None |
| 1 | Minimal | Minimal | Single cell |
| 2 | Mild | Mild | < 30% |
| 3 | Moderate | Moderate | 30%-60% |
| 4 | Severe | Severe | > 60% |

later and neutrophils being the main source in the very later stages^[10,11]. The role of these various cells, NADPH oxidase and mitochondrial depolarisation have been supported by knockout animal models^[12-15]. There are no xanthine oxidase knockout on liver IRI. These mice only survive up to 6 wk and are runted.

MICROCIRCULATORY DYSFUNCTION

Microcirculatory changes play an important part in hepatic IRI. Reduction in sinusoidal diameter and blood flow are among the earliest changes in reperfusion injury. This results from a combination of direct damage to sinusoidal endothelial cells (SECs), vasoconstriction and expression of adhesion molecules with accumulation of platelets and leucocytes.

Two of the key vasoactive substances that maintain sinusoidal vascular tone are endothelin-1 (ET-1), a vasoconstrictor, and nitric oxide (NO), a vasodilator and inhibitor of platelet aggregation. There appears to be a relative excess of ET-1 in the early stages of liver IRI.

Liver transplantation in pigs has provided evidence that after reperfusion Kupffer cell activation leads to increased release of ET-1 which binds to SEC and hepatocyte endothelin A (ETA) receptor, thereby reducing hepatic micro and macro-perfusion resulting in increased liver injury^[16,17]. The activation of this pathway is associated with increased expression of tumor necrosis factor- α (TNF- α), interleukin-6 (IL-6) and endothelial NOS (eNOS)^[16,17]. Knockout models for ETA receptor or heterozygote knockout for endothelin-1 have not been studied in liver ischaemia reperfusion injury. Double knockouts of ET-1, ET-2 and ETA receptor are lethal pre- or perinatally. It has become apparent products of heme oxygenase, namely carbon monoxide (CO) and biliverdin, and NO from nitric oxide synthase, which are all vasodilators, are likely play a role in reducing the severity of liver IRI *in vivo*.

CELL INJURY AND DEATH

Hepatocytes and SECs are the two main cell types that are injured in IRI. Hepatocytes are more sensitive to warm ischaemic injury (37 °C), while SECs are more sensitive to cold ischaemia (4 °C) found in cold preservation of donor liver grafts before transplantation. Physiologically, exclusive injury of one cell type is not found and

there is evidence that both cell types have been injured directly in both cold and warm IRI.

There has been debate about what the primary mode of cell death is in liver IRI: apoptosis or necrosis. Apoptosis is an energy dependent process, so in theory when there is greater depletion of ATP, necrosis should dominate. Also, necrosis takes longer to become apparent, normally more than 3 h. This is challenging to show experimentally *in vivo*, as tissue ATP before and after reperfusion would need to be measured as well as the change in metabolic state of the cell. Many of the same initiators and pathways are involved for both types of cell death, so there is much overlap. Some authors refer to the process as neuroapoptosis.

Different assays have been used to implicate apoptosis, including activation of various genes such as caspase-3 which is thought to be a specific indicator of apoptosis, and Bax. One isolated *ex vivo* perfused liver model using knockout of Bax showed reduced liver IRI (Table 2), apoptosis and caspase-3 activation in the knockouts compared to the normal wild type livers^[18]. The TUNEL assay has been used to indicate apoptosis, but it now appears that it does not specifically distinguish between apoptosis and necrosis. Varying degrees of necrosis and apoptosis have been shown in the literature for different ischaemia reperfusion protocols, but these conclusions on the different levels of necrosis versus apoptosis need to be interpreted with caution as the assays for apoptosis are relatively nonspecific.

ADHESION MOLECULES

The adhesion to the hepatic sinusoidal endothelial cells and transmigration into liver tissue require sequential steps in which many molecules are involved. The selectin family (P-, E- and L-selectin) of adhesion molecules are expressed by SECs early in reperfusion. They mediate loose or rolling adhesion of platelets and leucocytes. Knockout models indicate that there is an initial peak of P-selectin expression 20 to 30 min after reperfusion which is required for early IRI^[19,20]. Functionally, some groups have found that E-selectin expression, and not P-selectin, is required for IRI to occur^[21]. This is followed by firmer adhesion of leucocytes on SECs by upregulation of integrins, such as anti-CD11a and anti-CD11b, vascular cell adhesion molecule 1 (VCAM-1), and intercellular adhesion molecules (ICAM-1), respectively (Table 2).

PLATELETS

Platelets and leucocytes begin to adhere to SECs within 5 min of reperfusion (Table 2). Khandoga *et al.*^[22] used an ICAM-1 knockout model of early liver IRI and showed reduced IRI in the knockouts. Although ICAM-1 deficiency attenuated postischemic adherence of both platelets and leucocytes, the application of an anti fibrinogen antibody selectively reduced the number of adherent

platelets but did not influence leucocyte adhesion, which significantly reduced liver IRI. The study concluded that the very early phase of IRI is characterised by increased lipid peroxidation, apoptosis and reduced sinusoidal perfusion, depends on platelet rather than leucocyte adhesion on SECs and that this is mediated by fibrinogen deposited on the adhesion molecules: E-selectin, VCAM-1 and ICAM-1.

NEUTROPHILS

Neutrophils are important cellular mediators of liver IRI after 6 h of reperfusion as demonstrated using partial hepatic ischemia reperfusion (IR) models with histology and MPO assay of liver samples (myeloperoxidase, an enzyme expressed most abundantly in neutrophils) as endpoints^[23,24]. The neutrophil oxidative burst is the main source of reactive oxygen species in the later stages of IRI and contributes directly to hepatocellular injury. This has been supported by immunologically deficient knockout models of liver IRI using nude (nu/nu) mice which lack a thymus so cannot generate mature T lymphocytes and a knockout for gp91 phox, the glycosylated subunit of the heterodimer phagocyte NADPH oxidase(-/-). The knockouts have shown reduced liver IRI, reduced neutrophil infiltration and reduced oxidative burst (Table 2)^[13,25].

Leucocyte transmigration across endothelial and extracellular matrix (ECM) barriers is a complex process. Leucocyte migration across ECM proteins is dependent on matrix degradation, not only by increasing matrix permeability, but also for generating ECM-derived fragments, which are highly chemotactic for leucocytes. Matrix metalloproteinase (MMP)-9 is one of two major gelatinases in the MMP family responsible for the turnover and degradation of several ECM proteins, including fibronectin, a key ECM protein expressed by SECs in the early phase of IRI.

An MMP-9-/- knockout model of liver IRI showed reduced liver damage compared to normal mice and that neutrophil transmigration within liver sinusoids occurs over fibronectin in an MMP-9 dependent manner^[26]. These conclusions were based on correlations between assays of MMP-9 activity and liver histology from *in vivo* experiments and *in vitro* but not *in vivo* studies of neutrophil transmigration induced by fibronectin. The limitations of this study are that it did not assess other ECM proteins and that the conclusions are based on *in vitro* studies which may not reflect the *in vivo* mechanism of neutrophil transmigration. For instance, the role of SECs in leucocyte migration was not considered in this knockout model of IRI. SEC activation and injury has an important role in liver IRI as discussed earlier, by contributing to microcirculatory dysfunction. Neutrophil recruitment is mediated, at least in part, by macrophage inflammatory protein-2 (MIP-2) binding to the chemokine receptor (CXCR2) on neutrophils, supported by a study using a CXCR2 knockout model which showed reduced

Table 2 Summary of knockout models of liver ischemia reperfusion injury pertaining to reactive oxygen species, cellular metabolism/adenosine and cells involved in the injurious mechanisms

| Ref. | Knockout model | IR protocol | Outcome measure | Agent | Adaptive responses | Injurious responses |
|--|--|--|--|---|---|---|
| Kuboki <i>et al</i> ^[24] | OTII; TCRd deficient | 70% I 90 min/R 4, 8 h | Histology; serum ALT; MPO | AntiCD1d Ab; anti NK1.1 Ab; anti CD25+ Ab | | Antigen dependent CD4+ T cell activation <i>via</i> TCR and NKT cell activation increase IRI; GD T cell recruit PMN but not affect IRI |
| Evans <i>et al</i> ^[2] | ob/ob or double knockout of leptin and UCP2 | Total hepatic ischaemia 15 min/R 1, 24 h | Histology (Neil and Hubscher scoring); serum ALT; WB; liver ATP assay; lipid peroxidation; 24 h survival | | | In steatotic livers of ob/ob mice only, UCP-2 depletes liver ATP which increases IRI 1 h onwards |
| Hanschen <i>et al</i> ^[12] | IL6 (-/-); CD4 (-/-); TNFR1 (-/-) | Left lobe I 90 min/R 30 min, 2, 3, 4 h | Kupffer cell activity (fluorescent latex beads and intravital microscopy, IVM); IH; serum AST and ALT | GdCl ₃ or glutathione to wild types (WT) only | | Kupffer cells activation, ROS, IL6 and TNF- α increase SEC VAP-1 expression and CD4+ T cell sinusoidal recruitment which increase IRI; CD4+ T cells inhibit Kupffer cell phagocytic activity |
| Kim <i>et al</i> | Adenosine A1 receptor (A1AR) (-/-) | 70% I 1 h/R 24 h | Histology; serum ALT; IH; semiquantitative PCR; WB; TUNEL | CCPA (A1AR agonist); DPCPX (A1AR antagonist) | Endogenous adenosine <i>via</i> A1AR reduces IRI | Exogenous adenosine increase IRI most likely <i>via</i> a different adenosine receptor subtype to A1AR |
| Ben-Ari <i>et al</i> ^[18] | Bax (-/-); Bax (+/-) | Isolated liver perfused in environmental chamber: Global I 90 min/R 1 or 15 min | Histology (apoptosis features); serum ALT, AST, LDH; TUNEL and caspase-3 assay; WB | | | Bax activation after 15 min reperfusion activates caspase-3 which increases liver apoptosis |
| Lapps <i>et al</i> ^[30] | Rag1 (-/-), i.e., lack mature lymphocytes A2AR (-/-); IFN γ (-/-) | 70% I 72 min/R 2, 24 h | Histology; serum ALT; intracellular IFN γ | <i>ip</i> ATL146 (A2AR agonist); PK136 (NK1.1 depletion); CD1d Ab (inhibit NKT cell); NKT cell adoptive transfer from WT, A2AR and IFN γ KO to Rag1 KO | Exogenous and endogenous adenosine acts through AZAR to reduce NKT cell recruitment | NKT cell recruitment increases IRI through release of IFN γ from at least 2 h reperfusion onwards and increased neutrophil recruitment from at least 24 h after reperfusion |
| Shimamura <i>et al</i> ^[25] | Cd1d (-/-); nu/nu (no NKT cell, normal NK cells); perforin (-/-); gld/gld (Fas ligand deficient) | Total hepatic ischaemia 30 min/R 2, 6, 12, 24, 48 h | Serum ALT; peroxide assay; cytotoxic assay; IH; ELISA | Anti-NK and anti-NKT Ab | | NKT cell activation 1 to 24 h after reperfusion releases IFN γ and PMN activation 6 to 12 h after reperfusion with increased oxidative burst lead to increased apoptosis and necrosis in IRI |
| Caldwell <i>et al</i> ^[26] | CD4 (-/-); B cell (-/-) | 70% I 90 min/R 1, 2, 4, 8 h | Histology; serum ALT; MPO | Adoptive transfer CD4+ T cell to CD4(-/-); anti-IL17 Ab | CD4+ T cell only 1-4 h after reperfusion secrete IL17 releasing MIP-2 increasing neutrophil infiltration, but inhibiting their oxidative burst, and reducing necrosis 8 h reperfusion onwards | |
| Baskin-Bey <i>et al</i> | Cathepsin B (-/-) | Two weeks fed methionine choline deficient (MCD) diet to induce steatosis; liver stored 24 h 4 °C UWS then perfused in isolated apparatus at 37 °C for 1 h | Histology; electron microscopy (EM); TUNEL; IH; liver tissue ALT and LDH | R-3032 <i>ip</i> 2 h preop (cathepsin B inhibitor) | | Reduced lysosomal integrity more pronounced in steatotic livers with increased cathepsin B release into cytosol associated with increased apoptosis and necrosis |

| | | | | | |
|--|---|--|--|---|---|
| Khandoga <i>et al.</i> ^[21] | ICAM (-/-) | Left lobe I 90 min/R 20 min | Serum AST and ALT; IH; caspase-3 assay; lipid peroxidation assay; IVM | Anti-fibronectin Ab | Platelets bind fibronectin deposited on ICAM-1 expressed on SECs, associated with reduced sinusoidal perfusion, increased lipid peroxidation and apoptosis |
| Shen <i>et al.</i> ^[33] | nu/nu; CD154 (-/-) | 70% I 90 min/R 4 h | Serum ALT; histology; MPO; WB | Anti-CD154 Ab to WT; adoptive transfer spleen lymphocytes into KO or Ab treated group | CD4-CD154 T cell costimulation is associated with increased IRI |
| Wyllie <i>et al.</i> ^[69] | Natural resistance associated macrophage protein 1 (Nrampl) (-/-) | 70% I 45 min/R 30, 60 min | Plasma GOT and TNF- α ; histology; WB; Northern Blot; IH; EMSA (NF κ B) | | Macrophage activation after reperfusion increases TNF- α release and NF κ B activity which increases IRI |
| Young <i>et al.</i> ^[21] | P-selectin/ ICAM-1 double KO | 70% I 90 min/R 1.5, 3, 6 h | Serum ALT; histology | | |
| Ozaki <i>et al.</i> ^[13] | gp91 phox component of phagocyte NADPH oxidase (-/-) | 70% I 60 min/R 5, 8, 24 h +/- <i>in vivo</i> injection 3 d preop of adenovirus | Serum GOT; histology (HE); ELISA for DNA histone fragments; TUNEL; IH; WB; assays for lipid peroxidation, hydrogen peroxide and superoxide; EMSA (NF κ B) | Replication deficient adenovirus encoding Rac1 (control: Ad β gal) | P-selectin and ICAM-1 do affect the severity of IRI up to 6 h reperfusion in this model, although PMN infiltration is slightly increased in midzonal area Liver tissue releases ROS within 5 min of reperfusion and PMN from 8 h onwards, associated with increased lipid peroxidation, apoptosis and necrosis. NF κ B DNA binding is associated with increased IRI; NADPH oxidase regulated by Rac1 small GTP binding protein is a source of ROS in IRI |
| Sawaya <i>et al.</i> ^[90] | P-selectin (-/-) | Left lobe I 30 min/R 15, 30, 60, 120 min | Serum AST, ALT, LDH; histology; IVM in terminal hepatic venule (THV) | Radiolabelled anti P-selectin Ab | P selectin expression on SECs increases rolling, saltating and adherent leucocytes in THV peaking at 30 min reperfusion |
| Singh <i>et al.</i> ^[20] | P-selectin (-/-) | Left lobe I 30 min/R 20 min, 2, 5, 12, 24 h | Serum AST, ALT, LDH; histology; WB | Radiolabelled anti P-selectin Ab | P-selectin expression peaks at 20 min and 5 h after reperfusion and is associated with worse IRI |

KO: Transgenic knockout; I: Ischemia; R: Reperfusion; IR: Ischemia reperfusion injury; ROS: Reactive oxygen species; ATP: Adenosine triphosphate; IH: Immunohistochemistry; HE: Hematoxylin and eosin; WB: Western blotting; MPO: Myeloperoxidase assay; PCR: Polymerase chain reaction; ELISA: Enzyme labelled immunosorbent assay; EMSA: Electrophoretic mobility shift assay; AST: Aspartate transaminase; ALT: Alanine transaminase; LDH: Lactate dehydrogenase; GOT: Glutamic oxaloacetic transaminase; NADPH: Nicotinamide adenine dinucleotide phosphate; IR: Ischemia reperfusion; IVM: Intravital microscopy; A2AR: Adenosine (subtype 2A) receptor; PMN: Polymorphonuclear cell; NKT: Natural killer T cell; IFN: Interferon; Ab: Antibody; TNF: Tumour necrosis factor; TNFRI: Tumour necrosis factor receptor (subtype 1); TCR: T cell receptor; TUNEL: Terminal deoxynucleotidyl transferase dUTP nick end labeling (assay for cell death); IL: Interleukin; ICAM: Intercellular adhesion molecule; VAP: Vascular adhesion protein.

neutrophil transmigration and hepatocellular injury in the knockout animals^[27]. These responses are coordinated by a complex mixture of substances including cytokines, chemokines and adhesion molecules produced by other leucocytes and various liver cell types. These will be discussed further.

T CELL RESPONSES IN LIVER WARM IRI

CD4+ T cells are activated and recruited into liver sinusoids in liver IRI (Figure 1). They have a dual role either contributing to injury or reducing the extent of injury depending on the CD4+ subtype and mechanism of cellular activation. The majority of CD4+ T cells can be subdivided into $\alpha\beta$ TCR (the most common subtype) expressing cells, $\gamma\delta$ TCR expressing cells, NKT cells and regulatory T cells (Tregs). B cells, CD8+ T cells^[28] and NK cells^[25,29] do not have an important role in modulating IRI.

NKT cells contribute to liver injury in the early stages from 1 h of reperfusion onwards. This has been supported by immunologically deficient knockout models, such as nu/nu, CD1d-/- (a non classical MHC that presents glycolipid and phospholipid to NKT cell TCR activating NKT cells) and RAG-1/- (recombination activation gene-1 required for the maturation of lymphocytes) knockout mice with up to 50% reduction in liver injury in the knockouts^[25,29,30]. NKT cells are also thought to contribute to neutrophil activation mediated by cytokines which NKT cells release, such as interferon gamma^[30]. A study using T cell subtype specific knockouts showed Treg cells are not in-

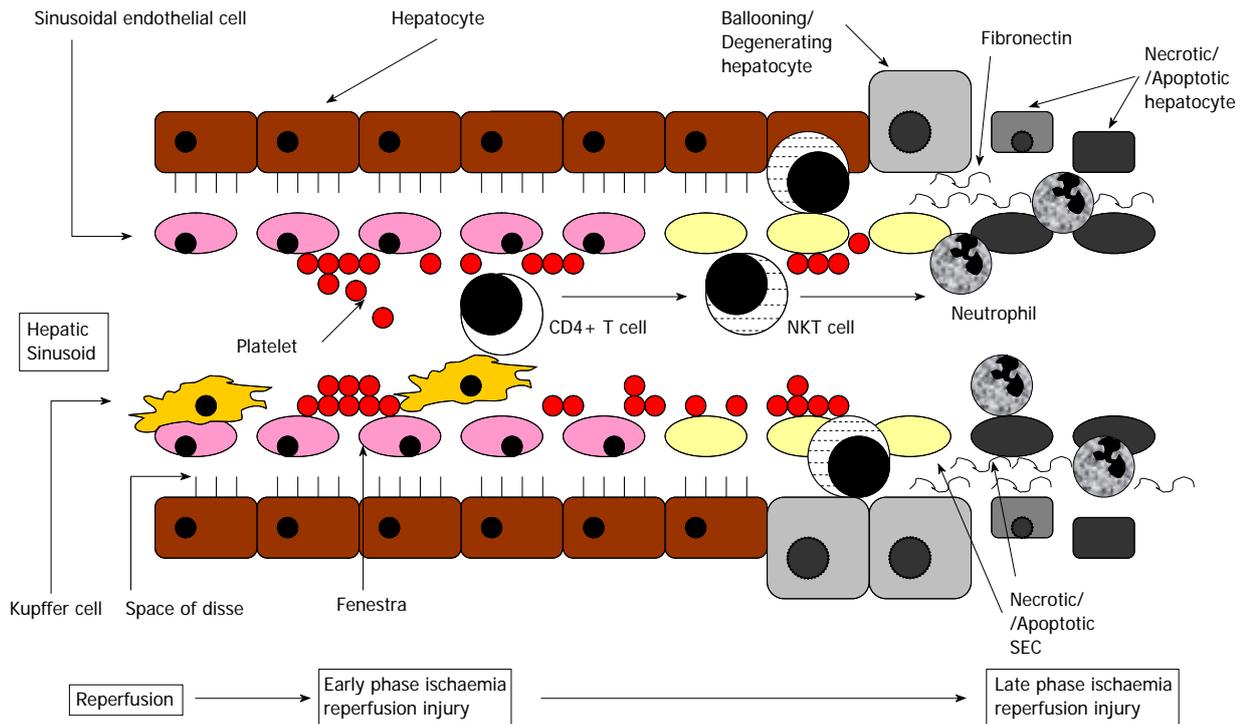


Figure 1 Schematic diagram of cellular mechanisms of liver ischaemia reperfusion injury within a liver sinusoid and the surrounding area containing hepatocytes. Initial sinusoidal perfusion failure from platelet plugging, then Kupffer cells activate CD4+ T cells that activate natural killer T (NKT) cell which cause sinusoidal endothelial cells (SEC) and hepatocyte injury, followed by neutrophil activation, adhesion and transmigration causing more cell injury.

involved in IRI. $\gamma\delta$ TCR T cells recruit neutrophils but this does not affect the severity of IRI^[29].

The CD4+ T cell activation in IRI is by an antigen independent pathway^[31]. This is supported by knockout models of the Toll-like receptor 4 (TLR 4) in which TLR 4 knockouts show reduced IRI and in normal animals the TLR 4 is activated on Kupffer cells resulting in IRI^[32].

One study on CD4+ T cell related liver IRI suggested co-stimulatory activation of the CD4 with the CD154 receptor on activated T cells^[33]. There is emerging evidence that there are also antigen dependent pathways activated in liver IRI. One partial hepatic IR (ischaemia reperfusion) model with 90 min ischaemia and 8 h reperfusion using a knockout for a TCR specific for ovalbumin self antigen showed reduced IRI in the knockout, with serum ALT reduced by 15% and reduced histological injury although this was not quantified, indicating that a small subset of T cells sensitised to self antigen contribute directly to liver IRI at least up to 8 h into reperfusion^[29].

CD4+ T cells of the $\alpha\beta$ TCR variety are recruited into liver sinusoids within 1 h of reperfusion. CD4+ T cell knockout models of liver IRI with adoptive transfer of functional CD4+ T cells into the knockout mice indicate that these cells are involved in neutrophil recruitment *via* cytokines such as interleukin 17 (IL 17) and MIP-2, these T cells inhibit the neutrophil oxidative burst. In a model of partial hepatic ischaemia for 90 min followed by reperfusion, CD4+ knockouts showed greater IRI than normal mice, with serum ALT approximately 25% higher in the knockouts and more severe histological injury in the knockouts although this was not numerically

quantified^[28]. They reduce the extent of liver IRI both indirectly *via* cytokines they release affecting other leucocytes and directly acting on hepatocytes^[28].

CYTOKINES AND CHEMOKINES

The complex interplay between cytokines and chemokines in liver IRI is not fully understood. The most extensively studied cytokines are TNF- α , interferon (IFN)- β , IFN- γ and IL-6 (Figure 2).

TNF- α is raised in serum within 30 min of reperfusion and persists for up to 8 h^[34,35]. TNF- α has ischaemic but not normal liver tissue^[36]. Release of TNF- α is stimulated by a cytokine cascade involving activation of interferon regulatory factor-1 (IRF-1), as shown using a double knockout of this factor in a partial hepatic IR (ischaemia reperfusion) model with 60 min ischaemia and 6 h reperfusion, where hepatocellular injury was 60% less in the IRF-/- knockout^[37].

There is some evidence from knockout studies that antigen independent macrophage/Kupffer cell TLR-4 activation stimulates TNF- α secretion^[32,38]. The effects of TNF- α are mediated by binding to its receptor Tumour necrosis factor receptor subtype 1 (TNFR1) leading to increased apoptosis^[39,40] and increased CD4+ T cell sinusoidal recruitment within 30 min of reperfusion^[12]. One TNFR1-/- knockout model of mouse liver transplantation with liver transplantation of either normal or TNFR1-/- livers into normal or TNFR-/- mice showed the deleterious effects of TNF- α are mediated by TNFR outside the liver, most likely infiltrating leucocytes, but

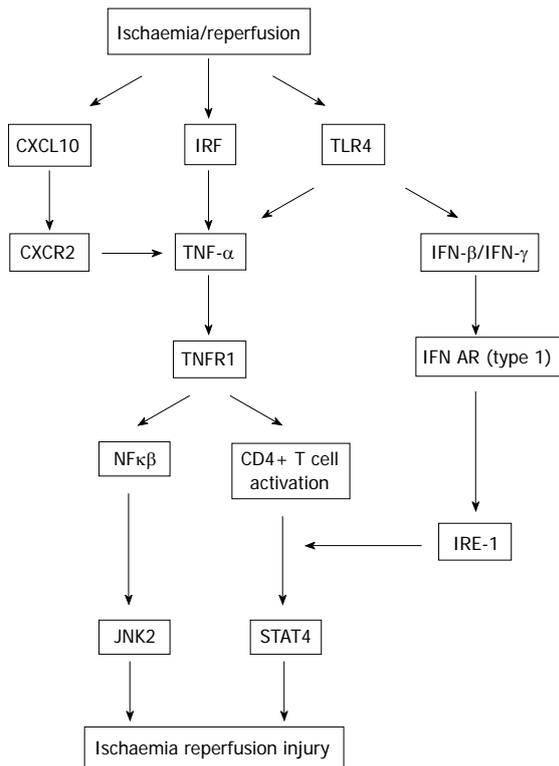


Figure 2 Cytokine and downstream signalling pathways in liver ischaemia reperfusion injury. Following liver ischaemia reperfusion, there is activation of tumor necrosis factor- α (TNF- α) by chemokine (CXCL) 10, interferon regulatory factor (IRF) and toll-like receptor (TLR) 4 in parallel. TNF- α activates downstream hepatocyte/sinusoidal endothelial cells (SEC) nuclear factor κ B (NF κ B) and CD4+ T cells separately which activate c-Jun N-terminal protein kinase-2 (JNK-2) and signal transducer activator of transcription-4 (STAT4), respectively leading to increased cell injury. A parallel pathway of cell injury occurs where TLR4 activation stimulates interferon (IFN)- β and IFN- γ expression, which acting through their receptor IFN receptor subtype (AR) activate interferon regulatory element (IRE)-1 which in turn activate CD4+ T cells. CXCR: Chemokine receptor.

TNFR on liver cells appear to reduce IRI in this model (Table 3).

IFN- β is a cytokine which is involved throughout the reperfusion period in liver IRI, a finding substantiated by work using knockout mice (Table 3). The damaging effects of IFN- β are mediated by binding to the interferon receptor subtype IFN AR (Type 1)^[41] with no hepatocellular liver injury demonstrated in IFN AR-/- knockouts (Figure 2)^[37]. Knockout models support other studies showing that IFN- γ produced by NKT cells contribute to liver IRI from early on in reperfusion^[25,27,30,32,37,42,43]. Activation of innate immune pathways *via* TLR 4 stimulate release of IFN- β and IFN- γ in liver IRI, confirmed by TLR 4 knockout models^[32].

Some cytokines released during liver IRI appear to reduce the severity of injury. The best evidence for this from knockout studies is for IL6^[34]. Camargo *et al*^[34] showed worse IRI in livers of IL6 knockout mice than wild type mice, which was restored to the wild type injury patterns by administration of recombinant IL6 to the knockout mice before ischaemia. There is evidence that IL4 and IL10 may also be protective in IRI, but knock-

outs of these cytokines have not been used in liver IRI models to substantiate this.

Chemokines (CXCL), like cytokines, are a family of locally produced factors, but chemokines are smaller molecules, which act locally forming concentration gradients that guide leucocyte chemotaxis. A CXCL 10 knockout model found that this chemokine contributed to liver IRI (Figure 2 and Table 3) from 1 h of reperfusion onwards with associated activation of neutrophils, Kupffer cells and increased TNF- α and IL 1 β release^[44]. A study using knockouts of chemokine receptor 2 (CXCR 2) showed CXCR 2 activation contributes to liver IRI and neutrophil recruitment^[26].

Plasminogen activator inhibitor type-1 (PAI-1) inhibits plasmin generation by inhibiting activation of plasminogen activators which play a role in diverse proteolysis related processes. One group used a model of liver ischemia reperfusion using wild type normal and PAI-1 knockout mice based on controlled intravenous haemorrhage maintaining MAP 25-30 mmHg for 2.5 h followed by controlled resuscitation intravenously with shed blood/Ringer's lactate maintaining MAP > 80 mmHg for 4 h. They demonstrated liver IRI in wild type animals reflected by raised serum ALT, histological periportal and pericentral injury (zone 2) and electron microscopic SEC injury with loss of fenestra. DNA microarray showed PAI-1 mRNA was most elevated after haemorrhage-resuscitation and immunohistochemistry showed PAI-1 expression was localised to SEC. PAI-1 knockouts had no liver injury following haemorrhage-resuscitation. In normal mice PAI-1 mediated IRI was associated with reduced urokinase plasminogen activator (u-PA) in zymogens of hepatocytes, reduced hepatocyte growth factor (HGF) and increased TGF- β 1, but no differences in IL6 and IL10. These changes with haemorrhage-resuscitation are associated with reduced phosphorylation activation of the ERK-1/-2 MAP kinase pathway which were reversed in PAI-1 knockouts. Based on these results, the study concluded that liver IR activates SEC PAI-1 which inhibits u-PA which reduces levels of active HGF and increases levels of TGF- β 1 that together reduce activation of downstream ERK MAP kinase pathway, mediating liver IRI. The group used a systemic model of liver IRI (systemic hypotension) rather than direct liver ischaemia and reperfusion so PAI-1 may not have the same role in liver IRI resulting from total or partial inflow occlusion. The knockout animals which showed no liver IRI with haemorrhage-resuscitation, were anaesthetised with isoflurane, while wild type animals which did show liver IRI with haemorrhage-resuscitation were anaesthetised with sodium pentobarbital. These results need to be interpreted with caution as isoflurane preconditions against liver IRI, so the protective effect may not be due to lack of PAI-1.

COMPLEMENT SYSTEM

Activation of the complement system has been shown

| Ref. | Knockout model | IR protocol | Outcome measure | Agent | Adaptive responses | Injurious responses |
|---|--|--|--|---|---|---|
| Kuboki <i>et al</i> ^[26] | CXCR2 | 70% I 90 min/R 12, 24, 48, 96 h | Histology; MPO; serum ALT, TNF- α , IL6; WB and NF- κ B activity | | CXCR2 activates STAT3 hepatocyte proliferative pathway | MIP2 activates CXCR2 which increases neutrophil recruitment and IRI. Nuclear factor (NF) κ B activity reduced in IRI |
| Zhai <i>et al</i> ^[44] | IFNAR type1 (-/-); IFNAR type 2 (-/-) | 70% I 90 min/R 6 h | Histology; quantitative PCR | | | IFN β (not IFN γ) mediates IRI by binding to IFNAR type 1 |
| Zhao <i>et al</i> | CXCL10 (-/-) | 70% I 90 min/R 1, 2, 4, 8 h | Histology; serum ALT; IH; quantitative PCR; WB | | | CXCL10 activation increases TNF- α , IL6, IL1b, iNOS, MIP-2 mRNA and PMN and Kupffer cell activation contributes to IRI |
| Fondevilla <i>et al</i> | C6 deficient rats | Donor/recipient: WT/WT, KO/WT, WT/KO, KO/KO; | Serum GOT; histology; MPO; IH; TUNEL; WB; PCR; ELISA | | | Membrane attack complex (C5b-C9) activation in this OLT model of cold/warm IRI increases apoptosis, necrosis, PMN and macrophage infiltration and TNF- α , IFN γ and IFN β expression |
| Shen <i>et al</i> ^[32] | Toll like receptor 4 (TLR4) (-/-) | OLT and organ storage 24 h 4 °C UWS Donor/recipient: WT/WT, KO/WT/WT, KO, KO/KO; OLT with dearterialisation, organ stored 24 h 4 °C UWS | Histology; IH; MPO; quantitative PCR; caspase-3 activity; WB | | TLR4 activation increases IL4 and IL10, but inhibits HO-1 | TLR4 activation increases TNF- α , IL1b, IL2, IFN γ , ICAM1, CXCL10, PMN and CD4+ T cell recruitment leading to increased liver necrosis and apoptosis |
| Conzelmann <i>et al</i> | TNFR (-/-) | Donor/recipient: WT/WT, KO/WT/WT, KO, KO/KO; organ storage 12 h 4 °C UWS; 8 h graft harvest | Histology; serum ALT; MPO; TUNEL and caspase-3 assay; IH | | TNFR within liver mediates reduced IRI | TNFR outside liver increases IRI in terms of necrosis, apoptosis and neutrophil infiltration |
| Tsung <i>et al</i> ^[37] | Interferon regulatory factor-1 (IRF-1) (-/-) | 70% I 60 min/R 1, 3, 6, 12 h | Histology; serum ALT; WB; PCR | Adenovirus IRF-1 vector | Increased IL6 | IFN γ , TNF- α , IL1b all activate IRF-1 which increase JNK (not p38 MAPK) and TNF- α and iNOS expression in IRI |
| Tian <i>et al</i> ^[40] | TNFR1 (-/-); IL6 (-/-) | Donor/recipient: WT/WT, KO/WT, WT/KO, KO/KO; OLT: 50% or small for size 30% arterialised graft | Histology; serum AST; portal flow measurement; IVM; IH; PCR; 30 d mortality | GdCl3 (ip to donor); pentoxifylline (to donor and recipient sc); recombinant IL6 to KO only | | Increased activation of Kupffer cells and TNF- α mediated activation of IFNRI from 3 h reperfusion onwards increases liver necrosis, nonperfused sinusoids, adherent leucocytes and reduces hepatocyte regeneration |
| Shen <i>et al</i> ^[38] | TLR4 (-/-); TLR2 (-/-) | 70% I 90 min/R 6 h | Histology; serum ALT; MPO; WB; PCR | Snp (inhibit HO-1); CoPP | HO-1 is expressed which inhibits TLR4 | TLR4 activation increases TNF- α expression associated with increased IRI |
| Lagoa <i>et al</i> ^[81] | PAL-1 (-/-) | MAP 25-30 mmHg for 2.5 h (2.25 mL/100 g blood withdrawn)/ Resuscitation MAP > 80 mmHg for 4 h (30 min with shed blood and crystalloid) | Serum ALT, IL6, IL10; histology; Electron microscopy; IH; zymography for plasminogen activators; DNA microarray; PCR; WB | PAL-1 to PAL-1 (-/-) mice | | PAL-1 expression in SEC contributes to IRI with periportal/pericentral injury, loss of sinusoidal fenestra and prominent SEC injury; PAL-1 inhibits u-PA which reduces formation of active HGF and increases active TGF- β 1, but no effect on IL6 or IL10; this is associated with reduced activation of ERK-1/-2 pathway. |
| Teoh <i>et al</i> ^[36] | TNF- α (-/-) | 70% I 90 min/R 2, 4, 24 h | Serum ALT; IH; serum TNF- α ; EMSA (NF- κ B); WB | Low dose or high dose TNF- α ip | | TNF- α from at least 2 h reperfusion onwards is injurious to ischaemic but not normal liver, increasing NF- κ B DNA binding |
| Inderbitzin <i>et al</i> ^[7] | CI inhibitor overexpressed | Total hepatic ischaemia 30 min/R 2 h | Endothelial permeability index (measured using radiolabelled albumin iv into inferior vena cava) of liver, lung and gut | | CI inhibitor overexpression is protective in IRI | Classical complement pathway is activated in IRI; liver ischaemia and reperfusion causes liver and gut, but not lung, IRI in this model |

| | | | | |
|--------------------------------------|-----------------------------------|--|--|--|
| Zhai <i>et al</i> | TLR4 (-/-); TLR2 (-/-) | 70% I 90 min/R 6 h | Serum ALT; histology; PCR | TLR4 activation increases expression of IRF3 which upregulates IFN β associated with increased IRI |
| Rudiger <i>et al</i> ^[69] | TNFR (-/-); Fas (-/-); FasL (-/-) | 70% I 75 min/R 3 h | Serum AST; TUNEL; caspase-3 assay; ELISA; WB | TNF- α binds to TNFR1 which increases apoptosis in IRI; Fas and FasL not involved in this model |
| Kato <i>et al</i> ^[62] | IL1R (-/-) | 70% I 90 min/R 1, 2, 4, 8, 16, 24 h | Serum ALT, IL1 β , TNF- α and MIP-2; histology (PMN score); MPO; EMSA (NF- κ B); PCR | IL1R not involved in IRI |
| Calmargo <i>et al</i> | IL6 (-/-) | Median lobe (45%) I 90 min/R 30, 60, 90, 120 min | Serum AST and ALT; histology; PCR | TNF- α expression during reperfusion is associated with protective IL6 released in IRI is worse IRI |

KO: Transgenic knockout; WT: Wild type (normal animals); IF: Immunohistochemistry; WB: Western blotting; MPO: Myeloperoxidase assay; PCR: Polymerase chain reaction; ELISA: Enzyme labelled immunosorbent assay; EMSA: Electrophoretic mobility shift assay; AST: Aspartate transaminase; ALT: Alanine transaminase; GOT: Glutamic oxaloacetic transaminase; I: Ischemia; R: Reperfusion; IR: Ischemia reperfusion injury; IVM: Intravital microscopy; IFN: Interferon; Ab: Antibody; TNF: Tumour necrosis factor; TNFR1: Tumour necrosis factor receptor (subtype 1); TUNEL: Terminal deoxynucleotidyl transferase dUTP nick end labeling (assay for cell death); IL: Interleukin; CXCR: Chemokine receptor; PAI: Plasminogen activator inhibitor; NF: Nuclear factor; C1-9: Complement protein 1 to 9; MAP: Mean arterial pressure; MIP: Major intrinsic protein.

to occur in local and remote IRI in many organs^[45]. This system consists of around 30 soluble and membrane bound proteins which are activated by one of three pathways: the antibody dependent classical pathway, the alternate pathway and the mannose binding lectin pathway. Activated complement acts both directly through the formation and deposition of membrane attack complexes (C5b-C9) and indirectly following activation by cytokines and chemokines in IRI^[46]. Knockout models have been used to consolidate the results of other studies showing activation of complement by all three pathways having direct and indirect effects on cellular reperfusion injury. The best examples of these complement knockout studies of IRI are in the gut^[47-51], kidneys^[52,53] and heart^[54]. Studies on the liver have looked at nonspecific blockade of all parts or the final common pathways of the complement system, so based on current evidence there is little understanding of the relative importance of the different pathways of complement activation in liver IRI.

Inhibition of complement formation before hepatic ischaemia in studies of liver IRI have shown a reduction in the severity of injury within an 1 h of reperfusion when cobra venom factor (to inhibit all parts of the complement system)^[11] was used, but liver IRI and polymorphonuclear cell accumulation were also reduced in the late phase of IRI 24 h following ischaemia when animals were pretreated with sCRI (a complement inhibitor derived from the family of complement regulatory glycoproteins and inhibits activation of C3, which is common to all pathways of complement activation, and so blocks the generation of both C3a and C5a and the MAC)^[11]. Scozaec *et al*^[11] found local complement activation in human liver allografts correlated to cell injury. One pharmacological *in vivo* and *in vitro* study of a warm liver IRI model in a rat showed that complement is involved in the induction of Kupffer cell-induced oxidant stress, the priming of Kupffer cells and neutrophils for enhanced reactive oxygen generation, and the continuous accumulation of neutrophils in the liver during reperfusion. In a pig whole liver to canine left liver xenotransplantation model where a control group was compared against a gadolinium chloride (GdCl₃) (which depletes Kupffer cells) and cobra venom treated group, there was less liver injury following transplantation in those with complement inhibition using the cobra venom. This provided a large animal model supporting the role of complement in activation of Kupffer cells in liver ischaemia reperfusion injury. The groups were small (3 animals each) and the conclusions would be more robust if there had been groups treated with only GdCl₃ or cobra venom^[55].

One group used wild type rats and rats deficient in C6 in all combinations of donor and recipient in a liver transplantation model to show that there was less injury in recipients of C6-/- grafts, implicating that the membrane attack complex (C5b-9) is involved in cold ischaemia related liver IRI in this model^[56]. One model of liver IRI using mice overexpressing the inhibitor of the classical complement pathway, C1 inhibitor, showed C1 inhibitor reduces liver IRI and remote IRI in the lungs and gut followed liver ischaemia and reperfusion^[57]. This would appear to indicate that the classical pathway of complement activation is the most important complement activation pathway involved in liver IRI (Table 3), although further studies using C1 inhibitor knockouts and mannose binding lectin knockouts to substantiate the relative contribution of the various activation pathways, as well as specific knockouts of other complement components to assess the significance of different complement mediated injurious pathways.

MATRIX METALLOPROTEINASE-9

MMP-9 is a zinc dependent secreted gelatinase which catalyses degradation of type IV collagen and gelatin. MMP-9 knockout models of liver IRI have shown liver IRI is reduced by up to 80% in MMP-9^{-/-} knockouts and that in normal animals increased expression of MMP-9 on macrophages and neutrophils occurs during reperfusion (Table 4) which increases neutrophil transmigration over fibronectin in liver sinusoids and increases TNF- α and interferon γ secretion and CD4⁺ T cell activation by mechanisms that remain to be elucidated^[12,27]. The mechanisms by which MMP-9 expression is increased in liver IR were not investigated in these knockout studies, but a possible pathway involves induction of MMP-9 by ROS and TNF- α .

NITRIC OXIDE SYNTHASE

NOS catalyses formation of NO from L-arginine. NO is a versatile molecule which is vasoactive, is involved in activating molecular signalling pathways in cell survival, has immunological effects as well as directly injurious effects in high levels as a free radical itself. There are three isoforms of NOS: constitutive calcium (Ca²⁺) dependent forms which are eNOS and neuronal NOS (nNOS), and an inducible calcium independent form, namely inducible NOS (iNOS). Only eNOS and iNOS are expressed in liver. Most studies agree that eNOS is upregulated in liver IRI and this reduces the severity of IRI. This is confirmed by knockout models of IRI (Table 4), where eNOS expression is related to reduced liver necrosis, apoptosis, leucocyte infiltration and increasing liver sinusoidal diameter and liver blood flow^[23,24,58,59].

The role of iNOS is more controversial (Table 4)^[58-64]. Some knockout models of iNOS show no role for iNOS in liver IRI^[24,60,61]. One knockout study of iNOS showed in the liver IRI model used that iNOS was protective which was at least partly mediated by activation of iNOS by eNOS^[23]. Yet another set of knockout studies conclude iNOS contributes to IRI^[27,37,58]. Hamada *et al.*^[27] used separate iNOS and MMP-9 knockout mice to show in their model of liver IRI that iNOS is upregulated in macrophages which increases IFN- γ release and NO which increases MMP-9 expression on the macrophages and neutrophils. This signalling cascade contributes to increased liver IRI. However, one study using the partial (70%) hepatic IR model with 45 min lobar ischaemia found increased IR injury in iNOS^{-/-} mice demonstrated by increased hepatocellular and histological injury and increased liver sinusoid neutrophil infiltration compared to wild type animals, but they found no iNOS mRNA expression in wild type livers^[24]. This led the authors to conclude that there may be genetic compensation in iNOS^{-/-} animals, although there was no reference to which genes may have been involved in this compensation. Genetic compensation is a rare phenomenon where after a gene is mutated and its function is lost, compensatory genes are upregulated, although the mechanisms for

this is unclear^[65].

Large animal studies using pigs and various inhibitors of iNOS (AG, ONO-1714) on both a warm liver ischaemia reperfusion model and an orthotopic liver transplantation model have shown that iNOS expression is stimulated by IR in Kupffer cells and neutrophils in the centrilobular region resulting in higher levels of serum nitrite/nitrate, reduced capillary perfusion with more thrombi and ultimately increased liver injury and increased mortality^[62-64].

The conflicting results of the role of iNOS in early phase liver IRI may reflect different roles of iNOS and the regulation of its function in liver IRI depending on the duration of the liver ischaemia. Partial hepatic IR models with prolonged ischaemia of 60 min or longer have found that iNOS does have a role in liver IRI^[27], while models using shorter ischaemia times of 45 min have shown no role of iNOS^[24]. Several studies that have used iNOS^{-/-} knockout animal models of hepatic IR, but there is evidence, that iNOS^{-/-} knockouts show genetic compensation^[24], so conclusions based on models of liver IR using iNOS^{-/-} animals need to be interpreted with caution.

The overall conclusions from the current literature is that eNOS and iNOS are both induced during liver IRI from 1 h reperfusion onwards (for mRNA and 2 h reperfusion onwards for protein), eNOS reduces injury and low levels of iNOS induction are probably protective while high expression of iNOS contributes to increased injury and the overall effect of iNOS physiologically depends on how ischaemia and reperfusion is produced.

HAEM OXYGENASE-1

Haem oxygenase-1 (HO-1 or heat shock protein 32, HSP 32) is the inducible isoform of HO-1, the constitutive isoform being HO-2. This enzyme catalyses the formation of CO, biliverdin and Fe²⁺ from haem degradation. HO-1 has been implicated as having a protective role in IRI^[66] through CO and biliverdin being vasodilators and reducing apoptosis and necrosis^[67]. HO-1 is typically expressed three or more h after liver reperfusion^[38,68]. The protective effects of HO-1 are supported by knockout models of liver IRI, where knockouts of HO-1 have more severe IRI than normal animals (Table 4)^[38,43]. These knockout models also provide evidence that HO-1 acts at least partly by inhibiting TLR4 (Toll-like receptor 4) activation and the resulting release of TNF- α and IFN- γ ^[38,43,69].

DOWNSTREAM PATHWAYS

A wide range of downstream pathways have been studied in liver IRI. The majority of systems which are activated in ischaemia reperfusion are effective through these pathways. Some of the key mediators TNF- α , IFN- β , IFN- γ and CXCL10 (Figure 2). In particular, the roles of the transcription factors nuclear factor $\kappa\beta$ (NF $\kappa\beta$)^[70], the survival kinases (JNK, MAPK³s, PKC, PI3K/Akt), signal

Table 4 Nitric oxide synthase, HSP/heme oxygenase-1, matrix metalloproteinase knockout models of liver ischemia reperfusion injury

| Ref. | Knockout model | IR protocol | Outcome measure | Agent | Adaptive responses | Injurious responses |
|---|-------------------------|---|--|--|---|---|
| Hamada <i>et al.</i> ^[26] | iNOS (-/-); MMP-9 (-/-) | 70% I 90 min/R 3, 6, 24 h | Histology; serum ALT, NO2-/NO3-; myeloperoxidase activity (MPO); immunohistochemistry; PCR; Western blotting; MMP-9 activity assay; MMP-9 protein levels; neutrophil (PMN) migration assay; TUNEL and caspase-3 activity | ONO-1714 (iNOS inhibitor); NO donor (DETA NONOate) | | Increased macrophage iNOS producing NO increases PMN MMP-9 and PMN transmigration over fibronectin |
| Hamada <i>et al.</i> ^[22] | MMP-9 (-/-) | 70% I 90 min/R 6, 24 h | Histology; serum GPT and GOT; MPO; IH; PCR | Anti MMP-9 <i>in vivo</i> ; MMP-2/9 inhibitor; anti MMP-2 (all to WT only) | | MMP-9 (not MMP-2) increase TNF- α , IFN γ , IL2, IL6 and increase PMN and CD4+ T cell recruitment leading to increased liver necrosis No involvement of HSP70 in IRI; NF κ B activity associated with IRI |
| Kuboki <i>et al.</i> ^[23] | HSP70 (-/-) | 70% I 90 min/R 1, 8 h | Histology; serum AST; TNF- α ; IL6; MIP-2; MPO; WB; EMSA (NF κ B) | Sodium arsenite <i>in vivo</i> to induce HSP70; recombinant HSP70 | eNOS activation reduces necrosis and apoptosis, with associated inhibition of macrophage infiltration, increased sinusoidal diameter and blood flow | |
| Theruvath <i>et al.</i> ^[20] | eNOS (-/-) | Donor (WT/KO) to WT recipient; organ stored 18 h, 4 °C, UWS | Histology; serum ALT; iVM; TUNEL; IH (macrophage infiltration) | | | |
| Tsuchiashi <i>et al.</i> | HO-1 (+/-); HO-1 (-/-) | 70% I 90 min/R 6 h | Histology; serum GOT; MPO; quantitative real time RT-PCR; WB; TUNEL | CoPP (induces HO-1) 24 h preop | HO-1 upregulated which inhibits expression of cytokines TNF- α and IFN γ | TNF- α and IFN γ expression increased overall in IRI associated with increased apoptosis and necrosis |
| Hines <i>et al.</i> ^[21] | eNOS (-/-); iNOS (-/-) | 70% I 45 min/R 1, 3 h | Serum ALT; histology; PCR | | Increased eNOS expression in IRI inhibits TNF- α and IL12 expression; iNOS activates eNOS in this model | No PMN infiltration at 3 h reperfusion |
| Lee <i>et al.</i> ^[28] | eNOS (-/-); iNOS (-/-) | 70% I 1 h/R 1, 3, 6 h | Serum ALT and AST; perfusion studies; PCR | | eNOS activated during IRI is protective | Increased iNOS mRNA expression from 3 h reperfusion onwards regulates reperfusion and is associated with worse IRI |
| Hines <i>et al.</i> ^[60] | iNOS (-/-) | 70% I 45 min/R 1, 3, 6 h | Serum ALT; histology; MPO | L-NIL (iNOS inhibitor) | | Reduced IRI in iNOS (-/-), but no iNOS mRNA or L-NIL effect in WT; may be genetic compensation effect in KO |
| Kawachi <i>et al.</i> ^[24] | eNOS (-/-); iNOS (-/-) | 70% I 45 min/R 5 h | Serum ALT; histology; MPO | | eNOS is activated in IRI and is protective | There is no PMN infiltration up to 5 h reperfusion and iNOS is not activated in IRI in this model |

IH: Immunohistochemistry; WB: Western blotting; MPO: Myeloperoxidase assay; RT-PCR: Reverse transcriptase polymerase chain reaction; ELISA: Enzyme labelled immunosorbent assay; EMSA: Electrophoretic mobility shift assay; AST: Aspartate transaminase; ALT: Alanine transaminase; GOT: Glutamic oxaloacetic transaminase; I: Ischemia; R: Reperfusion; IR: Ischemia reperfusion injury; iVM: Intravital microscopy; IFN: Interferon; Ab: Antibody; TNF: Tumour necrosis factor; TNFR1: Tumour necrosis factor receptor (subtype 1); TUNEL: Terminal deoxynucleotidyl transferase dUTP nick end labeling (assay for cell death); IL: Interleukin; NF: Nuclear factor; MMP: Matrix metalloproteinase; HSP: Heat shock protein; eNOS: Endothelial nitric oxide synthase; iNOS: Inducible nitric oxide synthase; HO-1: Heme oxygenase (subtype 1).

transducer and activator of transcription (STATs)^[71], poly ADP ribose polymerase (PARP), peroxisome proliferator-activated receptor (PPAR) from pharmacological studies have been supported by knockout models of liver IRI (Table 5).

Table 5 Knockouts of downstream mediators and models of liver ischemia reperfusion injury

| Ref. | Knockout model | IR protocol | Outcome measure | Agent | Adaptive responses | Injurious responses |
|---|---|--|---|---|--|--|
| Theruvath <i>et al</i> ^[61,63] | JNK (-/-) | 70% I 1 h/R 4, 8 h | Histology; serum ALT; IVM (dyes to probe mitochondrial function and cell death); survival 14 d | | | JNK2 activation leads to mitochondrial depolarization and increased necrosis only |
| Theruvath <i>et al</i> ^[61,63] | JNK (-/-) | WT or KO donor; 30 h, 4 °C, UWS preservation; WT recipient | Histology 8 h posttransplant; serum ALT, TUNEL and caspase-3 assay; IVM; IH; lipid peroxidation | | NFκB activity reduces necrosis and apoptosis, inhibits TNF-α, JNK and iNOS | JNK2 activation leads to caspase-3 activation, mitochondrial depolarisation and release of cytochrome c, lipid peroxidation which all translate into reduced survival posttransplant |
| Beraza <i>et al</i> ^[72] | Conditional hepatocyte specific NEMO knockout | 70% I 1 h (caudate lobe resected)/R 3, 6 h | TUNEL and caspase-3 assay; WB; IH; Southern blotting; EMSA (NFκB) | | | |
| Okaya <i>et al</i> ^[65] | PPARα (-/-) | 70% I 90 min/R 4, 8 h | Serum ALT; TNF-α; MIP-2; MPO; liver NO; NO ₂ ; WB; EMSA (AP-1, NFκB) | WY14643 <i>in vivo</i> (PPARα agonist) | PPARα protective in IRI | PPARα independent release of TNF-α and MIP-2 and increased NO; NO ₂ associated with IRI |
| Shen <i>et al</i> ^[78] | STAT4 (-/-); STAT6 (-/-); nu/nu | 70% I 90 min/R 6 h | Serum ALT; histology; MPO; WB; PCR | Adoptive transfer of CD4+ T cells from WT or other KO to nu/nu; SnPP <i>in vivo</i> | HO-1 expressed at very low levels after 6 h in this model, but protective in IRI | CD4+ T cell activation involving T cell STAT4 activation, but not STAT6 associated with increased IRI |
| Khandoga <i>et al</i> ^[61] | PARP (-/-) | Left lobe I 90 min/R 30 min | Serum ALT; IVM; IH; PCR | | | PARP activation in IRI upregulates E-selectin, ICAM1 and VCAM1, associated with increased platelet and leucocyte endothelial interaction and reduced sinusoidal perfusion |
| Kato <i>et al</i> ^[62] | P50 NFκB (-/-) | 70% I 90 min/R 1, 8 h | Serum ALT; histology; MPO WB; EMSA (p50 and p65 subunits of NFκB) | | | No effect of p50 subunit deletion, but increased p50/p65 heterodimer in WT and some p65 in KO, so there may be some functional redundancy of NFκB subunits |
| Kato <i>et al</i> ^[63] | STAT4 (-/-) | 70% I 90 min/R 30 min, 1, 2, 4, 8 h | Serum ALT; histology; MPO; WB | Anti IL12 Ab | | IL12 expression associated with IRI. STAT4 not activated in IRI in this model |
| Kato <i>et al</i> ^[70] | STAT6 (-/-) | 70% I 90 min/R 1, 4, 8 h | Serum ALT, TNF-α; MPO; PCR; EMSA (NFκB) | IL4 or IL13 <i>in vivo</i> | | STAT6 is not activated in IRI in this model, although STAT6 activation by <i>in vivo</i> IL4 or IL13 is protective. IRI is associated with increased NFκB DNA binding |

KO: Transgenic knockout; WT: Wild type (normal animals); IH: Immunohistochemistry; WB: Western blotting; MPO: Myeloperoxidase assay; PCR: Polymerase chain reaction; EMSA: Electrophoretic mobility shift assay; ALT: Alanine transaminase; I: Ischemia; R: Reperfusion; IR: Ischemia reperfusion injury; IVM: Intravital microscopy; IFN: Interferon; Ab: Antibody; TNF: Tumour necrosis factor; TNFR1: Tumour necrosis factor receptor (subtype 1); TUNEL: Terminal deoxynucleotidyl transferase dUTP nick end labeling (assay for cell death); IL: Interleukin; NF: Nuclear factor; STAT: Signal Transducer and Activator of Transcription; JNK: A survival kinase; PARP: Poly (ADP-ribose) polymerase.

Most studies show that NFκB DNA binding increases after liver ischemia and contributes to liver IRI^[13,36,67,70,71]. TNF-α increases NFκB activity^[36,70]. One knockout study, in contrast, showed a CXCR2 dependent fall in NFκB activity following liver reperfusion^[60], although a longer period of ischemia and reperfusion was used than other studies. Another group used a conditional NFκB knockout to study liver IRI and showed NFκB activity was protective, reducing necrosis, apoptosis, JNK expression and TNF-α expression. Unlike the other studies, the caudate lobe was resected in their protocol, which alters how IR occurs compared to a model of hepatic IR without resection. The role of NFκB, therefore, is unclear in liver IRI, but it may be that the specific way in which IR is executed modulates NFκB activity and the resulting activation of downstream sig-

nalling pathways in IRI.

Theruvath *et al.*^[14,15] used a JNK 2 knockout to show JNK 2 contributes to IRI in both a mouse liver transplantation model and warm ischaemia reperfusion model of liver IRI by increasing mitochondrial depolarisation and caspase-3 activity leading to liver injury manifested as hepatocyte cell membrane lipid peroxidation, necrosis and apoptosis. A knockout model has provided evidence that JNK activity, but not p38 MAPK contributes to liver IRI after reperfusion (Table 5)^[37].

Knockout models of PI3K and Protein kinase C (PKC) have been used in cardiac IRI, but not in the liver IRI. One group used a porcine liver transplant model using chelerythrine (a PKC inhibitor) and/or ischaemic preconditioning (IPC) of the donor liver before cold storage to show that PKC activity was not affected by IRI alone, although PKC was strongly activated by IPC reducing the severity of IRI^[73-79].

In a rat model of warm partial hepatic ischaemia reperfusion using a caspase-3 inhibitor (Z-Aspmk *in vivo* 2 min before 2 h of ischaemia) it was found that IRI was associated with reduced PI3K/Akt activity and increased hepatocyte apoptosis, with the converse pattern in the caspase-3 inhibitor treated group^[77-79]. In contrast, in another rat model of IRI, hepatocytes isolated from rats which underwent partial warm hepatic ischaemia/reperfusion or sham laparotomy cultured and treated with IL-1 β provided evidence that during IR, IL-1 β binding to IL-1 β receptor-1 increased NF κ B activity and phosphorylated Akt which acted in parallel to increase iNOS expression (mRNA and protein) with a resulting increase in NO release^[77-79].

Activation of the STAT family of transcription factors is mediated by extracellular signalling molecules such as cytokines which bind to membrane receptors which activate intracellular Janus kinases on the cytoplasmic face of the plasma membrane, which in turn activate a STAT protein which is then transported to the nucleus where they bind DNA to affect gene expression (Jak/STAT signalling pathway). STAT6 activation does not appear to be involved in liver IRI based on results from knockout models^[71,72,80]. Kato *et al.*^[72] found in their model of IRI using STAT4 knockouts that STAT4 did not affect the extent of liver injury after 8 h reperfusion. In contrast, Shen *et al.*^[80] showed STAT4 expression was related to IRI after 6 h of reperfusion, although it was specifically its expression within CD4+ T cells that mediated the liver injury (Table 5). This was demonstrated by reduced IRI in mice lacking mature lymphocytes (nu/nu mice), which was restored to normal animal IRI severity in the nu/nu mice by adoptive transfer of CD4+ T cells from spleens of normal mice but not from spleens of STAT4 knockouts. The reasons for this discrepancy between Kato *et al.*^[72] and Shen *et al.*^[80] are not clear, as both groups used a partial (70%) hepatic IR model of 90 min ischaemia and reperfusion including the same timeframe, the same endpoints and double knockouts. The transgenic knockouts had different wild type backgrounds in the two studies of

C57Bl6 or Balb/c wild types by Kato *et al.*^[72] and Shen *et al.*^[80] respectively, which may have affected the IRI results. Genetic compensation in the knockouts of either study is a possibility that may explain the discrepancy, although STAT4 protein expression was not assessed by Shen *et al.*^[80] and there is little detail on how the knockouts were generated in both studies.

A knockout of PARP has been used to show that PARP activation contributes to early liver IRI (Table 5) and activates signalling pathways increasing expression of adhesion molecules on SECs^[61]. A liver IRI model using a PPAR knockout demonstrated background PPAR activity reduces the severity of liver reperfusion injury acting *via* signalling pathways that remain to be elucidated but appear not to involve NO or TNF- α , both of which act independently of PPAR in this model^[55].

CONCLUSION

Liver IRI is a clinically relevant phenomenon in a wide range of settings including trauma surgery, hepatic resection and transplantation, affecting clinical outcome. Laboratory work using knockout models and large animal studies have provided insights into the mechanisms of liver IRI. Liver IRI occurs as a continuum beginning from the moment of reperfusion onwards for up to a week.

There is a complex interplay between cellular mediators, ROS, the complement system, cytokines/chemokines and other secreted factors that activate several parallel intracellular pathways that include transcription factors, nitric oxide synthase and haem oxygenase-1, all of which is beginning to be unravelled. Further laboratory work using knockout models and large animal studies of liver IRI will provide further mechanistic insights into this phenomenon and identify pharmacological agents that could be entered into clinical trials for reducing IRI.

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Thiopurines in inflammatory bowel disease revisited

Florian Bär, Christian Sina, Klaus Fellermann

Florian Bär, Christian Sina, Klaus Fellermann, Medical Department 1, University Hospital Schleswig Holstein, 23538 Lübeck, Germany

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Correspondence to: Klaus Fellermann, MD, Medical Department 1, University Hospital Schleswig Holstein, Ratzeburger Allee 160, 23538 Lübeck, Germany. fellermann@uk-sh.de

Telephone: +49-451-5002398 Fax: +49-451-5006242

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Abstract

Although a great variety of new drugs have been introduced for the therapy of inflammatory bowel diseases so far, a definite cure of the disease is still out of scope. An anti-inflammatory approach to induce remission followed by maintenance therapy with immunosuppressants is still the mainstay of therapy. Thiopurines comprising azathioprine and its active metabolite mercaptopurine as well as tioguanine, are widely used in the therapy of chronic active inflammatory bowel disease (IBD). Their steroid sparing potential and efficacy in remission maintenance are out of doubt. Unfortunately, untoward adverse events are frequently observed and may preclude further administration or be life threatening. This review will focus on new aspects of thiopurine therapy in IBD, its efficacy and safety.

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Key words: Thiopurines; Mercaptopurine; Tioguanine; Azathioprine; Ulcerative colitis; Crohn's disease

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INTRODUCTION

The main aim in treating patients with inflammatory bowel disease (IBD) taking into account the armamentarium of available therapeutic agents is to choose the most efficient therapeutic regimen on an individual basis in order to achieve remission of disease. Two aspects have to be kept in mind to achieve this goal: first is to treat the patient with the greatest efficacy regarding disease remission and probably obtaining mucosal healing. And in second place treat with the greatest safety and minimal toxicity. One integral part of this management strategy is the use of immunosuppressants after remission induction. Most commonly used among this group are the thiopurines azathioprine (AZA) and mercaptopurine (MP). AZA and MP need to be activated by metabolism to 6-thioguanine nucleotides (6-TGN). Although these nucleotides disturb proper DNA synthesis it has not conclusively shown, that 6-TGN are the one and only molecules responsible for proper action. However, immunosuppressive function seems to be mediated in part by induction of apoptosis in lymphocytes. A correlation of therapeutic benefit and 6-TGN levels has been put into question. Although thiopurines are widely used, several safety concerns remain. Tioguanine has been proposed as an alternative to overcome such problems, as it skips the metabolic conversion to 6-methyl mercaptopurine (6-MMP) which is responsible for hepatotoxicity. This review will discuss efficacy and safety of thiopurine therapy in IBD.

PHARMACOLOGY OF THIOPURINES

Oral bioavailability is variable and inversely proportional to the oral dose (AZA 27% to 83%, MP 5% to 37%). After oral intake 88% of AZA is rapidly converted non-enzymatically to 6-MP and S-methyl-4-nitro-5-thioimidazole in erythrocytes and human cells. This process relies on sulphydryl-containing compounds such as cysteine and glutathione. Deposition and consumption of the latter is in part controlled by glutathione-S-transferase activ-

ity^[1]. 6-MP as the active metabolite undergoes a complex biotransformation into active and inactive metabolites that easily transfer membranes. At first 6-MP undergoes extensive first pass catabolism by xanthine oxidase (XO) forming 6-thiouric acid which is excreted by the kidneys. Allopurinol is a well known inhibitor of XO. In addition, 6-MP is a substrate for thiopurine methyltransferase (TPMT), the rate limiting enzyme of detoxification. Methylation results in the inactive metabolite 6-MMP. The active metabolites of 6-MP are initially formed by hypoxanthine phosphoribosyltransferase (HPRT). The first active intermediate thioinosine monophosphate is then rapidly converted to the 6-TGN which are accredited to be the paramount effectors of thiopurines. Their cytotoxic and immunosuppressive effect is attributed to their incorporation into cellular nucleic acids resulting in inhibition of lymphocyte proliferation. Beside this action, 6-TGN might play an important role in the signaling cascade of apoptosis in lymphocytes by inhibiting Rac 1 activation in T-cells^[2]. Competing with this intracellular activation is the thiol-methylation by TPMT. The individual capacity of this enzyme influences the relative proportion of intracellular active 6-TGN produced by a given individual and has important implications in predicting toxicity. TPMT polymorphism will be discussed elsewhere (see below). Pharmacology of thiopurines is comprehensively summarized in^[3,4].

USE OF THIOPURINES IN CROHN'S DISEASE

The efficacy of thiopurines has been demonstrated in several trials both for induction and maintenance of disease free remission in Crohn's disease (CD). The first study demonstrating the efficacy of MP to induce remission was reported by Present *et al*^[5]. Sixty-seven percent of the patients treated with 1.5 mg/kg MP daily responded as compared to 8% in the placebo group. The today well known delayed onset of action was additionally well described in this study with a mean time to response of 3.1 mo. The efficacy in remission maintenance was clearly shown in the landmark withdrawal trial by Candy *et al*^[6]. Several other studies reported variable responses ranging from 36%-100%^[7-11]. A major drawback of some of these studies was their short duration. Due to the thiopurines' time lag of action the therapeutic gain might have been underestimated. Despite these varying results updated meta analysis favour therapy over placebo with an odds ratio of 2.43 (95%CI: 1.62-3.64) regarding remission induction with AZA or MP^[12,13]. In case of remission maintenance the OR for AZA is 2.32 (95%CI: 1.55-3.49) with a number needed to treat (NNT) of 6 and with MP 3.32 (95%CI: 1.40-7.87) with a NNT of 4, including 7 trials^[14].

As one third of the patients become steroid dependent and 20% of the patients loose response to steroids after one year^[15], an early introduction of immunomodulators seems mandatory, at least in patients who fail to taper off steroids. An elegant study by Markowitz *et al*^[16] in

children with new onset of CD underlines the potential of early MP administration for remission maintenance. Steroid pulse therapy in combination with MP resulted in a reduction of relapse rates from 28% to 4% after 6 mo and from 47% to 9% after 18 mo compared to steroids alone. Additionally, a significant steroid sparing effect was observed. These results suggest a short term steroid use for induction of remission and thiopurines for long term steroid-free maintenance therapy.

A recent randomised controlled trial assessed steroid free remission in active CD after 26 wk of treatment with AZA, infliximab or the combination of both^[17]. While AZA was less beneficial than infliximab, the best results were obtained with combined treatment. Similar findings were reported regarding mucosal healing. The inferior performance of AZA as a single agent may be related to the defined primary aim and time point.

USE OF THIOPURINES IN ULCERATIVE COLITIS

Although thiopurines are widely used in the treatment of patients suffering from ulcerative colitis (UC) controlled data are limited^[18-21]. If thiopurines have any place in the treatment of UC it is for remission maintenance or steroid-dependence. A recent meta-analysis including 6 studies reported an OR of 2.56 in favour of thiopurines (95%CI: 1.51-4.34)^[22]. Moreover, a steroid sparing potential of thiopurines in UC is obvious^[20,21].

In patients presenting with steroid refractory disease thiopurines play a role as they enhance the beneficial effect of the rescue therapy with cyclosporine^[23]. Despite fundamental evidence, thiopurines remain a therapeutic option for UC patients failing 5-ASA monotherapy or requiring multiple steroid courses^[24]. One half of the UC patients responding to a first course of corticosteroids will require immunosuppressives mainly because of steroid-dependence^[25]. In accordance with the SONIC trial for CD, a similar study was conducted in UC^[26]. The results and drawbacks are comparable though the difference between AZA and infliximab monotherapy vanished. Thus, combination treatment was stated to be the most effective with regard to steroid-free remission and mucosal healing at 16 wk.

OPTIMAL DURATION OF TREATMENT?

An unresolved question after successful initiation of thiopurine treatment is, how to assess the optimal duration of treatment. Bouhnik *et al*^[27] reported that the beneficial effects disappear after 4 years of AZA treatment, based on a small number of patients. In a controlled study AZA withdrawal was not equivalent to continued therapy with AZA for maintenance of remission in patients with CD who had been in remission on AZA for more than 3.5 years^[26,28]. In case of UC the data are sparse. A retrospective survey reported that relapse rates were higher in UC patients with a short duration of AZA administra-

tion indicating a favourable longevity of treatment^[29]. In accordance with a large observational study in England AZA sustains remission in CD as well as UC for at least 5 years^[30].

Thus far patients should be informed about indefinite treatment especially in complicated cases and after recurrent surgery. The decision to withdraw the drug has to be made on an individual relapse-risk assumption.

MUCOSAL HEALING

The surrogate marker mucosal healing is increasingly acknowledged as a treatment goal despite the lack of prospective data to assume disease course prediction. First insight in endoscopic healing was given by D' Haens *et al*^[31] who found that the majority of AZA treated CD patients were able to achieve near to complete mucosal healing. Mantzaris *et al*^[32] found that after 1 year of maintenance therapy in clinically quiescent CD, AZA was superior to budesonide in improving endoscopic healing and histological remission. In steroid dependent UC AZA was more effective than 5-ASA in achieving clinical and endoscopic remission^[33]. More data can be extracted from the SONIC and US SUCCESS trials (endoscopic healing with AZA in CD at 26 wk 16.5%, UC at 16 wk 37%) contradicting former results^[17,26]. As shown in CD thiopurines are effective drugs to induce remission and mucosal healing and to maintain it in UC as well^[34].

SAFETY OF THIOPURINES

Adverse events during the use of thiopurines in the treatment of patients with IBD can be categorized as non-dose dependent (allergic/idiosyncratic) side effects on one hand and dose dependent ones on the other^[35-37]. In general the number needed to harm is 15.

Five percent to ten percent of the patients do not tolerate thiopurines regardless of the dose and their underlying drug metabolism. Most common reactions are flu-like illness, fever, nausea, rash, abdominal pain, pancreatitis and allergic reactions that typically occur within 2-4 wk after initiating therapy. This has not been conclusively related to TPMT polymorphisms but wildtype glutathione-S-transferase is overrepresented in those patients^[38]. About half of those patients can be successfully re-challenged with MP which lacks an imidazole ring. This switch cannot generally be recommended for patients who experienced pancreatitis. Patients with an allergic reaction should be allocated to alternative immunomodulators such as methotrexate. In the following a few severe adverse events shall be enlightened in detail.

Myelotoxicity

The most common side effect in the treatment of IBD patients with thiopurines is myelosuppression, which is mostly manifested as leucopenia and occurs in 2.2% to 15% of the patients^[37-39]. The majority of those events respond to dose reduction, but infectious complications in-

crease if the white blood cell count falls below 2000/ μ L. Therefore special care is warranted in those patients and in patients on multiple immunosuppressants. TPMT deficiency accounts for one fourth of the leukopenia in CD treated with thiopurines whereas the rest is obscure^[40]. A drop in platelets may occur in conjunction with leucopenia or as a single event. If it is not reversible after dose reduction or discontinuation of therapy patients need to be further evaluated with a special focus on hepatotoxicity.

Hepatotoxicity

The exact mechanisms of hepatotoxicity by thiopurines are not clarified^[41,42]. Some of the patients develop a mild elevation in their liver function tests and most of them respond to dose reduction. Hence, a cessation of therapy is not necessary. In those patients whose liver enzymes do not normalize over time further exploration is necessary. Potentially serious hepatic side effects occur with the development of nodular regenerative hyperplasia in patients treated with tioguanine^[43] as well as AZA^[42-44] resulting in progressive liver damage and portal hypertension. An alternative is the use of the XO-inhibitor allopurinol in combination with 1/4 of the standard dose of a thiopurine which increases 6-MP bioavailability and reduces 6-MMP formation thereby limiting the risk of hepatotoxicity but long-term prospective studies are lacking^[45-48].

Malignancy

The incidence of cancer and lymphoma in IBD and the influence of thiopurines is still a controversial topic^[49-53]. 6-Thioguanine accumulates in the DNA of thiopurine treated patients and is able to interact with UVA to generate reactive oxygen species. It has recently been shown that the UVA/DNA 6-TG interaction irreversibly inhibits transcription in cultured human cells and provokes polyubiquitylation of the major subunit of RNA polymerase II. This persistent transcription-blocking DNA lesions seem to be responsible for acute skin responses to sunlight and predispose for the development of skin cancer^[54]. Recently published data point to an increased risk for non-melanoma skin cancer (NMSC). The incidence rate ratio was higher among patients with IBD compared with controls (1.64, 95%CI: 1.51-1.78). Persistent thiopurine use (> 365 d) was even stronger associated with NMSC (adjusted OR 4.27, 95%CI: 3.08-5.92)^[55]. There is one study that contradicts these results^[56], but three others confirm the data and report an increased risk for NMSC in patients with IBD treated with thiopurines^[57-59]. To summarize, up to now these patients should be protected against UV radiation and should undergo lifelong dermatologic screening.

The issue of lymphoma is open for discussion. There is an ongoing debate if the disease itself already increases the risk for the development of lymphoma. Additionally a confounding factor should be kept in mind as patients with an aggressive course of the disease do have a greater

innate risk for lymphoma and besides a higher likelihood to receive thiopurine treatment^[60]. There are some studies finding no association between thiopurine treatment and lymphoma in IBD while others suggest an increased incidence^[61-69]. A meta-analysis of the above mentioned data revealed a relative risk of four for all lymphomas in patients on thiopurine treatment compared with those with IBD not receiving thiopurines^[70]. This increase was confirmed in a recent prospective evaluation of french IBD patients with a hazard ratio of 5.28 for ongoing thiopurine treatment^[71,72]. Of interest is the potentially overt risk in patients with double immunosuppression but the data are lacking so far. The rare variant of hepatosplenic lymphomas with fatal outcome has to be acknowledged and is related to thiopurines as well as TNF blockers.

Thiopurines have been demonstrated to be risk-neutral in the context of the development of colorectal cancer in IBD patients^[73]. And there are various studies available with regard to cervical cancer related to HPV 16 and 18 infection. So far there is no clear picture due to the paucity of data and the risk of cervical cancer seems to be comparable to the general population. Although there is no evidence for an increased risk of other solid tumors the lack of data always needs to be taken into consideration^[61].

Taken together thiopurine treatment in patients with IBD has a potential to induce or propagate neoplasia. This relative risk increase is most significant for lymphoma and NMSC in second place but the risk-benefit ratio supports the continuation of treatment in IBD.

Use in pregnancy

Women with IBD have similar rates of fertility compared to the general population. Unfortunately they have a greater rate of adverse pregnancy outcomes which are related to disease activity. Therefore an immunosuppressive treatment of patients with severe disease course is mandatory during pregnancy. Although thiopurines are rated D (positive evidence of human fetal risk, but potential benefits may warrant its use) by the Food and Drug Administration they seem to be safe and well tolerated during pregnancy^[74]. There are smaller studies that found an increased risk of congenital malformations, perinatal mortality, and preterm birth^[75,76]. Opposing results are presented by Francella *et al*^[77] in their analysis on 155 patients who conceived after IBD was diagnosed. There was no statistical difference regarding rates of spontaneous abortion, abortion as a result of a birth defect, major congenital malformations, neoplasia or increased infections after the intake of MP. This is now confirmed by the registered data from the CESAME study^[78].

OPTIMIZING SAFETY AND EFFICACY

Due to their complex metabolism and genetic polymorphisms in metabolizing enzymes there is a wide inter- and intra-patient variation in the concentrations of active and toxic metabolites. In 9%-25% of patients serious drug

toxicity leads to a cessation of therapy and therapeutic efficacy is unachievable in about 15% of patients^[79].

TPMT measurement

TPMT competes with XO and HPRT for the substrate 6-MP. The TPMT gene carries genetic polymorphisms that lead to a nearly 50-fold variation in the enzyme activity between individual patients^[80]. Hence, low TPMT activity leads to a greater conversion of 6-MP to 6-TGN (the predominant active metabolite) *via* the HPRT pathway. This is not only associated with greater therapeutic activity but also a higher likelihood of myelotoxicity. On the other hand high TPMT activity results in greater 6-MMP production at the expense of active metabolites.

A growing number of single nucleotide polymorphisms within the TPMT gene are identified, but the frequency among approximately 30 known alleles is very low except for the 3A/C alleles. There are population differences in the frequency of abnormal TPMT alleles especially in relation to the 3A and 3C genotypes, with more than 90% of the patients carrying the 1/1 wild type genotype and normal enzyme activity. 10%-11% of the patients have a reduced enzyme activity due to the heterozygous 1/3 (TPMT3A or 3C) genotype. Only 1 in 300 patients carries the homozygous 3/3 genotype with absent TPMT activity. Together this distribution in a population follows a classical trimodal pattern^[80], although very high metabolizers superimpose this view without clinical relevance. The TPMT status can be determined by measuring enzyme activity (radiochemical or HPLC) or by genotyping. Allelic frequencies in IBD may be somewhat higher^[81-83]. In patients with TPMT deficiency a tiny dose of thiopurines (1/10) can be used under careful monitoring. Patients with intermediate TPMT activity should receive half or one third of the initial dose, normal to high TPMT activity is in need of up to very high doses. Other adverse drug effects are unrelated to TPMT pheno- or genotype. However, a recent finding is the association of some type 2 adverse events (flu-like illness, rash) and inositol pyrophosphohydrolase (ITPase, ITPA) polymorphisms. This has not been implemented in the recommendations so far but warrants attention^[84]. At present determination of TPMT activity is questionable in the clinical setting although cost effectiveness has been noted. A recent position paper summarizes the recommendations of TPMT testing and monitoring of 6-TGN levels^[85].

Monitoring metabolites

Metabolite monitoring is not mandatory for patients with IBD who are treated with thiopurines. Although several trials have observed an association between high 6-TGN levels and a favourable response this has not been unequivocally reproduced (summarized in^[86]). However, monitoring is particularly useful in those patients that do not respond to a standard dose of thiopurine drugs after a meaningful duration of treatment. The combination of erythrocyte 6-TGN and 6-MMP concentrations can

be helpful to detect the reason for a lack of response. Absent 6-TGN and 6-MMP levels unmask poor compliance. If the concentration of both metabolites is low, the patient is probably under-dosed. A low 6-TGN level in conjunction with a high 6-MMP concentration identifies a patient who may respond to a rechallenge with low dose AZA in combination with allopurinol. Otherwise he has to be termed thiopurine resistant. In case of high concentrations of both metabolites the patient suffers from thiopurine refractory disease.

TIOGUANINE AS AN ALTERNATIVE TO AZA AND MP-REAL BENEFIT?

In order to overcome problems of toxicity and delayed action of thiopurines, tioguanine, originally established for treatment of leukemias mainly in children, was investigated for remission induction and maintenance in IBD. It serves as a direct precursor to 6-TGN, the proposed active metabolite of thiopurines. In one group with 37 patients with active CD tioguanine appeared to be effective with acceptable short-term toxicity^[87,88]. 6-TGN levels were far above those documented for classical thiopurines. However, neither toxicity nor efficacy increased. Recent data has shown that hepatotoxicity does not reoccur during tioguanine treatment in most IBD patients who failed conventional thiopurines due to 6-MMP associated hepatotoxicity. Hence, tioguanine appears to be a justifiable alternative in these IBD patients^[89]. Another investigation by Dubinsky *et al.*^[92] found opposing results and stated that NRH is a common finding in tioguanine treated patients with IBD. As progression or reversibility of NRH remains unknown the authors do not recommend tioguanine therapy for patients with IBD. Tioguanine related hepatotoxicity in the Dubinsky study was surprisingly frequent whereas in other studies this rate is nearly comparable to that of AZA as NRH has been described under therapy with AZA as well (see chapter hepatotoxicity). A lower dose of 10-20 mg tioguanine for remission maintenance has been proposed to be safe in the long term^[90]. Accordingly, tioguanine should not be abandoned but more surveillance data are needed.

CONCLUSION

The role of conventional thiopurines, 6-MP and AZA, still evolves in the treatment of patients with IBD. The above stated data underline the important role of thiopurines in remission maintenance. Understanding the metabolic pathways has greatly optimized treatment and lead to greater safety and efficacy. Long-term observational studies regarding the still controversial issues of hepatotoxicity, nodular regenerative hyperplasia and malignant potential of the drugs are still warranted. Tioguanine may be an effective alternative in patients who are intolerant to AZA or MP but the incidence and fate of NRH is still unresolved.

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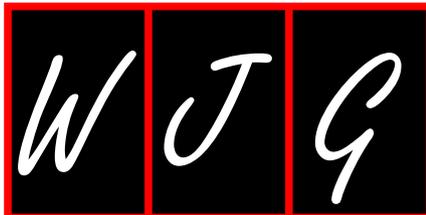
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Current concepts on the role of nitric oxide in portal hypertension

Liang Shuo Hu, Jacob George, Jian Hua Wang

Liang Shuo Hu, Jacob George, Jian Hua Wang, Storr Liver Unit, Westmead Millennium Institute and Westmead Hospital, University of Sydney, Westmead, NSW 2145, Australia
Author contributions: Hu LS, George J and Wang J contributed equally to this paper.

Supported by Australian NH and MRC, AP1004595
Correspondence to: Jian Hua Wang, PhD, Storr Liver Unit, Westmead Millennium Institute and Westmead Hospital, University of Sydney, Westmead, NSW 2145,
Australia. jianhua.wang@sydney.edu.au

Telephone: +61-2-98459131 Fax: +61-2-98459103

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Abstract

Portal hypertension (PHT) is defined as a pathological increase in portal venous pressure and frequently accompanies cirrhosis. Portal pressure can be increased by a rise in portal blood flow, an increase in vascular resistance, or the combination. In cirrhosis, the primary factor leading to PHT is an increase in intra-hepatic resistance to blood flow. Although much of this increase is a mechanical consequence of architectural disturbances, there is a dynamic and reversible component that represents up to a third of the increased vascular resistance in cirrhosis. Many vasoactive substances contribute to the development of PHT. Among these, nitric oxide (NO) is the key mediator that paradoxically regulates the sinusoidal (intra-hepatic) and systemic/splanchnic circulations. NO deficiency in the liver leads to increased intra-hepatic resistance while increased NO in the circulation contributes to the hyperdynamic systemic/splanchnic circulation. NO mediated-angiogenesis also plays a role in splanchnic vasodilation and collateral circulation formation. NO donors reduce PHT in animals models but the key clinical challenge is the development of an NO donor or drug delivery system that selectively targets the liver.

INTRODUCTION

Portal hypertension (PHT) is a common clinical consequence of chronic liver disease that is associated with significant morbidity and mortality. PHT is classified as either pre-hepatic, intra-hepatic or post-hepatic, with intra-hepatic PHT being the form most often caused by cirrhosis, irrespective of etiology^[1]. The extent of PHT is quantified in clinical practice by measuring the hepatic portal vein pressure gradient (HPVG)^[2], representing the difference between the wedged hepatic vein pressure (a measure of pressure at the level of the hepatic sinusoid), and the free hepatic vein pressure. Thus, HPVG is often used to assess the effects of pharmacological therapy in reducing portal pressure^[3].

Based on hydromechanics, fluid pressure in a hollow tube is determined by fluid resistance and flow. In PHT, therefore, the intra-hepatic vascular resistance (IHVR) and splanchnic blood flow are the two main contributors to portal pressure^[4]. Under normal circumstances, post-prandial increases in splanchnic blood flow is always associated with an autonomous down-regulation of IHVR, leading to no alteration in portal pressure. In contrast, IHVR is significantly up-regulated by mechanical and hemodynamic factors in the setting of cirrhosis, which is further aggravated by splanchnic vasodilation^[5]. Clinically, this increase in portal pressure is the antecedent to

variceal bleeding with its associated morbidity and high mortality^[6,7].

IHVR is influenced by both hepatic fibrotic architectural distortion in cirrhosis leading to obstruction to blood flow, as well as by dynamic hepatic stellate cell (HSC) contraction around sinusoidal blood vessels. Angiogenesis, or the formation of new blood vessels, is also an important component of the pathophysiology of PHT. The resulting alterations in vascular contractility and angiogenesis contribute to PHT in both the intrahepatic and splanchnic circulation.

Endothelin 1 (ET-1), angiotensin II, norepinephrine, prostaglandin F₂, thromboxane A₂, and thrombin can trigger liver sinusoidal contraction. In contrast, substances such as acetylcholine, vasointestinal peptides, nitric oxide (NO), carbon monoxide, prostaglandin E₂, and adrenomedullin relax the sinusoidal vasculature^[8,9]. Among these agents, ET-1 and NO are the most important regulators of the sinusoidal microcirculation^[8,9]. In PHT, an insufficient release of vasodilators particularly NO from endothelial cells is critical to the genesis of the dynamic and modifiable component of increased vascular resistance^[8,9]. Consistent with this, improvements in intrahepatic NO availability is beneficial for the treatment of PHT in animals and patients^[10-14]. Hence, this review will focus on an update on the mechanisms whereby NO mediates PHT and on the potential to modulate this system to reduce portal pressure.

SYNTHESIS AND FUNCTION OF NO

NO is synthesized by nitric oxide synthase (NOS) through a series of redox reactions involving L-arginine (the main substrate), oxygen and nicotinamide adenine dinucleotide phosphate. There are 4 major isoforms of NOS: endothelial nitric oxide synthase (eNOS), inducible nitric oxide synthase (iNOS), neuronal nitric oxide synthase (nNOS) and mitochondrial nitric oxide synthase^[15]. Following synthesis by NOS, the half-life of endogenously generated NO is extremely short, about 1 s. Thus, endogenous NO production is intimately regulated by the activity of NOS.

The generated NO molecule has a large diffusion coefficient and can therefore freely penetrate cellular membranes in an autocrine or paracrine manner. Within the cell, NO stimulates the conversion of guanosine 5'-triphosphate (GTP) to cyclic guanosine 3'-5'-monophosphate (cGMP), thereby regulating calcium balance through the cGMP-dependent protein kinase pathway (Figure 1). This leads to vasodilatation^[16]. The end products of NO metabolism *in vivo* are nitrate (NO₃⁻) and nitrite (NO₂⁻) that are an indirect measure of the total NO concentration^[17].

NO is also highly reactive with other molecules including superoxide anion (O₂⁻), oxygen (O₂) and hemoproteins such as hemoglobin and myoglobin. The intermediate products of these reactions are known as reactive nitrogen species, which promotes many pathophysiologi-

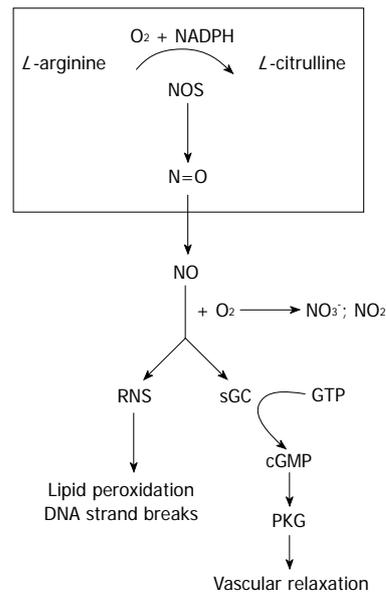


Figure 1 Nitric oxide formation and function. Nitric oxide synthase (NOS) catalyzes the biosynthesis of nitric oxide (NO) from L-arginine, nicotinamide adenine dinucleotide phosphate (NADPH) and O₂. NO freely diffuses into cells where it mediates vascular relaxation by stimulating the cyclic guanosine 3'-5'-monophosphate (cGMP)/cGMP-dependent protein kinase G (PKG) pathway. It also forms reactive nitrogen species (RNS) which leads to many damaging reactions including lipid peroxidation and DNA strand breaks. GTP: Guanosine 5'-triphosphate; sGC: Soluble guanylyl cyclase.

cally damaging reactions including lipid peroxidation, DNA strand breaks, and the generation of nitrosamines, nitrotyrosine and nitro guanosine.

MOLECULAR MECHANISMS REGULATING NOS

eNOS serves a key role in maintaining circulatory homeostasis and is expressed mainly in endothelial cells and to a lesser extent in cardiac myocytes and platelets^[15]. The enzyme localizes to small invaginations of the plasma membrane named caveolae in quiescent cells. eNOS protein is constitutively expressed in the cell and activation mostly comprises post-translational regulation and modifications in its subcellular localization^[18].

Within cells, eNOS closely associates with several proteins that impact on its function, including caveolin. Caveolin negatively regulates eNOS by directly abrogating the enzyme's activation and blocking the binding site for calmodulin^[19]. In contrast, calmodulin acts as an indispensable protein competing with caveolin for binding with, and activating eNOS^[20,21]. Other relevant proteins in relation to NO production include heat shock protein 90 and tetrahydrobiopterin (BH4) that are positive regulators of eNOS^[22-24]. Finally, eNOS interacting protein and eNOS trafficking inducer protein participate in the subcellular trafficking of eNOS when eNOS translocates from caveolae into the cytoplasm^[25-27].

Phosphorylation at key serine residues is the major post-translational modification that is required for eNOS

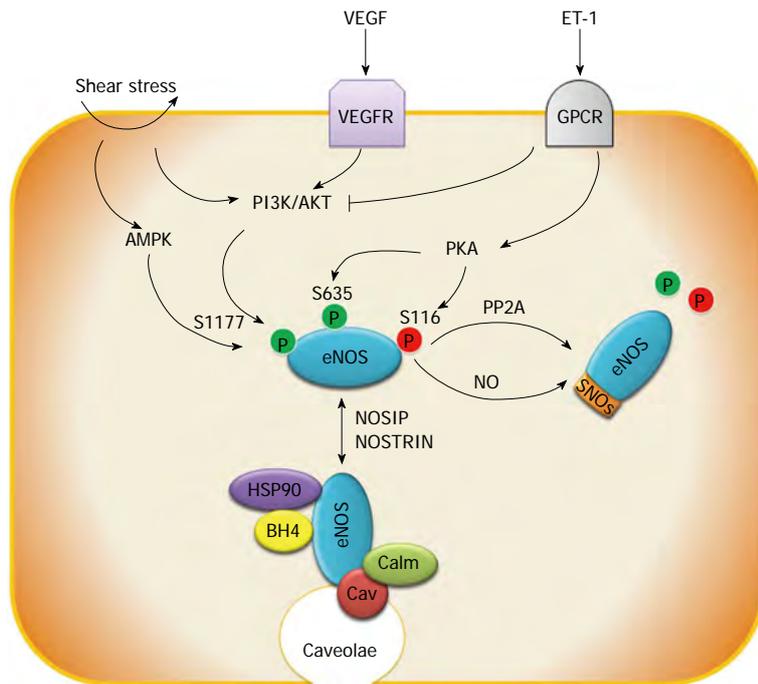


Figure 2 The molecular regulation of endothelial nitric oxide synthase activity. Endothelial nitric oxide synthase (eNOS) phosphorylation can be triggered by shear stress, vascular endothelial growth factor (VEGF), endothelin 1 (ET-1) and other factors through adenosine monophosphate-activated protein kinase (AMPK), protein kinase B (AKT) and protein kinase A (PKA) pathways, whereas protein phosphatase 2 (PP2A) de-phosphorylates eNOS. In addition, S-nitrosylation (SNOs) by eNOS-derived nitric oxide (NO) inhibits eNOS activity. Endothelial nitric oxide synthase interacting protein (NOSIP) and endothelial nitric oxide synthase trafficking inducer protein (NOSTRIN) regulate the sub-cellular location of eNOS protein between the caveolae and cytoplasm. The principal location of eNOS is in caveolae where its function is inhibited by binding to caveolin (Cav). HSP90, calmodulin (Calm) and tetrahydrobiopterin (BH4) are indispensable proteins and cofactors for catalyzing NO production. PI3K: Phosphatidylinositol-3-kinase; GPCR: G protein-coupled receptor.

function. Phosphorylation of Ser 1177, Ser 635 and Ser 617 activates eNOS whereas phosphorylation of Thr 495 and Ser 116 inhibits eNOS activity^[28]. Phosphorylation at Ser 1177 can be initiated by activation of several intracellular pathways including phosphatidylinositol-3-kinase (PI3K/AKT), cAMP-dependent protein kinase A (PKA), adenosine monophosphate-activated protein kinase, cGMP-dependent protein kinase G (PKG) and *Calmodulin Kinase II*-dependent pathway (CaM kinase II)^[29-32], while Ser 635 and Ser 116 is activated *via* a PKA-dependent pathway^[33,34]. Additionally, shear stress, vascular endothelial growth factor (VEGF) and high-density fatty acids can phosphorylate and activate eNOS (Figure 2)^[35]. In contrast, phosphatases like protein phosphatase 2 dephosphorylates and inactivates eNOS^[36]. S-Nitrosylation inhibits eNOS activity by modifying its steric configuration, whereas de-nitrosylation is associated with an increase in eNOS activity^[37,38].

Unlike eNOS, iNOS is more widely expressed, including in macrophages, vascular smooth muscle cells, HSCs and Kupffer cells after stimulated by lipopolysaccharide (LPS) or inflammatory cytokines. iNOS produces a relatively high level of NO compared to eNOS^[39]. In contrast to eNOS, iNOS expression is principally modulated by transcriptional mechanisms. Many transcription factors regulate the expression of iNOS including nuclear factor- κ B (NF- κ B), activator protein, signal transduction and activation of transcription 1a, specificity protein 1, CCAAT/enhancer-binding protein (C/EBP), *cAMP*

response element-binding, GATA binding transcription factor, hypoxia-inducible factor, interferon regulatory transcription factor, nuclear factor of activated T-cells, nuclear factor-interleukin 6, octamer-1 transcription factor, poly [ADP-ribose] polymerase 1, polyomavirus enhancer activator 3, tumor protein 53 and serum response factor^[40]. Among these, NF- κ B is considered the primary mediator for iNOS induction. In turn, NF- κ B can be activated by a range of stimuli including LPS, interleukin-1 β , tumor necrosis factor (TNF)- α and oxidative stress^[41,42]. iNOS can also be post-translationally regulated through mRNA stabilisation by RNA-binding proteins such as A+U rich RNA binding factor, human antigen R, K-homology splicing regulator protein, polypyrimidine tract-binding protein and tristetraprolin^[40].

nNOS is principally expressed in neurons localized to the nervous system including the brain, the autonomic nervous system and neurons around interlobular arteries. The portal vein endothelial cells also express nNOS^[43]. Like eNOS, nNOS is regulated by post-translational mechanisms and both Hsp90 and calmodulin are involved in the process of nNOS activation^[44-46].

MOLECULAR REGULATION OF NOS IN LIVER CIRRHOSIS AND PHT

Regulation of intra-hepatic eNOS

In cirrhosis and PHT, there is reduced NO production

by hepatic endothelial cells that is attributed to dysfunction of the eNOS system^[47-50]. Many factors contribute to intra-hepatic eNOS dysfunction/reduced eNOS activity. These include increases in oxidative stress, caveolin-1, RhoA, thromboxane A₂ (TXA₂), G-protein-coupled receptor kinase-2 (GRK2) and asymmetric dimethylarginine (ADMA) as well as decreased AKT and BH4 activity.

Reduced AKT activity and increased binding ability of caveolin-1 to eNOS in cirrhosis attenuates eNOS expression^[51,52]. Liu *et al.*^[51], reported that ET-1 activates G-protein-coupled receptor kinase-2 (GRK2) which directly interacts with and inhibits AKT phosphorylation. They also noted that the IHVR was significantly reduced in bile duct ligation (BDL) mice genetically deficient in GRK2^[52]. In another study of eNOS expression during BDL, Morvarid *et al.*^[53], noted that total eNOS protein was unchanged, but that functional, phosphorylated eNOS protein was decreased. Similarly, AKT expression was down-regulated in a time dependent manner. In contrast, caveolin-1 was increased^[53].

Intrahepatic oxidative stress is a key mediator of sinusoidal endothelial dysfunction and impairment of eNOS/NO expression^[54-57]. For example, Gracia *et al.*^[58], noted that increased intrahepatic oxidative stress (increased ROS and O₂⁻) was associated with reduced NO production and NO bioavailability. The authors went on to demonstrate that cyclooxygenase (COX) attenuated eNOS activation by stimulating TXA₂ which inhibits AKT phosphorylation in endothelial cells^[59]. A superoxide dismutase mimetic, Tempol significantly decreased superoxide, and increased NO in cultured hepatic endothelial cells. As expected, Tempol administration also resulted in a decline of portal pressure^[60].

ADMA, an endogenous inhibitor of NOS, causes uncoupling of NOS leading to generation of RNS, such as peroxynitrite. In BDL rats, a higher serum ADMA level was observed^[61]. Further, impaired endothelial cell-mediated relaxation in perfused livers of BDL rats was exacerbated by ADMA and was associated with a decreased rate of ADMA removal^[61,62].

BH4, a cofactor of eNOS, has been reported to be associated with dysfunction of the NO system. BH4 expression is down-regulated in liver cirrhosis and can further be oxidized and inactivated by O₂⁻. In the absence of BH4, eNOS cannot generate NO but instead produces O₂⁻, thereby leading to further decreases in NO production^[24,63]. In an *in vivo* study, Matei *et al.*, observed that in rats rendered cirrhotic after the administration of carbon tetrachloride (CCl₄), exogenous BH4 resulted in hepatic NOS and cGMP activation and a reduction in portal pressure^[64].

Rho-associated protein kinase (ROCK) is a kinase belonging to the AGC (PKA/PKG/PKC) family of serine-threonine kinases. It is mainly involved in regulating the shape and movement of cells by acting on the cytoskeleton. Rho-kinase is substantially involved in the contraction of activated HSCs^[65,66]. In BDL rats, fasudil (a potent Rho-kinase inhibitor) significantly suppressed liver

Rho-kinase activity and increased eNOS phosphorylation compared with controls^[67]. Fasudil also reduced the binding of the serine/threonine AKT to Rho-kinase and increased the binding of AKT to eNOS^[67].

Regulation of extra-hepatic vascular eNOS, iNOS and nNOS in cirrhosis

In contrast to the hypoactive SECs in the intrahepatic microcirculation, hyperactive endothelial cells with increased NO production play a critical role in modulating the vascular changes observed in the splanchnic and systemic circulation. For example, increased activity of peripheral vascular AKT signaling is noted, while constitutive AKT inhibition by an inactive mutant decreases aortic eNOS and improves systemic hemodynamics, splanchnic perfusion pressure and renal excretory function without affecting portal pressure^[68]. Other studies reported that VEGF induces NO production by activation of eNOS protein expression and activity^[69,70]. Likewise, in portal hypertensive rats, NO production is increased in response to shear stress^[71]. LPS detoxification is limited in liver with PHT thereby increasing plasma LPS. Resident macrophages in the splanchnic circulation respond to this circulating LPS with the production of proinflammatory cytokines, such as TNF- α ^[72] that then induces iNOS in extrahepatic vasculature^[73-75]. Bacteria-derived TNF- α also triggers the expression and activity of the key enzyme involved in the regulation of BH4, GTP-cyclohydrolase I, thereby increasing eNOS-derived NO in the mesenteric vasculature^[76,77]. Finally, nNOS expression is augmented in mesenteric nerves in portal hypertensive rats (portal vein ligation), an effect mediated by HSP-90^[46,78,79].

THE ROLE OF NO/NOS IN THE REGULATION OF IHVR

An increase in IHVR can be induced by reversible hemodynamic modifications to vascular tone which may represent 28%-40% of the increase in portal pressure in cirrhosis^[80-82]. Anatomic structures leading to this change include vascular smooth muscle cells surrounding branches of the portal vein, and HSCs located in the space of Disse. Both cells types have contractile properties and thus modulate IHVR^[82-84].

The role of NO in the modulation of IHVR has been well documented^[85-87]. eNOS dysfunction in sinusoidal endothelial cells and consequent reduction in NO production (or bioavailability) plays an essential role^[51]. This results in reduced vasodilation and a decreased capacity for antagonizing contractile factors such as ET-1, angiotensin II, norepinephrine, prostaglandin F₂, and thromboxane A₂^[83,88].

Recently, gene delivery techniques have been used to increase NOS (eNOS or nNOS) delivery to the liver of CCl₄ treated mice. In one study, a plasmid eukaryotic expression vector (liposome-pcDNA3/eNOS) or control vector was injected into rat portal vein, leading to increased eNOS mRNA and protein in liver. Hepatic

NO production was enhanced and IHVR and portal vein pressure (PVP) reduced^[89]. In another study, recombinant adenovirus carrying the nNOS gene (Ad.nNOS) or control vector was administered *via* the femoral vein to rats. Again, Ad.nNOS reduced IHVR and portal pressure^[90]. These data indicate that NO deficiency in cirrhotic liver contributes to the elevation in IHVR and conversely that NO delivery may play a therapeutic role^[89-92].

Activation and contraction of HSCs also contributes significantly to the dynamic and reversible component of IHVR. Indeed, activated HSCs are more susceptible to vasoconstrictor substances than quiescent cells^[83,92,93]. Under physiological conditions, NO produced by hepatic endothelial cells inhibits the growth, migration and contraction of HSCs through paracrine pathways^[94,95]. However, reduced NO production and/or impaired NO bioavailability in cirrhosis promotes HSCs activation and contraction, leading to sinusoidal remodeling and elevation of the IHVR.

iNOS has also been suggested to contribute to the hyperdynamic status seen in PHT. However, its role in mediating IHVR is unclear. In one study, liver iNOS was increased in BDL rats and reduction of portal pressure by ursodeoxycholic acid was associated with iNOS down-regulation^[96,97].

ROLE OF NO/NOS IN THE REGULATION OF SPLANCHNIC BLOOD FLOW

A hyperdynamic splanchnic circulatory state is a major accompaniment of PHT. The increase in splanchnic blood flow and the subsequent increase in portal venous inflow aggravates and perpetuates PHT. The mechanisms underlying this phenomenon are not fully understood, but overproduction of endogenous vasodilators and decreased vascular reactivity to vasoconstrictors has been suggested^[98].

Overproduction of NO in the splanchnic and systemic circulation contributes to this phenomenon as NOS inhibition effectively ameliorates splanchnic hyperemia^[99,100]. eNOS up-regulation and increased NO release by the superior mesenteric arteries endothelium occur before the development of the hyperdynamic splanchnic circulation^[101]. Juan *et al.*^[70], noted increased eNOS expression in portal-hypertensive rats with even mild increases in portal pressure. In another study, phosphorylated eNOS protein was increased, whereas caveolin-1 was decreased in the aorta of BDL rats^[52]. In contrast, in eNOS knockout mice injected with CCL4, attenuated splanchnic blood flow was observed. However, this was associated with an increase in IHVR, presumably due to the reduced NO within the liver^[102]. Taken together, these results suggest up-regulated eNOS expression during splanchnic hyperemia, contrasts with the relative eNOS deficiency in liver.

There are also several studies demonstrating the im-

portance of iNOS in the hyperdynamic circulation of cirrhosis^[64,72,73,103,104]. In cirrhosis, endotoxins, cytokines and bacterial infection promote iNOS formation and overproduction of NO^[64,105-107]. The increased splanchnic iNOS appears to reside in resident macrophages of the superior mesenteric artery^[73,108]. Supporting this concept, Ferguson *et al.*^[64], observed that a selective iNOS inhibitor, *N*-[3-(aminomethyl) benzyl]acetamide, caused peripheral vasoconstriction in patients with cirrhosis. It is interesting to note that there also exists an interaction between eNOS and iNOS in the vasculature. For example, in cirrhosis, increased and dominant expression of eNOS in large arteries results in systemic hypotension and increased blood flow. These effects could be abrogated by activated iNOS in the small vessels of the splanchnic circulation as iNOS activation inhibited eNOS expression in the small vessels^[109]. nNOS may likewise promote vasodilation of the splanchnic circulation, though its contribution is overall less significant^[110,111].

NO AND ANGIOGENESIS IN PHT

It is now established that angiogenesis is associated with the progression of PHT^[112,113]. Angiogenic factors stimulate collateral vessel formation both in the liver and in extrahepatic locations, manifesting as the reopening of pre-existing shunts^[114,115]. This pathological angiogenesis may directly participate in the development of liver fibrosis^[56,116,117].

Again, NO is an important mediator of intrahepatic microcirculatory remodeling^[114,115]. Thus, NO inhibition prevents angiogenesis and diminishes mesenteric vascular proliferation in animals with PHT^[118,119]. Shaki *et al.*^[120], found that NO-mediated angiogenesis was mediated by endothelial VEGF and VEGF receptor-1. Most recently, Huang *et al.*^[121], reported that through mesenteric eNOS and COX1 down-regulation, the cannabinoid receptor 2 agonist JWH 015, could alleviate mesenteric and intrahepatic angiogenesis, PHT, the severity of portosystemic collaterals and the extent of fibrosis in BDL cirrhotic rats.

NO-BASED PHARMACOTHERAPY

As discussed, NO is paradoxically regulated in PHT. There is excessive production of NO in the splanchnic circulation (thereby leading to vasodilation), while in the intra-hepatic microcirculation, a deficit of NO production is associated with increased IHVR. These paradoxical roles of NO initially raised concerns about the use of NO inhibitors or donors as therapy for PHT. However, inhibition of NO release has been shown in animals and humans to attenuate the hyperdynamic circulation of cirrhosis^[122-125]. No significant reduction in portal pressure was achieved^[122-125]. This is likely a consequence of reductions in portal venous inflow induced by the NO inhibitors being offset by an increase in intra-hepatic resistance.

In recent years, many animal and clinical studies have

demonstrated that NO donors result in a substantial reduction in portal pressure^[10-14]. These agents could theoretically aggravate the cirrhotic vasodilatory syndrome leading to harmful effects such as systemic hypotension and renal dysfunction^[126,127]. For these reasons, the ideal NO drug for the treatment of PHT should act to decrease IHVR without worsening splanchnic/systemic vasodilatation^[128].

NCX-1000 is a drug synthesized by adding an NO-releasing moiety to ursodeoxycholic acid. The compound is selectively metabolized by hepatocytes to release NO in the liver^[129,130]. Animal studies demonstrate that this drug alleviates IHVR and portal pressure without changes in systemic hemodynamics^[129-131]. However, human clinic trials were disappointing as NCX-1000 failed to decrease HVP, there were postprandial increases in portal pressure and systolic blood pressure was reduced in a dose-dependent manner^[132].

O₂-vinyl-1-(pyrrolidin-1-yl)diazen-1-ium-1,2-diolate (V-PYRRO/NO) was designed as a liver-selective NO-producing pro-drug activated by hepatic P450s^[133]. The drug has a short half-life and may additionally alleviate liver injury by NO-mediated protection of hepatocytes^[134-136]. Continuous administration of V-PYRRO/NO to BDL rats was shown to improve liver fibrosis and splanchnic hemodynamics without adverse systemic effects^[137]. However, in another study in mice using the CCl₄ model, V-PYRRO/NO significantly lowered mean arterial pressure making it less suitable for use in humans^[138].

AVE-9488(4-fluoro-*N*-indan-2-yl-benzamide) is a novel agent that up-regulates eNOS expression^[139]. Biecker *et al*^[139], reported that oral application of AVE 9488 ameliorated portal pressure by 24% in BDL rats, without any impact on the mean arterial pressure. Additional experiments confirmed that AVE 9488 increased hepatic eNOS protein synthesis, but not in the aortic and superior mesenteric artery^[139]. However, following 3-d use, AVE 9488 increased blood flow in the collateral circulation^[139].

Recently, an inorganic gold and silica nanoparticle mediated drug delivery system using SNAP (*S*-nitroso-*N*-acetyl-DL-penicillamine), an NO donor was reported^[140]. This system inhibited HSC proliferation and HSC tube formation, though the relevance of the latter to the situation *in vivo* is unclear. The methodology described however, does provide a novel approach to deliver NO into specific liver cell types. Whether this drug modulates PHT *in vivo* is unclear. Taken together, the data presented indicates that there are no liver-selective NO donors/drugs with demonstrated efficacy for the treatment of PHT.

CONCLUSION

NO plays a pivotal role in the pathogenesis of PHT. NO levels are differentially altered in cirrhosis, with reduced production in the intrahepatic circulation and increased NO production in the splanchnic bed. Ideally, a NO do-

nor or drug delivery system that selectively targets liver cells (HSCs or SECs) without actions on the systemic circulation is required to reduce PHT without adverse systemic effects.

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Cancer detection by ubiquitin carboxyl-terminal esterase L1 methylation in pancreatobiliary fluids

Norihiro Kato, Hiroyuki Yamamoto, Yasushi Adachi, Hirokazu Ohashi, Hiroaki Taniguchi, Hiromu Suzuki, Mayumi Nakazawa, Hiroyuki Kaneto, Shigeru Sasaki, Kohzoh Imai, Yasuhisa Shinomura

Norihiro Kato, Hiroyuki Yamamoto, Yasushi Adachi, Hirokazu Ohashi, Mayumi Nakazawa, Shigeru Sasaki, Yasuhisa Shinomura, First Department of Internal Medicine, Sapporo Medical University School of Medicine, Sapporo 060-8543, Japan

Hiroaki Taniguchi, Kohzoh Imai, The Section of Antibody, Vaccine and Molecular Targeted Therapy Research, The Institute of Medical Science, The University of Tokyo, Tokyo 108-8639, Japan
Hiromu Suzuki, Department of Molecular Biology, Sapporo Medical University School of Medicine, Sapporo 060-8556, Japan

Hiroyuki Kaneto, Division of Gastroenterology, Muroran City General Hospital, Muroran 051-8512, Japan

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Correspondence to: Hiroyuki Yamamoto, MD, FJSM, PhD, First Department of Internal Medicine, Sapporo Medical University School of Medicine, S-1, W-16, Chuo-ku, Sapporo 060-8543, Japan. h-yama@sapmed.ac.jp

Telephone: +81-11-6112111 Fax: +81-11-6112282

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Abstract

AIM: To evaluate the utility of measuring epigenetic alterations in pancreatic and biliary fluids in determining molecular markers for pancreatobiliary cancers.

METHODS: DNA was extracted from undiluted pancreatic and biliary fluids. As a surrogate for a genome-wide hypomethylation assay, levels of long interspersed nuclear element-1 (LINE-1) methylation were analyzed

using bisulfite pyrosequencing. CpG island hypermethylation of 10 tumor-associated genes, aryl-hydrocarbon receptor repressor, adenomatous polyposis coli, calcium channel, voltage dependent, T type α 1G subunit, insulin-like growth factor 2, O-6-methyl-guanine-DNA methyltransferase, neurogenin 1, CDKN2A, runt-related transcription factor 3 (RUNX3), secreted frizzled-related protein 1, and ubiquitin carboxyl-terminal esterase L1 (UCHL1), was analyzed using MethylLight. To examine the role of CpG methylation and histone deacetylation in the silencing of UCHL1, human gallbladder carcinoma cell lines and pancreatic carcinoma cell lines were treated with 2 or 5 μ mol/L 5-AZA-dC for 72 h or 100 nmol/L Trichostatin A for 24 h. After the treatment, UCHL1 expression was analyzed by real-time reverse transcription-polymerase chain reaction.

RESULTS: Pancreatobiliary cancers exhibited significantly lower LINE-1 methylation levels in pancreatic and biliary fluids than did noncancerous pancreatobiliary disease ($58.7\% \pm 4.3\%$ vs $61.7\% \pm 2.2\%$, $P = 0.027$; $53.8\% \pm 6.6\%$ vs $57.5\% \pm 1.7\%$, $P = 0.007$); however, LINE-1 hypomethylation was more evident in pancreatic cancer tissues than in pancreatic fluids ($45.4\% \pm 5.5\%$ vs $58.7\% \pm 4.3\%$, $P < 0.001$). CpG island hypermethylation of tumor-associated genes was detected at various frequencies, but it was not correlated with LINE-1 hypomethylation. Hypermethylation of the *UCHL1* gene was cancer-specific and most frequently detected in pancreatic (67%) or biliary (70%) fluids from patients with pancreatobiliary cancer. As a single marker, hypermethylation of the *UCHL1* gene in pancreatic and biliary fluids was most useful for the detection of pancreatic and pancreatobiliary cancers, respectively (100% specificity). Hypermethylation of the *UCHL1* and *RUNX3* genes in pancreatic and biliary fluids was the most useful combined marker for pancreatic (87% sensitivity and 100% specificity) and pancreatobiliary (97% sensitivity and 100% specificity) cancers. Treatment with a demethylating agent, 5-AZA-

2'-deoxycytidine, restored UCHL1 expression in pancreatobiliary cancer cell lines.

CONCLUSION: Our results suggest that hypermethylation of UCHL1 and RUNX3 in pancreatobiliary fluid might be useful for the diagnosis of pancreatobiliary cancers.

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Key words: Pancreatobiliary cancers; DNA methylation; Pancreatobiliary fluids; Ubiquitin carboxyl-terminal esterase L1; Runt-related transcription factor 3

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INTRODUCTION

Despite recent advances in diagnosis and treatment, the prognosis of patients with pancreatobiliary cancer is still poor. Surgical resection is possible in only a small proportion of patients^[1,2]. Consequently, elucidation of the biological characteristics of pancreatobiliary carcinomas is necessary to improve the prognosis of patients and to devise better treatment strategies. Various genetic and epigenetic alterations play a role in pancreatobiliary cancer^[3-6].

Two contradicting epigenetic alterations often coexist in cancer: global or genome-wide hypomethylation, which is mainly observed in repetitive sequences within the genome, and regional hypermethylation, which is frequently associated with CpG islands within gene promoters^[7]. Hypermethylation of CpG islands is a common feature of cancer that is associated with gene silencing^[7,8]. A number of genes are aberrantly methylated and silenced in pancreatobiliary cancer that are rarely methylated in non-neoplastic counterparts^[4-6,8], and this methylation is detectable in pancreatic and/or biliary fluids^[9-12]. The detection and/or quantification of these alterations in pancreatic and/or biliary fluids has promise for facilitating the differentiation of benign and malignant pancreatic and/or biliary strictures.

In contrast to CpG islands, repetitive DNA elements are normally heavily methylated in somatic tissues. Approximately 45% of the human genome is composed of repetitive sequences, including long interspersed nuclear elements (LINEs) and short interspersed nuclear elements^[13]. Liquid chromatography-mass spectrometry analysis has shown that levels of LINE-1 methylation strongly correlate with methyl cytosine content. This strong correlation enables LINE-1 methylation to be

used as a proxy for genome-wide methylation^[14]. Moreover, LINE-1 hypomethylation is known to occur during the development and progression of various human malignancies^[15,16]. Additionally, we recently reported that LINE-1 hypomethylation correlates significantly with the aggressiveness of gastrointestinal stromal tumors and that LINE-1 methylation could be a useful marker for risk assessment^[17]. Array comparative genomic hybridization analysis revealed a significant correlation between LINE-1 hypomethylation and chromosomal aberrations^[17]. Chromosomal gains and losses are also common in pancreatic and biliary cancers^[18,19]; their detection by fluorescence *in situ* hybridization modestly improves the prediction of cancer using biliary brushings^[20,21]. Gene hypomethylation has been reported to be a frequent epigenetic event in pancreatic cancer and is commonly associated with the overexpression of affected genes^[22]. A previous study showed that hypomethylation is more common in carcinoid tumors than in pancreatic endocrine tumors and is associated with clinicopathologic features, including lymph node metastasis, as well as genetic and epigenetic alterations in these tumors^[23].

To date, however, only a few groups have reported the methylation of LINE-1 and/or other repetitive sequences in pancreatobiliary cancer^[23,24], and there are no published studies analyzing LINE-1 methylation in pancreatic and/or biliary fluids. We found correlations between the level of LINE-1 methylation and the methylation of other repetitive sequences^[17]. In this study, we analyzed LINE-1 methylation and its relationship with hypermethylation of CpG islands in pancreatic and biliary fluids, and we investigated whether the detection and/or quantification of these epigenetic alterations can be used as markers for pancreatobiliary cancer.

MATERIALS AND METHODS

Clinical samples and cell lines

Pancreatic and biliary fluids were obtained at the time of endoscopic retrograde cholangiopancreatography (ERCP) and ERCP/percutaneous transhepatic cholangiography and drainage, respectively^[9-12]. Pancreatic and biliary fluids were collected from 30 and 48 patients, respectively. Informed consent was obtained from each subject. Tumors were classified according to the tumor-node-metastasis classification system of the International Union Against Cancer. The absence of cancer was based on clinical evaluation and follow-up of one or more years. Human gallbladder carcinoma cell lines TGBC1TKB and TGBC2TKB and pancreatic carcinoma cell lines PANC-1, PK-1, PK-45P and PK59 were purchased from Riken Cell Bank (Tsukuba, Japan). Cells were cultured in RPMI1640 or DMEM supplemented with 10% fetal bovine serum.

Extraction and bisulfite treatment of DNA

DNA was extracted from undiluted pancreatic and bili-

ary fluids using a DNeasy Tissue Kit. Extracted DNA was quantified, and 500 ng of DNA was modified with sodium bisulfite using a Methylamp™ DNA modification kit.

Bisulfite-pyrosequencing

Bisulfite-pyrosequencing analysis was performed as described previously^[17,24]. Briefly, polymerase chain reaction (PCR) was run in a 25 μ L volume containing 50 ng bisulfite-treated DNA, 1 \times MSP buffer, 1.25 mmol/L dNTP, 0.4 μ mol/L of each primer and 0.5 U of Jump-Start REDTaq DNA Polymerase. The PCR protocol for bisulfite sequencing entailed 5 min at 95 $^{\circ}$ C; 40 cycles of 1 min at 95 $^{\circ}$ C, 1 min at 60 $^{\circ}$ C and 1 min at 72 $^{\circ}$ C; and a 7 min final extension at 72 $^{\circ}$ C. The biotinylated PCR product was purified, made single-stranded and used as a template in a pyrosequencing reaction run according to the manufacturer's instructions. The PCR products were bound to Streptavidin Sepharose beads HP; then, the beads containing the immobilized PCR product were purified, washed and denatured using a 0.2 mol/L NaOH solution. After adding 0.3 μ mol/L sequencing primer to the purified PCR product, pyrosequencing was performed using a PSQ96MA system and Pyro Q-CpG software. Primer sequences for LINE-1 methylation were as previously described^[25].

MethylLight assay

The MethylLight assay was performed as previously described^[25,26]. Based on previous studies and our preliminary results, we analyzed 10 promoter CpG island loci: aryl-hydrocarbon receptor repressor, adenomatous polyposis coli, calcium channel, voltage dependent, T type α 1G subunit, insulin-like growth factor 2, O-6-methylguanine-DNA methyltransferase, neurogenin 1, CDKN2A, runt-related transcription factor 3 (RUNX3), secreted frizzled-related protein 1, and ubiquitin carboxyl-terminal esterase L1 (UCHL1). β -actin was used as the internal reference gene to quantify modified DNA levels in the samples^[26]. Primers, probes and the percentage of methylated reference (PMR, *i.e.*, the degree of methylation) were as previously described^[27-29]. We used a PMR cutoff of 4 to distinguish methylation-positive (PMR > 4) from methylation-negative (PMR \leq 4) samples based on previously validated data^[29].

5-AZA-2'-deoxycytidine and/or Trichostatin A treatment

To examine the role of CpG methylation and histone deacetylation in the silencing of UCHL1, cancer cells were treated with 2 or 5 mol/L 5-AZA-2'-deoxycytidine (5-AZA-dC) (Sigma) for 72 h or 100 nmol/L Trichostatin A (TSA) for 24 h. The cells were also treated with 2 μ mol/L 5-AZA-dC for 72 h, followed by 100 nmol/L TSA for an additional 24 h. The timing and sequencing of 5-AZA-dC and/or TSA were based on similar preliminary studies, as well as published studies^[30]. After the treatment, UCHL1 expression was analyzed by real-time RT-PCR.

Real-time quantitative PCR

Total RNA from cell lines was extracted using an extraction kit. cDNA was synthesized from 1 μ g of total RNA using SuperScript III reverse transcriptase with random hexamers. qRT-PCR was performed using the TaqMan real-time PCR system as previously described^[31]. A comparative threshold cycle (C_T) was used to determine the gene expression relative to the control (calibrator). Control reactions were performed without reverse transcriptase.

Statistical analysis

Mean methylation levels of LINE-1 were compared using *t* tests, the Welch test, or one-way ANOVA with a post hoc Games-Howell test. LINE-1 methylation levels and hypermethylation of tumor-associated genes were assessed for associations with clinicopathological parameters using *t* tests, the Welch test, the χ^2 two-tailed test, Fisher's exact test, the Mann-Whitney test, or one-way ANOVA. A *P* value < 0.05 was considered statistically significant. A *P* value between 0.05 and 0.10 was considered to indicate a trend toward an association.

RESULTS

Hypomethylation of LINE-1 in pancreatic and biliary fluids from patients with pancreatobiliary cancers

We performed bisulfite pyrosequencing to quantitatively analyze LINE-1 promoter methylation as a surrogate for genome-wide methylation (Figure 1). The mean level of LINE-1 methylation in pancreatic fluids was slightly but significantly lower in patients with pancreatic cancer than in those with noncancerous pancreatic disease (Figure 2). The mean level of LINE-1 methylation in biliary fluids was significantly lower in patients with pancreatobiliary cancer than in those with noncancerous pancreatobiliary disease. There was no correlation between LINE-1 methylation levels and clinicopathological characteristics in patients with pancreatic or pancreatobiliary cancers. The mean level of LINE-1 methylation in pancreatic cancer tissues was significantly lower than that in pancreatic fluids from the corresponding patients.

Analysis of CpG island hypermethylation of tumor-related genes

We next assessed the methylation levels of CpG islands of well-characterized tumor-suppressor and tumor-associated genes. Using MethylLight assays, we analyzed 10 genes. CpG island hypermethylation of tumor-associated genes was detected at various frequencies. The results are summarized on the basis of each individual marker (Tables 1 and 2). Methylation of several genes, such as UCHL1 and RUNX3, was cancer-specific (Figure 3). Some cancer samples showed methylation in many genes, suggesting that these cancers have a CpG island hypermethylator phenotype. We failed to find any significant correlation between the methylation of these genes and clinicopathological features or between CpG island meth-

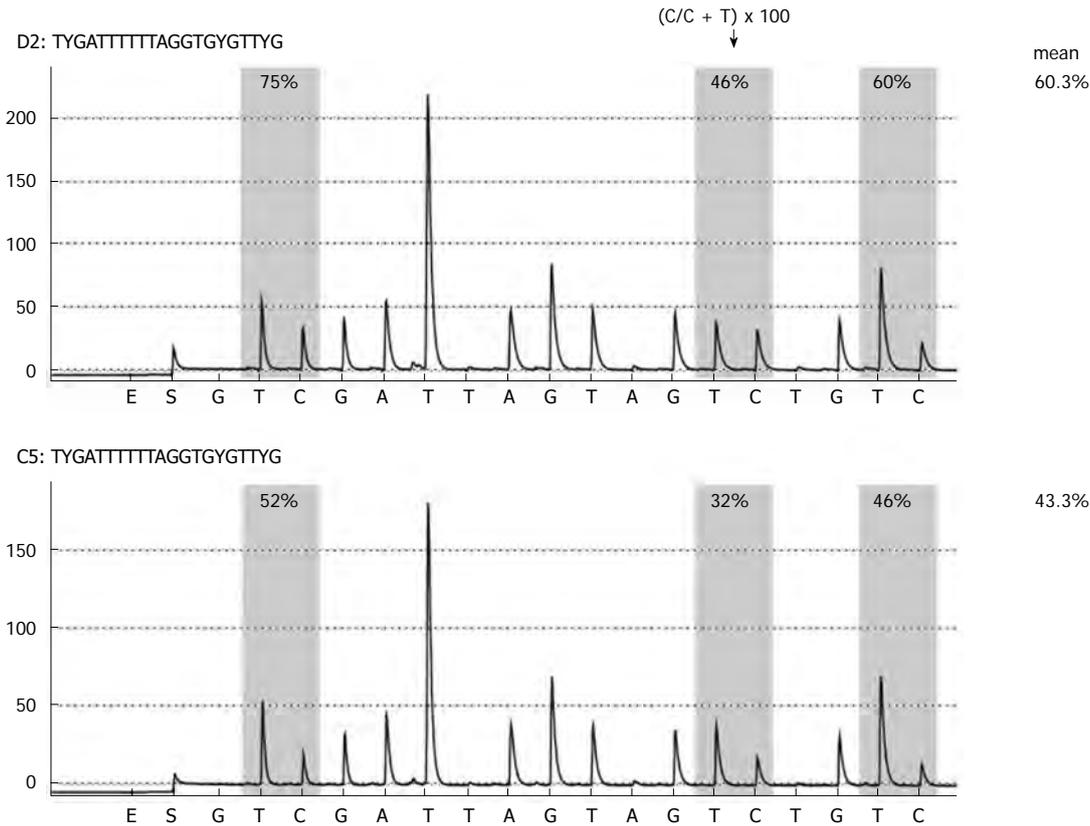


Figure 1 Representative long interspersed nuclear element-1 methylation analysis by pyrosequencing. Long interspersed nuclear element-1 methylation analysis in biliary fluids from patients with noncancerous pancreatic disease (chronic pancreatitis, upper panel) and pancreatic cancer (lower panel) is shown.

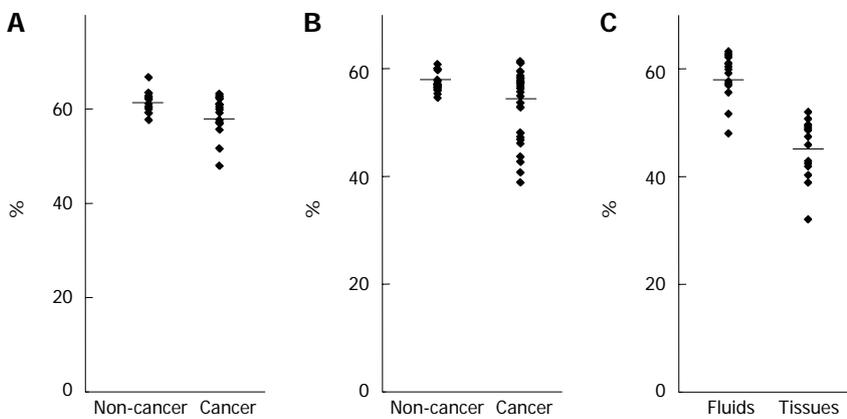


Figure 2 Analysis of long interspersed nuclear element-1 methylation levels using pancreatobiliary fluids. A: Comparison of the long interspersed nuclear element-1 (LINE-1) methylation levels in pancreatic fluids between pancreatic cancer and noncancerous pancreatic disease; B: Comparison of the LINE-1 methylation levels in biliary fluids between pancreatobiliary cancer and noncancerous pancreatobiliary disease; C: Comparison of the LINE-1 methylation levels in pancreatic cancer between pancreatic fluids and tissues.

ylation and LINE-1 methylation levels.

Among the cancer-specific hypermethylated genes in pancreatic fluids, the *UCHL1* gene was most frequently (67%) detected in pancreatic cancer and served as the most useful single marker for the detection of pancreatic cancer (Figure 3A). Hypermethylation of the *UCHL1* and *RUNX3* genes in pancreatic fluids was the most useful combined marker for the detection of pancreatic cancer.

Among the cancer-specific hypermethylated genes in biliary fluids, the *UCHL1* gene was most frequently (70%) detected in pancreatobiliary cancer and served as the most useful single marker for the detection of pancreatobiliary cancer (Figure 3C). Hypermethylation of the *UCHL1* and *RUNX3* genes in biliary fluids was the most useful combined marker for detection of pancreatobiliary cancer.

The pancreatic and biliary fluids obtained from pan-

Table 1 CpG islands hypermethylation of tumor-associated genes in pancreatic fluids

| | Age (yr) | Sex | Stage | UCHL1 | RUNX3 | CDKN2A | IGF2 | CACNA1G | AHRR | SFRP1 | MGMT | APC | NEUROG1 |
|------|----------|-----|-------|-------|-------|--------|------|---------|------|-------|------|-----|---------|
| NC1 | 68 | M | | MN | MN | MN | MN | MN | MN | MP | MP | MN | MN |
| NC2 | 65 | M | | MN | MN | MN | MN | MP | MN | MN | MN | MN | MN |
| NC3 | 59 | M | | MN | MN | MP | MN | MN | MN | MN | MN | MN | MP |
| NC4 | 70 | F | | MN | MN | MN | MP | MN | MN | MN | MN | MN | MN |
| NC5 | 43 | F | | MN | MN | MN | MN | MN | MN | MN | MP | MN | MN |
| NC6 | 55 | M | | MN | MN | MN | MN | MP | MN | MN | MN | MN | MN |
| NC7 | 49 | M | | MN | MN | MN | MN | MN | MP | MN | MN | MP | MN |
| | | | | MN | MN | MN | MN | MN | MN | MN | MN | MN | MN |
| IP1 | 71 | F | | MN | MN | MP | MN | MN | MN | MN | MN | MN | MN |
| IP2 | 79 | F | | MN | MN | MN | MN | MN | MN | MN | MN | MN | MP |
| IP3 | 76 | M | | MN | MP | MN | MN | MN | MN | MN | MP | MN | MN |
| IP4 | 75 | F | | MN | MN | MN | MP | MN | MP | MP | MN | MP | MN |
| IP5 | 84 | F | | MN | MN | MN | MN | MN | MN | MP | MN | MN | MN |
| IP6 | 52 | M | | MN | MN | MP | MN | MN | MN | MP | MP | MN | MN |
| IP7 | 62 | M | | MN | MN | MN | MP | MN | MN | MN | MP | MP | MN |
| IP8 | 63 | M | | MN | MN | MP | MN | MN | MN | MN | MP | MN | MN |
| | | | | MN | MN | MN | MN | MN | MN | MN | MN | MN | MN |
| PC1 | 66 | F | II A | MP | MN | MN | MN | MN | MN | MN | MP | MN | MN |
| PC2 | 54 | F | III | MN | MP | MN | MN | MN | MN | MN | MN | MN | MN |
| PC3 | 66 | M | II B | MP | MP | MN | MN | MN | MN | MP | MN | MP | MP |
| PC4 | 67 | M | II B | MP | MN | MN | MN | MP | MN | MN | MN | MP | MN |
| PC5 | 71 | M | II B | MN | MP | MP | MN | MN | MN | MN | MN | MN | MN |
| PC6 | 49 | F | II A | MP | MP | MN | MN | MN | MN | MP | MN | MP | MP |
| PC7 | 73 | F | II B | MP | MN | MN | MN | MN | MP | MP | MN | MN | MN |
| PC8 | 75 | M | III | MP | MP | MN | MN | MN | MN | MN | MN | MP | MN |
| PC9 | 59 | M | II B | MN | MN | MN | MN | MP | MN | MP | MP | MP | MP |
| PC10 | 78 | F | II A | MP | MP | MP | MN | MN | MN | MP | MP | MN | MP |
| PC11 | 75 | F | II B | MN | MN | MN | MP | MN | MN | MP | MN | MN | MN |
| PC12 | 66 | M | IV | MP | MP | MP | MN | MN | MN | MP | MN | MP | MP |
| PC13 | 70 | F | IV | MP | MN | MN | MN | MN | MP | MP | MP | MP | MP |
| PC14 | 68 | M | I B | MN | MP | MN | MN | MN | MN | MN | MP | MN | MN |
| PC15 | 62 | M | II B | MP | MN | MP | MN | MN | MN | MN | MN | MN | MN |

NC: Non-cancer; IP: Intraductal papillary and mucinous pancreatic tumour; pancreatic cancer (PC) 1, PC5, PC7, PC8 and PC13 are identical in Tables 1 and 2. MP: Methylation-positive; MN: Methylation-negative.

creatic cancer patients were compared with regard to the methylation patterns of 10 tumor-associated genes ($n = 5$). The methylation patterns were similar in both the pancreatic and biliary fluids from the same patients (Tables 1 and 2). The methylation patterns of the *UCHL1* and *RUNX3* genes were identical in the pancreatic and biliary fluids from the same patients.

Reactivation of *UCHL1* expression by 5-AZA-dC/Trichostatin A treatment in pancreatobiliary cancer cell lines

To further examine the role of CpG methylation and histone deacetylation in silencing *UCHL1*, cancer cells were treated with 5-AZA-dC and/or TSA. 5-AZA-dC restored *UCHL1* expression, and combined treatment with 5-AZA-dC and TSA restored *UCHL1* expression synergistically at the mRNA level in pancreatobiliary cancer cell lines (Figure 4 and data not shown). TSA alone did not restore *UCHL1* expression in cell lines.

DISCUSSION

In the present study, we found that the levels of methylation of LINE-1 were reduced in pancreatobiliary cancers compared to those in noncancerous pancreatobiliary

disease. Genome-wide hypomethylation is known to be a common feature of human cancer, and genome-wide hypomethylation has recently been studied in various human malignancies using LINE-1 and other repetitive sequences as surrogates. Our results suggest that pancreatobiliary cancers exhibit a pattern of genome-wide hypomethylation that can be detected using pancreatic and biliary fluids.

Genome-wide hypomethylation is thought to be associated with tumor malignancy through a variety of mechanisms. For example, global hypomethylation is associated with genomic instability^[32], which may confer a poor prognosis. Hypomethylation can also lead to the activation of proto-oncogenes, endogenous retroviruses or transposable elements; such transcriptional dysregulation could affect tumor aggressiveness. Although we found correlations between the level of LINE-1 methylation and the methylation of other repetitive sequences^[17], it is possible that there are functional and/or biological differences in the regulation of repetitive DNA sequences. Further analysis is necessary to clarify the role of genome-wide hypomethylation in pancreatobiliary cancers.

The LINE-1 hypomethylation in pancreatic cancers determined using pancreatic fluids was less significant than that determined using tissue samples, although the

Table 2 CpG islands hypermethylation of tumor-associated genes in biliary fluids

| | Age (yr) | Sex | Stage | <i>UCHL1</i> | <i>RUNX3</i> | <i>CDKN2A</i> | <i>IGF2</i> | <i>CACNA1G</i> | <i>AHRR</i> | <i>SFRP1</i> | <i>MGMT</i> | <i>APC</i> | <i>NEUROG1</i> |
|------|----------|-----|-------|--------------|--------------|---------------|-------------|----------------|-------------|--------------|-------------|------------|----------------|
| NC8 | 77 | M | | MN | MN | MN | MN | MN | MN | MN | MP | MN | MN |
| NC9 | 69 | F | | MN | MN | MN | MN | MN | MN | MP | MN | MN | MN |
| NC10 | 76 | M | | MN | MN | MN | MN | MN | MN | MN | MP | MN | MN |
| NC11 | 58 | M | | MN | MN | MN | MN | MN | MN | MN | MN | MN | MN |
| NC12 | 41 | M | | MN | MN | MN | MN | MN | MP | MN | MP | MN | MN |
| NC13 | 66 | M | | MN | MN | MN | MN | MN | MP | MP | MP | MP | MP |
| NC14 | 69 | F | | MN | MN | MN | MN | MN | MP | MP | MP | MN | MP |
| NC15 | 71 | F | | MN | MN | MN | MN | MN | MN | MN | MP | MP | MN |
| NC16 | 59 | M | | MN | MN | MN | MN | MN | MP | MP | MN | MN | MN |
| NC17 | 54 | F | | MN | MN | MN | MN | MN | MN | MN | MN | MP | MP |
| NC18 | 67 | M | | MN | MN | MN | MN | MN | MP | MP | MN | MN | MN |
| NC19 | 85 | M | | MN | MN | MN | MN | MN | MN | MN | MN | MN | MN |
| NC20 | 73 | F | | MN | MN | MN | MN | MN | MN | MP | MN | MN | MN |
| NC21 | 80 | F | | MN | MN | MN | MN | MN | MN | MN | MP | MN | MN |
| NC22 | 74 | F | | MN | MN | MN | MN | MN | MN | MN | MP | MN | MN |
| NC23 | 52 | M | | MN | MN | MN | MN | MN | MN | MN | MN | MN | MN |
| NC24 | 64 | M | | MN | MN | MN | MN | MN | MN | MP | MP | MP | MN |
| NC25 | 64 | F | | MN | MN | MN | MN | MN | MP | MP | MN | MN | MN |
| GB1 | 75 | F | III B | MP | MP | MP | MP | MP | MP | MN | MN | MN | MN |
| GB2 | 76 | F | III B | MN | MP | MP | MP | MP | MP | MN | MN | MP | MN |
| GB3 | 62 | M | III A | MP | MP | MP | MP | MN | MN | MP | MP | MN | MN |
| GB4 | 67 | M | IV A | MN | MP | MN | MN | MN | MN | MN | MN | MN | MN |
| GB5 | 59 | F | III B | MP | MN | MP | MN | MN | MP | MP | MN | MP | MP |
| GB6 | 63 | F | II | MP | MN | MP | MN | MN | MP | MP | MN | MP | MP |
| GB7 | 77 | M | I | MN | MP | MP | MN | MN | MN | MP | MP | MN | MN |
| GB8 | 78 | M | III A | MP | MN | MP | MN | MN | MN | MP | MP | MN | MN |
| BC1 | 73 | M | I | MN | MP | MN | MP | MN | MP | MP | MN | MN | MN |
| BC2 | 80 | F | II A | MP | MP | MN | MN | MN | MP | MP | MP | MN | MN |
| BC3 | 71 | M | II B | MP | MN | MN | MN | MP | MP | MP | MN | MN | MN |
| BC4 | 75 | M | III | MP | MN | MN | MN | MN | MN | MP | MP | MN | MN |
| BC5 | 77 | M | II B | MN | MP | MN | MN | MN | MN | MN | MP | MN | MN |
| BC6 | 65 | M | II A | MP | MN | MP | MN | MN | MN | MN | MN | MN | MN |
| BC7 | 72 | M | II A | MP | MN | MN | MN | MN | MN | MP | MN | MN | MN |
| BC8 | 73 | F | IV | MP | MP | MN | MN | MN | MP | MN | MN | MN | MN |
| BC9 | 76 | F | II A | MP | MP | MN | MP | MP | MP | MP | MN | MN | MN |
| BC10 | 74 | M | II B | MP | MN | MN | MN | MN | MP | MN | MP | MN | MP |
| BC11 | 66 | M | III | MP | MN | MN | MN | MN | MN | MN | MN | MP | MN |
| BC12 | 58 | F | II B | MN | MP | MN | MN | MN | MN | MN | MN | MN | MN |
| PC1 | 66 | F | II A | MP | MN | MN | MN | MN | MN | MP | MP | MN | MN |
| PC5 | 71 | M | II B | MN | MP | MP | MN | MN | MN | MN | MP | MN | MN |
| PC7 | 73 | F | II B | MP | MN | MN | MN | MN | MP | MN | MN | MN | MN |
| PC8 | 75 | M | III | MP | MP | MN | MN | MN | MN | MN | MP | MN | MN |
| PC13 | 70 | F | IV | MP | MN | MN | MN | MN | MP | MP | MP | MP | MN |
| PC16 | 59 | M | II A | MP | MP | MN | MN | MP | MN | MN | MN | MN | MN |
| PC17 | 80 | F | II B | MP | MP | MP | MP | MN | MP | MP | MN | MN | MN |
| PC18 | 67 | M | I B | MN | MP | MN | MN | MN | MP | MN | MP | MN | MN |
| PC19 | 63 | M | II A | MP | MN | MN | MN | MN | MN | MN | MP | MP | MN |
| PC20 | 78 | F | II B | MN | MN | MP | MN | MN | MP | MN | MN | MN | MN |

NC: Non-cancer; GB: Gallbladder cancer; BC: Biliary cancer; pancreatic cancer (PC) 1, PC5, PC7 and PC8 and PC13 are identical in Tables 1 and 2. MP: Methylation-positive; MN: Methylation-negative.

number of samples analyzed was limited. It is possible that some of the pancreatic fluid samples did not contain sufficient concentrations of cancer DNA^[12]. Given the relatively poor diagnostic yield of cytology in this setting, a problem that is likely to be related to the highly scirrhous nature of pancreatic ductal adenocarcinomas, sample adequacy is likely to be one of the limiting factors in the molecular analysis of these samples^[12]. Serum LINE-1 hypomethylation has been reported to be a potential prognostic marker for hepatocellular carcinoma^[33]. It would be interesting to analyze serum LINE-1 meth-

ylation levels in patients with pancreatobiliary cancers.

CpG island hypermethylation of tumor-associated genes was detected at various frequencies in pancreatobiliary cancers using pancreatobiliary fluids. Although genome-wide hypomethylation and regional hypermethylation of 5' CpG islands are common features of neoplasias, the link between the two remains controversial^[17]. In the current study, we did not find a significant correlation between 5' CpG island hypermethylation of tumor-associated genes and global hypomethylation.

Hypermethylation of the *UCHL1* gene was cancer-

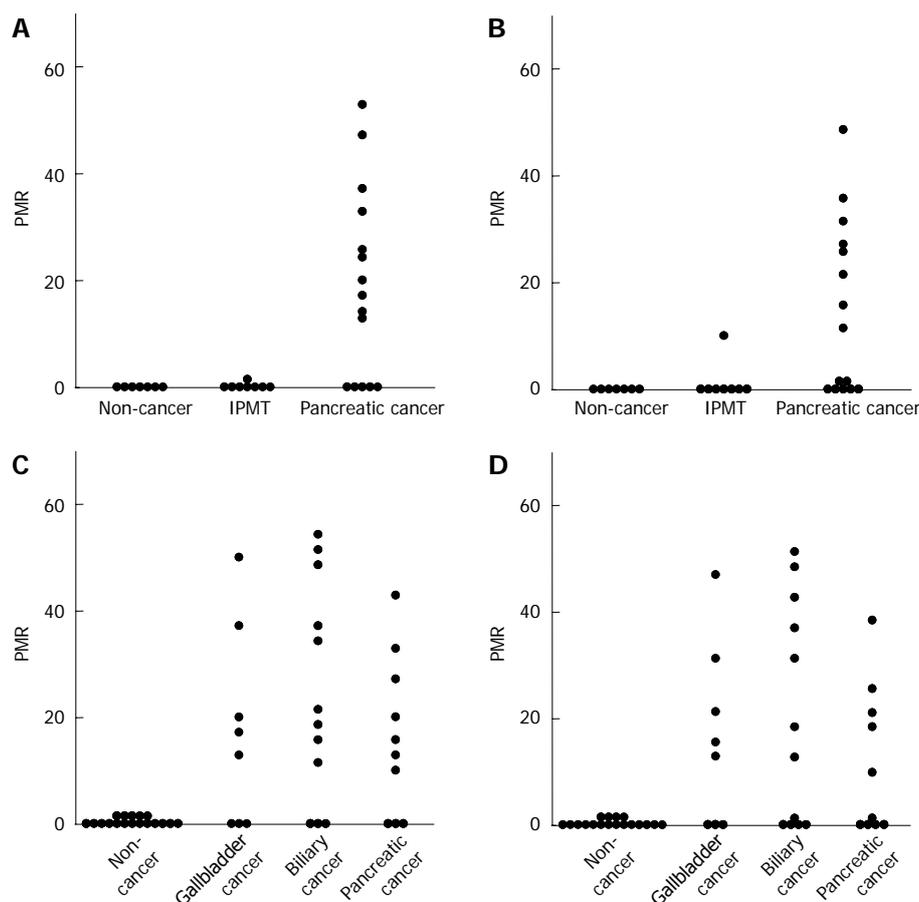


Figure 3 Analysis of methylation levels of ubiquitin carboxyl-terminal esterase L1 and runt-related transcription factor 3 using pancreatobiliary fluids. Comparison of the ubiquitin carboxyl-terminal esterase L1 (UCHL1) (A) and runt-related transcription factor 3 (RUNX3) (B) methylation levels in pancreatic fluids between pancreatic cancer and noncancerous pancreatic disease; Comparison of the UCHL1 (C) and RUNX3 (D) methylation levels in biliary fluids between pancreatobiliary cancer and noncancerous pancreatobiliary disease. PMR: Percentage of methylated reference.

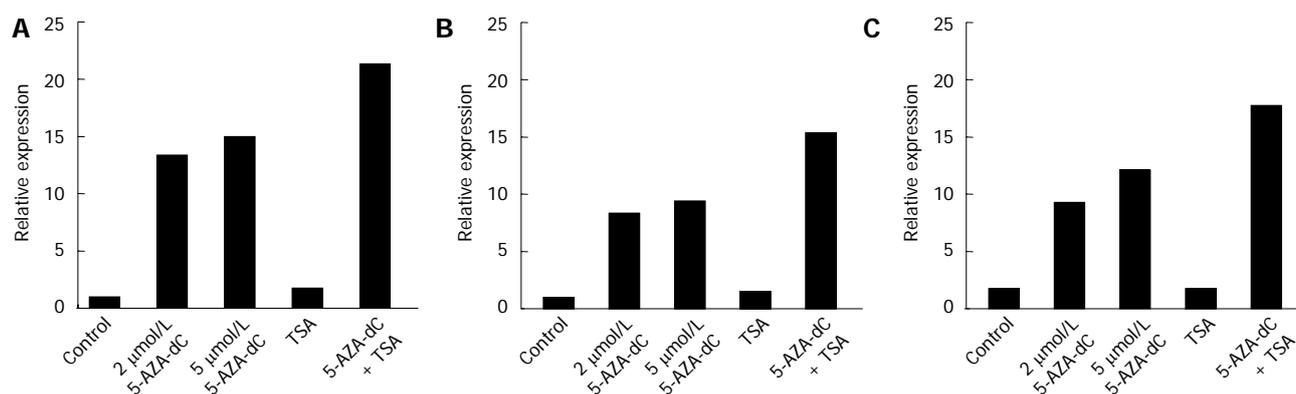


Figure 4 Reactivation of ubiquitin carboxyl-terminal esterase L1 by 5-AZA-2'-deoxycytidine and/or Trichostatin A treatment in pancreatic and biliary cancer cell lines. A: PK-1 cells; B: PK45P cells; C: TGBC1TKB cells. To examine the roles of CpG methylation and histone deacetylation in the silencing of ubiquitin carboxyl-terminal esterase L1, cancer cells were treated with 2 or 5 μmol/L 5-AZA-2'-deoxycytidine (5-AZA-dC) for 72 h or 100 nmol/L Trichostatin A (TSA) for 24 h. The cells were also treated with 2 μmol/L 5-AZA-dC for 72 h, followed by 100 nmol/L TSA for an additional 24 h.

specific and most frequently detected in pancreatobiliary cancers. Hypermethylation of the *UCHL1* gene in pancreatic and biliary fluids was the most useful single marker of pancreatic and pancreatobiliary cancers, respectively. Hypermethylation of the *UCHL1* and *RUNX3* genes in pancreatic and biliary fluids was the most useful combined marker for pancreatic and pancreatobiliary cancers,

respectively. Epigenetic inactivation of *UCHL1* has been reported in a variety of human cancers^[34]. Epigenetic inactivation of *RUNX3* is known to play an important role in the pathogenesis of pancreatobiliary cancer^[35,36].

LINE-1 and SAT2 methylation levels have been reported to be significantly lower in extrahepatic cholangiocarcinoma than in normal duct and biliary intraepithelial

neoplasias (BilINs). BilINs showed a decrease of SAT2 methylation levels, but no decrease of LINE-1 methylation levels was found compared to those in normal samples^[24]. Most of the cancer-specific CpG island hypermethylation is thought to occur in the BilIN stage, before LINE-1 hypomethylation. Our results also suggest that CpG island hypermethylation analyzed in pancreatobiliary fluids is more useful than LINE-1 methylation for the detection of pancreatobiliary cancer.

Importantly, the methylation patterns of 10 tumor-associated genes were similar in both the pancreatic and biliary fluids from the same patients with pancreatic cancer. Moreover, the methylation patterns of the *UCHL1* and *RUNX3* genes were identical in both the pancreatic and biliary fluids from the same patients. These results further support the notion that hypermethylation of *UCHL1* and *RUNX3* in pancreatobiliary fluids is a useful marker for the detection of pancreatobiliary cancer.

To confirm the role of epigenetic alterations in transcriptional repression of the *UCHL1* gene, we treated pancreatobiliary cancer cell lines, in which *UCHL1* was methylated, with 5-AZA-dC alone or in combination with TSA. Treatment with 5-AZA-dC restored the *UCHL1* expression in cancer cell lines. Moreover, combined treatment with 5-AZA-dC and TSA restored *UCHL1* expression synergistically, indicating that CpG methylation and histone deacetylation play important roles in silencing the *UCHL1* gene.

Not only the clinical utility but also the pathobiological effects of nucleic acids in circulation (nucleosomes, DNA, RNA, microRNA *etc.*) are receiving increasing attention^[37,38]. Further analysis is necessary to clarify the possible detrimental effects of nucleic acids in the tumor microenvironment, including the contribution of methylated DNA in pancreatobiliary fluids to disease progression.

In conclusion, our results suggest that hypermethylation of the *UCHL1* gene plays a key role in the pathogenesis of pancreatobiliary cancers and that detection of hypermethylation of *UCHL1* and *RUNX3* in pancreatobiliary fluids is useful for the diagnosis of these malignancies. Our MethyLight panel (*UCHL1* and *RUNX3*) is simple and accurate for differentiating between neoplastic and non-neoplastic samples and compares favorably with other quantitative MSP panels and with the identification of mutant *KRAS* or telomerase, which have been used previously to differentiate between malignant and benign pancreatic samples^[39,40]. Moreover, newer assays that can detect low concentrations of mutations in pancreatic juice^[41], as well as novel assays and technologies, are likely to improve the detection of low concentrations of mutant DNA for cancer diagnosis in the future. Although we focused on epigenetic alterations in the current study, a combination of highly specific epigenetic and genetic markers might provide the best diagnostic utility.

with pancreatobiliary cancer is still poor. Elucidation of the biological characteristics of these carcinomas has become necessary to improve the prognosis of patients and to devise better treatment strategies.

Research frontiers

Roles of epigenetic alterations in pancreatobiliary cancer are receiving increasing attention. Two contradicting epigenetic alterations often coexist in cancer: global or genome-wide hypomethylation, which is mainly observed in repetitive sequences within the genome, and regional hypermethylation, which is frequently associated with CpG islands within gene promoters. Long interspersed nuclear element-1 (LINE-1) methylation status and its relationship with the hypermethylation of CpG islands in pancreatic and biliary fluids taken from patients with pancreatobiliary cancer is not known.

Innovations and breakthroughs

This is the first study to report that pancreatobiliary cancers exhibit a pattern of genome-wide hypomethylation that can be detected using pancreatic and biliary fluids. CpG island hypermethylation of tumor-associated genes was detected at various frequencies. Hypermethylation of the ubiquitin carboxyl-terminal esterase L1 (*UCHL1*) gene may play a key role in the pathogenesis of pancreatobiliary cancers.

Applications

Hypermethylation of *UCHL1* and runt-related transcription factor 3 in pancreatobiliary fluids might be useful for the diagnosis of pancreatobiliary cancers. A combination of highly specific epigenetic and genetic markers might provide the best diagnostic utility.

Terminology

LINEs are 6-8 kb long, GC-poor sequences encoding an RNA-binding protein and a reverse transcriptase/endonuclease; these sequences constitute approximately 20% of the human genome. LINE-1 elements are most abundant, and over half a million copies of these elements are present in the human genome; *UCHL1*, which is also known as PARK5/PGP9.5, is a member of the ubiquitin carboxy terminal hydrolase family targeting the ubiquitin-dependent protein degradation pathway. With both ubiquitin hydrolase and dimerization-dependent ubiquitin ligase activities, *UCHL1* plays important roles in multiple cellular processes. *UCHL1* is a tumor-suppressor gene that is inactivated by promoter methylation or gene deletion in several types of human cancers.

Peer review

The presence of such high amounts of methylated DNA in pancreatobiliary fluid is intriguing. The study has translational significance.

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COMMENTS

Background

Despite recent advances in diagnosis and treatment, the prognosis of patients

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Short-type single balloon enteroscope for endoscopic retrograde cholangiopancreatography with altered gastrointestinal anatomy

Hiroshi Yamauchi, Mitsuhiro Kida, Kosuke Okuwaki, Shiro Miyazawa, Tomohisa Iwai, Miyoko Takezawa, Hidehiko Kikuchi, Maya Watanabe, Hiroshi Imaizumi, Wasaburo Koizumi

Hiroshi Yamauchi, Mitsuhiro Kida, Kosuke Okuwaki, Shiro Miyazawa, Tomohisa Iwai, Miyoko Takezawa, Hidehiko Kikuchi, Maya Watanabe, Hiroshi Imaizumi, Wasaburo Koizumi, Department of Gastroenterology, Kitasato University East Hospital, Sagami-hara, Kanagawa 252-0380, Japan

Author contributions: Yamauchi H and Kida M contributed equally to this work; Yamauchi H and Kida M designed study concept; Yamauchi H and Okuwaki K performed the research; Yamauchi H, Kida M, Okuwaki K, Miyazawa S, Iwai T, Takezawa M, Kikuchi H, Watanabe M and Imaizumi H contributed technical support; Kida M and Koizumi W contributed critical revision of the manuscript for important intellectual content; Yamauchi H analyzed the data and wrote the paper.

Supported by A Prototype Single Balloon Enteroscope from Olympus Medical Systems, Tokyo, Japan

Correspondence to: Dr. Hiroshi Yamauchi, Department of Gastroenterology, Kitasato University East Hospital, 2-1-1 Asamizodai, Minami-ku, Sagami-hara, Kanagawa 252-0380,

Japan. yhiroshi@kitasato-u.ac.jp

Telephone: +81-42-7489111 Fax: +81-42-7498690

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Abstract

AIM: To evaluate the effectiveness of a short-type single-balloon-enteroscope (SBE) for endoscopic retrograde cholangiopancreatography (ERCP) in patients with a reconstructed intestine.

METHODS: Short-type SBE was developed to perform ERCP in postoperative patients with a reconstructed intestine. Short-type SBE is a direct-viewing endoscope with the following specifications: working length, 1520 mm; total length, 1840 mm; channel diameter, 3.2 mm. In addition, short-type SBE has a water-jet channel. The study group comprised 22 patients who underwent

31 sessions of short-type SBE-assisted ERCP from June 2011 through May 2012. Reconstruction was performed by Billroth-II (B-II) gastrectomy in 6 patients (8 sessions), Roux-en-Y (R-Y) gastrectomy in 14 patients (21 sessions), and R-Y hepaticojejunostomy in 2 patients (2 sessions). We retrospectively studied the rate of reaching the blind end (papilla of Vater or choledochojejunal anastomosis), mean time required to reach the blind end, diagnostic success rate (defined as the rate of successfully imaging the bile and pancreatic ducts), therapeutic success rate (defined as the rate of successfully completing endoscopic treatment), mean procedure time, and complications.

RESULTS: Among the 31 sessions of ERCP, the rate of reaching the blind end was 88% in B-II gastrectomy, 91% in R-Y gastrectomy, and 100% in R-Y hepaticojejunostomy. The mean time required to reach the papilla was 18.3 min in B-II gastrectomy, 21.1 min in R-Y gastrectomy, and 32.5 min in R-Y hepaticojejunostomy. The diagnostic success rates in all patients and those with an intact papilla were respectively 86% and 86% in B-II gastrectomy, 90% and 87% in R-Y gastrectomy, and 100% in R-Y hepaticojejunostomy. The therapeutic success rates in all patients and those with an intact papilla were respectively 100% and 100% in B-II gastrectomy, 94% and 92% in R-Y gastrectomy, and 100% in R-Y hepaticojejunostomy. Because the channel diameter was 3.2 mm, stone extraction could be performed with a wire-guided basket in 12 sessions, and wire-guided intraductal ultrasonography could be performed in 8 sessions. As for complications, hyperamylasemia (defined as a rise in serum amylase levels to more than 3 times the upper limit of normal) occurred in 1 patient (7 sessions) with a B-II gastrectomy and 4 patients (19 sessions) with an R-Y gastrectomy. After ERCP in patients with an R-Y gastrectomy, 2 patients (19 sessions) had pancreatitis, 1 patient (21 sessions) had

gastrointestinal perforation, and 1 patient (19 sessions) had papillary bleeding. Pancreatitis and bleeding were both mild. Gastrointestinal perforation improved after conservative treatment.

CONCLUSION: Short-type SBE is effective for ERCP in patients with a reconstructed intestine and allows most conventional ERCP devices to be used.

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Key words: Endoscopic retrograde cholangiopancreatography; Single-balloon-enteroscope; Short type; Billroth-II gastrectomy; Roux-en-Y gastrectomy

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INTRODUCTION

Endoscopic retrograde cholangiopancreatography (ERCP) is often difficult to perform in postoperative patients with a reconstructed intestine because of altered anatomical features. However, the advent of balloon enteroscopy has improved the outcome of ERCP in patients with a reconstructed intestine, and many recent studies have found this procedure to be useful^[1-20]. However, during ERCP, several problems remain to be solved. For example, these scopes do not have a lifting device, and the narrow channel diameter precludes the use of wire-guided devices. Moreover, the development of special devices is required because a single balloon enteroscope (SBE) has a long working length. Therefore, the development of a balloon endoscope with a short working length and a large working channel is eagerly anticipated^[9]. The development of short-type double balloon enteroscopy (DBE) (models EC-450B15 and EI-530B, Fujifilm, Osaka, Japan) allowed many ERCP devices to be used. However, because the channel diameter of most conventional balloon enteroscopy is 2.8 mm, wire-guided devices cannot be used. We describe our experience with 22 postoperative patients with a reconstructed intestine who underwent ERCP (31 sessions) using a short-type SBE that was specifically developed for ERCP (model SIF-Y0004; Olympus Medical Systems, Tokyo, Japan). To the best of our knowledge, this is the first report to describe ERCP performed with this SBE.

MATERIALS AND METHODS

Patients

From June 2011 to May 2012, 37 sessions of short-type SBE-assisted ERCP were performed in 28 postoperative

Table 1 Baseline characteristics of the patients *n* (%)

| | Billroth-II gastrectomy (<i>n</i> = 8) | Roux-en-Y gastrectomy (<i>n</i> = 21) | Roux-en-Y hepatico- jejunostomy (<i>n</i> = 2) | Total No. of SIF-Y0004- assisted ERCP (<i>n</i> = 31) |
|--|---|--|--|---|
| Age ¹ , mean (range), yr | 75.4 (62-82) | 71.3 (50-85) | 65 (65) | 72.0 (50-85) |
| Sex ² : | | | | |
| Male | 5 (83) | 13 (93) | 2 (100) | 20 (91) |
| Female | 1 (17) | 1 (7) | 0 (0) | 2 (9) |
| Reasons for surgery ¹ | | | | |
| Gastric ulcer | 2 (25) | 0 (0) | 0 (0) | 2 (6.5) |
| Duodenum ulcer | 2 (25) | 2 (9.5) | 0 (0) | 4 (13) |
| Carcinoma of the stomach | 4 (50) | 19 (90.5) | 0 (0) | 23 (74) |
| Carcinoma of the pancreas | 0 (0) | 0 (0) | 2 (100) | 2 (6.5) |
| Reasons for ERCP ¹ | | | | |
| AOSC (due to CBD stone) | 1 (12.5) | 1 (5) | 0 (0) | 2 (6.5) |
| Carcinoma of the pancreas | 2 (25) | 0 (0) | 0 (0) | 2 (6.5) |
| Carcinoma of the gallbladder | 1 (12.5) | 0 (0) | 0 (0) | 1 (3.3) |
| CBD stone | 3 (37.5) | 14 (66) | 2 (100) | 19 (61) |
| IPMN | 0 (0) | 1 (5) | 0 (0) | 1 (3.3) |
| IPNB | 0 (0) | 1 (5) | 0 (0) | 1 (3.3) |
| suspected | | | | |
| Metastasis of L/N in porta hepatis | 1 (12.5) | 4 (19) | 0 (0) | 5 (16) |

¹No. of procedures; ²No. of patients. AOSC: Acute obstructive suppurative cholangitis; CBD: Common bile duct; IPMN: Intraductal papillary mucinous neoplasm; IPNB: Intraductal papillary neoplasm of the bile duct; ERCP: Endoscopic retrograde cholangiopancreatography.

patients who had a reconstructed intestine in our hospital. Six patients were excluded from the study because the passage of the scope to the blind end (papilla of Vater or choledochojejunal anastomosis) was precluded by proximal narrowing or occlusion of the intestine caused by tumor invasion. The remaining 22 patients (31 sessions) were studied. Reconstruction was performed by Billroth II (B-II) gastrectomy in six patients (eight sessions), Roux-en-Y (R-Y) gastrectomy in 14 (21 sessions), and R-Y hepaticojejunostomy in two (two sessions). B-II gastrectomy was performed only in patients with a long afferent loop or Braun's anastomosis in whom a direct-viewing multipurpose endoscope could not reach the blind end. Table 1 shows the detailed demographic characteristics of the patients.

Endoscope and ERCP Instruments

The SIF-Y0004 is a direct-viewing endoscope with the following specifications: angle of view, 120°; bending section, up 180°, down 180°, right 160°, and left 160°; working length, 1520 mm; total length, 1840 mm; outer diameter of distal end, 9.2 mm; outer diameter of insertion end, 9.2 mm; and working channel diameter, 3.2 mm. The SIF-Y0004 has a water-jet channel.

A sliding tube with a working length of 880 mm was

Table 2 Specifications of two types of single-balloon endoscopes

| | SIF-Y0004 | SIF-Q260 |
|-------------------------------|-----------------|-----------------|
| Direction of view | Forward viewing | Forward viewing |
| Angle of view | 120° | 140° |
| Outer diameter (mm) | | |
| Distal end | 9.2 | 9.2 |
| Insertion end | 9.2 | 9.2 |
| Bending section | | |
| Up/down | 180°/180° | 180°/180° |
| Right/left | 160°/160° | 160°/160° |
| Working length (mm) | 1520 | 2000 |
| Total length (mm) | 1840 | 2345 |
| Working channel diameter (mm) | 3.2 | 2.8 |
| Water-jet channel | Yes | No |

used. The specifications of the SIF-Y0004 are compared with those of a conventional SBE (SIF-Q260; Olympus Medical Systems, Tokyo, Japan) in Table 2 and Figure 1.

For biliary cannulation and injection of contrast media, a conventional ERCP catheter (PR-4Q-1; Olympus Medical Systems, Tokyo, Japan) and a bending cannula (PR-233Q; Olympus Medical Systems) were used. If cannulation was difficult to perform with a catheter alone, a 0.035-inch guide wire (RF-GA35403; Radifocus®, Terumo Corporation, Tokyo, Japan) was used. For stent placement and insertion of guide-wire instruments, a 0.035-inch Jag-wire™ (Boston Scientific; Natick, MA, United States) and a 0.025-inch disposable Visiglide™ guide wire (G-240-2545A, Olympus Medical Systems, Japan) were mainly used.

Endoscopic papillary balloon dilation was done using a Quantum TTC® Biliary Balloon Dilator (QBD-8X3, Cook Medical, Bloomington, IN, United States) and a CRE™ Balloon Dilation Catheter (Boston Scientific), measuring 10 to 15 mm, was used to perform endoscopic papillary large balloon dilation. Lithotripsy was performed with the following retrieval baskets: models FG-V435P (Flower Basket V®, Olympus Medical Systems), FG-V436P (Tetra Catch V®, Olympus Medical Systems), and EBL-15-200 (Escort II Extraction Balloon, Cook Medical).

Intraductal ultrasonography was carried out with a miniature ultrasound probe (UM-G20-29R, Olympus Medical Systems), inserted using a guide wire. As for stents, a 7-French pigtail plastic stent (PBD-203 series, Olympus Medical Systems) and metallic WallFlex™ Biliary RX Fully Covered and RX Partially Covered Stents Systems (Boston Scientific) were used.

Methods

Many studies have reported that balloon enteroscopy-assisted ERCP is therapeutically useful; however, a system for this procedure is not commercially available. We explained to patients that treatment outcomes and the incidence of complications have not been reported, and received written informed consent from all patients. Patients were sedated with pethidine (50 mg) and midazolam

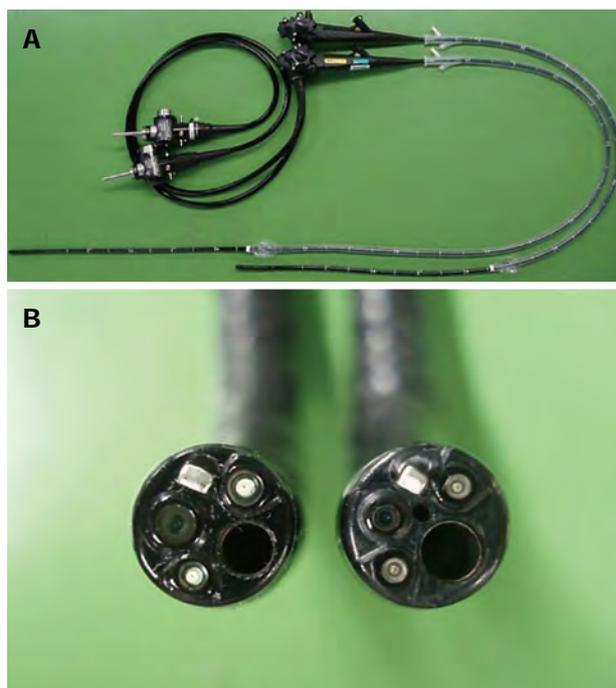


Figure 1 Comparison of two types of single-balloon endoscopes. A: Comparison of working lengths of two types of single balloon endoscopes (SBEs). Left: SIF-Q260 (working length 2000 mm); Right: SIF-Y0004 (working length 1520 mm); B: Comparison of working channel diameters of two types of SBE. Left: SIF-Q260 (working channel diameter 2.8 mm); Right: SIF-Y0004 (working channel diameter 3.2 mm and water-jet channel).

(3 to 10 mg), and vital signs were intermittently monitored during all procedures. Propofol was used if necessary. The same operator performed all examinations from the time of endoscope insertion to treatment. The endoscope was inserted with the patient in the prone position, and abdominal compression was applied manually if a scope was technically difficult to insert deeply. Butylscopolamine (20 to 40 mg) or glucagon (1 to 2 mg) was administered as an antispasmodic. Three very experienced endoscopists (with experience in > 1200 sessions of ERCP) conducted the examinations. After ERCP, all patients received nafamostat mesilate (10 mg) to prevent pancreatitis, with the exception of those in whom the drug was contraindicated because of allergies or other reasons.

We retrospectively studied the rate of reaching the blind end, time required to reach the papilla, diagnostic success rate, therapeutic success rate, procedure time, and complications according to the reconstruction method. The diagnostic success rate was defined as the rate of successfully imaging of the bile and pancreatic ducts. The therapeutic success rate was defined as the rate of successfully completing the endoscopic treatment. The procedure time was defined as the interval from the start of cannulation to removal of the endoscope. Pancreatitis and bleeding occurring after ERCP were evaluated according to the 1991 Consensus Guidelines (Cotton Classification)^[21]. Hyperamylasemia was defined as a rise in serum amylase levels to more than three times the upper limit of normal. In our hospital, we routinely measure

Table 3 Summary of results *n* (%)

| | Billroth-II gastrectomy (<i>n</i> = 8) | Roux-en-Y gastrectomy (<i>n</i> = 21) | Roux-en-Y Hepatico- jejunostomy (<i>n</i> = 2) | Total No. of SIF-Y0004- assisted ERCP (<i>n</i> = 31) |
|--|--|---|--|---|
| Reaching the blind end | 7 (88) | 19 (91) | 2 (100) | 28 (90) |
| Mean time (min) to reach the blind end (range) | 18.3 (5-37) | 21.1 (10-37) | 32.5 (32-33) | 21.2 (5-37) |
| Diagnostic success | | | | |
| Total | 6 (86) | 17 (90) | 2 (100) | 25 (89) |
| Intact papilla | 6 (86) | 13 (87) | - | 19 (86) |
| Therapeutic intervention required | | | | |
| Total | 5 (83) | 16 (94) | 2 (100) | 23 (92) |
| Intact papilla | 5 (83) | 12 (92) | - | 17 (89) |
| Therapeutic success | | | | |
| Total | 5 (100) | 15 (94) | 2 (100) | 22 (96) |
| Intact papilla | 5 (100) | 11 (92) | - | 16 (94) |
| Mean procedure time (min) (range) | 35.4 (7-65) | 43.3 (12-125) | 46.5 (26-41) | 40.2 (7-125) |

ERCP: Endoscopic retrograde cholangiopancreatography.

serum amylase 3 h after the completion of ERCP, as well as the next morning. In patients in whom the blind end was not reached, SBE-assisted ERCP was not attempted a second time, except if the patient strongly requested to undergo the procedure again. If SBE-assisted ERCP was unsuccessful, open surgery or percutaneous cholangioscopy was performed.

RESULTS

Rate of reaching the blind end (papilla of Vater or choledochojejunal anastomosis)

The rate of reaching the blind end was 88% (7/8) in patients with a B-II gastrectomy, 91% (19/21) in those with a R-Y gastrectomy, and 100% (2/2) in those with a R-Y hepaticojejunostomy (Table 3).

Mean time required to reach the blind end

The mean time required to reach the blind end was 18.3 min (range, 5 to 37 min) in B-II gastrectomy, 21.1 min (range, 10 to 37 min) in R-Y gastrectomy, and 32.5 min (range, 32 to 33 min) in R-Y hepaticojejunostomy (Table 3).

Diagnostic success rates

The diagnostic success rate was 86% (6/7) in B-II gastrectomy, 90% (17/19) in R-Y gastrectomy, and 100% (2/2) in R-Y hepaticojejunostomy. All the patients with a B-II gastrectomy and 15 (80%) of the 19 patients with an R-Y gastrectomy had an intact papilla. The diagnostic success rate among patients with an intact papilla was 86% (6/7) in B-II gastrectomy and 87% (13/15) in R-Y gastrectomy (Table 3).

Therapeutic success rate

The therapeutic success rate was 100% (5/5) in B-II gastrectomy, 94% (15/16) in R-Y gastrectomy, and 100% (2/2) in R-Y hepaticojejunostomy. Among patients with an intact papilla, the therapeutic success rate was 100% (5/5) in B-II gastrectomy and 92% (11/12) in R-Y gastrectomy (Table 3).

Treatment procedures

The channel diameter of 3.2 mm allowed 12 sessions of stone extraction with a wire-guided basket, and eight sessions of wire-guided intraductal ultrasonography to be performed (Figures 2, 3). Residual stones could thus be confirmed, and biliary strictures evaluated. All treatment procedures are summarized in Table 4.

Mean procedure time

The mean procedure time was 35.4 min (range, 7 to 65 min) in B-II gastrectomy, 43.3 minutes (range, 12 to 125 min) in R-Y gastrectomy, and 29.5 min (range, 26 to 33 min) in R-Y hepaticojejunostomy (Table 3).

Complications

Hyperamylasemia, pancreatitis, and gastrointestinal perforation were the main complications encountered (Table 5). Pancreatitis and bleeding were both mild. Gastrointestinal perforation improved after conservative treatment.

DISCUSSION

When performing ERCP in postoperative patients with a reconstructed intestine, whether the scope can reach the papilla or choledochojejunal anastomosis is an important concern. However, recent studies have reported that the blind end is reached in more than 80% of patients^[3-5,9-15,17]. After reaching the blind end, imaging and treatment of the papilla or biliary anastomosis become the most important issues. In particular, the outcomes of ERCP in patients with an intact papilla differ substantially among hospitals and remain far from satisfactory. Shimatani *et al*^[12] reported that the diagnostic success rate of DBE-assisted ERCP exceeded 90% in patients with an intact papilla, as compared with 25% to 80% in general^[8,9,11]. In several small studies, the diagnostic success rate of SBE-assisted ERCP in patients with an intact papilla ranged from 25% to 80%^[1,4,5]. In our study, the diagnostic success rate was 86%, indicating relatively good results. As for the relative advantages and disadvantages of SBE and DBE, May *et al*^[22] reported that setting up a DBE system requires 15.2

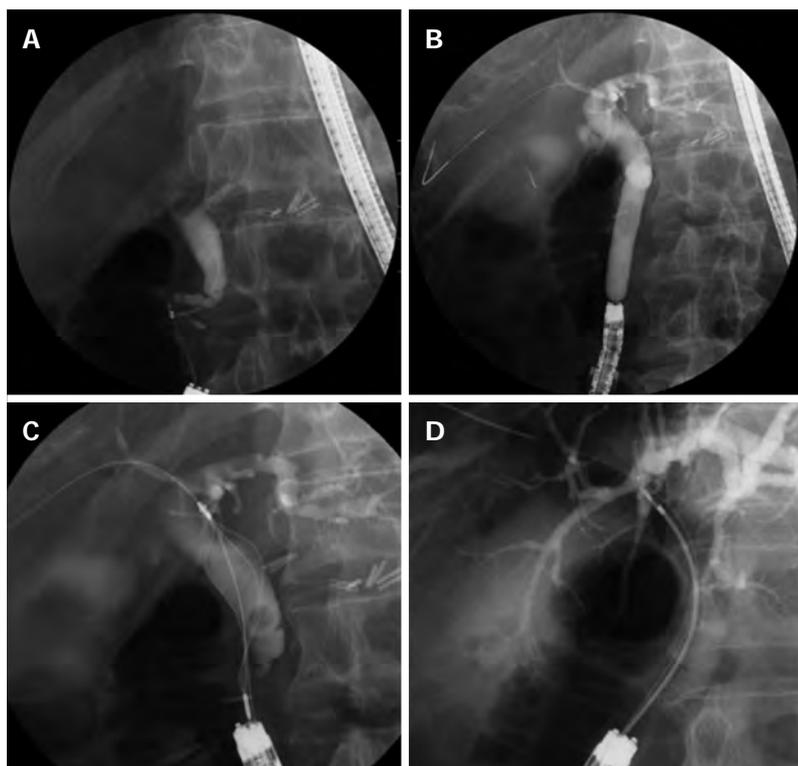


Figure 2 Therapeutic intervention. A: Cholangiogram showing a bile duct stone; B: Radiographic image showing papillary dilation using a large balloon catheter; C: Radiographic image showing wire-guided 4-wire retrieval basket; D: Radiographic image showing wire-guided intraductal ultrasonography.

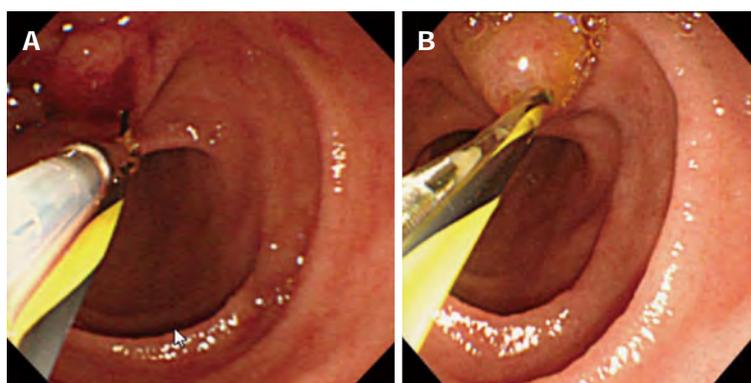


Figure 3 Wire-guided devices. A: A wire-guided 4-wire retrieval basket was inserted; B: A wire-guided intraductal ultrasonography was inserted.

min, whereas Kawamura *et al*^[23] showed that an SBE system could be set up within 5 min because it is simpler. A DBE system requires two balloons; therefore, the technique is complex, and the balloon at the distal end may interfere with passage of the scope through the intestine. However, one study reported that a DBE is easier to pass through an intestine with sharp angles than an SBE^[24].

Although several studies have compared SBE-assisted ERCP with DBE-assisted ERCP, and such a comparison would be challenging, these procedures are reported to be similarly clinically useful^[1,11,18-20].

The SIF-Q260, a conventional SBE, has a working length of 2000 mm and a channel diameter of 2.8 mm. Therefore, most conventional ERCP devices cannot be used, and the types of devices that could be used are limited. A short-type DBE has a working length of 1520 mm and a channel diameter of 2.8 mm, making passage of devices through the forceps channel difficult. Itoi *et al*^[3] replaced an SBE with a conventional forward-viewing

endoscope after reaching the papilla. We have used this technique in several patients in our hospital. However, even if the papilla is reached, some patients have severe bowel adhesion associated with complex loops that are difficult to resolve. Replacement of the scope is therefore not necessarily successful.

In this study, we used an SIF-Y0004, a new enteroscope that was developed to perform ERCP in post-operative patients with a reconstructed intestine. This enteroscope has a working length of 1520 mm and a channel diameter of 3.2 mm, allowing most conventional ERCP devices to be employed. In our hospital, the use of the SIF-Y0004 permitted the delivery of 8.5-French or smaller devices. The SIF-Y0004 also allowed the use of wire-guided devices that could not be passed through enteroscopy with a 2.8 mm channel diameter, as well as the insertion of a 7-French stent after the placement of two guide wires. A decreased rate of reaching the blind end was an important concern, because the SIF-Y0004

Table 4 Treatment procedures

| | Billroth-II gastrectomy | Roux-en-Y gastrectomy | Roux-en-Y Hepatico- jejunostomy | Total No. of SIF-Y0004- assisted ERCP |
|------------------|----------------------------|--------------------------|---------------------------------------|--|
| EST | 0 | 3 | 0 | 3 |
| EPBD | 2 | 5 | 0 | 7 |
| EPLBD | 1 | 4 | 1 | 6 |
| EBD | | | | |
| Plastic stent | 1 | 3 | 0 | 4 |
| Metallic stent | 2 | 2 | 0 | 4 |
| Stone extraction | | | | |
| Wire-guided | 1 | 9 | 2 | 12 |
| basket | | | | |
| Balloon catheter | 0 | 1 | 0 | 1 |
| Wire-guided IDUS | 1 | 7 | 0 | 8 |

EBD: Endoscopic biliary drainage; EPBD: Endoscopic papillary balloon dilatation; EPLBD: Endoscopic papillary large balloon dilatation; EST: Endoscopic sphincterotomy; IDUS: Intraductal ultrasonography; ERCP: Endoscopic retrograde cholangiopancreatography.

has a shorter effective length than a conventional SBE. However, the rate of reaching the blind end in our study was 90%, which was similar to that of ERCP performed with a SIF-Q260 in our hospital (89%).

The SIF-Y0004 permitted the use of devices that could not be used with the SIF-Q260. Compared with a short-type DBE, more devices and diagnostic instruments could be used with SIF-Y0004. Conventional models have a channel diameter of 2.8 mm; therefore, ERCP for stone extraction requires free-hand cannulation and the use of a 4-wire retrieval basket. However, the SIF-Y0004 allowed wire-guided devices to be used for stone extraction; successfully accomplished in 12 patients in our study. In addition, this new SBE allowed wire-guided intraductal ultrasonography to be easily performed to confirm residual stones.

The incidence of complications after ERCP in patients with a reconstructed intestine has been reported to be 0% to 11% for perforation, 0% to 20% for pancreatitis, and 0% to 32% for bleeding, and differed considerably among hospitals^[1-5,8,9,12-16]. Many patients who underwent ERCP in our hospital had undergone gastrectomy and had an intact papilla; therefore, cannulation was expected to be difficult, potentially leading to a high incidence of pancreatitis. However, the incidence of pancreatitis in this study was 7.7%, which is not particularly higher compared with previous reports. Further studies of larger numbers of patients are required because our study group was small.

The patient with gastrointestinal perforation had duodenal ulcer postoperatively and had undergone abdominal surgery three times. The initial procedure was Roux-en-Y reconstruction after distal gastrectomy, and a duodenum-transverse colon fistula developed. Subsequently, abdominal surgery was performed twice to repair the fistula site. Marked adhesion was found in the horizontal portion of the duodenum. Although a defect apparently caused by a stone in the bile duct was found on endoscopic retro-

Table 5 Complications *n* (%)

| Complications | Billroth-II gastrectomy (<i>n</i> = 8) | Roux-en-Y gastrectomy (<i>n</i> = 21) | Roux-en-Y Hepatico- jejunostomy (<i>n</i> = 2) | Total No. of SIF-Y0004- assisted ERCP (<i>n</i> = 31) |
|-------------------------------------|---|--|--|---|
| Hyperamylasemia ¹ | | | | |
| Total | 1 (14.3) | 4 (21.1) | - | 5 (19.2) |
| Intact papilla | 1 (14.3) | 3 (20) | - | 4 (18.2) |
| Pancreatitis ² | | | | |
| Total | 0 (0) | 2 (10.5) | - | 2 (7.7) |
| Intact papilla | 0 (0) | 2 (13.3) | - | 2 (9.1) |
| Perforation of digestive tract | 0 (0) | 1 (4.8) | 0 (0) | 1 (3.2) |
| Bleeding of papilla ² | 0 (0) | 1 (5.3) | 0 (0) | 1 (3.4) |

¹Hyperamylasemia: More than 3 times the upper limit of normal;

²According to Cotton's criteria. ERCP: Endoscopic retrograde cholangiopancreatography.

grade cholangiography, the scope was retracted during the examination and did not reach the blind end. The scope was inserted several times, but the results were the same. Lithotripsy was therefore not performed, and the examination was terminated. After endoscopy, the patient had abdominal pain and increased amylase levels. Post-ERCP pancreatitis was suspected. Enhanced computed tomography showed a small amount of free air around the horizontal portion of the duodenum, and perforation was diagnosed. There were no symptoms of peritoneal irritation. Computed tomography performed 5 d after surgery showed disappearance of the free air. As for the common bile duct stones, there was no dilation of the bile duct, and a percutaneous approach was precluded. We therefore recommended open surgery. However, ERCP with the SIF-Y0004 was repeated after 10 d at the strong request of the patient. Lithotripsy was successfully performed without perforation.

In the patient with papillary bleeding, melena occurred 12 h after endoscopic papillary large balloon dilatation. The hemoglobin level decreased by 2 g/dL. Bleeding from the papilla was suspected, and endoscopic examination with the SIF-Y0004 showed blood clots at and around the papilla. There was no active bleeding from the papilla. The examination was completed without any treatment. Subsequently, there was no melena or progression of anemia. Hemostasis was achieved spontaneously, with no need for blood transfusion.

The present study was small. The SIF-Y0004 has a channel diameter of 3.2 mm, currently the largest available among balloon endoscopes, and a short working length. The water-jet channel of this model helps to maintain a field of view during scope insertion and treatment. It will be particularly useful in the presence of bleeding.

Further studies in larger numbers of patients are needed to confirm our results. However, in postoperative patients with a reconstructed intestine who underwent ERCP, the rate of the reaching the blind end with the SIF-Y0004 was similar to that with the SIF-Q260. This

new model SBE permitted the use of most ERCP devices. Our results suggest that the SIF-Y0004 enables high-quality diagnosis and treatment to be provided reliably.

ACKNOWLEDGMENTS

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COMMENTS

Background

Endoscopic retrograde cholangiopancreatography (ERCP) with duodenal scope or conventional forward viewing endoscope for reconstructed intestine (Billroth-II reconstruction, Roux-en-Y reconstruction) has often been described as unsatisfactory because of altered anatomical features. Balloon enteroscopy has improved the outcomes of ERCP in patients with a reconstructed intestine.

Research frontiers

Balloon enteroscopy has improved the outcomes of ERCP in patients with a reconstructed intestine. These patients may avoid the need for surgical treatment in case of biliopancreatic problems.

Innovations and breakthroughs

Balloon-enteroscope assisted-ERCP is an important endoscopic breakthrough for the therapeutic management of the conventionally inaccessible biliopancreatic ducts. However, several problems remain to be solved. For example, wire-guided devices cannot be used because these scopes have a narrow channel diameter, 2.8 mm. In this study, the authors used a newly designed short-type single balloon enteroscope (SBE) that was developed to perform ERCP in postoperative patients with a reconstructed intestine. This short-type SBE has a working length of 1520 mm, and a channel diameter of 3.2 mm, allowing most conventional ERCP devices to be employed. Among all balloon enteroscopy, these specifications are limited to this scope. As for treatment, this scope permitted the use of devices that could not be used with conventional SBE. Compared with a short-type double balloon enteroscopy, more devices and diagnostic instruments could be used with this scope. Conventional models have a channel diameter of 2.8 mm; therefore, ERCP for stone extraction requires free-hand cannulation. However, this scope allowed wire-guided devices to be used for stone extraction. In addition, this scope allowed wire-guided intraductal ultrasonography to be easily performed to confirm residual stones.

Applications

This study suggests that short-type SBE enables high-quality diagnosis and treatment to be provided reliably.

Terminology

Billroth-II reconstruction is a surgical technique in gastrectomy. Roux-en-Y reconstruction of the small intestine is a frequently performed surgical technique in gastrointestinal surgery, hepatobiliary and pancreatic surgery. The location of the Vater's papilla and choledochojejunal anastomosis of the most patients who are underwent these operation are differed from normal position that was accessible with conventional endoscope.

Peer review

The authors investigated the efficacy of new short-type SBE for ERCP in patients with altered gastrointestinal anatomy, and concluded that short-type SBE is effective for ERCP in patients with a reconstructed intestine. This study clearly shows the efficacy of short-type SBE as a clinical trial.

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Endoplasmic reticulum stress sensitizes human esophageal cancer cell to radiation

Xue-Li Pang, Gang He, Yang-Bo Liu, Yan Wang, Bo Zhang

Xue-Li Pang, Yang-Bo Liu, Department of Oncology, Southwest Hospital, Third Military Medical University, Chongqing 400038, China

Gang He, Yan Wang, Bo Zhang, Department of Medical Genetics, Third Military Medical University, Chongqing 400038, China

Author contributions: Pang XL and Zhang B designed the research; He G, Zhang B, and Liu YB performed the majority of the experiments; He G, Liu YB, and Wang Y contributed new reagents and analytic tools; Pang XL, Wang Y, and Zhang B analyzed data; Zhang B wrote the manuscript.

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Correspondence to: Dr. Bo Zhang, Department of Medical Genetics, Third Military Medical University, 30 Gaotanyan Street, Shapingba District, Chongqing 400038, China. bo_zhang@yahoo.com

Telephone: +86-23-68753670 Fax: +86-23-65318610

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Abstract

AIM: To investigate the role of endoplasmic reticulum (ER) stress in cancer radiotherapy and its molecular mechanism.

METHODS: Tunicamycin (TM) was applied to induce ER stress in human esophageal cancer cell line EC109, and the radiosensitization effects were detected by acute cell death and clonogenic survival assay. Cell cycle arrest induced by TM was determined by flow cytometric analysis after the cellular DNA content was labeled with propidium iodide. Apoptosis of EC109 cells induced by TM was detected by annexin V staining and Western blotting of caspase-3 and its substrate poly ADP-ribose polymerase. Autophagic response was determined by acridine orange (AO) staining and Western blotting of microtubule-associated protein-1 light chain-3 (LC3) and autophagy related gene 5 (ATG5).

In order to test the biological function of autophagy, specific inhibitor or Beclin-1 knockdown was used to inhibit autophagy, and its effect on cell apoptosis was thus detected. Additionally, involvement of the phosphatidylinositol-3 kinase (PI3K)/Akt/mammalian target of the rapamycin (mTOR) pathway was also detected by Western blotting. Finally, male nude mice inoculated subcutaneously with EC109 cells were used to confirm cell model observations.

RESULTS: Our results showed that TM treatment enhanced cell death and reduced the colony survival fraction induced by ionizing radiation (IR), which suggested an obvious radiosensitization effect of TM. Moreover, TM and IR combination treatment led to a significant increase of G2/M phase and apoptotic cells, compared with IR alone. We also observed an increase of AO positive cells, and the protein level of LC3-II and ATG5 was induced by TM treatment, which suggested an autophagic response in EC109 cells. However, inhibition of autophagy by using a chemical inhibitor or Beclin-1 silencing led to increased cell apoptosis and decreased cell viability, which suggested a cytoprotective role of autophagy in stressed EC109 cells. Furthermore, TM treatment also activated mTORC1, and in turn reduced Akt phosphorylation, which suggested the PI3K/Akt/mTOR signal pathway was involved in the TM-induced autophagic response in EC109 cells. Tumor xenograft results also showed synergistic retarded tumor growth by TM treatment and IR, as well as the involvement of the PI3K/Akt/mTOR pathway.

CONCLUSION: Our data showed that TM treatment sensitized human esophageal cancer cells to radiation *via* apoptosis and autophagy both *in vitro* and *in vivo*.

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Key words: Endoplasmic reticulum stress; Tunicamycin; Esophageal cancer; Radiosensitivity; Autophagy; Apoptosis

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INTRODUCTION

Esophageal cancer, which principally consists of esophageal squamous cell carcinoma (ESCC) and esophageal adenocarcinoma, is one of the leading causes of cancer-related death, and its worldwide incidence is increasing annually^[1]. Historically, treatment of esophageal cancer involved radiotherapy, which was often combined with other treatments, such as surgery and chemotherapy^[2]. However, there is differential sensitivity to radiation in tumors of the same grade, which to some extent has limited the clinical application of radiotherapy. How to enhance the radiosensitivity of esophageal cancer is still an unresolved problem.

Enhancing radiosensitivity can be accomplished by increasing tumor-specific cell death induced by radiation. Following ionizing radiation (IR), many types of tumor cells primarily undergo apoptosis, which is often significant even at very low doses^[3]. Recently, however, another type of cell death, termed type II cell death or cell death with autophagy, has been observed following radiation^[4]. Induction of autophagy leads to the formation of a double membrane-bound structure called the autophagosome. The autophagosome subsequently fuses with a lysosome, creating an autolysosome, whose contents and inner membrane are degraded and recycled^[5,6]. Recent studies have reported a role for autophagy in a variety of pathophysiological conditions, including cancer, defense against infections, and as a response to radiation. Although the fundamental role of induced autophagy is controversial, the current literature appears to support the role of autophagy as a mode of radiosensitization rather than radioprotection. Radiation-induced up-regulation of autophagic responsive genes has suggested a new mechanism of cell death and a new target for cancer therapy^[7-9].

The endoplasmic reticulum (ER) is an essential intracellular organelle with multiple roles including the synthesis of nascent proteins, Ca²⁺ storage, glycosylation, and the trafficking of newly-synthesized membrane and secretory proteins. Perturbations of these processes have been demonstrated to interfere with the proper functioning of ER, thus leading to a condition defined as ER stress^[10,11]. Following ER stress, specific signaling pathways have evolved in eukaryotic cells and are collectively termed the unfolded protein response (UPR). The UPR consists of three signal transduction pathways: protein kinase RNA dependent-like ER kinase (PERK), activating transcription factor 6 (ATF6), and inositol-requiring protein-1 (IRE1). Failure to relieve ER stress can result in cellular dysfunction and disease^[12]. The end result of sustained ER stress

and the UPR is usually cell death involving apoptosis and autophagy^[13,14]. Signaling through PERK, IRE1, and ATF6 pathways can trigger pro-apoptotic signals *via* the activation of downstream molecules such as the C/EBP homologous protein (CHOP, also known as growth arrest and DNA damage 153, GADD153), Jun kinase (JNK), and members of the Bcl-2 protein family^[15,16]. Cell death for a given cell is dependent on its genetic background and the treatment given. Radiation in the absence of the pro-apoptotic Bcl-2 family members Bax and Bak results in increased autophagy and cell death. This radiosensitization response is blocked by inhibitors of autophagy such as 3-methyladenine (3-MA)^[17].

In our previous work, we found that IR-induced up-regulation of ER stress markers glucose-regulated protein 78 (GRP78) and 94 (GRP94), both at the level of protein and mRNA. PERK and IRE1 signaling pathways were also activated by radiation, which suggested that IR could induce an ER stress response^[18]. However, its biological significance remained unknown. Tunicamycin (TM) is a naturally-occurring antibiotic that induces ER stress in a range of cell contexts^[19,20]. However, whether it could sensitize esophageal cancer cells to radiation was unknown. In order to explore the role of ER stress and the molecular mechanisms invoked following radiation treatment, TM was applied to induce ER stress in the human esophageal cancer cell line EC109. Our results showed that TM treatment sensitized esophageal cancer cells to radiation *via* apoptosis and autophagy both *in vitro* and *in vivo*.

MATERIALS AND METHODS

Materials

The ER stress inducers tunicamycin and autophagy inhibitor 3-MA were obtained from Merck. The annexin V staining kit was purchased from the Beyotime Institute of Biotechnology. Acridine orange (AO) was purchased from Sigma-Aldrich. All primary antibodies except Beclin-1 (Santa Cruz Biotechnology, sc10086) were purchased from Cell Signaling Technology. Specific siRNA of Beclin-1 (BECN1-homo-358) was obtained from Shanghai GenePharma Co., Ltd. Lipofectamine 2000 was obtained from Invitrogen. DMEM cell culture media and fetal bovine serum were obtained from Thermo Scientific.

Cell culture and radiation

The human esophageal cancer cell line EC109 was obtained from Shanghai Cell Bank (<http://www.cellbank.org.cn/>), and cultured in DMEM supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin (complete media). Cells were treated with 10 Gy of gamma radiation using a ⁶⁰Co source. The medium was immediately replaced after irradiation, and the dishes were returned to the incubator for the indicated times. Cells were harvested by scraping in PBS.

Cell growth and colony forming assays

For cell growth curves, exponentially growing cells were cultivated in 96-well plates, with 5×10^3 cells in each well. Twelve hours later, cells were treated with TM at the indicated concentrations, and further cultivated. Viable cells were detected using the cell counting kit 8 (Beyotime Institute). Briefly, 10 μ L of cell counting kit-8 (CCK-8) solution (10 mg/mL) was added to the media, and cells were incubated for two more hours. Plates were read using a microplate reader (Bio-Tek Instruments) set to 450 nm (wavelength correction set to 540 nm). Relative abundance was normalized to that of the control.

For colony formation assays, cells in the logarithmic growth phase were seeded in 6-well plates at concentrations predetermined to give 25-200 colonies. Cells were then pretreated with TM (5 μ g/mL) for 24 h, and then irradiated at the indicated dose. After changing the media, cells were further cultivated for 10-14 d. Cultures were fixed and stained with 0.5% crystal violet in absolute methanol. The number of colonies with > 50 cells was counted using a dissecting microscope. The percentage of cell survival was calculated and normalized to that of the control.

Cell cycle analysis

Exponentially growing EC109 cells were cultivated and synchronized for 24 h in serum-free media. The media with or without TM (0.5 μ g/mL) was replaced with complete media before irradiation with 10 Gy. Twenty four hours later, cells were harvested by trypsinization, washed with ice-cold PBS, and fixed in 70% ethanol. Before analysis, DNA was labeled with propidium iodide (PI) in the presence of RNase (1 mg/mL) for 30 min at room temperature. Cell cycle distributions were analyzed on a FACSsort (Becton Dickinson) with Cell Quest software (version 313) for the proportions of cells in the G1, S, and G2/M phases of the cell cycle.

Apoptosis assays

Apoptosis was measured using the annexin V fluorescein isothiocyanate (FITC) Apoptosis Kit (Beyotime Institute) according to the manufacturer's instructions. Briefly, TM or TM plus radiation-treated cells were trypsinized and washed twice with cold PBS. Cells were then stained for 15 min at room temperature, and analyzed on a FACSsort (Becton Dickinson). Caspase-8 activity was measured using the human active Caspase-8 immunoassay (Beyotime Institute). Optical density (A) was detected by using the microplate reader (Bio-Tek Instruments). Enzymatic activities were expressed as arbitrary A units, and relative activity was normalized to that of control.

AO and Hoechst 33342 staining

Cells were treated with TM for the indicated times, washed with PBS, trypsinized, and then collected in PBS. Cells were then stained with AO (100 μ g/mL) for 15 min at room temperature. Green (510 to 530 nm) and red (650 nm) fluorescence emissions from 1×10^5 cells illuminat-

ed with blue (488 nm) excitation light were analyzed on a FACSsort. For Hoechst 33342 staining, EC109 cells were stained for 15 min at room temperature, and then visualized with a fluorescence microscope.

siRNA transfection

EC109 cells were transfected with siRNA against Beclin-1 (5' GGAGCCAUUUUUUGAAACUTT) or control siRNA using Lipofectamine 2000 according to the manufacturer's instructions. Cells were collected and used for Western blotting 48 h after transfection. For cell viability assays, cells were further treated with TM for a further 24 or 48 h.

RNA extraction and quantitative real-time PCR

RNA was extracted with TRIzol reagent (Invitrogen) and converted to cDNA using the reverse transcription kit (Applied Biosystems). Quantitative real-time PCR (qRT-PCR) was carried out using the ABI 5700 real-time PCR system (Applied Biosystems) using specific primers. Reactions were done in triplicate from the same cDNA reaction. The PCR conditions were: initial denaturation at 95 $^{\circ}$ C for 5 min; 40 cycles of denaturation at 95 $^{\circ}$ C for 20 s; annealing at 60 $^{\circ}$ C for 30 s; and elongation at 72 $^{\circ}$ C for 30 s. Gene expression of ATG5 and Beclin-1 was normalized to the corresponding β -actin level and the comparative CT method was used to calculate relative gene expression.

Western blotting

Total protein was resolved on SDS-PAGE and transferred onto a nitrocellulose membrane. After blocking in 3% non-fat milk (in PBS) for 30 min, membranes were incubated with antibodies against GRP78 (#3177), GRP94 (#2104), LC3 (#4108), ATG5 (#2630), PARP (#9542), cleaved Caspase-3 (#9662), and Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (#8884), followed by incubation with corresponding secondary antibodies (Zhongshan Corp.). Expression of individual proteins was normalized to that of GAPDH. Western blotting were repeated at least three times.

Tumor xenograft model

All mouse experiments were approved by the University Committee on the Use and Care of Animals of the Third Military Medical University. Male nude mice were inoculated subcutaneously with 1×10^6 human EC109 cells in the dorsal aspect of the neck. Three weeks after tumor inoculation, all mice developed a large tumor (approximately 100 mg). Mice with established 3-wk-old tumors were randomly divided into four groups: Groups 1 and 2 were injected intraperitoneally (IP) with PBS or TM (1.5 mg/kg), respectively; Group 3 was given a single dose of localized irradiation (10 Gy); and the final group were irradiated and then given an IP injection of TM. All experiments used five mice per group. Tumor volume (TV) was measured every 3 d post-irradiation (day 0), and was calculated according to the formula $TV = L \times W^2/2$,

where L and W are the length and width, respectively. Tumor tissues and other organs (liver and kidney) were removed and processed for Western blotting analysis on day 15.

Statistical analysis

The one-sample *t*-test was used to statistically analyze the differences in the expression ratios of irradiated versus non-irradiated samples. A *P*-value less than 0.05 was considered significant.

RESULTS

Radiosensitization effect of TM in EC109 cells

TM has been used to induce ER stress in various studies. We first determined that TM induced ER stress in EC109 cells. TM treatment increased protein levels of GRP78 and GRP94, hallmarks of ER stress, in a dose- and time-dependent manner. The mRNA level of these two genes also increased after TM treatment, which was consistent with the changes in protein levels. Another indicator of ER stress is the selective splicing of X-box binding protein 1 (XBP1), which can be detected by RT-PCR. TM treatment induced an additional product of XBP1, indicating that a 26-bp intron was spliced from its normal transcript. Together, these results showed that TM treatment induced an ER stress response in EC109 cells.

The effect of TM treatment on the growth of cells was determined by a CCK-8 assay. TM treatment resulted in a reduction of cell growth in a dose-dependent manner, which became apparent after 24 h of treatment. Compared with other concentrations, 5 $\mu\text{g}/\text{mL}$ of TM induced obvious growth inhibition in EC109 cells, and was used in further experiments (Figure 1A). To evaluate if TM could increase radiosensitivity, EC109 cells were treated with various concentrations of TM alone or combined with IR at a dose of 10 Gy for 24 or 48 h. Cell viability was then determined using the CCK-8 assay. Cell viability decreased significantly when IR was combined with TM (Figure 1B). Cell viability at 48 h after IR was more pronounced than at 24 h, which indicated a time-dependent effect. To further confirm radiosensitization by TM, clonogenic survival assays were performed to examine long-term survival. Compared with IR alone, cell survival was significantly decreased in cells treated with TM and IR (Figure 1C). These results indicated that TM could sensitize EC109 cells to radiation.

Cell cycle arrest induced by TM

As TM treatment decreased cell viability, we wondered whether this was due to cell cycle arrest. To void its intrinsic cytotoxic effect, we decided to use TM at 0.5 $\mu\text{g}/\text{mL}$, a concentration that was able to consistently inhibit cell growth. Flow cytometric analysis of DNA content was conducted to assess changes in the proportion of cells in each phase of the cell cycle. TM treatment for 24 h indicated no significant changes in the cell cycle compared to the control. However, IR alone induced a G2/M

arrest in EC109 cells, which was consistent with our previous observation^[21]. TM treatment combined with IR greatly enhanced G2/M arrest (Figure 2). This indicated that the radiosensitization effect of TM was associated with cell cycle arrest.

Apoptosis is enhanced by TM in irradiated cells

Both IR and prolonged ER stress can induce an apoptotic response. We therefore studied whether TM enhanced apoptosis of irradiated EC109 cells. Apoptotic signaling pathways (both extrinsic and intrinsic) lead to activation caspases, which cleave essential cell substrates such as PARP. IR and ER stress have been shown to activate executioner caspases Caspase-3 and -8. The protein levels of active (cleaved) Caspase-3 were determined by Western blotting. Levels of cleaved Caspase-3 increased after TM treatment or IR (Figure 3A). Combined TM and IR treatment induced an increase greater than that of radiation alone. Furthermore, cleaved PARP and Caspase-8 activity (Figure 3B) showed a similar pattern of increase compared to Caspase-3.

To evaluate the proportion of apoptotic cells, EC109 cells were stained with Annexin V-FITC and PI, and analyzed by FACS (Figure 3C). TM treatment increased the amount of apoptotic cells and occurred in a time-dependent manner. Combined treatment significantly increased the number of apoptotic cells compared with that of radiation alone. Hoechst 33342 staining of TM-treated cells showed a similar result (Figure 3D). These results indicated that TM treatment enhanced cell apoptosis induced by IR.

Autophagy response induced by TM

TM treatment has been reported to induce cell death with autophagy in some cell lines. Upon induction of autophagy, cytosolic LC3- I is converted into LC3- II, which decorates the autophagosome and often serves as a marker of autophagy. Simultaneously, the gene expression of ATGs, such as ATG5, ATG7, and ATG12, were up-regulated under the condition of autophagy. To determine if TM induces an autophagic response in EC109 cells, Western blotting for LC3 and ATG5 protein levels were performed. As expected, the protein levels of autophagic markers increased after TM treatment in a dose-dependent manner. Consistent with the increase in protein levels, mRNA levels of Beclin-1 and ATG5 also increased after TM treatment. In order to verify that the molecular response to TM resulted in the morphological characteristics of autophagy, EC109 cells were treated with TM and then stained with AO. TM increased the number of cells displaying red fluorescence in a dose-dependent manner (Figure 4A).

We then determined the autophagic response following radiation. EC109 cells were irradiated and the protein levels of LC3 and ATG5 were detected by Western blotting. TM treatments increased protein levels of LC3 and ATG5, and the levels of these proteins were further increased by TM and radiation combined treatment (Figure

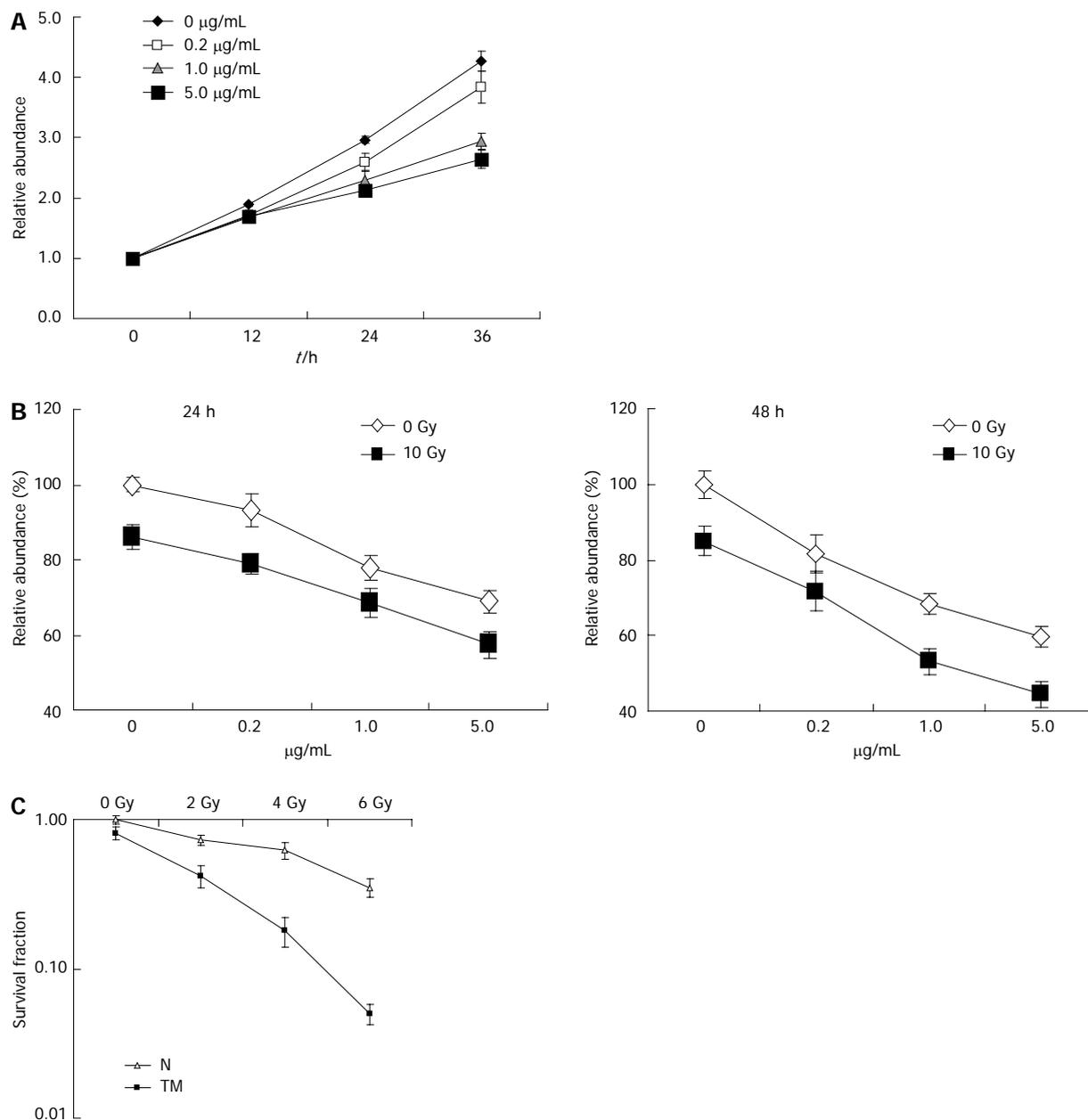


Figure 1 Radiosensitization effect of tunicamycin in EC109 cells. A: Exponentially growing EC109 cells were treated with tunicamycin (TM) at the indicated concentrations. Cell growth was determined using cell counting kit-8 (CCK-8) and relative growth rate was calculated using the absorption at 0 h as a baseline; B: EC109 cells were pretreated with or without TM (5 $\mu\text{g/mL}$), followed by a single dose of 10 Gy. 24 h (left panel) or 48 h (right panel) later, relative cell viability was measured using CCK-8; C: EC109 cells were pretreated with or without TM (5 $\mu\text{g/mL}$) for 24 h. After irradiation with 2, 4 and 6 Gy, cells were further cultivated for 10-14 d. The number of colonies with > 50 cells was counted under a dissecting microscope. N: Untreated with TM.

4B). Similarly, mRNA levels of Beclin-1 and ATG5 also increased after combined treatment, which suggested an augmented autophagic response (Figure 4C). Moreover, the percentage of AO positive cells was synergistically enhanced by radiation and TM treatment (Figure 4D).

As autophagy is thought to be involved in cell survival or cell death, we wanted to understand the significance of the autophagy response induced by TM. 3-MA, an inhibitor of autophagy, was used to specifically inhibit the autophagic response of EC109 cells to TM treatment. The addition of 3-MA resulted in an increase of LC3-II for-

mation in accordance with previously published data^[22]. Western blotting analysis revealed that the protein level of ATG5 decreased in EC109 cells after 3-MA treatment, compared with TM treatment alone (Figure 4E). This indicated that the autophagic response induced by TM treatment was inhibited by 3-MA. We then determined if 3-MA affected cell viability. As shown in Figure 4F, when cells were treated with inhibitor alone, cell viability after radiation did not change significantly. However, it decreased significantly when the cells were co-treated with TM and inhibitor. This suggested that inhibition of

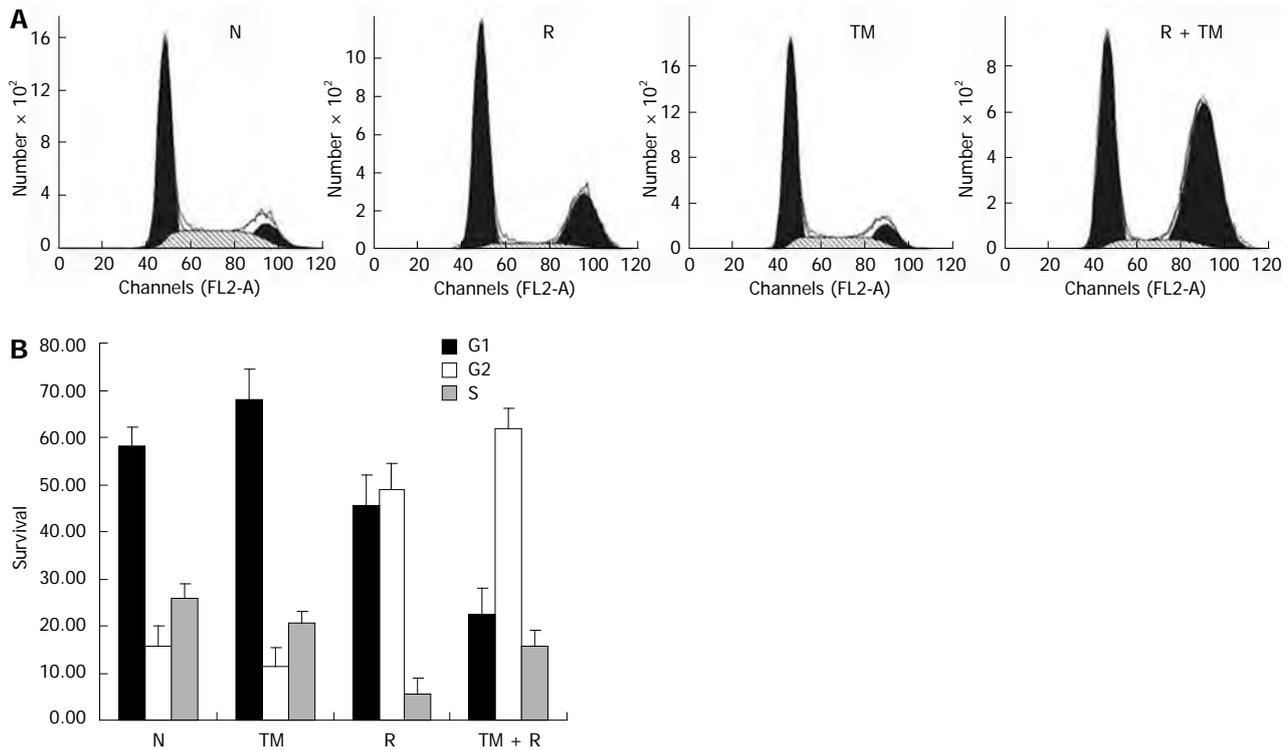


Figure 2 Cell cycle arrest induced by tunicamycin. A: EC109 cells were pretreated with or without tunicamycin (TM) (0.5 μg/mL), followed by a single dose of 10 Gy. 24 h later, cells were harvested, fixed, and labeled with propidium iodide. Cell cycle distributions were analyzed on a FACSsort; B: Data are the mean ± SD of three independent experiments. N: Sham-treated with TM; R: 10 Gy irradiation.

autophagy sensitized cells to radiation.

The effect of autophagy inhibition on apoptosis

Recent studies indicated a cross-talk between apoptosis and autophagy, yet the reciprocal relationship between them is far from clearly elucidated^[23]. Many studies have shown that blockage of autophagy with inhibitors increased apoptosis. Thus, we studied whether apoptosis of EC109 cells treated with TM would be augmented by an autophagy inhibitor.

To this end, autophagy was inhibited using 3-MA in cells treated with TM and IR, and then cells were analyzed by annexin V staining. 3-MA treatment significantly increased cell apoptosis (TM *vs* TM + 3-MA, Figure 5A). Similarly 3-MA treatment increased apoptosis in EC109 cells synergistically treated with TM and radiation. Western blotting for cleaved Caspase-3 and its substrate PARP showed that 3-MA treatment after radiation and TM induced a substantial increase in the active form of Caspase-3 and cleaved PARP (Figure 5B). Autophagy was subsequently inhibited by silencing Beclin-1, which has pivotal role in autophagy. siRNA against Beclin-1 was transfected into EC109 cells, and protein levels of Beclin-1 were detected 24 h later. Beclin-1 was significantly knocked down by the siRNA (Figure 5C). As expected, the autophagic response induced by TM treatment was attenuated when Beclin-1 was knocked down, which indicated a suppression of LC3II and ATG5 (Figure 5D). To determine the impact of Beclin siRNA on TM-induced apoptosis, Western blotting for cleaved

Caspase-3 and PARP was carried out. Similarly, increased levels of cleaved Caspase-3 and PARP were observed after silencing of Beclin-1 (Figure 5E). In addition, the levels of CHOP were also augmented. These results indicated that inhibition of autophagy increased apoptosis induced by TM.

Cell signaling pathways involved in TM response

Recent studies have shown that the PI3K/Akt/mTOR pathway is involved in ER stress-triggered apoptosis, and is also involved in the regulation of autophagy^[24,25]. Upon induction of ER stress, activation of mTORC1 reduces Akt phosphorylation, an event upstream of IRE-JNK signaling and subsequent apoptosis. We first explored whether TM treatment would activate mTORC1, using phosphorylation of p70S6K as a marker. Basal activity of mTORC1 was observed in untreated cells, and was rapidly upregulated following TM treatment in a dose- and time-dependent manner (Figure 6A). Accordingly, the protein levels of PI3K and phosphorylated Akt (p-Akt) declined. This suggested that the PI3K/Akt/mTOR signaling pathway was involved in TM treatment, which led to an autophagic response in EC109 cells. Western blotting for levels of PI3K and p-Akt were carried out to further explore the effect on this pathway after the combined treatment of TM and IR. Protein levels of PI3K and p-Akt showed a further decrease after combined treatment (Figure 6B). This suggested that this signal pathway was involved in the enhancement of radiosensitivity induced by TM treatment.

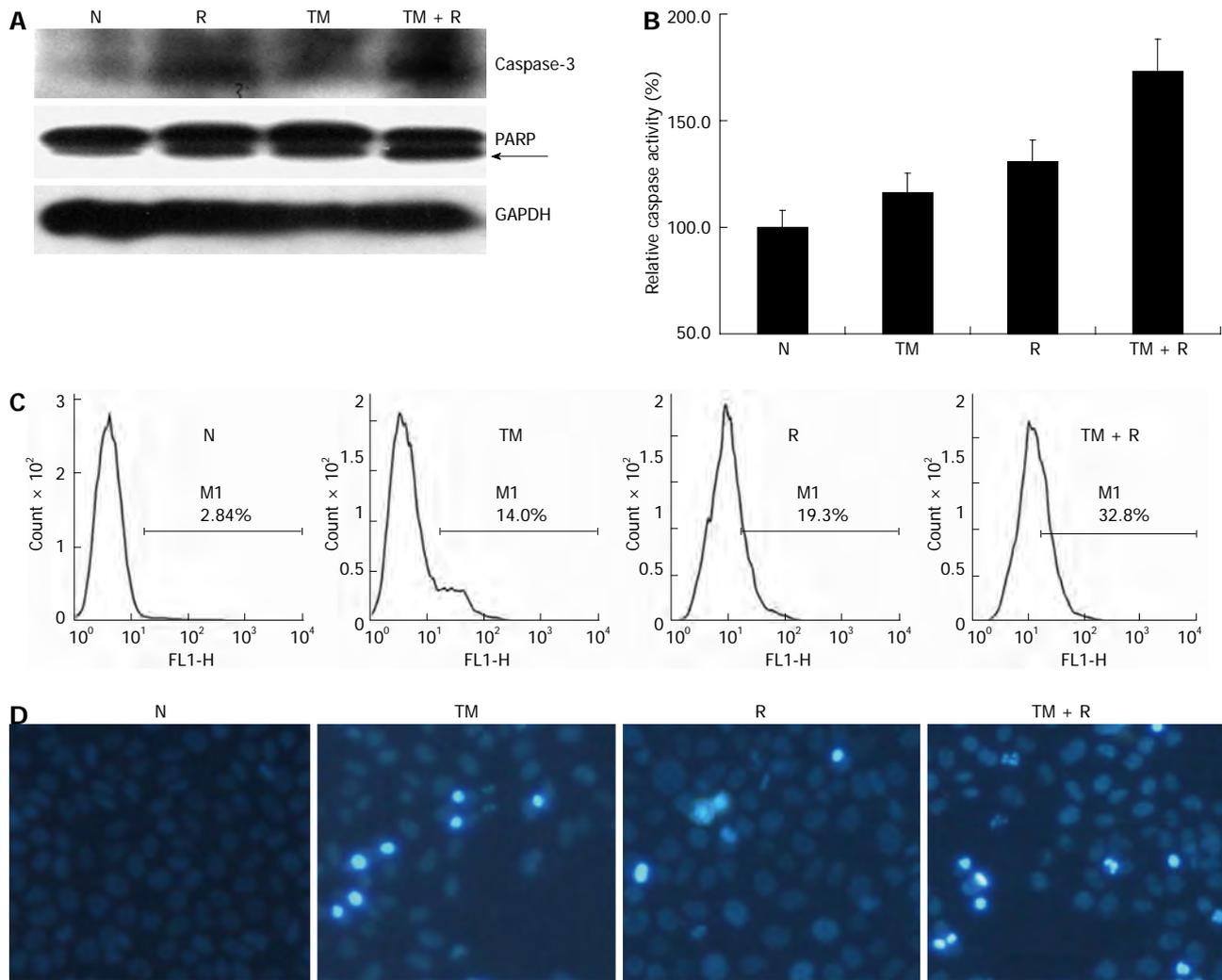


Figure 3 Apoptosis is enhanced by tunicamycin in irradiated cells. EC109 cells were pretreated with or without tunicamycin (TM) (5 $\mu\text{g}/\text{mL}$), followed by a single dose of 10 Gy. **A:** Western blotting analysis for poly ADP-ribose polymerase (PARP), Caspase-3, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH); **B:** Caspase-3 activity of treated cells; **C:** Apoptosis was determined by annexin V labeling; **D:** Representative images of cells stained with Hoechst 33343. N: Sham-treated with TM; R: 10 Gy irradiation.

In vivo studies

To determine if radiosensitization of TM *in vitro* could be recapitulated *in vivo*, nude mice with established tumor xenografts were treated with TM and local radiotherapy. To assess the effect of TM alone on ER stress markers, samples from tumor xenografts were analyzed by Western blotting. TM treatment induced an increase of GRP78 and GRP94 (Figure 7A). In addition, TM treatment increased the protein levels of ATG5 and LC3II, which indicated that TM treatment upregulated the autophagy response *in vivo* (Figure 7A). Similar results were observed in liver and kidney samples.

Simultaneously, the growth of tumors was determined. In control groups, tumors grew progressively to an average volume of 300 mm^3 , and all mice were sacrificed at day 12. The results of tumor growth curves showed that tumor growth could be retarded by radiation alone (Figure 7B, C). TM treatment also significantly inhibited the growth of tumor xenografts, compared with the control group. Moreover, the inhibition of tu-

mor xenograft growth was synergistically enhanced by combined radiation and TM treatment. Furthermore, the protein level of PI3K and p-Akt was also determined in tumor xenografts. TM treatment combined with radiation induced a significant decrease of the PI3K/Akt/mTOR signaling pathway, which showed that TM functioned similarly to that of the cell model (Figure 7D). These results suggested that TM treatment sensitized EC109 cells to radiation *in vivo*.

DISCUSSION

Here we have shown that the classic ER stress inducer TM could sensitize esophageal cancer cells to radiation *in vitro* and *in vivo*. TM causes ER stress by specifically inhibiting N-linked glycosylation of peptides in the ER, which leads to protein misfolding that exceeds the capacity of protein chaperones. Cells benefit from moderate ER stress to alleviate damage, while sustained ER stress induces cell death^[20]. In this study, we treated EC109 cells

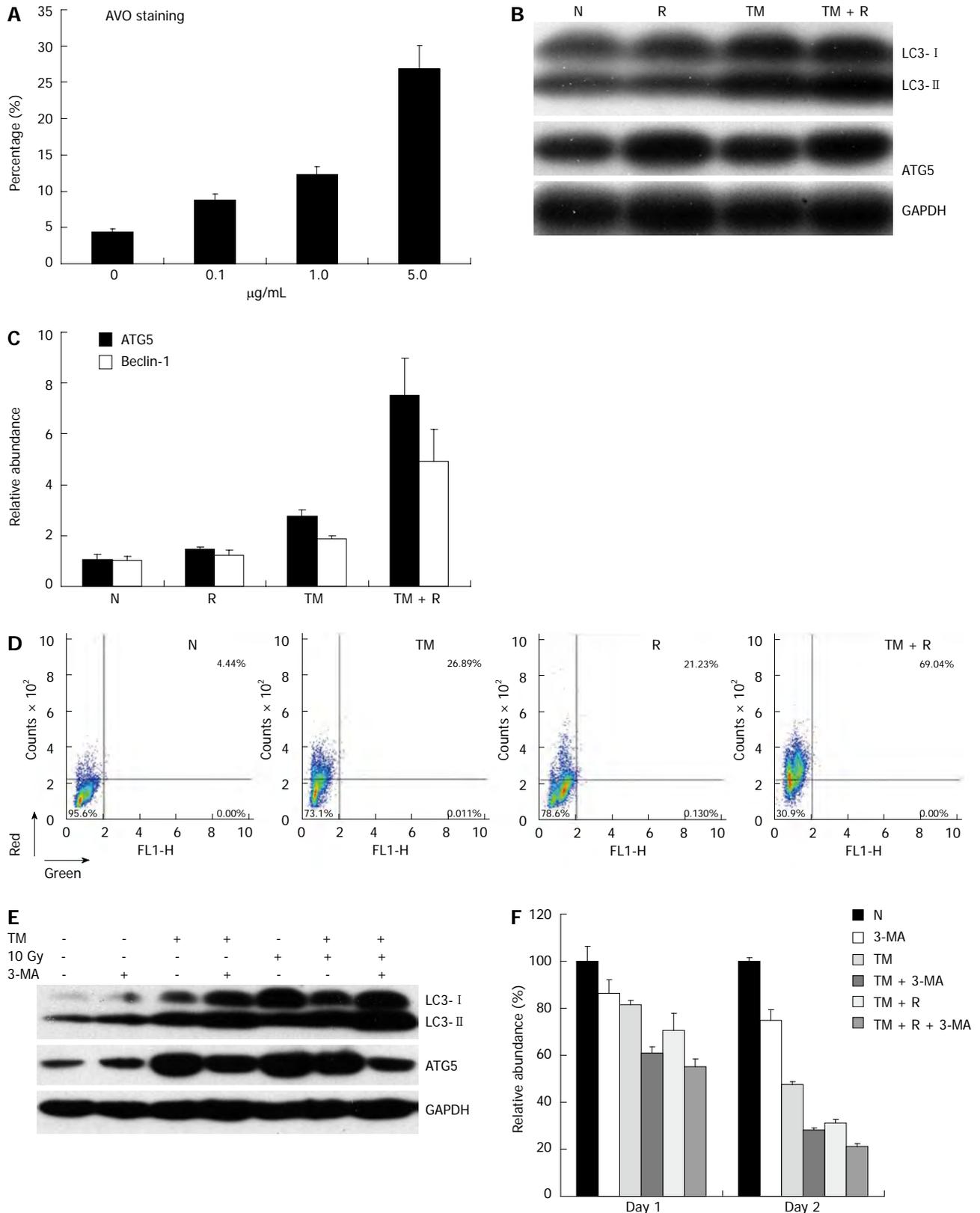


Figure 4 Autophagy response induced by tunicamycin. **A:** EC109 cells were exposed to increasing concentrations of tunicamycin (TM), and stained with acridine orange (AO); **B:** EC109 cells were pretreated with or without TM (5 µg/mL), followed by a single dose of 10 Gy and analyzed by Western blotting for LC3 and ATG5; **C:** Relative mRNA expression of Beclin-1 and ATG5 was assessed by quantitative polymerase chain reaction; **D:** After AO staining, cells were quantitatively analyzed on a FACSsort; **E:** Cells were treated as indicated, and protein levels of LC3 and ATG5 were assessed by Western blotting; **F:** Relative cell viability of cells treated with TM, radiation and autophagy inhibitor 3-MA for 24 or 48 h. N: Sham-treated with TM; R: 10 Gy irradiation. GAPDH: Glyceraldehyde-3-phosphate dehydrogenase.

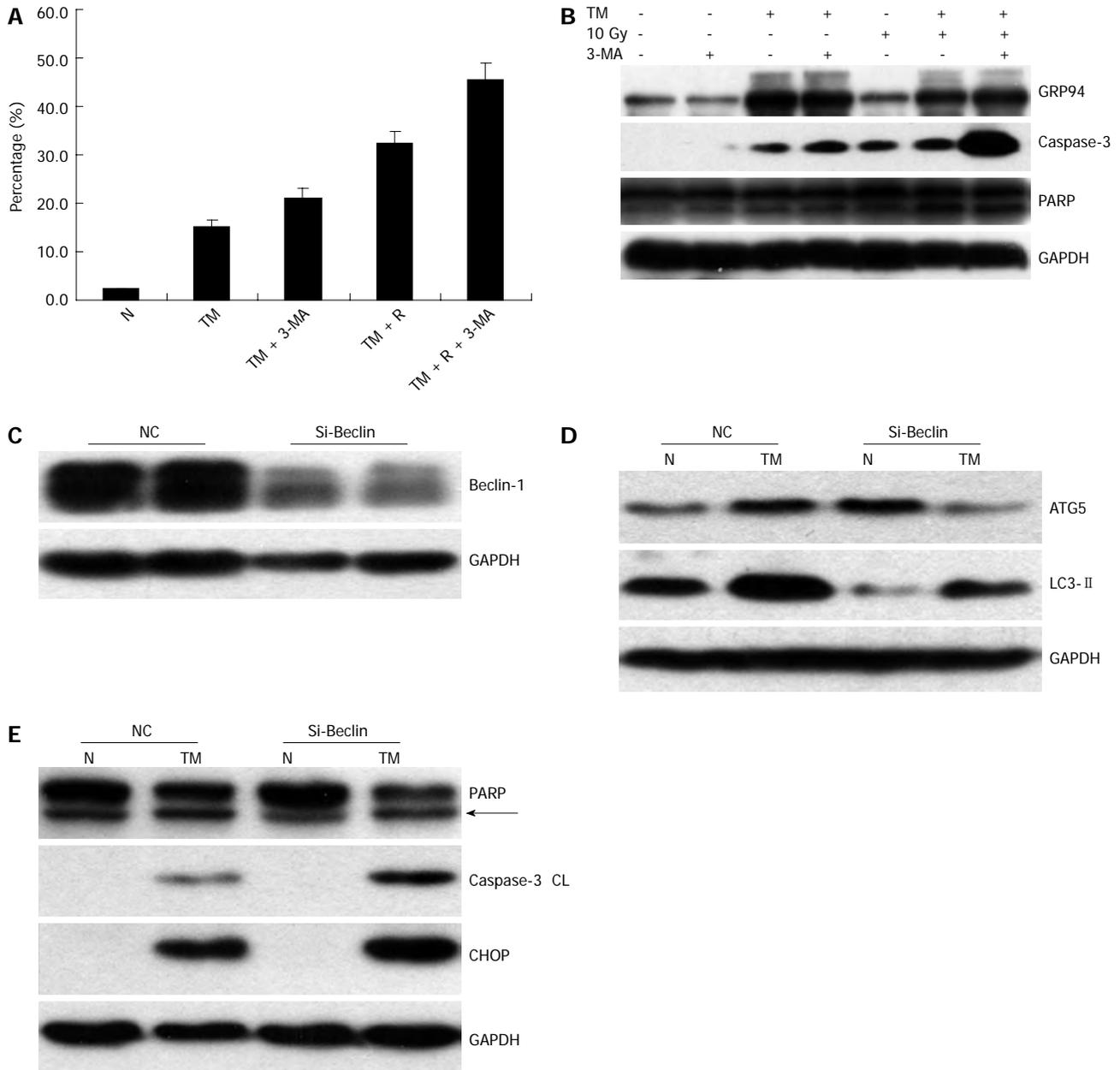


Figure 5 The effect of autophagy inhibition on cell apoptosis. EC109 cells were treated with tunicamycin (TM), radiation, and 3-MA for 24 h. **A**: The proportion of annexin V positive cells was measured and analyzed on a FACSsort; **B**: Protein levels of GRP94, cleaved Caspase-3, and its substrate poly ADP-ribose polymerase (PARP) were assessed by Western blotting; **C**: EC109 cells were transfected with Beclin-1 siRNA. Two days after transfection, protein levels of Beclin-1 and ATG5 were determined by Western blotting; **D**, **E**: Western blotting of cells transfected with siRNA and treated with TM. Levels of LC3, ATG5, C/EBP homologous protein (CHOP), cleaved Caspase-3, and PARP were shown. N: Sham-treated with TM; R: 10 Gy irradiation; GAPDH: Glyceraldehyde-3-phosphate dehydrogenase.

with TM for 24 h, which led to sustained ER stress and induced cell death. Increased apoptosis induced by TM was observed in a time-dependent manner. Besides inducing ER stress, TM was found to induce autophagy in renal proximal tubular cells. In this work, we established that TM induced an autophagic response in esophageal cancer cells (Figures 4B, 7A). Other classic ER stress inducers, such as DTT and MG132, were also found to induce autophagy. These studies implied a causal link between ER stress and autophagy. However, the biological significance of ER stress-induced autophagy largely remained unresolved.

In our previous work, we found that radiation alone also induces moderate ER stress, which was verified by other studies^[27,28]. The ER stress induced by radiation could also be interpreted as an adaptive response, as the ER stress inhibitor salubrinal blocked 50% of apoptosis induced by X-rays in pulmonary artery endothelial cells. However, sustained ER stress leads to cell death. In this work, sustained ER stress induced by TM treatment enhanced radiation sensitivity *in vitro* and *in vivo*. This suggests a potential strategy for maximizing the efficiency of cancer radiotherapy. The mechanism underlying ER stress-induced cell death needs to be further studied. In

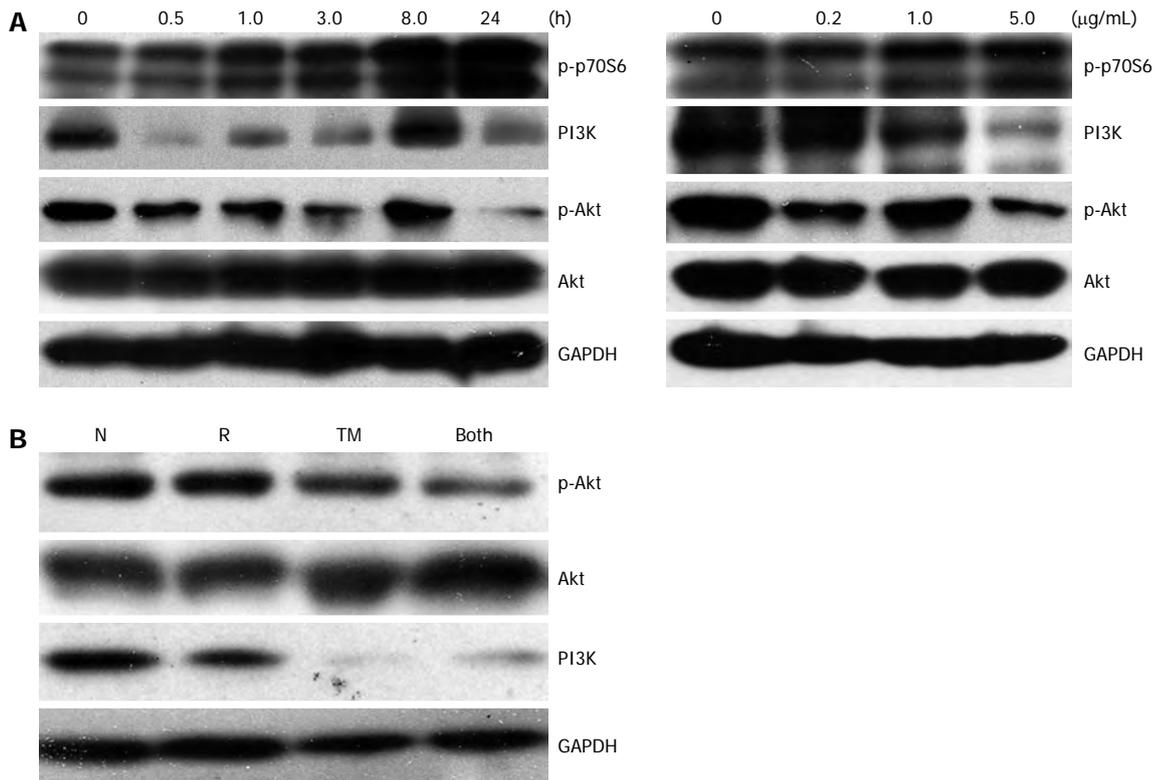


Figure 6 Cell signaling pathways associated with tunicamycin. A: EC109 cells were exposed to tunicamycin (TM) (5 µg/mL) for the indicated time periods, subjected to Western blotting analysis (left panel) or treated with TM at the indicated concentrations for 24 h, and subjected to Western blotting analysis (right panel); B: Western blotting analysis of cells pretreated with or without TM (5 µg/mL), followed by a single dose of 10 Gy. N: Sham-treated with TM; R: 10 Gy irradiation; GAPDH: Glyceraldehyde-3-phosphate dehydrogenase.

this work, we focused on autophagy and its relationship with cell apoptosis.

Induction of autophagy can be associated with cell death, and has greatly attracted researchers' interest. Initial work showed that nutrient starvation induced autophagy^[29]. Autophagy is also involved in physiological processes, such as development and differentiation. Moreover, recent studies have demonstrated a fundamental role of autophagy in a variety of pathophysiological conditions, including cancer, virus infection, heart failure, and neurodegeneration.

Many studies have found that autophagy might have a function in cancer treatment. However, contradictory findings about the role of autophagy were observed. Autophagy has been considered a mechanism by which the cell protects itself from various stresses, including nutrient starvation and chemotherapy. For example, it was reported that autophagy could protect glioma and fibrosarcoma cells from cisplatin-mediated apoptosis^[30]. But, there are also multiple examples in the literature where autophagy is the mode of cell death in tumor cells. Akar *et al.*^[31] reported that doxorubicin promoted autophagic cell death in MCF-7 cells at a clinically appropriate dose. It was also reported that autophagy was the main type of cell death in DNA-PK deficient human malignant glioma cells after radiation, while normal control cells could survive and proliferate, although a small portion of the cells underwent apoptosis^[32]. Another study reported that

autophagy (but not apoptosis) was the primary response to IR and radiosensitization induced by inhibition of NF-kappa B activation associated with autophagy^[33]. Conversely, pharmacological inhibition of autophagy using 3-MA blocked radiosensitization. Rapamycin, an inhibitor of the mTOR pathway that ordinarily induces autophagy in various types of cancer cells, increased the sensitivity of MCF-7 cells to radiation^[17]. Thus, the cytoprotective or cytotoxic role of autophagy depends on the cell context. In this work, we found that blockage of autophagy by the inhibitor 3-MA led to enhanced apoptosis, which suggested a cytoprotective role for autophagy in TM treatment. In addition, the autophagy response induced by TM treatment could be inhibited by Beclin-1 siRNA. It was reported that the inhibition of Beclin-1 diminished survival in radioresistant cancer cell lines after radiation, whereas survival in more radiosensitive carcinoma cells was enhanced^[34]. We did not assess the radiosensitivity of Beclin-1 knockdown alone. However, Beclin-1 knockdown enhanced cell apoptosis induced by TM.

Different cell signaling pathways are associated with TM treatment. The PI3K/Akt/mTOR pathway is critical for ER stress-induced apoptosis and autophagy induced by TM. Akt (also known as protein kinase B) functions as a principle mediator for many biological functions, including cell proliferation, differentiation, and survival. Dysregulation of Akt has been frequently detected in many types of cancer. The relationship between Akt and

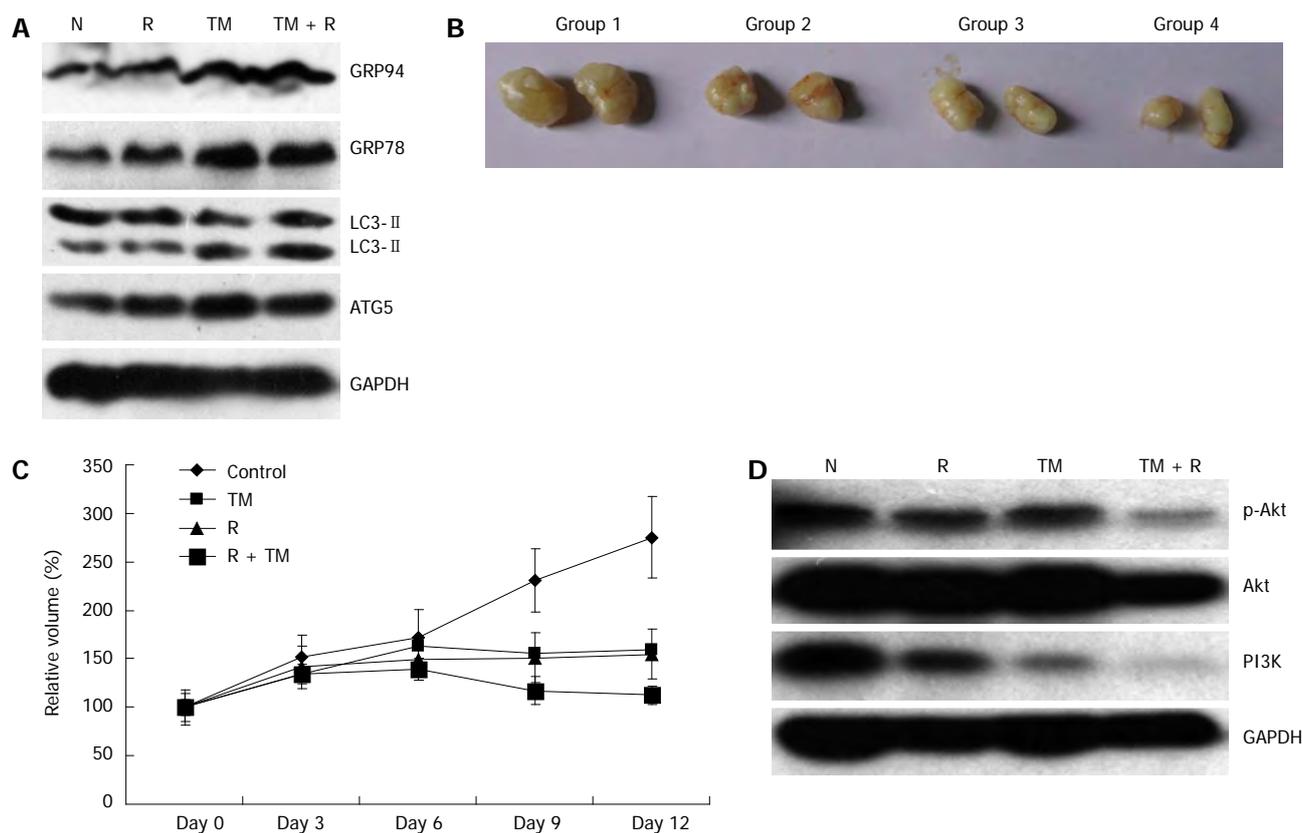


Figure 7 Radiosensitization by tunicamycin *in vivo*. A: Tumor xenografts removed from each group were subjected to Western blotting analysis for GRP78, GRP94, LC3, and ATG5; B: Examples of tumor xenografts removed from each group; C: Growth curves of tumor xenografts; D: Samples of tumor xenografts were subjected to Western blotting analysis for Akt, p-Akt, and PI3K. N: Sham-treated with tunicamycin (TM); R: 10 Gy irradiation; GAPDH: Glyceraldehyde-3-phosphate dehydrogenase.

mTOR is very intricate. Akt activates mTOR through the inactivation of tuberous sclerosis complex 2 (TSC2) within the TSC1-TSC2 complex. Increased activation of mTOR triggers a negative feedback loop for the PI3K/Akt pathway, leading to suppression of Akt^[35]. TM led to a sharply decreased level of p-Akt in mouse embryonic fibroblasts, while inducing a transient and biphasic activation of p-Akt in the rat renal tubular epithelial cell line NRK-52E^[20,24]. In this study, TM treatment led to decreased levels of p-Akt, which was further diminished following radiation. This unexpected observation could be related to the different cell context of these experiments.

In summary, cellular apoptosis and autophagy were found to be associated with TM treatment in EC109 cells and tumor xenografts. TM induced sustained ER stress, which sensitized cancer cells to radiation *in vitro* and *in vivo*. Our findings help to understand the molecular mechanisms associated with TM treatment, and suggest a potential strategy to maximize the efficiency of cancer radiotherapy.

COMMENTS

Background

The endoplasmic reticulum (ER) is an essential intracellular organelle with multiple roles, including the synthesis of nascent proteins, Ca²⁺ storage, gly-

cosylation, and the trafficking of newly-synthesized membrane and secretory proteins. Perturbations of these processes have been demonstrated to interfere with the proper functioning of ER, thus leading to a condition defined as ER stress. ER stress plays an important role in many cellular processes; it can activate an adaptive response aimed at neutralizing these threats and re-establishing homeostasis, which leads to cell survival. However, if these countermeasures are unsuccessful and prolonged stress persists, the ER stress response may abandon its pro-survival efforts and initiate a pro-apoptotic program to eliminate the faulty cells. In the authors' previous work, it was found that radiation could induce ER stress, which was associated with the protein kinase RNA dependent-like ER kinase and inositol-requiring protein-1 signaling pathways. However, the biological significance of ER stress remained unknown. The ER stress induced by radiation could also be interpreted as an adaptive response, as the ER stress inhibitor salubrinal blocked cell apoptosis induced by X-rays in pulmonary artery endothelial cells. However, sustained ER stress leads to cell death.

Research frontiers

According to a recent review, ER stress can be viewed as a "yin" and "yang" principle based on its relative levels. Moderate levels of ER stress favor the pro-survival, cell-protective module ("yang") while severe levels of ER stress will abandon their protective efforts and instead will trigger cell death ("yin"). For cancer chemotherapy and radiotherapy, those approaches which block the pro-survival function and/or enhance the pro-apoptotic process will benefit the therapeutic effects. Based on this delicate sensitivity of ER function, a large number of compounds which cause ER stress are tested for cancer therapy. However, its molecular mechanisms have not been fully elucidated.

Innovations and breakthroughs

Previous studies showed tunicamycin (TM) inhibited N-Acetylglucosamine transferases, and thus prevented the formation of N-linked glycoproteins, which ultimately induced sustained ER stress in various types of cells. A number of *in vitro* studies have investigated the chemosensitizing properties of TM. However, the radiosensitizing properties of TM are less investigated, and its

molecular mechanisms remained unknown. In this study, the authors found that sustained ER stress induced by TM sensitized human esophageal cancer cells to radiation. This finding was confirmed both *in vitro* and *in vivo*. The authors also showed that the PI3K/Akt/mTOR pathway was involved in the TM-induced autophagic response, and inhibition of autophagy increased apoptosis induced by TM.

Applications

The results of this study suggest that TM induces sustained ER stress, which sensitized cancer cells to radiation *in vitro* and *in vivo*. The authors' findings help to add to the understanding of the molecular mechanisms associated with TM treatment, and suggest a potential strategy for maximizing the efficiency of cancer radiotherapy.

Peer review

This is a good descriptive study in which the authors analyzed the radiosensitizing effect of TM on human esophageal cancer cells. The results are interesting and suggest that TM might be a potential therapeutic substance that could be used for cancer radiotherapy.

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Effects of hypoxia-inducible factor-1 α silencing on the proliferation of CBRH-7919 hepatoma cells

Lin-Feng Xu, Jia-Yan Ni, Hong-Liang Sun, Yao-Ting Chen, Yu-Dan Wu

Lin-Feng Xu, Jia-Yan Ni, Hong-Liang Sun, Yao-Ting Chen, Department of Interventional Radiology, Sun Yat-Sen Memorial Hospital, Sun Yat-Sen University, Guangzhou 510210, Guangdong Province, China

Yu-Dan Wu, Department of Hematology, Sun Yat-Sen Memorial Hospital, Sun Yat-Sen University, Guangzhou 510210, Guangdong Province, China

Author contributions: Xu LF and Ni JY contributed equally to this work; Ni JY performed the majority of experiments; Sun HL and Chen YT provided vital reagents and analytical tools and were also involved in editing the manuscript; Xu LF and Wu YD provided the financial support for this work and designed the study; Ni JY wrote the manuscript.

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Correspondence to: Yu-Dan Wu, MD, Department of Hematology, Sun Yat-Sen Memorial Hospital, Sun Yat-Sen University, Guangzhou 510210, Guangdong Province, China. wu_yudan@126.com

Telephone: +86-20-81332442 Fax: +86-20-81332442

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Abstract

AIM: To study the effects of hypoxia-inducible factor-1 α (HIF-1 α) silencing on the proliferation of hypoxic CBRH-7919 rat hepatoma cells.

METHODS: The CBRH-7919 rat hepatoma cell line was used in this study and the hypoxic model was constructed using CoCl₂. The HIF-1 α -specific RNAi sequences were designed according to the gene coding sequence of rat HIF-1 α obtained from GeneBank. The secondary structure of the *HIF-1 α* gene sequence was analyzed using RNA draw software. The small interfering RNA (siRNA) transfection mixture was produced by mixing the siRNA and Lipofectamine2000™, and transfected into the hypoxic hepatoma cells. Real time reverse transcription-polymerase chain reaction (RT-PCR) and Western blotting assay were used to detect

the expression levels of mRNA and protein. HIF-1 α and vascular endothelial growth factor (VEGF) mRNA was determined using real time RT-PCR; the protein expression levels of AKT, p-AKT, p21 and cyclinD1 were determined using Western blotting. The proliferation of hepatoma cells was observed using the methyl thiazolyl tetrazolium (MTT) assay and the bromodeoxyuridine (BrdU) incorporation cell proliferation assay.

RESULTS: Under induced hypoxia, the viability of the hepatoma cells reached a minimum at 800 μ mol/L CoCl₂; the viability of the cells was relatively high at CoCl₂ concentrations between 100 μ mol/L and 200 μ mol/L. Under hypoxia, the mRNA and protein expression levels of HIF-1 α and VEGF were significantly higher than that of hepatoma cells that were cultured in normoxia. HIF-1 α -specific RNAi sequences were successfully transfected into hepatoma cells. The transfection of specific siRNAs significantly inhibited the mRNA and protein expression levels of HIF-1 α and VEGF, along with the protein expression levels of p-AKT and cyclinD1; the protein expression of p21 was significantly increased, and there was no significant difference in the expression of AKT. The MTT assay showed that the amount of hepatoma cells in S phase in the siRNA transfection group was obviously smaller than that in the control group; in the siRNA transfection group, the amount of hepatoma cells in G1 phase was more than that in the control group. The BrdU incorporation assay showed that the number of BrdU positive hepatoma cells in the siRNA transfection group was less than that in the control group. The data of the MTT assay and BrdU incorporation assay suggested that HIF-1 α silencing using siRNAs significantly inhibited the proliferation of hepatoma cells.

CONCLUSION: Hypoxia increases the expression of HIF-1 α , and HIF-1 α silencing significantly inhibits the proliferation of hypoxic CBRH-7919 rat hepatoma cells.

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Key words: RNA interference; Hypoxia-inducible factor-1 α ; Vascular endothelial growth factor; Protein kinase B; CBRH-7919 hepatoma cells

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INTRODUCTION

Hepatocellular carcinoma (HCC) is one of the most common malignancies. HCC accounts for more than 660 000 new cases of cancer arising annually all around the world^[1,2]. HCC is a highly malignant tumor and the majority of HCC patients are diagnosed at the intermediate or advanced stages of tumor growth. HCC is often accompanied by poor liver function, which makes surgical resection impossible. Liver transplantation is one treatment option for HCC patients. However, the availability of organ donors limits the application of this treatment. Currently, nonsurgical therapy has been accepted as the main treatment for HCC, and gene therapy for the treatment of HCC has begun to attract increasing attention. RNA interference is a powerful method for the knock-down of pathologically relevant genes. Small interfering RNAs (siRNAs) have been widely demonstrated as effective biomedical genetic-therapy applications for many diseases^[3,4]. siRNAs induce sequence-specific gene silencing of the target mRNAs to which they are perfectly complementary by directing the RNA-induced silencing complex to mediate site-specific cleavage, thus destroying the targeted mRNA^[5]. In view of its powerful gene-silencing properties, RNAi has been proposed as an important option to validate new therapeutic targets and to develop innovative anticancer therapies.

Intratumoral hypoxia is a common finding in human cancers and associated with an increased risk of tumor regeneration, invasion, metastasis and patient mortality^[6]. Hypoxia inducible factor-1 (HIF-1) functions as a master regulator of oxygen homeostasis in almost all nucleated mammalian cells and is composed of the constitutively expressed HIF-1 β subunit and the highly regulated HIF-1 α subunit. The HIF-1 α subunit is the functional subunit of HIF-1 and plays a critical role in cellular adaptation to change in oxygen availability^[7-9]. HIF-1 α is a central transcription factor produced by tumor cells under hypoxic conditions and is a key regulator of several genes that are important in cancer biology. Over-expression of HIF-1 α in human tumors is associated with poor prognosis and poor therapeutic outcomes, and HIF-1 α has been suggested as a significant target for cancer therapy^[10,11].

According to previous studies^[12-14], it is possible to silence HIF-1 α using siRNA interference technology. Thus, in this study we constructed specific HIF-1 α siRNA in-

terference sequences, and transfected these into hypoxic CBRH-7919 rat hepatoma cells to study the effects of HIF-1 α silencing on the activation of the phosphatidylinositol 3'-kinase(PI3K)/AKT signaling pathway and the proliferation of hepatoma cells. Consequently, we found that the HIF-1 α silencing significantly inhibits the activation of the PI3K/AKT signaling pathway and the proliferation of hypoxic CBRH-7919 rat hepatoma cells.

MATERIALS AND METHODS

Cell lines and cell culture

The CBRH-7919 rat hepatoma cell line was provided by the Central Laboratory of Sun Yat-Sen University (Guangzhou, China). The hepatoma cells were routinely grown in RPMI1640 medium, supplemented with 10% fetal bovine serum, 100 U/mL penicillin and 100 U/mL streptomycin in a 37 °C incubator with 5% CO₂. The cells were passaged every 2-3 d to maintain exponential growth.

Hypoxia model construction

Cells were plated in 60-mm dishes or 6-well plates according to the experimental requirements and were cultured at 37 °C with 5% CO₂. In preparation for the experiment, the medium was replaced with a thin layer of fresh medium which contained 10% FCS to decrease the diffusion distance of the ambient gas. Different densities of CoCl₂ (100, 150, 200 and 300 μ mol/L) were then mixed into the culture dishes, and the samples were processed for 12 and 24 h. A control sample containing 0 μ mol/L CoCl₂ was also processed.

Design of siRNA and transfection studies

siRNAs targeted against HIF-1 α were designed according to the gene coding sequence of rat HIF-1 α obtained from GeneBank. The secondary structure of the HIF-1 α gene sequence was analyzed using RNA draw software. Three potential target mRNA sequences were selected and siRNA sequences were determined using online design software (www.ambion.com/techlib/misc/siRNA_finder.htm): 1:P1:5'-AGUGACUGAUUCUG-GCAGCTT-3', P2: 5'-GCUGCCAGAAUCAGUCACUTT-3'; 2:P1: 5'-GGAUGACUUUAAGCAAGAATT-3', P2: 5'-UUCUUGCUUAAAGUCAUCCTT-3'; 3:P1: 5'-GAAACUCUCCAAGCAAUUTT-3', P2: 5'-AAU-UGCUUGGAAGAGUUUCTT-3'. Based on the process of siRNA target sequence design, the silencer siRNAs were produced according to the sequence of the selected target mRNA. Then the siRNAs transfection mixture was produced by mixing the siRNA and Lipofectamine2000TM (Invitrogen, Carlsbad, CA, United States). The mixture containing siRNA sequences was then transfected into cultured cells, according to the manufacturer's protocol. The cells were harvested 24 h after transfection for analyses. A subset of CBRH-7919 cells was treated only with Lipofectamine 2000TM reagent as a control group.

Real time reverse transcription-polymerase chain reaction

RNA was extracted directly from all samples using TRIzol reagent (Invitrogen) followed by isopropanol precipitation. An RNA polymerase chain reaction (PCR) kit (TaKaRa) was used to obtain template cDNA for real-time PCR (ABI Prism 7500, Perkin Elmer, Foster City, CA, United States) using SYBR Premix Ex Taq (TaKaRa). Specific primer sequences were designed by TaKaRa as follows: HIF-1 α : 5'-ACTGCACAGGCCACATTCAT-3' (sense) and 5'-CGAGGCTGTGTCTGACTGAGA-3' (antisense); vascular endothelial growth factor (VEGF): 5'-AGGCGAGGCAGCTTGAGTTA-3' (sense) and 5'-CTGTCTGACGGTGACGATGGT-3' (antisense); β -actin: 5'-CCTAGGCACCAGGGTGTGAT-3' (sense) and 5'-TTGGTGACAATGCCGTGTTTC-3' (antisense). The initial denaturation phase was 3 min at 95 °C followed by 39 cycles of denaturation at 95 °C for 10 s and annealing at 55 °C for 30 s. Relative quantification of PCR products was performed after normalization to β -actin.

Western blotting

Cellular proteins were extracted with RIPA lysis buffer, and protein concentrations were measured by the Bradford method. Protein samples (20 μ g/well) were separated by 10% sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis, and electrophoretically transferred to nitrocellulose membranes. The membranes were then blocked for 60 min and subsequently incubated with primary antibodies (1:3000) overnight at 4 °C prior to incubation with anti-mouse IgG conjugated to horseradish peroxidase (1:6000) for 120 min at room temperature. Finally, after developing with enhanced chemiluminescence detection reagents, the protein bands of membranes were visualized by exposure to x-ray film. Protein expression was quantified by densitometry and normalized to β -actin expression. Anti-HIF-1 α , anti-Akt/phosphorylated Akt, anti-VEGF, anti-p21, cyclinD1, and anti- β -actin, antibodies were obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, United States).

Methyl thiazolyl tetrazolium cell proliferation assay

Cells were seeded into 96-well plates (Costar; Corning Inc., Corning, NY, United States) for 9 h. Following exposure to 0.5% O₂, the cells were exposed to various concentrations of cisplatin or doxorubicin for 48 h in a CO₂ incubator. Methyl thiazolyl tetrazolium (MTT) (10 μ L; 5 g/L in PBS) was added to each well followed by incubation for 4 h at 37 °C. Subsequently, the formazan crystals were solubilized with 150 μ L of 10% SDS in 0.01 mol/L HCl for 24 h. The absorbances at 500 nm relative to a reference wave length of 490 nm were determined with a microplate reader (Bio-Rad 680, Bio-Rad, United States). The absorbance values were expressed as percentages relative to the untreated controls, and the concentrations resulting in 50% inhibition of cell growth (IC₅₀ values) were calculated.

Bromodeoxyuridine incorporation assay

CBRH-7919 cells were seeded in 96-well plates and treated with various drugs as indicated in each experiment for 48 h. At the end of treatment, bromodeoxyuridine (BrdU) incorporation was assayed by incubating the cells with BrdU for 0.5-1 h using a BrdU Cell Proliferation Assay Kit (Calbiochem, San Diego, CA, United States) according to the manufacturer's instructions.

Statistical analysis

All experimental data were expressed as mean \pm SD. Comparisons between two groups were made using the Student's *t*-test. Statistical significance was determined by analysis of variance followed by Fisher's least significant difference analysis using the SPSS 19.0 software package. The statistical significance level was set at $P < 0.05$.

RESULTS

Viability of hepatoma cells under hypoxia

CBRH-7919 hepatoma cells were cultured in a hypoxia model, which was constructed using various concentrations of CoCl₂ (Figure 1). The viability of hepatoma cells under hypoxia was observed using MTT, and the results of the analysis indicated that the viability of hepatoma cells was gradually decreased as CoCl₂ concentrations increased from 100 μ mol/L to 800 μ mol/L. The viability of the hepatoma cells reached a minimum at 800 μ mol/L CoCl₂; however, the viability of the cells was relatively high at CoCl₂ concentrations between 100 μ mol/L and 200 μ mol/L (Figure 1).

Hypoxia increased the expressions of HIF-1 α and VEGF

Hepatoma cells were cultured under 0, 100, 150, 200 and 300 μ mol/L CoCl₂ using processing times of 12 and 24 h, respectively. CoCl₂ at 0 μ mol/L was the control group. The results of real-time RT-PCR assay indicated that the mRNA levels of HIF-1 α and VEGF gradually increased as the concentrations of CoCl₂ increased from 0 μ mol/L to 200 μ mol/L with a 12 h processing time and the expression levels of HIF-1 α and VEGF reached a maximum at 200 μ mol/L CoCl₂ (Figure 2). However, the expressions of HIF-1 α and VEGF were declined as the concentration of CoCl₂ reached 300 μ mol/L. When the processing time was increased to 24 h, the expression levels of HIF-1 α and VEGF were higher than those observed at 12 h. Additionally, the protein expression levels of HIF-1 α and VEGF demonstrated the same patterns as their respective mRNA expression levels (Figure 3).

Expressions of HIF-1 α and VEGF were inhibited by siRNA interference

Based on the previous phase of our study, the optimal hypoxia-mimicking condition was determined to be the concentration of 150 μ mol/L CoCl₂ with a 24 h processing time. Thus, CBRH-7919 hepatoma cells were cultured under these conditions prior to the next step of the ex-

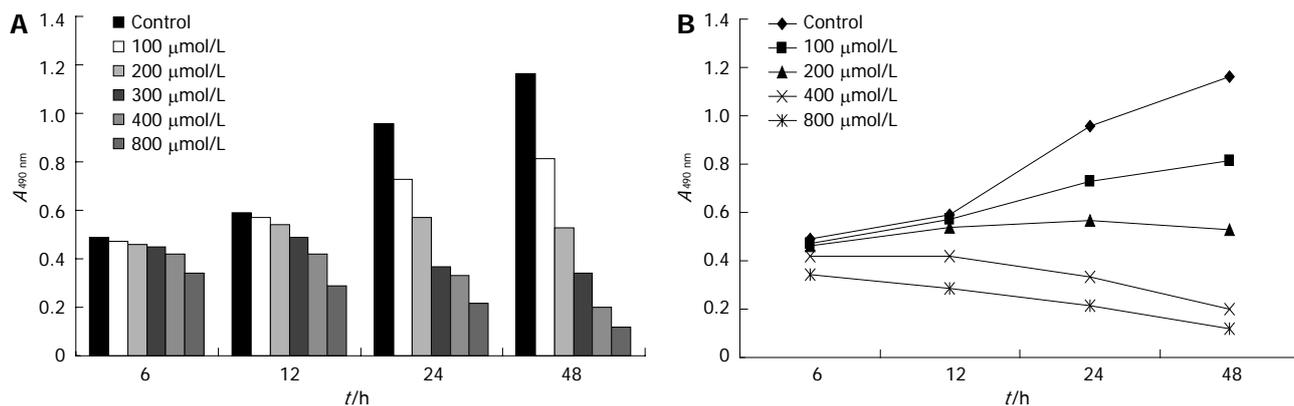


Figure 1 Viability of hepatoma cells under hypoxia was measured using methyl thiazolyl tetrazolium cell proliferation assay. A: Histograms; B: Polygon.

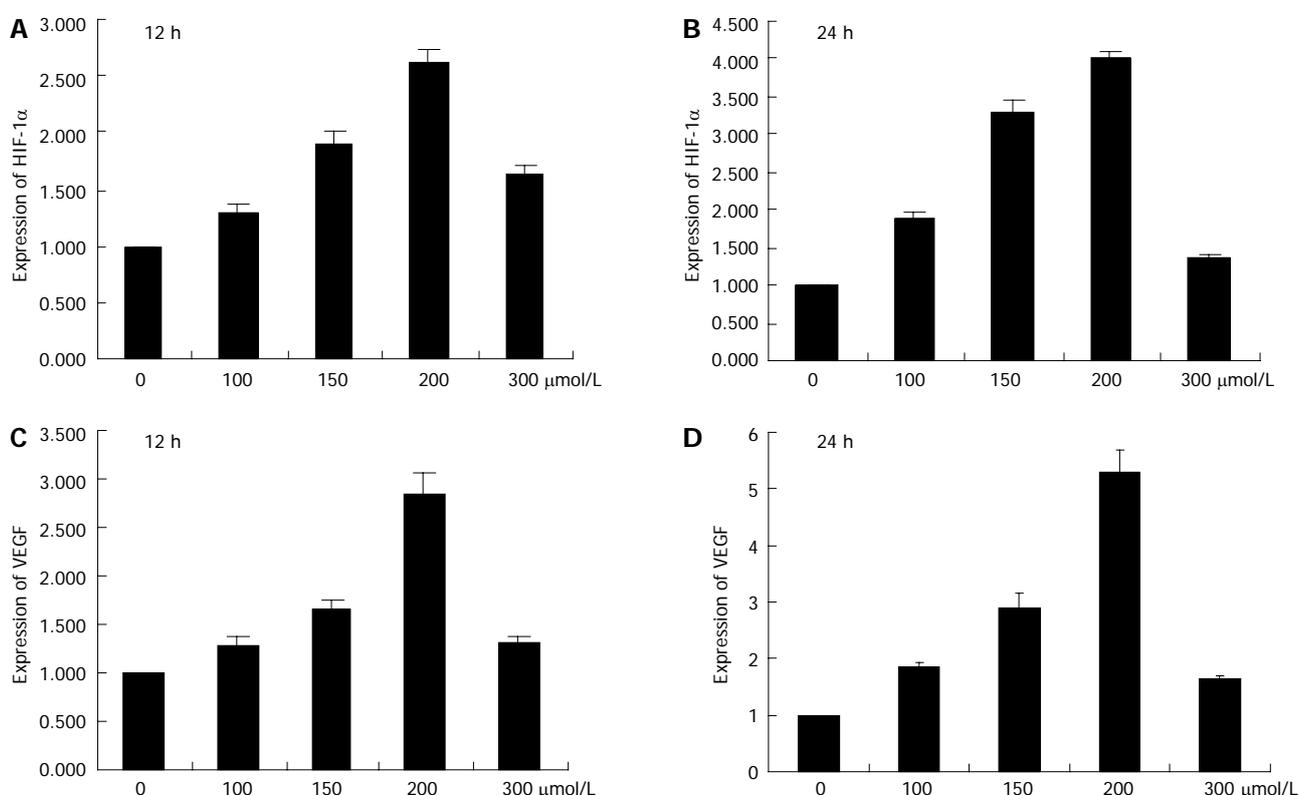


Figure 2 mRNA expression levels of hypoxia-inducible factor-1 α and vascular endothelial growth factor. A, B: Histograms illustrating hypoxia-inducible factor-1 α (HIF-1 α) mRNA expression after exposure to various concentrations of CoCl₂ for 12 h and 24 h; C, D: Histograms illustrating vascular endothelial growth factor (VEGF) mRNA expression after exposure to various concentrations of CoCl₂ for 12 h and 24 h.

periment. The specific siRNA sequences were designed and the hepatoma cells were transfected using three different groups of specific siRNA vectors. The protein and mRNA expression levels of HIF-1 α and VEGF were measured using real-time RT-PCR and western blot analysis, respectively. The results indicated that both the mRNA and protein expression levels of HIF-1 α and VEGF were significantly decreased after the transfection with specific siRNA sequences with a processing time of 24 h (Figures 4 and 5). However, there were no differences in the mRNA and protein expression levels of HIF-1 α and VEGF of hepatoma cells in the control group.

Effects of HIF-1 α silencing on the expressions of VEGF, AKT, p-AKT, p21 and cyclinD1

Based on the previous phases of our study, the specific siRNA sequence “Ri3” was selected for the next phase of this study. “Ri3” was transfected into the hepatoma cells cultured with 150 μ mol/L CoCl₂ with a 24 h processing time, and Western blotting analysis was performed to detect the protein expression levels of HIF-1 α , VEGF, AKT, p-AKT, p21 and cyclinD1. The results indicated that the protein expression levels of HIF-1 α , VEGF, p-AKT and cyclinD1 were significantly decreased compared with those of the control group; however, the

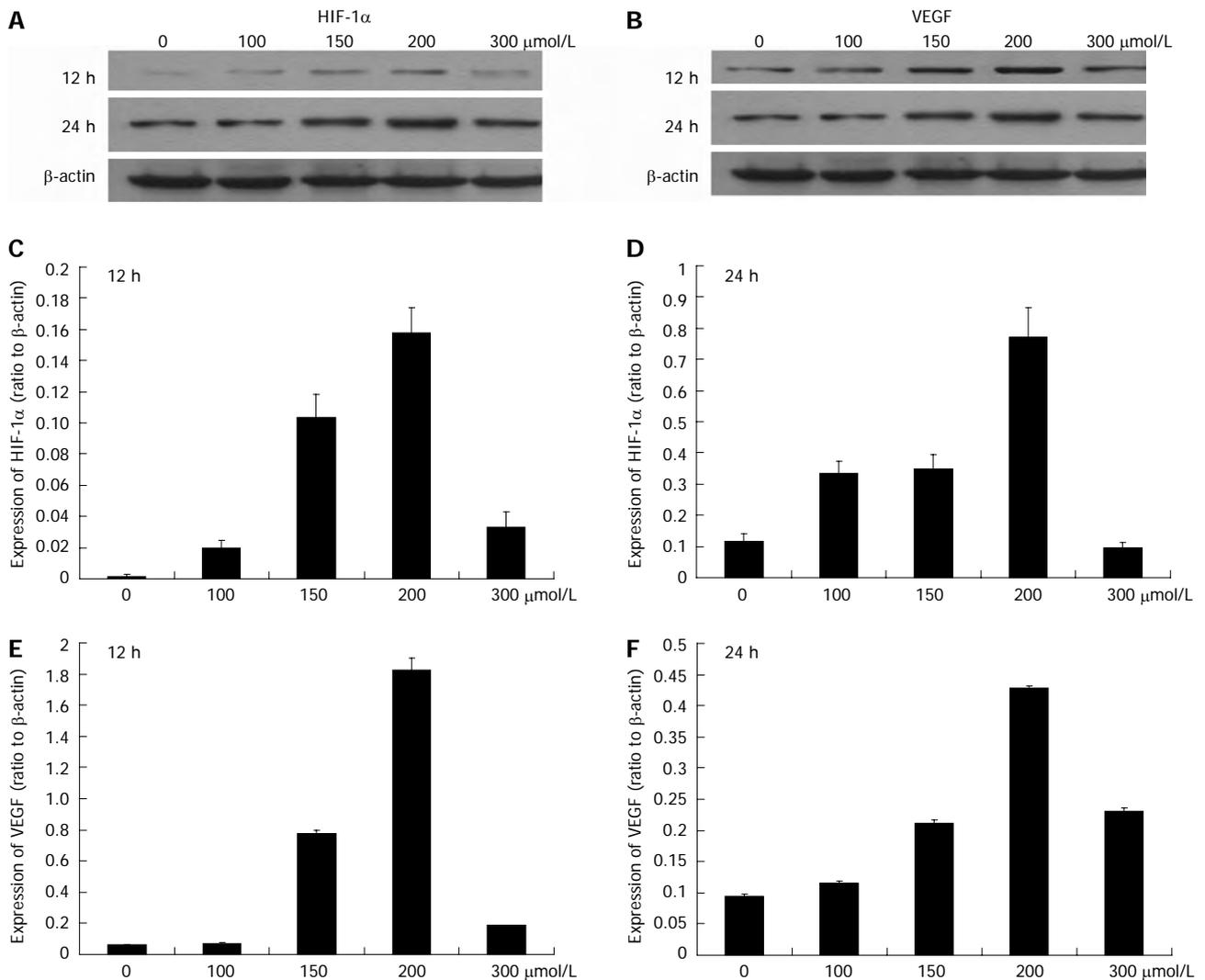


Figure 3 Protein expression levels of hypoxia-inducible factor-1 α and vascular endothelial growth factor after exposure to 0-300 μ mol/L CoCl₂ for 12 and 24 h. A, B: The Western blotting analysis of protein expression levels of hypoxia-inducible factor-1 α (HIF-1 α) and vascular endothelial growth factor (VEGF); C, D: Histograms illustrating HIF-1 α protein expression after exposure to various concentrations of CoCl₂ for 12 h and 24 h; E, F: Histograms illustrating VEGF protein expression after exposure to various concentrations of CoCl₂ for 12 h and 24 h.

expression of p21 protein was significantly increased and there was no significance difference in the expression of AKT protein (Figure 6).

Effects of HIF-1 α silencing on the proliferation of hepatoma cells

In consideration of the functions of p21 and cyclinD1 in the cell cycle, the decrease in the expressions of p21 and cyclinD1 caused by HIF-1 α silencing suggested that the knockdown of HIF-1 α may suppress the proliferation of the hepatoma cells. Thus, we studied the effects of HIF-1 α -specific siRNAs on the proliferation of the CBRH-7919 hepatoma cells. HIF-1 α -specific siRNAs or controls were transfected using Lipofectamine 2000TM and the proliferation of hepatoma cells was determined using the MTT assay and the BrdU incorporation assay at 24 h after the transfection. The results of the MTT assay and BrdU incorporation assay indicated that the HIF-1 α silencing significantly inhibited the proliferation

of CBRH-7919 hepatoma cells when compared with the control group at the processing time of 24 h (Figures 7 and 8).

DISCUSSION

The hypoxia inducible transcription factors mediate the primary transcriptional responses to hypoxic stress in normal and transformed cells^[15]. HIF-1 α was firstly described by Semenza in 1992 and was shown to play a critical role in mediating O₂-dependent transcriptional responses. HIF-1 activity in tumors depends on the availability of the HIF-1 α subunit, the expression level of which is increased under hypoxic conditions and is associated with the activation of oncogenes and/or the inactivation of tumor suppressor genes. Over-expression of HIF-1 α has been correlated with increased angiogenesis, tumor progression, invasion, metastasis and poor patient prognosis, and this has led to the current interest in HIF-

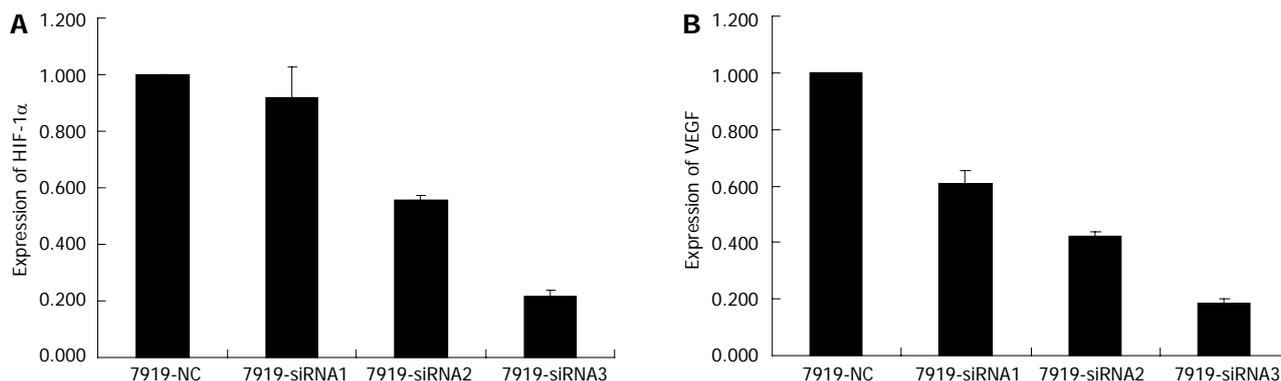


Figure 4 Histograms illustrating hypoxia-inducible factor-1 α (A) and vascular endothelial growth factor (B) mRNA expression after small interfering RNAs transfection (24 h processing time). siRNA: Small interfering RNA.

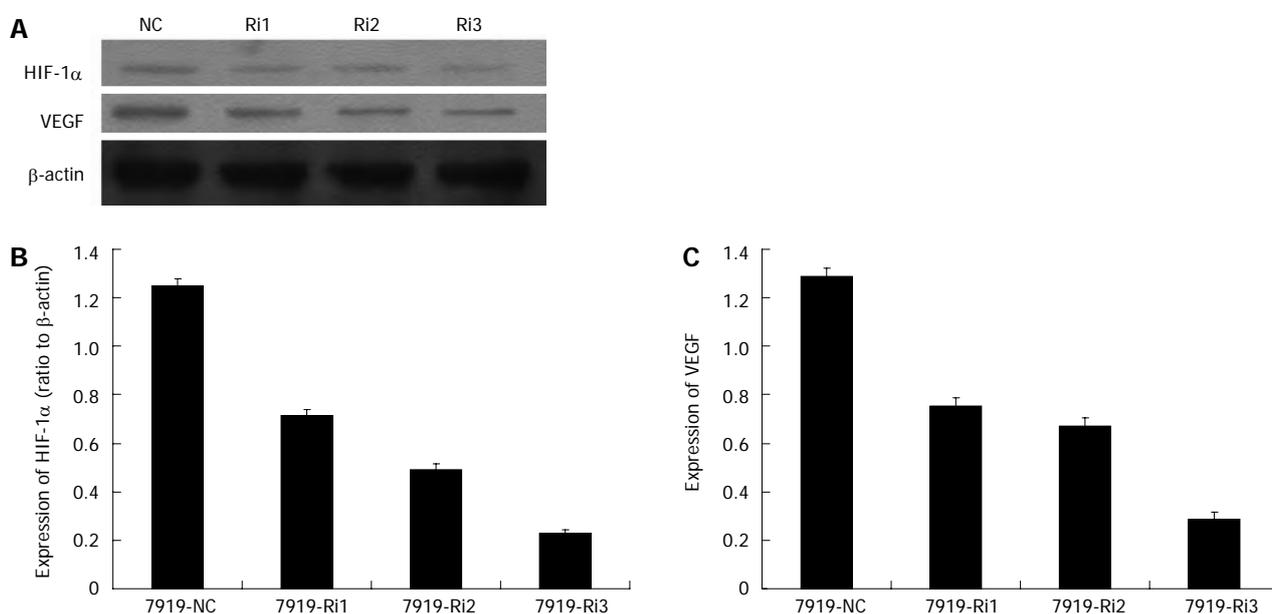


Figure 5 Protein expression levels of hypoxia-inducible factor-1 α and vascular endothelial growth factor after small interfering RNAs transfection (24 h processing time). A: The Western blotting analysis of protein expression levels of hypoxia-inducible factor-1 α (HIF-1 α) and vascular endothelial growth factor (VEGF) after small interfering RNA (siRNA) transfection; B, C: Histograms illustrating HIF-1 α (B) and VEGF (C) protein expression after siRNA transfection. NC: Control group without siRNA transfection; Ri1, Ri2, Ri3: Three different specific siRNA sequences.

1 α as a promising anticancer genetic target for anticancer therapies^[16,17].

In this study, rat CBRH-7919 hepatoma cells were cultured in a hypoxic model constructed using CoCl₂, and specific HIF-1 α siRNA sequences were designed and transfected into hypoxic hepatoma cells. Our study demonstrated that the expressions of HIF-1 α , VEGF, p-AKT and cyclinD1 were significantly decreased compared with those of the control group; however, the expression of p21 was significantly increased and there was no significant difference in the expression of AKT. This finding indicates that silencing of HIF-1 α using specific siRNA can inhibit the expressions of VEGF, p-AKT and cyclinD1 while up-regulating the expression of p21. The subsequent MTT cell proliferation and BrdU incorporation assays revealed that the proliferation of hepatoma cells was significantly inhibited by HIF-1 α silencing.

The PI3K/AKT signaling transduction pathway is

one of the most important pathways inside human cancer and plays a critical role in various cellular functions, such as proliferation, adhesion, migration, invasion, metabolism and survival^[18-20]. The activation of the PI3K/AKT signaling pathway depends on the expression of p-AKT (phosphorylated AKT). Over-expression of p-AKT is one of the most common indications of human malignancies, such as gastric cancer, liver cancer, colorectal cancer, pancreatic cancer and breast cancer^[21,22]. Additionally, p-AKT is believed to play an important role in cancer cell survival and chemotherapy resistance. In our study, although there was no significant difference in the expression of AKT, the expression of p-AKT was significantly inhibited after the specific siRNA sequences were transfected into hepatoma cells. This indicated that the silencing of HIF-1 α using specific siRNAs inhibited the activation of the PI3K/AKT signaling transduction pathway. The inhibition of the PI3K/AKT signaling pathway may be

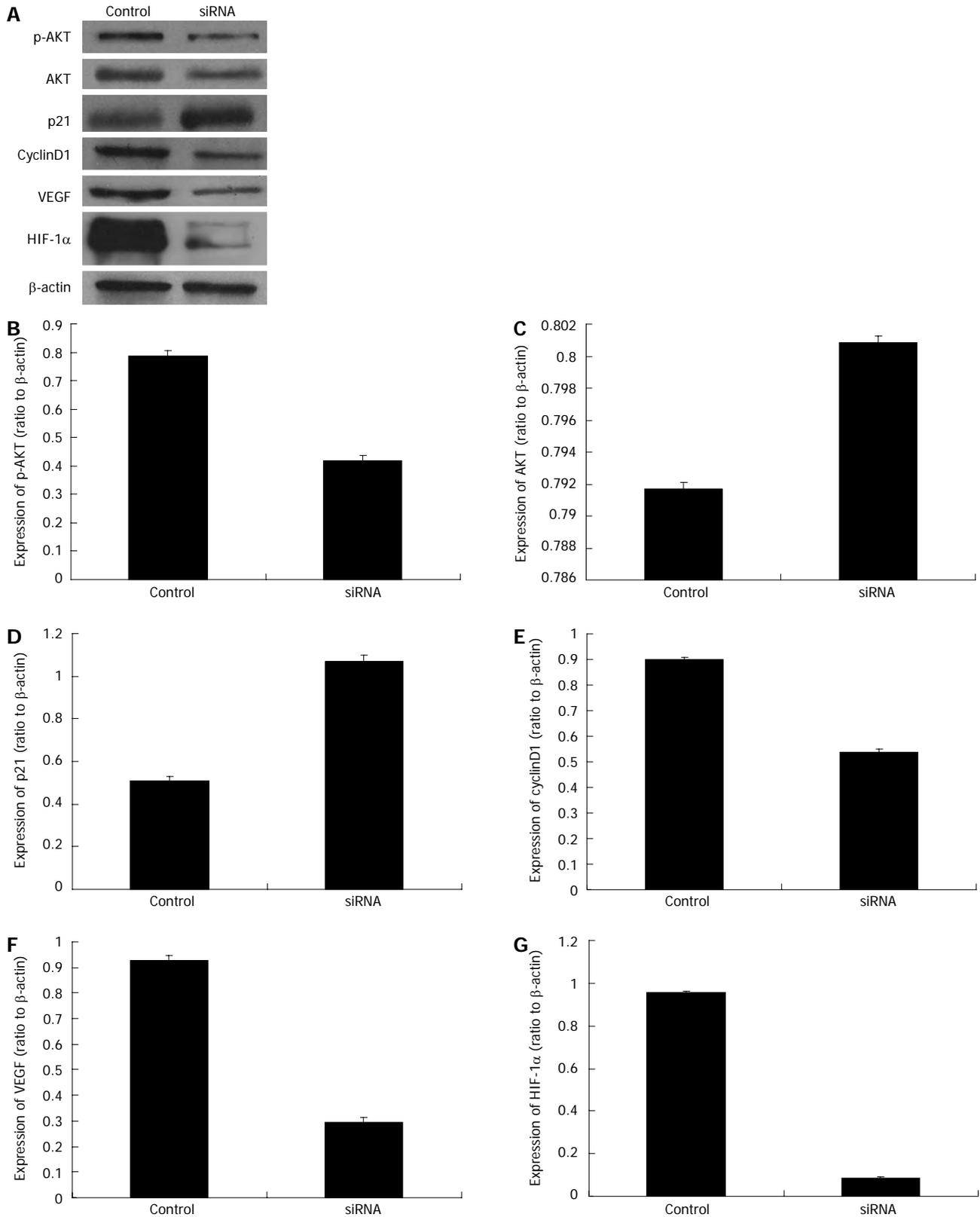


Figure 6 Protein expression levels of p-AKT, AKT, p21, cyclinD1, vascular endothelial growth factor and hypoxia-inducible factor-1 α after the transfection with specific small interfering RNAs (processing time of 24 h). A: The Western blotting analysis of protein expression levels of p-AKT, AKT, p21, cyclinD1, vascular endothelial growth factor (VEGF) and hypoxia-inducible factor-1 α (HIF-1 α) after specific small interfering RNA (siRNA) transfection; B-G: Histograms illustrating p-AKT (B), AKT (C), p21 (D), cyclinD1(E), VEGF (F) and HIF-1 α (G) protein expression after specific siRNA transfection.

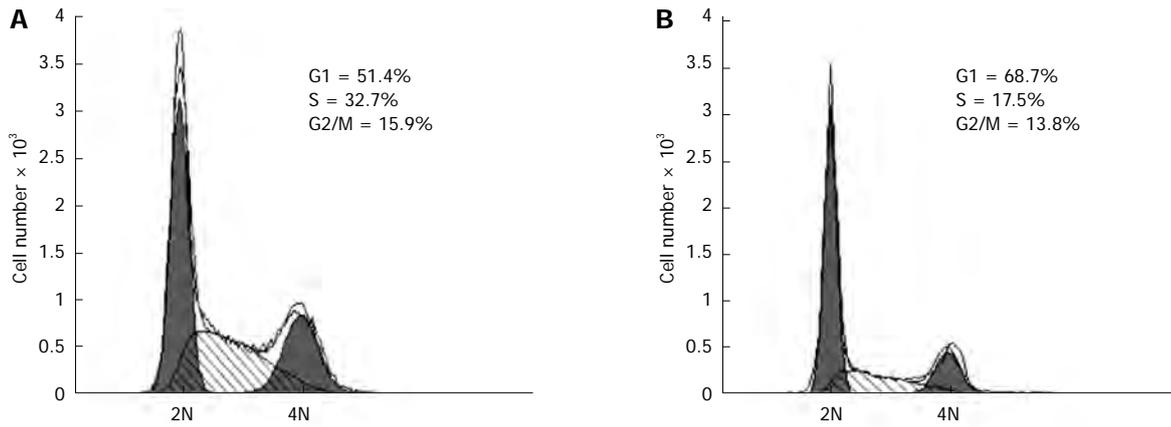


Figure 7 Methyl thiazolyl tetrazolium cell proliferation assay indicated that the hypoxia-inducible factor-1 α silencing *via* specific small interfering RNAs significantly inhibited the proliferation of CBRH-7919 hepatoma cells. A: Contro group; B: Processing time of 24 h.

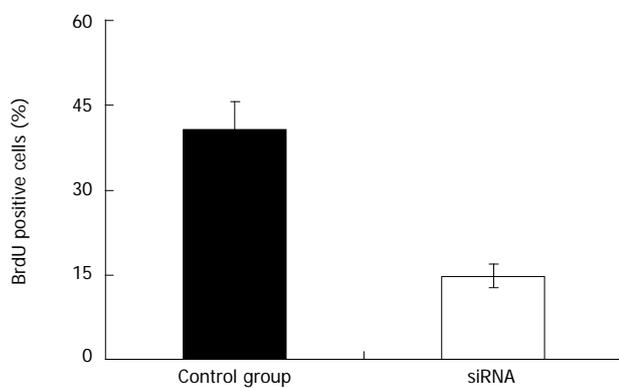
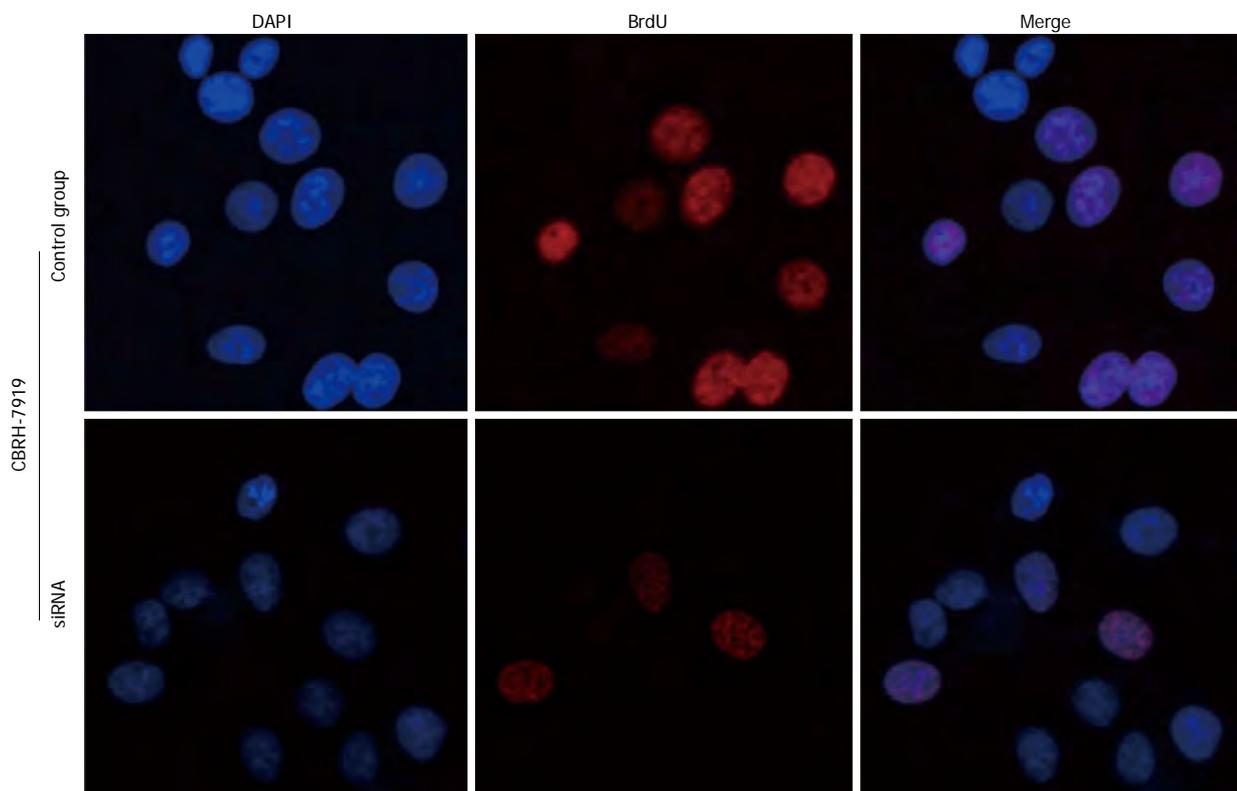


Figure 8 Proliferation of hepatoma cells detected by bromodeoxyuridine incorporation illustrates that hypoxia-inducible factor-1 α silencing *via* specific small interfering RNAs significantly inhibited the proliferation of CBRH-7919 hepatoma cells. DAPI: 4',6'-diamidino-2-phenylindole hydrochloride; BrdU: Bromo-deoxyuridine.

associated with inhibition of the activation of the PI3K-independent pathway. Under hypoxia or within tumor cells, the normal degradation of HIF-1 α is inhibited and HIF-1 α accumulates in the nucleus, which promotes the activation of downstream targeted genes and activates AKT^[23-25]. Over-expression of VEGF is also observed in various types of human cancer cells. VEGF promotes the proliferation of vessel endothelial cells, inhibits the apoptosis of vessel endothelial cells, and stimulates the formation of blood vessels. Previous studies reported that VEGF alone can activate the PI3K/AKT pathway^[26,27]. Hence, it is indicated that inhibition of the expression of VEGF by HIF-1 α silencing may affect the activation of the PI3K/AKT pathway. p21 and cyclinD1 are the downstream targets of the PI3K/AKT signaling transduction pathway and their expressions are also regulated by the pathway^[28,29]. Our data revealed that the expression of p21 was significantly increased; however, the expression of cyclinD1 was significantly decreased after HIF-1 α was silenced by specific siRNAs. The results of our study provide strong evidence to support our hypothesis that the activation of the PI3K/AKT signaling pathway can be inhibited by HIF-1 α silencing.

Considering the functions of p21 and cyclinD1 in cell cycle processing^[30-34], HIF-1 α silencing may have an effect on PI3K/AKT signaling pathway-dependent proliferation of hepatoma cells as well. In our study, the results of the MTT cell proliferation and BrdU incorporation assays indicated that the transfection of specific siRNAs effectively increased the number of cells in G0/G1 phase, and the numbers of cells in S and G2/M phases were significantly decreased compared with those of the control group. Additionally, the proportion of BrdU-positive cells in the siRNA interference group was smaller than that of the control group. Taken together, these data indicate that HIF-1 α silencing by the specific siRNAs can significantly inhibit the activation of the PI3K/AKT signaling transduction pathway and the proliferation of hepatoma cells.

HIF-1 α is currently accepted as one of the most important genetic targets for anticancer therapy. However, to our knowledge, there have only been a few studies examining the effects of HIF-1 α on the proliferation of hepatoma cells. Our study demonstrated that HIF-1 α silencing can inhibit the proliferation of hypoxic rat CBRH-7919 hepatoma cells. However, the correlation between HIF-1 α and the PI3K/AKT signaling transduction pathway is still contested^[35-38] and further research is urgently needed to provide new evidence. The evidence obtained in our study indicated that HIF-1 α can be silenced using specific siRNAs and that the silencing of HIF-1 α can significantly inhibit the activation of the PI3K/AKT signal transduction pathway.

Transcatheter arterial chemoembolization (TACE) is the most commonly used palliative treatment for HCC in clinical practice^[39-41]. However, the hypoxic microenvironment of tumor tissue after TACE often induces high expression levels of HIF-1 α and promotes tumor progres-

sion, invasion and metastasis of tumor, providing a poor prognosis for patients with HCC. Thus, the long-term results of TACE are not satisfying. The combination of TACE and siRNA interference for the treatment of HCC can be expected to down-regulate the expression of HIF-1 α after TACE. Unfortunately, it is unclear whether the effects of siRNA interference *in vivo* are the same as (or similar to) that which are observed *in vitro*. Thus, further studies are required to explore the effects of siRNA interference *in vivo*.

COMMENTS

Background

The over-expression of hypoxia-inducible factor-1 α (HIF-1 α) has been demonstrated in multiple types of malignant tumors. HIF-1 α contributes to tumor angiogenesis and metastasis and plays a critical role in mediating O₂-dependent transcriptional responses. Preliminary tests showed that it is possible to silence HIF-1 α using small interfering RNA (siRNA) interference technology.

Research frontiers

Gene therapy is a potential treatment for malignant tumors and some other diseases. siRNAs have been widely demonstrated as effective biomedical genetic-therapy applications for many diseases. In this study, the authors demonstrate that HIF-1 α silencing using siRNAs can significantly inhibit the proliferation of hepatoma cells, which could be a potential gene therapy for anti-cancer treatment.

Innovations and breakthroughs

Previous studies reported that HIF-1 α contributes to tumor angiogenesis and metastasis and plays a critical role in mediating O₂-dependent transcriptional responses. However, the relationship between HIF-1 α and the PI3K/AKT signaling pathway, and the effects of HIF-1 α on the proliferation of hepatoma cells, have not been unequivocally addressed. The results of this study showed that HIF-1 α silencing can significantly inhibit the activation of the PI3K/AKT signaling pathway and the proliferation of hepatoma cells. This study proves that HIF-1 α could be an effective and important gene target for anti-cancer treatment.

Applications

By understanding the effect of HIF-1 α silencing on the PI3K/AKT signaling pathway and the proliferation of hepatoma cells, an effective gene target has been identified for the gene treatment of hepatocellular carcinoma (HCC), providing an experimental basis for the clinical practice of gene therapies for malignant tumors.

Terminology

HIF-1 α has been correlated with increased angiogenesis, tumor progression, invasion, metastasis and poor patient prognosis. In this study, HIF-1 α was silenced using siRNA technology. It demonstrated that HIF-1 α silencing significantly inhibited the activation of the PI3K/AKT signaling pathway and the proliferation of hepatoma cells, and HIF-1 α was proved to be an effective gene target for the gene therapy of HCC.

Peer review

This paper reported that hypoxia induces HIF-1 α expression, and knocking-down HIF-1 α by siRNA inhibits the PI3K/AKT signaling pathway and the proliferation of hypoxic CBRH-7919 rat hepatoma cells. Those findings indicate HIF-1 α could be a potential drug target for cancer therapy.

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Effects of small interfering RNA inhibit Class I phosphoinositide 3-kinase on human gastric cancer cells

Bao-Song Zhu, Li-Yan Yu, Kui Zhao, Yong-You Wu, Xiao-Li Cheng, Yong Wu, Feng-Yun Zhong, Wei Gong, Qiang Chen, Chun-Gen Xing

Bao-Song Zhu, Li-Yan Yu, Kui Zhao, Yong-You Wu, Yong Wu, Feng-Yun Zhong, Wei Gong, Qiang Chen, Chun-Gen Xing, Department of General Surgery, The Second Affiliated Hospital, Soochow University, Suzhou 215004, Jiangsu Province, China

Xiao-Li Cheng, Department of Gastroenterology, Qianfoshan Hospital, Shandong University, Jinan 250014, Shandong Province, China

Author contributions: Xing CG and Zhu BS designed the research; Xing CG, Zhu BS, and Zhao K wrote the paper; Zhao K and Yu LY collected and analyzed data; Zhu BS, Wu YY, Zhong FY, and Cheng XL selected the color figures in the paper; all authors contributed to the intellectual context and approved the final version of the manuscript.

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Correspondence to: Chun-Gen Xing, Professor, Department of General Surgery, The Second Affiliated Hospital of Soochow University, 1055 San Xiang Road, Suzhou 215004, Jiangsu Province, China. xingcg@126.com

Telephone: +86-512-67784106 Fax: +86-512-67784106

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Abstract

AIM: To investigate the effects of small interfering RNA (siRNA)-mediated inhibition of Class I phosphoinositide 3-kinase (Class I PI3K) signal transduction on the proliferation, apoptosis, and autophagy of gastric cancer SGC7901 and MGC803 cells.

METHODS: We constructed the recombinant replication adenovirus PI3K(I)-RNA interference (RNAi)-green fluorescent protein (GFP) and control adenovirus NC-RNAi-GFP, and infected it into human gastric cancer cells. MTT assay was used to determine the growth rate

of the gastric cancer cells. Activation of autophagy was monitored with monodansylcadaverine (MDC) staining after adenovirus PI3K(I)-RNAi-GFP and control adenovirus NC-RNAi-GFP treatment. Immunofluorescence staining was used to detect the expression of microtubule-associated protein 1 light chain 3 (LC3). Mitochondrial membrane potential was measured using the fluorescent probe JC-1. The expression of autophagy was monitored with MDC, LC3 staining, and transmission electron microscopy. Western blotting was used to detect p53, Beclin-1, Bcl-2, and LC3 protein expression in the culture supernatant.

RESULTS: The viability of gastric cancer cells was inhibited after siRNA targeting to the Class I PI3K blocked Class I PI3K signal pathway. MTT assays revealed that, after SGC7901 cancer cells were treated with adenovirus PI3K(I)-RNAi-GFP, the rate of inhibition reached 27.48% \pm 2.71% at 24 h, 41.92% \pm 2.02% at 48 h, and 50.85% \pm 0.91% at 72 h. After MGC803 cancer cells were treated with adenovirus PI3K(I)-RNAi-GFP, the rate of inhibition reached 24.39% \pm 0.93% at 24 h, 47.00% \pm 0.87% at 48 h, and 70.30% \pm 0.86% at 72 h ($P < 0.05$ compared to control group). It was determined that when 50 MOI, the transfection efficiency was 95% \pm 2.4%. Adenovirus PI3K(I)-RNAi-GFP (50 MOI) induced mitochondrial dysfunction and activated cell apoptosis in SGC7901 cells, and the results described here prove that RNAi of Class I PI3K induced apoptosis in SGC7901 cells. The results showed that adenovirus PI3K(I)-RNAi-GFP transfection induced punctate distribution of LC3 immunoreactivity, indicating increased formation of autophagosomes. The results showed that the basal level of Beclin-1 and LC3 protein in SGC7901 cells was low. After incubating with adenovirus PI3K(I)-RNAi-GFP (50 MOI), Beclin-1, LC3, and p53 protein expression was significantly increased from 24 to 72 h. We also found that Bcl-2 protein expression down-regulated with the treatment of adenovirus PI3K(I)-RNAi-GFP (50 MOI). A number of

isolated membranes, possibly derived from ribosome-free endoplasmic reticulum, were seen. These isolated membranes were elongated and curved to engulf a cytoplasmic fraction and organelles. We used transmission electron microscopy to identify ultrastructural changes in SGC7901 cells after adenovirus PI3K(I)-RNAi-GFP (50 MOI) treatment. Control cells showed a round shape and contained normal-looking organelles, nucleus, and chromatin, while adenovirus PI3K(I)-RNAi-GFP (50 MOI)-treated cells exhibited the typical signs of autophagy.

CONCLUSION: After the Class I PI3K signaling pathway has been blocked by siRNA, the proliferation of cells was inhibited and the apoptosis of gastric cancer cells was enhanced.

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Key words: Gastric cancer cells; Class I phosphoinositide 3-kinase; RNA interference; Apoptosis; Autophagy

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INTRODUCTION

Gastric cancer is the fourth most common cancer and the second leading cause of cancer death worldwide^[1], with nearly a million new cases diagnosed each year. The phosphatidylinositol 3-kinases (PI3Ks) are a family of lipid kinases whose primary biochemical function is to phosphorylate the 3-hydroxyl group of phosphoinositides^[2]. Phosphorylation results in the activation of second messenger molecules, with consequent signal transduction that sets in motion a variety of physiological cellular metabolic and survival functions.

The PI3K-serine/threonine kinase (AKT)-mammalian target of the rapamycin (mTOR) pathway is an important cellular pathway involved in cell growth, tumorigenesis, cell invasion, and drug response^[3-5]. This pathway is frequently activated in many cancers, and uncontrolled PI3K-AKT-mTOR signaling may also result in poor clinical outcome in lung, cervical, ovarian, and esophageal cancers^[3,4,6-8].

PI3Ks are grouped into three Classes (I-III), with varying structure and substrate preference. The functions of Class I PI3Ks relate to glucose homeostasis, metabolism, growth, proliferation, and survival. Isoform-specific roles are described, albeit with degrees of overlap, with potential implications for toxicity and efficacy of novel inhibitors of Class I PI3Ks^[9]. A substantial body of evidence exists in support of the notion that not only does PI3K pathway activation promote cell survival and tumor

progression, but also can predict for therapeutic resistance to a broad range of anticancer therapies.

The discovery of Class I PI3K revealed a novel role for autophagy in induced cell death, and Class I PI3K is believed to be a crucial modulator in both apoptosis and autophagy. Our aim was to detect the effects of adenovirus PI3K(I)-RNA interference (RNAi)-green fluorescent protein (GFP) on the growth and apoptosis of gastric cancer cells *in vitro*. To compare transduction efficiency, biological and molecular mechanisms of adenovirus PI3K(I)-RNAi-GFP on gastric cancer cell lines will be detected.

MATERIALS AND METHODS

Reagents

SGC7901 and MGC801 gastric cancer cells were purchased from the Shanghai Institute of Cell Biology, Chinese Academy of Sciences (Shanghai, China). RPMI1640 medium was purchased from Gibco (Rockville, MD, United States). Fetal bovine serum was obtained from Hangzhou Sijiqing Biological Engineering Material Co., Ltd. (Hangzhou, China), L-glutamine and MTT were provided by Sigma (St Louis, MO, United States). Antibodies against p53, Bcl-2, Beclin-1, and LC3 were provided by Cell Signaling Technology (Beverly, MA, United States).

Adenoviral vectors and infections

RNAi sequence design against Class I PI3K and the construction of vectors expressing Class I PI3K short hairpin RNA (shRNA). The Class I PI3K-specific target sequence was chosen according to online shRNA tools of Invitrogen (<http://www.invitrogen.com/rnai>) using the Class I PI3K reference sequence (GenBank accession No. NM_006218). The target sequence was designed as follows: Class I PI3K (base 3090-3118), "5'-AGAG-GTTTCAGGAGATGTGTT ACAAG GCT-3'". shRNAs were then chemically synthesized and a lentiviral vector was constructed. The exact insertion of the specific shRNA was further confirmed by sequencing. The recombinant adenovirus vector that expresses shRNA against Class I PI3K was synthesized by Shanghai Genesil Co., Ltd. Stocks of replication-defective adenoviral vectors expressing green fluorescent protein [adenovirus PI3K(I)-RNAi-GFP and control adenovirus NC-RNAi-GFP] were stored at -80 °C. Infections were performed at 70% to 75% confluence in DMEM supplemented with 2% FCS. Cells were subsequently incubated at 37 °C for at least 4 h, followed by the addition of fresh medium. Cells were subjected to functional analyses at fixed time points following infection as described for individual experimental conditions.

Determination of optimal multiplicity of infection

1×10^4 SGC7901 and MGC803 cells/well were seeded in 96 well plates to 60%-70% cultured adherent cells. Different multiplicities of infection (MOI = 10, 20, 30,

50, 100) of the adenovirus NC-RNAi-GFP (100 μ L) and diluted infected cells were then added. Eight hours later, 10% fetal bovine serum for RPMI1640 culture medium was added, and 48 h later was counted under a fluorescence microscope to calculate the number of cells that expressed GFP.

Cell culture and viability assay

SGC7901 and MGC803 cells were maintained in RPMI1640 medium containing 10% heat-inactivated fetal bovine serum and 0.03% *L*-glutamine incubated in a 5% CO₂ atmosphere at 37 °C. Cells in a mid-log phase were used in experiments. Cell viability was assessed by MTT assay. To determine the response of SGC7901 cells to adenovirus PI3K(I)-RNAi-GFP, SGC7901 cells were plated into 96-well microplates (7×10^4 cells/well) and adenovirus PI3K(I)-RNAi-GFP was added to a culture medium and cell viability was assessed with MTT assay 24 h after adenovirus PI3K(I)-RNAi-GFP treatment. MTT (Sigma, St Louis, MO, United States) solution was added to a culture medium (500 mg/L final concentration) for 4 h before the end of treatment, and the reaction was stopped by the addition of 10% acidic SDS (100 μ L). The absorbance value (*A*) at 570 nm was read using an automatic multiwell spectrophotometer (Bio-Rad, Richmond, CA, United States). The percentage of cell proliferation was calculated as follows: cell proliferation (%) = $(1 - A \text{ of experiment well} / A \text{ of positive control well}) \times 100\%$.

Visualization of monodansylcadaverine-labeled vacuoles

Exponentially-growing cells were plated onto 24-chamber culture slides, cultured for 24 h, and then incubated with the drug in 10% FCS/RPMI 1640 for 12 and 24 h. Autophagic vacuoles were labeled with MDC^[10] (Sigma, St Louis, MO, United States) by incubating cells with 0.001 mmol/L MDC in RPMI1640 at 37 °C for 10 min. After incubation, cells were washed three times with phosphate-buffered saline (PBS) and immediately analyzed with a fluorescence microscopy (Nikon Eclipse TE 300, Japan) equipped with a filter system (V-2A excitation filter: 380-420 nm, barrier filter: 450 nm). Images were captured with a CCD camera and imported into Photo-shop.

Immunofluorescence staining LC3

MGC803 cells were seeded onto 24-chamber culture slides and treated with adenovirus PI3K(I)-RNAi-GFP (50 MOI) and adenovirus NC-RNAi-GFP. After fixation in methanol for 10 min and blocked with a buffer containing 1% bovine serum albumin (BSA) and 0.1% Triton X-100 for 1 h, cells were incubated with either the primary antibody against LC3 from Cell Signaling Technology (Beverly, MA, United States) or diluted at 1:200 with PBS containing 1% BSA at 4 °C overnight. Cells were then incubated for 1 h with 1:500 secondary fluorescence conjugated antibodies (Sigma) to visualize the binding sites of the primary antibody under a laser confocal microscope

(Leisa, Germany).

Detection of mitochondrial potential

Mitochondrial $\Delta\psi$ was determined using the KeyGEN Mitochondrial Membrane Sensor Kit (KeyGEN, Nanjing, China). The MitoSensor dye aggregates in the mitochondria of healthy cells and emits red fluorescence against green monomeric cytoplasmic background staining. However, in cells with a collapsed mitochondrial $\Delta\psi$, the dye cannot accumulate in the mitochondria and remains as monomers throughout the cells with green fluorescence^[11]. SGC7901 cells were briefly incubated with adenovirus PI3K(I)-RNAi-GFP in 24-well plates for the indicated times, then pelleted, washed with PBS, and resuspended in 0.5 mL of diluted MitoSensor reagent (1 mmol/L in incubation buffer). After incubating cells with MitoSensor reagent for 20 min, 0.2 mL of incubation buffer was added and cells were centrifuged then resuspended in 40 μ L of incubation buffer. Finally, cells were washed and resuspended in 1 mL PBS for flow cytometry analysis.

Total cell protein extraction and Western blotting analysis

For extraction of total cell proteins, cells were washed with pre-cooled PBS and subsequently lysed in pre-cooled RIPA lysis buffer (50 mmol Tris-HCl, pH 7.4, 150 mmol NaCl, 1 mmol dithiothreitol, 0.25% sodium deoxycholate, and 0.1% NP-40) containing 1 mmol/L phenylmethylsulfonyl fluoride, 50 mmol sodium pyrophosphate, 1 mmol/L Na₃VO₄, 1 mmol NaF, 5 mmol EDTA, 5 mmol EGTA, and a protease inhibitors cocktail. Cell lysis was performed on ice for 30 min. Clear protein extracts were obtained by centrifugation for 30 min at 4 °C. Protein extraction from SGC7901 gastric cancer cells was performed as previously described. Protein concentration was determined with a Bradford protein assay kit. Proteins were resolved on 8.5% polyacrylamide gels and subsequently transferred onto nitrocellulose membranes. For immunoblotting, nitrocellulose membranes were incubated with specific antibodies recognizing target proteins overnight at 4 °C. The membranes were then incubated with horseradish peroxidase-conjugated secondary antibody (1:3000) for 1 h at room temperature, and subsequently analyzed by an enhanced chemiluminescence detection system (Amersham Pharmacia Biotech) and visualized by autoradiography. Protein β -actin (1:5000; Sigma) was used as a loading control.

Transmission electron microscopic examination

Pursuant to treatment with adenovirus PI3K(I)-RNAi-GFP, cells were fixed in ice-cold 2.5% glutaraldehyde in 0.1 mol/L PBS, and preserved at 4 °C for further processing. When processing resumed, cells were post-fixed in 1% osmium tetroxide in the same buffer, dehydrated in graded alcohols, embedded in Epon 812, sectioned with an ultra-microtome, and stained with uranyl acetate and lead citrate, followed by examination with a transmission

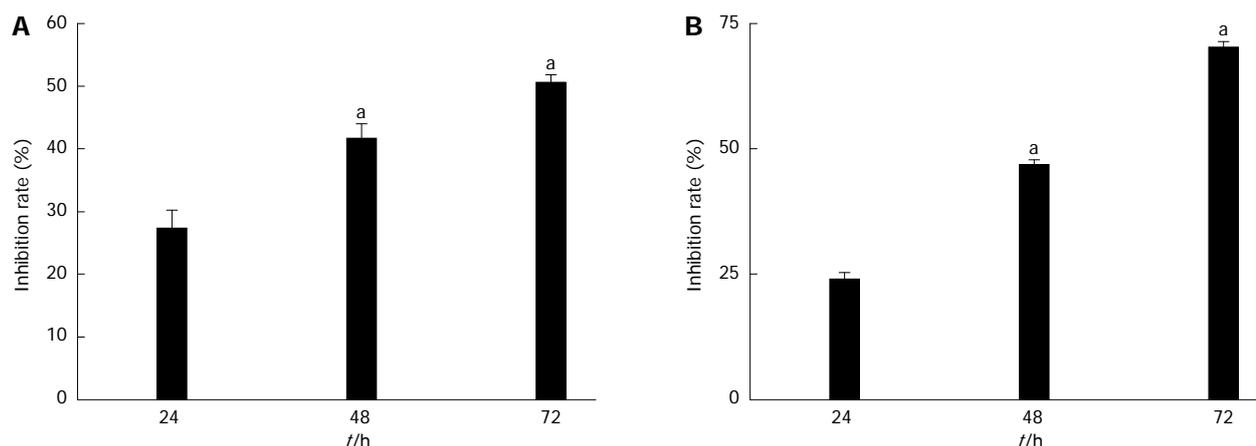


Figure 1 Reduced viability of SGC7901 and MGC803 cells after adenovirus Class I phosphoinositide 3-kinase-RNA interference-green fluorescent protein treatment. A: SGC7901 cells (7×10^4 cells/mL); B: MGC803 cells (7×10^4 cells/mL) cultured with adenovirus Class I phosphoinositide 3-kinase [PI3K(I)]-RNA interference-green fluorescent protein (RNAi-GFP) (50 MOI) and adenovirus negative control-RNAi-GFP for 24, 48, and 72 h. Cell viability was analyzed by MTT assay. Values were given as mean \pm SD of three independent experiments. ^a $P < 0.05$ vs control group.

electron microscope (Philips CM120, Dutch).

Statistical analysis

All data were presented as mean \pm SD. Statistical analysis was carried out by ANOVA, followed by a Dennett's-test, with $P < 0.05$ being considered significant.

RESULTS

Cell viability was detected after adenovirus PI3K(I)-RNAi-GFP treatment

MTT assay showed that the inhibition rate of gastric cancer cells transfected with adenovirus PI3K(I)-RNAi-GFP was significantly higher than adenovirus NC-RNAi-GFP (50 MOI) ($P < 0.05$). Adenovirus PI3K(I)-RNAi-GFP inhibited the proliferation of SGC7901 and MGC803 cancer cell viability. MTT assays revealed that, after 24 h of treatment with adenovirus PI3K(I)-RNAi-GFP, the rate of inhibition for SGC7901 cancer cells had reached $27.48\% \pm 2.71\%$. The rate of inhibition rose when the incubation time was prolonged, reaching $41.92\% \pm 2.02\%$ at 48 h, and $50.85\% \pm 0.91\%$ at 72 h after treatment (Figure 1). It was also revealed that, after 24 h of treatment with adenovirus PI3K(I)-RNAi-GFP, the rate of inhibition for MGC803 cancer cells had reached $24.39\% \pm 0.93\%$. The rate of inhibition rose when the incubation time was prolonged, reaching $47.00\% \pm 0.87\%$ at 48 h, and $70.30\% \pm 0.86\%$ at 72 h after treatment (Figure 1).

Transfection efficiency and cell morphology were detected by fluorescence microscope

With the treatment of adenovirus PI3K(I)-RNAi-GFP (50 MOI) transfected SGC7901 and MGC803 cells after 24 h, we found that the cell body was swollen and rounded, with the cells showing further deformation after 48 h. For fragments of recombinant adenovirus containing GFP, after transfection with 72 h, SGC7901 and MGC803 cells can be counted under a fluorescence

microscope due to the green fluorescence of the tumor cells (Figure 2). It was determined that when 50 MOI, the transfection efficiency was $95\% \pm 2.4\%$.

Adenovirus PI3K(I)-RNAi-GFP transfection increased autophagic vacuoles:

The autofluorescent substance MDC has been recently shown to be a marker for late autophagic vacuoles (L-AVs), but not endosomes^[10]. The dye is trapped in acidic, membrane-rich organelles, and also exhibits increased fluorescence quantum yield in response to the compacted lipid bilayers present in L-AVs^[12]. When cells are viewed with a fluorescence microscope, AVs stained by MDC appear as distinct dot-like structures distributed within the cytoplasm or localizing in the perinuclear regions. We found that there was an increase in the number of MDC-labeled vesicles after treatment of adenovirus PI3K(I)-RNAi-GFP (50 MOI) from 24 to 72 h (Figure 3).

Adenovirus PI3K(I)-RNAi-GFP transfection increased punctate LC3:

Microtubule-associated protein 1 light chain 3 (LC3), the mammalian ontology of Atg8, targets to the autophagosomal membranes in an Atg5-dependent manner and remains there even after Atg12-Atg5 dissociates. LC3 is considered to be the only credible marker of the autophagosome in mammalian cells^[13]. We used immunofluorescence staining to detect the expression and localization of LC3. The results showed that adenovirus PI3K(I)-RNAi-GFP transfection induced punctate distribution of LC3 immunoreactivity, indicating an increased formation of autophagosomes by adenovirus PI3K(I)-RNAi-GFP (Figure 4).

Adenovirus PI3K(I)-RNAi-GFP transfection induced mitochondrial dysfunction:

In the present study, mitochondrial membrane potential was examined using the fluorescent dye JC-1. We detected a collapse in mitochondrial membrane potential ($\Delta\psi$) as early as 24 h after adenovirus PI3K(I)-RNAi-GFP (50 MOI) treatment, as indicated by the increased emission of green fluorescence.

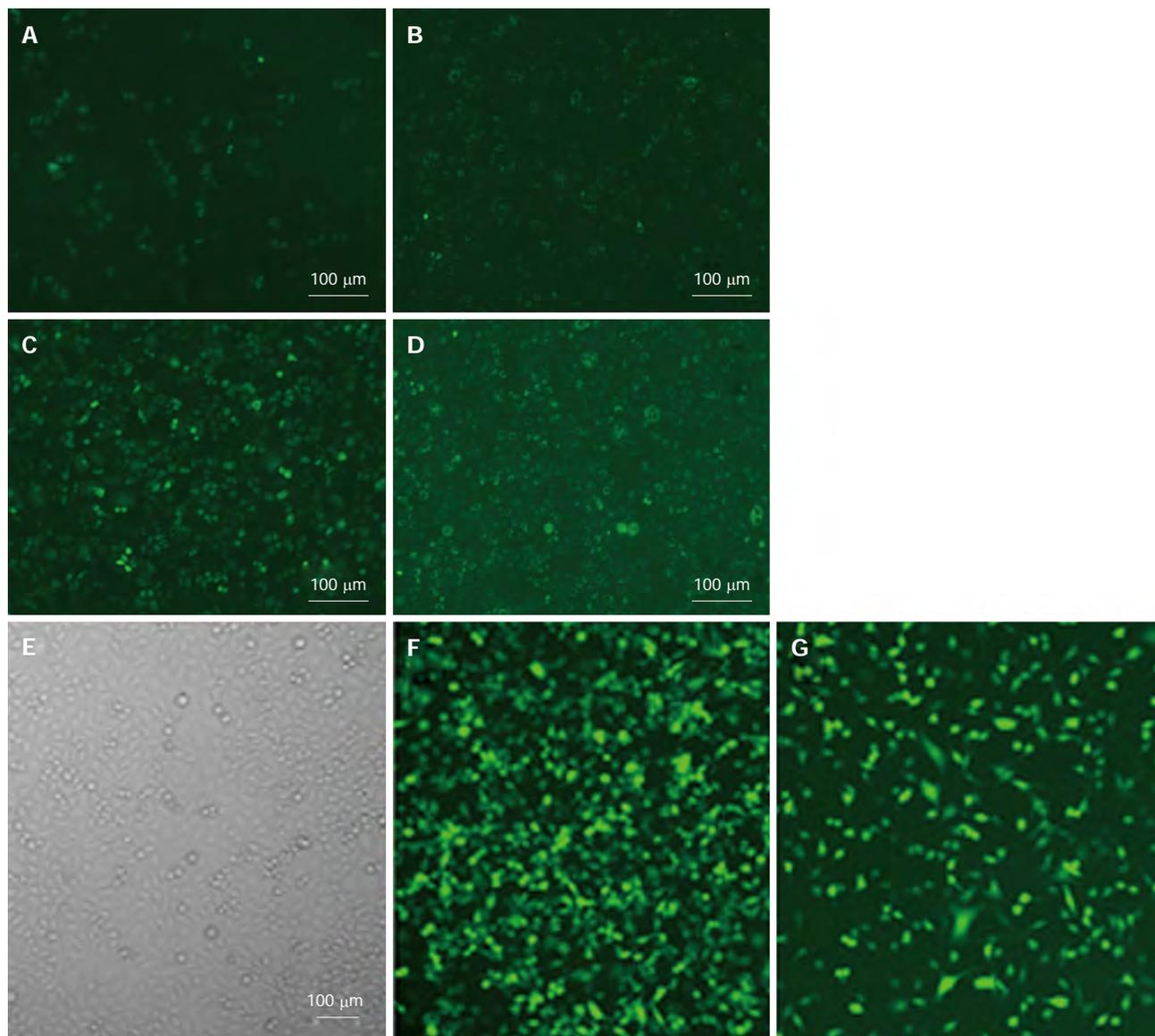


Figure 2 Transfection efficiency and cell morphology were detected by fluorescence microscope after adenovirus Class I phosphoinositide 3-kinase-RNA interference-green fluorescent protein and adenovirus negative control-RNA interference-green fluorescent protein treatment. A-D: SGC7901 cells; E-G: MGC803 cells incubated with adenovirus Class I phosphoinositide 3-kinase [PI3K(I)]-RNA interference-green fluorescent protein (RNAi-GFP) (50 MOI) for the indicated time. A and E: Control group; B and F: 24 h after adenovirus PI3K(I)-RNAi-GFP treatment; C and G: 48 h adenovirus PI3K(I)-RNAi-GFP (50 MOI) treatment; D: 72 h after adenovirus PI3K(I)-RNAi-GFP (50 MOI) treatment ($\times 200$) ($n = 3$).

This change reached its maximum at 24 h after adenovirus PI3K(I)-RNAi-GFP (50 MOI) treatment (Figure 5). A collapse in mitochondrial membrane potential always indicates cell apoptosis or necrosis. Adenovirus PI3K(I)-RNAi-GFP (50 MOI) induced mitochondrial dysfunction and activated cell apoptosis in SGC7901 cells, and the results described here prove that RNAi of Class I PI3K induced apoptosis in SGC7901 cells.

Adenovirus PI3K(I)-RNAi-GFP transfection up-regulated the expression of Beclin-1 and LC3: To assay if adenovirus PI3K(I)-RNAi-GFP (50 MOI) transfection increases the expression of autophagic relative protein, Western blotting analysis was used to detect the expression of LC3 and Beclin-1. The results showed that the

basal level of Beclin-1 and LC3 protein in SGC7901 cells was low. After incubating with adenovirus PI3K(I)-RNAi-GFP(50 MOI), Beclin-1 and LC3 protein expression was significantly increased from 24 to 72 h (Figure 6).

Adenovirus PI3K(I)-RNAi-GFP transfection increased the expression of p53 and decreased the expression of Bcl-2: To assay if adenovirus PI3K(I)-RNAi-GFP (50 MOI) transfection influences the expression of apoptotic relative protein, Western blotting analysis was used to detect the expression of Bcl-2 and p53. The results showed that the basal level of p53 protein in SGC7901 cells was low. After incubating with adenovirus PI3K(I)-RNAi-GFP, p53 protein expression was significantly increased from 24 to 72 h. We found that Bcl-2 protein expression down-regulated

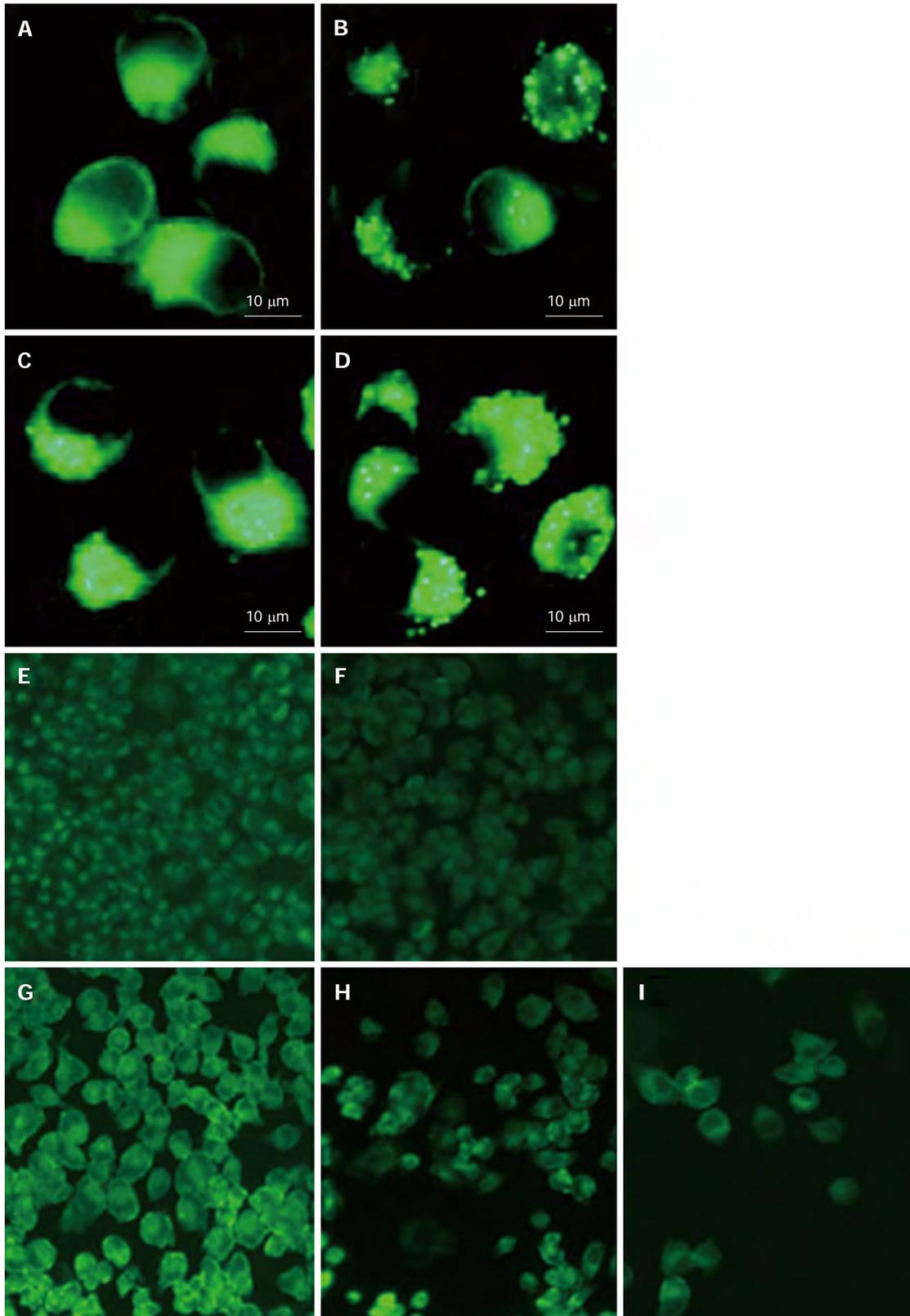


Figure 3 Monodansylcadaverine staining showed autophagy was activated after adenovirus Class I phosphoinositide 3-kinase-RNA interference-green fluorescent protein (50 MOI) treatment. A-D: SGC7901 cells; E-I: MGC803 cells incubated with adenovirus Class I phosphoinositide 3-kinase [PI3K(I)]-RNA interference-green fluorescent protein (RNAi-GFP) (50 MOI) and adenovirus negative control (NC)-RNAi-GFP for the indicated time and stained with monodansylcadaverine (100 $\mu\text{mol/L}$). Fluorescence particles showed L-acoustic vector sensor. A and E: Control; B and F: Adenovirus NC-RNAi-GFP; C and G: 24 h after adenovirus PI3K(I)-RNAi-GFP (50 MOI) treatment ($\times 200$) ($n = 3$); D and H: 48 h after adenovirus PI3K(I)-RNAi-GFP (50 MOI) treatment; I: 72 h after adenovirus PI3K(I)-RNAi-GFP (50 MOI) treatment ($\times 1000$) ($n = 3$).

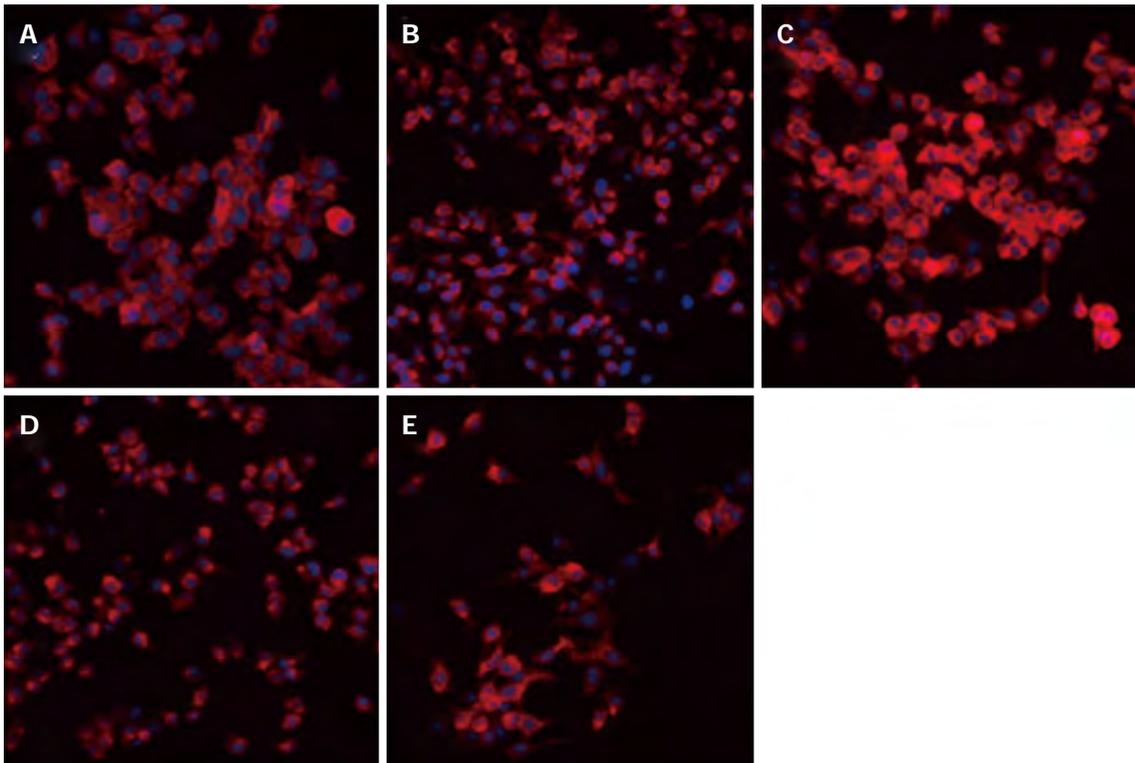


Figure 4 Microtubule-associated protein 1 light chain 3 expression and location in MGC803 cells after treatment with adenovirus Class I phosphoinositide 3-kinase-RNA interference-green fluorescent protein. Cells were treated with adenovirus Class I phosphoinositide 3-kinase [PI3K(I)]-RNA interference-green fluorescent protein (RNAi-GFP) (50 MOI) for 24 h (C), 48 h (D), and 72 h (E), and analyzed with an immunofluorescence microscope. A: Control; B: Adenovirus negative control-RNAi-GFP ($\times 400$) ($n = 3$). Adenovirus PI3K(I)-RNAi-GFP increased the punctate distribution of light chain 3 from 24 to 72 h.

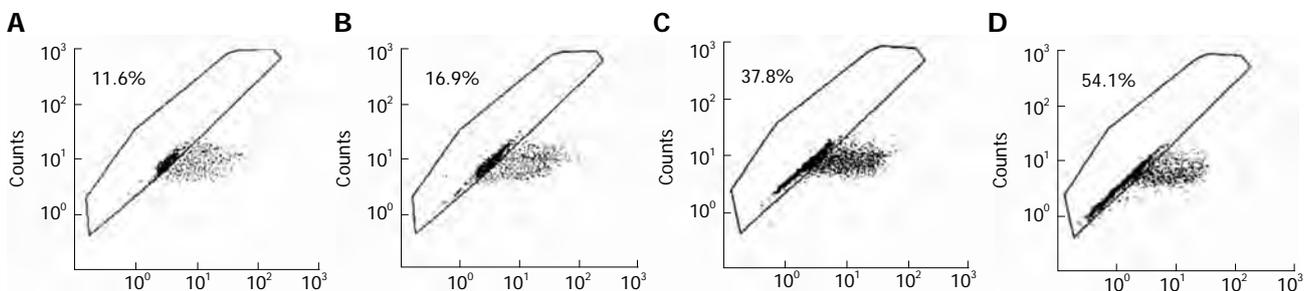


Figure 5 Flow cytometric analysis of mitochondria membrane potential in the control and adenovirus Class I phosphoinositide 3-kinase-RNA interference-green fluorescent protein-treated SGC7901 cells. A: Control: adenovirus negative control RNA interference-green fluorescent protein (RNAi-GFP), cells were treated with adenovirus Class I phosphoinositide 3-kinase [PI3K(I)]-RNAi-GFP (50 MOI) for 24 h (B), 48 h (C) and 72 h (D), and were then stained with JC-1 (5 $\mu\text{mol/L}$) for 30 min.

with the treatment of adenovirus PI3K(I)-RNAi-GFP (50 MOI) (Figure 6).

Activation of autophagy/lysosomes and impairment of mitochondria with adenovirus PI3K(I)-RNAi-GFP treatment: We used transmission electron microscopy to identify ultrastructural changes in SGC7901 cells after adenovirus PI3K(I)-RNAi-GFP (50 MOI) treatment. Control cells showed a round shape and contained normal-looking organelles, nucleus, and chromatin (Figure 7), while adenovirus PI3K(I)-RNAi-GFP (50 MOI)-treated cells exhibited the typical signs of autophagy (Figure 4B-D). A number of isolated membranes, possibly derived from ribosome-free endoplasmic reticulum,

were seen. These isolated membranes were elongated and curved to engulf a cytoplasmic fraction and organelles (Figure 7C and D). These membrane structures formed autophagosome traits with double or multi-membranes, and then fused with lysosomes in the formation of autolysosomes. The lysosome staining darkened, indicating the activation of lysosomal enzymes (Figure 7C and D). The loss of organelles and cytoplasm vacuolization were also observed when the incubation time was prolonged (Figure 7C and D).

DISCUSSION

In the present study, we showed that the RNAi of Class I

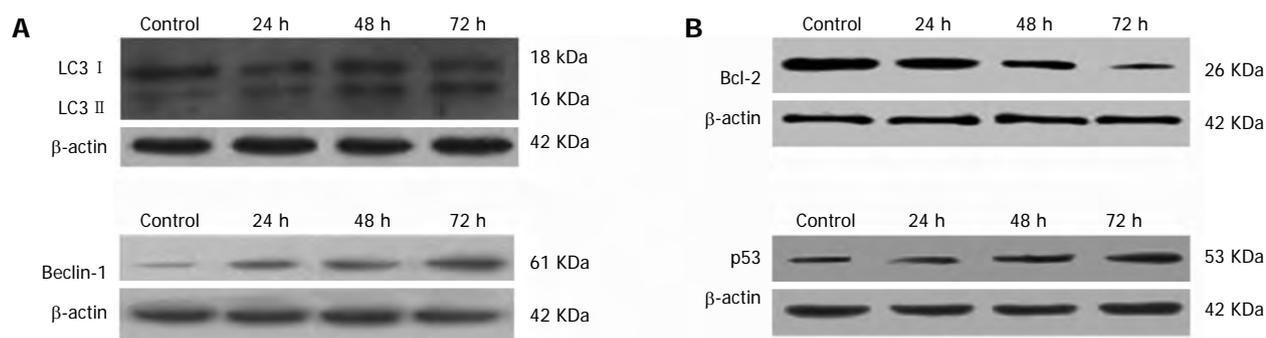


Figure 6 Effects of adenovirus Class I phosphoinositide 3-kinase-RNA interference-green fluorescent protein on light chain 3 and Beclin-1 protein expression/Bcl-2 and p53 protein expression in SGC7901 cells. A: Effect of adenovirus Class I phosphoinositide 3-kinase [PI3K(I)]-RNA interference-green fluorescent protein (RNAi-GFP) (50 MOI) and adenovirus negative control (NC)-RNAi-GFP on light chain 3 (LC3) and Beclin-1 protein expression. Control: Adenovirus NC-RNAi-GFP. SGC7901 cells were treated with adenovirus PI3K(I)-RNAi-GFP (50 MOI) for 24 to 72 h then harvested for the extraction of total proteins. Adenovirus PI3K(I)-RNAi-GFP up-regulates the expression of LC3 and Beclin protein; B: Adenovirus PI3K(I)-RNAi-GFP (50 MOI) and adenovirus NC-RNAi-GFP on Bcl-2 and p53 protein expression. Control: Adenovirus NC-RNAi-GFP. SGC7901 cells were treated with adenovirus PI3K(I)-RNAi-GFP (50 MOI) for 24 to 72 h then harvested for the extraction of total proteins. Adenovirus PI3K(I)-RNAi-GFP up-regulates the expression of p53 and down-regulates the expression of Bcl-2 protein.

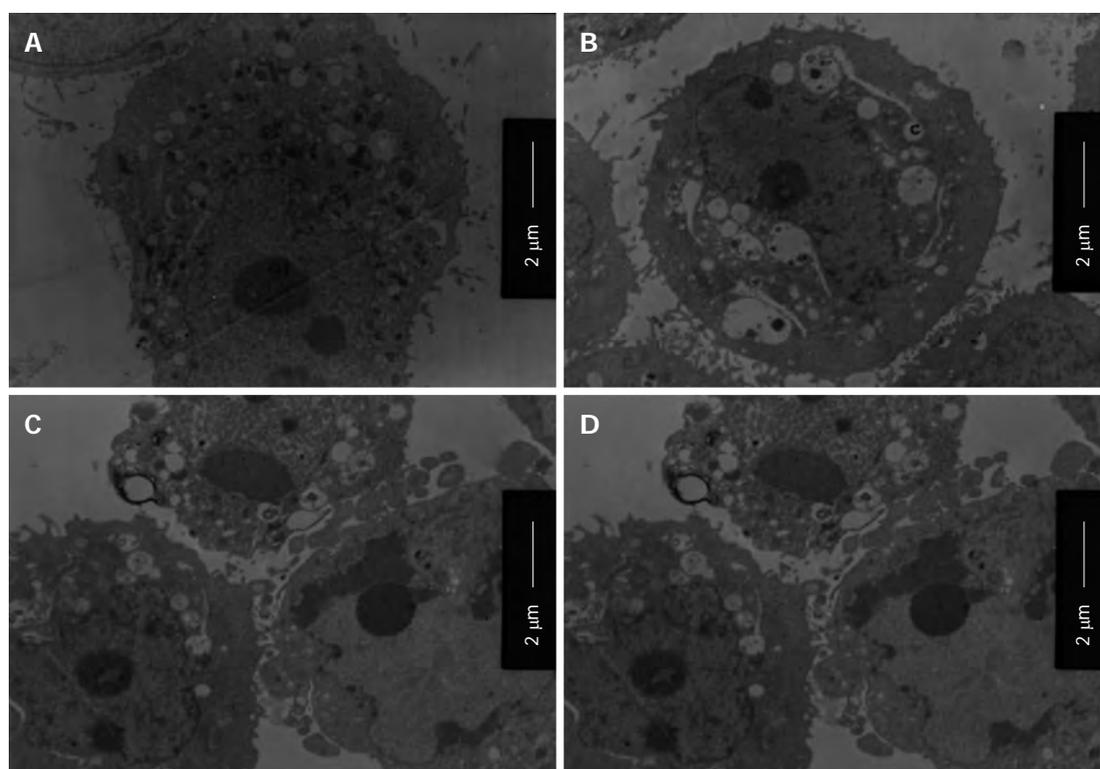


Figure 7 Ultrastructure of SGC7901 cells undergo autophagy, apoptosis and necrosis after adenovirus Class I phosphoinositide 3-kinase-RNA interference-green fluorescent protein treatment. A: Control: Adenovirus negative control RNA interference-green fluorescent protein (RNAi-GFP); B: Adenovirus Class I phosphoinositide 3-kinase [PI3K(I)]-RNAi-GFP (50 MOI)-treated (24 h); C: Adenovirus PI3K(I)-RNAi-GFP (50 MOI)-treated (48 h); D: Adenovirus PI3K(I)-RNAi-GFP (50 MOI)-treated (72 h).

PI3K reduced viability and induced apoptosis in SGC7901 and MGC803 gastric cancer cells, thus demonstrating the cytotoxic effects of adenovirus PI3K(I)-RNAi-GFP. We also showed that adenovirus PI3K(I)-RNAi-GFP increased the expression of p53, Beclin-1, and LC3, while decreasing the expression of Bcl-2. These findings suggest that RNAi of the Class I PI3K signaling pathway is a potential strategy for managing gastric cancers.

The mitochondria play critical roles in integrating cell death signals. Apoptosis is a cellular process involving the

selective degradation of membranous organelles such as the mitochondria. The mitochondrial permeability transition (MPT) represents an important event in initiating apoptosis. Thus, it is not surprising that apoptosis, and even necrosis, share a common mechanism through induction of the MPT. Observations made in the present study suggest that the mitochondrial $\Delta\psi$ collapsed after treatment of adenovirus PI3K(I)-RNAi-GFP; thus mitochondria may have initiated an apoptotic pathway.

The tumor suppressor p53 plays a central role in sensing

various genotoxic stresses. The basal levels of p53 were low in SGC7901 gastric cancer cells, and adenovirus PI3K(I)-RNAi-GFP upregulated the expression of p53. Upregulation of p53 after treatment with adenovirus PI3K(I)-RNAi-GFP induced apoptotic cell death. Moll and Zaika have proposed that the induction of apoptotic cell death by p53 occurs *via* both target gene activation and transactivation-independent mechanisms in mitochondria^[14]. In response to various forms of cellular stress, the levels of p53 increase, and a proportion of p53 rapidly localizes to the mitochondria^[15]. In the present study, the mitochondrial $\Delta\psi$ collapse after adenovirus PI3K(I)-RNAi-GFP treatment may have been caused by upregulation of p53. p53 accumulates in the nucleus, where it transactivates a number of proapoptotic target genes^[16], and induces apoptotic cell death.

Beclin-1 is monoallelically deleted in human breast and ovarian cancers, where it is expressed at reduced levels^[17,18]. The present results suggest that autophagy induced by adenovirus PI3K(I)-RNAi-GFP may contribute to anti-tumor effects. We also found that adenovirus PI3K(I)-RNAi-GFP increased the expression of Beclin-1, particularly the production of p53. Bcl-2 and Bcl-xL associate with the evolutionarily-conserved autophagy inducer Beclin-1, a haploinsufficient tumor suppressor^[19].

Inhibition may require Bcl-2 localized on the endoplasmic reticulum^[20,21]; notably, a BH3 domain within Beclin-1 mediates their association^[22]. In our research, we also found that the expression of Beclin-1 up-regulated with the treatment of adenovirus PI3K(I)-RNAi-GFP and the expression of Bcl-2 decreased. This indicated that autophagy activated and apoptosis induced after adenovirus PI3K(I)-RNAi-GFP treatment decreased the expression of Bcl-2.

All these observations suggest that autophagy and apoptosis activation may have significant contributions to adenovirus PI3K(I)-RNAi-GFP-induced death of SGC7901 and MGC803 cells. Further investigation of upstream signal regulation of autophagy and apoptosis may provide new insights into the mechanisms accommodating or contributing to autophagy and apoptosis, thereby unveiling new strategies for tumor therapy.

COMMENTS

Background

The discovery of Class I phosphoinositide 3-kinase (Class I PI3K) revealed a novel role for autophagy in induced cell death, and it is believed to be a crucial modulator in both apoptosis and autophagy. The authors predicted that activation of autophagy by blocking Class I PI3K may contribute to the anti-tumor actions of Class I PI3K inhibitors.

Research frontiers

The anti-tumor activity of Class I PI3K short hairpin RNA (shRNA) might be related to the induction of apoptosis of tumor cells, but the precise mechanism of its anti-tumor activity is not well understood.

Innovations and breakthroughs

Blocking Class I PI3K increases the expression of p53 and Beclin-1, and induces apoptotic and autophagic proteins, which contribute to the Class I PI3K inhibitor-induced apoptosis of cancer cells through both apoptotic and autophagic mechanisms. Further investigation of the relationship between autophagy activation and the anti-tumor effects of Class I PI3K inhibitors will unveil new

strategies for tumor therapy.

Applications

Blocking Class I PI3K increases the expression of p53 and Beclin-1, and induces apoptotic and autophagic proteins, which contribute to Class I PI3K shRNA-induced apoptosis of cancer cells by activating autophagic mechanisms; thereby providing new ideas for tumor treatment.

Terminology

Autophagy is a general term for the degradation of cytoplasmic components within lysosomes. There are three types of autophagy: macroautophagy, microautophagy, and chaperone-mediated autophagy. The term "autophagy" usually indicates macroautophagy.

Peer review

The authors examined the effects of Class I PI3K shRNA on the activation of apoptosis and autophagy, and the contribution of autophagy to the cytotoxic effects of Class I PI3K shRNA in gastric cancer cell line SGC7901. The results showed that shRNA Class I PI3K leads to the activation of apoptotic and autophagic pathways, and autophagy activation contributes to Class I PI3K shRNA-induced death of cancer cells.

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Psychosocial factors and their association with reflux oesophagitis, Barrett's oesophagus and oesophageal adenocarcinoma

Paul Denver, Michael Donnelly, Liam J Murray, Lesley A Anderson

Paul Denver, Michael Donnelly, Liam J Murray, Lesley A Anderson, Centre for Public Health, Queen's University Belfast, Belfast BT12 6BJ, Northern Ireland, United Kingdom

Author contributions: Murray LJ was the PI of the FINBAR study; Anderson LA was the project co-ordinator for the FINBAR Study; Donnelly M conceptualised and designed the sub-study of psychological factors including their assessment and supported by Donnelly M; Anderson LA supervised the analysis and write-up of results undertaken by Denver P. All authors contributed to the writing and production of the manuscript.

Correspondence to: Lesley A Anderson, PhD, Lecturer in Epidemiology, Centre for Public Health, Queen's University Belfast, Institute of Clinical Sciences, Block B, Grosvenor Road, Belfast BT12 6BJ, Northern Ireland, United Kingdom. l.anderson@qub.ac.uk

Telephone: +44-28-90632315 Fax: +44-28-90248017

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Abstract

AIM: To investigate the role of psychological characteristics as risk factors for oesophageal adenocarcinoma (OAC), as well as the reflux-mediated precursor pathway.

METHODS: An all-Ireland population-based case-control study recruited 230 reflux oesophagitis (RO), 224 Barrett's oesophagus (BO) and 227 OAC patients and 260 controls. Each case/control group completed measures of stress, depression, self-efficacy, self-esteem, repression and social support. A comparative analysis was undertaken using polytomous logistic regression adjusted for potential confounders.

RESULTS: Compared to controls, OAC patients were almost half as likely to report high stress levels over their lifetime ($P = 0.010$, OR 0.51; 95%CI: 0.29-0.90)

and 36% less likely to report having experienced depression (OR 0.64; 95%CI: 0.42-0.98). RO patients reported significantly higher stress than controls particularly during middle- and senior-years (P for trends < 0.001). RO patients were 37% less likely to report having been highly emotionally repressed (OR 0.63; 95%CI: 0.41-0.95). All case groups (OAC, RO and BO) were more likely than controls to report having had substantial amounts of social support (OR 2.84; 95%CI: 1.63-4.97; OR 1.97; 95%CI: 1.13-3.44 and OR 1.83; 95%CI: 1.03-3.24, respectively).

CONCLUSION: The improved psychological profile of OAC patients may be explained by response shift. The role of psychological factors in the development of OAC requires further investigation.

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Key words: Reflux oesophagitis; Barrett's oesophagus; Oesophageal adenocarcinoma; Adjustment; Psychological; Psychosocial factors

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INTRODUCTION

Gastro-oesophageal reflux comprises a series of chronic symptoms caused by abnormal reflux of gastric and intestinal digestive juice^[1] which may cause inflammation of the oesophageal mucosa reflux oesophagitis (RO)^[2] or cause a squamous to columnar cell metaplasia within the

distal oesophagus Barrett's oesophagus (BO)^[3]. Prevalence rates for gastro-oesophageal reflux disease in western countries range from 10%-20%^[4,5]. Incidence of BO in Northern Ireland is 567/100 000 of the population (unpublished data, Northern Ireland BO Registry). Although the absolute risk remains low (0.5% per patient per year)^[6], BO confers an increased risk of developing oesophageal adenocarcinoma (OAC)^[7], the incidence of which has increased by 600% in western countries in recent decades^[8]. The incidence rate for oesophageal cancer in Northern Ireland is similar to rates reported elsewhere^[9].

Previous studies have found associations between hypothesized risk factors including diet, obesity and smoking^[10-14] and the development of OAC though the demographic distribution of risk factors does not fully align with OAC incidence^[15]. Stress appears to affect the neuroendocrine and immune systems as well as the sympathetic nervous system^[16,17]. In population-based studies, job strain-related stress has been positively associated with symptomatic reflux and an increased risk of OAC^[17-19]. However, the role of psychosocial factors in the development of OAC and its precursor lesions has not been investigated.

Data from an all-Ireland population-based, case-control study (the FINBAR Study) was used to investigate the nature and extent to which different psychosocial factors (*e.g.*, stress and depressed mood) affected the development of OAC, including the influence such factors may have on the reflux-mediated precursor pathway.

MATERIALS AND METHODS

The FINBAR study was an all-Ireland population-based case-control study comprising three patient groups with (1) OAC; (2) long-segment BO; and (3) RO and a group of normal population controls. All participants were Caucasian and aged between 35-85 years. The design of the FINBAR Study has been described elsewhere^[10,20]. Briefly, OAC cases had histological confirmation of adenocarcinoma within the oesophagus. They were identified using electronic pathology records from all pathology laboratories in Northern Ireland and from the main hospitals involved in the treatment of oesophageal cancer in the Republic of Ireland. BO patients had histological confirmation of specialized intestinal metaplasia within the oesophagus and at least 3 centimeters of BO observed at endoscopy. RO patients were those diagnosed with macroscopically visible erosive oesophagitis (grades 2-4 in the Savary Miller/Hetzel-Dent classification or grades B, C or D in the Los Angeles classification) at upper gastro-intestinal (GI) endoscopy. BO and RO patients were frequency matched (within 5-year age- and sex-strata) to the distribution of OAC patients. Control subjects had no history of oesophageal or other GI cancer or BO. They were frequency matched by sex, and 5-year age bands to OAC patients.

For the psychosocial assessment patients were asked to reflect on their life as a whole when answering the

questions. Self-perceived stress or daily strain was assessed using the 4-item Reed Stress Inventory. Responses to items were scored as follows: 0, no response on one or more statements; 1, "not at all" for all four statements; 2, "not at all" for any three statements with any other response for the fourth; 3, "not at all" for any two statements with "hardly true" for the other two; 4, "not at all" for any one or two statements with any other responses for the remainder but not those for a score of 3; 5, all other response sets not specified under 0, 1, 2, 3, 4, 6, 7 or 8; 6, "moderately true" to all four statements, or "moderately true" for three statements with "exactly true" for the fourth; 7, "exactly true" for any three statements with "moderately true" or "hardly true" for the fourth; and 8, "exactly true" in response to all four statements. Scores from 1 to 8 were categorised as representing high (6-8), medium (4-5), or low (1-3) levels of stress.

A 4-part item was used to assess self-reported stress across the lifespan from: (1) childhood/teenager (up to 19 years); (2) young adulthood (20-39 years); (3) midlife (40-59 years); and (4) senior or later years in life (60-85 years). Each life period was considered a separate variable in the analyses in order to gauge approximately the extent to which any particular developmental period in an individual's life had been stressful and to assess its significance for patients compared to controls.

Depressed mood was assessed using an adapted 2-item case-finding instrument^[21]. A score of 1-2 on either question indicated a "not depressed" status while a score of 3-4 on either question indicated that the respondent was likely to be "depressed" or to have a depressed mood.

Self-efficacy and coping ability were measured using a 10-item scale designed to assess the extent to which a respondent had a self-belief that they had the capacity to overcome difficult tasks and cope with adversity. Responses on the 4-point likert scale were summed yielding a score of between 10 and 40 in the direction of increasing self-efficacy and coping ability. Self-efficacy was categorized as low (scores of 10-29), medium (30-34) and high (35-40) based on the tertile distribution of scores among the controls.

A single-item was used to measure self-esteem with a score of 1-2 indicating low self-esteem and a score of 3-4 indicating high self-esteem.

A single-item was used to assess emotional repression. A high score (3-4) indicated a tendency to share feelings and emotions relatively easily and a low score (1-2) was endorsed by respondents who were reticent or repressive in nature.

The responses to three questions on social support and loneliness were summed and categorized as indicating varying degrees of support from hardly any (scores 0-8), some (score 9), moderate (score 10) and substantial (scores 11-12) based on the quartile distribution of scores among the control group.

Ethical approval

Ethical approval for the FINBAR study was obtained

Table 1 Characteristics of participants

| Variables | Controls | RO | | BO | | OAC | |
|--------------------------------------|-------------|-------------|---------|-------------|---------|-------------|---------|
| | n (%) | n (%) | P value | n (%) | P value | n (%) | P value |
| Gender | | | | | | | |
| Male | 220 (84.6) | 189 (82.2) | 0.47 | 185 (82.6) | 0.55 | 192 (84.6) | 0.99 |
| Female | 40 (15.4) | 41 (17.8) | | 39 (17.4) | | 35 (15.4) | |
| Age (yr) | 63.0 | 61.7 | 0.22 | 62.4 | 0.57 | 64.2 | 0.28 |
| Education (yr) | 12.0 | 10.8 | < 0.001 | 11.3 | 0.01 | 10.7 | < 0.001 |
| Job type | | | | | | | |
| Manual | 119 (48.0) | 107 (48.2) | 0.71 | 130 (59.1) | 0.02 | 128 (59.5) | 0.01 |
| Non-manual | 129 (52.0) | 115 (51.8) | | 90 (40.9) | | 87 (40.5) | |
| GOR symptoms | | | | | | | |
| Never | 211 (81.2) | 140 (60.9) | < 0.001 | 60 (26.8) | < 0.001 | 117 (51.5) | < 0.001 |
| Ever | 49 (18.8) | 90 (39.1) | | 164 (73.2) | | 110 (48.5) | |
| Smoking status | | | | | | | |
| Never | 102 (40.2) | 109 (48.4) | 0.03 | 87 (39.2) | 0.40 | 45 (20.4) | < 0.001 |
| Ex-smoker | 107 (42.1) | 68 (30.2) | | 85 (38.3) | | 99 (44.8) | |
| Current | 45 (17.7) | 48 (21.3) | | 50 (22.5) | | 77 (34.8) | |
| Alcohol (g/d) | 26.1 | 22.0 | 0.15 | 22.3 | 0.21 | 19.2 | 0.01 |
| Body mass index (kg/m ²) | 27.0 | 27.8 | 0.05 | 27.0 | 0.90 | 28.7 | < 0.001 |
| Reed stress inventory, mean ± SD | | | | | | | |
| Mean score (range 2-8) | 4.50 ± 2.08 | 4.11 ± 1.88 | 0.014 | 4.28 ± 2.08 | 0.187 | 3.80 ± 2.09 | < 0.001 |
| Stress teenage years | | | | | | | |
| Mean score (range 1-5) | 1.80 ± 1.05 | 1.67 ± 0.95 | 0.146 | 1.86 ± 1.18 | 0.539 | 1.85 ± 1.17 | 0.655 |
| Stress young adulthood | | | | | | | |
| Mean score (range 1-5) | 2.45 ± 1.23 | 2.67 ± 1.11 | 0.301 | 2.34 ± 1.25 | 0.328 | 2.37 ± 1.24 | 0.486 |
| Stress midlife | | | | | | | |
| Mean score (range 1-5) | 2.49 ± 1.26 | 3.33 ± 1.20 | < 0.001 | 2.75 ± 1.34 | 0.038 | 2.50 ± 1.33 | 0.928 |
| Stress senior years | | | | | | | |
| Mean score (range 1-5) | 2.07 ± 1.17 | 2.80 ± 1.42 | < 0.001 | 2.34 ± 1.35 | 0.08 | 2.33 ± 1.37 | 0.088 |
| Depression | | | | | | | |
| Mean score (range 2-8) | 4.11 ± 1.74 | 4.42 ± 2.37 | 0.107 | 4.38 ± 2.25 | 0.147 | 3.82 ± 2.31 | 0.109 |
| Self efficacy | | | | | | | |
| Mean score (range 10-40) | 32.0 ± 4.76 | 33.8 ± 4.32 | < 0.001 | 31.8 ± 5.52 | 0.794 | 34.3 ± 4.89 | < 0.001 |
| Self esteem | | | | | | | |
| Mean score (range 1-4) | 2.95 ± 0.81 | 2.93 ± 1.10 | 0.828 | 2.76 ± 1.04 | 0.022 | 3.13 ± 0.97 | 0.034 |
| Repression | | | | | | | |
| Mean score | 2.80 ± 0.89 | 2.64 ± 1.17 | 0.097 | 2.86 ± 1.05 | 0.501 | 2.86 ± 1.17 | 0.554 |
| Social support | | | | | | | |
| Mean score (range 1-4) | 3.57 ± 0.60 | 3.82 ± 0.50 | < 0.001 | 3.62 ± 0.63 | 0.40 | 3.76 ± 0.48 | < 0.001 |

RO: Reflux oesophagitis; BO: Barrett's oesophagus; OAC: Oesophageal adenocarcinoma; GOR: Gastroesophageal reflux.

from the Research Ethics Committee of the Queen's University Belfast, the Clinical Research Ethics Committee of the Cork Teaching Hospitals and the Research Ethics Committee Board of St. James's Hospital, Dublin.

Statistical analysis

Group *t*-tests and Pearson χ^2 tests were used to compare cases and controls. Polytomous multivariate logistic regression was used to compare the psychosocial factors in each case group with the control group, adjusting for potential confounders including gender, age, years of full-time education, job type (manual, non-manual), Gastroesophageal reflux (GOR) symptoms (never, ever), smoking status (never, ex-smoker, current smoker), alcohol consumption (g/d) and BMI (self-reported weight 5-years before interview divided by height measured at

interview).

RESULTS

In total, 227 OAC patients, 224 BO patients, 230 RO patients and 260 controls were recruited into the study with participation rates of 64%, 82%, 69% and 42% respectively. Case groups and controls were similar regarding gender and age due to frequency matching. Other characteristics are displayed in Table 1.

There were no significant trends between reported stress levels as measured by the Reed Stress Inventory and risk of RO or BO (Table 2). However, OAC patients were half as likely to report high stress than controls (OR 0.51; 95%CI: 0.29-0.90). Stress levels were similar during childhood/teenage years for RO, BO, OAC cases and

Table 2 Self-reported stress levels during lifetime

| Stress levels | Controls | RO | | BO | | OAC | |
|------------------------------|--------------|--------------|--------------------------|--------------|-------------------------|--------------|--------------------------|
| | <i>n</i> (%) | <i>n</i> (%) | AOR (95%CI) | <i>n</i> (%) | AOR (95%CI) | <i>n</i> (%) | AOR (95%CI) |
| Reed stress inventory levels | | | | | | | |
| Low | 45 (17.6) | 63 (27.9) | 1.00 | 60 (27.2) | 1.00 | 81 (36.3) | 1.00 |
| Medium | 154 (60.2) | 122 (54.0) | 0.92 (0.51-1.63) | 102 (46.2) | 0.51 (0.31-0.86) | 91 (40.8) | 0.37 (0.23-0.61) |
| High | 57 (22.3) | 41 (18.1) | 1.43 (0.64-3.22) | 59 (26.7) | 0.60 (0.33-1.09) | 51 (22.9) | 0.51 (0.29-0.90) |
| | | | <i>P</i> for trend 0.48 | | <i>P</i> for trend 0.10 | | <i>P</i> for trend 0.010 |
| Teenage years | | | | | | | |
| 1 | 135 (52.7) | 132 (58.7) | 1.00 | 122 (55.5) | 1.00 | 127 (57.5) | 1.00 |
| 2 | 67 (26.1) | 53 (23.6) | 1.05 (0.64-1.71) | 42 (19.1) | 0.73 (0.43-1.23) | 32 (14.5) | 0.63 (0.37-1.06) |
| 3 | 31 (14.1) | 26 (11.6) | 0.77 (0.40-1.49) | 31 (14.1) | 1.06 (0.57-1.96) | 42 (19.0) | 1.50 (0.84-2.67) |
| 4 | 16 (6.3) | 11 (4.9) | 0.67 (0.26-1.70) | 14 (6.4) | 0.75 (0.31-1.79) | 9 (4.1) | 0.58 (0.23-1.48) |
| 5 | 7 (2.7) | 3 (1.3) | 0.28 (0.06-1.26) | 11 (5.0) | 0.76 (0.25-2.29) | 11 (5.0) | 1.18 (0.40-3.48) |
| | | | <i>P</i> for trend 0.10 | | <i>P</i> for trend 0.55 | | <i>P</i> for trend 0.85 |
| Young adulthood | | | | | | | |
| 1 | 64 (25.0) | 38 (16.9) | 1.00 | 76 (34.7) | 1.00 | 70 (32.3) | 1.00 |
| 2 | 67 (26.2) | 61 (27.1) | 2.04 (1.11-3.76) | 47 (21.5) | 0.59 (0.34-1.04) | 52 (24.0) | 0.80 (0.47-1.38) |
| 3 | 82 (32.0) | 75 (33.3) | 1.92 (1.06-3.49) | 57 (26.0) | 0.47 (0.27-0.81) | 55 (25.4) | 0.67 (0.39-1.14) |
| 4 | 32 (12.5) | 39 (17.3) | 2.65 (1.27-5.55) | 23 (10.5) | 0.46 (0.22-0.96) | 24 (11.1) | 0.71 (0.35-1.44) |
| 5 | 11 (4.3) | 12 (5.3) | 3.20 (1.07-9.55) | 16 (7.3) | 0.86 (0.33-2.28) | 16 (7.4) | 1.22 (0.47-3.18) |
| | | | <i>P</i> for trend 0.01 | | <i>P</i> for trend 0.05 | | <i>P</i> for trend 0.49 |
| Midlife | | | | | | | |
| 1 | 68 (28.7) | 20 (9.1) | 1.00 | 52 (25.0) | 1.00 | 58 (27.9) | 1.00 |
| 2 | 57 (24.1) | 38 (17.2) | 2.56 (1.22-5.37) | 39 (18.8) | 0.87 (0.48-1.59) | 62 (29.8) | 1.25 (0.73-2.16) |
| 3 | 55 (23.2) | 50 (22.6) | 4.04 (1.97-8.25) | 48 (23.1) | 1.07 (0.59-1.94) | 38 (18.3) | 0.95 (0.53-1.71) |
| 4 | 41 (17.3) | 76 (34.4) | 8.50 (4.14-17.46) | 47 (22.6) | 1.23 (0.65-2.30) | 25 (12.0) | 0.70 (0.36-1.36) |
| 5 | 16 (6.8) | 37 (16.7) | 9.82 (4.11-23.45) | 22 (10.6) | 1.27 (0.55-2.89) | 25 (12.0) | 1.60 (0.72-3.53) |
| | | | <i>P</i> for trend 0.001 | | <i>P</i> for trend 0.33 | | <i>P</i> for trend 0.97 |
| Senior years | | | | | | | |
| 1 | 64 (42.4) | 30 (24.4) | 1.00 | 47 (39.2) | 1.00 | 47 (37.3) | 1.00 |
| 2 | 38 (25.1) | 28 (22.8) | 1.76 (0.86-3.61) | 23 (19.2) | 0.71 (0.35-1.45) | 32 (25.4) | 1.08 (0.56-2.09) |
| 3 | 30 (19.9) | 22 (17.9) | 1.77 (0.82-3.83) | 22 (18.3) | 0.93 (0.44-1.96) | 19 (15.1) | 0.84 (0.40-1.78) |
| 4 | 12 (8.0) | 23 (18.7) | 4.28 (1.72-10.67) | 18 (15.0) | 2.15 (0.85-5.43) | 14 (11.1) | 1.71 (0.67-4.35) |
| 5 | 7 (4.6) | 20 (16.3) | 5.92 (1.72-16.77) | 10 (8.3) | 1.48 (0.48-4.54) | 14 (11.1) | 2.35 (0.82-6.70) |
| | | | <i>P</i> for trend 0.001 | | <i>P</i> for trend 0.20 | | <i>P</i> for trend 0.14 |

RO: Reflux oesophagitis; BO: Barrett's oesophagus; OAC: Oesophageal adenocarcinoma; 1: Low; 2-4: Medium; 5: High; AOR: Adjusted odds ratio.

controls, respectively. Regarding young adulthood, there was a significant trend between having RO and reported stress; RO cases also reported significantly much higher stress during middle- and senior-years than controls (almost 10-fold and 6-fold more, respectively) with a significant linear trend (*P* for trend < 0.001) observed for each point on the life-span stress scale.

OAC cases were 36% less likely than controls to report depression; no significant association was observed between depression and BO or RO (Table 3). Self-efficacy levels were 3-times higher in RO patients and 2-times higher in OAC patients compared to controls. No significant association was observed for self-efficacy and BO. OAC patients were 58% more likely than controls to report high self-esteem though the association was not statistically significant (95%CI: 0.99-2.52). Self-esteem did not differ significantly between RO or BO cases respectively and controls. RO patients were 37% less likely than controls to report being repressed; significant associations were not observed between repression and BO or OAC status. RO, BO and OAC patients were more likely to report high levels of social support than controls.

DISCUSSION

This is the first study to investigate psychological characteristics of RO, BO and OAC patients as possible risk factors for the development of these conditions. OAC patients compared to controls reported significantly lower stress levels throughout their life in contrast to comparisons between RO and BO patients and controls. RO patients reported significantly higher stress levels in later life. OAC patients were also less likely than controls to report depression and to have higher (albeit non-significant) self-esteem and significantly better coping skills. All three case groups reported more social support than controls.

It has been hypothesized that psychosocial factors such as stress and social support may mediate or moderate cancer risk through, for example, influencing neuroendocrine and immune functioning^[22]. Long-term exposure to stress causes persistent activation of the hypothalamic-pituitary-adrenal axis reducing tumour suppressor capability and suppressing DNA repair functions^[23-26] through suppression of lymphocyte activity^[27] and cytotoxic T-cell

Table 3 Psychosocial factors by group status

| | Controls | RO | | BO | | OAC | |
|----------------|------------|------------|------------------|------------|------------------|------------|------------------|
| | n (%) | n (%) | AOR (95%CI) | n (%) | AOR (95%CI) | n (%) | AOR (95%CI) |
| Depression | | | | | | | |
| No | 163 (63.7) | 131 (58.0) | 1.00 | 118 (52.2) | 1.00 | 147 (65.9) | 1.00 |
| Yes | 93 (36.3) | 95 (42.0) | 0.92 (0.61-1.40) | 104 (46.9) | 1.12 (0.74-1.70) | 76 (34.1) | 0.64 (0.42-0.98) |
| Self-efficacy | | | | | | | |
| Low | 84 | 35 | 1.00 | 79 | 1.00 | 43 | 1.00 |
| Medium | 74 | 55 | 1.65 (0.92-2.98) | 54 | 0.61 (0.36-1.05) | 49 | 1.06 (0.60-1.87) |
| High | 97 | 135 | 3.14 (1.86-5.31) | 86 | 0.78 (0.48-1.27) | 128 | 2.17 (1.32-3.57) |
| Self-esteem | | | | | | | |
| Low | 67 (26.2) | 63 (27.9) | 1.00 | 77 (35.0) | 1.00 | 46 (20.6) | 1.00 |
| High | 189 (73.4) | 163 (72.1) | 0.93 (0.59-1.47) | 143 (65.0) | 0.76 (0.48-1.18) | 177 (79.4) | 1.58 (0.99-2.52) |
| Repression | | | | | | | |
| Low | 83 (32.4) | 100 (44.4) | 1.00 | 71 (32.0) | 1.00 | 72 (32.3) | 1.00 |
| High | 173 (67.6) | 125 (55.6) | 0.63 (0.41-0.95) | 151 (68.0) | 1.20 (0.78-1.86) | 151 (67.7) | 1.10 (0.72-1.67) |
| Social support | | | | | | | |
| Hardly any | 89 | 78 | 1.00 | 77 | 1.00 | 60 | 1.00 |
| Some | 89 | 53 | 0.72 (0.43-1.21) | 61 | 1.12 (0.67-1.86) | 58 | 1.28 (0.77-2.14) |
| Moderate | 37 | 30 | 0.93 (0.49-1.77) | 30 | 1.26 (0.66-2.39) | 38 | 1.89 (1.02-3.48) |
| Substantial | 41 | 64 | 1.97 (1.13-3.44) | 54 | 1.83 (1.03-3.24) | 67 | 2.84 (1.63-4.97) |

RO: Reflux oesophagitis; BO: Barrett's oesophagus; OAC: Oesophageal adenocarcinoma; AOR: Adjusted odds ratio.

and natural killer cell activity^[28,29]. However, perceived stress over one's lifetime appeared to be significantly lower in OAC patients. Other factors such as recall bias and response shift may explain these reported lower levels of stress. Retrospective reporting may be skewed by recall bias as patients may lose their memory trace with time and their salience following a cancer diagnosis. Also, the current study identified an overall positive psychological profile among OAC patients including less depression, higher self-efficacy, higher self-esteem and more social support. The phenomenon of "response shift" may help to explain the findings in so far as a perceptual adjustment may occur in how a patient views stress or adversity and friends; and family tend to be sympathetic, caring and more likely to offer support at times of illness^[30-32]. Furthermore, a "cancer experience" has the potential to effect positive psychological change or post-traumatic growth (PTG)^[33]. For example, studies have found that PTG is inversely associated with emotional distress^[34] and positively associated with happiness^[35]. The majority of studies that have investigated the role of PTG have been conducted with patients with breast cancer, a form of cancer which has relatively high survival rates. In Europe, five and ten year survival rates of women diagnosed with breast cancer between 2000 and 2002 were 82% and 72%, respectively^[35]. In contrast, survival rates for OAC patients are much lower between 13% and 17% for males and females, respectively, in Ireland^[36]. A diagnosis of a cancer such as OAC with a poor prognosis and survival rate might be expected to result in despair, pessimism, reduce active coping and increase dependence on more an emotional-based style; and, in turn, these factors might be expected to reduce potential for PTG. In addition, OAC presents a unique symptom complex and the psychosocial issues and disabilities that arise from the treatment modalities involved with this cancer might be expected

to influence a patient's benefit finding ability and diminish their capacity for PTG. However, this study - the first to investigate PTG and response shift in OAC patients - suggests that benefit finding and positive well-being may be experienced by OAC patients despite the often hopeless prognosis. High levels of self-efficacy reported by OAC patients may serve to regulate the stress process, relieve depression and improve cancer symptoms^[37,38]. For example, OAC patients were less depressed than controls (OR 0.64; 95%CI: 0.42-0.98).

Perceived stress was higher in RO patients (particularly among older patients) in line with other studies [that demonstrate a link between psychosocial factors and gastro-oesophageal reflux disease (GORD)^[19,39]] even though RO patients were more likely than controls to report high self-efficacy. The association between GORD and somatisation disorder and depression reported elsewhere^[40] was not supported by the results of this study. The voluntary self-selection to participate in the study may have led to a form of ascertainment bias, whereby individuals with lower self-efficacy may have been less likely to participate in the study. Also, recall bias may have over-reported prior levels of self-efficacy, based on their current ability to cope with adversity.

Repression has been linked with poor health and cancer^[41-45]. However, there was no significant association found between repression and OAC. The single-item measure of repression used in this study may not have been sufficiently sensitive^[46]. RO patients were less likely than controls to report high repression. This relationship may have been moderated by social support - RO patients were more likely than controls to report that they had good social support.

This is a large, all-Ireland, population-based study, for which there was a relatively high response rate among case groups. The low control group response rate (42%),

however, merits a cautious approach to the interpretation of results (*e.g.*, representativeness of the population) though adjusted analyses were performed on potential confounders such as GOR, gender, age at interview, smoking status, alcohol consumption, occupation and BMI.

The retrospective nature of this study meant that it was susceptible to recall bias; this problem may be overcome by using a prospective study design. The resource-heavy nature, however, of these kinds of studies means that a retrospective study design, which can generate large data sets relatively quickly and efficiently, is usually more practicable. In any case, the incidence of OAC is still relatively low^[8] and the sample population is not large enough for a prospective study.

In conclusion, our results suggest that there may be a complex interaction between psychological factors regarding the development of RO, BO and OAC. Furthermore, the results presented here indicate that these conditions have significant psychological health consequences. Further research with enhanced methodological rigor is required to clarify the role of psychological factors in the development of OAC.

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COMMENTS

Background

Incidence of oesophageal adenocarcinoma (OAC) is rising more rapidly than that of any other form of cancer in the western world. The causes of OAC are largely unknown. Psychological factors are thought to mediate cancer risk for other forms of malignancies, but have not been investigated with regards to OAC or the conditions predisposing to it, including reflux oesophagitis (RO) and Barrett's oesophagus (BO).

Research frontiers

Several risk factors for OAC have been investigated, including diet, obesity and smoking. These factors mediate cancer risk in a variety of ways and have been shown to confer an increased risk of OAC. These risk factors, however don't fully explain the dramatic increase that has been seen over the last three decades (600% in Western countries) and psychosocial factors are thought to play a role.

Innovations and breakthroughs

Stress has been shown to mediate immune function, and subsequently cancer

risk in several forms of cancer, through the hypothalamic-pituitary-adrenal axis. Other psychosocial risk factors for cancer, such as depression, anxiety and job strain have also been investigated, but it remains unclear to what extent these factors are influencing the risk of OAC; either independently or through RO or BO.

Applications

Survival rates for OAC patients are relatively low and the comorbidities and treatment regimens for this form of cancer can greatly diminish quality of life in these patients. Identification of unambiguous risk factors for both OAC itself as well as the premalignant, reflux-mediated pathway, would provide the potential for early intervention and greatly improve survival and quality of life for RO, BO and OAC patients.

Terminology

Oesophageal adenocarcinoma is a cancer of the oesophagus that originates in the glandular tissue of the epithelium. Barrett's oesophagus is a squamous to columnar cell metaplasia within the distal oesophagus and is the main risk factor for OAC. Reflux oesophagitis is an inflammation of the oesophageal mucosa and is caused by abnormal gastro-oesophageal reflux; it also increases the risk of developing OAC.

Peer review

This is a very well written original manuscript assessing relationship between psychosocial factors and reflux esophagitis, Barrett's esophagus and esophageal adenocarcinoma.

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Evaluation of the relationship between dietary factors, *CagA*-positive *Helicobacter pylori* infection, and *RUNX3* promoter hypermethylation in gastric cancer tissue

Yan-Wei Zhang, Sang-Yong Eom, Dong-Hyuk Yim, Young-Jin Song, Hyo-Yung Yun, Joo-Seung Park, Sei-Jin Youn, Byung-Sik Kim, Yong-Dae Kim, Heon Kim

Yan-Wei Zhang, Sang-Yong Eom, Dong-Hyuk Yim, Yong-Dae Kim, Heon Kim, Department of Preventive Medicine and Medical Research Institute, College of Medicine, Chungbuk National University, Cheongju 361-763, South Korea

Young-Jin Song, Hyo-Yung Yun, Department of Surgery, College of Medicine, Chungbuk National University, Cheongju 361-763, South Korea

Joo-Seung Park, Department of Surgery, College of Medicine, Eulji University, Daejeon 461-713, South Korea

Sei-Jin Youn, Department of Internal Medicine, College of Medicine, Chungbuk National University, Cheongju 361-763, South Korea

Byung-Sik Kim, Department of Surgery, Asan Medical Center, College of Medicine, Ulsan University, Seoul 138-736, South Korea

Author contributions: Zhang YW and Kim H designed the study protocol; Eom SY and Yim DH performed the statistical analysis and data interpretation; Song YJ, Yun HY, Park JS, Youn SJ and Kim BS equally contributed to this study by selection of subjects, interviews, cancer tissue sampling and clinical data acquisition; Zhang YW and Kim YD drafted the manuscript.

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Correspondence to: Dr. Heon Kim, Department of Preventive Medicine and Medical Research Institute, College of Medicine, Chungbuk National University, 52 Naesudong-ro, Hungdok-gu, Cheongju, Chungbuk 361-763, South Korea. kimheon@cbu.ac.kr
Telephone: +82-43-2612864 Fax: +82-43-2742965

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Abstract

AIM: To evaluate the relationship among *Helicobacter pylori* (*H. pylori*) infection, *CagA* status, and dietary factors with *RUNX3* promoter hypermethylation.

METHODS: Gastric cancer tissue samples were collected from 184 South Korean patients. All patients were interviewed following a semi-quantitative food frequency questionnaire. The average frequencies of intake and portion sizes of 89 common food items were documented, and total intakes of calories, nutrients, vitamins, and minerals were calculated for each subject. DNA was extracted from gastric cancer tissue samples, and amplification of the *HSP60* gene was performed to detect *H. pylori* infection. Nested polymerase chain reaction (PCR) was used to detect the presence of the *CagA* gene. *RUNX3* gene expression was measured by reverse transcription-PCR, and *RUNX3* methylation status was evaluated by methylation-specific PCR. The odds ratios (ORs) and 95%CI associated with *RUNX3* promoter hypermethylation status were estimated for each of the food groups, lifestyle factors, and the interaction between dietary and lifestyle factors with *CagA* status of *H. pylori* infection.

RESULTS: Overall, 164 patients (89.1%) were positive for *H. pylori* DNA, with the *CagA* gene detected in 59 (36%) of these *H. pylori*-positive samples. In all, 106 (57.6%) patients with gastric cancer demonstrated CpG island hypermethylation at the *RUNX3* promoter. *RUNX3* expression was undetectable in 52 (43.7%) of the 119 gastric cancer tissues sampled. A high consumption of eggs may increase the risk of *RUNX3* methylation in gastric cancer patients, having a mean OR of 2.15 (range, 1.14-4.08). A significantly increased OR of 4.28 (range, 1.19-15.49) was observed with a high consumption of nuts in patients with *CagA*-positive *H. pylori* infection. High intakes of carbohydrate, vitamin B1, and vitamin E may decrease the risk of *RUNX3* methylation in gastric cancer tissue, particularly in *CagA*- or *H. pylori*-negative infection, with OR of 0.41 (0.19-0.90), 0.42 (0.20-0.89), and 0.29 (0.13-0.62),

respectively. A high consumption of fruits may protect against *RUNX3* methylation.

CONCLUSION: These results suggest that the *CagA* status of *H. pylori* infection may be a modifier of dietary effects on *RUNX3* methylation in gastric cancer tissue.

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Key words: Gastric cancer; *RUNX3*; *Helicobacter pylori*; *CagA*; Dietary factors

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INTRODUCTION

The incidence of gastric cancer has declined dramatically in most countries over the past 70 years, yet it remains the second most common cause of cancer-related deaths worldwide. The estimated incidence in 2008 was 989 600, and the majority of new cases occurred in developing countries^[1]. Asian countries, including Japan, South Korea and China, have a particularly high incidence of gastric cancer; it is the second most frequently diagnosed cancer in the Korean population.

The development of gastric cancer appears to be the result of a complex interaction between environmental and genetic factors. Extensive epidemiology studies have shown that *Helicobacter pylori* (*H. pylori*) infection is a major risk factor for gastric cancer and its precursor lesions^[2]. The risk of developing gastric cancer is estimated to increase 2 to 6 times in patients with *H. pylori* infection, as determined by retrospective case-control and prospective epidemiology studies^[3]. As such, the World Health Organization and the International Agency for Research on Cancer consensus group have classified *H. pylori* as a Class I human carcinogen. More than half of the world's population is infected with *H. pylori*; its prevalence ranges from 25% in developed countries to more than 90% in developing regions^[4]. Among individuals infected with *H. pylori*, a small percentage develop gastric cancer by a process influenced by bacterial virulence. The most widely studied *H. pylori* virulence factor is the *CagA* antigen, a 96-to 138-kDa protein^[5]. The *CagA* gene, found on a genomic region called the *cag* pathogenicity island (PAI), is considered as a marker for enhanced virulence. Moreover, individuals infected with *CagA*-positive *H. pylori* strains have a higher risk of developing peptic ulcers and gastric cancer compared to those harboring *CagA*-negative *H. pylori* strains^[6].

The human runt-related transcription factors (*RUNX*) are important targets of the transforming growth factor (TGF)- β superfamily signaling pathway^[7]. Three different *RUNX* genes have been identified as human homologues of the *Drosophila* genes *runt* and *lozenge*^[8]. *RUNX1* (*AML1/CBEA2/PEBP-2aB*) is believed to be essential in hematopoiesis and is located in chromosome 21q22.3^[8]. Moreover, it is targeted for translocation or mutation in acute and chronic leukemias and in myelodysplastic syndromes^[9]. *RUNX2* (*AML3/CBEA1/PEBP2aA*), located on chromosome 6q21, is regarded as indispensable for the development of the musculoskeletal system and has been associated with the human bone disease cleidocranial dysplasia^[10]. In humans, *RUNX3* (*AML2/CBEA3/PEBP2aC*) is found at chromosomal locus 1p36 in a region that is frequently deleted in many types of cancers. This region is therefore postulated to contain an important tumor suppressor gene^[11]. The *RUNX3* protein combines with Smads and acts synergistically to regulate various target genes^[7]. Little or no expression of *RUNX3* has been observed in gastric cancer^[12] or in carcinomas of the liver, lung, breast, prostate, endometrium, and colon^[13]. Several mechanisms are thought to be responsible for downregulating *RUNX3*, including promoter hypermethylation, loss of heterozygosity, hemizygous deletion, and mutation, and these mechanisms have been linked to carcinogenesis in a wide range of human solid tumors. Recent studies have demonstrated that loss of *RUNX3* expression is causally related to the genesis and progression of gastric cancer. Approximately 45% to 60% of surgically resected gastric cancer specimens and cell lines derived from these cancers do not express *RUNX3* due to either hemizygous deletion of the gene or hypermethylation of its promoter region^[14]. Therefore, *RUNX3* is considered a tumor suppressor gene, and hypermethylation of its promoter is thought to play an important role in gastric carcinogenesis. Diets low in methyl-contributing folate, vitamins B6 and B12, and methionine and a high consumption of alcohol have been hypothesized to affect DNA methylation at CpG islands and confer an increased risk of cancer^[15]. CpG island DNA methylation is also thought to occur as a result of inflammation^[16]. Infection with *CagA*-positive *H. pylori* has been associated with higher grades of gastric mucosal inflammation as well as severe atrophic gastritis and is believed to play a role in the development of gastric carcinoma^[17].

The purpose of this study was to evaluate the associations between dietary factors and *RUNX3* promoter hypermethylation status and to assess the combined contribution of dietary factors and *H. pylori CagA* status to *RUNX3* promoter hypermethylation in patients with gastric cancer.

MATERIALS AND METHODS

Study subjects

Between September 2003 and March 2006, 184 South Korean patients with gastric cancer were enrolled in the

Table 1 Characteristics of the study subjects

| Variables | n (%) |
|--------------------------------------|----------------------------|
| All subjects | 184 |
| Age (mean ± SD), yr | 59.8 ± 11.3 (range: 23-83) |
| Gender | |
| Male | 124 (67.4) |
| Female | 60 (32.6) |
| <i>Helicobacter pylori</i> infection | |
| Negative | 20 (10.9) |
| Positive | 164 (89.1) |
| <i>CagA</i> status | |
| Negative | 125 (67.9) |
| Positive | 59 (32.1) |
| <i>RUNX3</i> methylation | |
| Negative | 78 (42.4) |
| Positive | 106 (57.6) |
| <i>RUNX3</i> expression | |
| Positive | 67 (56.3) |
| Negative | 52 (43.7) |

study. All patients were diagnosed at Chungbuk National University Hospital and Eulji University Hospital, both of which are located in the middle of the South Korean peninsula. Gastric cancer tissue samples were collected from all patients with prior consent. Patient characteristics are shown in Table 1. Tissue samples were obtained during resection surgery, immediately frozen in liquid nitrogen, and stored at -80 °C until needed for DNA and RNA extraction.

Patient demographics and other potential risk factors for gastric cancer were collected during direct interviews with subjects. Trained personnel interviewed the subjects using a structured questionnaire within one month of the diagnosis of gastric cancer and other benign diseases. Control subjects were questioned while undergoing routine medical examinations during hospital visits. Dietary data were collected using a semiquantitative food frequency table that had been previously evaluated for its validity and reliability^[18]. The average frequency of intake and portion size of 89 common food items was documented. These items were classified into 21 food groups based on their ingredients: cereals; potatoes; nuts; noodles (pasta); breads and cakes; vegetables; mushrooms; fruits; red meats; eggs; fish and shellfish; stews; chicken; kimchi; soybean foods; soybean pastes; milk and dairy products; jams, honey, sweets and chocolates; coffee and tea; seaweeds; and alliums. Each food item was divided into high and low groups according to the median value of its distribution.

The amount of calories, nutrients, vitamins, and minerals consumed for each food item was estimated by multiplying the amount of the food item consumed by its nutrient value. Total intakes of calories, nutrients, vitamins, and minerals were calculated for each subject by summing the respective calories, nutrients, vitamins, and minerals for each food item^[19]. The intake amounts of these factors were adjusted for caloric intake using the method of Willett *et al.*^[20].

Polymerase chain reaction amplification of heat-shock protein 60 and *CagA* gene

DNA was extracted from gastric cancer tissue samples using a commercially available kit (Wako, Osaka, Japan). To detect *H. pylori* infection in these samples, a nested PCR protocol was developed against heat-shock protein (*HSP60*). *HSP60* was chosen due to its high degree of sequence conservation in all biota^[21].

DNA amplification of the *HSP60* gene was performed according to the nested polymerase chain reaction (PCR) method described by Varsha *et al.*^[22]. Forward and reverse oligonucleotides were derived from the conserved region located between bases 8085 and 8675 of the *HSP60* gene of *H. pylori* (ATCC 26695). Internal primers sets were derived from the region between bases 8162 and 8663 (Gene Bank Accession number NC-0091; gene ID 899089). The first amplification was performed using HSP1, the sense primer (5'-AAGGCATGCAATTGATAGAGGCT-3'), and HSP2, the antisense primer (5'-CTTTTCTCTTTCATTTCCACTT-3'), in a 25-μL reaction mixture containing 2.5 μL of 10 × PCR buffer, 5 pmol of each primer, 10 ng of DNA, 200 μmol/L aliquots of each dNTP, and 1 unit of *Taq* polymerase. This PCR resulted in a 590-base pair DNA fragment.

For the second amplification, 2 μL of the primary amplification product was used in a 15-μL reaction mixture with HSPN1, the sense primer (5'-TTGATAGAGGCTACCTCTCC-3'), and HSPN2, the antisense primer (5'-TGTCATAATCGCTTGTCGTGC-3'). Both PCR amplifications consisted of 35 cycles of denaturation at 94 °C for 30 s, annealing at 58 °C for 40 s, and elongation at 72 °C for 30 s. The final amplicon (expected size, 501 base pairs) was analyzed by electrophoresis on a 2% agarose gel.

Nested PCR was also chosen as a sensitive and specific method to detect the presence of the *CagA* gene. Forward and reverse oligonucleotides were derived from the conserved region located between bases 544680 and 545161 of the *CagA* gene of *H. pylori* (ATCC 26695). Internal primers sets were derived from the region between bases 544833 and 545131 (Gene Bank Accession number NC-00921; gene ID 899089). The first amplification was performed with *CagA* N1, the sense primer (5'-TG-GCAGTGGGTTAGTCATAGCAG-3'), and *CagA* N2, the antisense primer (5'-AGGACTCTTGCAGGCGTTGGTG-3'), in a 15-μL reaction mixture containing 1.5 μL of 10 × PCR buffer, 5 pmol of each primer, 10 ng of DNA, 200 μmol/L aliquots of each dNTP, and 1 unit of *Taq* polymerase, resulting in a 481-base pair DNA fragment. For the second amplification, 2 μL of the primary amplification product was used in a 15 μL reaction mixture with *CagA* D1, the sense primer (5'-ATAATGCTA-AATTAGACAACCTTGAGCGA-3'), and *CagA* R1, the antisense primer (5'-TTAGAATAATCAACAAACAT-CACGCCAT-3'). Both PCR amplifications consisted of 35 cycles of denaturation at 94 °C for 30 s, annealing at

55 °C for 30 s, and elongation at 72 °C for 30 s. The final amplicon (expected size, 298 base pairs) was analyzed by electrophoresis on a 2% agarose gel.

***RUNX3* hypermethylation analysis**

The methylation status of the CpG island in the promoter region of *RUNX3* was determined by bisulfite treatment of DNA (Imprint DNA Modification Kit, Sigma-Aldrich, St. Louis, MO, United States) in order to convert all unmethylated cytosines to uracils while leaving methylated cytosines unchanged, followed by methylation-specific PCR. Amplifications were carried out in a 96-well plate. DNA extracted from the leukocytes of healthy individuals and treated with *SssI* methyltransferase (New England Biolabs, Beverly, MA, United States) prior to bisulfite modification was used as a positive control for the methylated alleles. Bisulfite-modified DNA extracted from leukocytes of a healthy individual served as a positive control for the unmethylated alleles, and water was used as a negative control.

Amplification was carried out in a 20 µL reaction volume containing 10 ng of bisulfite-modified DNA and 2 µL of 10 × PCR buffer with 20 mmol/L MgCl₂, 4 µL of a GC-rich solution, 5 pmol of each primer for *RUNX3*, 200 µmol/L aliquots of each dNTP, and 1 unit of Faststart *Taq* DNA polymerase (Roche Applied Science, Mannheim, Germany). PCR was performed in a TaKaRa PCR Thermal Cycler Dice Gradient (Otsu, Japan) for methylated *RUNX3* with *RUNX3*-5M, the sense primer (5'-TTACGAGGGGCGGTCGTACGCGGG-3'), and *RUNX3*-3M, the antisense primer (5'-AAAACGACC-GACGCGAACGCCTCC-3'), using an initial denaturation at 95 °C for 5 min, followed by 35 cycles of 95 °C for 1 min, 69.1 °C for 1 min, 74 °C for 1 min and a final extension at 74 °C for 7 min. For unmethylated *RUNX3*, PCR was performed using *RUNX3*-5U, the sense primer (5'-TTATGAGGGGTGGTTGTATGTGGG-3') and *RUNX3*-3U, the antisense primer (5'-AAAACAACCAA-CACAAACACCTCC-3') following an initial denaturation at 95 °C for 5 min, followed by 35 cycles of 95 °C for 1 min, 61.8 °C for 1 min, 72 °C for 1 min and a final extension at 72 °C for 7 min. The PCR product (expected size, 218 base pairs) was separated on a 3% agarose gel. To verify the PCR product, the methylation-specific PCR amplicons from 5 samples were purified with HiYield PCR DNA Extraction Kit (Real Biotech, Banqiao, Taiwan) and subjected to direct DNA sequencing (ABI 3100, Applied Biosystems, Foster City, CA, United States).

RNA isolation and reverse transcription-PCR

Total RNA was extracted from 137 gastric cancer tissue samples using a commercially available kit (Easy-Blue, Intron, Seongnam, South Korea). All samples were assayed for *RUNX3* expression. The assay failed for 18 samples due to RNA degradation. The RT reaction was performed using 1 µg of total RNA with SuperScript III First-strand cDNA Synthesis Kit (Invitrogen, Carls-

bad, CA, United States). The following primer sets were used: 5'-TCTGCTCCGTGCTGCCCTCGCACTG-3' and 5'-AGGCATTGCGCAGCTCAGCGGAGTA-3' for *RUNX3* (151 bp) and 5'-ACCCACTCCTCACCTTTG-3' and 5'-CTCTTGTGCTCTTGCTGGG-3' for *GAPDH* (178 bp). Amplification was carried out in a 15-µL reaction volume containing 0.1 µg of cDNA and 1.5 µL of 10 × PCR buffer with 20 mmol/L MgCl₂, 3 µL of a GC-rich solution, 5 pmol of each primer, 200 µmol/L aliquots of each dNTP, and 1 unit of Faststart *Taq* DNA polymerase. PCR was performed in the TaKaRa PCR Thermal Cycler Dice Gradient with an initial denaturation at 95 °C for 5 min, followed by 30 cycles (for *RUNX3*) or 25 cycles (for *GAPDH*) of 95 °C for 1 min, 62.3 °C for 1 min, 72 °C for 1 min and a final extension at 72 °C for 7 min. The PCR product was separated on a 3% agarose gel.

Statistical analysis

The amount of calories, nutrients, vitamins and minerals for each particular food type was calculated by multiplying their respective values by the level of intake. The total intake of calories, nutrients, vitamins, and minerals was calculated as the sum of each value across all food types consumed by each individual^[19]. Each value was adjusted for caloric intake using the method of Willet *et al*^[20]. The median value calculated for each variable was used as the grouping criterion in the unmethylated *RUNX3* group.

The odds ratios (ORs) and 95% CIs associated with the *RUNX3* methylation status were estimated for each of the food groups and lifestyle factors and for the interaction between dietary and lifestyle factors with *CagA* status of *H. pylori* infection using an unconditional logistic model that controlled for age, sex and total energy intake. The homogeneities of the ORs according to the *CagA* status were evaluated using the Breslow-Day test. *P* values less than 0.05 were considered significant.

RESULTS

Overall, 164 patients (89.1%) were found to be positive for *H. pylori* DNA, with the *CagA* gene detected in 59 (36%) of the *H. pylori*-positive samples. Overall, *CagA*-positive expression was detected in 32.1% of all patients included in our study. In all, 106 (57.6%) patients with gastric cancer demonstrated CpG island hypermethylation at the *RUNX3* promoter. *RUNX3* expression was undetectable in 52 (43.7%) of the 119 gastric cancer tissues sampled.

We found a significant difference in *RUNX3* expression status between patients with and without hypermethylated *RUNX3*. *RUNX3* gene expression was detected in 71.7% of patients with unmethylated *RUNX3*. In contrast, *RUNX3* gene expression was detected in only 46.6% of patients with methylated *RUNX3* (*P* = 0.0070) (Table 2).

A high consumption of eggs was associated with a higher risk of *RUNX3* methylation, with an OR of 2.15

Table 2 Distribution of age, gender, *Helicobacter pylori* infection, *CagA* status, and *RUNX3* expression according to *RUNX3* methylation status *n* (%)

| Variables | <i>RUNX3</i> methylation (+) | <i>RUNX3</i> methylation (-) | <i>P</i> value |
|--------------------------------------|------------------------------|------------------------------|----------------|
| Age (mean ± SD), yr | 60.5 ± 11.2 | 58.7 ± 11.5 | 0.290 |
| Gender | | | 0.648 |
| Male | 70 (56.5) | 36 (67.4) | |
| Female | 54 (43.5) | 24 (40.0) | |
| <i>Helicobacter pylori</i> infection | | | 0.234 |
| Negative | 14 (13.2) | 6 (7.7) | |
| Positive | 92 (86.8) | 72 (92.3) | |
| <i>CagA</i> status | | | 0.997 |
| Negative | 72 (67.9) | 53 (68.0) | |
| Positive | 34 (32.1) | 25 (32.0) | |
| <i>RUNX3</i> expression | | | 0.007 |
| Positive | 34 (46.6) | 33 (71.7) | |
| Negative | 39 (53.4) | 13 (28.3) | |

(95%CI, 1.14-4.08). In contrast, high fruit consumption was associated with a lower risk of *RUNX3* methylation, with an OR of 0.50 (95%CI, 0.27-0.93). A large consumption of eggs or chicken increased the risk of *RUNX3* methylation in *CagA*- or *H. pylori*-negative cancer patients. A high intake of unfermented seaweeds or aliums conferred larger protective effects against *RUNX3* methylation in *CagA*-positive cancer patients compared to *CagA*-negative cancer patients. In homogeneity tests, however, the OR of high consumption of these food groups was similar between those with *CagA*-positive and *CagA*-negative status. In contrast, a high consumption of nuts was a significant risk factor for *RUNX3* methylation in *CagA*-positive patients. The OR of high nut intake in *CagA*-positive patients was 4.28 (95%CI, 1.19-15.49), and this value significantly differed from that in *CagA*-negative or *H. pylori*-negative cases (Table 3).

Large intakes of carbohydrates and vitamin E decreased the risk of *RUNX3* methylation in gastric cancer, with odd ratios of 0.48 (95%CI, 0.25-0.91) and 0.47 (95%CI, 0.26-0.88), respectively. When stratified according to the *CagA* status, high intakes of carbohydrates, vitamin B1, or vitamin E conferred a protective effect in *CagA*-negative or *H. pylori*-negative patients but not in *CagA*-positive patients, with statistically significant differences demonstrated for vitamins B1 and E (Table 4).

DISCUSSION

RUNX3, a member of the *RUNX* family, is a candidate tumor suppressor gene that is thought to play a role in the progression of gastric cancer. *RUNX3* is unique in that it is inactivated primarily by epigenetic silencing, unlike many other tumor suppressors, such as *p53*, that are inactivated mainly by deletion and mutation^[23]. Molecular epidemiology studies have investigated the relationship between DNA hypermethylation-induced inactivation of *RUNX3* and individual risk of cancer development in various organs, including the lungs, pancreas, urinary

bladder, and gastric cancers^[12,24-26].

In the present study, we found that *RUNX3* methylation was observed in 58% (106/184) of our gastric cancer patients. Consistent with our results, *RUNX3* methylation was previously observed in 52.6% (30/57) of the gastric cancer specimens in a Japanese study^[27]. Other investigators have reported the prevalence of *RUNX3* methylation in primary gastric cancers to be 71% (57/80) and 64% (48/75)^[13,28]. These differences in methylation frequency can be explained by the fact that different regions were examined within the *RUNX3* CpG island. Homma *et al*^[29] suggested that the methylation status of the regions spanning the transcription start site (No 6-8) is critical for the loss of *RUNX3* expression. In our study, we examined CpG islands within regions No 6-7 of *RUNX3* and observed levels of methylation similar to those reported by Homma *et al*^[29]. Previous studies have shown that, among primary gastric cancer samples and gastric cancer cell lines, 45%-60% showed loss of *RUNX3* expression due to hypermethylation of the CpG island located in the P2 promoter region^[12,30]. We found that *RUNX3* expression was undetectable in 43.7% of primary gastric cancer tissues. The significant association between *RUNX3* methylation status and its expression is consistent with the fact that promoter hypermethylation downregulates the expression of this gene.

H. pylori infection and *CagA* status did not reveal any significant association with *RUNX3* promoter hypermethylation or with *RUNX3* expression levels (Table 2). This finding suggests that *H. pylori* infection, regardless of *CagA* status, does not induce *RUNX3* methylation and that the ability of *RUNX3* to function as a tumor suppressor is not specific to *H. pylori*-related gastric cancer. A Japanese study reported a marginally significant association between *H. pylori* infection and *RUNX3* promoter hypermethylation; however, no significance was found between *CagA* positive *H. pylori* infection and *RUNX3* promoter hypermethylation^[27]. Our results are further supported by a German study that demonstrated that the level of *RUNX3* mRNA expression in the gastric epithelium is not influenced by *H. pylori* infection^[31]. Interestingly, it has previously been shown that *H. pylori* infection induces the ubiquitination and degradation of *RUNX3* and suppresses *RUNX3* expression in cultured gastric epithelial cells and mouse gastric epithelial cells. This discordance may be due to differences between *in vivo* and *in vitro* systems, and differences between mouse and human diseases. Furthermore, we tested the association of *H. pylori* infection with *RUNX3* promoter hypermethylation, not with *RUNX3* expression. It is possible that *H. pylori* infection suppresses the expression of *RUNX3* in gastric epithelial cells *via* pathways other than promoter hypermethylation. For example, Liu *et al*^[32] reported that *CagA* can inhibit the expression of *RUNX3* *via* Src/MEK/ERK and p38 MAPK pathways.

Several dietary factors, including folate, methionine, and vitamins B12 and B6 have been reported to be involved either directly or indirectly in DNA methylation^[15]. Re-

Table 3 Associations between dietary factors and *RUNX3* methylation risk, according to *CagA* status

| | Total subjects | | <i>CagA</i> (-) or <i>H. pylori</i> (-) | | <i>CagA</i> (+) | |
|------------|-----------------------------|-------------------------------|---|-------------------------------|-----------------------------|----------------------------------|
| | Methylated/ unmethylated | OR (95%CI) ¹ | Methylated/ unmethylated | OR (95%CI) ¹ | Methylated/ unmethylated | OR (95%CI) ¹ |
| Nuts | | | | | | |
| Low | 36/40 | 1.0 | 26/23 | 1.0 | 10/17 | 1.0 |
| High | 70/38 | 1.65 (0.86-3.18) | 46/30 | 1.10 (0.49-2.49) | 24/8 | 4.28 (1.19-15.49) ^{2,3} |
| Vegetables | | | | | | |
| Low | 65/39 | 1.0 | 47/26 | 1.0 | 18/13 | 1.0 |
| High | 41/39 | 0.58 (0.31-1.07) | 25/27 | 0.49 (0.23-1.03) | 16/12 | 0.78 (0.27-2.36) |
| Fruits | | | | | | |
| Low | 69/39 | 1.0 | 51/31 | 1.0 | 18/8 | 1.0 |
| High | 37/39 | 0.50 (0.27-0.93) ³ | 21/22 | 0.55 (0.26-1.19) | 16/17 | 0.32 (0.10-1.06) |
| Meat | | | | | | |
| Low | 39/40 | 1.0 | 26/28 | 1.0 | 13/12 | 1.0 |
| High | 67/38 | 1.68 (0.91-3.12) | 46/25 | 1.83 (0.87-3.87) | 21/13 | 1.30 (0.41-4.10) |
| Egg | | | | | | |
| Low | 32/40 | 1.0 | 21/29 | 1.0 | 11/11 | 1.0 |
| High | 74/38 | 2.15 (1.14-4.08) ³ | 51/24 | 2.64 (1.21-5.75) ³ | 23/14 | 1.57 (0.48-5.23) |
| Chicken | | | | | | |
| Low | 34/39 | 1.0 | 20/26 | 1.0 | 14/13 | 1.0 |
| High | 72/39 | 1.78 (0.93-3.41) | 52/27 | 2.24 (1.02-4.95) ³ | 20/12 | 0.95 (0.27-3.39) |
| Seaweeds | | | | | | |
| Low | 64/39 | 1.0 | 43/30 | 1.0 | 21/9 | 1.0 |
| High | 42/39 | 0.63 (0.35-1.16) | 29/23 | 0.89 (0.43-1.87) | 13/16 | 0.23 (0.07-0.79) ³ |
| Alliums | | | | | | |
| Low | 66/40 | 1.0 | 43/28 | 1.0 | 23/11 | 1.0 |
| High | 40/38 | 0.55 (0.30-1.01) | 29/25 | 1.71 (0.34-1.48) | 11/14 | 0.28 (0.09-0.92) ³ |
| Fish | | | | | | |
| Low | 61/39 | 1.0 | 40/29 | 1.0 | 21/10 | 1.0 |
| High | 45/39 | 0.69 (0.38-1.27) | 32/24 | 0.89 (0.43-1.85) | 13/15 | 0.44 (0.14-1.33) |

¹Adjusted for age, gender, total energy intake; ²Odds ratio (OR) was significantly different according to *CagA* status in a homogeneity test; ³*P* value < 0.05 statistically significant difference.

verse associations between these dietary factors and cancer risk, observed in epidemiological studies, have led to the hypothesis that folate, methionine, and other dietary factors confer an altered risk of gastric cancer due to their role in DNA methylation^[33]. However, few data exist to support this hypothesis. Many studies have investigated potential associations between dietary factors and DNA promoter methylation in colon cancer^[34,35]; however, these studies have failed to establish a consistent association. Methionine is considered to be an essential dietary component because it is the penultimate methyl donor for mammalian methylation reactions and it cannot be manufactured by the body. Consuming excessive quantities of methionine may affect DNA promoter CpG island methylation and thereby cause dysregulation of gene expression. In the present study, a high consumption of methionine-rich foods such as eggs and chicken increased the risk of *RUNX3* promoter methylation, and a high consumption of nuts increased the risk when combined with *CagA*-positive *H. pylori* infection.

Two major forms of *CagA* have been identified: East Asian *CagA* and Western *CagA*. In countries such as Japan, South Korea and China, most *H. pylori* infections contain East Asian *CagA*. The degree of inflammation, activity of gastritis, and atrophy are significantly higher in patients infected with East Asian *CagA*-positive strains compared to patients infected with *CagA*-negative or

Western *CagA*-positive strains^[36]. *CagA*-positive *H. pylori* infection induces progressive inflammatory changes in the gastric mucosa that ultimately lead to gastric cancer: superficial gastritis, atrophic gastritis, intestinal metaplasia, dysplasia, and carcinoma^[37]. In the present study, we observed that *CagA*-positive *H. pylori*-infected individuals who consume a large quantity of nuts may increase the risk of *RUNX3* methylation by 4-fold. The risk of *RUNX3* methylation was higher in *CagA*-positive *H. pylori*-infected subjects than in *CagA*- or *H. pylori*-negative patients of the same age.

The association of certain vitamin deficiencies and cancer is well established and is usually associated with environmental conditions that affect all tissues. High intakes of folate and vitamins B6 and B12 may protect against DNA methylation in colorectal cancer^[35]. In the present study, however, we did not find a protective effect of these vitamins. Instead, we found that high intakes of vitamin B1 and carbohydrates provide a protective effect against *RUNX3* methylation in gastric cancer, but this was not demonstrated in individuals with *CagA*-positive status. Vitamin B1 is essential for the body to utilize carbohydrates for energy and for metabolizing amino acids. The main biologically active vitamin B1 derivative is thiamine diphosphate, which is involved in universal metabolic pathways including glycolysis, the pentose phosphate pathway and the tricarboxylic acid cycle. Several studies

Table 4 Associations between micronutrients and mineral salts intake and *RUNX3* methylation risk, according to *CagA* status

| | Total subjects | | <i>CagA</i> (-) or <i>H. pylori</i> (-) | | <i>CagA</i> (+) | |
|--------------|-----------------------------|-------------------------------|---|---------------------------------|-----------------------------|-------------------------------|
| | Methylated/ unmethylated | OR (95%CI) ¹ | Methylated/ unmethylated | OR (95%CI) ¹ | Methylated/ unmethylated | OR (95%CI) ¹ |
| Protein | | | | | | |
| Low | 54/39 | 1.0 | 35/26 | 1.0 | 19/13 | 1.0 |
| High | 52/39 | 0.90 (0.50-1.64) | 37/27 | 0.95 (0.46-1.95) | 15/12 | 0.86 (0.29-2.57) |
| Fat | | | | | | |
| Low | 60/39 | 1.0 | 38/23 | 1.0 | 22/16 | 1.0 |
| High | 46/39 | 0.74 (0.21-1.35) | 34/30 | 0.66 (0.32-1.36) | 12/9 | 0.98 (0.30-3.25) |
| Carbohydrate | | | | | | |
| Low | 71/39 | 1.0 | 53/28 | 1.0 | 18/11 | 1.0 |
| High | 35/39 | 0.48 (0.25-0.91) ³ | 19/25 | 0.41 (0.19-0.90) ³ | 16/14 | 0.53 (0.15-1.91) |
| Vitamin A | | | | | | |
| Low | 57/39 | 1.0 | 36/27 | 1.0 | 21/12 | 1.0 |
| High | 49/39 | 0.73 (0.40-1.35) | 36/26 | 0.88 (0.42-1.86) | 13/13 | 0.51 (0.17-1.56) |
| Vitamin B1 | | | | | | |
| Low | 65/40 | 1.0 | 48/24 | 1.0 | 17/15 | 1.0 |
| High | 41/38 | 0.67 (0.36-1.22) | 24/29 | 0.42 (0.20-0.89) ^{2,3} | 17/10 | 2.04 (0.63-6.62) ² |
| Vitamin B2 | | | | | | |
| Low | 56/39 | 1.0 | 37/25 | 1.0 | 19/14 | 1.0 |
| High | 50/39 | 0.81 (0.44-1.48) | 35/28 | 0.77 (0.37-1.62) | 15/11 | 0.97 (0.33-2.85) |
| Vitamin C | | | | | | |
| Low | 66/39 | 1.0 | 50/31 | 1.0 | 16/8 | 1.0 |
| High | 40/39 | 0.56 (0.30-1.04) | 22/22 | 0.59 (0.27-1.29) | 18/17 | 0.38 (0.11-1.28) |
| Vitamin E | | | | | | |
| Low | 73/39 | 1.0 | 54/24 | 1.0 | 19/15 | 1.0 |
| High | 33/39 | 0.47 (0.26-0.88) ³ | 18/29 | 0.29 (0.13-0.62) ^{2,3} | 15/10 | 1.30 (0.42-4.05) ² |

¹Adjusted for age, sex, total energy intake; ²Odds ratio (OR) was significantly different according to *CagA* status in a homogeneity test; ³*P* value < 0.05 statistically significant difference.

have reported that a high intake of carbohydrates protects against esophageal cancer and gastric cancer^[38]. Our study suggests that carbohydrates may protect against *RUNX3* promoter CpG island hypermethylation.

Vitamin E is a lipid-soluble antioxidant found in cell membranes where it prevents lipid peroxidation of polyunsaturated fatty acids. An Italian study reported that individuals with a high intake of vitamin E reduce their risk of gastric cancer by 50%^[39]. In the present study, the protective effect of high vitamin E intake on *RUNX3* methylation was observed in *CagA*-negative or *H. pylori*-negative gastric cancer patients but not in individuals with *CagA*-positive *H. pylori* infection. However, adverse effects of vitamin E have also been reported^[40]. Because vitamin E is metabolized using the same pathway as xenobiotics and may induce drug-metabolizing enzymes in rodents, it is hypothesized that high doses of vitamin E may lead to bioactivation of carcinogens within the human body^[41].

The association between high fruit consumption and protection from gastric cancer has been reported in many studies^[42,43]. The European Prospective Investigation into Cancer and Nutrition (EPIC-EURGAST) has reported similar results: fresh fruit and citrus fruit consumption may protect against diffuse and cardia gastric cancer, respectively^[44], but consistent results have yet to be achieved. Fruits contain vitamins C and E, which may protect cell membranes and DNA from oxidative damage. In our study, a protective effect of high fruit intake on *RUNX3*

methylation was observed, as well as a marginal inverse association between high vitamin C intake and *RUNX3* methylation.

We used nested PCR methods with DNA from gastric cancer tissues to detect *H. pylori* infection and *CagA* status. Nested PCR has the advantages of high sensitivity and specificity, quick results, and the ability to type bacteria without the requirement for special transport conditions. In our study, 89% of the gastric cancer patients were found to be positive for *H. pylori* DNA, and 32% were positive for *CagA* DNA. A Japanese study in which PCR methods were used to detect *H. pylori* infection and *CagA* status reported a 31.6% *CagA*-positive rate in 57 gastric cancer tissues^[27], which is very similar to our result. Several South Korean studies documented a higher prevalence of *CagA*-positive *H. pylori* infection^[45,46]. However, one study used an immunoblot method and the other included a smaller sample size. Because of the potential for false-positive immunoblot test results and the fact that individuals who had the infection in the past, irrespective of current infection, were classified as *CagA*-positive by this test, the actual *CagA* prevalence was likely lower than reported (97%). The difference between the *CagA* positive rates in our study and the previous one could be explained by the different detection methods. Moreover, a majority of the previous studies only investigated the role of *RUNX3* promoter methylation or *H. pylori* infection and *CagA* status in gastric cancer. We demonstrate, for the first time, that the interaction between *CagA*-positive

H. pylori infection and a high methionine intake contributes to *RUNX3* methylation in gastric cancer.

This study has some limitations. Because non-cancerous mucosa from gastric cancer patients or normal mucosal tissues from healthy controls were not included in this study, we were unable to determine *RUNX3* hypermethylation status or *RUNX3* expression level in those tissues. In addition, because this study is retrospective, we could not determine the temporal sequence of *H. pylori* infection and *RUNX3* promoter methylation. Finally, our sample size was not large enough to allow us to draw firm conclusions, especially regarding the effects of dietary factors on *RUNX3* promoter methylation.

In summary, a high consumption of chicken, eggs, or nuts rich in methionine may increase the risk of *RUNX3* promoter methylation in gastric cancer patients, whereas diets rich in fruits, carbohydrates, and vitamins B1 and E may decrease that risk. However, these inverse associations were observed only in patients negative for *CagA* or *H. pylori*. Moreover, a high intake of nuts combined with *CagA*-positive *H. pylori* infection significantly increased the risk of *RUNX3* promoter methylation. The *CagA* status of the *H. pylori* infection may be an important modifier of *RUNX3* methylation in gastric cancer patients.

COMMENTS

Background

Helicobacter pylori (*H. pylori*) infection is a major risk factor for gastric cancer, and the cytotoxin-associated gene A (*CagA*) is considered a marker for enhanced *H. pylori* virulence. Individuals infected with *CagA*-positive *H. pylori* strains have a higher risk of developing gastric cancer. *RUNX3* is a candidate tumor suppressor gene, and loss of *RUNX3* expression is considered a critical step in the genesis and progression of gastric cancer.

Research frontiers

Recent epidemiological studies have investigated the relationship between inactivation of *RUNX3* and the risk of cancer development in various organs. *RUNX3* inactivation has been reported to be frequently, but not always, found in gastric cancer tissues. One of the most important mechanisms for silencing *RUNX3* expression is *via* hypermethylation of the gene promoter, but little is known regarding the causative factors of *RUNX3* promoter hypermethylation. The authors cannot rule out the possibility that other risk factors for gastric cancer, such as dietary factors and *CagA*-positive *H. pylori* infection, can induce promoter hypermethylation of the *RUNX3* gene. In this study, the authors found that *H. pylori* infection, regardless of *CagA* status, is not associated with *RUNX3* methylation and that the ability of *RUNX3* to function as a tumor suppressor is not specific to *H. pylori*-related gastric cancer. In addition, the authors identified dietary factors that, in combination with *CagA*-positive *H. pylori* infection, modify the risk of *RUNX3* promoter methylation.

Innovations and breakthroughs

The authors determined the relationship between *CagA*-positive *H. pylori* infection and certain dietary factors with *RUNX3* promoter hypermethylation positive in gastric cancer patients. The authors adopted this case-case study design to maximize comparability and statistical power. To the best of our knowledge, this is the first study of the interaction between *CagA*-positive *H. pylori* infection and *RUNX3* methylation in gastric cancer tissues.

Applications

The results of this study suggest that the *CagA* status of *H. pylori* infection may modify dietary effects on *RUNX3* promoter hypermethylation in gastric cancer tissue. Further studies are needed to determine the mechanisms by which *CagA* and dietary factors interact and influence the development of gastric cancer.

Terminology

CagA, a 120-145 kDa protein encoded on the 40 kb *Cag* pathogenicity island, is

a *Helicobacter pylori* virulence factor. *RUNX3* is a member of the runt domain-containing family of transcription factors and is encoded by the *RUNX3* gene.

Peer review

In this study, the authors evaluated associations of *Helicobacter pylori* infection, *CagA* status, and dietary factors with *RUNX3* promoter hypermethylation in 184 Korean patients with gastric cancer.

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Common bile duct stones on multidetector computed tomography: Attenuation patterns and detectability

Chang Whan Kim, Jae Hyuck Chang, Yeon Soo Lim, Tae Ho Kim, In Seok Lee, Sok Won Han

Chang Whan Kim, Jae Hyuck Chang, Tae Ho Kim, In Seok Lee, Sok Won Han, Department of Internal Medicine, College of Medicine, The Catholic University of Korea, 505 Banpo-Dong, Seocho-Gu, Seoul 137-701, South Korea

Yeon Soo Lim, Department of Radiology, College of Medicine, The Catholic University of Korea, 505 Banpo-Dong, Seocho-Gu, Seoul 137-701, South Korea

Author contributions: Chang JH and Kim CW designed the study and analyzed the data; Lim YS and Chang JH analyzed the CT scan; Kim TH, Lee IS, and Han SW reviewed and revised the manuscript; and Chang JH and Kim CW wrote the manuscript.

Correspondence to: Jae Hyuck Chang, MD, PhD, Department of Internal Medicine, College of Medicine, The Catholic University of Korea, 505 Banpo-Dong, Seocho-Gu, Seoul 137-701, South Korea. wwjaang@catholic.ac.kr

Telephone: +82-32-3407086 Fax: +82-32-3402255

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Abstract

AIM: To investigate the attenuation patterns and detectability of common bile duct (CBD) stones by multidetector computed tomography (MDCT).

METHODS: Between March 2010 and February 2012, 191 patients with suspicion of CBD stones undergoing both MDCT and endoscopic retrograde cholangiopancreatography (ERCP) were enrolled and reviewed retrospectively. The attenuation patterns of CBD stones on MDCT were classified as heavily calcified, radiopaque, less radiopaque, or undetectable. The association between the attenuation patterns of CBD stones on MDCT and stone type consisting of pure cholesterol, mixed cholesterol, brown pigment, and black pigment and the factors related to the detectability of CBD stones by MDCT were evaluated.

RESULTS: MDCT showed CBD stones in 111 of 130 patients in whom the CBD stones were demonstrated

by ERCP with 85.4% sensitivity. The attenuation patterns of CBD stones on MDCT were heavily calcified 34 (26%), radiopaque 31 (24%), less radiopaque 46 (35%), and undetectable 19 (15%). The radiopacity of CBD stones differed significantly according to stone type ($P < 0.001$). From the receiver operating characteristic curve, stone size was useful for the determination of CBD stone by MDCT (area under curve 0.779, $P < 0.001$) and appropriate cut-off stone size on MDCT was 5 mm. The factors related to detectability of CBD stones on MDCT were age, stone type, and stone size on multivariate analysis ($P < 0.05$).

CONCLUSION: The radiopacity of CBD stones on MDCT differed according to stone type. Stone type and stone size were related to the detectability by MDCT, and appropriate cut-off stone size was 5 mm.

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Key words: Common bile duct gallstones; Gallstones; Multidetector computed tomography; Endoscopic retrograde cholangiography

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INTRODUCTION

Traditionally, endoscopic retrograde cholangiopancreatography (ERCP) was the gold standard for investigation of bile duct diseases, but its role has been limited to therapeutic use due to the invasiveness for the procedure^[1-4]. Therefore, multiple imaging tests have been used to diagnose stones in the common bile duct (CBD) instead of

ERCP. Commonly performed imaging tests are abdominal sonography, computed tomography (CT), magnetic retrograde cholangiopancreatography (MRCP), and endoscopic ultrasonography (EUS). Abdominal sonography is easy to perform, but it cannot evaluate the overall CBD. MRCP and EUS have been reported to be accurate for the diagnosis of choledocholithiasis^[5-8]. However, they both have some limitations that prevent active use on patients; metal in the body, claustrophobia, and lack of rapidity for MRCP; operator dependency for EUS; high cost and the differences of the facilities among different centers for MRCP and EUS.

CT scans are being used more frequently and performed as the initial imaging technique in patients with abnormal liver function test results or possible symptoms related to the biliary tract because it can be performed rapidly, is equipped in most centers, has relatively low cost, and gives extensive information on pancreaticobiliary structures including CBD stones. However, CT has been reported to have lower accuracy for the diagnosis of CBD stones than MRCP or EUS^[9-11], which does not make CT the imaging technique of choice for patients with clinical suspicion of choledocholithiasis. The development of multi-detector computed tomography (MDCT) has shown promise in increasing the accuracy of CT in the diagnosis of pancreaticobiliary diseases. With an almost universal use of MDCT, acquisition of images with high spatial resolution is now routine and the advent of more recent MDCT technology may further advance the use of CT for these diagnoses given its ability to acquire an isotropic data set with minimal motion artifacts^[12]. A recent study showed that MDCT was comparable with MRCP or EUS for the detection of CBD stone with 87% sensitivity, 85% specificity, and 86% accuracy^[13].

Gallstones are classified into cholesterol stones (pure cholesterol, combination, or mixed) and pigment stones (black or brown) according to the National Institutes of Health (NIH)-International Workshop on Pigment Gallstone Disease^[14] and the Gallstone Research Committee from the Japanese Society of Gastroenterology^[15]. CT can reveal the heterogeneous nature of these biliary stones in attenuation patterns ranging from being heavily calcified and radiopaque, to being slightly less radiopaque than bile due to cholesterol, to having gas attenuation due to locules of nitrogen gas^[16]. The association between the attenuation patterns of CBD stones on MDCT and the gallstone type has not been clarified. It is also not fully elucidated how the types and size of stones affect the detectability of MDCT. Therefore, we investigated the attenuation patterns and the detectability of CBD stones by MDCT according to stone type and stone size, and intended to find the factors related to the detectability of CBD stones by MDCT.

MATERIALS AND METHODS

Patients

We consecutively enrolled patients with suspicion of

choledocholithiasis undergoing both MDCT and ERCP during the period from March 2010 and February 2012 at a single institution. Hematologic and biochemical tests were performed at the time of admission. Suspected choledocholithiasis was defined as follows: recent abdominal pain, leukocytosis, and/or abnormal blood chemistry findings including total bilirubin, alanine aminotransaminase, alkaline phosphatase, or γ -glutamyl transferase levels. Exclusion criteria were as follows: CT from other institution, more than one month duration between MDCT and ERCP, failure in removing CBD stones by ERCP, and patients suspected of cystic duct stones. After MDCT, MRCP or EUS was performed for further determination of CBD stones if possible. ERCP was performed when the imaging study showed CBD stones or CBD stones were highly suspected despite negative imaging studies. Patient anonymity was preserved and the Institutional Review Board of our hospital approved this study (HC12RISI0038). This study protocol was in complete compliance with the Declaration of Helsinki, as revised in Seoul in 2008.

Multidetector computed tomography

CT studies were performed either a 64-slice MDCT scanner (Somatom Sensation 64; Siemens, Erlangen, Germany) with a detector collimation of 24 mm \times 1.2 mm, a table feed of 28.8 mm per rotation, a rotation time of 1 s, a tube current of 200 effective mAs, and a tube voltage of 120 kV or a 16-slice MDCT scanner (Somatom Sensation 16; Siemens, Erlangen, Germany) with a detector collimation of 16 mm \times 1.5 mm, a table feed of 24 mm per rotation, a rotation time of 1 s, a tube current of 200 effective mAs, and a tube voltage of 120 kV. Precontrast scan ranged from the diaphragm to the iliac crest and postcontrast scan ranged from the diaphragm to the symphysis pubis. Contrast material (Ultravist 300, Bayer, Berlin, Germany) with a volume of 120 mL and an injection rate of 2 mL/s was injected into the antecubital vein. Image acquisition was initiated after 90 s of contrast injection. Pre and post contrast axial images were reconstructed by 5 mm without overlap and postcontrast coronal images by 3 mm without overlap.

Endoscopic retrograde cholangiopancreatography

ERCP was performed with a duodenoscope (JF 240; Olympus Optical Co., Ltd., Tokyo, Japan). Two experienced gastroenterologists who had performed > 1000 ERCPs conducted the procedures. Conscious sedation was achieved with midazolam and pethidine hydrochloride. Contrast media (iopromide; 1:1 dilution with saline) was injected to obtain a cholangiogram after cannulation of the common bile duct without papillotomy. If the cholangiogram was interpreted as positive for CBD stones, endoscopic sphincterotomy was performed and the bile duct was swept with a Dormia basket and a retrieval balloon catheter to remove the calculi. Following this, the calculi were taken out for the evaluation of stone

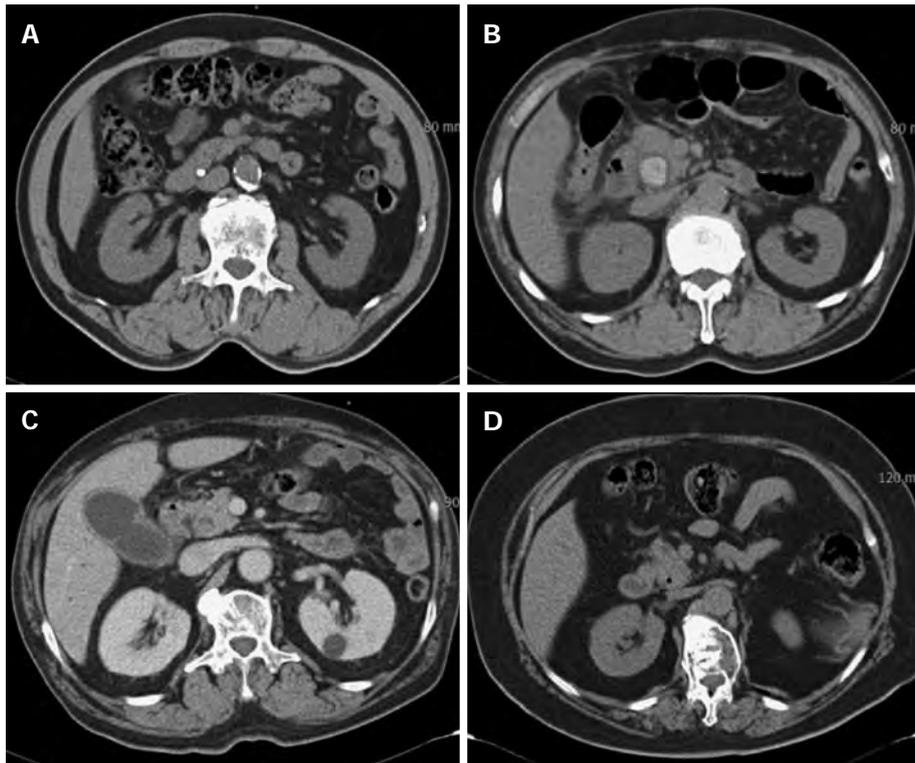


Figure 1 Attenuation patterns of common bile duct stones on multidetector computed tomography. A: Heavily calcified; B: Radiopaque; C: Less radiopaque; D: Gas attenuation in the stone.

type. If the cholangiogram was considered as negative for CBD stones, the absence of stones were confirmed again by a basket and a retrieval balloon catheter.

Analysis of images on multidetector computed tomography

CT scan data sets were transferred to picture archiving and communication system (PACS) workstations for analysis, and CT images were interpreted by one radiologist who had more than ten years of experience interpreting gastroenterological images. The radiologist received no clinical information or results of ERCP and used pre-contrast and portal venous phase images. Pre-contrast images were used for the initial detection of CBD stones. If the stones could not be discriminated in pre-contrast images, portal venous phase images served as references. The radiologist was free to use the window settings he preferred, which included narrow settings if a common duct stone was not initially identified on soft tissue window settings. The mean Hounsfield units of the stones were measured on PACS workstations. The attenuation patterns of CBD stones were classified as follows (Figure 1): (1) heavily calcified as having very high attenuation (mean Hounsfield unit > 150); (2) radiopaque as having distinctly higher attenuation than surrounding structures ($150 \geq \text{mean Hounsfield unit} > 80$); (3) less radiopaque as having slightly higher attenuation than surroundings (mean Hounsfield unit ≤ 80); and (4) gas attenuation as having a gas in or around the

stones^[16]. Common bile duct caliber was measured using the axial image of CT.

Type and size of common bile duct stones

Types of stones were classified by morphology as black pigment, brown pigment, and cholesterol stones according to the NIH-International Workshop^[14]. The color and shape on the external appearance and cross sectional shape on the internal structure were used as indexes. Cholesterol stones were subclassified into pure cholesterol stones, mixed stones, and combination stones according to the classification of the Japanese Society of Gastroenterology^[15]. To reduce the confusion in discrimination between mixed and combination stones, they both were classified as mixed stone. For measuring the stone size and number, MDCT (axial and coronal), MRCP, EUS, and/or ERCP were utilized together. The largest diameter of the stone, as the representative of size, was measured using electronic calipers on the workstation. If there were more than two stones, the largest stone was selected for the evaluation of stone type and stone size.

Statistical analysis

A Pearson's χ^2 test or Fisher's exact test was used to compare categorical data and Student's *t*-test or Mann-Whitney *U*-test was used for comparisons of continuous data to analyze the attenuation patterns and the detectability of CBD stones. A linear by linear association was used to analyze the trend between the attenuation patterns and the

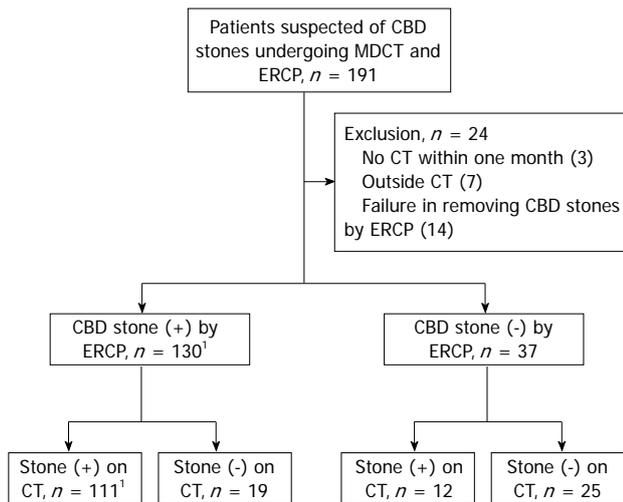


Figure 2 Flow chart of patients with suspicion of common bile duct stones undergoing multidetector computed tomography and endoscopic retrograde cholangiopancreatography. The sensitivity of multidetector computed tomography (CT) for common bile duct stones was 85.4%. ¹Two patients had recurrent common bile duct stones during the study period. CBD: Common bile duct; ERCP: Endoscopic retrograde cholangiopancreatography.

stone type. Receiver operating characteristic curve for CT detectability of CBD stone according to stone size was plotted. The area under the curve and the optimal cut-off value of stone size for CT detectability were calculated. Multivariate analysis for the related factors to the detectability of CBD stones by MDCT was performed with the significant factors identified from univariate analysis using binary logistic regression analysis (enter method). Statistical analyses were performed with SPSS, version 14 (SPSS, Inc., Chicago, IL, United States). *P*-values < 0.05 were considered significant.

RESULTS

Patients

One hundred ninety-one patients with suspicion of CBD stones undergoing MDCT and ERCP were consecutively enrolled from March 2010 and February 2012 (Figure 2). Twenty-four patients were excluded because they did not undergo CT within 1 mo (3), received CT from other institution (7), and failed to remove CBD stones by ERCP (14). This resulted in a study population of 167 patients consisting of 86 males and 81 females with a mean age of 65.7 years (SD, ± 15.9 years). The mean time between MDCT and ERCP was 2.4 d (SD, ± 4.5 d). MDCT was performed with 64-channel or 16-channel in 76% and 23%, respectively. CBD stones were demonstrated by ERCP in 130 patients. Of them, 54 (42%) patients had accompanying gallbladder stones and 31 (24%) previously received cholecystectomy. Thirteen patients (10%) underwent endoscopic sphincterotomy previously. The mean number of CBD stones was 2.0 (SD, ± 1.6). The types of stones revealed by ERCP was as follows: 23

Table 1 Correlation between the attenuation patterns of common bile duct stones on multidetector computed tomography and stone type (*n* = 111)

| Attenuation patterns | Stone type, <i>n</i> (size, mean ± SD, mm) | | | | Overall |
|------------------------------|--|-------------------|---------------|------------------|--------------|
| | Black pigment | Mixed cholesterol | Brown pigment | Pure cholesterol | |
| Heavily calcified | 15 | 14 | 5 | 0 | 34 |
| Radiopaque | (6.1 ± 3.4) | (7.0 ± 3.2) | (9.2 ± 4.1) | | (6.9 ± 3.5) |
| Less radiopaque ¹ | 3 | 12 | 16 | 0 | 31 |
| Overall | (7.0 ± 1.0) | (10.2 ± 4.3) | (13.6 ± 4.8) | | (11.6 ± 4.8) |
| Less radiopaque ¹ | 3 | 10 | 33 | 0 | 46 |
| Overall | (5.7 ± 4.0) | (7.6 ± 3.5) | (9.9 ± 5.1) | | (9.1 ± 4.8) |
| Overall | 21 | 36 | 54 | 0 | |
| | (6.2 ± 3.1) | (8.2 ± 3.6) | (10.9 ± 5.1) | | |

¹Including six less radiopaque stones with gas attenuation. *P* < 0.001.

(18%) black pigment stones, 38 (29%) mixed cholesterol stones, 60 (46%) brown pigment stones, and 9 (7%) pure cholesterol stones. The mean size of stones were 10.1 mm (SD, ± 5.4 mm), 8.0 mm (SD, ± 3.9 mm), 5.8 mm (SD, ± 3.2 mm), and 5.7 mm (SD, ± 3.8 mm) in brown pigment, mixed cholesterol, black pigment, and pure cholesterol stones, respectively. The brown pigment stones were significantly larger than other types of stones (*P* < 0.05).

Attenuation patterns of common bile duct stones

The attenuation patterns of CBD stones consisted of heavily calcified 34 (31%), radiopaque 31 (28%), and less radiopaque (41%, Table 1). The mean Hounsfield units were 421 (range, 156-1552), 104 (range, 81-141), and 59 (range, 11-80) in heavily calcified, radiopaque, and less radiopaque patterns, respectively. The radiopacity of stones differed significantly according to stone type (*P* < 0.001). An increasing trend in the radiopacity was observed among brown pigment, mixed cholesterol, and black pigment stones by analysis with a linear-by-linear association (*P* < 0.001). The mean size of heavily calcified stones, radiopaque stones, and less radiopaque stones differed significantly (*P* < 0.05). The order of mean stone size according to the radiopacity was radiopaque stones, less radiopaque stones, and heavily calcified stones. Heavily calcified and radiopaque stones were well discriminated on the pre-contrast images of MDCT. For less radiopaque stones, the images of portal venous phase were helpful in discriminating CBD stones because bile duct was well shown in that phase. The coronal reconstructed CT scan was useful in one patient. One mixed cholesterol stone with less radiopacity was ambiguous on portal venous-phase images, but the coronal reconstructed CT scan showed less radiopaque stone near the major ampulla (Figure 3). Of 46 less radiopaque stones, six stones had a pattern of gas attenuation in or around the stones which were less radiopaque or not well discriminated. All patients with gas attenuated stones had previously undergone endoscopic sphincterotomy.

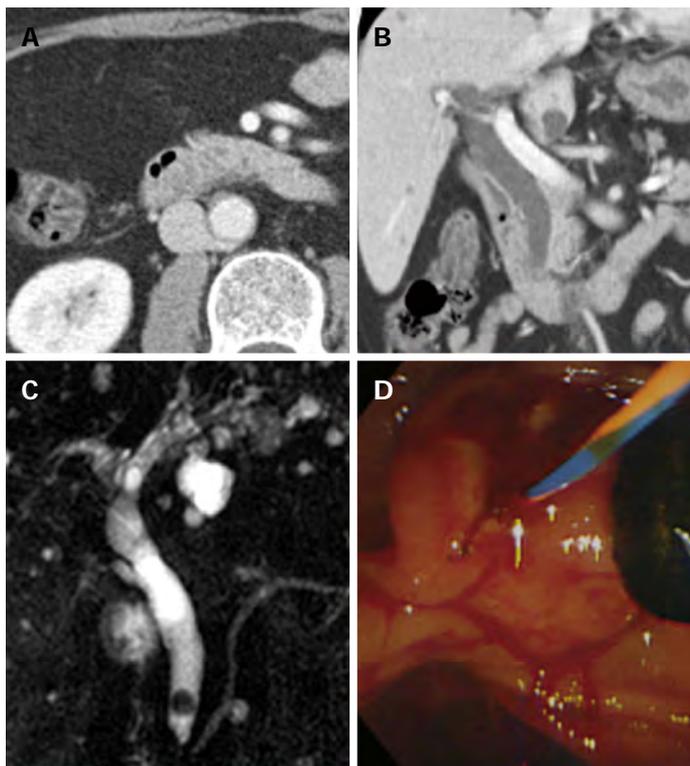


Figure 3 A case of difficult discrimination of common bile duct stone on the axial image of multidetector computed tomography. The coronal reconstructed image was helpful. A: The stone was ambiguous on the portal venous-phase axial computed tomography (CT) scan; B: The coronal reconstructed CT scan showed a less radiopaque stone near the major ampulla; C, D: Magnetic retrograde cholangiopancreatography and endoscopic retrograde cholangiopancreatography showed a six mm, mixed common bile duct stone.

Table 2 Detectability of common bile duct stones according to stone size and stone type *n* (%)

| | <i>n</i> | Detectable (<i>n</i> = 111) | Undetectable (<i>n</i> = 19) | <i>P</i> value |
|---------------------------|----------|---------------------------------|----------------------------------|----------------|
| Size (mm) | | | | |
| < 3 | 9 | 4 (44) | 5 (56) | < 0.001 |
| 3-5 | 40 | 29 (74) | 11 (26) | |
| 6-10 | 45 | 43 (96) | 2 (4) | |
| 11-15 | 26 | 25 (96) | 1 (4) | |
| > 15 | 10 | 10 (100) | 0 (0) | |
| ≤ 5 | 49 | 33 (67) | 16 (33) | < 0.001 |
| > 5 | 81 | 78 (96) | 3 (4) | |
| Type | | | | |
| Black pigment | 23 | 21 (91) | 2 (9) | < 0.001 |
| Mixed cholesterol | 38 | 36 (95) | 2 (5) | |
| Brown pigment | 60 | 54 (90) | 6 (10) | |
| Pure cholesterol | 9 | 0 (0) | 9 (100) | |
| Black or mixed | 61 | 57 (93) | 4 (7) | 0.023 |
| Brown or pure cholesterol | 69 | 54 (78) | 15 (22) | |

Detectability of common bile duct stones

The detectability of CBD stones by MDCT increased according to stone size (Table 2). 96% of stones larger than 5 mm were detectable by MDCT, but 67% of stones smaller than 5 mm were detectable (*P* < 0.001). Three stones were not detected by MDCT despite having a size of more than 5 mm and they all were determined to be pure cholesterol stones. The detection rate of CBD stones less than 3 mm was 44% (4/9). Although this rate was less than the detection rate of CBD stones between 3-5 mm (75%, 29/40), it was not statistically significant. From the receiver operating characteristic curve, stone size was useful for the determination of

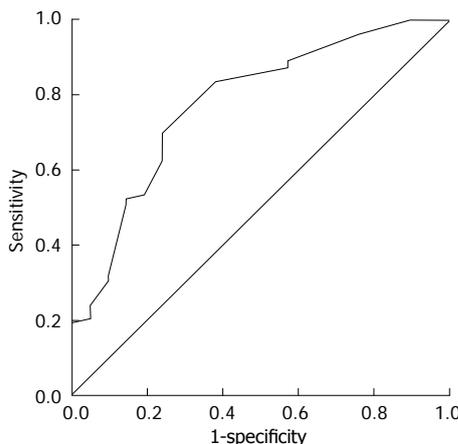


Figure 4 Receiver operating characteristic curve of the detectability of multidetector computed tomography for common bile duct stones according to stone size. The area under the curve was 0.779 and the optimal cut-off value of stone size was 5 mm.

CBD stone by MDCT (area under curve 0.779, *P* < 0.001, Figure 4). Appropriate cut-off stone size considering sensitivity and specificity was 5 mm. The detection rate of black pigment or mixed cholesterol stones (93%) was higher than that of brown pigment or pure cholesterol stones (78%, *P* = 0.023). However, the detection rate did not differ among black pigment, mixed cholesterol, and brown pigment stones. Nineteen undetected CBD stones by MDCT consisted of nine pure cholesterol stones, six brown pigment stones, two black pigment stones, and two mixed cholesterol stones. Of them, the sizes of all stones except for the pure cholesterol stones were less

Table 3 Univariate analysis of the factors related to the detectability of common bile duct stones by multidetector computed tomography *n* (%)

| Factors | Detectable (<i>n</i> = 111) | Undetectable (<i>n</i> = 19) | <i>P</i> value |
|----------------------------------|---------------------------------|----------------------------------|----------------|
| Age (yr) | 68.3 ± 14.2 | 58.0 ± 16.2 | 0.009 |
| Male sex | 58 (52) | 9 (47) | 0.966 |
| Accompanying GB stones | 43 (39) | 11 (58) | 0.117 |
| Previous cholecystectomy | 28 (25) | 3 (16) | 0.561 |
| Previous sphincterotomy | 13 (12) | 0 (0) | 0.213 |
| Black or mixed stone | 57 (51) | 4 (21) | 0.009 |
| Stone size (mm) | 9.1 ± 4.8 | 4.3 ± 2.9 | < 0.001 |
| CBD diameter (mm) | 12.4 ± 4.6 | 9.2 ± 3.5 | 0.005 |
| WBC (× 10 ⁹ /L) | 10.9 ± 6.01 | 9.86 ± 3.07 | 0.251 |
| Alanine aminotransaminase (IU/L) | 210 ± 250 | 250 ± 208 | 0.513 |
| Total bilirubin (mg/dL) | 3.8 ± 3.9 | 3.3 ± 2.8 | 0.565 |
| Alkaline phosphatase (IU/L) | 215 ± 153 | 209 ± 114 | 0.862 |
| γ-glutamyl transferase (IU/L) | 412 ± 342 | 500 ± 465 | 0.327 |

CBD: Common bile duct; WBC: White blood cell.

than 5 mm. The size of two black pigment stones which were undetected by MDCT were as small as 1 and 3 mm.

The related factors to the detectability of CBD stones were age, stone type, stone size, and CBD diameter in univariate analysis ($P < 0.01$, Table 3). In multivariate analysis, age, stone size, and stone type were significant independent factors for CBD stone detectability by MDCT ($P < 0.05$, Table 4). Of them, stone size and stone type had large odds ratios of 8.851 (95%CI: 2.249–34.84) and 1.437 (95%CI: 1.153–1.791), respectively.

DISCUSSION

Abdominal CT has been a very popular procedure in routine clinical practice. Rapidity, relatively low cost, extensive information about the abdomen, and wide availability facilitate the use of CT. Especially in the emergency department, the utilization of CT has increased for the rapid detection of pancreato-biliary diseases. CT has been developed from conventional to helical CT. Recently, multi-detector CT was introduced and has been used in many institutions. Helical CT using MDCT technology can use thin slice images in a single breath-hold and reconstruct those slices retrospectively using a variable overlap. It reduces much of the image degradation previously experienced from motion artifacts and volume averaging^[13]. As CT technology continues to improve with the increasing use of 16-, 32-, 64-, and 256-MDCT, it is likely that the accuracy for the detection of choledocholithiasis in patients undergoing routine abdominal CT will improve^[12]. MDCT showed better results than previous studies that showed various sensitivities for the detection of choledocholithiasis by CT range

Table 4 Multivariate analysis of the factors related to the detectability of common bile duct stones by multidetector computed tomography

| Factors | <i>P</i> value | Odds ratio (95%CI) |
|---|----------------|---------------------|
| Age | 0.032 | 1.049 (1.004-1.095) |
| Stone type (black or mixed <i>vs</i> brown or pure cholesterol) | 0.002 | 8.851 (2.249-34.84) |
| Stone size | 0.001 | 1.437 (1.153-1.791) |
| CBD diameter | 0.538 | |

CBD: Common bile duct.

from 71% to 93%^[17-21], with a mean sensitivity of approximately 80%. In the present study, MDCT displayed a sensitivity of 85.4% for the detection of CBD stones. Although MDCT has a better resolution and can detect greater number of small stones, there is a theoretic limitation for the detectability of choledocholithiasis by CT due to the iso or slightly hypoattenuating nature of pure cholesterol stones relative to bile, making them difficult to detect^[13,22-24].

The detectability of CBD stones by MDCT depends on several factors. The present study showed that the factors related to the detectability of CBD stone were age, stone size, and stone type. Although the optimal size of CBD stones for MDCT detection was determined as 5 mm, 67% of CBD stones smaller than 5 mm were detectable by MDCT. This showed that MDCT had some role in the initial screening even for small CBD stones less than 5 mm. The radiopacity of stones differed significantly according to stone type. Black pigment and mixed cholesterol stones were more detectable than brown and pure cholesterol stones. However, attenuation patterns could not discriminate between specific stone types because of the overlap in CT attenuation. A previous study showed that the mean CT attenuation of cholesterol stones was lower than that of pigment gallstones, and CT attenuation measurement was not useful for the determination of gallstone composition due to the overlap of CT attenuation values^[25]. Besides the type and size of stones, the position of stones can influence the detectability. It is recognized that small stones impacted at the ampulla are difficult to identify, particularly in non-dilated biliary ducts. One patient in the present study has a CBD stone near the ampulla, and it was detected by a coronal image rather than an axial image. Unfortunately, a previous study revealed that CT coronal images did not show significant improvement of diagnosis for CBD stones^[11]. We consider that coronal images do not increase the general detectability of CBD stones, but it may become helpful in special situations such as stones near the ampulla. The phase of CT image is another factor concerning the detectability of CBD stones. It was reported that portal venous phase CT images are specific and sensitive for the detection of biliary duct narrowing and choledocholithiasis^[26]. Radiopaque stones are well demarcated on a precontrast image, but

less radiopaque stones are not easily discriminated on a precontrast image. Portal venous phase images show common bile duct clearly, so it helps discriminate CBD stones especially for less radiopaque stones. Peak voltage setting also affects the detection of gallstones. In a study to evaluate the effect of four peak voltage settings (80, 100, 120, and 140 kV) on the *in vitro* conspicuity of gallstones in an anthropomorphic phantom by CT, the sensitivity for gallstone detection was significantly higher at 140 kV^[25]. We used 120 kV for the peak voltage setting on MDCT. 140 kV would increase the sensitivity of MDCT according to a previous study. However, the danger of increasing radiation hazard cannot be neglected. In the present study, the diameter of the bile duct affected the CT detectability of CBD stones in univariate analysis, but not in multivariate analysis. This was probably because the diameter of the bile duct is related to stone size. Although age was a significant factor related to the detectability, the odds ratio of age was as small as 1.049 and the lower limit of 95% confidence interval was close to 1 (1.004). Therefore, it can be stated that age showed little clinical significance. With regard to age, it could be considered that younger patients had a tendency of small stones and cholesterol stones. However, this needs the further investigation.

Formation of pigment stones in the bile duct is a late complication of endoscopic sphincterotomy^[27]. Sphincterotomy permits chronic bacterial colonization of the bile duct that results in deconjugation of bilirubin and precipitation of pigment stones. In the present study, all six patients with gas attenuated stones had previously undergone endoscopic sphincterotomy. According to our results, if CBD stones were indistinct by CT and air attenuations were found in the CBD in patients previously undergoing endoscopic sphincterotomy, CBD stones would be strongly suspected and further studies should be performed for the confirmation of CBD stones.

Gallstones are extremely common in Western countries, where the prevalence of bile-duct stones is relatively low. In contrast, primary choledocholithiasis and hepatolithiasis appear to be more frequent in East Asian countries than in Western societies^[28,29]. Primary bile duct stones are predominantly composed of calcium bilirubinate, namely brown pigment stones. The pathogenesis of primary bile duct stones is based on bile stasis and infection, which are associated with bile duct strictures, extrahepatic anomalies, and biliary parasites. In contrast, secondary stones are considered to originate from gallbladder stones, and are commonly composed of cholesterol. In the present study, the proportion of cholesterol stones, black pigment stones, and brown pigment stones were 36%, 18%, and 46%, respectively. Tazuma^[28] reported that the proportion of cholesterol stones, black pigment stones, and brown pigment stones in the CBD were 31%, 12%, and 54%, respectively in

Japan. This was similar to our study. In a Korean population study of bile duct stones 14 years ago, the majority were brown pigment stones (76%), and the remaining were cholesterol stones (18%) and black pigment stones (4%)^[30]. The discrimination of stone type is sometimes difficult. Atypical stones with a black or brown colored surface, but with a radial fashioned surface and a high cholesterol content are not rare^[15,31,32]. These stones are frequently confused by their external appearance with black pigment stones originating from the gallbladder. We used color and shape on the external appearance and cross sectional shape on the internal structure as the indexes to reduce such confusion. Mixed and combination stones were classified as mixed stones to make a clear classification. Some investigators classify mixed or combination stone as stones of the intermediate group which are difficult to classify into either cholesterol stones or pigment stones^[15].

There were some limitations in the present study. First, this was a retrospective study. Some disparity in the interpretation of CBD stones between the original time of the examination and the time of the study can exist. The interpreter in the present study who was primed for the detection of choledocholithiasis likely led to a little improved sensitivity for detection for bile duct stones compared with real-time interpretations at the time of the examination. Second, stone composition analysis was not performed. Analysis using spectroscopy would help determine stone type more precisely. However, we exerted the greatest effort to determine stone type by using the color and shape on the external appearance and cross sectional shape on the internal structure. Third, reformation of MDCT images was not performed. Reformation process such as multiplanar reformation or minimum intensity reformation helps with the detection of gallstones^[33,34]. However, reformation needs an additional process which requires much time and effort. Most hospitals do not perform reformation as a routine procedure due to this restraint.

In conclusion, MDCT showed a moderately high sensitivity for the detection of CBD stones, and the radiopacity of CBD stones by MDCT differed according to stone type. Type and size of stones were significant factors related to the detectability of CBD stones. We expect further prospective studies with a larger cohort of patients to demonstrate the characteristics of CBD stones by MDCT.

COMMENTS

Background

Multidetector computed tomography (MDCT) can reveal the heterogeneous nature of the biliary stones in attenuation patterns ranging from being heavily calcified and radiopaque, to being slightly less radiopaque than bile due to cholesterol, to having gas attenuation due to locules of nitrogen gas. The association between the attenuation patterns of common bile duct (CBD) stones on MDCT and the gallstone type has not been clarified. It is also not fully elucidated how the types and size of stones affect the detectability of MDCT.

Research frontiers

This study demonstrated that the radiopacity of CBD stones differed significantly according to stone type. Stone size was important for the determination of CBD stone by MDCT, and appropriate cut-off stone size was 5 mm. The factors related to detectability of CBD stones on MDCT were age, stone type, and stone size.

Innovations and breakthroughs

This is the first report the radiopacity of CBD stones according to stone type. MDCT showed moderately high sensitivity for the detection of CBD stones. Although magnetic retrograde cholangiopancreatography or endoscopic ultrasonography is considered more accurate method for the detection of CBD stones, MDCT also had good sensitivity and can give information about stone type.

Applications

MDCT is useful method for detection of CBD stones, especially when CBD stones are more than 5 mm. Stone type can be estimated by the radiopacity of CBD stones on MDCT

Peer review

The author evaluated the role of MDCT in detection of CBD stones, and found a moderately high sensitivity for the detection of CBD stones, and the radiopacity of CBD stones by MDCT differed according to stone type. The article is of great significance in clinical evaluation of CBD stones by this approach.

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Routine defunctioning stoma after chemoradiation and total mesorectal excision: A single-surgeon experience

Shao-Chieh Lin, Po-Chuan Chen, Chung-Ta Lee, Hong-Ming Tsai, Peng-Chan Lin, Helen HW Chen, Yuan-Hwa Wu, Bo-Wen Lin, Wen-Pin Su, Jenq-Chang Lee

Shao-Chieh Lin, Po-Chuan Chen, Bo-Wen Lin, Jenq-Chang Lee, Division of Colorectal Surgery, Department of Surgery, National Cheng Kung University Hospital, College of Medicine, National Cheng Kung University, Tainan 704, Taiwan

Chung-Ta Lee, Department of Pathology, National Cheng Kung University Hospital, College of Medicine, National Cheng Kung University, Tainan 704, Taiwan

Hong-Ming Tsai, Department of Radiology, National Cheng Kung University Hospital, College of Medicine, National Cheng Kung University, Tainan 704, Taiwan

Peng-Chan Lin, Wen-Pin Su, Department of Oncology, National Cheng Kung University Hospital, College of Medicine, National Cheng Kung University, Tainan 704, Taiwan

Helen HW Chen, Yuan-Hwa Wu, Department of Radiation Oncology, National Cheng Kung University Hospital, College of Medicine, National Cheng Kung University, Tainan 704, Taiwan

Author contributions: Lin SC and Chen PC made equal contributions to the writing of this manuscript; Lee CT, Tsai HM, Lin PC, Chen HHW, Wu YH, Lin BW, Su WP made substantial contributions to study design, data analysis and interpretation; Lee JC originated study conception and made final approval of the version to be published.

Correspondence to: Jenq-Chang Lee, MD, Division of Colorectal Surgery, Department of Surgery, National Cheng Kung University Hospital, College of Medicine, National Cheng Kung University, No. 138, Sheng Li Road, East District, Tainan 704, Taiwan. leejc@mail.ncku.edu.tw

Telephone: +886-6-2353535 Fax: +886-6-2250586

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Abstract

AIM: To investigate the 10-year results of treating low rectal cancer by a single surgeon in one institution.

METHODS: From Oct 1998 to Feb 2009, we prospectively followed a total of 62 patients with cT2-4 low rectal cancer with lower tumor margins measuring at 3 to 6 cm above the anal verge. All patients received neo-

adjuvant chemoradiation (CRT) for 6 wk. Among them, 85% of the patients received 225 mg/m²/d 5-fluorouracil using a portable infusion pump. The whole pelvis received a total dose of 45 Gy of irradiation in 25 fractions over 5 wk. The interval from CRT completion to surgical intervention was planned to be approximately 6-8 wk. Total mesorectal excision (TME) and routine defunctioning stoma construction were performed by one surgeon. The distal resection margin, circumferential resection margin, tumor regression grade (TRG) and other parameters were recorded. We used TRG to evaluate the tumor response after neoadjuvant CRT. We evaluated anal function outcomes using the Memorial Sloan-Kettering Cancer Center anal function scores after closure of the defunctioning stoma.

RESULTS: The median distance from the lower margin of rectal cancer to the anal verge was 5 cm: 6 cm in 9 patients, 5 cm in 32 patients, 4 cm in 10 patients, and 3 cm in 11 patients. Before receiving neoadjuvant CRT, 45 patients (72.6%) had a cT3-4 tumor, and 21 (33.9%) patients had a cN1-2 lymph node status. After CRT, 30 patients (48.4%) had a greater than 50% clinical reduction in tumor size. The final pathology reports revealed that 33 patients (53.2%) had a ypT3-4 tumor and 12 (19.4%) patients had ypN1-2 lymph node involvement. All patients completed the entire course of neoadjuvant CRT. Most patients developed only Grade 1-2 toxicities during CRT. Thirteen patients (21%) achieved a pathologic complete response. Few post-operative complications occurred. Nearly 90% of the defunctioning stomas were closed within 6 mo. The local recurrence rate was 3.2%. Pathologic lymph node involvement was the only prognostic factor predicting disease recurrence (36.5% vs 76.5%, $P = 0.006$). Nearly 90% of patients recovered sphincter function within 2 year after closure of the defunctioning stoma.

CONCLUSION: Neoadjuvant CRT followed by TME,

combined with routine defunctioning stoma construction and high-volume surgeon experience, can provide excellent surgical quality and good local disease control.

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Key words: Rectal cancer; Neoadjuvant chemoradiation; Total mesorectal excision; Pathologic complete response; Defunctioning stoma

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INTRODUCTION

Since total mesorectal excision (TME) was first described by Heald in 1982, low rectal cancer treatment has experienced a revolutionary advancement. The traditional abdominoperineal resection procedure has gradually been replaced by TME and coloanal anastomosis for resectable rectal cancers. Improved overall survival and decreased local recurrence rates have been achieved with the combined progression of knowledge about surgical anatomy and technique, new chemotherapy regimens and radiotherapy technology. For locally advanced rectal cancers, randomized controlled trials have shown that neoadjuvant chemoradiation therapy (CRT) leads to a decrease in tumor size and enhances the possibilities of tumor resectability and sphincter preservation^[1,2]. Furthermore, with new chemotherapy regimens developing at a rapid pace, every new clinical trial has attempted to incorporate these new drugs into their protocols to gain better oncologic results. However, the complications related to CRT overuse can be devastating, including treatment toxicity, anastomotic leakage, or any type of postoperative complications, such as persistent fistula^[3] and subsequent permanent colostomy. These complications can adversely affect patient quality of life and even make patients abandon the treatment course before the scheduled operation. In addition, many large series have reported that chemotherapy regimens can differ between series and surgeon experience can be variable between the institutions, making a uniform comparison difficult. To avoid these problems, our team has adopted a less toxic chemotherapy regimen and routine defunctioning stoma construction after TME surgery since the initiation of the treatment protocol 10 year ago. Because surgical resection remains the only curative treatment for locally advanced rectal cancer, we believe patients will have better chance of cure if they can benefit from neoadjuvant CRT without associated complications and

proceed to surgical operations in good physical condition. The aim of this report is to present our recent 10 years' experience of 62 consecutive low rectal cancer patients undergoing neoadjuvant CRT and TME surgery at National Cheng Kung University Hospital where all operations were performed in a standard fashion by a single surgeon.

MATERIALS AND METHODS

From Oct 1998 to Feb 2009, we prospectively followed patients with locally advanced low rectal cancer who underwent a radical operation by a single surgeon, Jenq-Chang Lee. Patients with clinical tumor category cT2-4 or clinical node category cN1-2 low rectal cancer and a lower tumor margin within 3-6 cm of the anal verge were offered the choice of receiving neoadjuvant CRT and were included in the study. The exclusion criteria included other synchronous malignancies, previous chemotherapy or radiotherapy to the pelvis, contraindications to CRT and unwillingness to receive neoadjuvant CRT. All patients had a confirmed pathological diagnosis before undergoing any treatment. Among a total of 302 rectal cancers operated by Dr. Lee during this time period, 63 consecutive patients were included according to the above criteria. All but one patient received neoadjuvant CRT and then underwent a radical operation. All operations achieved R0 resection. One patient was excluded because she refused the radical operation after CRT. In the end, a total of 62 patients were included for retrospective data analysis. All of them were followed for more than 3 year.

A clinical staging evaluation was performed before neoadjuvant CRT with a digital examination and computed tomography. Magnetic resonance imaging, endorectal ultrasonography and positron emission tomography were used selectively. All patients were well informed before treatment initiation. All patients received neoadjuvant CRT for 6 wk. Among them, 85% of the patients received 225 mg/m²/d 5-fluorouracil (5-FU) using a portable infusion pump. The whole pelvis received a total dose of 45 Gy of irradiation in 25 fractions over 5 wk. The duration from CRT completion to surgical intervention was planned to be approximately 6-8 wk. The clinical staging was re-evaluated just before the operation. Each rectal cancer was reviewed and staged according to the American Joint Committee on Cancer Staging Manual^[4]. The distal resection margin (DRM), circumferential resection margin (CRM), tumor regression grade (TRG) and other parameters were recorded. We used the TRG to evaluate the tumor response to neoadjuvant CRT^[5]. The follow-up protocol was based on the National Comprehensive Cancer Network (NCCN) guidelines^[6]. We evaluated anal function outcomes using the Memorial Sloan-Kettering Cancer Center anal function scores (MSK-AF)^[7] after closure of the defunctioning stoma.

Table 1 Clinical demographics (n = 62) n (%)

| Variable | |
|---|-------------------------|
| Age (yr) (mean ± SD) | 58.7 ± 12.8 |
| Gender (male/female) | 40/22 (64.5/35.5) |
| Distance of tumor from anal verge (cm) (median, range) | 5 (3-6) |
| Pre-treatment stage | |
| cT2/T3/T4 | 17/42/3 (27.4/67.7/4.8) |
| cN0/cN1/N2 | 41/16/5 (66.1/25.8/8.1) |
| Tumor response to CRT (tumor size reduction) ¹ | |
| 100% response | 8 (12.9) |
| 50%-99% | 22 (35.5) |
| < 50% | 32 (51.6) |
| Toxicity of neoadjuvant CRT | |
| Skin ² | 1 (1.6) |
| Nausea/vomiting ² | 7 (11.3) |
| Diarrhea ³ (Grade II/Grade III) | 20/1 (32.3/1.6) |
| Hematological toxicity | 0 |
| Adjuvant chemotherapy | 57 (91.9) |

¹Evaluated by digital examination and computed tomography; ²Grade I - II;

³Grade I - III. CRT: Chemoradiation therapy.

Surgical technique

Total mesorectal excision (TME) was performed in every patient. The methods of TME^[8,9] have previously been described in detail. Low anterior resection with the double-stapling technique and straight coloanal anastomosis was attempted in every patient if feasible. If double-stapling anastomosis could not be performed for low-lying rectal cancer, intersphincteric resection was performed for patients with adequate oncological margins. Abdominoperineal resection (APR) was chosen for patients with inadequate oncological margins or by patients themselves. All patients who underwent sphincter-saving procedures underwent the routine construction of a defunctioning loop colostomy or ileostomy. The defunctioning loop colostomy construction was performed by simply pulling the transverse colon out through the right upper quadrant abdominal wall incision and was matured immediately without placing a fixation suture between the colonic serosa and abdominal wall peritoneum or fascia layer.

Post-operative adjuvant chemotherapy

Postoperative adjuvant chemotherapy was proposed to every patient. A total of five patients (8.1%) refused adjuvant chemotherapy. Forty-one patients (71.9%) received a short-term infusion of 5-FU/leucovorin. Fourteen patients (24.6%) received either the FOLFOX or FOLFIRI regimen. Two patients (3.5%) received oral capecitabine. Two patients (3.5%) did not complete adjuvant chemotherapy due to personal reasons.

Statistical analysis

The survival rates were assessed by Kaplan-Meier analysis, and survival curves were compared by the log-rank test. The analysis was performed using Prism 4 software. A *P* value < 0.05 (two tailed) was regarded as statistically significant.

Table 2 Peri-operative and pathological characteristics (n = 62) n (%)

| Variable | |
|--|-------------------------|
| Interval between CRT and TME (d) (median, range) | 49 (28-101) |
| Types of surgical procedure | |
| TME + straight coloanal anastomosis with | |
| Double stapling | 44 (71.0) |
| Intersphincteric resection | 12 (19.4) |
| APR | 6 (9.7) |
| DRM (cm) (median, range) | 2.5 (2.0-4.5) |
| Positive CRM | 2 (3.2) |
| Retrieved lymph node number (median, range) | 8 (1-20) |
| Pathologic TNM stage | |
| ypCR | 13 (21.0) |
| ypT0/ypT1/ypT2/ypT3/ypT4 | 2/5/9/32/1 |
| | (3.2/8.1/14.5/51.6/1.6) |
| ypN0/ypN1/ypN2 | 37/8/4 |
| | (59.7/12.9/6.5) |
| TRG ¹ | |
| 1 | 13 (21.0) |
| 2-3 | 41 (66.1) |
| 4-5 | 8 (12.9) |

¹Tumor regression grade (TRG): Grade 1, no residual tumor; Grade 2, rare residual cancer; Grade 3, fibrosis outgrowing residual cancer; Grade 4, residual cancer outgrowing fibrosis; Grade 5, absence of regressive change. DRM: Distal resection margin; CRM: Circumferential resection margin; APR: Abdominoperineal resection; TME: Total mesorectal excision.

RESULTS

Patient demographics

The patient demographics are summarized in Table 1. The median distance from the lower margin of the rectal cancer to the anal verge was 5 cm: 6 cm in 9 patients, 5 cm in 32 patients, 4 cm in 10 patients, and 3 cm in 11 patients. Before neoadjuvant CRT, 45 patients (72.6%) had a cT3-4 tumor and 21 (33.9%) patients had a cN1-2 lymph node status. After CRT, 30 patients (48.4%) had a greater than 50% clinical reduction in tumor size. The final pathology reports revealed that 33 patients (53.2%) had a ypT3-4 tumor and 12 (19.4%) patients had ypN1-2 lymph node involvement. All patients completed the entire course of neoadjuvant CRT.

Toxicity associated with chemoradiation

The toxicity profiles are summarized in Table 1. Twenty-seven patients (43.5%) experienced Grade 1-2 toxicity. No Grade 4-5 toxicity was reported. Adverse events were graded according to the National Cancer Institute Common Terminology Criteria^[10].

Surgical and pathological characteristics

The peri-operative characteristics are summarized in Table 2. All patients who underwent sphincter-preserving surgery also underwent temporary defunctioning stoma construction. Among them, fifty-four patients (96.4%) underwent a loop transverse colostomy, and two patients underwent a loop ileostomy.

Table 3 Post-operative morbidity and mortality (*n* = 62) *n* (%)

| | Case number |
|--------------------------------|-------------|
| Mortality | 0 (0) |
| Early complication | |
| Anastomotic leakage | 1 (1.6) |
| Wound infection | 2 (3.2) |
| Delayed perineal wound healing | 1 (1.6) |
| Pelvic abscess | 0 (0) |
| Rebleeding | 0 (0) |
| Adhesion ileus | 1 (1.6) |
| Central venous port infection | 1 (1.6) |
| Pneumonia | 0 (0) |
| Deep vein thrombosis | 0 (0) |
| Urinary tract infection | 1 (1.6) |
| Late complication | |
| Anastomotic stenosis | 1 (1.6) |
| Fistula formation | 0 (0) |
| Incisional hernia | 1 (1.6) |
| Anal bleeding | 2 (3.2) |

Operative morbidity and mortality

The post-operative morbidity and mortality are summarized in Table 3. One patient had subclinical Grade A anastomotic leakage by the definition of the International Study Group of Rectal Cancer^[11]. No patient developed Grade B or C anastomotic leakage. One patient developed post-operative adhesion ileus and recovered after conservative treatment. Late post-operative complications occurred in three patients, including incisional hernia, anastomotic stenosis, and occasional anal bleeding related to irradiation proctitis, which were all managed uneventfully and without the necessity of creating a permanent defunctioning stoma.

Anal function outcome

Six patients received APR. Among the 56 patients who underwent a sphincter-saving procedure, 49 patients also underwent defunctioning stoma closure, and their post-operative anal functions are summarized in Table 4. We performed the defunctioning stoma closure 5-6 mo after the sphincter-saving procedure and the completion of adjuvant chemotherapy. Among the seven patients who did not undergo stoma closure, four patients had early disease recurrence and one patient had a major co-morbidity. The other two patients died from noncancerous causes. The MSK-AF scores revealed that 32 patients (65.3%) had fair or poor anal function one month immediately after stoma closure. However, the proportion of patients with fair or poor anal function decreased at 12 (24.5%) and 24 (12.2%) mo. At 12 and 24 mo after defunctioning stoma closure, the proportion of patients with excellent or good anal function improved from 75.5% to 87.8%, meaning that most patients had recovered anal function within 1 to 2 year after stoma closure.

Recurrence and survival

The median follow-up period of our patients was 58 mo. All patients were followed up for more than 3 year. One

Table 4 Post-operative anal function (*n* = 49) *n* (%)

| | 1 ² | 12 ² | 24 ² |
|--------------------------|----------------------|----------------------|----------------------|
| MSK-AF ¹ (mo) | | | |
| Excellent/good | 8/9 (16.3/18.4) | 23/14 (46.9/28.6) | 23/20 (46.9/40.8) |
| Fair/poor | 12/20 (24.5/40.8) | 10/2 (20.4/4.1) | 4/2 (8.2/4.1) |

¹Memorial Sloan-Kettering Cancer Center anal function score (MSK-AF): Excellent: 1-2 bowel movements/d and no soilage; Good: 3-4 bowel movement/d or mild soilage; Fair: More than 4 bowel movements/d or moderate soilage; Poor: More than 6 bowel movement/d or significant leakage or enema dependent; ²Periods after stoma closure.

patient (1.6%) had only local recurrence. One patient (1.6%) had both local recurrence and distant metastasis, and another thirteen patients (21%) had distant metastases. For patients with distant metastasis, the lungs were the most frequent site of metastasis (12/14, 85.7%). Seven patients with isolated lung metastasis underwent pulmonary metastasectomy, and four are still alive. For these four patients, lung metastasis occurred at 16, 17, 34 and 46 mo, respectively, after TME, and they have lived for an additional 21, 63, 41 and 20 mo, respectively, to date. Among the thirteen patients with a PCR, twelve patients (92.3%) remain disease free and one patient who refused adjuvant chemotherapy developed lung metastasis 18 mo after surgery.

Six patients (10%) died of disease recurrence. Four patients died from noncancerous causes. Nine patients (14.5%) had disease recurrence or distant metastasis but are still alive. The five-year overall survival and disease-free survival rates were 83.6% and 69.3%, respectively (Figure 1A). Our data show that, if patients achieved a pathologic complete response (PCR), a favorable disease-free survival trend could be observed in the TRG1 group, although no statistical significance was reached (Figure 1B, *P* = 0.07). Our data also demonstrate that persistent pathologic lymph node involvement after neoadjuvant CRT increases the risk of subsequent disease recurrence (Figure 1C, *P* = 0.006). However, neither TRG nor pathologic lymph node status affects overall survival.

DISCUSSION

This study investigated the treatment results of neoadjuvant CRT with protracted venous infusion of 5-FU followed by TME and routine defunctioning stoma construction in locally advanced low rectal cancer patients by a single surgeon at one institution. Beginning in 1998, we treated our locally advanced low rectal cancer patients with neoadjuvant CRT followed by TME surgery. In accordance with the NCCN guidelines, we have prescribed protracted venous infusion of 5-FU as a neoadjuvant radio-sensitizer for most patients with concurrent radiotherapy, which has resulted in less treatment toxicity and made all patients more likely to complete the entire

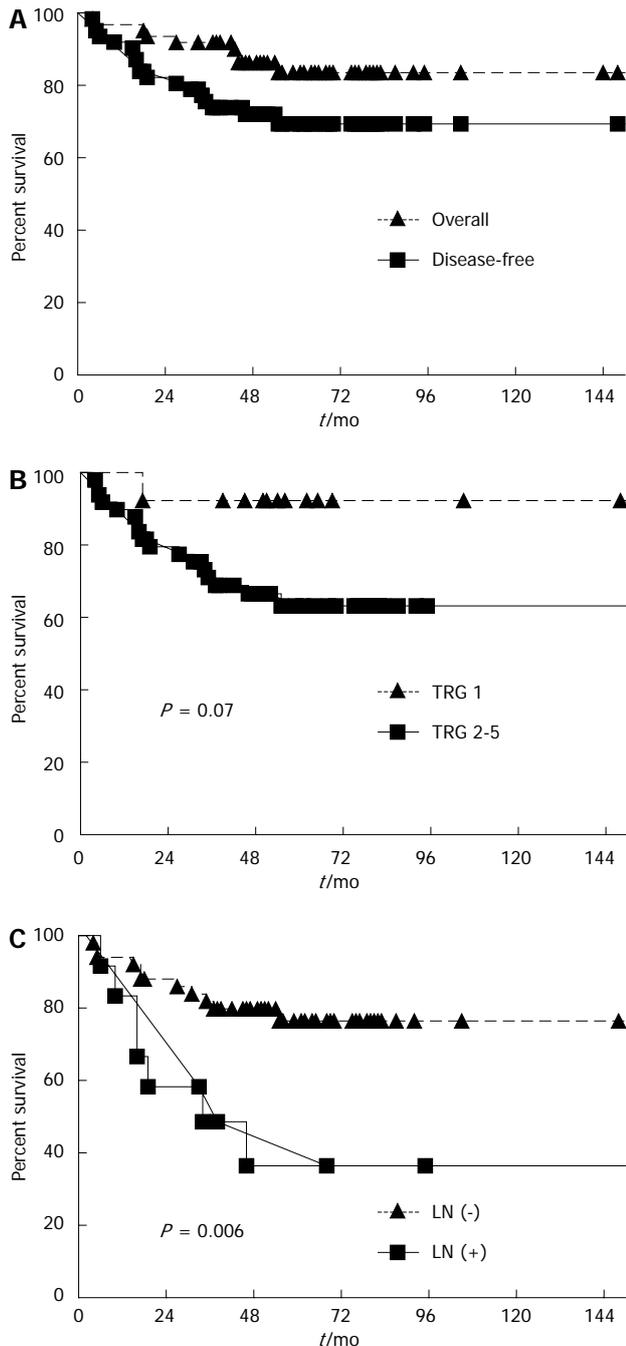


Figure 1 Five-year survival rate of the low rectal cancer patients treated with neoadjuvant chemoradiation and total mesorectal excision. A: The 5-year overall and disease-free Kaplan-Meier survival curves; B: The 5-year disease-free survival rates of patients with tumor regression grade (TRG) 1 and TRG 2-5 are 92.3% and 63.2%, respectively; C: The 5-year disease-free survival rates of patients with positive and negative lymph node (LN) involvement are 36.5% and 76.5%, respectively.

course of CRT and subsequent radical surgery after a 6-8 wk interval. Our protocol is identical to that of the Cleveland Clinic and has achieved very similar PCR and completion rates to theirs^[12]. With this neoadjuvant CRT protocol, we achieved a PCR rate of 21%, a nearly 50% decrease in preoperative tumor size, and only 1.6% associated Grade 3 toxicity, with a 100% CRT completion

rate. Long-term follow-up also demonstrated a five-year overall survival rate of 83.6%, a five-year disease-free survival rate of 69.3%, and a local recurrence rate of approximately 3%. These outcomes were comparable to other prospective trials using different neoadjuvant CRT regimens, including short-term 5-FU/leucovorin infusion and 5-FU plus oxaliplatin, cetuximab, or bevacizumab^[13]. Most of these trials reported PCR rates of 8%-20%. Very few reports using stronger regimens achieved more than 30% PCR rates^[14,15], but these also incurred more serious treatment toxicities, which could possibly make patients abandon the entire treatment protocol. Clearly, our protocol achieved lower treatment toxicity without compromising the desirable treatment effects of neoadjuvant CRT.

The DRM has been identified as one of the prognostic factors affecting local recurrence. In our series, the median DRM was 2.5 cm. All our patients achieved a DRM of more than 2 cm. However, in some studies, 1 cm was postulated to be a sufficient DRM in low rectal cancer patients receiving neoadjuvant CRT^[16]. The CRM is another important prognostic factor^[17]. Tumor involvement over the CRM has a negative impact on the five-year local recurrence, distant metastasis, and overall survival rates in patients with low rectal cancer^[18,19]. In our series, two patients (3.2%) acquired positive CRMs and developed local and distant metastases, corresponding to the negative impact on survival. In addition, recent studies have shown that a PCR is associated with favorable outcomes with regard to local control, disease-free survival and overall survival^[20-22]. In our series, we also achieved the same favorable trend.

Frequently, fewer than 12 lymph nodes can be harvested despite maintaining vigorous surgical standards in low rectal cancer patients if neoadjuvant CRT was performed^[23,24]. However, the persistence of lymph node metastasis after neoadjuvant radiotherapy is known to be associated with poorer prognosis and survival and may serve as a marker for more aggressive tumor behavior^[25]. In our series, the median number of retrieved lymph nodes was 8, which is the same as the median number of retrieved lymph nodes published in EORTC trial 22 921^[26]. Our result confirms that the persistence of lymph node metastasis after neoadjuvant CRT is a poor prognostic factor and a possible predictor of future recurrence.

Routine temporary defunctioning stoma construction after TME was, at one time, a controversial issue^[27]. However, current opinions have gradually shifted to support the routine construction of defunctioning stoma to decrease post-operative complications^[28-30]. A recent meta-analysis has also demonstrated that anastomotic leakage can have a negative impact on local recurrence and cancer-specific survival in colorectal cancers^[31]. In our series, a defunctioning loop stoma was created for every low rectal cancer patient treated with neoadjuvant CRT and a sphincter-saving procedure, achieving very

low rates of early and late surgical complications. Only one patient who underwent a sphincter-saving procedure suffered from sub-clinical anastomotic leakage. We believe that our good result was the consequence of stool diversion by the defunctioning stoma. In addition, we did not encounter major complications associated with the colostomy takedown procedure because we only trimmed the colocolic junction and closed the colonic wall directly, instead of routinely performing segmental resection. No sutures were used to attach the colon serosa to the abdominal wall fascia or peritoneum during the colostomy construction stage, and we therefore did not need to perform segmental resection of the colon at the colostomy reversal stage. Our method of colostomy construction and reversal was proven to be safe and feasible by other authors as well^[32]. Only patients dying shortly after the procedure due to early recurrence or noncancerous causes did not have their stomas closed. Based on our experience, we believe that routine defunctioning stoma construction prevents significant postoperative complications, including fistula formation due to anastomotic leakage and subsequent permanent colostomy, and guarantees a smooth and safe treatment course.

For patients undergoing TME and coloanal anastomosis, post-operative anal function recovery is crucial, and sphincter preservation without adequate function can be troublesome or even meaningless to patient quality of life. In addition, neoadjuvant radiotherapy has been reported to delay postoperative anorectal function recovery^[33]. In our series, one month after closure of the defunctioning stoma, only one third of the patients reported good to excellent sphincter function, but most recovered to good to excellent sphincter function within 2 year. Only 6 patients (12.2%) reported fair or poor anal sphincter function 2 year after closure of the defunctioning stoma. Our result was consistent with other reports noting that patients could achieve equal anal sphincter function two year after a straight coloanal anastomosis procedure to those who underwent J-Pouch coloanal anastomosis or T-coloplasty^[34].

TME is the integral part of low rectal cancer treatment in which surgeon experience plays the most critical role. However, current large series, both prospective and retrospective in nature, have to enroll a large numbers of patients and involve many surgeons with different training backgrounds and techniques. In this situation, surgeon experience is arbitrarily unified, and complications related to personal skill become difficult to evaluate. There is a paucity of evidence in the literature regarding the volume-outcome relationship in the field of rectal surgery. Two systemic reviews and a meta-analysis reveal that surgeons with more experience are associated with decreased mortality, decreased local recurrence, better overall survival, lower permanent stoma, and lower APR rates^[35,36]. The important surgical quality parameters of our team, including the DRM, CRM, retrieved lymph

node number, anastomotic leakage rate, and local recurrence rate, are in line with the current standards throughout the world. Our results can be largely attributed to the experience of the surgeon.

In conclusion, our experience demonstrates that neoadjuvant CRT with protracted venous infusion of 5-FU can minimize associated morbidities and achieve a comparable pathologic response for patients with locally advanced low rectal cancer. TME at 6-8 wk after CRT and routine defunctioning stoma construction can achieve excellent local control and better quality of life. The whole process can be safely executed in a standard fashion by experienced surgeons.

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COMMENTS

Background

Rectal cancer incidence has increased steadily year by year. Although surgical resection remains the only chance of cure, low rectal cancer surgery is still relatively difficult because the lesion is located in a confined pelvic space and is near the anal sphincters.

Research frontiers

The procedure of total mesorectal excision (TME) and pre-operative chemoradiation have gradually become a standard procedure for low rectal cancer. Clinical trials have successfully demonstrated that this procedure can decrease local recurrence rates and make tumor shrink before surgery, achieving better chance of anal sphincter preservation.

Innovations and breakthroughs

Although the procedure is popular in the western society, this is still not a common practice in the eastern society. The authors' ten-year single-surgeon experience demonstrates that this procedure can be safely performed with continuous infusion chemotherapy, routine defunctioning stoma construction and sufficient surgeon experience.

Terminology

TME is a surgical procedure involving resection of all perirectal soft tissue, which contains rectal lymphatic drainage and is the origin of local recurrence. Defunctioning stoma is a surgical procedure involving pulling out a part of bowel segment for stool diversion to protect the distal bowel anastomosis site.

Peer review

This is a paper addressing a single-surgeon's experiences regarding treatment for patients with mid-low rectal cancer. The authors emphasized the importance of routine defunctioning stoma after chemoradiation and TME. It is a reasonable paper although there are no novel points reported compared to pertinent literatures.

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Esophagogastroduodenoscopy with conscious sedation does not interfere with catheter-based 24-h pH monitoring

Yung-Kuan Tsou, Jau-Min Lien, Chin-Kuo Chen, Cheng-Hui Lin, Hsing-Yu Chen, Mu-Shien Lee

Yung-Kuan Tsou, Jau-Min Lien, Cheng-Hui Lin, Mu-Shien Lee, Department of Gastroenterology and Hepatology, Chang Gung Memorial Hospital, Chang Gung University College of Medicine, Taoyuan 333, Taiwan

Chin-Kuo Chen, Department of Otolaryngology, Chang Gung Memorial Hospital, Chang Gung University College of Medicine, Taoyuan 333, Taiwan

Hsing-Yu Chen, Center for Traditional Chinese Medicine, Chang Gung Memorial Hospital, Chang Gung University College of Medicine, Taoyuan 333, Taiwan

Author contributions: Tsou YK conceptualized and designed the study, performed 24-h pH monitoring, analyzed and interpreted the data, and wrote the manuscript; Lien JM revised the manuscript and gave final approval of the version to be published; Chen CK, Lin CH, and Lee MS collected data; Chen HY performed the statistical analyses.

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Correspondence to: Yung-Kuan Tsou, MD, Department of Gastroenterology and Hepatology, Chang Gung Memorial Hospital, Chang Gung University College of Medicine, 5, Fu-Shin Street, Kweishan, Taoyuan 333, Taiwan. flying3454@xuite.net
Telephone: +886-3-3281200 Fax: +886-3-3272236

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Abstract

AIM: To investigate the impact of esophagogastroduodenoscopy with conscious sedation on the subsequent 24-h catheter-based pH monitoring.

METHODS: Fifty patients with extra-esophageal symptoms of gastroesophageal reflux disease undergoing ambulatory dual-probe 24-h pH monitoring were enrolled from March 2010 to August 2011. All of the data were collected prospectively and analyzed retrospectively. Thirty-six patients (72%, group A) underwent pH monitoring shortly after esophagogastroduodenoscopy (EGD) with conscious sedation, and 14 patients (28%, group B) underwent pH monitoring without conscious

sedation. The 24-h pH data from two time periods were analyzed: the first 4 h (Period I) and the remaining time of the study (Period II).

RESULTS: The mean age of the patients was 49.6 ± 12.5 years; 20 patients (40%) were men. The baseline data, including age, sex, body mass index, reflux esophagitis, the Reflux Symptom Index, and the Reflux Findings Score, were comparable between the two groups. The percentage of total time with a pH < 4 and the frequency of acid reflux during Period I were not significantly different between the two groups, as measured using both pharyngeal ($0.03\% \pm 0.10\%$ vs $0.07\% \pm 0.16\%$, $P = 0.32$; and 0.07 ± 0.23 episodes/h vs 0.18 ± 0.47 episodes/h, $P = 0.33$, respectively) and esophageal probes ($0.96\% \pm 1.89\%$ vs $0.42\% \pm 0.81\%$, $P = 0.59$; and 0.74 ± 1.51 episodes/h vs 0.63 ± 0.97 episodes/h, $P = 0.49$, respectively). The percentage of total time with a pH < 4 and the frequency of acid reflux were also not significantly different between Periods I and II in group A patients, as measured using both pharyngeal ($0.03\% \pm 0.10\%$ vs $0.23\% \pm 0.85\%$, $P = 0.21$; and 0.07 ± 0.23 episodes/h vs 0.29 ± 0.98 episodes/h, $P = 0.22$, respectively) and esophageal probes ($0.96\% \pm 1.89\%$ vs $1.11\% \pm 2.57\%$, $P = 0.55$; and 0.74 ± 1.51 episodes/h vs 0.81 ± 1.76 episodes/h, $P = 0.55$, respectively).

CONCLUSION: EGD with conscious sedation does not interfere with the results of subsequent 24-h pH monitoring in patients with extra-esophageal symptoms of gastroesophageal reflux disease.

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Key words: Esophagogastroduodenoscopy; Conscious sedation; pH monitoring; Gastroesophageal reflux disease; Extraesophageal symptoms

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interfere with catheter-based 24-h pH monitoring. Esophago-gastroduodenoscopy with conscious sedation does not interfere with catheter-based 24-h pH monitoring. *World J Gastroenterol* 2013; 19(11): 1805-1810 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v19/i11/1805.htm> DOI: <http://dx.doi.org/10.3748/wjg.v19.i11.1805>

INTRODUCTION

The manifestation of gastroesophageal reflux disease (GERD) can be either esophageal or extra-esophageal^[1]. Extra-esophageal reflux, or laryngopharyngeal reflux (LPR), refers to the backflow of gastric contents into the larynx and pharynx^[1,2]. Ambulatory dual-probe 24-h pH monitoring is currently considered the gold-standard diagnostic modality for the evaluation of patients with LPR^[2-4]. This catheter-based pH measurement is performed with a pH sensor located within 2 cm above the proximal border of the upper esophageal sphincter (UES)^[5]. Prior manometry-guided localization is required to determine UES positioning relative to the nostril. The two trans-nasal procedures are usually performed without intravenous sedation. Therefore, they are unpleasant and uncomfortable tests for patients^[6,7].

Conscious sedation improves the quality of the test and increases the patient's willingness to undergo a gastrointestinal examination^[8]. Benzodiazepine administration and opiate-based sedation are the most common practices for conscious sedation^[9]. Meperidine causes a reduction in the LES pressure in animal and human studies^[10,11]. It may induce or exacerbate gastroesophageal reflux (GER), causing interference with pH studies^[11]. However, capsule-based (Bravo) wireless pH measuring system is usually performed during or shortly after an esophagogastroduodenoscopy (EGD) with conscious sedation^[12]. Therefore, it is unclear whether conscious sedation during EGD has an impact on the results of the subsequent esophageal pH test. To increase patient willingness to undergo 24-h pH monitoring, we attempted to perform a catheter-based pH study in a manner similar to the Bravo system in our patients. The aim of this study was to investigate whether EGD with conscious sedation interfered with subsequent catheter-based 24-h pH monitoring in patients with extra-esophageal symptoms of GERD.

MATERIALS AND METHODS

From March 2010 to August 2011, 53 patients with LPR-like complaints underwent ambulatory 24-h pH monitoring at the Chang Gung Memorial Hospital. These complaints included globus ($n = 27$), sore throat ($n = 13$), chronic cough ($n = 9$), hoarseness ($n = 3$), and dysphagia ($n = 1$). Three patients were excluded from the analysis because of numerous artifacts (pH out of range) on their pH records ($n = 2$) and intolerance of the procedure ($n = 1$). All of the patients were instructed to discontinue any

medications that affect gastric acid secretion and esophageal motility at least 7 d prior to the 24-h pH monitoring. This cohort study was incorporated into a prospective study (CMRPG390591) that was originally designed to evaluate the effects of different doses of proton pump inhibitors on the treatment of LPR. All of the data were collected prospectively and analyzed retrospectively. The study protocol was approved by the ethics committee at Chang Gung Memorial Hospital (IRB No: 99-3494C).

A total of 50 patients were enrolled in the current study. The mean patient age was 49.6 ± 12.5 years (range, 20-76 years). There were 20 (40%) men and 30 (60%) women. The mean body mass index (BMI) was 23.0 ± 3.2 kg/m² (range, 18.1-30.0 kg/m²). Group A included 36 patients (72%) who underwent EGD with conscious sedation 30 min prior to pH monitoring. Group B included the remaining 14 patients (28%) who did not undergo EGD or conscious sedation on the same day.

EGD with conscious sedation

All of the patients in group A underwent EGD with intravenous sedation using meperidine and midazolam on the same day prior to their 24-h pH monitoring study. The dosage of meperidine was 0.52 ± 0.10 mg/kg (range, 0.32-0.81 mg/kg), and the dosage of midazolam was 33.1 ± 7.1 g/kg (range, 16.1-53.6 g/kg). Hyoscine butylbromide was not given before or during the endoscopy in any of the participants.

Esophageal manometry and 24-h pH monitoring

All of the patients underwent esophageal manometry using a station pull-through technique to locate the UES in reference to the nostril. Subsequently, 24-h pH monitoring was performed using antimony electrodes and fitting recorders (Orion II, Medical Measurement Systems, The Netherlands). The pH catheter had two or four sensors that were 15 cm or 5 cm apart. The pH electrodes were calibrated before and after the test using reference buffer solutions with a pH of 4 or 7. The most proximal electrode (pharyngeal probe) was placed in the hypopharynx 2 cm above the manometry-defined proximal border of the UES. Each patient wore a data-logger with a sampling frequency of 1 Hz during the test period. Symptom occurrence, meal times and body positions (supine or upright) were written down in a diary and recorded in a data log. The patients were advised to eat their usual meals and engage in their usual activities on the day of the test. After a period of 24 h, they returned the data log, and the data were downloaded onto a computer using software provided by the manufacturer. The data from the diaries were extracted for interpretation.

A single pharyngeal event (pH < 4) preceded by a precipitous pH drop of the same magnitude in the esophageal probe was defined as a positive result for LPR. The most distal pH sensor in the esophagus (esophageal probe) was 15 cm away from the pharyngeal probe. Pathologic GER was defined as a percentage of total time with a pH < 4 greater than 4.2% as measured

Table 1 Comparisons of baseline data and data obtained during the first 4 h between patients in group A and group B

| Variables | Group A (n = 36) | Group B (n = 14) | P value ¹ |
|--|---------------------|---------------------|----------------------|
| Baseline data | | | |
| Age (yr) | 49.5 ± 13.2 | 49.9 ± 11.0 | 0.91 |
| Sex (F) | 15 (41.7) | 5 (35.7) | 0.70 |
| Body mass index | 22.8 ± 3.2 | 23.3 ± 3.4 | 0.75 |
| Presence of heartburn or regurgitation | 13 (36.1) | 7 (50) | 0.37 |
| Erosive esophagitis on EGD | 6 (16.7) | 2 (14.3) | 1 |
| Reflux symptom index | 15.9 ± 7.5 | 16.8 ± 9.2 | 0.62 |
| Reflux findings score | 8.1 ± 3.7 | 6.4 ± 2.4 | 0.19 |
| Positive for LPR | 12 (33.3) | 5 (35.7) | 0.87 |
| Positive for pathologic GER | 3 (8.3) | 1 (7.1) | 1 |
| Data obtained during the first 4 h | | | |
| Frequency of symptoms onset (episodes/h) | 0.04 ± 0.1 | 0.14 ± 0.22 | 0.08 |
| Pharyngeal probe | | | |
| Total time of pH < 4 (%) | 0.03 ± 0.10 | 0.07 ± 0.16 | 0.32 |
| Mean frequency of reflux (episodes/h) | 0.07 ± 0.23 | 0.18 ± 0.47 | 0.33 |
| Patients with long reflux | 0 | 0 | 1 |
| Esophageal probe | | | |
| Total time of pH < 4 (%) | 0.96 ± 1.89 | 0.42 ± 0.81 | 0.59 |
| Mean frequency of reflux (episodes/h) | 0.74 ± 1.51 | 0.63 ± 0.97 | 0.49 |
| Patients with long reflux | 1 (2.8) | 0 | 1 |

Data are expressed as absolute numbers (percentage) or mean ± SD. ¹Statistical significance was defined as a $P < 0.05$. EGD: Esophagogastro duodenoscopy; LPR: Laryngopharyngeal reflux; GER: Gastroesophageal reflux.

by the esophageal probe^[13].

Analysis of the pH data

The elimination half-life of meperidine is 3.2-3.7 h, and the half-life of midazolam is approximately 3 h^[14,15]. To analyze the effect of conscious sedation on 24-h pH monitoring, computer software provided by MMS was used to analyze the 24-h pH data of each patient during two periods: the first 4 h (Period I) and the remaining time of the study (Period II). Meal times and sleep h were excluded from the analysis. Thus, only data recorded when the patients assumed an upright position were used for the comparisons. The mean duration of each period was 3.5 ± 0.5 h for Period I and 10.1 ± 2.1 h for Period II.

An acid reflux event was defined as an episode of pH < 4 detected on the pharyngeal or esophageal probe. Long reflux was defined as a reflux event lasting more than 5 min. The variables used for the comparisons were percentage of total time with a pH < 4, frequency of acid reflux events (episodes/h), and presence of long reflux.

Statistical analysis

Continuous variables are expressed as the mean ± SD in the text and tables. The differences in the variables between patients in group A and B were compared using a t test for age and a Mann-Whitney U test for BMI, Reflux Symptom Index (RSI), Reflux Finding Score (RFS),

percentage of total time with a pH < 4, and frequency of acid reflux. Either a χ^2 or Fisher's exact test (when χ^2 test was inappropriate) was used to analyze differences in sex, presence of heartburn and regurgitation, erosive esophagitis, LPR, pathologic GER, and long reflux. The differences in the variables between Period I and Period II in group A patients were compared using a Wilcoxon Signed Rank test for the percentage of total time with a pH < 4, frequency of symptoms, and the presence of acid reflux. A McNemar's test was used to analyze differences in the presence of long reflux. Statistical significance was defined as $P < 0.05$. The statistical analyses were performed using the SPSS version 17.0 for Windows.

RESULTS

Comparisons between patients with and without sedated EGD

The demographic, clinical, and endoscopic data from the patients in groups A and B were compared (Table 1). There were no significant differences between the two groups. Characteristic symptoms of GERD, heartburn and acid regurgitation were present in both groups (13/36 vs 7/14, $P = 0.37$). Six patients (16.7%) in group A and 2 (14.3%) in group B had erosive esophagitis (all were grade A, except one patient in group A who was grade B based on the Los Angeles classification) on the EGD ($P = 1$). The RSI is a validated patient-administered questionnaire for the diagnosis of LPR^[16]. A total score greater than 13 is regarded as a positive result. The patients in group A scored 15.9 ± 7.5, and the patients in group B score 16.8 ± 9.2 ($P = 0.62$). The RFS is an 8-item scale listing common physical findings in LPR patients^[17]. A total score of greater than 7 is regarded as a positive result. The RFS was 8.1 ± 3.7 in group A and 6.4 ± 2.4 in group B ($P = 0.19$). Twelve patients (33.3%) in group A and 5 (35.7%) in group B met the pH criteria for LPR ($P = 0.87$). There were three (8.3%) patients in group A and one (7.1%) in group B who had pathologic GER ($P = 1.00$).

The pH data obtained from Period I were compared between the two groups (Table 1). Using the pharyngeal probe, the percentage of total time with a pH < 4 was 0.03% ± 0.10% (range, 0%-0.4%) in group A and 0.07% ± 0.16% (range, 0%-0.5%) in group B ($P = 0.32$). The frequency of acid reflux events was 0.07 ± 0.23 episodes/h (range, 0-1.1 episodes/h) in group A and 0.18 ± 0.47 episodes/h (range, 0-1.7 episodes/h) in group B ($P = 0.33$). None of the patients in either group had long reflux events. Using the esophageal probe, the mean percentage of total time with a pH < 4 was 0.96% ± 1.89% (range, 0%-7%) in group A and 0.42% ± 0.81% (range, 0%-2.9%) in group B ($P = 0.59$). The frequency of acid reflux events was 0.74 ± 1.51 episodes/h (range, 0-6.3 episodes/h) in group A and 0.63 ± 0.97 episodes/h (range, 0-3.1 episodes/h) in group B ($P = 0.49$). One patient in group A and no patients in group B had long reflux ($P = 1$).

Table 2 Comparisons between Period I and Period II in group A patients (mean ± SD)

| Variables | Period I | Period II | P value ¹ |
|---------------------------------------|-------------|-------------|----------------------|
| Frequency of symptom (episodes/h) | 0.04 ± 0.10 | 0.03 ± 0.06 | 0.29 |
| Pharyngeal probe | | | |
| Total time of pH < 4 (%) | 0.03 ± 0.10 | 0.23 ± 0.85 | 0.21 |
| Mean frequency of reflux (episodes/h) | 0.07 ± 0.23 | 0.29 ± 0.98 | 0.22 |
| Patients with long reflux | 0 | 0 | 1 |
| Esophageal probe | | | |
| Total time of pH < 4 (%) | 0.96 ± 1.89 | 1.11 ± 2.57 | 0.55 |
| Mean frequency of reflux (episodes/h) | 0.74 ± 1.51 | 0.81 ± 1.76 | 0.55 |
| Patients with long reflux | 1 (2.8%) | 5 (13.9%) | 0.22 |

¹Statistical significance was defined as a *P* < 0.05.

Comparisons between different periods in patients who underwent EGD with conscious sedation

The pH data for Period I and Period II from the group A patients are shown in Table 2. The frequency of symptom occurrence was 0.04 ± 0.10 episodes/h (range, 0-0.3 episodes/h) during Period I and 0.03 ± 0.06 episodes/h (range, 0-0.3 episodes/h) during Period II (*P* = 0.29). Based on the pharyngeal probe measurements, the percentage of total time with a pH < 4 was 0.03% ± 0.10% (range, 0%-0.4%) in Period I and 0.23% ± 0.85% (range, 0%-5%) in Period II (*P* = 0.21, Figure 1A). The frequency of acid reflux was 0.07 ± 0.23 episodes/h (range, 0-1.1 episodes/h) in Period I and 0.29 ± 0.98 episodes/h (range, 0-5.7 episodes/h) in Period II (*P* = 0.22). None of the patients had a long reflux event. Based on the esophageal probe measurements, the percentage of total time with a pH < 4 was 0.96% ± 1.89% (range, 0%-7%) in Period I and 1.11% ± 2.57% (range, 0%-13.6%) in Period II (*P* = 0.55, Figure 1B). The frequency of acid reflux was 0.74 ± 1.51 episodes/h (range, 0-6.29 episodes/h) in Period I and 0.81 ± 1.76 episodes/h (range, 0-9.78 episodes/h) in Period II (*P* = 0.55). One patient had a long reflux event during Period I and five had a long reflux event during Period II (*P* = 0.22).

DISCUSSION

EGD is usually performed without conscious sedation in Taiwan. Some of our patients reported that nasally passed procedures for esophageal manometry and 24-h pH monitoring were less tolerable than EGD without conscious sedation. Because moderate conscious sedation may be helpful to facilitate gastrointestinal procedures, we performed a catheter-based pH study in a manner similar to the Bravo system in our patients^[8,12]. Our study results showed that EGD with conscious sedation does not have an immediate effect on subsequent catheter-based pH monitoring with regard to the relevant parameters assessed with pH monitoring techniques^[6,18].

The Bravo capsule is usually placed during an EGD with conscious sedation^[19-23]. The Bravo pH system allows for the measurement of esophageal acid exposure

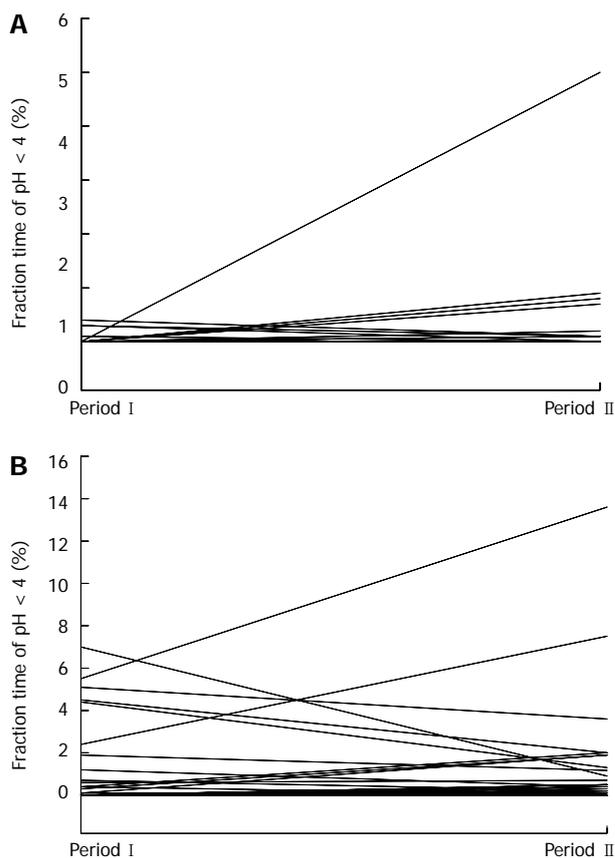


Figure 1 Individual comparison of the fraction of total time with a pH < 4 between Period I and Period II in group A patients. A: Using pharyngeal probe measurements; B: Using esophageal probe measurements.

over a 48-h period. Therefore, many studies have investigated whether there is day-to-day discrepancy during Bravo pH monitoring^[19-23]. The results are conflicting. Bechtold *et al*^[19] and Bhat *et al*^[20] reported more acid reflux events on day 1 than on day 2 using the Bravo system, suggesting that endoscopy and the associated sedatives may be responsible for the day-to-day discrepancy. Other studies showed that patients who underwent same-day EGD with intravenous sedation did not demonstrate any significant differences in reflux variables between day 1 and day 2 using Bravo pH monitoring^[21-23].

Bhat *et al*^[20] further analyzed their pH data by dividing the study period into the first 6 h and the remaining 18 h on both day 1 and day 2. They found an increase in esophageal acid exposure during the first 6 h after capsule insertion on day 1 compared to the corresponding period on day 2. There was no such difference during the remaining 18 h on day 1 and day 2. They concluded that EGD with conscious sedation interferes with subsequent capsule-based Bravo pH measurements. In this study, we divided the study period into the first 4 h and the remaining 20 h because the elimination half-life is nearly 4 h for meperidine and approximately 3 h for midazolam^[14,15]. Our results showed no interference in patients who underwent EGD with sedation. Our data further revealed that three pH variables were not significantly different between the patients with and without prior sedation (us-

ing both the pharyngeal and laryngeal probes). Ayazi *et al.* observed a similar day-to-day discrepancy in patients receiving capsule-based Bravo pH monitoring without prior EGD and conscious sedation^[24]. Their results argue for an iatrogenic effect of sedated EGD on the pH monitoring.

There are some limitations in the present study. First, the study cohort had extra-esophageal symptoms (LPR) but not esophageal symptoms (classical GERD). The mechanism of LPR may be different from that of classical GERD^[2,25]. Therefore, whether the study results can be applied to pH monitoring in patients with characteristic symptoms of GERD needs further investigation. Second, the esophageal probe was placed at a variable distance above the proximal border of the LES of participants. The results of the pH parameters obtained from the esophageal probe may be suboptimal.

In conclusion, our results suggest that EGD with conscious sedation does not interfere with the results of subsequent catheter-based pH monitoring in patients with extra-esophageal manifestations of GERD. Catheter-based 24-h pH monitoring can be performed shortly after EGD with conscious sedation, especially for those patients who are intolerable to the procedures.

COMMENTS

Background

One of the limitations of catheter-based pH monitoring is discomfort. Whether it can be performed shortly after esophagogastroduodenoscopy (EGD) with conscious sedation like the performance of Bravo pH system is unknown. Authors aimed to investigate the impact of sedated EGD on the subsequent 24-h catheter-based pH monitoring.

Research frontiers

In order to increase patients' willingness to receive 24-h pH monitoring, authors have attempted to perform the catheter-based pH study in a similar way to Bravo system in our patients.

Innovations and breakthroughs

EGD with conscious sedation does not interfere with the results of the subsequent 24-h pH monitoring in patients with extraesophageal symptoms of gastroesophageal reflux disease.

Applications

Catheter-based 24-h pH monitoring can be performed shortly after sedated EGD especially for those patients who are intolerable to the procedures.

Terminology

Laryngopharyngeal reflux is backflow of gastric content into larynx and pharynx which has been widely used by otolaryngologists, while extraesophageal reflux is commonly used by gastroenterologist.

Peer review

The paper is well composed, documented. The results are interesting and suggest that EGD with conscious sedation does not interfere with the results of subsequent 24-h pH monitoring in patients with extra-esophageal symptoms of gastroesophageal reflux disease.

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Value of α -fetoprotein in association with clinicopathological features of hepatocellular carcinoma

Chang Liu, Guang-Qin Xiao, Lu-Nan Yan, Bo Li, Li Jiang, Tian-Fu Wen, Wen-Tao Wang, Ming-Qing Xu, Jia-Yin Yang

Chang Liu, Guang-Qin Xiao, Lu-Nan Yan, Bo Li, Li Jiang, Tian-Fu Wen, Wen-Tao Wang, Ming-Qing Xu, Jia-Yin Yang, Department of Liver and Vascular Surgery, West China Hospital, Sichuan University, Chengdu 610041, Sichuan Province, China
Author contributions: Liu C, Xiao GQ contributed equally to this work and performed the majority of experiments; Liu C, Xiao GQ, Yan LN, Yang JY designed the research; Jiang L, Wen TF, Li B performed the research; Wang WT, Xu MQ contributed analytic tools; Jiang L analyzed the data; and Liu C wrote the paper.

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Correspondence to: Jia-Yin Yang, MD, Professor of Medicine, Department of Liver and Vascular Surgery, West China Hospital, Sichuan University, Guoxuexiang 37, Chengdu 610041, Sichuan Province, China. docjackyang@163.com

Telephone: +86-28-85422867 Fax: +86-28-85422469

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Abstract

AIM: To explore the relationship between α -fetoprotein (AFP) and various clinicopathological variables and different staging system of hepatocellular carcinoma (HCC) thoroughly.

METHODS: A retrospective cohort study of consecutive patients diagnosed with HCC between January 2008 and December 2009 in West China Hospital was enrolled in our study. The association of serum AFP values with the HCC clinicopathological features was analysed by univariate and multivariate analysis, such as status of hepatitis B virus (HBV) infection, tumor size, tumor number, vascular invasion and degree of tumor differentiation. Also, patients were divided into four groups at the time of enrollment according to different cutoff values for serum value of AFP (≤ 20 $\mu\text{g/L}$, 21-400 $\mu\text{g/L}$, 401-800 $\mu\text{g/L}$, and ≥ 801 $\mu\text{g/L}$), to compare the positive rate of patient among four groups stratified by vari-

ous clinicopathological variables. And the correlation of different kinds of tumor staging systems, such as TNM, Barcelona Clinic Liver Cancer (BCLC) staging classification and China staging, were compared with the serum concentration of AFP.

RESULTS: A total of 2304 HCC patients were enrolled in this study totally; the mean serum level of AFP was 555.3 ± 546.6 $\mu\text{g/L}$. AFP levels were within the normal range (< 20 $\mu\text{g/L}$) in 27.4% ($n = 631$) of all the cases. 81.4% ($n = 1875$) patients were infected with HBV, and those patients had much higher serum AFP level compared with non-HBV infection ones (573.9 ± 547.7 $\mu\text{g/L}$ vs 398.4 ± 522.3 $\mu\text{g/L}$, $P < 0.001$). The AFP level in tumors ≥ 10 cm (808.4 ± 529.2 $\mu\text{g/L}$) was significantly higher ($P < 0.001$) than those with tumor size 5-10 cm (499.5 ± 536.4 $\mu\text{g/L}$) and with tumor size ≤ 5 cm (444.9 ± 514.2 $\mu\text{g/L}$). AFP levels increased significantly in patients with vascular invasion (694.1 ± 546.9 $\mu\text{g/L}$ vs 502.1 ± 543.1 $\mu\text{g/L}$, $P < 0.001$). Patients with low tumor cell differentiation (559.2 ± 545.7 $\mu\text{g/L}$) had the significantly ($P = 0.007$) highest AFP level compared with high differentiation (207.3 ± 420.8 $\mu\text{g/L}$) and intermediate differentiation (527.9 ± 538.4 $\mu\text{g/L}$). In the multiple variables analysis, low tumor cell differentiation [OR 6.362, 95%CI: 2.891-15.382, $P = 0.006$] and tumor size (≥ 10 cm) (OR 5.215, 95%CI: 1.426-13.151, $P = 0.012$) were independent predictors of elevated AFP concentrations (AFP > 400 $\mu\text{g/L}$). Serum AFP levels differed significantly ($P < 0.001$) in the D stage of BCLC (625.7 ± 529.8 $\mu\text{g/L}$) compared with stage A (506.2 ± 537.4 $\mu\text{g/L}$) and B (590.1 ± 551.1 $\mu\text{g/L}$).

CONCLUSION: HCC differentiation, size and vascular invasion have strong relationships with AFP, poor differentiation and HCC size ≥ 10 cm are independent predictors of elevated AFP. BCLC shows better relationship with AFP

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Key words: α -fetoprotein; Hepatocellular carcinoma; Tumor markers; Clinical features; Pathological features

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INTRODUCTION

Hepatocellular carcinoma (HCC) is the fifth most common cancer and third most significant cause of cancer mortality in the world, with 5-year survival rates at a mere 7% in patients^[1-3]. When patients with obvious clinical symptoms come to the hospital for treatment, the HCC has already reached the mild to advanced stages and is usually large in size. Given the subsequent rapid growth and vascular invasion, patients have to face serious progress and poor prognosis. There is no doubt that a thorough comprehension of the pathobiological features of HCC will definitely help clinicians diagnose HCC at earlier stages, thus improving patient outcomes.

Currently, the most commonly used methods for screening and diagnosing HCC are ultrasound imaging and serum α -fetoprotein (AFP) concentration measurements. AFP has been used worldwide as the golden standard compared to other serum markers, especially in poor, remote areas. However, the diagnostic value of AFP is still controversial given that its sensitivity and specificity are unstable^[4,5]. Moreover, researchers have studied AFP-related parameters, such as AFP mRNA^[6-8] and AFP glycoforms^[9-11]. Recently, both of these AFP-related parameters have been proven to have diagnostic potential in a way, and even they are recommended as complementary tests. Nevertheless, their usage is limited due to financial and technological reasons; it is unlikely that they can replace serum AFP as the golden standard of diagnostic serum markers for hepatocellular carcinoma.

The serum AFP level also plays an important role in representing the pathobiological features of HCC identified as prognostic factors^[12-14]. Some clinicians have reported that the elevation of serum AFP levels is consistent with increased tumor sizes^[15-17] and that small tumors seem to excrete less AFP into the blood. They also report that there is no clear increasing tendency between tumor size and AFP levels^[18]. Others have reported that highly differentiated small tumors express undetectable levels of serum AFP^[14], whereas lowly differentiated tumor cells, in most cases, had AFP levels of $> 400 \mu\text{g/L}$ ^[19]. Similarly, other factors such as tumor number^[14,16] and vascular invasion^[14,20-22] have been found to be correlated with serum AFP levels to a certain extent. Nonetheless, these studies failed to research the relationship

between various variables thoroughly, and the sample size of some cohort studies was not large enough.

The objects of this study were to comprehensively evaluate the relationship between AFP and clinicopathological features of HCC, to compare the clinical practicality of different tumor staging systems, and to state the clinical importance of measuring AFP concentrations in cases of HCC in China.

MATERIALS AND METHODS

Ethics

Informed consent was obtained from all subjects for participation in the study and the usage of sera. The study protocol conformed to the ethical guidelines of the World Medical Association Declaration of Helsinki and was approved by the ethical committee of the West China Hospital, Sichuan University. All participants provided their written informed consent to participate in this study.

Patient tissue

The base population consisted of 2381 consecutive patients who received a final diagnosis of HCC at the department of liver and vascular surgery, West China Hospital, Sichuan University, between January 2008 and December 2009. Of these 2381 patients, 31 were excluded from the study because of the absence of tumor size data, and 46 were excluded due to missing serum AFP level data. Therefore, a total of 2304 HCC patients were included in this study. The final diagnosis of HCC was made in two main ways according to whether the patients had a hepatectomy or not. HCC diagnosis was made by the pathological examination of the resected specimens for the 1825 patients (79.2%) who received hepatic resection or fine needle aspiration. The remaining 479 patients (20.8%) had advanced HCC with impaired liver function, with portal vein invasion or metastasis, and thus did not undergo an invasive operation. Instead, this group of patients had their HCC confirmed by a variety of combined imaging techniques, identification of a focal lesion $> 2 \text{ cm}$ in diameter in 2 imaging modalities, such as ultrasonography (US), enhanced computed tomography (CT) image, magnetic resonance imaging (MRI), and/or angiography, and showing arterial hypervascularization in at least 1 of imaging modalities. A mass lesion within a cirrhotic liver in the presence of a serum AFP level $> 400 \mu\text{g/L}$ was also diagnostic^[23]. For lesions $< 2 \text{ cm}$ in diameter, diagnosis of HCC required hepatitis B surface antigen (HBsAg) positive and AFP $> 400 \mu\text{g/L}$. The severity of liver dysfunction was assessed *via* the Child-Pugh classification. Serum samples were collected and stored at $-70 \text{ }^\circ\text{C}$ until examined. Hepatitis B virus (HBV) infection status was determined on the basis of HBsAg, hepatitis B surface antibody, hepatitis B core antibody, hepatitis B e antigen, and hepatitis B e antibody using the electrochemiluminescence immuno-

Table 1 Background characteristics of 2304 patients with hepatocellular carcinoma *n* (%)

| Variables | <i>n</i> = 2304 |
|-----------------------------|-----------------|
| Sex | |
| Male | 1987 (86.2) |
| Female | 317 (13.8) |
| Age (yr), mean ± SD | 51.9 ± 12.9 |
| AFP value (μg/L), mean ± SD | 555.31 ± 546.69 |
| HBV infection | |
| No | 429 (18.6) |
| Yes | 1875 (81.4) |
| HCV infection | |
| No | 2287 (99.3) |
| Yes | 17 (0.7) |
| Child-Pugh grade | |
| A | 1790 (77.7) |
| B | 495 (21.5) |
| C | 19 (0.8) |

HCC: Hepatocellular carcinoma; AFP: α -fetoprotein; HBV: Hepatitis B virus; HCV: Hepatitis C virus.

assay (ECLIA) method, and HBsAg positive was defined as HBV infection. Background characteristics of the patients are listed in Table 1.

AFP assay and assessment

Serum samples for the detection of AFP were taken upon entry into the study before initial treatment, and AFP was determined by ECLIA, which was intended for use on Roche MODULAR ANALYTICS E170 immunoassay analyzers (Roche Diagnostics GmbH, Mannheim, Germany). The time between collecting serum samples and performing an operation or getting a CT, US, MRI and/or angiography image was less than seven days. Detected serum AFP values ranged from 0 to 1210 $\mu\text{g/L}$, and all of the AFP values more than 1210 $\mu\text{g/L}$ were recorded as 1210 $\mu\text{g/L}$ in our study. The cutoff for normal AFP levels ($< 20 \mu\text{g/L}$) was chosen on the basis of the EASL guidelines^[24] and on the data reported in the majority of previous studies^[14,25]. Four different cutoff values for serum AFP were set: $\leq 20 \mu\text{g/L}$, 21-400 $\mu\text{g/L}$, 401-800 $\mu\text{g/L}$, and $\geq 801 \mu\text{g/L}$. Thus, patients were classified into four groups based on their level of serum AFP at the time of enrollment.

Clinicopathological variables

We assessed the association of AFP values with the clinicopathological variables that have been reported as prognostic factors for HCC^[16,26]. The investigated variables are shown in Table 2. All variables were assessed either pathologically, *via* resected specimens and fine needle biopsy, or by imaging techniques. Vascular invasion was defined as the presence of portal vein invasion, venous invasion and/or biliary invasion. Tumor size was defined by the longest axis and estimated with US, CT or MRI. When multiple HCC tumors were present, the axis of the largest one was measured and taken as the representative HCC diameter. The diagnosis was histologically

Table 2 Serum α -fetoprotein level of different tumor related factors *n* (%)

| Variables | <i>n</i> = 2304 | AFP ($\mu\text{g/L}$) | <i>P</i> value |
|------------------------------------|-----------------|-------------------------|----------------|
| HBV infection | | | < 0.001 |
| Yes | 1875 (81.4) | 573.9 ± 547.7 | |
| No | 429 (18.6) | 398.4 ± 522.3 | |
| Tumor size(cm) | | | < 0.001 |
| ≤ 5 | 871 (37.8) | 444.9 ± 514.2 | |
| 5-10 | 804 (34.9) | 499.5 ± 536.4 | |
| ≥ 10 | 629 (27.3) | 808.4 ± 529.2 | |
| Tumor number | | | 0.451 |
| 1 | 1666 (72.2) | 531.9 ± 18.9 | |
| 2 | 288 (12.5) | 532.2 ± 44.7 | |
| ≥ 3 | 350 (15.3) | 556.0 ± 42.2 | |
| Vascular invasion | | | < 0.001 |
| No | 1714 (74.4) | 502.1 ± 543.1 | |
| Yes | 590 (25.6) | 694.1 ± 546.9 | |
| Tumor differentiation ¹ | | | 0.007 |
| High | 139 (7.5) | 207.3 ± 420.8 | |
| Intermediate ^b | 963 (52.3) | 527.9 ± 538.4 | |
| Low | 741 (40.2) | 559.2 ± 545.7 | |
| TNM staging | | | 0.306 |
| I | 495 (21.5) | 578.6 ± 549.3 | |
| II | 289 (12.5) | 493.3 ± 542.2 | |
| III A | 726 (31.5) | 548.6 ± 544.3 | |
| III B | 214 (9.3) | 585.3 ± 553.4 | |
| III C | 101 (4.3) | 558.9 ± 551.7 | |
| IV | 479 (20.8) | 534.0 ± 545.9 | |
| BCLC staging | | | 0.008 |
| A | 869 (37.7) | 506.2 ± 537.4 | |
| B | 251 (10.9) | 590.1 ± 551.1 | |
| C | 1028 (44.6) | 607.3 ± 553.3 | |
| D ^d | 156 (6.8) | 625.7 ± 529.8 | |
| China staging | | | 0.386 |
| I | 137 (5.9) | 487.8 ± 542.2 | |
| II a | 198 (8.6) | 528.8 ± 533.9 | |
| II b | 893 (38.7) | 560.8 ± 545.7 | |
| III | 1076 (46.7) | 527.9 ± 544.8 | |

¹We assessed 1843/2304 patients who had underwent hepatectomy or fine needle aspiration. ^b $P < 0.001$ vs high and low differentiation groups. ^d $P < 0.001$ vs stage A and B of BCLC. AFP: α -fetoprotein; HBV: Hepatitis B virus; TNM: Tumor-node-metastasis; BCLC: The Barcelona-Clinic Liver Cancer Group.

confirmed using resected specimens or fine needle aspiration biopsy, and the grading followed the methodology reported by Edmonson *et al*^[27] (high, intermediate, or low differentiation). Similarly, if multiple tumors had more than two grades of histological differentiation, the most dedifferentiated grade was recorded.

Among the several tumor staging systems used in the world, the tumor-node-metastasis (TNM) system is the most widely accepted^[28,29]. The American Joint Committee on Cancer (AJCC) published its 7th edition in 2009^[30], and the main difference from the 6th edition of the AJCC staging system is the separation of the T3 stage into two subgroups, T3a and T3b. The definition of the T3a stage is the presence of multiple tumors, any $> 5 \text{ cm}$, while the T3b stage is defined as having tumors of any size involving a major portal or hepatic vein. The tumor staging was also determined by the Barcelona Clinic Liver Cancer (BCLC) staging classification, which

Table 3 Positive rate of patients with different tumor-related factors in four α -fetoprotein level intervals *n* (%)

| Variables AFP ($\mu\text{g/L}$) | ≤ 20 | 20-400 | 401-800 | ≥ 801 | <i>n</i> = 2304 | <i>P</i> value |
|-----------------------------------|-------------|-------------|------------|-------------|-----------------|----------------|
| HBV infection | | | | | | < 0.001 |
| Yes | 507 (26.7) | 441 (23.2) | 126 (6.4) | 801 (42.4) | 1875 (81.4) | |
| No | 202 (47.0) | 82 (19.2) | 18 (4.3) | 127 (29.5) | 429 (18.6) | |
| Tumor size (cm) | | | | | | < 0.001 |
| ≤ 5 | 243 (27.90) | 361 (41.45) | 66 (7.58) | 201 (23.08) | 871 (37.80) | |
| 5-10 | 262 (32.59) | 206 (25.62) | 43 (5.35) | 293 (36.44) | 804 (34.90) | |
| ≥ 10 | 117 (18.60) | 84 (13.35) | 27 (4.29) | 401 (63.75) | 629 (27.30) | |
| Tumor number | | | | | | 0.593 |
| 1 | 469 (28.15) | 461 (27.67) | 103 (6.18) | 622 (38.00) | 1666 (72.31) | |
| 2 | 66 (22.92) | 97 (33.68) | 14 (4.86) | 111 (38.54) | 288 (12.50) | |
| ≥ 3 | 87 (24.86) | 93 (26.57) | 19 (5.43) | 151 (43.14) | 350 (15.19) | |
| Vascular invasion | | | | | | < 0.001 |
| No | 510 (29.75) | 498 (29.05) | 101 (5.89) | 605 (35.30) | 1714 (74.39) | |
| Yes | 112 (19.98) | 153 (25.93) | 35 (5.93) | 290 (49.15) | 590 (25.61) | |
| Tumor differentiation | | | | | | 0.012 |
| High | 57 (42.22) | 60 (44.44) | 5 (3.70) | 16 (11.85) | 135 (5.99) | |
| Intermediate | 285 (29.75) | 263 (27.45) | 81 (8.46) | 335 (34.97) | 958 (41.84) | |
| Low | 263 (35.93) | 134 (18.31) | 43 (5.87) | 301 (41.12) | 732 (32.16) | |
| TNM staging | | | | | | 0.767 |
| I | 125 (25.25) | 139 (28.08) | 23 (4.65) | 208 (42.02) | 495 (21.48) | |
| II | 90 (31.14) | 81 (28.03) | 18 (6.23) | 100 (34.60) | 289 (12.54) | |
| III A | 197 (27.13) | 201 (27.69) | 50 (6.89) | 278 (38.29) | 726 (31.51) | |
| III B | 50 (28.36) | 64 (29.91) | 10 (4.67) | 90 (42.06) | 214 (9.29) | |
| III C | 27 (26.73) | 28 (27.72) | 5 (4.95) | 41 (40.59) | 101 (4.38) | |
| IV | 133 (27.77) | 138 (28.81) | 30 (6.26) | 178 (37.16) | 479 (20.79) | |
| BCLC staging | | | | | | < 0.001 |
| A | 261 (30.03) | 266 (30.61) | 56 (6.44) | 286 (32.91) | 869 (37.72) | |
| B | 59 (23.51) | 78 (31.08) | 15 (5.98) | 99 (39.44) | 251 (10.89) | |
| C | 271 (23.36) | 272 (24.46) | 53 (5.16) | 432 (42.02) | 1028 (44.62) | |
| D | 31 (19.87) | 35 (22.44) | 12 (7.69) | 78 (50.00) | 156 (6.77) | |
| China staging | | | | | | 0.124 |
| I | 34 (24.82) | 54 (39.42) | 3 (2.19) | 46 (33.58) | 137 (5.95) | |
| II a | 48 (24.24) | 70 (35.35) | 7 (3.54) | 73 (36.87) | 198 (8.59) | |
| II b | 231 (25.87) | 237 (26.54) | 65 (7.28) | 360 (40.31) | 893 (38.76) | |
| III | 309 (28.72) | 290 (29.95) | 61 (5.67) | 416 (38.66) | 1078 (46.70) | |

Patients were divided into four groups according to the serum α -fetoprotein (AFP) level, they were ≤ 20 , 20-400, 401-800 and ≥ 801 $\mu\text{g/L}$, the positive rate of patients in each interval of AFP were calculated. HBV: Hepatitis B virus; TNM: Tumor-node-metastasis; BCLC: The Barcelona-Clinic Liver Cancer Group.

was updated in 2010^[31], and not involving the Cancer of the Liver Italian Program (CLIP) staging^[32] because it includes AFP as a stage criteria. We also adopted the China Staging system, which was updated in 2001^[33].

Statistical analysis

Values are presented as the mean \pm SD. Correlations between markers values were analyzed by Spearman's rank correlation. Categorical binary variables were compared by χ^2 . Associations between marker values and clinicopathological variables were analyzed by the Wilcoxon rank-sum test, and a binary logistic regression model for multiple variables analysis. *P* values of < 0.05 were accepted as statistically significant. All statistical analyses were performed using SPSS 17.0 (SPSS Inc., Chicago, IL, United States).

RESULTS

The distribution of patients with respect to the etiology of the disease is reported in Table 1. Of the 2304 cases,

1987 patients were men and 317 patients were women (male-to-female ratio 6.27: 1). The mean serum level of AFP was 555.31 ± 546.69 $\mu\text{g/L}$. The mean patient age was 51.9 ± 12.9 years old. AFP levels were within the normal range (< 20 $\mu\text{g/L}$) in 27.4% ($n = 631$) of the cases. 81.4% ($n = 1875$) patients were infected with HBV, and those patients had much higher AFP level ($P < 0.001$) compared with non-HBV infection ones.

AFP as a marker for clinicopathological variables representatives of tumor-related features

The correlation of AFP levels with tumor-related features is depicted in Tables 2, 3 and 4. The AFP level in patients with tumor sizes ≥ 10 cm was significantly higher ($P < 0.001$) than those patients with smaller tumors (tumor size < 10 cm). There was no significant difference between patients with one or multiple tumors ($P = 0.451$). AFP levels were remarkably higher in patients with vascular invasion, such that the AFP level was clearly higher than those levels in patients without evidence of vascular invasion ($P < 0.001$). Notably, there is also a

Table 4 Binary logistic regression analysis of tumor related factors elevating the level of α -fetoprotein ($> 400 \mu\text{g/L}$)

| Variables | P value | Odds ratio (95%CI) |
|-----------------------|---------|----------------------|
| HBV infection | 0.156 | 4.162 (0.991-16.557) |
| Vascular invasion | 0.060 | 3.963 (0.529-7.384) |
| ≤ 5 | - | - |
| 5-10 | 0.341 | 0.862 (0.317-9.284) |
| ≥ 10 | 0.012 | 5.215 (1.426-13.151) |
| Tumor number | | |
| 1 | - | - |
| 2 | 0.513 | 0.487 (0.056-4.204) |
| ≥ 3 | 0.122 | 0.146 (0.013-1.671) |
| Tumor differentiation | | |
| High | - | - |
| Intermediate | 0.017 | 3.951 (1.501-9.917) |
| Low | 0.006 | 6.362 (2.891-15.382) |

The level status of α -fetoprotein ($> 400 \mu\text{g/L}$) was used as a dependent variable. HBV: Hepatitis B virus.

similar pattern with increasing tumor cell differentiation—patients with low tumor cell differentiation have the significantly highest AFP levels ($P = 0.007$). In the BCLC staging classification, AFP levels at various stages are significantly different ($P = 0.008$), with stage D having the highest level of AFP. No relationship or difference was found in the TNM staging and China staging classification systems.

Positive rates of patients with HCC in four AFP intervals

The positive rate of patients in four AFP intervals with and without HBV infection were quite different ($P < 0.001$), about half of the HBV infected patients with the AFP levels greater than $801 \mu\text{g/L}$. On the contrary, only one fifth of the non-HBV infection patients had AFP levels more than $801 \mu\text{g/L}$, AFP levels in half of the uninfected patients were less than $20 \mu\text{g/L}$ approximately. With respect to different tumor sizes, the positive rate of patient was significantly different ($P < 0.001$). The patients with the largest tumor size, ≥ 10 cm, accounted for most of the cases in the interval where the AFP cutoff value was more than $801 \mu\text{g/L}$ (63.75%). Patients with tumor sizes either ≤ 5 cm, 5-10 cm, or ≥ 10 cm had the lowest rate of serum AFP in the AFP interval from 401 to $800 \mu\text{g/L}$. The positive rate of serum AFP levels in different intervals was not significantly different in patients with solitary or multiple tumors ($P = 0.593$). Whether the patients with vascular invasion or not, the portion of AFP values beyond $801 \mu\text{g/L}$ accounted for the most cases (49.15% and 35.30% respectively), and the positive rate of AFP value was significantly different ($P < 0.001$). The high, intermediate, and low tumor cell differentiation had significant differences in each serum AFP interval. The low differentiation group accounted for the majority of patients in the AFP interval of more than $801 \mu\text{g/L}$ (41.12%), and the patients with high tumor cell differentiation had the highest negative rate (42.22%). The value of AFP had a statistically significant

correlation with BCLC, and the positive rate of the AFP level in the interval greater than $801 \mu\text{g/L}$ was found to be significantly different ($P < 0.001$). However, AFP levels greater than $801 \mu\text{g/L}$ tended to exist in the highest percentage of patients among all stages under the BCLC and China staging systems.

Multiple variables analysis of tumor related factors elevating the level of AFP

In the binary logistic multiple-regression models, the level status of AFP ($> 400 \mu\text{g/L}$) was used as a dependent variable. Both low tumor cell differentiation (OR 6.362, 95%CI: 2.891-15.382, $P = 0.006$) and tumor size (≥ 10 cm) (OR 5.215, 95%CI: 1.426-13.151, $P = 0.012$) were independent predictors of elevated AFP concentrations.

DISCUSSION

Serum tumor markers are useful in detecting malignant carcinoma rapidly and simply using biochemical methods. In this study, we focused on AFP, which is the most commonly used tumor marker in HCC. To date, many previous studies have evaluated the relationship between serum AFP levels and tumor-related clinicopathological factors. However, in the majority of those studies, researchers^[14,19,21,26] only took one or two clinicopathological variables into consideration, such as tumor size, tumor number, or portal venous invasion. Moreover, few of these researchers investigated these relationships in a comprehensive manner. With these facts in mind, we obtained a thorough understanding of the correlation between tumor markers and various clinicopathological variables.

It is known to all that, HBV is the major hepatocarcinogen which is responsible for up to 80% of HCC worldwide. There are over 400 million patients infected with chronic HBV globally, which equals to over 5% of the world's whole population; and it is estimated that about 20% of these infected individuals may eventually develop HCC^[34]. Just as the relationship between HBV and HCC has been clarified, AFP is found higher in HBV-related HCC than in non-HBV-related HCC^[35]. Hann *et al*^[36] conducted a clinic based longitudinal cohort study (617 cases and followed for up to 22 years) retrospectively to determine the predictive role of baseline AFP value in the prediction of the long-term risk of developing HCC in HBV patients. Their conclusion was that elevated serum AFP was significantly associated with increased risk of HCC in HBV patients and that high levels of serum AFP were associated with the higher risk of developing HCC in non-cancer HBV patients. Our result indicated that 81.4% patients was in status of HBV infection and their serum AFP level raised up at quite a high level compared with non-HBV infection patient. Mutual influence of the HCC and HBV might have effect on the level of AFP.

Our study showed that patients' serum AFP concen-

trations were progressively higher with increasing tumor sizes, which is consistent with the results of previous studies^[15-17], and the same pattern could be observed for the incidence rate of serum AFP greater than 801 µg/L. Specifically, the negative rates of serum AFP concentrations (< 20 µg/L) decreased as tumor sizes increased. This particular focus on tumor sizes and different cutoff values might be useful in suggesting criteria values for the screening and diagnosing of HCC. Moreover, it is still controversial as to whether AFP concentration has a positive correlation with tumor size worldwide^[14,26,37]. One possible reason for this difference may be that the size of HCC tumors included in other studies was relatively smaller than in our study. We divided patients into three groups according to the longest axis: ≤ 5 cm, 5-10 cm and ≥ 10 cm. Nevertheless, the conventional division manner of tumor sizes in previous studies focused on a diameter ≤ 5 cm. Another possible reason for these differences may be due to the difference in the tumor stages of the patients enrolled in these studies. As for the tumor sizes, the serum AFP level showed no tendency to increase with increasing tumor number, and the incidence of different intervals of the AFP level showed no clear correlation with tumor number as well. This result was not consistent with other studies^[12], which showed a significant relationship between AFP levels and tumor number. The main difference was that the interindividual variation of secretion ability of tumor cell played a more important role in elevating the serum AFP concentration compared with tumor cell number.

The cause of cancer recurrence in HCC patients is mainly due to tumor cells spreading *via* the portal and hepatic veins. Additionally, vascular invasion has been proven to be an adverse prognostic factor for HCC recurrence. It is important to detect vascular invasion at earlier stages of the cancer. In our study, there was a relationship between AFP concentration and the presence of indices of tumor vascular invasiveness, that is, the serum AFP value was significantly higher in patients with various vascular invasions compared to those patients without vascular invasion. Among the patients with vascular invasion, the majority of them had AFP values greater than 801 µg/L (49.15%). Some early studies have shown that high serum AFP levels are a good indication of the higher incidence of vascular invasion in patients with HCC, but the total incidence of vascular invasion was 25.61% in all of our cases, which is slightly lower than some other studies^[21,38,39]. This deviation from the literature might be due to the definition of vascular invasion in our study, which focused on the involvement of the vessels within the portal and hepatic vein and biliary ducts, not including the vessels within the fibrous tumor capsule.

Before patients underwent hepatectomy, it is impossible to exactly distinguish the degree of differentiation of the tumor cell by imaging techniques, and fine needle aspiration is not recommended because it has the risk of

the cancer seeding. Hence, a serum AFP value greater than 400 µg/L might be a useful criterion for predicting the histological tumor grade^[19]. In the present study, the serum AFP value increased according to the change of tumor cell differentiation from high to low differentiation, which strongly suggests that the tumor cell with elevated malignant potential will relatively enhance the secretion capacity. Previous studies that investigated the relationship between AFP and tumor cell differentiation, namely Kentaroh Yamamoto^[14], Koicci Oishie^[19] and Chaur-Shine Wang^[16] and Fabio Farinati^[40], reached the same conclusion. Together, these findings highlighted that during the course of the back and forth liver cell necrosis and regeneration, cell proliferation and serum AFP values are abnormally elevated. With respect to the tumor staging system, several staging systems are available, such as Okuda, CLIP, BCLC, Child-Pugh staging, TNM staging and the French classification. In terms of our study, the main function of tumor staging is presented in three main concepts: the malignant characteristics of tumor cells, the degree of liver impairment and the patients' general condition. As a result, BCLC and TNM staging are used in our study. Another staging system that was included was China staging. With our data, BCLC had the dominant advantage compared to the other two staging systems given that it showed a clear relationship with increasing AFP levels. This result may be due to BCLC integrating the Okuda and Child-Pugh staging systems, which consist of tumor features and conditions of liver functions. The serum AFP level of 400 µg/L was adopted as the cutoff value based on the Milan and Hangzhou criterion^[41] for liver transplantation. In our study, patients with AFP levels less than 400 µg/L had less vascular invasion and were at a lower tumor stage. Based on Hangzhou criteria, HCC patients with a simultaneous total tumor diameter of smaller than 8 cm, histopathology grade of I or II and a preoperative AFP level of less than or equal to 400 µg/L, can be selected for liver transplantation and have good outcomes.

The limitation of this study is that it does not include other serum tumor markers, such as AFP-L3 and des-gamma-carboxy prothrombin (DCP), to assess the correlation among them and to compare differences in their relationship with various clinicopathological variables. AFP-L3 and DCP have been proposed as complements or substitutes in the diagnosis of HCC. A high serum AFP-L3 fraction has been proven to be closely related to the poor differentiation of HCC^[42]. Research has also indicated that a high serum DCP is more useful than AFP in the differential diagnosis of HCC from other benign hematopathologist and is unique in detecting small tumors in patients^[43]. The reason we did not include AFP-L3 and DCP is that few cases have examined these markers in our hospital so far. Next, we plan to accumulate enough HCC cases with DCP and AFP-L3 data to investigate the detailed relationship with HCC and to compare them to the relationship with the serum AFP

concentration.

Although some clinical doctors regard serum AFP as 'obsolete', measuring AFP concentrations in the blood is still the easiest and preferred serum tumor marker for the current screening and diagnosing of HCC in China. Despite the promising results of these newly discovered serum tumor markers, such as DCP and AFP-L3, they are only regarded as a complementary test to the AFP value and currently cannot match the diagnostic and prognostic value of AFP. At least for the proportion of AFP seropositive patients, AFP is indeed demonstrated to be associated with larger tumor sizes, vascular invasion and low tumor differentiated grades, which confirmed the rationality of using BCLC with AFP values in our study. However, physicians should be aware that neither a positive nor a negative serum AFP result is conclusive for the final diagnosis of HCC.

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COMMENTS

Background

Hepatocellular carcinoma (HCC) is one of the most common and important cancers in the world that is associated with a poor prognosis. Although imaging studies are the most important and valuable diagnostic tool for HCC, α -fetoprotein (AFP) is also indispensable for diagnosing HCC. Regarding the clinicopathological features of HCC with AFP, several investigators have reported a high frequency of vascular invasions, intrahepatic metastasis, large tumor size, low differentiation, multiple tumor number, and worse survival in HCC patients with high levels of AFP.

Research frontiers

AFP has been the most widely used tumor marker worldwide and is still the golden standard amongst diagnostic markers for HCC. However, its diagnostic value is more and more questioned, due to poor sensitivity and specificity. To date, several other tumor markers have been investigated and compared with AFP, such as plasma des-c-carboxy prothrombin, also known as protein induced by vitamin K deficiency or antagonist-II, the lens culinaris agglutinin-reactive fraction of α -fetoprotein and Golgi protein-73 have been investigated as complements for AFP, despite the promising results of these new potential markers, at this moment, they are only recommended as complementary tests to the conventionally diagnostic methods used and cannot (yet) replace serum AFP as the gold standard of tumour markers for HCC. As a result, a comprehensive investigation with large samples in regard to the relationship between clinicopathological features of HCC and AFP is needed urgently.

Innovations and breakthroughs

The authors analyzed the correlation between serum AFP levels and characteristics of the patients with HCC. Together with the sample size of the study, which is large enough to obtain statistically relevant results, this article could be useful for the physicians at the clinical practice, keeping in mind that represent a guidelines for the clinicopathological features of HCC, as long as the AFP result is accompanied by other diagnosis techniques.

Applications

The present study provides a whole description of the relationship of various clinicopathological features of HCC with serum level of AFP, these finding in the article will play an important role in clinical decision making in future.

Terminology

AFP is a glycoprotein and synthesized by the yolk sac during early foetal life and later on by the foetal liver. In adult life, AFP synthesis is repressed. Elevated serum levels of AFP are only seen in maternal serum during pregnancy, in certain tumors (gastric cancer, lung cancer, pancreatic cancer, testicular car-

cinoma and mainly in HCC) and hepatic non-tumor disease (chronic hepatitis and liver cirrhosis).

Peer review

This is a large study about the diagnostic and prognostic abilities of AFP in hepatocellular carcinoma. The series is impressive and various interesting conclusions are reached. In this sense, the article has merit and would represent a valuable contribution to the literature.

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Double-balloon enteroscopy for mesenchymal tumors of small bowel: Nine years' experience

Qiong He, Yang Bai, Fa-Chao Zhi, Wei Gong, Hong-Xiang Gu, Zhi-Min Xu, Jian-Qun Cai, De-Shou Pan, Bo Jiang

Qiong He, Yang Bai, Fa-Chao Zhi, Wei Gong, Hong-Xiang Gu, Zhi-Min Xu, Jian-Qun Cai, De-Shou Pan, Bo Jiang, Guangdong Provincial Key Laboratory of Gastroenterology, Department of Gastroenterology, Nanfang Hospital, Southern Medical University, Guangzhou 510515, Guangdong Province, China
Author contributions: He Q and Bai Y performed this study; He Q carried out data analysis and wrote the paper; Bai Y, Zhi FC, Gong W, Gu HX, Xu ZM, Cai JQ, Pan DS, Jiang B performed capsule endoscopy and double balloon enteroscopy; and Bai Y and Zhi FC designed the study.

Correspondence to: Fa-Chao Zhi, MD, Guangdong Provincial Key Laboratory of Gastroenterology, Department of Gastroenterology, Nanfang Hospital, Southern Medical University, Guangzhou 510515, Guangdong Province, China. zfc@fimmu.com
Telephone: +86-20-61641532 Fax: +86-20-61641532
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Abstract

AIM: To assess the value of double-balloon enteroscopy (DBE) for the diagnosis of gastrointestinal mesenchymal tumors (GIMTs) in the small bowel and clarify their clinical and endoscopic characteristics.

METHODS: A retrospective review in a total of 783 patients who underwent a DBE procedure from January 2003 to December 2011 was conducted. Data from patients with pathologically confirmed GIMTs were analyzed at a single tertiary center with nine years' experience. The primary outcomes assessed included characteristics of patients with GIMTs, indications for DBE, overall diagnostic yield of GIMTs, endoscopic morphology, positive biopsy, comparison of diagnosis with capsule endoscopy, and subsequent interventional management.

RESULTS: GIMTs were identified and analyzed in 77 patients. The mean age was 47.74 ± 14.14 years

(range: 20-77 years), with 63.6% being males. The majority of individuals presented with gastrointestinal bleeding, accounting for 81.8%, followed by abdominal pain, accounting for 10.4%. Small bowel pathologies were found in 71 patients, the detection rate was 92.2%. The diagnostic yield of DBE for GIMTs was 88.3%. DBE was superior to capsule endoscopy in the diagnosis of GIMTs ($P = 0.006$; McNemar's χ^2 test). Gastrointestinal stromal tumor was the most frequent and leiomyoma was the second frequent GIMT. Single and focal lesions were typical of GIMTs, and masses with smooth or unsmooth surface were the most common in the small bowel. GIMTs were removed from all the patients surgically except one patient treated with endoscopic resection.

CONCLUSION: DBE is a safe and valuable procedure for patients with suspected GIMTs, and it provides an accurate position for subsequent surgical intervention.

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Key words: Small bowel tumor; Mesenchymal tumor; Double-balloon enteroscopy; Capsule endoscopy

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INTRODUCTION

Small bowel diseases (SBDs) are less common in the entire digestive tract^[1]. As a result of deep anatomical location of the small bowel (SB) and nonspecific clinical manifestations of SBDs, the diagnosis and management

of SBDs are frequently delayed, leading to considerable medical cost and poor prognosis^[2]. Early identification, diagnosis and intervention for SBDs become extremely important in clinical practice. With the development of capsule endoscopy (CE) and balloon-assisted enteroscopy (BAE), a new era has been created for the diagnosis of SBDs.

Gastrointestinal mesenchymal tumors (GIMTs), including myogenic tumors, neurogenic tumors and gastrointestinal stromal tumors (GISTs), account for less than 10% of gastrointestinal tumors^[5-5]. Radiological imaging, such as barium study, computed tomography and angiography, is usually performed to examine GIMTs without the advent of CE and double-balloon enteroscopy (DBE). Traditional examination by barium study is feasible for bigish intraluminal SBTs^[6,7]. CT is used to locate the lesion, assess for invasion and detect metastasis of SBTs^[8,9]. Angiography is effective for detecting SBTs with active bleeding. GIMTs are common in the SB, varying from the duodenum to the ileum^[10]. Their true incidences might be higher than those reported, as novel methods such as CE and BAE are much more sensitive and specific in diagnosing GIMTs than conventional methods^[7]. CE is performed to detect SBTs and produce a higher detection rate due to its advantage of invasiveness^[11-14].

Several studies reported DBE for the diagnosis of SBTs, indicating that DBE is a safe and effective procedure that enables accurate diagnosis of SBTs^[15-19]. To date, few studies have reported the diagnosis of GIMTs by DBE and described their clinical and endoscopic features. This study was conducted retrospectively to evaluate the usefulness and safety of DBE for the diagnosis of GIMTs and to understand their clinical and endoscopic characteristics.

MATERIALS AND METHODS

Patients

Retrospective chart review was conducted in 783 consecutive patients who were suspected to have SBDs and investigated by DBE between January 2003 and December 2011 at a single center (a university teaching hospital). The data of the patients were reviewed, including demographic data, examinations prior to DBE, indications for DBE, the locations of GIMTs, endoscopic findings, removal mode of GIMTs, histopathological findings and postoperative management.

Written informed consents were obtained from each patient and/or their guardians. The study was approved by the Institutional Review Board of Nanfang Hospital, Southern Medical University, Guangzhou, China.

DBE procedure

All DBE procedures were performed with no absolute contraindications. A low residue and liquid diet was prescribed for the patients undergoing this procedure, and colored food was avoided at least one day prior to the procedure. All the patients completed bowel cleansing

Table 1 Demographic data of patients undergoing double-balloon enteroscopy

| Characteristics | |
|--------------------------------------|---------------------------|
| Sex (M/F) | 49/28 |
| Age (mean \pm SD, range, yr) | 47.74 \pm 14.14 (20-77) |
| Duration (mean \pm SD, range, mo) | 25.0 \pm 37.7 (0.2-156) |
| Prior blood transfusion (Y/N) | 46/31 |
| Prior abdominal/pelvic surgery (Y/N) | 8/69 |
| Previous examination before DBE | |
| Gastroduodenoscopy | 73 |
| Colonoscopy | 66 |
| Push enteroscopy | 1 |
| Barium study | 10 |
| CT | 12 |
| MRI | 2 |
| Angiography | 2 |
| Meckel's scan | 3 |
| Bone marrow aspiration | 2 |
| Indications for DBE | |
| Melena/hematochezia | 63 |
| Abdominal pain | 8 |
| Debilitation | 1 |
| Vomiting | 1 |
| Distention | 1 |
| Weight loss | 1 |
| Physical examination | 2 |

M: Male; F: Female; Y: Yes; N: No; DBE: Double balloon enteroscopy; CT: Computed tomography; MRI: Magnetic resonance imaging.

preparation by ingesting a 1.8-2 L polyethylene-glycol solution followed by an overnight fasting, at least 6-10 h prior to the start of the procedure.

The Fujinon DBE system (Fujinon Inc, Japan) introduced in our center in 2003 was used and reported previously elsewhere^[20-22]. All procedures were carried out by experienced endoscopists. The selection of transoral or transanal approach was based on the clinical manifestations and/or suspected findings from prior examinations such as barium study, CT scan, and CE findings. The opposite routine was performed after making a positional mark by India ink if negative findings were detected by the peroral routine, and vice versa. If a lesion was detected by DBE, a positional mark was performed as well.

Statistical analysis

Statistical analysis was performed using the software SPSS Version 17.0 for Windows. Continuous data were presented as means, mean \pm SD or range, and categorical variables were expressed as frequency or percentages. The χ^2 test was used to compare differences in categorical variables examined. Agreement analysis was assessed by the Kappa statistic. A *P* value < 0.05 (two-sided) was considered statistically significant.

RESULTS

Demographics of clinical data

A total of 77 inpatients who underwent DBE were identified; their final diagnoses were confirmed as GIMTs by histopathology and/or surgery. Characteristics of all the

Table 2 Comparison of diagnosis in 31 patients evaluated by capsule endoscopy and double balloon enteroscopic investigation

| CE findings (<i>n</i> = 31) | DBE findings (<i>n</i> = 31) | | Total |
|------------------------------|-------------------------------|----------|-------|
| | Positive | Negative | |
| Positive | 18 | 1 | 19 |
| Negative | 11 | 1 | 12 |
| Total | 29 | 2 | 31 |

Kappa = 0.036. CE: Capsule endoscopy; DBE: Double-balloon enteroscopy.

patients are shown in Table 1. The mean age was 47.74 ± 14.14 years (range: 20-77 years), with 63.6% being males. The majority of patients presented with GI bleeding, accounting for 81.8%, followed by abdominal pain, accounting for 10.4%.

All the patients underwent other medical examinations prior to DBE, including gastroduodenoscopy (73 cases), colonoscopy (66 cases), and push enteroscopy (1 case), and yielded negative or suspected diagnoses. Barium study was conducted in 10 patients, only one patient was suspected of having a SBT. Twelve patients received CT scan, SBT was found in two patients and suspected SBT was found in one patient. Two patients were found to have suspected SBT by magnetic resonance imaging, 2 by angiography, 3 by Meckel's scan and 2 by bone marrow aspiration.

Thirty-one patients underwent CE examination before DBE within an interval of two weeks. All patients successfully completed CE procedures which reached the colon. Positive diagnoses were made in 11 patients, and suspected diagnoses in 8 patients. No lesion was detected in 12 patients. No complications occurred during and after the procedure. Thirty-seven DBE procedures were performed in 31 patients, including 22 antegrade approaches, 3 retrograde approaches, and 6 combinations of the two approaches. The sensitivity of DBE and CE for the diagnosis of GIMTs was 93.5% and 61.3%, respectively. DBE for the diagnosis of GIMTs was superior to CE ($P = 0.006$, McNemar's χ^2 test) (Table 2).

Endoscopic diagnosis and management

A total of 93 DBE procedures were performed in 77 patients, including 49 antegrade DBE approaches, 12 retrograde DBE approaches and 16 combinations of the two approaches. Total enteroscopy (TE) was achieved in 3 patients. Lesions were found in the small bowel in 71 patients, the detection rate for GIMTs being 92.2%. Clear diagnosis was established in 68 patients, and the diagnostic yield of DBE for GIMTs was 88.3%. Multiple tissue samplings were made in 41 cases; positive diagnoses were obtained in 5 cases. Only one therapeutic procedure was performed in one patient, *i.e.*, a leiomyoma (8 mm) was removed by DBE. All the patients successfully completed the entire DBE procedure, without any complications occurring during and after the procedure.

Among 9 patients with unclear diagnosis by DBE,

one was found with overt, ongoing bleeding, and two were found with single ulcerative lesions in proximal small bowel, respectively. No abnormality was found in six patients, including two patients treated with the combination of the two approaches (neither completed TE), one with the antegrade approach, the other three with the retrograde approach. Patients with indefinite diagnoses underwent surgical procedures (laparotomy or laparoscopic exploration) because of persistent symptoms. Five patients had GIMTs with extraluminal growth confirmed by surgery; one patient undergoing the antegrade approach had a GIMT located in the ileum.

Endoscopic and clinical features

Endoscopic diagnosis was established in the overwhelming majority of the patients. Most GIMTs presented as a single lesion under the endoscopic view, protruding into the intra-luminal mass in the small bowel. The unsmooth surface of the tumor was seen most frequently, showing the appearance of erosion or ulcer (Figure 1). The second frequent morphology was a mass with smooth surface, indicating a tumor with sessile base in a rounded or oval shape (Figure 2). Rare GIMTs presented with irregular shapes under endoscopic view.

In this study, GIMTs with confirmed diagnoses included GIST (60 cases), leiomyoma (6 cases), lipoma (3 cases), hemangioma (3 cases), lymphangioma (3 cases), fibrous histiocytoma (1 case), and angiosarcoma (1 case). Based on the primary sites of tumors, GIMTs in our study were all primary tumors verified surgically and pathologically. Two kinds of GIMTs were detected on the basis of site, including intra- and extra-luminal tumors. Intra-luminal GIMTs were detected most frequently and verified by endoscopy and surgery (Figures 1, 2). A single lesion was most frequently examined, except in two patients who had multiple lymphangiomas. GIMTs were detected most frequently in the jejunum (60 cases), and next in the ileum (16 cases) and duodenum (1 case). No spread and metastasis was investigated and confirmed after surgical removal.

Postoperative management and follow-up

The findings of DBE changed the therapeutic plan and enabled all the patients to receive early intervention. The clinical symptoms disappeared after surgery and all the patients felt an improvement in their conditions. They received an average follow-up time of 14.5 mo after intervention, and important improvements were obtained in the patients after DBE and surgical intervention. No complication was reported.

DISCUSSION

Since the introduction of CE and DBE, the blind spot of the entire GI tract has been revealed and investigated thoroughly. Our study reported the diagnosis of GIMTs using the DBE technique and their clinical characteristics. We analyzed the data of all the subjects registered in the

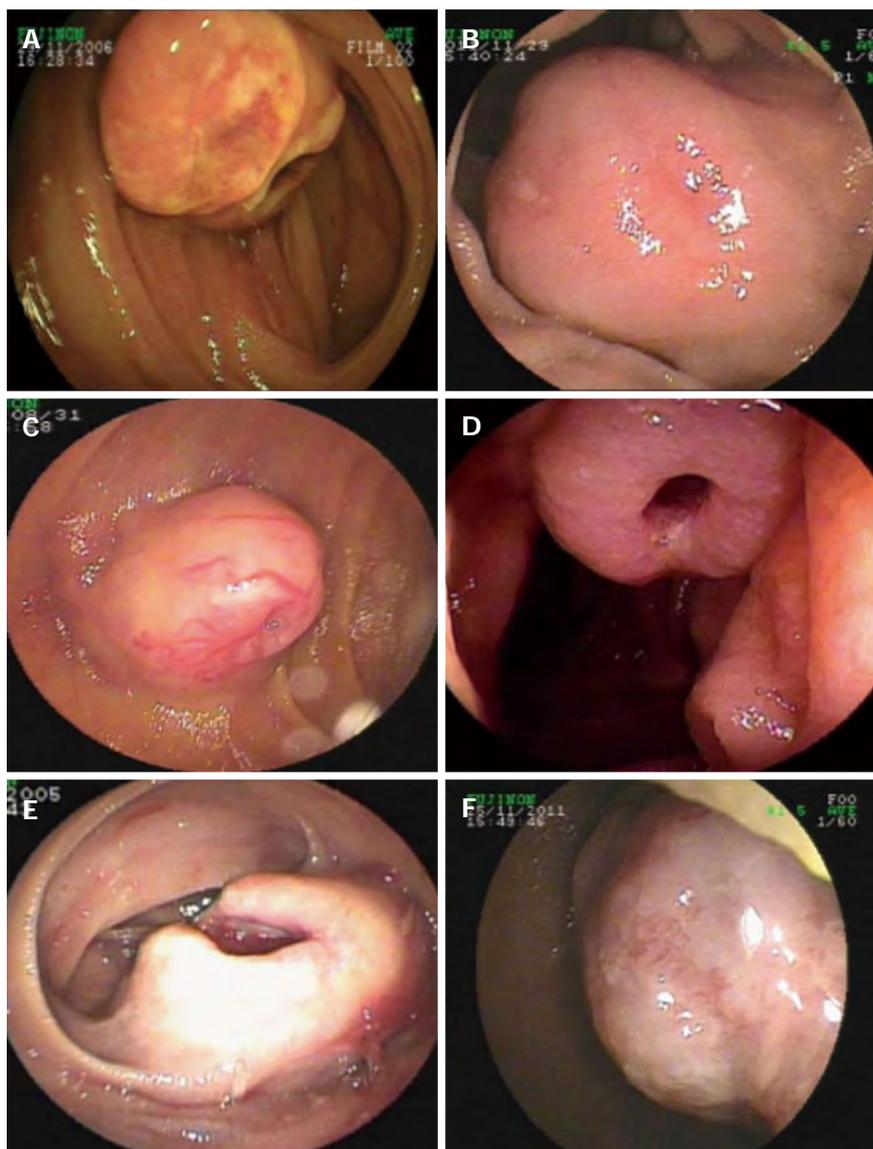


Figure 1 Unsmooth surface of verified gastrointestinal mesenchymal tumors, showing the appearance of erosion or ulcer. A, B, F: Ulcerative lesions in the surface of the tumors; C, D, E: Ulcerative and depressed pits (A-D: Gastrointestinal mesenchymal tumor; E: Leiomyoma; F: Lipoma).

DBE database in China, which represented more than 780 patients investigated by DBE for a variety of indications after the introduction of this modality. DBE produced a higher detection rate because GIMTs were diagnosed in 77 (9.8%) of 783 subjects who were suspected to have SBDs.

Other diagnostic modalities such as barium study and CT scan were used to detect SBTs prior to the introduction of CE and DBE. However, confirmative analysis is unfeasible for GIMTs. Moreover, surgical intervention should be performed cautiously in patients with indefinite diagnosis by these examinations. Small lesions are difficult to examine by these traditional examinations. DBE is performed to permit real-time visualization of the tumors and make a positional mark, which helps the surgeons to reveal the lesions. In our study, prior examinations only established clear diagnoses in a few patients. This may result in delayed interventions for GIMTs in patients

with negative diagnoses. Most GIMTs in the small bowel may be malignant and invasive. Early detection and diagnosis of GIMTs using DBE would be conducive to early intervention and improvement of prognosis. Therefore, DBE is a reliable method as a complementary tool for traditional methods (such as barium meal and CT) or as a direct means for detecting GIMTs in the small bowel.

As a noninvasive and pain-free tool for investigating the small bowel, these advantages have made CE more competitive than DBE. Previous studies have shown that using CE to diagnose SBTs produced a higher accuracy in suspected patients^[2,11,14,23]. Even though a small proportion of subjects received CE examinations before DBE, clear diagnosis for GIMTs established by CE was significantly lower than that by DBE in this study. This may be because of the nature of CE and confirmation of previous reports. CE is performed to visualize the GI tract according to bowel movement, this feature is

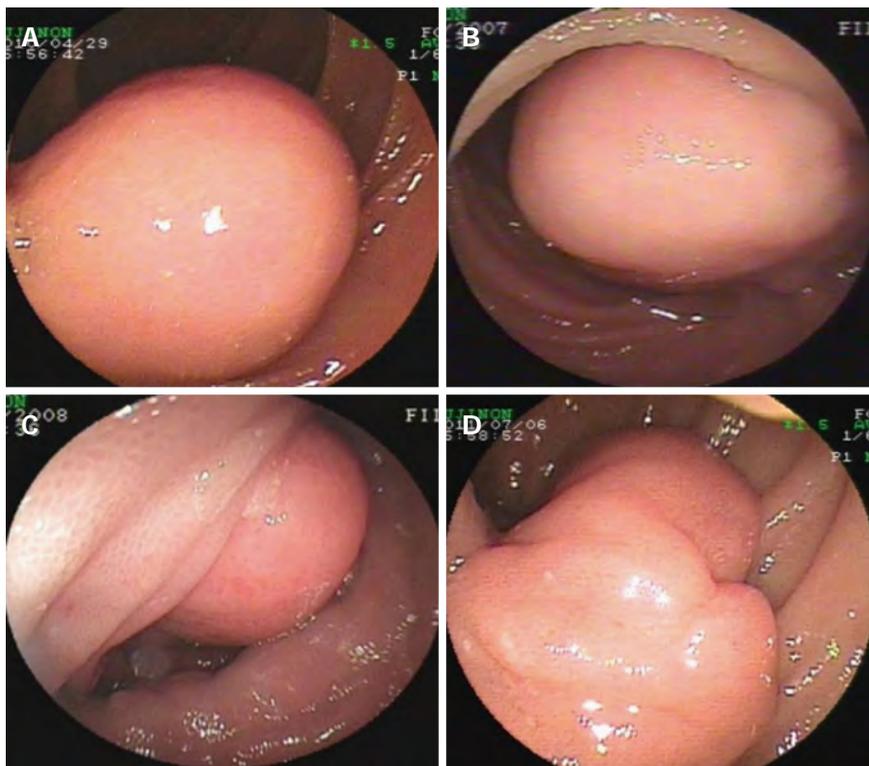


Figure 2 Morphology of verified tumors with smooth surface, indicating tumors with sessile base in round or oval shape. A-C: Single tumor with round shape and smooth surface (A, B: Gastrointestinal mesenchymal tumor; C: Lipoma; D: A polyp-like tumor with expanded tail, and hemangioma was confirmed by post-surgical pathology).

both an advantage and a disadvantage. False positive or false negative findings for SBTs are the significant limitations of CE^[24,25]. Missed diagnosis may occur during the examination because of only forward movement^[26]. An intra-luminal tumor without mucosal damage or with less protuberance into the lumen is a significant challenge for CE to establish a clear diagnosis. In diagnosis of GIMTs in this study, CE failed to detect the presence of tumors in some patients. This shortcoming can be overcome by DBE through straightening the intestinal tube, which can reduce greatly the possibility of missed tumors. Moreover, severe complications, such as intestinal obstruction or CE retention, may occur during the procedure^[27-31]. The most important aspect is that biopsy and histopathological establishment are unavailable for CE. These shortcomings of CE can be overcome by BAE.

Although previous studies have reported the diagnosis of DBE for SBTs and characterized the features of SBTs,^[15,17-19,32] there are differences in distinct types of tumors arising from different tissues. Furthermore, a few patients with confirmed GIMTs were detected in these studies. The present study exclusively focused on the diagnosis and characteristics of GIMTs investigated by DBE. In theory, tumors from mesenchymal tissues have similar clinical and endoscopic characteristics. As reported in the literature^[17-19,32], most SBTs are detected in adult patients and GI bleeding is a major indication for the DBE procedure. We found that patients with GIMTs were all adults. Males predominantly accounted for more

than half of the patients. GI hemorrhage is the most frequent symptom of GIMTs. The main site of SBTs reported in the literature is the ileum^[33]. Confirmed GIMTs from the current findings were almost exclusively located in the jejunum. Moreover, our rate of GIMTs (77/783) is higher than that previously reported^[18]. In fact, GISTs represented 77.9% of GIMTs in our series. It is reported that this type of tumor is more frequently seen in the proximal small bowel^[18,19].

We found that a single and focal lesion is typical of GIMTs, and that masses with a smooth or unsmooth surface are the most common in the small bowel. As far as GIMTs were concerned, precise diagnosis is readily concluded using the DBE procedure before histopathological analysis, which is judged by the endoscopic characteristics of GIMTs. According to our experiences and the findings of the present study, preoperative endoscopic diagnosis is consistent with the final histological diagnosis. As reported by Mitsui *et al*^[18] 40.9% of patients with GISTs were positively diagnosed by the biopsy specimen. In our series, the rate of positive diagnosis by biopsy pathology was lower in patients with GIMTs (12.2%) than that reported previously. Biopsy diagnosis for GIMTs is not as effective as postoperative pathology of the excised specimen. Therefore, endoscopic diagnosis by BAE for GIMTs is effective and significant in clinical practice.

In conclusion, DBE is a safe and valuable procedure for patients with suspected GIMTs, and provides accurate position for subsequent surgical intervention.

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COMMENTS

Background

Previous studies reported that double balloon enteroscopy (DBE) is a safe and effective procedure for the diagnosis of small bowel tumors. To date, few reports have focused on the diagnosis of gastrointestinal mesenchymal tumors (GIMTs) detected by DBE and described their clinical and endoscopic features.

Research frontiers

A growing number of studies have been conducted on the diagnosis of small bowel diseases (SBDs) since the introduction of double-balloon enteroscopy. The current study exclusively focuses on the diagnosis of mesenchymal tumors in the small bowel and their clinical characteristics.

Innovations and breakthroughs

This study reported the diagnosis of GIMTs using the DBE technique and their clinical characteristics in 780 patients investigated by DBE for a variety of indications. DBE produced a higher detection rate because GIMTs were diagnosed in 77 (9.8%) out of 783 subjects who were suspected to have SBDs.

Applications

DBE is a safe and valuable procedure for patients with suspected GIMTs, and provides an accurate position for subsequent surgical intervention.

Peer review

This study is interesting, representing one of the largest DBE series ever reported and provides very good information for gastroenterologists. DBE is a very useful diagnostic tool for SBDs, particularly for the diagnosis of GIMTs for both gastroenterologists and surgeons.

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Multicenter case-control study of the risk factors for ulcerative colitis in China

Yu-Fang Wang, Qin Ou-yang, Bing Xia, Li-Na Liu, Fang Gu, Kai-Fang Zhou, Qiao Mei, Rui-Hua Shi, Zhi-Hua Ran, Xiao-Di Wang, Pin-Jin Hu, Kai-Chun Wu, Xin-Guang Liu, Ying-Lei Miao, Ying Han, Xiao-Ping Wu, Guo-Bing He, Jie Zhong, Guan-Jian Liu

Yu-Fang Wang, Qin Ou-yang, Department of Gastroenterology, West China Hospital, Sichuan University, Chengdu 610041, Sichuan Province, China

Bing Xia, Department of Gastroenterology, Zhongnan Hospital, Wuhan University School of Medicine, Wuhan 430071, Hubei Province, China

Li-Na Liu, Department of Gastroenterology, First Affiliated Hospital of Dalian Medical University, Dalian 116021, Liaoning Province, China

Fang Gu, Department of Gastroenterology, Third Hospital of Beijing University, Beijing 100191, China

Kai-Fang Zhou, Department of Gastroenterology, Union Hospital Tongji Medical College, Huazhong Science and Technology University, Wuhan 430074, Hubei Province, China

Qiao Mei, Department of Gastroenterology, First Affiliated Hospital of Anhui Medical University, Hefei 230022, Anhui Province, China

Rui-Hua Shi, Department of Gastroenterology, First Affiliated Hospital of Nanjing Medical University, Nanjing 210029, Jiangsu Province, China

Zhi-Hua Ran, Department of Gastroenterology, School of Medicine, Renji Hospital, Shanghai Jiaotong University, Shanghai 200127, China

Xiao-Di Wang, Department of Gastroenterology, Beijing Sino-Japan Friendship Hospital, Beijing 10029, China

Pin-Jin Hu, Department of Gastroenterology, First Affiliated Hospital, Zhongshan Medical University, Guangzhou 528000, Guangdong Province, China

Kai-Chun Wu, State Key Laboratory of Cancer Biology and Xijing Hospital of Digestive Diseases, Fourth Military Medical University, Xi'an 710032, Shaanxi Province, China

Xin-Guang Liu, Department of Gastroenterology, First Affiliated Hospital, Peking University, Beijing 100034, China

Ying-Lei Miao, Department of Gastroenterology, First Affiliated Hospital of Kunming Medical College, Kunming 650032, Yunnan Province, China

Ying Han, Department of Gastroenterology, the Military General Hospital of Beijing PLA, Beijing 10026, China

Xiao-Ping Wu, Department of Gastroenterology, Xiangya Hospital, Second Affiliated Hospital of Zhongnan University, Changsha 410008, Hunan Province, China

Guo-Bing He, Department of Gastroenterology, Affiliated Hospi-

tal of North Sichuan Medical College, Nanyun 637000, Sichuan Province, China

Jie Zhong, Department of Gastroenterology, Ruijin Hospital, Shanghai Jiaotong University, Shanghai 200025, China

Guan-Jian Liu, Department of Epidemiology, West China Hospital, Sichuan University, Chengdu 610041, Sichuan Province, China

Author contributions: All these authors contributed equally to this paper; all authors designed the questionnaire and performed the investigation; Wang YF and Ou-yang Q designed the study and wrote the manuscript; Liu GJ analyzed the data.

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Correspondence to: Qin Ou-yang, Professor, Department of Gastroenterology, West China Hospital, Sichuan University, Chengdu 610041, Sichuan Province, China. qin.ouyang@163.com

Telephone: +86-28-85422387 Fax: +86-28-85423387

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Abstract

AIM: To evaluate potential risk factors in the development of ulcerative colitis (UC) in China.

METHODS: A total of 1308 patients with UC and 1308 age-matched and sex-matched controls were prospectively studied in China. The UC cases were collected from 17 hospitals in China from April 2007 to April 2010. Uniform questionnaires were designed to investigate risk factors including smoking, appendectomy, stress, socio-economic conditions, nonsteroidal anti-inflammatory drugs (NSAIDs), oral contraceptives, diet, breastfeeding, infections and family sanitary conditions. Group comparisons by each factor were done using simple logistic regression analysis. Conditional logistic regression was used for multivariate analysis.

RESULTS: By univariate analysis, the variables predictive of UC included feeling stress, light and heavy alcoholic drinking, spicy food, sugar consumption and infectious diarrhea, while heavy tea intake and tap water consumption were protective against UC. On multivariate analysis, the protective factor for UC was tap water consumption [odds ratios (OR) = 0.424, 95%CI: 0.302-0.594, $P < 0.001$]; while the potential risk factors for UC were heavy sugar consumption (OR = 1.632, 95%CI: 1.156-2.305, $P < 0.001$), spicy food (light intake: OR = 3.329, 95%CI: 2.282-4.857, $P < 0.001$; heavy intake: OR = 3.979, 95%CI: 2.700-5.863, $P < 0.001$), and often feeling stress (OR = 1.981, 95%CI: 1.447-2.711, $P < 0.001$). Other factors, such as smoking habit, appendectomy, breastfeeding, a history of measles, rural or urban residence, education, oral contraceptives, and NSAID use have not been found to have a significant association with the development of UC in the present study.

CONCLUSION: Our study showed tap water consumption was a protective factor for UC, while spicy food, heavy sugar consumption and often feeling stress were risk factors for UC in this Chinese population.

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Key words: Ulcerative colitis; Risk factors; Case-control study

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INTRODUCTION

The causes of inflammatory bowel diseases (IBD), including Crohn's disease (CD) and ulcerative colitis (UC), remain unknown. IBD is thought to arise in genetically predisposed individuals who mount an aberrant immune response to gut microbiota secondary to some environmental trigger^[1]. Genetic susceptibility in the population should be relatively stable; it cannot account for the rapid rise of IBD incidence in China. Environmental factors may play an important role in the pathogenesis of IBD.

IBD has been primarily characterized as a disease of industrialized nations, emerging in the early 20th century in developed countries. In the past 20 years, incidence rates of traditionally high incidence areas such as North America and Europe have remained relatively stable or even decreased^[2], while the diseases have become more prevalent in previously low incidence areas as they have become industrialized^[3]. Recent surveys in China have

also shown that IBD, especially UC, has been increasing in China^[4-7].

A number of environmental risk factors have been explored in Western countries. There is no such epidemiologic study of large sample groups in Chinese populations. China presents an opportunity to study an environment that is evolving more to a Western-style based on changes in socioeconomics, diet habit, *etc.* The present study aims to assess the potential risk factors for the development of UC in the Chinese population by a multicenter case-control study.

MATERIALS AND METHODS

Patients and controls

A multicenter case-control study was undertaken in China during a 3-year period from April 2007 to April 2010. Patients were collected from 17 hospitals in 12 areas of China including Beijing and Dalian, which were chosen from Northern China; Shanghai, Nanjing and Hefei from Eastern China; Guangzhou from Southern China; Chengdu, Xian, Nanchong, Kunming from Western China; Wuhan and Changsha from Central China. The investigators were invited to take part in the program if their medical centers fulfilled the following requirements: (1) the medical center should be a university hospital or grade A tertiary hospital; and (2) diagnostic facilities for high-quality endoscopy, radiology and pathology should be available. The diagnosis of UC was made by clinical, laboratory, endoscopic, and histologic examinations in accordance with the suggested guidelines for the diagnosis and treatment of IBD, which were approved in China in 2007^[8].

Controls were randomly selected in the same area as friends, neighbors or colleagues of the patients and matched by sex and age during the study. The controls were healthy volunteers with no bowel disease or other severe disease.

Questionnaire survey

UC patients and controls were interviewed to complete a questionnaire. A uniform questionnaire was designed by the IBD collaborative group of the Chinese Digestive Association. The questionnaire included an exploration of dietary habits, smoking, alcohol use, oral contraceptives, nonsteroidal anti-inflammatory drugs (NSAIDs), previous appendectomy, breast-feeding in infancy, childhood measles virus infection, parasite infection, infectious gastroenteritis, education, employment, stress and family sanitary conditions (including lavatory and water conditions). Subjects were instructed to answer questions in such a way that the information reflected their behavior and characteristics prior to the appearance of disease symptoms. Participating investigators or supervised physicians were in charge of the questionnaire.

In the questionnaire, study subjects were asked about the consumption of milk, green tea, alcohol, spicy food,

Table 1 Social characteristics and household factors between ulcerative colitis and healthy controls

| Variables | UC | Control | P value | OR | 95%CI |
|--------------------------|-------|---------|---------|-------|-------------|
| Living area | | | | | |
| Rural | 16.4% | 15.7% | | | |
| Town | 16.6% | 12.9% | 0.617 | 0.930 | 0.702-1.234 |
| City | 67.0% | 71.4% | 0.054 | 0.711 | 0.569-1.887 |
| Education | | | | | |
| Primary or below | 14.3% | 13.2% | | | |
| Secondary | 39.7% | 36.8% | 0.959 | 0.994 | 0.781-1.265 |
| College or above | 46.0% | 50.0% | 0.164 | 0.846 | 0.669-1.071 |
| Feeling stress | | | | | |
| None | 21.5% | 21.3% | | | |
| Occasionally | 55.6% | 66.5% | 0.056 | 0.828 | 0.682-1.005 |
| Often | 22.9% | 12.2% | 0.000 | 1.858 | 1.440-2.398 |
| Water source | | | | | |
| Boiled well or tap water | 82.0% | 68.5% | | | |
| Well water | 3.4% | 1.9% | 0.118 | 1.494 | 0.903-2.471 |
| Tap water | 5.4% | 21.6% | 0.000 | 0.21 | 0.159-0.279 |
| Mineral water | 9.2% | 8.0% | 0.758 | 0.957 | 0.723-1.267 |
| Lavatory | | | | | |
| Closestool | 11.3% | 9.8% | | | |
| Squat pan | 39.4% | 41.6% | 0.173 | 0.829 | 0.634-1.085 |
| Flush toilet | 49.3% | 48.6% | 0.368 | 0.886 | 0.680-1.154 |
| Refrigerator | 78.7% | 81.1% | 0.181 | 0.86 | 0.690-1.073 |

UC: Ulcerative colitis; OR: Odds ratio.

and sugar in the year before the appearance of symptoms. Consumption degree of each food item was assessed using the following three categories: none or rare, light (1-2 times a week) and heavy (3-6 times a week or every day for a period of at least 2 mo). Consumption of meat and vegetables was classified into the following 3 categories: vegetarian, meat-eater, and balanced diet. Vegetarian was defined as eater of fruits and vegetables, and never or hardly eats meat or any animal products. Meat-eater was defined as eater of meat and never or hardly eats fruits and vegetables. Balanced diet was defined as consumption of both meat and vegetables.

Oral contraceptive use was defined as use for at least 1 mo for any indication including birth control, hormone replacement therapy, regulation of menstrual disorders, or other reasons. NSAID use was defined as taking related drugs at least twice per week for a period of at least one month.

The questionnaire ascertained whether subjects were non-smokers, current smokers and ex-smokers. Non-smokers were defined as those who never or rarely smoked. Current smokers were those who had smoked more than 1 cigarette per day within 6 mo before the diagnosis of UC. Ex-smokers were defined as patients who quit smoking more than 6 mo before the diagnosis of UC.

The frequency of feeling stress was classified into none, occasionally (feeling stress 1-2 times a week) and often (feeling stress 3-6 times a week or every day for a period of at least one month).

Statistical analysis

Statistical analyses were performed using SPSS17.0 software. We compared the background characteristics of UC with those of control subjects by two-sample *t* tests or χ^2 tests. Group comparisons by each factor were done using simple logistic regression analysis. Multiple logistic regression analysis was performed to propose a final set of independent risk factors for UC. Odds ratios (OR) and 95%CI were calculated. Odds ratios are provided for associations that were statistically significant at a *P* value < 0.05.

RESULTS

Demographic characteristics

A total of 1308 UC patients and 1308 age-matched and sex-matched controls were enrolled. The age of the patients at diagnosis ranged from 16 to 70 years. The average age was 41.6 ± 12.3 years for UC patients and 41.4 ± 13.5 years for healthy controls. The male to female ratio was 1.23:1 both for UC patients and controls. There were no statistically significant differences between UC and the controls in age and sex ratio (*P* > 0.05).

Univariate analysis of risk factors for UC

Sociodemographic characteristics and family sanitary conditions for both UC patients and controls are shown in Table 1. Patients with UC were more likely to feel stress (22.9%) than controls (12.2%) (OR = 1.858, 95%CI: 1.440-2.398, *P* < 0.001). However, UC patients tended to be less likely to have used tap water as their primary water source compared with boiled well or tap water *vs* controls (OR = 0.210, 95%CI: 0.159-0.279, *P* < 0.001). There were no statistically significant differences between UC and the controls in living area in recent 5 years, educational status, lavatory and refrigerator details.

Dietary factors between the two groups are shown in Table 2. Compared with the group of none or rare tea intake, heavy tea intake before the diagnosis seemed to have a protective effect on the development of UC (OR = 0.738, 95%CI: 0.591-0.922, *P* = 0.007). In contrast to nonalcoholic drinkers, light and heavy alcoholic drinkers before the diagnosis were at a higher risk of developing the disease (light drinkers: OR = 1.264, 95%CI: 1.073-1.490, *P* = 0.005; heavy drinkers: OR = 1.453, 95%CI: 1.122-1.882, *P* = 0.005). Compared with the group of none or rare spicy food intake, UC patients were significantly more likely to ingest spicy food than controls (light intake: OR = 2.432, 95%CI: 1.943-3.043, *P* < 0.001; heavy intake: OR = 3.189; 95%CI: 2.513-4.046, *P* < 0.001). Compared with the group of none or rare sugar intake, subjects with UC were also significantly more likely to ingest sugar than controls (light intake: OR = 3.162, 95%CI: 2.480-4.032, *P* < 0.001; heavy intake: OR = 3.390, 95%CI: 2.921-5.288, *P* < 0.001). There were no significant differences in milk, vegetable and meat consumption between two groups. Medical histories and

Table 2 Dietary factors between ulcerative colitis and healthy controls

| Variables | UC | Control | P value | OR | 95%CI |
|------------------------|-------|---------|---------|-------|-------------|
| Milk | | | | | |
| None or rare | 23.9% | 24.5% | | | |
| Light | 55.8% | 58.5% | 0.845 | 0.981 | 0.806-1.293 |
| Heavy | 20.3% | 16.9% | 0.096 | 1.237 | 0.963-1.590 |
| Tea intake | | | | | |
| None or rare | 18.5% | 17.7% | | | |
| Light | 50.5% | 42.3% | 0.231 | 1.139 | 0.921-1.409 |
| Heavy | 30.9% | 40.0% | 0.007 | 0.738 | 0.591-0.922 |
| Alcohol | | | | | |
| None or rare | 40.5% | 47.0% | | | |
| Light | 46.9% | 43.0% | 0.005 | 1.264 | 1.073-1.490 |
| Heavy | 12.5% | 10.0% | 0.005 | 1.453 | 1.122-1.882 |
| Spicy food consumption | | | | | |
| None or rare | 11.1% | 25.2% | | | |
| Light | 50.6% | 47.4% | 0.000 | 2.432 | 1.943-3.043 |
| Heavy | 38.3% | 27.4% | 0.000 | 3.189 | 2.513-4.046 |
| Sugar | | | 0.000 | 1.989 | 1.718-2.301 |
| None or rare | 12.3% | 31.8% | | | |
| Light | 64.8% | 53.1% | 0.000 | 3.162 | 2.480-4.032 |
| Heavy | 23.0% | 15.2% | 0.000 | 3.390 | 2.921-5.288 |
| Vegetable and meat | | | | | |
| Vegetarian | 13.3% | 12.4% | | | |
| Meat-eaters | 10.4% | 9.4% | 0.421 | 0.909 | 0.720-1.147 |
| Balanced diet | 76.3% | 78.2% | 0.848 | 1.032 | 0.745-1.432 |

UC: Ulcerative colitis; OR: Odds ratio.

other issues between the two groups are shown in Table 3. As shown in Table 3, UC patients (14.1%) were more likely to report that they had ever had infectious diarrhea than controls (9.3%) (OR = 1.610, 95%CI: 1.256-2.064, $P < 0.001$). There were no significant differences in smoking status, breast feeding during infancy, appendectomy, measles and parasite infection, NSAID and oral contraceptive use between the two groups.

Multivariate analysis of risk factors for UC

The multivariate analysis by logistic regression analysis showed that consumption of tap water was a protective factor for UC (OR = 0.424, 95%CI: 0.302-0.594, $P < 0.001$); while the potential risk factors for UC were often feeling stress (OR = 1.981, 95%CI: 1.447-2.711, $P < 0.001$), spicy food consumption (light intake: OR = 3.329, 95%CI: 2.282-4.857, $P < 0.001$; heavy intake: OR = 3.979, 95%CI: 2.700-5.863, $P < 0.001$), and heavy sugar consumption (OR = 1.632, 95%CI: 1.156-2.305, $P < 0.001$).

DISCUSSION

This is the first multicenter study to investigate the risk factors of UC patients in the Chinese population. We conducted a case-control study during a 3-year period and evaluated a number of potential risk factors in a group of UC patients and a group of age-matched and sex-matched controls.

The major finding of the present study (on multivariate analysis) was to demonstrate that some dietary habits

Table 3 Smoking, breast feeding and medical history between ulcerative colitis and healthy controls

| Variables | UC | Control | P value | OR | 95%CI |
|-------------------------|-------|---------|---------|-------|-------------|
| Smoking | | | | | |
| Nonsmoker | 74.8% | 74.5% | | | |
| Current smoker | | | | | |
| < 10 cigarettes per day | 13.5% | 13.4% | 0.982 | 1.003 | 0.798-1.259 |
| > 10 cigarettes per day | 7.7% | 8.9% | 0.292 | 0.859 | 0.648-1.139 |
| Ex-smoker | 4.0% | 3.2% | 0.272 | 1.264 | 0.832-1.922 |
| Breast feeding | 46.5% | 53.5% | 0.628 | 1.080 | 0.790-1.477 |
| Infectious diarrhea | 14.1% | 9.3% | 0.000 | 1.610 | 1.256-2.064 |
| Appendectomy | 3.4% | 3.2% | 0.737 | 0.929 | 0.602-1.432 |
| NSAIDs | 9.0% | 7.2% | 0.209 | 1.269 | 0.875-1.841 |
| Measles | 9.4% | 8.2% | 0.286 | 1.162 | 0.882-1.532 |
| Parasite | 15.7% | 11.2% | 0.053 | 1.486 | 0.946-1.926 |
| Oral contraceptive | 6.3% | 5.2% | 0.082 | 2.734 | 0.880-8.495 |

UC: Ulcerative colitis; NSAIDs: Nonsteroidal anti-inflammatory drugs; OR: Odds ratio.

including sugar consumption, spicy food and tap water consumption were related to UC development.

First of all, our study confirmed that sugar consumption was related to an increased risk of UC. Previous studies have found strong positive associations between foods with a relatively high amount of sugar and CD^[9,10]. Tragnone *et al*^[11] confirmed that both UC and CD patients had a higher intake of total carbohydrate, starch, and refined sugar than did healthy controls. Russel *et al*^[12] reported consumption of cola drinks and chocolate were positively associated with developing ulcerative colitis. However, a negative relationship was found for carbohydrate consumption in a Canadian study^[13]. The possible reasons for this finding were studied in CD. Rashid *et al*^[14] report that high dietary starch intake increases the growth of intestinal microflora, among which *Klebsiella* microbes constituted an important part. Increased exposure to *Klebsiella* in the gut leads to high production of anti-*Klebsiella* antibodies as well as autoantibodies to the cross-reactive self-antigens with resultant inflammation at the pathological sites. Another study^[15] shows that translocation of *Escherichia coli* is reduced by soluble non-starch polysaccharide, but increased by the emulsifier Polysorbate-80. Although all studies above were related to CD, perhaps sugar is having the same deleterious effects in UC as it does in CD through effects on the composition of intestinal microflora or on the permeability of the gastrointestinal mucosa.

Secondly, spicy food is confirmed to be a risk factor for UC in our study. Another study from China also shows spicy food may contribute to the progress of UC^[16]. Capsaicin is the spicy component of hot peppers. Capsaicin can promote intestinal vasodilation, stimulate mucus secretion, and sometime even lead to diarrhea. Therefore, capsaicin may lead to changes in mucosal barrier function or colonic motility. Some studies also show capsaicin can modulate the lymphocyte prolifera-

tive response and induce tumor necrosis factor- α secretion^[17,18]. There is no such study about spicy food and UC. The exact effect and mechanism still needs further study.

Thirdly, our study shows consumption of tap water has a protective effect for UC. A systematic environmental factor study from the EPIMAD registry reported that the regular consumption of tap water decreased the risk of CD^[19]. Another two case-control studies revealed a more frequent hot water supply among patients presenting with CD than among controls^[20,21], but failed to find such an association with UC. Although findings are inconsistent, consumption of tap water may have the same protective effect in UC as it does in CD.

This is consistent with the hygiene hypothesis of IBD, which theorizes that a lack of exposure to enteric pathogens makes one susceptible to IBD. A possible explanation is that tap water may contain non-pathogenic bacteria which thrive in untreated water, but are absent from boiled water. This kind of bacteria may play an immunoregulatory role and reduce the risk of UC or CD by helping to establish the body's normal intestinal flora.

The data above showed that some dietary habits are related to UC development. Diet is probably the single most important factor influencing the composition and metabolic behavior of the microbiota. Some dietary habits may have a consistent impact on the gut microbiome or gastrointestinal mucosa permeability, and maybe trigger an aberrant immune response in genetically predisposed individuals.

Another finding of this study on multivariate analysis was to demonstrate that often feeling stress was a risk factor for UC. A case-control study observed an excess of life events in the 6-mo period prior to the onset of CD and UC^[22]. However, results remain controversial. The different results may be attributed to no standard method to measure stress and recall bias. Quantification and identification of stress remains a difficult task. Our study quantified "stress" by the frequency of feeling stress. As in most case-control studies, recall bias may be also a concern in this study.

In the present univariate analysis, a prior episode of infectious gastroenteritis and alcohol use were related to an increased risk of UC, while heavy tea consumption was shown to be a protective factor for UC. However, these positive associations failed to be demonstrated in the multivariate analysis.

Two large-scale case-control studies have also shown that IBD risk is higher in patients with a prior episode of infectious gastroenteritis, but specific pathogenic agents cannot be found in IBD^[23,24]. The hypothesis is that an enteric infection may trigger an initial change in the gut epithelial barrier resulting in exposure to microflora and disturbed adaptive and innate immune responses, leading to disease in a genetically susceptibility individual. Another possible reason for this finding may be related to antibiotics. Other studies^[25,26] have shown that exposure to antibiotics in childhood could hypothetically interfere

with the normal process of developing tolerance to enteric bacteria and may lead to IBD. However, we could not include this possible factor in the present study. Thus, further such studies are necessary to be done in China.

Alcohol has been shown to disrupt gut barrier function and increase intestinal permeability^[27,28]. However, another study reported an inverse relation between alcohol use and UC^[29]. Some studies also showed potential anti-inflammatory properties of green tea^[30,31]. It is likely that these factors are simply cofactors with other variables. Further studies are needed to examine these findings.

Other factors, such as smoking habit, appendectomy, breast-feeding in infancy, a history of measles, rural or urban residence, education, oral contraceptives, and NSAID use have not been found to have a significant association with the development of UC in the present study.

A main disadvantage of the study is that it is retrospective, relying on recall of childhood events and ingestions. There is a potential recall bias on some variables such as dietary ingestions. However, the recall issues should equally apply to cases with IBD and to controls. These data should be tested prospectively.

Exploring UC risk factors in China will be a great opportunity to advance our understanding of disease pathogenesis by investigating this aspect when the disease is newly emerging. Our study showed that often feeling stress, and a high intake of spicy food and sugar may enhance the risk of developing UC, while consumption of tap water may reduce the rate of presenting with UC. Smoking and appendectomy, which have been demonstrated to be associated with the development of UC in western studies, could not be confirmed in our study. These differences provide further evidence that environmental factors may influence development of UC and may give us some valuable clues to the cause of the disease. Thus, further studies are necessary to better understand the environmental determinants of IBD.

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COMMENTS

Background

Ulcerative colitis (UC) and Crohn's disease (CD) are collectively referred to as inflammatory bowel disease (IBD). The etiology of IBD has been extensively studied; however, causative factors are not yet fully understood. IBD is thought to arise in genetically predisposed individuals who mount an aberrant immune response to gut microbiota secondary to some environmental triggers. Genetic susceptibility in the population should be relatively stable, it cannot account for the rapid rise of IBD incidence in China. Environmental factors may play an important role in the pathogenesis of IBD. A number of environmental risk factors have been explored in western countries. However, there is a lack of such epidemiologic study in Chinese populations.

Research frontiers

A number of environmental risk factors of UC and CD have been explored in Western countries, including smoking, appendectomy, stress, socio-economic conditions, nonsteroidal anti-inflammatory drugs, oral contraceptives, diet, breastfeeding, infections/vaccinations, antibiotics, and childhood hygiene. However, most of these factors have demonstrated inconsistent findings.

Innovations and breakthroughs

The relationship between the risk of UC and environmental factors has been well studied, but the results are inconsistent in different racial populations. To date, there is no such epidemiologic study of large samples in Chinese populations. China presents an opportunity to study an environment that is evolving more to a Western-style based on changes in socioeconomics, diet habit, *etc.*

Applications

This study shows consumption of tap water is a protective factor for UC, while spicy food, heavy sugar consumption, and stress are risk factors for UC in Chinese populations.

Peer review

This is well done article. A great deal of work went into this study and should not be wasted. It is a clear paper with convincing results.

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Case of autoimmune hepatitis with markedly enlarged hepatoduodenal ligament lymph nodes

Hideki Fujii, Naoki Ohnishi, Kazuho Shimura, Masafumi Sakamoto, Tohru Ohkawara, Yoshihiko Sawa, Koichi Nishida, Yasuo Ohkawara, Tatsuro Kobata, Kanji Yamaguchi, Yoshito Itoh

Hideki Fujii, Naoki Ohnishi, Kazuho Shimura, Masafumi Sakamoto, Tohru Ohkawara, Yoshihiko Sawa, Koichi Nishida, Yasuo Ohkawara, Department of Internal Medicine, Aiseikai Yamashina Hospital, Shichouno-cho, Takehana, Yamashina-ku, Kyoto 602-8086, Japan

Tatsuro Kobata, Department of Gastroenterology and Hepatology, Uji Tokushukai Hospital, Kyoto 611-0042, Japan

Kanji Yamaguchi, Yoshito Itoh, Molecular Gastroenterology and Hepatology, Kyoto Prefectural University of Medicine, Graduate School of Medical Science, Kyoto 602-8566, Japan

Author contributions: Fujii H wrote the manuscript; Kobata T, Ohnishi N, Shimura K, Sakamoto M, Ohkawara T, Sawa Y, Nishida K, Ohkawara Y and Yamaguchi K took part in the discussion; Itoh Y designed the manuscript.

Correspondence to: Yoshito Itoh, MD, PhD, Associate Professor, Molecular Gastroenterology and Hepatology, Kyoto Prefectural University of Medicine, Graduate School of Medical Science, Kawaramachi-Hirokouji, Kamigyō-ku, Kyoto 602-8566, Japan. yitoh@koto.kpu-m.ac.jp

Telephone: +81-75-2515519 Fax: +81-75-2510710

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Abstract

Autoimmune hepatitis (AIH) is a necroinflammatory liver disease of unknown etiology. The disease is characterized histologically by interface hepatitis, biochemically by increased aspartate aminotransferase and alanine aminotransferase levels, and serologically by increased autoantibodies and immunoglobulin G levels. Here we discuss AIH in a previously healthy 37-year-old male with highly elevated serum levels of soluble interleukin-2 receptor and markedly enlarged hepatoduodenal ligament lymph nodes (HLLNs, diameter, 50 mm). Based on these observations, the differential diagnoses were AIH, lymphoma, or Castleman's disease. Liver biopsy revealed the features of interface hepatitis without bridging fibrosis along with plasma cell infiltration which

is the typical characteristics of acute AIH. Lymph node biopsy revealed lymphoid follicles with inflammatory lymphocytic infiltration; immunohistochemical examination excluded the presence of lymphoma cells. Thereafter, he was administered corticosteroid therapy: after 2 mo, the enlarged liver reached an almost normal size and the enlarged HLLNs reduced in size. We could not find AIH cases with such enlarged lymph nodes (diameter, 50 mm) in our literature review. Hence, we speculate that markedly enlarged lymph nodes observed in our patient may be caused by a highly activated, humoral immune response in AIH.

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Key words: Autoimmune hepatitis; Humoral immune response; Hepatoduodenal ligament lymph nodes; Corticosteroid; Hepatomegaly

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INTRODUCTION

Autoimmune hepatitis (AIH) is histologically characterized by inflammatory cell infiltration (plasma cell-dominant), piecemeal necrosis in the portal area of the liver, hypergammaglobulinemia, and autoantibodies in the serum^[1,2]. The onset is frequently insidious with nonspecific symptoms; however, the clinical spectrum is wide, ranging from an asymptomatic presentation^[3,4] to an acute severe disease such as fulminant hepatitis^[5,6]. The

diagnosis is based on typical histological changes in the liver and the presence of autoantibodies in the serum after excluding other etiologies that cause liver diseases. Because there can be a wide range of presentations at onset, a prompt diagnosis is required to achieve a favorable prognosis.

Here we report the case of a previously healthy patient who developed acute hepatitis with markedly enlarged hepatoduodenal ligament lymph nodes (HLLNs).

CASE REPORT

A 37-year-old male presented to a hospital with the complaints of general fatigue, loss of appetite, and icterus for the past two weeks. He was a non-smoker and non-drinker with no relevant medical history. Until that date, blood biochemistry (including liver function) was normal. He gave no history of previous trauma, indulgence in casual sex, or illicit drug abuse. He was suffering from mild atopic dermatitis that was not treated. Laboratory examination revealed a high serum total bilirubin (T-Bil) levels of 13.8 mg/dL, aspartate aminotransferase (AST) levels of 828 IU/L, alanine aminotransferase (ALT) levels of 823 IU/L and alkaline phosphatase (ALP) levels of 1055 IU/L. In addition, the HLLNs were markedly enlarged along with elevated serum levels of the soluble interleukin-2 receptor (sIL-2R, 2167 U/mL).

Eventually, he was referred to our hospital. Upon admission, his blood pressure, pulse rate, and body temperature were normal. Neurological examination did not reveal hepatic encephalopathy; however, severe icterus was observed. The liver was palpable > 5 cm below the costal margin and was smooth and hard. Mild lymphadenopathy of the axillary lymph nodes (diameter ≤ 10 mm) was observed, which were palpable but asymptomatic.

The laboratory data collected at the time of admission are summarized in Table 1. Following were the important parameters recorded for evaluation: AST, 1068 IU/L; ALT, 696 IU/L; T-Bil, 15.6 mg/dL; prothrombin time/international normalized ratio (PT/INR), 1.2; immunoglobulin (Ig) G, 3814 mg/dL; anti-nuclear antibody (ANA) × 2560; anti-mitochondrial antibody (AMA)-negative; anti-smooth muscle antibody-negative; serum sIL-2R, 2550 U/mL.

Extensive serological screening was conducted to identify liver injury caused by viral infection. Subsequently, the following tests were negative: hepatitis A virus IgM (HA-IgM), hepatitis B surface antigen (HBsAg), anti-hepatitis B core antibody (HBcAb), hepatitis C virus (HCV) RNA, fourth-generation human immunodeficiency virus screening assay, and Epstein-barr virus viral capsid antigen IgM (EBV VCA IgM). In addition, we excluded other potential causes of acute hepatitis (drug-induced liver injury, hereditary hemochromatosis, and Wilson's disease). Cytomegalovirus IgM (CMV IgM) was positive by enzyme-linked immunosorbent assay (2.06); however, CMV IgG and pp65-antigenemia (by immunofluorescent

Table 1 Laboratory data of the patient on admission

| | | | |
|---------------|----------------------------|--|--------------------------------|
| WBC | 2460/μL | IL-2 | 1.5 U/mL |
| Neut | 65% | IL-6 | 7.4 pg/mL (0-4.0 pg/mL) |
| Lym | 20% | IgG | 3814 mg/dL (820-1740 mg/dL) |
| Mono | 13% | IgA | 298 mg/dL (90-400 mg/dL) |
| RBC | 313 × 10 ⁴ /μL | IgM | 1738 mg/dL (31-200 mg/dL) |
| Hb | 10.1 g/dL | IgG4 | 97 mg/dL (4-108 mg/dL) |
| Plt | 17.5 × 10 ⁴ /μL | ANA | × 2560 |
| PT-INR | 1.2 | AMA | (-) |
| TP | 8.9 g/dL | Antismooth muscle antibody | (-) |
| Alb | 2.7 g/dL | sIL2R | 2550 U/mL |
| T-Bil | 15.6 mg/dL | HA IgM-Ab | (-) |
| D-Bil | 12.4 mg/dL | HBsAg | (-) |
| ALP | 847 IU/L | HBcAb | (-) |
| γ-GTP | 91 IU/L | IgM-HBcAb | (-) |
| AST | 1068 IU/L | HCVAb | (-) |
| ALT | 696 IU/L | HCV-RNA | (-) |
| LDH | 455 IU/L | Fourth- generation HIV screening assay | (-) |
| ChE | 130 IU/L | EBV VCA-IgM | (-) |
| Tch | 146 mg/dL | EBV VCA-IgG | (+) |
| CPK | 63 IU/L | CMV-IgM | 2.06 |
| CRP | 0.70 mg/dL | CMV-IgG | (-) |
| FBS | 95 mg/dL | pp65 antigenemia method | (-) |
| Ferritin | 504 ng/mL | | |
| Serum copper | 177 μg/dL | | |
| Ceruloplasmin | 40.2 mg/dL | | |

WBC: White blood cell; RBC: Red blood cell; PT/INR: Prothrombin time/international normalized ratio; ALP: Alkaline phosphatase; AST: Aspartate aminotransferase; ALT: Alanine aminotransferase; LDH: Lactic dehydrogenase; CPK: Creatine phosphokinase; CRP: cAMP receptor protein; FBS: Fatal bovine serum; IL: Interleukin; ANA: Anti-nuclear antibody; AMA: Anti-mitochondrial antibody; sIL-2R: Soluble interleukin-2 receptor; Ig: Immunoglobulin; HA-IgM: Hepatitis A virus IgM; HBsAg: Hepatitis B surface antigen; HBcAb: Anti-hepatitis B core antibody; HCV: Hepatitis C virus; EBV VCA: Epstein-barr virus viral capsid antigen; CMV: Cytomegalovirus; HIV: Human immunodeficiency virus.

assay of peripheral blood leukocytes) were negative. Immunoelectrophoresis revealed increased polyclonal immunoglobulins. The following types of human leukocyte antigen were detected: A24, B7, B71, DR1 and DR4.

Ultrasonography of the abdomen revealed slightly heterogeneous liver parenchyma and hypoechoic masses around the main trunk of the portal vein. Dynamic computed tomography (CT) of the abdomen revealed an enlarged liver and markedly enlarged HLLNs (50 mm in length along the major axis). Periportal edema, hepatomegaly and thickening of the gallbladder wall were apparent, thereby suggesting acute hepatitis. These lymph nodes displayed high intensity in diffusion-weighted magnetic resonance imaging. Magnetic resonance cholangiopancreatography revealed no dilation of the biliary tract. The positron emission tomography (PET)/CT using ¹⁸F-fluorodeoxyglucose (¹⁸F-FDG) revealed mild uptake of ¹⁸F-FDG in these lymph nodes. The maximum standard-

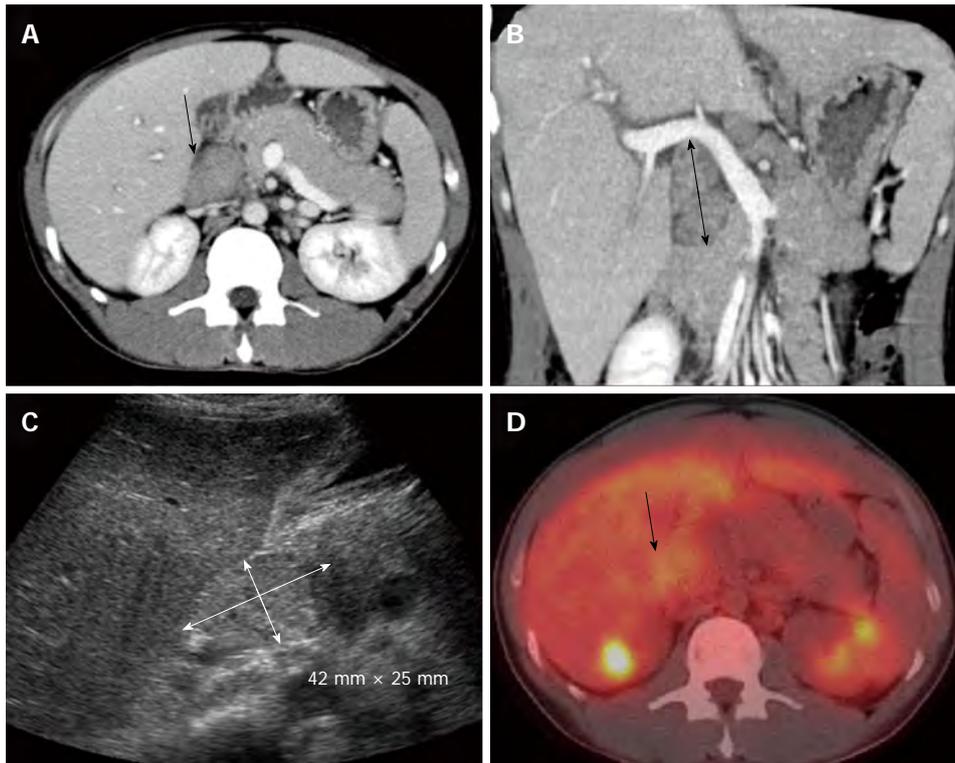


Figure 1 Imaging findings. A: Dynamic computed tomographic image (CT) of a transverse section; B: A coronal section revealing enlargement of the hepatoduodenal ligament lymph nodes (arrows); C: Ultrasonography of the abdomen revealing a large mass in the region of the hepatic portal vein (arrow); D: Positron emission tomography/CT using ^{18}F -fluorodeoxyglucose revealing mild accumulation. The maximum standardized uptake value was 3.98. The arrow shows the hepatoduodenal ligament lymph nodes.

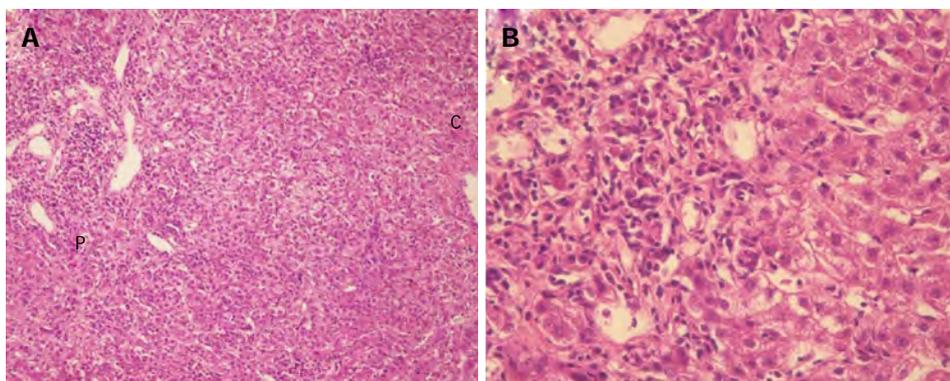


Figure 2 Liver biopsy. A: Liver biopsy specimen with hematoxylin and eosin staining ($\times 100$ magnification) revealing the histopathological appearance of acute hepatitis. Interface hepatitis and plasmacytic infiltrates are present; B: This is the same image at $\times 400$ magnification. P: Portal area; C: Central vein area.

ized uptake value was determined as 3.98 (Figure 1).

A high titer of ANA and high serum levels of IgG suggested the diagnosis of AIH; however, high serum levels of sIL-2R and markedly enlarged HLLNs prompted us to exclude the possibility of lymphoma before initiating the treatment. Biopsy of the liver and HLLNs were simultaneously performed. Liver biopsy revealed interface hepatitis and lymphocytic infiltration (plasma cell-dominant) without the formation of bridging fibrosis. Lymph node biopsy revealed lymphoid follicles with plasma cell infiltration; however, monoclonal prolifer-

ation of malignant cells was not observed by immunohistochemical staining. These observations confirmed the diagnosis of inflamed lymph nodes (Figures 2 and 3).

As per the international diagnostic criteria for AIH, our patient's score was 15^[7]. Using the simplified criteria for the diagnosis of AIH, the score was 8^[8]. These data were compatible with the final diagnosis of AIH. Moreover, the patient had a hyperbilirubinemia and a mildly reduced PT; thus, we had to consider the potential for severe acute hepatitis or fulminant hepatitis^[9]. Corticosteroid pulse therapy with 1000 mg of methylpredniso-

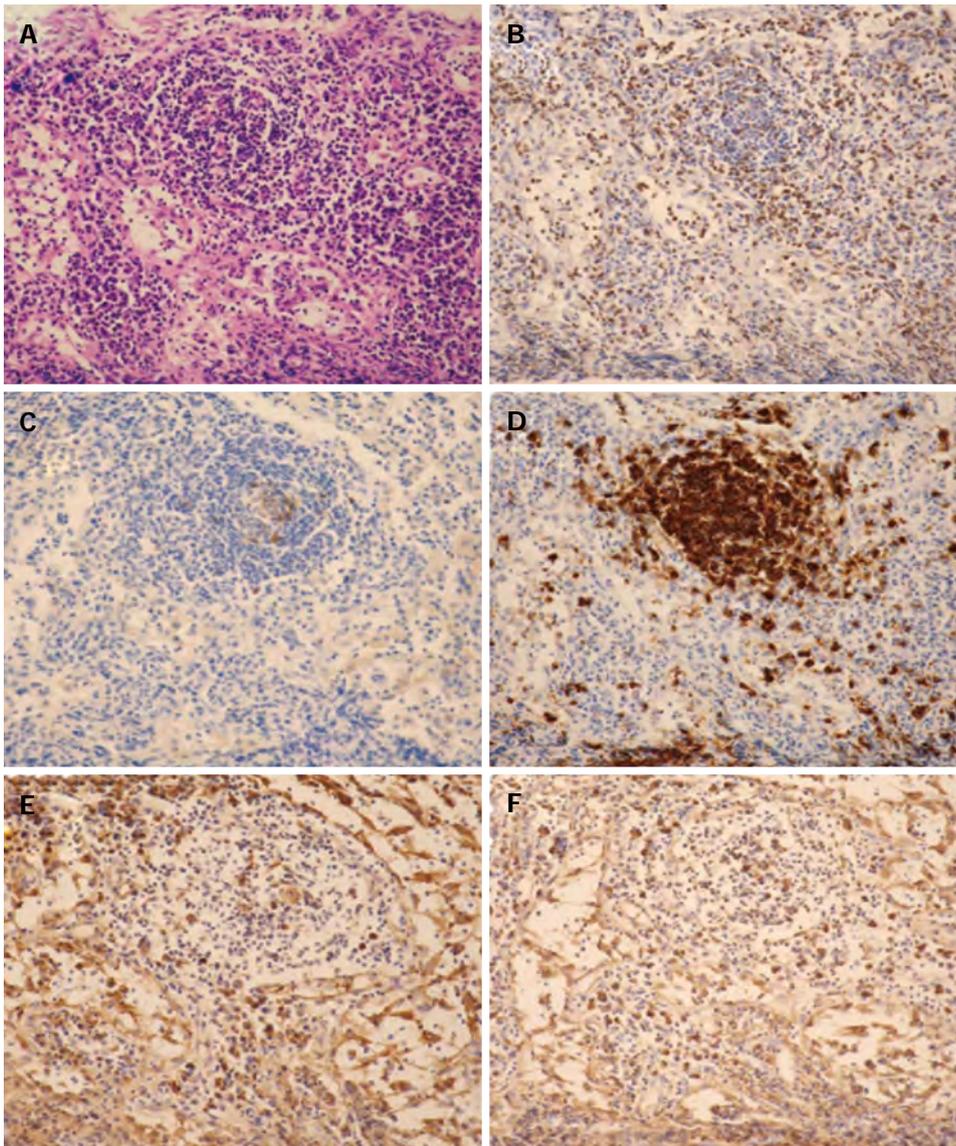


Figure 3 Histological sections of hepatoduodenal ligament lymph nodes ($\times 200$ magnification). A: The lymphoid follicles contain a reactive germinal center with hematoxylin and eosin staining. No evidences of granuloma or necrosis are visible; B: CD3 (T-cell marker) is positive in the interfollicular areas; C: CD10 (a marker of B-cell activation) is positive in the center of the follicles; D: CD20 (B-cell marker) is observed in the nodules; E: Kappa chain; F: Lambda chain. Neither the kappa nor the lambda chains predominate.

lone for 3 d was started followed by daily administration of 40 mg of prednisolone (per orally) with a dose reduction of 10 mg/d each week. Levels of AST, ALT and T-Bil improved gradually (Figure 4). At the time of discharge, the dose was 20 mg/d, thereafter the dose was reduced by 5 mg/d every 2 wk; subsequently, the levels of AST, ALT, T-Bil, and PT-INR improved. After 2 mo, the enlarged liver reached an almost normal size, and the markedly enlarged HLLNs reduced in size as well (Figure 5). Now, the levels of AST and ALT remain in the normal ranges with 5 mg/d of prednisolone after 8 mo and the size of HLLNs also presents normal size.

DISCUSSION

Here we present a case of AIH with markedly enlarged

HLLNs (50 mm in diameter). Liver biopsy revealed the features of acute phase AIH; lymph node biopsy revealed lymphoid follicles with inflammatory lymphocytic infiltration (plasma cell-dominant). He was successfully treated with oral prednisolone therapy. After 2 mo, the enlarged lymph nodes reduced in size and the serum AST and ALT levels lowered to normal ranges. In this case, a high serum titer of ANA and elevated IgG levels led us to a diagnosis of AIH. However, elevated serum levels of sIL-2R, markedly enlarged HLLNs, and accumulation of ^{18}F -FDG required us to exclude malignant lymphoma or Castleman's disease (CD).

A lymphoma in the abdominal cavity is not rare^[10], however, some cases of hepatic lymphoma (*e.g.*, lymphoplasmacytic lymphoma and T-cell/histiocyte-rich large B-cell lymphoma) have been reported to present as a dif-

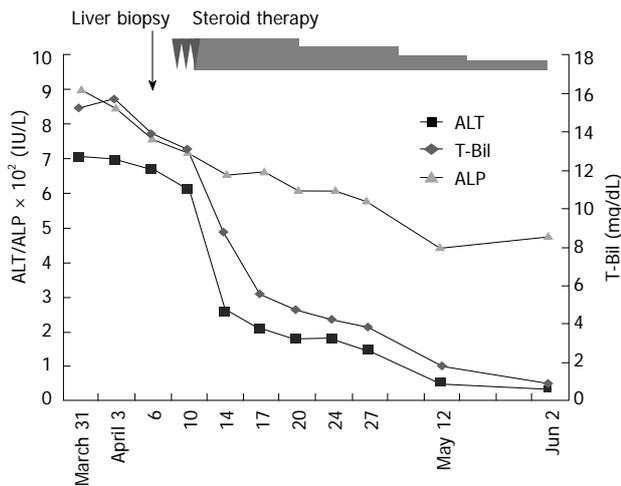


Figure 4 Clinical course. The patients showed high levels of alanine aminotransferase, alkaline phosphatase and total bilirubin. However, after the initial corticosteroid therapy, these levels improved gradually. ALT: Alanine aminotransferase; ALP: Alkaline phosphatase; T-Bil: Total bilirubin.

fuse infiltration of the liver parenchyma without forming a discrete mass^[11]. Although the imaging findings of lymphoma vary appreciably in each case^[12,13], ¹⁸F-FDG PET/CT has become widely used in the diagnosis of malignant lymphomas. However, only 67% of marginal zone lymphomas and 40% of peripheral T-cell lymphomas can take up FDG^[14]. The blood chemistry reports and markedly enlarged HLLNs with weakly positive ¹⁸F-FDG PET indicated that a malignant lymphoma could not be excluded until biopsy.

CD (also known as angiofollicular lymph node hyperplasia) is a rare non-neoplastic lymphoproliferative disorder of unknown etiology^[15]. Two clinical presentations can be distinguished. The localized (unicentric) variant of CD is the most common form of the disease and is confined to a single lymph node chain or area; histologically, it is usually a hyaline vascular form and is often asymptomatic and curable by surgical excision. The systemic (multicentric) variant of CD is less common and more aggressive; its corresponding histological pattern is a plasma cell variant and rarely the plasmablastic type^[16]. Extremely high levels of interleukin (IL)-6^[17] could be a characteristic of the multicentric variant of CD but not of IgG4-related systemic disease or other diseases presenting with lymphadenopathy^[18]. CD is usually localized to the chest (especially in the mediastinum and neck) and rarely occurs in the abdominal cavity^[19]. A differential diagnosis was necessary between AIH and the unicentric variant of CD. However, in this case, high serum levels of liver transaminases, lymph node findings, and slightly increasing levels of IL-6^[17] did not correspond to the unicentric variant of CD. Another differential diagnosis was IgG4-related disease but serum levels of IgG4 were substantially low.

A high incidence of swelling of the intra-abdominal lymph nodes has been reported in patients with non-ma-

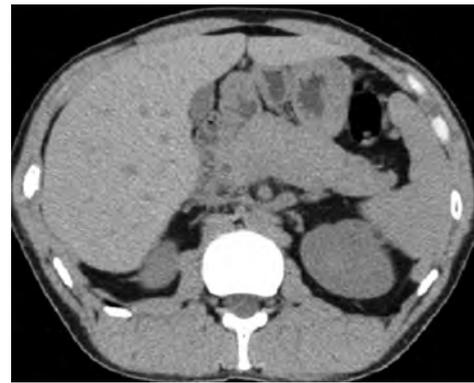


Figure 5 Computed tomography of the transverse section recorded 2 mo after starting corticosteroid. The hepatoduodenal ligament lymph nodes reduced in size.

lignant tumors, particularly subjects with chronic hepatitis B (CHB), chronic hepatitis C (CHC) or primary biliary cirrhosis (PBC)^[20]. Swelling of the lymph nodes near the common hepatic artery has been observed in 77%-91% of patients with CHC and 96% of patients with CHB. In addition, a higher incidence of lymph node swelling in PBC (74%-100%) and AIH (13%-73%) has been reported^[21]. Furthermore, some studies have reported that lymph node size can be correlated with an index of hepatocellular injury^[22,23]. However, the correlation between lymph node size and laboratory data is controversial^[24]. We could not find cases of AIH with such enlarged lymph nodes in the literature review similar to the ones described in our case. The pathological significance of enlarged lymph nodes in liver disease is unknown; however, further studies of such cases might help in clarifying its significance.

sIL-2R is an extracellular domain of a membrane-bound IL-2 receptor that is detectable on the cell surface of lymphoid cell lines such as activated T cells and natural killer cells, monocytes, eosinophils^[25-27], and on the cell surface of some tumor cells^[28] as well. The biological function of sIL-2R is incompletely understood; however, it is thought to be a marker of T-cell activation^[28]. Some studies have demonstrated that sIL-2R levels are increased in liver diseases^[29]. Liver damage in AIH is triggered by CD4+ T lymphocytes that recognize a certain autoantigenic epitope on the hepatocytes^[30,31]. An activated immune system is deeply involved in the pathophysiology of AIH; thus, highly elevated serum levels of sIL-2R could reflect the inflammatory activity of AIH, as observed in our patient.

In summary, we reported a case of AIH with elevated serum levels of sIL-2R and markedly enlarged HLLNs. Cases with such enlarged lymph nodes (50 mm in diameter) in AIH were not found in our literature review. We speculate that markedly enlarged lymph nodes might reflect a highly activated, humoral immune response in AIH, as observed in our patient.

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A case of colon perforation due to enteropathy-associated T-cell lymphoma

Jun Bong Kim, Seong Hwan Kim, Young Kwan Cho, Sang Bong Ahn, Yun Ju Jo, Young Sook Park, Ji Hyun Lee, Dong Hee Kim, Hojung Lee, Yun Young Jung

Jun Bong Kim, Seong Hwan Kim, Young Kwan Cho, Sang Bong Ahn, Yun Ju Jo, Young Sook Park, Ji Hyun Lee, Department of Internal Medicine, Eulji Medical Center, Eulji University, Seoul 139-711, South Korea

Dong Hee Kim, Department of Surgery, Eulji Hospital, Eulji Medical Center, Seoul 139-711, South Korea

Hojung Lee, Department of Pathology, Eulji Hospital, Eulji Medical Center, Seoul 139-711, South Korea

Yun Young Jung, Department of Radiology, Eulji Hospital, Eulji Medical Center, Seoul 139-711, South Korea

Author contributions: Kim JB and Kim SH contributed equally to this work; Cho YK and Ahn SB conceived and designed the study; Jo YJ, Park YS and Lee JH assisted in the research and revision of the paper; Kim DH performed operation; Lee H performed histologic and pathologic examination; Jung YY evaluated imaging studies; Kim JB and Kim SH wrote the paper; all of the authors have read and approved the final manuscript.

Correspondence to: Seong Hwan Kim, MD, PhD, Department of Internal Medicine, Eulji Medical Center, Eulji University, Seoul 139-711, South Korea. shkim@eulji.ac.kr

Telephone: +82-2-9708297 Fax: +82-2-9728621

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Abstract

Enteropathy-associated T-cell lymphoma (EATL) is an extremely rare disease, which is often related to gluten-sensitive enteropathy. It is an uncommon intestinal lymphoma with very poor prognosis and high mortality rate. In the absence of specific symptoms or radiological findings, it is difficult to diagnose early. Major complications of EATL have been known as intestinal perforation or obstruction, and only 5 cases of EATL are reported in South Korea. In this study, we report a case of 71-year-old male with symptoms of diarrhea, which later it progressed into cancer perforation of the colon. The initial colonoscopic findings were normal and computed tomography scan demonstrated a segmental

wall thickening of the distal ascending colon with non-specific multiple small lymphnodes, along the ileocolic vessels, but no signs of mass or obstruction. The histologic findings of resected specimen confirmed EATL type II. Patient expired two weeks after the operation. Therefore, we emphasize the need of random biopsy in the presence of normal mucosa appearance on colonoscopy for the early diagnosis of EATL.

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Key words: Enteropathy-associated T-cell lymphoma type II; Intestinal perforation; Gastrointestinal lymphoma; Celiac disease

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INTRODUCTION

Enteropathy-associated T-cell lymphoma (EATL) is a rare gastrointestinal non-Hodgkin's lymphoma, originating from intraepithelial T lymphocyte, which is strongly related with celiac disease^[1]. Due to its scarcity, EATL is accounted for less than 1% of all non-Hodgkin's lymphomas category, which primarily involves the small bowel^[2]. EATL has generally poor prognosis with high mortality rate. World Health Organization classified two types of EATL, defined as type I EATL, which comprises 80%-90% and type II EATL, a monomorphic variant^[3]. We have experienced the first case of EATL type II involving the whole tract of the colon in South Korea that was initially misdiagnosed as enterocolitis, resulting in the fatal colon perforation.

CASE REPORT

A 71-year-old man was admitted to the hospital with persistent diarrhea and abdominal pain for the past 6 mo. At that time, his abdominal computed tomography (CT) showed segmental wall thickening of distal ascending colon with nonspecific multiple small lymph nodes along the ileocolic vessels with colonoscopic findings, which revealed normal mucosa in the entire colon (Figure 1A). He continued to have diarrhea and diffuse abdominal pain when he arrived at our institution. He had chronic ill-looking appearance with alert mental status. The initial vital signs checked were normal, with increased bowel sound and right lower quadrant area tenderness. The laboratory findings revealed white blood cell count 3840/mm³, Hb 9.3 g/dL, and Plt 134 × 10³/mm³ with peripheral blood morphology, showing normocytic normochromic anemia. In liver chemistry, aspartate aminotransferase was increased to 61 IU/L, and C-reactive protein check 6.4 mg/dL. A plain abdominal radiography showed paralytic ileus in the large-bowel with gaseous distention. With the supportive treatment, abdominal symptoms and follow-up abdominal X-ray showed gradual improvement. He was discharged after one week of admission; however, few hours after the discharge, he revisited to emergency room with sudden onset of acute abdominal pain with high fever and drowsy mental status. Patient assumed to be at septic shock condition with blood pressure measuring 60/40 mmHg, heart-rate 104/min, respiration 27/min, and body temperature 39.7 °C. Plain abdomen radiography revealed free air on the diaphragm, which indicated the perforation of the intestine. Abdomen CT scan showed large irregular masses on the right ascending colon and the hepatic flexure was seen with hydropneumoperitoneum, indicating colon cancer perforation (Figure 1B). Also, multiple enhanced enlarged lymph nodes were found in pericolic, right iliac chain, preaortic, portal hepatis and peripancreatic area, suggesting metastatic lesions. Emergent right hemi-colectomy of large bowel was performed, and post-operative gross findings from the resected tumor showed 9 cm × 7 cm × 3 cm size mass in the ascending colon extension to serosa layer, without the invasion to the adjacent area (Figure 2). Histologic findings showed tumor cells diffusely infiltrating the whole intestinal mucosa, involving the resection margins of the terminal ileum to the colon. The lymphoma cells were small to medium-sized, with round, hyperchromatic nuclei, having a stippled chromatin pattern. Immunohistochemical staining of the tumor cells expressed CD56 and positive for CD3 and CD8 intraepithelial lymphocytes, but negative for CD20 and Epstein-Barr virus. The adjacent mucosa showed heavy lymphoid infiltrate invading in all layers of the colon layer (Figure 3). The final diagnosis confirmed type II EATL.

Two weeks after the operation, the follow up colonoscopy revealed normal mucosa and several random multiple biopsies were made in the entire colon (Figure 4). Histologic findings re-confirmed type II EATL. We recommend the treatment of cyclophosphamide, doxorubi-

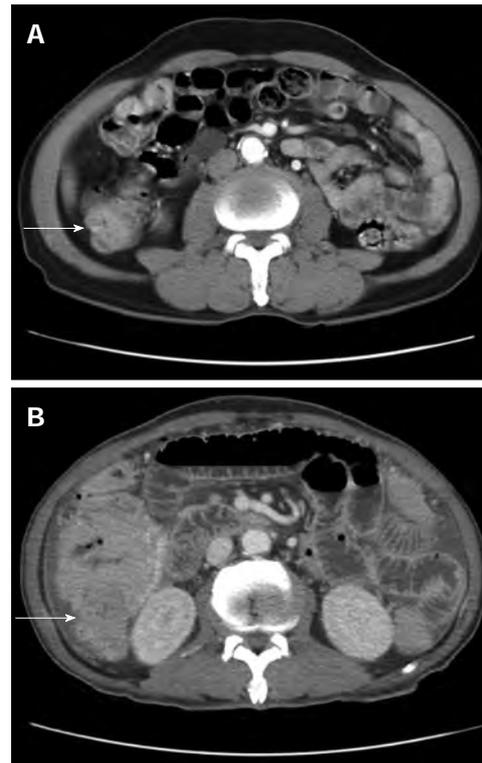


Figure 1 An abdominopelvic computed tomography shows segmental wall thickening of distal ascending with nonspecific multiple small lymph nodes along ileocolic vessels. Large irregular mass (A) in right ascending colon along hepatic flexure (B).



Figure 2 A gross specimen from the ascending colon after right hemicolectomy. In perforated site, there is an ulceroinfiltrative mass measuring 9 cm × 7 cm × 3 cm which is 13 cm away from ileocecal valve and distal resection margin, respectively. The tumor is grayish tan, fish-fresh and appears to extend to serosa.

cin, vincristine and prednisone chemotherapy to increase the chances of survival; however, he refused and wanted to go to a hospice facility near his hometown. He was transferred out and expired 3 d after the discharge from our institution.

DISCUSSION

Reports on the diffuse involvement of EATL type II are a rare finding, as described in this report. Recent ret-

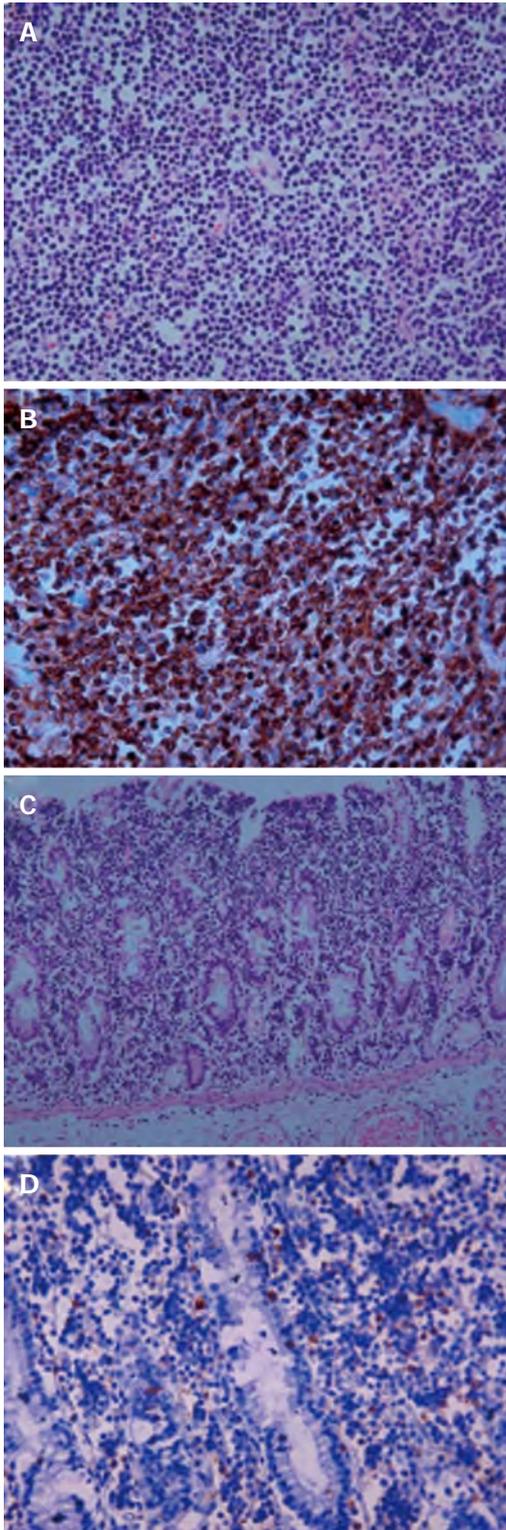


Figure 3 Histologic features in enteropathy-associated T-cell lymphoma type II. A: The lymphoma cells are small to medium-sized with round, hyperchromatic nuclei (x 400); B: The cells express CD56 (x 400); C, D: The adjacent mucosa shows heavy lymphoid infiltrate (x 200) (C) and CD8 positive intraepithelial lymphocytes (x 400) (D).

rospective study conducted by Asia Lymphoma Study Group have shown a few case series of EATL arising from an Asian population, site involving the whole colonic tract have not been reported yet. However, patho-



Figure 4 Colonoscopy revealed normal mucosa pattern in whole colonic tract.

logic features of type II EATL from Asian population were very different from those of classical EATL^[4].

Due to the absence of specific symptoms, EATL is often diagnosed after the intestinal perforation or obstruction, which makes it more difficult to give proper initial treatment. As reported in our case, the patient had an acute onset of lower abdominal pain, without other associated symptoms, and colonoscopic findings were normal with non-specific findings on radiologic studies. A similar case was reported recently on 39-year-old African-American male, who was diagnosed with EATL after emergent exploratory laparotomy^[5]. Among gastrointestinal tumors, primary gastrointestinal lymphomas comprise of 5%-10% of all non-Hodgkin's lymphoma and EATL accounts for less than 1% of all non-Hodgkin's lymphoma^[6]. The prevalence of the annual incidence of EATL is 0.5-1 per million people in Western countries^[7]. WHO classified the two types of EATL: Type I EATL more often arising in areas with the high prevalence of celiac disease, while type II EATL seen in regions where celiac disease prevalence is rare. Sharaiha *et al*^[8] reported that type I EATL is significantly increasing in the United States with close relation to celiac disease steadily rising every year. On the contrary, gluten sensitive celiac disease is rare in South Korea with very low prevalence^[9]. The immunophenotype of type II EATL show distinctive pattern, in which the tumor cells are positive in CD3, CD8+, CD56+ and TCR β + and the intraepithelial lymphocytes in the adjacent mucosa share the identical immunophenotype^[10]. The neoplastic cells have medium-sized, round, darkly staining nuclei, with a rim of a _ENREF_7 pale cytoplasm^[11]. There has been interesting case report by Okumura *et al*^[12] on the unusual type II EATL with MYC translocation in a Japanese patient, recently. As shown in our case, immunohistochemistry confirmed us to diagnose EATL and its subtype, rather than imaging study or colonoscopy. Therefore, it is crucial to require adequate biopsy and immunophenotyping to give early diagnosis and treatment to patients. With the introduction of a double balloon enteroscopy in 2003, diagnosis of celiac disease has been significantly advanced by non-surgical modalities^[13]. And a recent study on the usefulness of

diagnostic ¹⁸F-fluorodeoxyglucose-positron emission tomography (¹⁸F-FDG-PET) in distinguishing between the sites of lymphoma and refractory celiac disease over CT showed better sensitivity and specificity^[14]. Also, there was a case report using a capsule endoscopy to detect EATL involving the small bowel^[15]. As described above, double balloon enteroscopy, ¹⁸F-FDG-PET and capsule endoscopy might be all useful options to detect EATL in advanced to increase survival of patients. One-year survival rate of the intestinal T-cell non-Hodgkin's disease is 33%, and five-year survival to be 9%^[16]. The prognosis of EATL is more decimating; when treated with surgery and/or systemic chemotherapy, 80%-84% of patients die with a 5-year survival rate of 3.2%. Chemotherapy seems to be more beneficial to patients rather than surgery; however, there is no difference in the outcome between patients with type I and type II EATL^[17].

Our patient had chronic abdominal symptoms with normal colonoscopic findings. Diagnostic tools, such as plain abdominal X-ray, CT and colonoscopy findings were not effective to diagnose EATL in advance. And considering that most of colonoscopists do not perform a biopsy if mucosa shows normal pattern, this may delay from an early discovery of EATL.

In summary, we report a unique case of diffuse involvement of type II EATL of colon with perforation, who was presented with non-specific radiological findings. Therefore, we emphasize the need of a random biopsy or capsule endoscopy to diagnose EATL early. Due to the disease appearance resembling features of colitis, careful attention should be made to reveal its obscure clinical manifestations, and physicians should be aware to conduct through physical examination and adequate biopsy to rule out EATL_ENREF_10.

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Muco-submucosal elongated polyps of the gastrointestinal tract: A case series and a review of the literature

Char Loo Tan, Sze Hwa Tan, Jimmy BY So, Fredrik Petersson

Char Loo Tan, Sze Hwa Tan, Fredrik Petersson, Department of Pathology, National University Hospital System, Singapore 119074, Singapore

Jimmy BY So, Department of Surgery, National University Hospital System, Singapore 119074, Singapore

Author contributions: Tan CL, Tan SH, So JBY, Petersson F contributed to the manuscript writing and revision.

Correspondence to: Fredrik Petersson, MD, PhD, Department of Pathology, National University Health System, 5 Lower Kent Ridge Road, Singapore 119074,

Singapore. fredrikpetersson@live.se

Telephone: +65-97-714890 Fax: +65-67-780671

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Abstract

We present three cases of gastrointestinal muco-submucosal elongated polyps, two located in the duodenum and one in the descending colon. All three cases had a characteristic, “worm-like” endoscopic appearance and were lined by unremarkable mucosa. The vascular component was located in the submucosa and was composed of a mixture of variably dilated blood vessels (capillaries and veins) and lymphatics. The duodenal polyps displayed lipomatous metaplasia of the submucosal stroma. The dual vascular phenotype of the vascular component was confirmed by immunohistochemistry with D2-40 and CD31.

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Key words: Muco-submucosal elongated polyp; Vascular malformation; Endoscopic ultrasonography; Angioma; Endoscopy

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INTRODUCTION

Benign gastrointestinal polyps composed of mucosa and submucosa, the latter with a prominent vascular component are relatively unusual non-neoplastic lesions with uncertain etiopathogenesis. Such polyps have mainly been studied by Japanese investigators, but are increasingly being recognized by western investigators^[1,2]. The nomenclature pertaining to these polyps has varied. In the seminal paper on this entity, Mataka *et al*^[3] chose the term “colonic muco-submucosal elongated polyp”. Most of the reported cases have been located in the large bowel^[4-7], but three polyps with identical endoscopic and histological features have also been documented in the small bowel, including the duodenum^[8-10].

Irrespective of site, muco-submucosal elongated polyps display characteristic endoscopic features, with a “worm-like” appearance which are lined by unremarkable mucosa. Histologic examination confirms the presence of a normal mucosa and reveals a submucosal component with a variably prominent mixture of blood vessels and lymphatics and absence of significant inflammation. In this paper we present the clinicopathologic features of three additional cases of this entity and review the literature on these characteristic benign gastrointestinal polyps.

CASE REPORT

Case 1

A 55 year-old previously healthy female presented with postprandial abdominal discomfort of 3 mo duration. There were no signs or symptoms of gastrointestinal bleeding or malabsorption. Gastroduodenoscopy revealed a 4 cm long, slender, “worm-like” polyp in the second part of the duodenum (Figure 1A). The polyp was

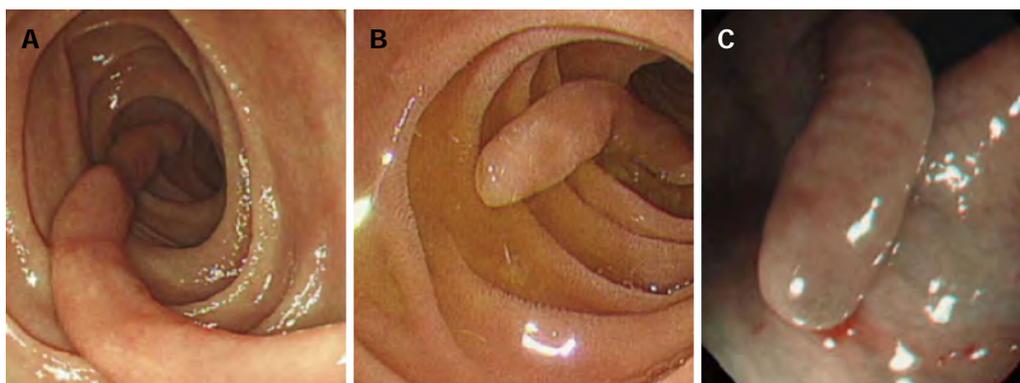


Figure 1 Endoscopic views of the polyps. A: Gastroduodenoscopy revealed a 4 cm long, slender, “worm-like” polyp in the second part of the duodenum; B: Gastroduodenoscopy showed a 2.2 cm long, slender polyp in the duodenum; C: Colonoscopy showed a 1.4 cm long, slender polyp in the descending colon.

removed endoscopically. The patient was well on follow up at 3 mo.

Case 2

A 70 year-old previously healthy man presented with reflux symptoms of unknown duration. Gastroduodenoscopy showed a 2.2 cm long, slender polyp in the duodenum (Figure 1B). The polyp was removed endoscopically.

Case 3

A 74 year-old female with a history of hypertension and iron deficiency anemia secondary to peptic ulcer disease presented with epigastric pain associated with constipation of 1 mo duration. Colonoscopy showed a 1.4 cm long, slender polyp in the descending colon (Figure 1C) and a distal rectal ulcer near the anal verge. The polyp was removed endoscopically. There was no evidence of diverticular disease. In addition, gastroduodenoscopy showed several benign gastric and duodenal ulcers (biopsies from the gastric ulcers showed mild chronic gastritis with reactive changes and no activity; no *Helicobacter pylori* were identified; the rectal biopsy showed features consistent with a solitary rectal ulcer).

The tissues were fixed in neutral formalin and both polyps were completely embedded in paraffin. 4 µm thick sections were cut and stained with hematoxylin and eosin (HE). An immunohistochemical study with commercially available antibodies (D2-40-podoplanin, CD31) using protocols according to the manufacturers’ recommendations were employed in Case 1 and 3. Immunohistochemistry with WT-1 were performed in all three cases.

Gross findings and histology

All three polyps were thin and elongated corresponding to the “worm-like” endoscopic appearance (Figure 2A). All polyps were lined by unremarkable mucosa. The submucosal components contained prominent vasculature including dilated, variably sized veins and lymphatic vessels running parallel to the long axis of the polyps and surrounded by loose collagenous stroma (Figure 2B and C). In addition, there was also focal lipomatous metaplasia in both duodenal polyps. No arterial vascular com-

ponent was identified. No vascular abnormalities were present in the mucosa. No significant inflammation was identified.

Immunohistochemistry

The endothelial cells of both vascular components in the polyps displayed immunoreactivity for CD31 throughout the lesion (Figure 3A). D2-40-podoplanin was selectively expressed by the endothelial cells of the lymphatic component (Figure 3B). In Case 1 and 2, the endothelial cells of both vascular components displayed patchy cytoplasmic positivity for WT-1 whereas Case 3 was completely negative (data not shown).

DISCUSSION

We herein report three cases of muco-submucosal elongated polyps, two in the duodenum and the other one in descending colon. This characteristic, albeit unusual entity, is not well covered in most standard textbooks in gastrointestinal pathology. Initially described by Japanese investigators, Matake *et al.*^{3,11} published two series of these polyps comprising 19 cases *in toto*. The polyps ranged in size from 1.2-16 cm (mean 2.9 cm) and displayed a characteristic appearance (elongated, slender, drumstick shaped). Histological examination revealed normal mucosa and a loose to dense submucosal layer containing a variably prominent vascular component composed of dilated blood vessels (veins and capillaries) and lymphatics. Subsequently, these polyps have been increasingly recognized and mostly documented in the large bowel (all parts) including rectum^{1,5,6,12,13}. In all 23 reported polyps from the large bowel, the size has ranged from 0.7 to 15 cm (mean 2.8 cm).

Two polyps with endoscopic and morphologic features conforming to a muco-submucosal elongated polyp have also been described in the small bowel^{9,10}.

In addition, in 2009, Kim *et al.*⁸ reported a case of a 5 cm long duodenal polyp with a “wormlike/drumstick” endoscopic appearance which they labelled as “polypoid vascular and lymphatic malformation of the duodenum”. The histologic features as described by the authors and the provided photomicrographs show, in our opinion, a

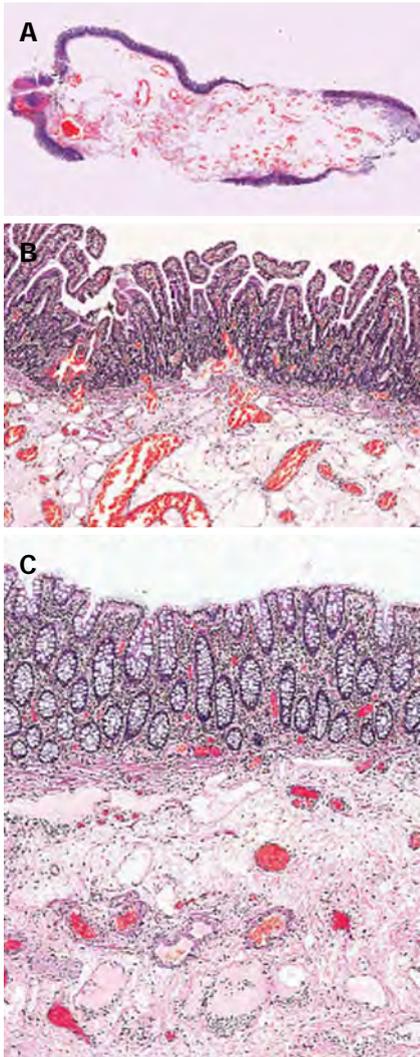


Figure 2 Histological section (hematoxylin and eosin staining). A: Case 1; B: Case 1 with normal small intestinal mucosal lining; C: Case 3 with normal large bowel mucosa overlying the submucosa which contains a prominent vascular component.

muco-submucosal elongated polyp.

Our Case 1 and 2 were of duodenal origin, which is a rare site and thus represents the third and fourth published cases of muco-submucosal elongated polyps of the small bowel.

A lesion resembling muco-submucosal elongated polyps has been described by Kelly^[14]. In this paper, the author characterized eight cases of swollen mucosal folds and broad based, ‘leaf-like’ polyps with mucosal and submucosal vascular congestion, thrombi, edema, hemorrhage and hemosiderin deposits in the sigmoid colon associated with diverticular disease in resection specimens. In addition to the submucosal component, these polypoid lesions showed mucosal prolapse-type changes. The author adequately labelled these lesions “polypoid prolapsing mucosal folds in diverticular disease” (PPMF). Interestingly, cases of muco-submucosal elongated polyps associated with diverticular disease have also been documented^[1,7]. In contrast to muco-submucosal

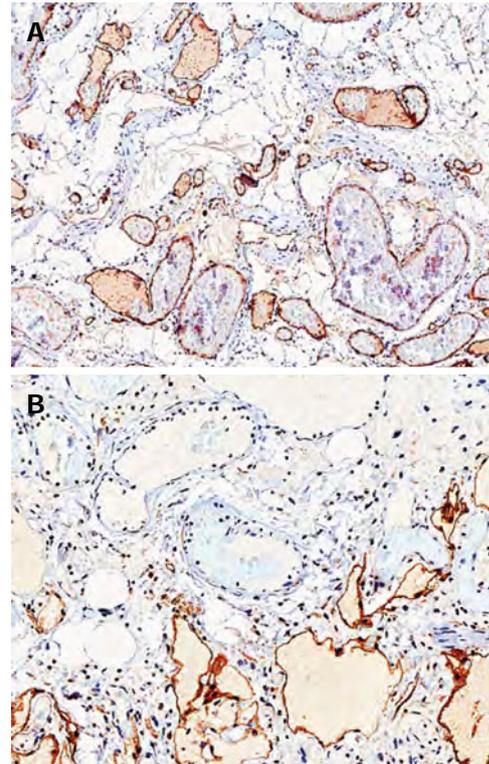


Figure 3 Immunohistochemistry. A: Positivity for CD31 in the endothelial cells of both vascular components; B: Positivity for D2-40/podoplanin selectively expressed by the endothelial cells in the lymphatic vessels.

elongated polyps, PPMFs do not display the “wormlike”, slender gross appearance with narrow attachment to the bowel mucosa. In addition, on histological examination, the mucosal component in PPMFs shows prolapse-type changes, which reportedly are absent in muco-submucosal elongated polyps, including the three cases presented herein.

Absence of significant inflammation in muco-submucosal elongated polyps also differentiate this entity from other mucosal prolapse associated polyps such as inflammatory “cap” polyps, inflammatory cloacogenic polyps and inflammatory myoglandular polyps^[15].

Filiform polyps (a subtype of inflammatory polyps) characterized by finger like projection covered by normal colonic mucosa with central core containing vessels and smooth muscle fibers can also mimic muco-submucosal elongated polyps. However, these polyps are usually multiple (“filiform polyposis”) and are associated with inflammatory bowel disease. Of note is that cases without apparent history of inflammatory bowel disease appear to exist^[16].

An inverted colonic diverticulum is a clinically important differential diagnosis. When these are large in size, they may resemble a pedunculated polyp and an endoscopic polypectomy may be performed which is associated with risk of perforation^[17]. A high index of clinical suspicion is important for these lesions and the key to its recognition is the endoscopic association with conventional diverticuli in the segment where the polyp

is located. A small biopsy from an inverted diverticulum shows non-specific features including normal to mildly inflamed mucosa and congested submucosa with variable degrees of chronic inflammation.

The residual stalk after a previously excised pedunculated adenoma or after autoamputation of any pedunculated polyp may also come into the endoscopic and histological differential diagnosis. Distinguishing, albeit non-specific features, are procedure related changes (granulation tissue and hemosiderin) and obviously, a history of previous polypectomy.

The etiology of muco-submucosal elongated polyps is unknown. It has been suggested that the gastrointestinal peristaltic movement may serve as mechanical traction for redundant mucosa, thereby making such areas of mucosa a nidus for polyp formation^[3,18]. In line with this, Alizart *et al*^[11] hypothesized that focal areas with prominent submucosal venous plexus may elevate the mucosa and serve as the leading point/nidus for the traction and hence polyp formation. A similar explanation has also been put forward by Kelly^[14] to explain the formation of PPMFs associated with diverticular disease.

We have immunohistochemically confirmed that the submucosal vascular component is composed of both blood vessels and lymphatics. Thus, we suggest that in addition to the criteria for the diagnosis of muco-submucosal elongated polyp as suggested by Alizart *et al*^[11]: (1) elongated, cylindrical shape with narrow base; (2) presence of submucosa; (3) absence of marked architectural disturbance of the overlying mucosa; and (4) absence of inflammatory infiltrate in both the mucosa and submucosa; the presence of a significant vascular submucosal component with a dual phenotype could be added to these criteria.

The endoscopic ultrasonographic features of muco-submucosal elongated polyps have been reported by Takahashi *et al*^[12] and Akahoshi *et al*^[13]. The ultrasonographic findings appear characteristic for this entity and in all lesions, elongated polypoid structures with both mucosal and submucosal layers were visualized. The “microcystic components” that the authors reported in the submucosal layer, most likely corresponds to the ectatic vascular component that is characteristically present in these lesions. The authors conclude that the distinctive ultrasonographic features allow for the diagnosis to be made and hence differentiate muco-submucosal elongated polyps from other submucosal lesions such as leiomyoma, lipoma and lymphangioma^[13]. Successful endoscopic removal of muco-submucosal elongated polyp (as in our cases) have previously been reported^[5,6,9,13].

Recently it has been suggested that endothelial cytoplasmic immunohistochemical expression of WT-1 is helpful in discriminating between cutaneous vascular neoplasms and vascular malformations. Trindade *et al*^[19] studied 117 cutaneous vascular neoplasms and 50 vascular malformations and found that all vascular neoplasms showed diffuse endothelial expression of WT-1 whereas all vascular malformations except for the arteriovenous

type were negative. The expression of WT-1 in a random manner in our first and second case and complete negativity in our third case lend support to the reactive nature of the vascular components in muco-submucosal elongated polyps. However, it is important to bear in mind that all the cases in Trindade *et al*^[19] study were of cutaneous origin and the underlying molecular mechanisms behind the development of cutaneous and intestinal vascular lesions may differ. Further studies are warranted to clarify this.

In conclusion, we report three cases of gastrointestinal muco-submucosal elongated polyps with prominent submucosal vascular components. One of these was located in the large bowel and the other two were of duodenal origin. We have determined by immunohistochemistry that the vascular component is a mixture of blood vessels and lymphatics. The endoscopic ultrasonographic appearance of these polyps appears to be characteristic allowing for a high degree of certainty in making this diagnosis by the endoscopists.

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Synchronous adenocarcinoma and extranodal natural killer/T-cell lymphoma of the colon: A case report and literature review

Chih-En Tseng, Ta-Wen Shu, Chih-Wen Lin, Kai-Sheng Liao

Chih-En Tseng, Kai-Sheng Liao, Department of Anatomic Pathology, Buddhist Dalin Tzu Chi General Hospital, Chiayi 62247, Taiwan

Chih-En Tseng, School of Medicine, Buddhist Tzu Chi University, Hualien 97004, Taiwan

Ta-Wen Shu, Department of General Surgery, Buddhist Dalin Tzu Chi General Hospital, Chiayi 62247, Taiwan

Chih-Wen Lin, Department of Medical Imaging, Buddhist Dalin Tzu Chi General Hospital, Chiayi 62247, Taiwan

Author contributions: Shu TW and Lin CW collected and interpreted the clinical data; Tseng CE and Liao KS wrote the paper.

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Correspondence to: Kai-Sheng Liao, MD, Department of Anatomic Pathology, Buddhist Dalin Tzu Chi General Hospital, 2, Min-Sheng Road, Dalin, Chiayi 62247, Taiwan. carl.liao@gmail.com

Telephone: +886-5-2648000 Fax: +886-5-2648999

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Abstract

Extranodal natural killer/T-cell lymphoma (ENKTL) is a distinct subtype of non-Hodgkin's lymphoma and is rare in the colon. Synchronous adenocarcinoma and ENKTL of the colon has not been reported in the literature. In the present study, we report a 63-year-old male who suffered from intermittent bloody stools for 2 mo. He did not have fever, body weight loss or night sweat. Endoscopic and imaging studies revealed a 4.5-cm ulcerative mass in the ascending colon and a 3.0-cm polypoid, easy bleeding mass in the sigmoid colon, respectively. Thought to have double carcinoma of the colon, he received simultaneous right hemicolectomy and sigmoidectomy. The pathological diagnosis was a synchronous ENKTL (ascending colon) and adenocarcinoma (sigmoid colon). The literature on synchronous adenocarcinoma and malignant lymphoma of the colon

was also reviewed.

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Key words: Synchronous cancers of the colon; Colonic adenocarcinoma; Colonic lymphoma; Extranodal natural killer/T-cell lymphoma; Epstein-Barr virus

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INTRODUCTION

Malignant lymphoma of the colorectum is uncommon with an incidence of less than 0.5% of all malignant large intestinal tumors^[1], and comprises 5.8% of all gastrointestinal lymphomas^[2]. Synchronous colonic adenocarcinoma and malignant lymphoma in the same patient is rare with an estimated incidence of around 0.0002%^[3]. Synchronous colonic adenocarcinoma and extranodal natural killer/T-cell lymphoma of the colon has not been reported. Herein, we present such a rare case and review the literature.

CASE REPORT

A 63-year-old male with a 2-year history of hypertension and gout under medical control visited our outpatient department on April 25, 2012. He complained of intermittent bloody stool passage in the previous 2 mo. There was no B symptoms (fever, night sweating, body weight loss greater than 10% in the past 6 mo) noted. Dark red blood in the upper rectum was noted during

digital examination and tumor bleeding was suspected. Colonoscopy was performed one week later, which revealed an approximately 4-cm ulcerated mass coated with necrotic tissue in the proximal ascending colon near the ileocecal valve (Figure 1A). Another 3.5-cm bleeding mass was located 30 cm from the anal verge (Figure 1B). Biopsies were taken from the two masses, which showed necrotic tissue and adenocarcinoma *in situ*, respectively. After discussion, the patient was admitted for colectomy to remove the two masses. On admission, his vital signs and routine blood and biochemical studies were all within normal limits. The tumor marker, carcinoembryonic antigen, was mildly elevated at 9.4 ng/mL (normal range: 0-7 ng/mL). During preoperative evaluation, a computed tomography (CT) scan of the chest and abdomen was performed and revealed an irregular mass in the proximal ascending colon and a polypoid, protruding mass located in the sigmoid colon (Figure 1C). A tiny questionable pulmonary nodule (about 1-2 mm) of unknown nature in the left upper lung lobe was also noted at this time. Thought to have double carcinoma of the colon with sparing transverse and descending colon and for keeping well post-operative life quality, he underwent laparoscopic right hemicolectomy and sigmoidectomy with anastomosis on May 4, 2012. Grossly, the specimen from laparoscopic right hemicolectomy demonstrated an ulcerative mass, measuring 4.5 cm × 3.5 cm × 1.2 cm, in the proximal ascending colon with ileocecal valve involvement (Figure 1D). The tumor was yellow-green in color, ulcerative and necrotic. On cutting, the mass showed a yellowish fleshy cut surface and invaded serosa. Some enlarged lymph nodes, measuring up to 1.0 cm were noted. Microscopic examination revealed an ulcerative tumor composed of sheets of large pleomorphic lymphoid cells with a scattered angiocentric pattern and patchy necrosis (Figure 1E and F). The tumor had metastasized to three of the twelve right pericolic lymph nodes. On immunohistochemical study, the neoplastic lymphocytes were positively stained for CD3, CD56 (Figure 1G), T cell intracellular antigen 1 and CD30, but negatively stained for pancytokeratin, CD20, CD246 (ALK), CD4, CD8 and myeloperoxidase. An *in situ* hybridization (ISH) study revealed positive Epstein-Barr virus encoded RNA (EBER) in tumor nuclei (Figure 1H). Other parts of intestinal tissue showed no evidence of atrophic mucosa or enteropathy. The pathologic diagnosis was extranodal NK/T-cell lymphoma, nasal type, of the proximal ascending colon. The specimen from sigmoidectomy showed a red fleshy and polypoid mass, measuring 3.0 cm × 2.0 cm × 1.2 cm, taken from the central part of the sigmoid colon (Figure 1I). On cutting, the mass showed a polypoid, fleshy cut surface and invaded subserosa grossly. Microscopically, this tumor revealed marked pleomorphic tumor cells with complex glands, foci of cribriform formation, and an extensive infiltrative pattern (Figure 1J). The pathologic diagnosis was a moderately-differentiated adenocarcinoma. Four pericolic lymph nodes with no evidence of metastasis were retrieved in this specimen. An additional ISH

study of the adenocarcinoma showed negative EBER. In accordance with these findings, a synchronous adenocarcinoma of the sigmoid colon and extranodal natural killer/T-cell lymphoma (ENKTL) of the proximal ascending colon was finally diagnosed. His post-operative course was uneventful and he was discharged 10 d after surgery.

On June 14, 2012, the patient was readmitted for further tumor staging and chemotherapy. His ECOG performance score was grade 1. A tumor survey using sinuscopy showed no tumor in the upper respiratory tract. Bone marrow biopsy performed on June 16, 2012 revealed neither tumor nor granuloma. Biochemical studies showed a lactate dehydrogenase (LDH) level of 176 IU/L (normal range: 85-227 IU/L). A CT scan of the head, neck, chest and abdomen was performed and showed multiple nodules in bilateral lungs with the largest nodule being 1.2 cm in diameter in the left upper lung lobe. He then received chemotherapy with cyclophosphamide, doxorubicin, vincristine, and prednisolone (CHOP). Unfortunately, a subsequent chest CT scan revealed rapid progression of bilateral lung nodules; the largest nodule being 5.4 cm in diameter in the right upper lung lobe on September 3, 2012 (Figure 1K). It was confirmed to be metastases of ENKTL by CT-guided biopsy (Figures 1L and M). In addition, serum LDH was markedly elevated at 1079 IU/L. Due to poor response to chemotherapy, the regimen was changed to dexamethasone, methotrexate, ifosfamide, *L*-asparaginase and etoposide but this was in vain. The patient died of respiratory failure secondary to lung metastasis on September 21, 2012. The survival time after diagnosis was only 4.5 mo.

DISCUSSION

The gastrointestinal tract is the most frequently involved extranodal site in non-Hodgkin lymphoma, stomach being the most common (50%-60%) followed by the small intestine (30%)^[4]. ENKTL is an extranodal lymphoma characterized by angioinvasion and damage, prominent necrosis, cytotoxic phenotype and associated with Epstein-Barr virus (EBV). Primary ENKTL of the gastrointestinal tract is very rare and carries a poor prognosis. The large intestine is the most common site in the gut affected by ENKTL followed by the small intestine^[5]. Synchronous adenocarcinoma and malignant lymphoma of the colon in the same patient is rare and only a few cases have been reported in the literature. Furthermore, synchronous colonic adenocarcinoma and ENKTL has never been reported.

The symptoms of colonic ENKTL are related to ulcerative plaques, or shallow multiple ulcerations, due to its angioinvasive nature, and may clinically manifest as abdominal pain and bloody stools, as seen in the present case. Due to its ulcerative nature in the colon, the differential diagnosis of ENKTL includes inflammatory bowel disease, tuberculous or cytomegaloviral infection, and neoplasm. There are no characteristic features to distin-

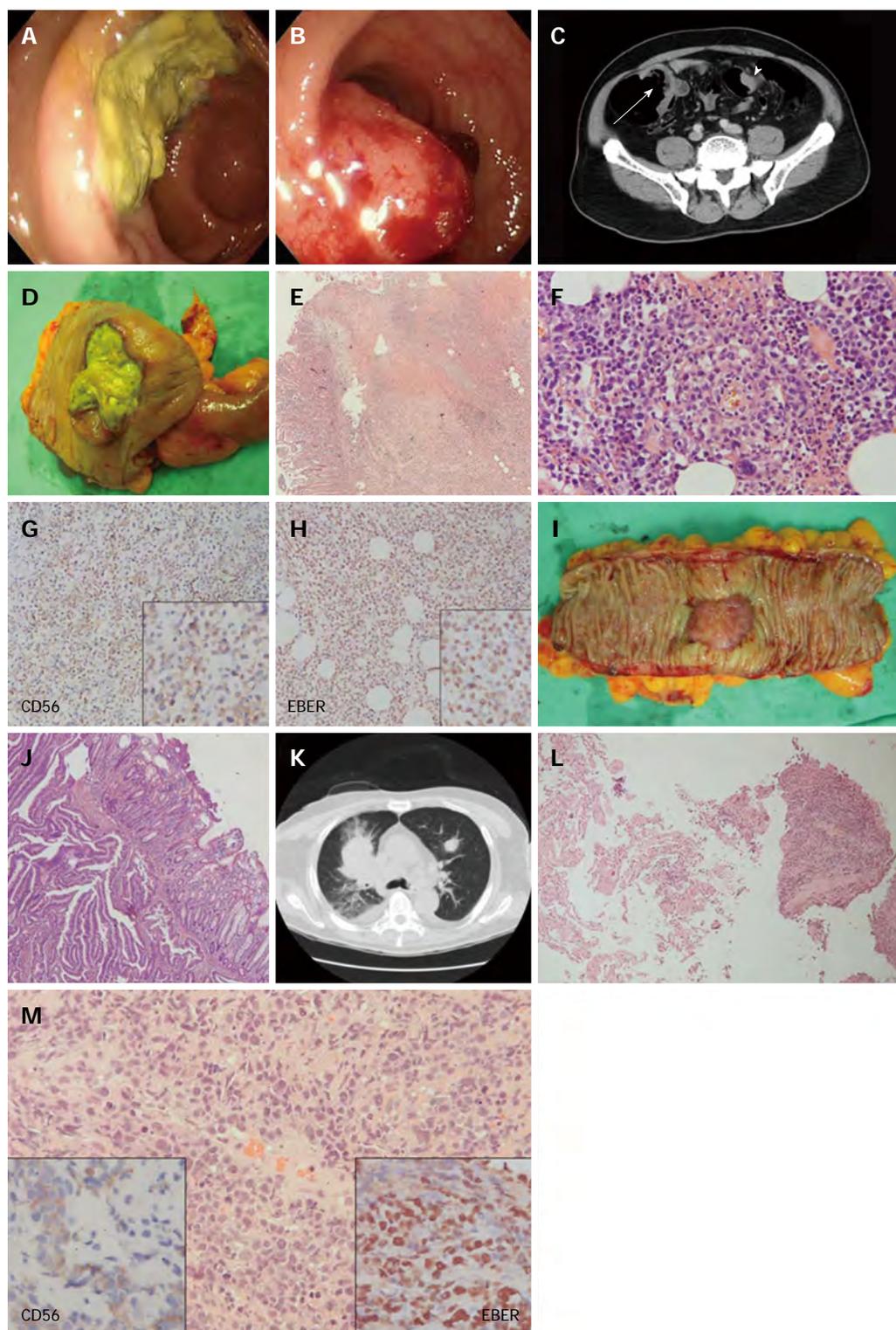


Figure 1 Clinical imaging studies and pathologic features of synchronous extranodal natural killer/T cell lymphoma and adenocarcinoma of the colon, as well as lung metastasis of the lymphoma. A: Appearance of the proximal ascending colon on colonoscopy: A marked ulcerative mass coated with necrotic tissue; B: Appearance of the sigmoid colon on colonoscopy: A polypoid bleeding mass; C: Computed tomography (CT) scan of the abdomen revealing a mass in the proximal ascending colon (arrow on the left) and another mass in the sigmoid colon (arrowhead on the right); D: Surgical specimen of the proximal ascending colon showing an ulcerative mass; E: Histologically, a section of the tumor from the proximal ascending colon showing neoplastic lymphoid cells (right lower part) infiltrating the colonic mucosa (left part) accompanied by a necrotic ulcer (upper middle part), hematoxylin and eosin (HE) stain, $\times 40$; F: Angioinvasion of the neoplastic lymphoid cells of the lymphoma, HE stain, $\times 400$; G: CD56 reactivity of the lymphoma cells, $\times 200$ (right lower inset, $\times 400$); H: Epstein-Barr virus encoded RNA (EBER) reactivity of the lymphoma cells, $\times 200$ (right lower inset, $\times 400$); I: Surgical specimen from the sigmoid colon revealing a polypoid, hemorrhagic mass; J: Histology of the sigmoid colon tumor showing adenocarcinoma with infiltrative neoplastic glands, HE stain, $\times 40$; K: Multiple lung metastases of the lymphoma on follow-up CT scan of the chest (the largest nodule on the right lung was biopsied); L: Histology of the lung biopsy showing lung parenchyma (on the left) and tumor cells (on the right), HE stain, $\times 100$; M: Histologically, a section of the lung biopsy showing the ovoid nucleated neoplastic cells with high N/C ratio, HE stain, $\times 400$, and reactivity of EBER (right lower inset, $\times 400$) and CD56 (left lower inset, $\times 400$).

Table 1 Synchronous adenocarcinoma and malignant lymphoma of colon in the literature

| Year | Age/ gender | Tumor1/tumor2 | Location1/location2 | Pre-OP Dx | OP | Die of | Survival time (mo) |
|-------------------------|----------------|-------------------------|---------------------------------------|---------------|---------------------------------------|--------------------------|-----------------------|
| 1984 | 14/M | Adenoca/large cell L | Rectosigmoid/cecum | Adenoca | R't hemicolectomy Sigmoidcolectomy | Sepsis | 1 |
| 1985 | 52/F | Adenoca/ML | Ascending/ascending | Adenoca | R't hemicolectomy | ML | 5 |
| 1995 | 74/F | Adenoca/MCL | Cecum, rectum/ileum, colon, rectum | Adenoca/MCL | Panproctectomy | - | - |
| 1997 | 32/F | Adenoca/MALToma | Sigmoid/cecum | Double cancer | Subtotal colectomy | MALToma | 17 |
| 2001 | 74/M | Adenoca/MCL | Rectum/sigmoid | Adenoca | LAR | - | 4 mo alive |
| 2001 | 54/M | Adenoca/MCL | Ascending/terminal ileum | Adenoca | R't hemicolectomy | - | 6 mo alive |
| 2003 | 85/M | Adenoca/MCL | Cecum/colon + terminal ileum | Adenoca | Ileocecal resection | Ruptured aneurysm | 1 |
| 2009 | 80/M | Adenoca/MCL | Sigmoid/pericolic LN | Adenoca | Sigmoidectomy | MCL | 14 |
| 2010 | 67/M | Adenoca + MALToma/FL | Ascending/terminal ileum | - | R't hemicolectomy | - | - |
| 2011 | 86/M | Adenoca/SLL/CLL | R't colon/mesenteric LN | Adenoca | R't hemicolectomy | Loss F/U | - |
| 2011 | 68/F | Adenoca/MALToma | Ascending/ascending | Adenoca | R't hemicolectomy | - | Alive |
| 2012 | 79/F | Adenoca/AITL | Cecum/pericolic LN | Adenoca | R't hemicolectomy | - | - |
| 2012 (the current case) | 63/M | Adenoca/ENKTL | Ascending/sigmoid | Double cancer | R't hemicolectomy + sigmoidectomy | ENKTL lung metastasis | 4.5 |

Adenoca: Adenocarcinoma; Large cell L: Large cell lymphoma; ML: Malignant lymphoma; MCL: Mantle cell lymphoma; MALToma: Mucosa-associated lymphoid tissue lymphoma; FL: Follicular lymphoma; SLL: Small cell lymphoma; CLL: Chronic lymphocytic leukemia; AITL: Angioimmunoblastic T-cell lymphoma; ENKTL: Extranodal natural killer/T-cell lymphoma; Rectosigmoid: Rectosigmoid colon; Ascending: Ascending colon; Sigmoid: Sigmoid colon; R't: Right; LAR: Low anterior resection; F/U: Follow-up.

guish colonic ENKTL from carcinoma from colofibrosopic and imaging viewpoints. This makes the preoperative diagnosis of colonic ENKTL challenging.

Synchronous colonic adenocarcinoma and malignant lymphoma is rare. There are only 13 reported cases, including the present case, in the literature (Table 1)^[6-16]. All these cases, with the exception of two (14-year-old and 32-year-old) were older than 50 years with a mean age of 63.7 years. Synchronous mantle cell lymphoma and adenocarcinoma (5 cases) were the most frequent diagnoses followed by synchronous extranodal marginal zone lymphoma of mucosa associated lymphoid tissue and adenocarcinoma (3 cases). The involved sections of the colon included the cecum, ascending colon, sigmoid colon and rectum. It is intriguing that the transverse colon and descending colon were spared in the reported cases including the present case. All cases except one of the synchronized colonic lymphoma were preoperatively diagnosed as colonic adenocarcinoma. This indicates that it is a challenge to preoperatively recognize synchronous colonic adenocarcinoma and malignant lymphoma. The most frequent surgical technique in these cases was right hemicolectomy (8 cases) since the right colon was the most common section involved. The overall survival time was relatively short with a mean of 7.6 mo (range: 1-17 mo). Four patients, including the present patient, out of the 13 cases died of lymphoma. In short, synchronous adenocarcinoma and malignant lymphoma of the colon involved aging patients, spared the transverse and descending colon and had a poor prognosis and short survival time.

The etiology of synchronous adenocarcinoma and malignant lymphoma of the colon is unknown. Chance coincidence is favored^[9,14]. However, local factors such

as absent immune surveillance in the lymphoma which possibly allowed carcinoma cells to grow has also been suggested^[8]. Recently, it was reported that EBV-derived latent membrane protein-1-induced protein may have an important role as a mediator in EBV-mediated neoplasms, including ENKTL^[17]. A low incidence of adenocarcinoma was also proven to be infected with EBV^[18]. Although EBV infection in adenocarcinoma of the sigmoid colon in our patient was not determined, the role of EBV in synchronous adenocarcinoma and ENKTL requires further elucidation.

In conclusion, synchronous adenocarcinoma and malignant lymphoma is uncommon. In particular, synchronous adenocarcinoma and ENKTL is extremely rare, with the present case being the only one reported, with poor prognosis. The etiology of this synchronization is still unknown. Chance coincidence or poor immunity in patients may contribute to the etiology, and the role of EBV needs further elucidation.

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EDITORIAL OFFICE
 Jin-Lei Wang, Director
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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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 Baishideng Publishing Group Co., Limited
 Flat C, 25/F, Lucky Plaza,
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Fax: +852-65557188
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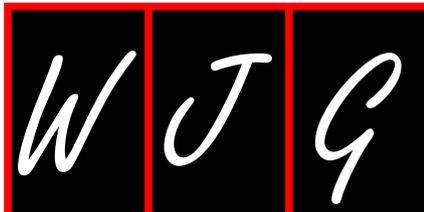
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New strategies for colorectal cancer screening

Maria Di Lena, Elisabetta Travaglio, Donato F Altomare

Maria Di Lena, Elisabetta Travaglio, Donato F Altomare, Department of Emergency and Organ Transplantation, University Aldo Moro of Bari, 11-70124 Bari, Italy

Author contributions: Di Lena M, Travaglio E and Altomare DF contributed equally to this paper.

Correspondence to: Donato F Altomare, MD, Professor, Department of Emergency and Organ Transplantation, University Aldo Moro of Bari, Piazza G Cesare, 11-70124 Bari, Italy. donatofrancesco.altomare@uniba.it

Telephone: +39-8-5592107 Fax: +39-8-5478735

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Abstract

Colorectal cancer (CRC) is still one of the leading causes of cancer-related death in Western countries, despite major improvements in its treatment. The dramatically high social and economic impact of CRC on human health makes the identification of a reliable screening tool of paramount importance. Current screening methods, such as the fecal occult blood test and colonoscopy do not adequately meet the ideal requisites of a screening test because, even if they are effective, they are limited first by too low specificity and sensitivity, or second by high invasiveness, costs and risk. Nowadays extended efforts are made by researchers to look for more reliable and effective screening tests based on a systems biology approach, using biological samples easily available, such as urine, breath, serum and feces. The effectiveness and reliability of several new attempts to screen these patients by non-invasive analysis of their biological samples using genomic (genetic and epigenetic alteration), transcriptomic (miRNA), proteomic (cancer-related antigens, new antibodies against tumor-associated antigens, mutated proteins) and metabolomic (volatile organic metabolites) methods are discussed in this review. Among the most interesting new screening tools, fecal fluorescent long-DNA, fecal miRNA and metabolomic evaluation in breath and/or serum seem to be most promising.

TRADITIONAL APPROACH TO COLORECTAL CANCER SCREENING

Colorectal cancer (CRC) is the second most commonly diagnosed cancer and the second leading cause of cancer death in Europe, with an incidence of 43 600 new cases between 2007 and 2008^[1]. The dramatically high social and economic impact of CRC on human health makes the identification of a reliable screening tool of paramount importance. CRC, as a cancer actually fulfills the World Health Organization conditions required for mass screening, since it is a very common disease, with major morbidity and mortality rates and is almost always preceded by a slow progressive premalignant lesion (the adenomatous polyp) which can readily be removed leading to true cancer prevention^[2]. Screening strategies for CRC involve the separation of the population into two main categories: average risk and high risk populations. Each of these categories is targeted using a different screening program. In the first group, adults over 50 years without a personal or family history of CRC, polyps or inflammatory bowel diseases (IBD) are screened. The high risk population includes subjects with a family history of CRC, a personal history of CRC or polyps or are index cases affected by IBD. There is, however, a third category, more specifically characterized by an hereditary risk and represented by hereditary cancer syndromes such

as familial adenomatous polyposis and hereditary non-polyposis CRC^[3,4]. Such cases should be screened directly with total colonoscopy (TC). The average risk population reflects the vast majority of the population and needs to be screened by less-invasive, low-cost techniques with acceptable patient compliance^[5]. For that reason, in the last decade, there has been a great interest and research effort in developing the optimal CRC screening tool.

Clinically validated screening strategies currently available in practice include fecal occult blood testing (FOBT), TC, flexible sigmoidoscopy (FS) and radiographic imaging, such as double contrast barium enema and virtual TC. FOBT is the most commonly used method for CRC screening. In this respect, it is non-invasive, inexpensive and matches patient compliance better than other screening tools. In 2008, Hewitson *et al*^[6] published a systematic review comparing the results of four randomized controlled trials, using FOBT as a screening tool, and in approximately 320 000 patients screened, there was an overall reduction of the relative risk of dying of CRC of 16%. Despite this, FOBT has demonstrated an unacceptably low specificity rate. To improve its reliability in this regard, fecal immunohistochemistry testing (FIT), which specifically detects non-degraded human globin using anti-human hemoglobin antibodies, has replaced the older guaiac-based FOBT (which identified the heme group by pseudoperoxidase). Despite this major improvement, the search for occult blood in the feces still has severe limitations as a screening tool, mainly because of its low specificity, hence leading to a high number of unnecessary colonoscopies^[7,8]. FS has been proposed as a balance between the invasiveness of a given test (such as low invasive tests like FOBT and FIT), their accuracy and their potential complications (*e.g.*, TC), considering that about two-thirds of the screened CRCs detected are located in the rectum and sigmoid colon. It may be possible to increase the performance characteristics of FS by combining it with FOBT/FIT, however, the risk of leaving undetected CRC in other colonic sites is currently unacceptable^[9,10].

TC still remains the gold standard for the diagnosis of both colorectal polyps and malignancies. The National Polyp Study demonstrated that the incidence of CRC was reduced from 76% to 90%^[11] after polypectomy. Although very effective for diagnosis and treatment, TC has the limitations of low patient compliance, high cost, a high level of invasiveness and a moderate incidence of serious complications in specific subgroups (an incidence of 0.1%-0.3% of life-threatening complications including bleeding and perforation). TC colonography (or virtual TC) involves the use of helical TC to generate high-resolution 3D images of the abdomen and pelvis, replacing the older barium enema in providing full structural evaluation of the entire colon. A study conducted by Fenlon *et al*^[12] in a high risk population, reported a sensitivity of 71% for TC colonography, although this was strongly influenced by polyp size where only 55% of polyps between 1 and 5 mm in maximal diameter were

correctly identified. The sensitivity for virtual diagnosis was significantly higher when polyps ranged between 6 and 9 mm or were larger than 10 mm in size (82% and 91%, respectively; $P = 0.001$)^[12]. This investigation, however, had the drawbacks of considerable exposure to ionizing radiation, discomfort of the bowel preparation and the necessity to complete the procedure by TC in cases of polyp or cancer detection, as well as being expensive (with inherent derivative costs) and currently not suitable for screening purposes.

From these considerations it is clear that current screening methods do not properly meet the ideal requisites of a screening test, so that extended effort has been dedicated by researchers at looking for more reliable and effective screening tests based on the systems biology approach using biological samples easily available such as urine, breath, serum and feces. Since the human genome was completely identified in 2003, the entire set of genes and proteins expressed have been extensively studied using genomic, transcriptomic or proteomic approaches.

GENOMIC APPROACH TO CRC SCREENING

Several authors have attempted to identify cancer-related mutated DNA/RNA, mutated proteins or normal proteins abnormally synthesized [*e.g.*, carcinoembryonic antigen (CEA), cytokeratins] in different biological samples as potential biomarkers for CRC. Colorectal carcinogenesis is characterized by genetic alteration (gene mutation or gene amplification) and epigenetic alteration (gene *hypermethylation* or *chromatin* modification), which both transform normal epithelial cells into cancer cells. CRC cells are continuously shed in the feces, due to a high proliferative rate, so that mutated DNA can be readily detected in the feces of these patients. This issue is complex, where mutation in the *APC*, *K-ras* and *p53* genes were initially investigated in stool samples of CRC patients, in accordance with the Volgenstein model of CRC genesis^[13]. Other markers have also been studied by Imperiale *et al*^[14] who conducted a large population-based study comparing the fecal DNA test with FOBT, using a DNA marker panel formed by 21 mutations and demonstrated a sensitivity of 52% for invasive cancers compared with 13% for FOBT in the same population. Fecal DNA testing has been commercially available in the United States since 2003, but so far has rarely been adopted for screening despite preliminary studies showing that the use of a large pool of genetic markers results in a sensitivity of 71%-91% and a specificity of more than 93%^[15]. A recent interesting approach involves the use of fluorescent long DNA (FL-DNA) measurement, designed to identify cancer DNA fragments greater than 150-200 db pairs. Changes are noted since cancer cells do not undergo apoptosis, which in normal epithelial cells typically initiate DNA cleavage and degradation producing small measurable fragments. This FL-DNA technique has shown a performance sensitivity up to 80% in detecting CRC^[16]. Such mutated DNA can also

be demonstrated in the urine of CRC patients. Human urine has been shown to contain two types of DNA: large type, greater than 1 kb, presumably derived from cells shed into the urine from the urinary tract and small type, between 150 bp and 250 bp, derived from the circulation, which can cross the renal barrier. Sample urine collection is non-invasive and isolation of DNA from urine is easier than from others specimens, due its low extraneous protein content. The comparison of mutated *K-ras* sequences, in particular the mutation in codon 12, between tumor, blood and urine from CRC patients and healthy controls showed an 83% correspondence of mutated DNA in urine and tumor tissue in the same patients^[17]. Epigenetic changes which characterize CRC cells have only been studied in urine samples; most notably, the *hypermethylated vimentin (m-VIM)* gene. The detection of *m-VIM* in urine samples is significantly associated with CRC when compared with healthy controls^[18].

TRANSCRIPTOMIC APPROACH TO CRC SCREENING

The most recent transcriptomic approach to identify potential biomarkers for CRC involves the study of microRNAs (miRNA), short non-coding 18-22 nucleotide RNA molecules involved in regulation of gene expression through post-transcriptional processing. Their expression is deregulated in cancer cells where altered miRNA expression leads to altered expression of their target gene including a range of potential oncogenes and oncosuppressors during carcinogenesis. Chen *et al*^[19] showed that levels of miRNA in the serum are stable, reproducible and consistent in humans, concluding that they can be potential biomarkers for different diseases. Recent studies have indicated that circulating microRNAs incorporated into microvesicles and exosomes may be involved in genetic informational exchange between cells and may regulate extracellular matrix degradation, immunologic response and angiogenic factors which favor cancer cell growth and metastasis^[20]. MiR-145, miR-143, miR-135a and b, miR-17-92, miR-21 have been most studied in CRC where Ng *et al*^[21] were able to identify a significant increase of miR92 in the plasma of CRC patients compared with controls. Similar results have been reported by Huang *et al*^[22] demonstrated a significant increase in miR29a and miR92a in patients with adenomas and CRC compared with controls, supporting the hypothesis that the miR17-92 cluster could have a role in cell proliferation, tumor angiogenesis and apoptotic suppression. Altered miRNA^[23] expression has been examined in the stools of CRC patients and could represent an optimal screening tool for this cancer where colonic cancer cells exfoliate in greater quantity and their nucleic acid can be extracted and distinguished from those of bacteria. In this regard, Link *et al*^[24] compared fecal specimens of patients with CRC, patients with adenomas and normal controls, showing a specific miRNA pattern in the three groups where miR21, miR106 were over expressed in

CRC patients compared with controls, but where levels were higher in patients with adenomas and tended to decrease in cancer cases. Other researchers, however, were unable to confirm the higher expression of miR21, whilst the clusters miR17-92 and miR135 have been found to be significantly higher in the feces of CRC patients when compared with controls^[25]. Another fecal mRNA frequently investigated as a potential CRC marker in stool is the prostaglandin-synthase 2, which showed a sensitivity between 50% and 90% and a specificity of 93% or higher in the diagnosis of CRC, although the reliability of this study was limited by the small number of CRC patients evaluated^[26,27].

PROTEOMIC APPROACH TO CRC SCREENING

A further method for early detection and screening of CRC is to look at the modified “proteome” as a direct effect of mutated gene expression or as the occurrence of new antibodies against tumor-associated antigens (TAAs) identified in CRC. Hundt *et al*^[28] have published a systematic review of 19 studies, in which 52 protein markers were analyzed, using common standard procedures such as enzyme-linked immunoassay, radioimmunoassay or more recent approaches like chromatographic and mass spectrometric assays based on surface-enhanced laser desorption/ionization time-of-flight (TOF) and matrix-assisted laser desorption/ionization TOF technologies. These compounds can be divided into antigens, antibodies, cytokines and other CRC-relevant proteins. CEA is the most investigated marker. High CEA levels are derived from embryonic tissues and CRC, but they also increase in other malignancies, including gastric and pancreatic cancer, as well as in IBD and in smokers. Its role for screening is limited because CEA evaluation has been shown to have a sensitivity of only 43%-69% in detecting early CRC, whilst its reliability increases in metastatic cancer where assessment lies outside the screening purpose. Carbohydrate antigens such as CA 19-9, CA195, CA 50 or CA 72-4 have been investigated in many studies, but with comparatively disappointing results. The best performance amongst these antigens is that of CA 19-9, with a sensitivity ranging between 18% and 65% and a specificity of over 90%. Other antigens considered for screening purposes include the sialylated Lewis antigen X, CO 29.11^[29], urokinase-type plasminogen activator^[30] and small intestinal mucin antigen^[31], but none of these serological antigens have so far demonstrated an acceptable reliability in clinical testing. Recently Matsubara *et al*^[32] studying the proteome of CRC patients compared with healthy controls, using label-free quantitative mass spectrometry and protein microarray, identified the adipophilin or adipose differentiation-related protein, a protein involved in the cancer pathway and normally expressed in cancer cells but not by the normal mucosa. This protein has been investigated as a potential plasma biomarker for early CRC stages, showing high receiver

operating characteristics^[32].

Other studies have focused on the use of autoantibodies antibodies against TAAs as serological markers for cancer diagnosis, because they are absent in healthy subjects and other non-cancer conditions. Many autoantibodies against known or unknown TAAs, have been found in the sera of patients with a range of malignancies^[33-35]. Various technologies such as serologic analysis of recombinant cDNA expression libraries, first described in 1995 by Sahin *et al*^[36], and protein arrays or phage display techniques have been used in their measurement. The occurrence of several serum autoantibodies against TAAs, such as epithelial cell adhesion molecule or cytokeratin, p53, p62, CEA, HER-2/neu, Ras, topoisomerase II -alpha, histone deacetylases 3 and 5, ubiquitin C-terminal hydrolase L3, tyrosinase, tropomyosin and cyclin B1 have all been evaluated in CRC patients^[37], but were detected only in a limited proportion of patients (< 40%). Mutated or abnormal proteins have been detected also in the feces as potential biomarkers for screening, including tumor pyruvate kinase type M2, which has good sensitivity for CRC (85%), but not for adenomas (28%)^[38], S100 calcium binding protein A12 and metalloproteinase inhibitor 1. The latter showed a sensitivity for cancer of around 85% and a specificity of 95%^[39] compared with healthy controls.

METABOLOMIC APPROACH TO CRC SCREENING

More recently, the study of specific metabolomic biomarkers for cancers has developed as a new frontier in cancer screening. Metabolomics are the endpoint of the “omics” cascade and incorporate the comprehensive study of low-molecular-weight metabolites, using high-throughput technologies, such as gas chromatography-mass spectrometry, or other analytical platforms. Ikeda *et al*^[40] investigated the differences in serum metabolite profiles of esophageal, gastric and CRC patients and healthy volunteers, using the metabolomic approach to determine specific metabolomic biomarker candidates. They showed a different distribution of L-alanine, glucuronic lactone and L-glutamine in CRC patients, with a sensitivity of 54.5%-81.8% and a specificity of 6.7%-91.6%^[40]. Specific metabolomes can be identified in several types of biological samples, including feces, urine, serum, sputum and breath. In this regard, breath analysis could be considered the favored option for medical diagnostic purposes mostly because of its non-invasive nature, its low cost and its ready patient compliance^[41]. Volatile organic compounds (VOCs) in exhaled breath were first isolated by Pauling *et al*^[42] in 1971, and alteration in VOC production in cancer patients has been postulated to relate to (per)oxygenation of cell membrane-based polyunsaturated fatty acids resulting from genetic and/or protein mutations within tumor cells and the increased relative prevalence of reactive oxygen species within cancer cells^[43,44]. Urine and serum are ideal tools for metabolomic analyses. Some studies using high-throughput techniques and artificial neural

network statistics have identified some volatile organic metabolites as potential biomarkers for CRC in urine^[45], and very recently, a Japanese group has developed a CRC-prediction model based on serum metabolomic analysis and which demonstrated a high sensitivity (82.8%) as a novel potential screening test for CRC^[46]. A similar metabolomic approach was carried out by our group^[47], looking at the VOCs contained in breath. In this study, 15 of the 58 VOCs identified formed a specific pattern in CRC patients and, using a probabilistic neural network, the ability to identify CRC patients showed a sensitivity of 86%, a specificity of 83% and an accuracy of 85% (area under the receiver operating characteristics curve: 0.85) for the diagnosis of CRC.

In conclusion, despite their usefulness and effectiveness, traditional methods for CRC screening are still far from fulfilling the optimal requisites for a screening test. The FOBT/FIT both have too low a sensitivity or specificity whilst the high sensitivity of CT is counterbalanced by its invasiveness and high cost. TC colonography is still improving its technical performance but is expensive and, in cases of positivity, a traditional TC is still required to remove polyps or for biopsies. New hopes are rapidly growing in this field with the application of the systems biology approach using biological samples which are readily available. Among these, the search for fecal FL-DNA, fecal miRNA and metabolomic evaluation in the breath and/or serum seems to be the most promising.

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Mechanisms, prevention and clinical implications of nonsteroidal anti-inflammatory drug-enteropathy

John L Wallace

John L Wallace, Farncombe Family Digestive Health Research Institute, McMaster University, Hamilton, ON L8S 4K1, Canada

John L Wallace, Inflammation Research Network, University of Calgary, Calgary, AB T2N 4N1, Canada

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Correspondence to: John L Wallace, PhD, MBA, FRSC, Farncombe Family Digestive Health Research Institute, McMaster University, 1280 Main Street West, HSC-3N4, Hamilton, ON L8S 4K1, Canada. altapharm@hotmail.com

Telephone: +1-905-5156132 Fax: +1-905-5289862

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Abstract

This article reviews the latest developments in understanding the pathogenesis, detection and treatment of small intestinal damage and bleeding caused by nonsteroidal anti-inflammatory drugs (NSAIDs). With improvements in the detection of NSAID-induced damage in the small intestine, it is now clear that this injury and the associated bleeding occurs more frequently than that occurring in the stomach and duodenum, and can also be regarded as more dangerous. However, there are no proven-effective therapies for NSAID-enteropathy, and detection remains a challenge, particularly because of the poor correlation between tissue injury and symptoms. Moreover, recent studies suggest that commonly used drugs for protecting the upper gastrointestinal tract (*i.e.*, proton pump inhibitors) can significantly worsen NSAID-induced damage in the small intestine. The pathogenesis of NSAID-enteropathy is complex, but studies in animal models are shedding light on the key factors that contribute to ulceration and bleeding, and are providing clues to the development of effective therapies and prevention



Biography

John L Wallace is a Professor in the Department of Medicine at McMaster University. He received his BSc and MSc from Queen's University (Canada) and his PhD from the University of Toronto. He completed his post-doctoral studies in the Department of Mediator Pharmacology at Wellcome Research Laboratories in London, England. That work was carried out in a group led by Sir John Vane, Sir Salvador Moncada and Dr. Brendan Whittle. In 2007, Dr. Wallace completed his MBA degree from the University of Birmingham (United Kingdom).

In 1996, he co-founded NicOX SA, based in Nice, France. He served as the Chair of the company's Scientific Advisory Board from 1996-2003, overseeing the development of nitric oxide-releasing drugs. NicOx went public on the Paris Stock Exchange in 1999, and has an ophthalmic drug in phase 3 clinical trials. In 2004, Dr. Wallace founded Antibe Therapeutics Inc., a company developing hydrogen sulfide-releasing drugs.

Dr. Wallace's research is focused on mediators of inflammation and their contribution to mucosal injury and dysfunction. He is also interested in the mechanisms of injury induced by the gastrointestinal tract by anti-inflammatory drugs, and the factors that regulate healing of ulcers. Dr. Wallace is attempting to develop gastrointestinal-sparing anti-inflammatory drugs.

He is a Fellow of the Royal Society of Canada, a member of the Brazilian Academy of Science and a Fellow of the British Pharmacological Society. He has won numerous international awards for his research, including the 2009 Premier's Summit Award (\$5 million) and the 2012 William Harvey Medal for Outstanding Contributions to Science. From 2000-2002, Dr. Wallace was President of the Canadian Association of Gastroenterology.

Dr. Wallace has published approximately 400 peer-reviewed papers 100 book chapters, and is among the top 0.5 percent of biomedical scientists in the world in terms of citations (Hirsch factor of 88, > 25 000 citations).

strategies. Novel NSAIDs that do not cause small in-

testinal damage in animal models offer hope for a solution to this serious adverse effect of one of the most widely used classes of drugs.

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Key words: Anti-inflammatory; Ulcer; Prostaglandin; Non-steroidal; Bleeding; Intestinal; Bile; Enterohepatic; Bacteria; Hydrogen sulfide; Aspirin; Hemorrhage

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INTRODUCTION

Nonsteroidal anti-inflammatory drugs (NSAIDs) are among the widely used prescription and over-the-counter medications. They are used to treat the symptoms of a variety of inflammatory conditions, most notably osteoarthritis, rheumatoid arthritis, ankylosing spondylitis and gout. In such conditions, NSAIDs are used chronically, and the affected patients frequently have co-morbidities such as hypertension, diabetes and obesity, as well as often also taking glucocorticoids or anti-coagulants.

By inhibiting the activity of cyclo-oxygenase (COX), NSAIDs prevent the formation of prostaglandin (PG) H₂, which is the precursor for the production of all other PG and thromboxane subtypes. Most NSAIDs inhibit COX activity in a competitive fashion, whereas aspirin is an irreversible inhibitor of the enzyme. Indeed, the ability of aspirin to irreversibly inhibit thromboxane synthesis by platelets, and the lack of capacity of platelets to synthesize more COX, underlie the utility of chronic, low-dose aspirin as an anti-thrombotic drug, reducing the incidence of several adverse cardiovascular events (*e.g.*, stroke, myocardial infarction).

Inhibition of COX is central to the major anti-inflammatory actions of NSAIDs. By inhibiting the production of PGs (particularly PGE₂ and PGI₂), NSAIDs reduce two key elements of inflammation: vasodilation and pain. By reducing blood flow to a damaged and inflamed site, NSAIDs also contribute to a reduction of edema.

Unfortunately, PGs do not only contribute to the cardinal signs of inflammation. They also play important roles in many physiological processes. In the gastrointestinal (GI) tract, PGs are very important mediators of mucosal defence and repair^[1]. Inhibition of their synthesis renders GI tissues much more susceptible to damage induced by luminal irritants (including gastric acid and bile), and less able to restore mucosal structure and function after injury^[1]. Suppression of PG synthesis is the key effect of NSAIDs that leads to gastro-duodenal ulceration and bleeding. However, several other effects of NSAIDs appear to be central to the ability of these drugs to cause damage in the small intestine.

OVERVIEW OF THE CLINICAL PROBLEM

For several decades, the ability of NSAIDs to induce significant damage to the small intestine was largely unappreciated, being over-shadowed by the attention paid to damage induced by these agents in the stomach and proximal duodenum. The prevalence and clinical significance of NSAID-enteropathy continues to be greatly under-recognized. NSAID-induced enteropathy and bleeding occur more frequently than NSAID-induced gastropathy^[2,3]. Significant small intestinal damage and bleeding can be observed in about 70% of chronic NSAID users^[4,5], and in the majority of patients the injury is sub-clinical^[6].

Unlike the case for NSAID-gastropathy, there are no proven-effective preventative therapies for NSAID-enteropathy, and the pathogenesis is poorly understood^[7]. Iron-deficient anemia is a common first presentation of NSAID-enteropathy, and serious complications can include massive bleeding, perforation and strictures, sometimes leading to death^[2,6,8].

Aspirin is the most commonly used NSAID, and it is a very frequent cause of small intestinal bleeding. In the United States and Europe, in over 50% of cases, aspirin has been identified as the precipitator of GI bleeding leading to hospital admissions^[3,9,10]. Aspirin-induced small intestinal damage appears to occur more frequently when the aspirin is enteric-coated^[8,11]. There is a lack of recognition of the frequency and potential severity of aspirin-induced lower GI injury, particularly when the aspirin is given at low doses for cardiovascular protection. In a recent clinical trial that involved over 1200 patients taking aspirin and another anti-platelet therapy for cardiovascular protection, lower GI bleeding was found to occur 3-times more frequently than upper GI bleeding^[12]. Zhu *et al*^[13] reported that only about 3.5% of patients prescribed low-dose aspirin also received a prescription for a proton pump inhibitor (PPI), histamine H₂ receptor antagonist (H₂RA) or muco-protective drug, suggesting that the prescribing physicians did not recognize the potential for GI adverse effects of low-dose aspirin. The pathogenesis of aspirin-induced small intestinal damage differs in several respects to that of the ulceration caused by other NSAIDs (discussed in more detail below).

Selective COX-2 inhibitors were introduced to the marketplace at the beginning of this century with a promise of GI safety^[14,15]. While some selective COX-2 inhibitors produce less gastroduodenal damage in some circumstances, the promise of these drugs has been largely unfulfilled^[16,17]. Selective COX-2 inhibitors cause small intestinal damage and bleeding (the latter effect is somewhat surprising given the minimal inhibitory effects these drugs of these drugs on platelet function). McCarthy^[3] noted that in the VIGOR study, the majority of the GI bleeds originated from lesions in the small intestine (distal to the ligament of Treitz): 58% of the GI bleeds in patients taking rofecoxib and 52% of the GI bleeds in patients taking naproxen^[13].

There are several reasons for the lack of recognition of the prevalence and seriousness of NSAID-enteropa-

thy. First, it is more difficult to detect small bowel damage than that induced by NSAIDs in the stomach and proximal duodenum: “The single most important reason for underestimating the clinical importance of NSAID enteropathy is the difficulty in making a diagnosis”^[2]. Second, there is a poor correlation between NSAID-induced small intestinal damage and clinical symptoms. The vast majority of NSAID-enteropathy is sub-clinical^[6], and when there are symptoms, they are largely non-specific (including iron deficiency anemia, occult blood, diarrhea, hypoalbuminemia, and malabsorption of vitamin B₁₂ and/or bile acids). Thirdly, some researchers have argued that the focus of large pharmaceutical companies on the development of “gastroprotective” drugs, such as H₂RA, PPI, and putative gastric-sparing drugs (selective COX-2 inhibitors, NSAID pro-drugs) has led to a preoccupation of physicians and researchers with the stomach and proximal duodenum, at the expense of consideration of the detrimental effects of NSAIDs in the small (and large) intestine. The fact that there are no proven-effective treatments for NSAID-enteropathy likely also contributes to the lack of recognition of this serious condition^[7].

DETECTION OF NSAID-ENTEROPATHY

Until recently, detection of NSAID-enteropathy has been very difficult, with most of the evidence for its occurrence coming from post-mortem studies or through indirect measures of intestinal bleeding^[4,18,19]. Several indirect methods for detecting and measuring the severity of NSAID-enteropathy were developed, prior to improved endoscopic techniques for viewing the small intestine becoming widely available. These included measuring small intestinal permeability with sugars^[20,21] or small molecular weight radioactive probes^[22], measuring bleeding (presumed of intestinal origin) with radiolabelled red blood cells^[23], and measuring leukocyte markers in the small intestine (radiographically)^[24] or in feces^[25]. All of these methods provide useful information, but none have become recognized as a “gold standard” for detecting and quantifying enteropathy, because of lack of specificity and/or sensitivity. However, with video capsule endoscopy (VCE) and double-balloon enteroscopy, it is now possible to directly visualize of NSAID-induced damage and bleeding throughout the small intestine. Using these methods, it has become clear that NSAID-enteropathy occurs frequently, even in low-risk subjects (healthy, young volunteers) with low-risk treatment protocols (short-term ingestion of NSAIDs, sometimes together with a “gastro-protective” agent). For example, using VCE, Graham *et al*^[5] found a high prevalence of ulcers in long-term NSAID users. More than 70% of these patients (taking NSAID for more than 3 mo) had intestinal inflammation accompanied by bleeding and protein loss. Symptoms persisted after stopping the therapy (by as long as 16 mo in some patients). Maiden *et al*^[25] reported gross damage in 68% of healthy volunteers taking diclofenac plus omeprazole for 2 wk. Even low-dose aspirin

was found to cause significant small intestinal damage with short-term administration; thus, Endo *et al*^[26] reported that 80% of patients taking low-dose aspirin for 2 wk had intestinal damage.

POLYPHARMACY CONUNDRUM: SHIFTING GI INJURY MORE DISTALLY

Animal studies of NSAID injury to the GI tract usually involve the use of healthy animals. Of course, the people most commonly taking NSAIDs on a chronic basis are those with chronic illnesses, and more often than not, they are affected by more than one disease. It is also the case that disorders such as rheumatoid arthritis, obesity and diabetes can increase the susceptibility of the patient to the GI (and other) adverse effects of NSAIDs^[27-29]. Moreover, these patients are often taking a number of different drugs, which can also affect susceptibility to NSAID-induced GI injury and bleeding. Polypharmacy is now commonplace, even in patients that do not have disorders other than the one for which NSAID therapy is indicated. Consider a disorder like osteoarthritis, which is more common in the elderly. Cardiovascular diseases are common in this group of patients, often leading to co-prescription of low-dose aspirin and sometimes of other anticoagulants. Low-dose aspirin is also frequently co-prescribed with selective COX-2 inhibitors and conventional NSAIDs because of concerns about the elevated risk of serious cardiovascular events in patients taking those drugs^[30]. Of course, co-administration of low-dose aspirin together with a selective COX-2 inhibitor essentially eliminates any advantage, in terms of upper GI safety, of the selective COX-2 inhibitor as compared to a conventional NSAID^[15,31-33]. To reduce the expected upper GI toxicity of the combination of an NSAID and low-dose aspirin, PPIs are typically prescribed as well. Indeed, there are now fixed-dose, enteric-coated, combination tablets available that contain an NSAID and a PPI^[34]. While there is strong evidence for PPIs reducing the severity of damage and bleeding in the stomach and duodenum, where the role of acid in the production of damage has been clearly demonstrated^[1,35], there is no evidence to suggest that a PPI (or other anti-secretory drug) would reduce the severity of NSAID-induced enteropathy. Indeed, antisecretory drugs have been described as “useless either in preventing or treating mucosal lesions” induced by NSAIDs in the intestine^[36]. It is worth repeating that the majority of damage and bleeding caused by NSAIDs occurs in the small intestine, distal to the ligation of Treitz^[3,13].

Using a rat model, we attempted to replicate common clinical scenarios of polypharmacy to determine the effects on the small intestine^[37]. Groups of rats were treated with combinations of anti-inflammatory doses of NSAIDs (naproxen, celecoxib or a novel hydrogen sulfide-releasing NSAID, ATB-346)^[38], a PPI (omeprazole or lansoprazole) and an anti-thrombotic dose of aspirin. In rats that received only the NSAID, the levels of small

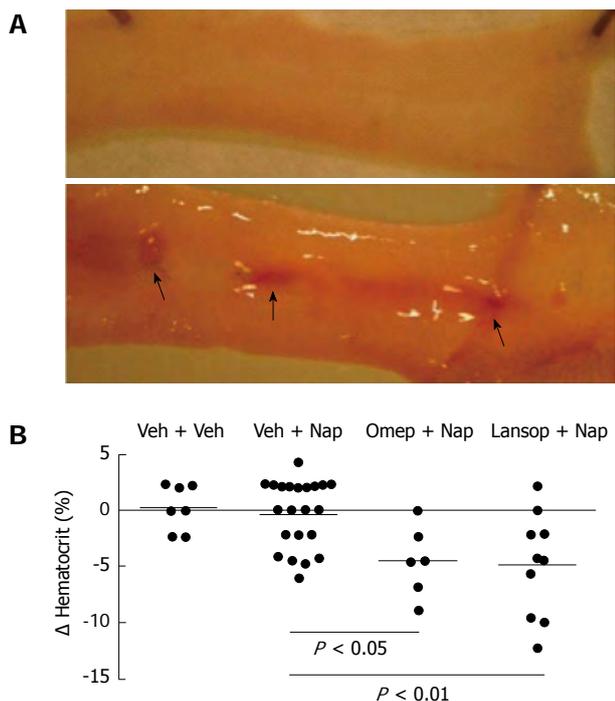


Figure 1 Proton pump inhibitors exacerbate naproxen-induced ulceration and bleeding. In panel A, the top image is of the jejunum of a rat treated with naproxen for 4.5 d (10 mg/kg twice-daily). There are no ulcers present. The bottom image is of a jejunum of a rat receiving the same naproxen treatment, but also treated with omeprazole at a dose that suppressed gastric acid secretion^[37]. The arrows indicate the numerous hemorrhagic ulcers that form with this combination of treatments; Panel B shows the change in hematocrit of rats treated with naproxen (Nap) plus vehicle (Veh), omeprazole (Omep) or lansoprazole (Lansop)^[37]. The two proton pump inhibitors significantly enhanced the decrease in hematocrit when co-administered with naproxen (no decrease in hematocrit was observed in rats treated with a proton pump inhibitors alone).

intestinal damage and bleeding were very low (Figures 1 and 2). However, when co-administered with a PPI or with low-dose aspirin, the levels of small intestinal damage and bleeding in rats treated with naproxen or celecoxib increased significantly (Figures 1 and 2). This effect has been confirmed in a recent study by Satoh *et al*^[39]. The combination of an NSAID with both a PPI and low-dose aspirin resulted in extensive damage and bleeding (the latter was evident post-mortem and also by marked decreases in hematocrit). ATB-346 did not produce small intestinal damage alone or in combination with a PPI and/or low-dose aspirin (Figure 2).

We then performed experiments to try to determine the mechanisms underlying the exacerbation of small intestinal damage by the PPIs. As discussed in more detail below, there is evidence that the bacteria residing in the small intestine play a significant role in the pathogenesis of NSAID-enteropathy. Given the evidence that marked suppression of gastric acid secretion by PPIs can alter the numbers of bacteria in the small intestine^[40-42], we focused our investigation on potential changes in intestinal microbiota. Treatment of rats with omeprazole resulted in a dramatic shift in the types of bacteria in the small intestine (dysbiosis). In particular, there was a marked reduction of the Actinobacteria, particularly of *Bifidobacteria*

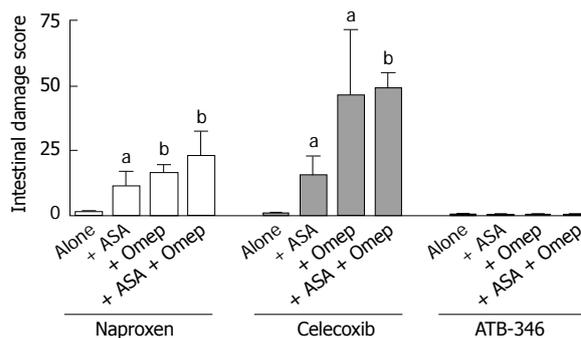


Figure 2 Proton pump inhibitors and low-dose aspirin significantly exacerbate nonsteroidal anti-inflammatory drug-induced small intestinal ulceration. Rats were treated orally, twice-daily for 4.5 d with equi-effective anti-inflammatory doses of naproxen (10 mg/kg), celecoxib (10 mg/kg) or ATB-346 (14.5 mg/kg). ATB-346 is a hydrogen sulfide-releasing derivative of naproxen^[38]. Starting 5 d before the nonsteroidal anti-inflammatory drugs (NSAIDs), the rats began receiving twice-daily treatments with omeprazole (Omep) (10 mg/kg) or vehicle. Starting 3 d before the NSAIDs, the rats began receiving daily doses of low-dose aspirin (10 mg/kg) or vehicle. The results are shown as the mean \pm SE of at least 6 rats per group. ^a $P < 0.05$, ^b $P < 0.01$ vs the corresponding group treated with the NSAID alone. No intestinal damage was observed in rats treated with aspirin (ASA) alone. The exacerbation of small intestinal ulceration with omeprazole was also observed with another proton pump inhibitor, lansoprazole^[37]. This figure was constructed using data from Blackler *et al*^[175].

spp. (> 80% reduction in the jejunum). This diminution of *Bifidobacteria* was an important factor in the PPI-induced increase in NSAID-induced intestinal damage: replenishment of intestinal *Bifidobacteria* in PPI-treated rats reduced levels of naproxen-induced intestinal damage those seen in rats not receiving a PPI. Further evidence that it was the dysbiosis induced by the PPI that resulted in elevated susceptibility to NSAID-enteropathy came from studies of germ-free mice^[37]. Groups of germ-free mice were colonized with intestinal contents from rats that had been treated with a PPI or vehicle. Beginning one week later, the mice were treated with naproxen for 4 d, and the severity of intestinal damage was then blindly evaluated. Mice that had been colonized with bacteria from PPI-treated rats developed significantly worse intestinal damage than those colonized with bacteria from vehicle-treated rats.

While no clinical studies have been published that directly tested the hypothesis that treatment with PPIs could cause dysbiosis and thereby exacerbate NSAID-induced intestinal damage, there are several reports with data that are consistent with our hypothesis, as summarized by Daniell^[43]. In addition to numerous studies documenting that PPIs altering the gut microbiota, resulting in diarrhea^[40-42,44], there is evidence from two studies for the presence of intestinal inflammation (detected by elevated fecal calprotectin levels) in patients taking PPIs^[45,46], and evidence for microscopic colitis in patients taking NSAIDs or PPIs^[47-49], and particularly in patients taking both types of drugs concurrently^[49]. In addition, two studies reported greater small intestinal damage in healthy volunteers taking an NSAID plus a PPI as compared to a group taking only a selective COX-2 inhibitor^[50,51], and it

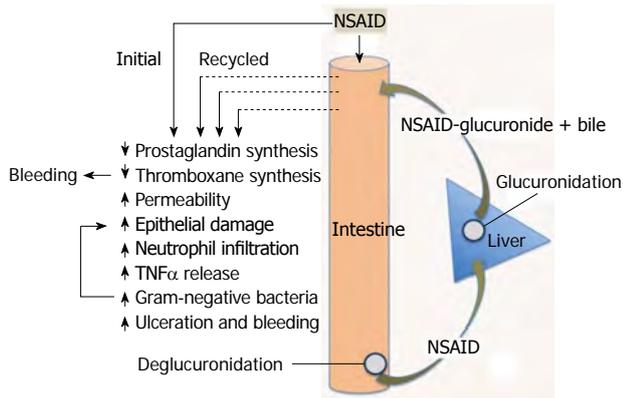


Figure 3 Pathogenesis of nonsteroidal anti-inflammatory drugs-induced enteropathy. Nonsteroidal anti-inflammatory drugs (NSAIDs) produce effects during their initial exposure to the small intestine, and when secreted back into the proximal small intestine, along with bile, following their absorption in the distal intestine, and glucuronidation in the liver. Suppression of thromboxane synthesis likely plays an important role in promoting bleeding (especially with aspirin, an irreversible inhibitor of platelet thromboxane synthesis). Repeated exposure of the intestinal epithelium to the combination of NSAIDs and bile will promote damage, and the damage is likely exacerbated by the shift in intestinal bacteria stimulated by the NSAID (elevated gram-negative bacteria). These effects appear to be mediated by endotoxin, acting at least in part through toll-like receptor-4. The interplay among bile, bacteria and recirculation of the NSAID is complex. For example, bacterial enzymes convert primary bile acids to secondary bile acids (which may be more damaging) and bacterial enzymes are necessary for deglucuronidation, which permits reabsorption and enterohepatic recirculation of NSAIDs. TNF α : Tumor necrosis factor- α .

is now clear that the ability of selective COX-2 inhibitors to damage the small intestine is comparable to that of non-selective NSAIDs^[17].

PATHOGENESIS

The key to development of treatments and prevention strategies for NSAID-enteropathy lies in better understanding of the pathogenesis of this injury. Fortunately, the animal models of NSAID enteropathy are very good, reproducible and simple, and can serve as useful tools for gaining a better understanding of the pathogenesis of this disorder and for testing potential therapeutic/preventative agents. Administration of NSAIDs to rats, for example, results in ulceration predominantly in the distal jejunum and ileum^[52], the same regions where ulcers are concentrated humans^[53,54]. While there will undoubtedly be some differences between rodent models and humans, the existing data suggest that the animal models will be predictive in terms of treatment and prevention strategies. Figure 3 shows some of the key mechanisms suggested to be involved in NSAID-enteropathy, which are discussed in more detail below.

Inhibition of cyclooxygenase activity

Flower *et al*^[55] first suggested the existence of more than one isoform of COX in 1972. It was almost 20 years later that the two isoforms, now known as COX-1 and COX-2, were sequenced^[56,57]. In the decade that followed, a tremendous amount of research was focused on un-

derstanding the physiology and pharmacology of these enzymes, largely fueled by the interest of several large pharmaceutical companies in the notion that selective inhibitors of COX-2 would provide all of the anti-inflammatory activities of NSAIDs without the major adverse effects. However, as the science of COX-2 caught up with the marketing of COX-2, it became evident that the delineation of functions of the two COX isoforms was not so clear-cut as had been proposed and heavily promoted. COX-1 contributes significantly to inflammation while COX-2 contributes significantly to many physiological functions, including mucosal defence^[58]. This was shown clearly both by studies of mice lacking the gene for one of the COX isoforms and pharmacological studies^[59-63]. A striking finding from our laboratory was that injection of carrageenan into the hind-paw of COX-2-deficient mice resulted in inflammation that did not resolve, as it would in a normal mouse^[60], suggesting an important role for COX-2-derived prostanoids in resolution of inflammation and healing. Gilroy *et al*^[61] provided compelling evidence from animal models of pleurisy showing the same, and identifying specific COX-2-derived prostanoids that contributed significantly to down-regulating inflammation. Serhan *et al*^[64] described a family of previously unrecognized lipid mediators (lipoxins, resolvins, protectins), some of which were derived from COX-2, that act at several levels of the inflammatory cascade to “turn off” inflammation and allow for a coordinated restoration of tissue homeostasis^[65].

The same was true in the GI tract, as COX-2-derived prostanoids were found to contribute significantly to maintenance of the integrity of the tissue, to repair of mucosal injury and to resolution of inflammation^[58]. Thus, COX-2 is the isoform that produces PGs at the margins of gastric ulcers, which contribute significantly to the healing of those ulcers^[66,67]. In the colon, prostaglandins derived from COX-2 play a very important role in down-regulating inflammation and promoting repair of mucosal injury^[52,68,69]. Suppression of COX-2 activity has been shown to exacerbate experimental colitis^[52,69]. Indeed, COX-2 is up-regulated throughout the GI tract when the tissue is injured or when there is insufficient PG production *via* COX-1^[52,63,70]. For example, COX-2 is rapidly induced in the stomach in response to suppression of COX-1 by aspirin^[70], and it helps to enhance mucosal defence in such circumstances. One of the mechanisms through which this is achieved is *via* the production, *via* COX-2, of a potent gastroprotective and anti-inflammatory substance, 15-epi-lipoxin A₄^[71,72]. Induction of damage in the stomach, in the absence of any other toxic challenge, requires suppression of both COX-1 and COX-2^[62], and this also appears to be the case in the small intestine^[63].

Clinical studies generally show that selective COX-2 inhibitors produce less gastroduodenal injury and bleeding than conventional NSAIDs, but the small intestinal damage may not differ substantially between the two sub-classes of NSAIDs. For example, Maiden *et al*^[73] performed a VCE study comparing the enteropathy

produced in patients on long-term NSAID or selective COX-2 inhibitor therapy, and the key finding was that NSAIDs and selective COX-2 inhibitors produced comparable levels of small bowel damage (small intestinal injury was observed in 50% of the patients treated with a selective COX-2 inhibitor *vs* 62% of patients treated with a conventional NSAID; not significantly different).

While suppression of COX activity undoubtedly contributes to the pathogenesis of NSAID-enteropathy, it is clear that other factors probably play a more significant role. Suppression of COX activity likely contributes to this disorder mainly through the impairment of repair processes, such as angiogenesis^[74], and through inhibition of platelet aggregation, leading to bleeding. The latter effect, however, is most apparent with aspirin, which irreversibly inhibits platelet COX-1, and with NSAIDs that have a long half-life.

Mitochondrial injury

One of the earliest changes that can be detected after NSAID administration, in addition to inhibition of COX activity, is mitochondrial injury^[75]. Morphological evidence of mitochondrial damage can be detected within 1 h of administration of an NSAID to rats, and *in vitro* studies of liver showed that the NSAID could rapidly cause uncoupling of oxidative phosphorylation^[75]. This provides a mechanistic explanation for the ability of NSAIDs to damage intestinal epithelial cells and to increase epithelial permeability, as have been demonstrated by several groups^[22,52,76]. On the other hand, this mechanism does not explain the localization of ulcers in the jejunum and ileum in animal models and in humans. In their endoscopic study of diclofenac-induced small intestinal injury, Fujimori *et al*^[54] observed denuded regions throughout the small intestine (perhaps indicative of a topical erosive effect), but ulcers were concentrated in the distal jejunum and ileum.

Role of bile and enterohepatic circulation

Several observations suggest important roles for bile and for enterohepatic circulation of NSAIDs in the pathogenesis of NSAID-enteropathy (Figure 3). Ligation of the bile duct in rats prevents NSAID-induced intestinal damage^[75-78]. There have also been reports that NSAIDs that do not re-circulate enterohepatically do not cause small intestinal damage^[52,75], although aspirin is a notable exception, at least when administered intraduodenally or in an enteric-coated formulation^[11,78]. Also, in rats lacking the hepatocanalicular conjugate export pump, which is required for excretion of conjugated NSAIDs into bile, but not for the flow of bile itself, intestinal damage induced by an NSAID (diclofenac) was prevented^[79]. On the other hand, induction of higher expression of the export pump aggravated NSAID-induced intestinal damage^[79]. A number of studies have demonstrated that a combination of an NSAID and bile is damaging to intestinal epithelial cells^[80,81] and non-GI cells^[82]. It is noteworthy that in all of these studies, it was secondary bile acids

that were found, in combination with NSAIDs, to be effective in damaging cells. Moreover, it has been shown that administration of an NSAID to rats results in increased concentrations of secondary bile acids in bile^[83]. Thus, when an NSAID recirculates enterohepatically, the intestinal epithelium is repeatedly exposed to a damaging combination of the NSAID and bile. If this were the primary mechanism of injury in NSAID-enteropathy, however, one would expect to see ulcers produced where the highest concentrations of NSAID and bile would be found (*i.e.*, near the Sphincter of Oddi), whereas the most severe tissue injury is concentrated in the more distal parts of the small intestine^[54]. It has been suggested that the sites of ulceration correspond to the sites of NSAID re-absorption, and related to the deconjugation of the NSAIDs at those sites by bacterial β -glucuronidases^[79,84-86].

Role of bacteria

There is an abundance of evidence that intestinal bacteria contribute to the pathogenesis of NSAID-enteropathy, but it remains unclear if there is a primary role, initiating the tissue damage, or just a secondary role, exacerbating tissue injury and impeding repair. One of the key observations leading some to propose a primary role of bacteria in NSAID-enteropathy is that germ-free rats and mice develop little or no intestinal damage when given an NSAID, but when colonized by gram-negative bacteria, these animals become susceptible to NSAID-enteropathy^[87,88]. Several studies have documented dramatic shifts in the types of bacteria in the small intestine following NSAID administration, with increases in gram-negative bacteria generally being observed, and a concomitant reduction in gram-positive bacteria^[89-93]. In some studies, there appeared to be an enrichment of specific bacteria, such as *Enterococcus faecalis*, *Clostridium*, *Bacteroides* and *Escherichia coli* (*E. coli*)^[89-91]. A number of studies reported protective effects of antibiotics against NSAID-enteropathy, particularly when the antibiotics were effective in reducing number of gram-negative bacteria^[88,89,93,94]. Similarly, some probiotics have been reported to reduce the severity of NSAID-enteropathy, especially when they prevent increases in the number of gram-negative bacteria in the intestine^[93,95,96]. Despite a considerable number of studies examining the potential contribution of bacteria to NSAID-enteropathy, there remains a lack of clear evidence for a primary role of bacteria in initiation of tissue injury. Bacteria rapidly colonize sites of ulceration and can interfere with ulcer healing^[97,98]. In one of the earliest papers on the pathogenesis of NSAID-enteropathy, Kent *et al*^[89] remarked "since the antibiotics do not prevent completely the ulceration, we think that these agents reduce the severity of the lesion by allowing healing to start sooner". A similar conclusion was drawn by Yamada *et al*^[99].

The apparent importance of gram-negative bacteria in the pathogenesis of NSAID-enteropathy is consistent with reports of a role for lipopolysaccharide (LPS) in driving tissue inflammation and impairment of ulcer healing. Hagiwara *et al*^[91] showed that heat-killed *E. coli*

and their purified LPS caused “deterioration” of NSAID-induced ileal ulcers, but could not cause ulcers themselves in the absence of the NSAID). Koga *et al*^[94] reported that systemic administration of LPS reversed the beneficial effects of an antibiotic in reducing the severity of NSAID-enteropathy in rats, and further demonstrated that T cell function was not required for NSAIDs to induce intestinal ulceration. Watanabe *et al*^[93] demonstrated that mice lacking the endotoxin receptor, toll-like receptor-4, developed much less (about 80%) intestinal damage when given an NSAID than the normal counterparts. These data are once again consistent with the notion that bacteria play a secondary role in NSAID-enteropathy, exacerbating tissue injury and interfering with ulcer healing. These effects may be in part attributable to activation of neutrophils in the mucosal microcirculation, which has been shown to contribute significantly to ulceration^[100-104], and local generation of tumor necrosis factor-alpha may be one of the main triggers leading to neutrophil recruitment and/or activation^[93,105-107].

As mentioned above, one of the key observations supporting an important role of bacteria in the pathogenesis of NSAID-enteropathy was that germ-free animals do not develop significant small intestinal damage following NSAID administration^[75-78]. However, one must bear in mind that ligation of the bile duct blocks the secretion of bile and the enterohepatic circulation of NSAIDs, both of which have been implicated in intestinal injury by these drugs (Figure 3). The conversion of primary bile acids to secondary bile acids is dependent on intestinal bacterial enzymes. Thus, germ-free animals lack secondary bile acids. As mentioned above, most studies that have shown that bile acids (alone or in combination with an NSAID) can cause damage to intestinal epithelial cells have used secondary, rather than primary bile acids^[80,81]. Moreover, the re-absorption of NSAIDs in the distal small intestine is largely dependent on bacterial β -glucuronidase activity, which de-conjugates NSAID-glucuronides, allowing the NSAID to be transported across the epithelium^[84]. Enterohepatic circulation of NSAIDs is negligible in animals that lack intestinal bacteria, resulting in decreased exposure of the intestine to the NSAID, and therefore decreased tissue injury. Recently, LoGuidice *et al*^[85] demonstrated that an inhibitor of bacterial β -glucuronidase could significantly reduce the severity of diclofenac-induced small intestinal injury in mice. β -glucuronidase has been shown to be expressed in *Clostridium*, *Peptostreptococcus*, *Staphylococcus* and *E. coli*^[85,108].

TREATING AND PREVENTING NSAID-ENTEROPATHY

In sharp contrast to NSAID-induced gastroduodenal damage, where several options are available to provide protection to a patient, no treatments or prevention strategies for NSAID-enteropathy have been convincingly shown to be effective. As outlined above, PPIs provide upper GI protection against NSAIDs but worsen NSAID-enteropathy

in animals, and there is emerging evidence that the same is the case in humans. There are novel NSAIDs in development that do not cause enteropathy in animals (discussed below).

Misoprostol, metronidazole and sulfasalazine have all been suggested to be beneficial in treatment or prevention of NSAID-enteropathy in humans, but the studies suggesting this had significant limitations (open-label, not controlled, and/or small sample sizes)^[22,24,109-111]. Misoprostol, H₂RA and sucralfate were found to be ineffective in reducing NSAID-induced intestinal permeability in humans^[112,113], though in one, open-label study misoprostol reduced the elevated intestinal permeability induced by indomethacin^[114]. Based on the animal data showing beneficial effects of metronidazole in reducing NSAID-enteropathy^[99], Bjarnason *et al*^[24] performed an open-label human study of chronic NSAID users. The patients took metronidazole for 2-12 wk while continuing their NSAID treatment. The endpoints were fecal excretion of ⁵¹Cr-labeled erythrocytes and ¹¹¹In-labelled neutrophils. Both markers declined significantly during metronidazole treatment, leading the authors to conclude that “these results suggest that the neutrophil is the main damaging effector cell in NSAID induced enteropathy” and that the main chemoattractant “may be a metronidazole sensitive microbe”.

The observations from animal studies that NSAID-enteropathy was accompanied by dramatic shifts in numbers and types of intestinal bacteria led to a number of studies of the potential value of probiotics for treatment or prevention of NSAID-enteropathy. In studies in rats, Kinouchi *et al*^[95] demonstrated that *Lactobacillus acidophilus* and *Bifidobacteria adolescentis* administration markedly reduced the severity of NSAID-induced ileal ulceration. Syer *et al*^[96] also showed a marked protective effect of *Bifidobacteria adolescentis* in a rat model of NSAID-enteropathy. Only two clinical trials of a probiotic for NSAID-enteropathy have been reported to date. Montalto *et al*^[115] performed a randomized, double-blind, placebo-controlled trial of VSL#3, a probiotic formulation consisting of 8 different live bacteria. Volunteers received indomethacin daily for 4 d, and fecal calprotectin levels were the endpoint. The placebo-treated volunteers exhibited markedly elevated fecal calprotectin levels during the period of indomethacin treatment, while during treatment with VSL#3 the fecal calprotectin levels remained within the normal range. In a study by Endo *et al*^[116], 25 patients with unexplained iron deficiency anemia who had been taking low-dose enteric-coated aspirin plus omeprazole for more than 3 mo were given either *Lactobacillus casei* (*L. casei*) or placebo for 3 mo while continuing the aspirin and omeprazole therapy. VCE at the end of the treatment period showed a significant reduction of mucosal breaks and “capsule endoscopy score” in the group receiving *L. casei*. The results of this small clinical study are consistent with a study of *L. casei* (strain Shirota) in a rat model of indomethacin-induced enteropathy^[117].

Lactoferrin has been shown to prevent NSAID-

induced bleeding in rodents^[118] and this effect may be related to its ability to promote the growth of *Bifidobacteria* in the small intestine^[119]. Oral treatment of healthy volunteers with recombinant lactoferrin was shown to reduce indomethacin-induced changes in small intestinal permeability^[120]. However, in this short-term study, only a very modest increase in intestinal permeability was seen, with only a single administration of lactoferrin that would have been unlikely to have significantly affected the intestinal microbiome.

Rebamipide is a quinolinone derivative that is used to promote the healing of GI ulcers and for mucosal protection. Its mechanism of action is not fully understood, though it appears to stimulate mucus secretion and PG synthesis^[121] and to scavenge oxygen-derived free radicals^[6]. It has been shown to significantly reduce the severity of NSAID-induced enteropathy in rats^[122]. Niwa *et al*^[123] performed a pilot study in healthy humans to examine the effectiveness of rebamipride in preventing NSAID-enteropathy. The volunteers received placebo or rebamipride together with diclofenac for 7 d. The small intestine was examined at the end of the study by VCE. Damage was observed in 8 of the 10 of placebo-treated group (2 ulcers, 1 bleed), but in only 2 of the 10 of rebamipride-treated group (no ulcers or bleeding). However, a larger study of healthy volunteers treated for 14 d with an NSAID (diclofenac), a PPI (omeprazole) and either rebamipride or placebo, failed to detect a significant benefit of rebamipride in terms of reducing the incidence of intestinal mucosal injury^[124]. Larger studies of rebamipride, ideally in patients receiving NSAID therapy for an inflammatory disorder, are needed to clarify if this drug will have benefit in reducing the incidence and/or severity of NSAID-enteropathy.

Studies in animal models have suggested other possible approaches to prevention of NSAID-enteropathy, but have not yet been assessed in humans. For example, in a mouse model of acute indomethacin-induced intestinal damage, Yasuda *et al*^[125] found that dopamine D2 receptor antagonists reduced the severity of damage, and these effects were mediated through the activation of endogenous anti-inflammatory pathways mediated by *via* α 7-nicotinic acetylcholine receptors, as had been observed previously^[126]. Using the same model, Kato *et al*^[127] demonstrated that certain 5-HT receptors could modulate susceptibility to NSAID-enteropathy. They reported that antagonists of the 5-HT₃ receptor (ondansetron and ramosetron) dose-dependently reduced intestinal damage, while a 5-HT₄ antagonist (GR113808) aggravated damage. A 5-HT₄ agonist (mosapride) significantly reduced damage. As in the case of protection with dopamine D2 receptor antagonists, the authors suggested that the beneficial effects the 5HT₄ agonist may be mediated through activation of α 7-nicotinic acetylcholine receptors. There have also been studies demonstrating a significant increase in intestinal motor activity after administration of NSAIDs, and have suggested that this contributes to the generation of injury, but pharmacological approaches targeting this 5-HT/ α 7-

nicotinic acetylcholine receptor axis have not yet been evaluated in human NSAID-enteropathy.

NSAID pro-drugs: The enteropathy remains

Pro-drugs have been defined as “bioreversible derivatives of drug molecules that undergo an enzymatic and/or chemical transformation *in vivo* to release the active parent drug, which can then exert the desired pharmacological effect”^[128,129]. A number of NSAID pro-drugs have been developed, based on the premise that if the drug can pass through the stomach in an inactive form, it will not inhibit PG synthesis in the stomach, and therefore will not be ulcerogenic. In essence, an NSAID pro-drug of this design does not differ significantly from an enteric coated NSAID, and the problems associated with the latter are well documented^[8,36]. Moreover, there are several problems with the premise upon which NSAID pro-drugs are based. First, once the drug is absorbed and transformed to release the parent drug, that drug will produce “the desired pharmacological effect”. That effect, reduction of pain and inflammation, is attributable to systemic inhibition of COX activity. In the absence of any “protective” intervention, systemic inhibition of COX activity will result in damage and bleeding in the upper GI tract. Thus, NSAIDs administered systemically induce significant gastrointestinal ulceration and bleeding^[130-132]. If a pro-drug is formulated such that it produces a marked delay in the release of the parent drug, there will be a similar delay in the onset of the desired activity. Second, the pro-drug approach is focused entirely on sparing the upper GI tract of injury, ignoring the potential of the drug to cause small intestinal injury, particularly if it undergoes enterohepatic recirculation. Once the pro-drug is metabolized to release the parent drug, the parent drug will behave, pharmacokinetically and pharmacodynamically, in the same way as if the parent drug itself had been administered. These are points that have been acknowledged on the website of a company that is developing a naproxen pro-drug: “the pro-drug approach will not address the GI damage associated with the systemic inhibition of COX after release of the parent drug, nor will it address the toxic effects of metabolites delivered into the gut lumen with bile”^[133].

Clinical trials of pro-drugs have often produced data that are very favourable to the pro-drug. However, this is largely because such studies have typically focused on acute gastric or gastroduodenal damage (erosions and “endoscopic ulcers”)^[134] that are of questionable clinical significance, since they do not necessarily predict the incidence of true ulcers^[135]. Indeed, the same is true for most of the trials of selective COX-2 inhibitors and of PPIs, which gave a false signal of the GI safety of those classes of drugs because of reliance on inappropriate endpoints for upper GI damage and lack of consideration of the potential damaging effects of these drugs on the small intestine. When examined in “real world” scenarios, using clinically meaningful endpoints^[135], there is little, if any, evidence of significant benefit of NSAID pro-drugs over

the parent drugs or over other NSAIDs. This topic has been very well reviewed by Graham^[135]. Thus, while the pro-drug sulindac rarely caused erosions or “endoscopic ulcers” in short-term studies of human volunteers^[136,137], longer term studies in at-risk patients showed this drug to offer no upper GI safety benefit as compared to other NSAIDs^[138]. Likewise, nabumetone was purported to be a GI-safe pro-drug, and acute upper GI studies suggested that this was the case^[139], but in at-risk patients the drug did not offer any benefit over other NSAIDs^[140]. Neither sulindac nor nabumetone have been specifically examined with respect to their propensity to cause small intestinal ulceration and bleeding. Moreover, there are suggestions in the literature^[132,141], supported by animal studies^[130,131], that systemic administration of NSAIDs, which completely avoids contact of the drug with the lining of the stomach and duodenum, does not offer significant benefit in terms of reducing the incidence of significant GI ulceration and bleeding.

Novel intestinal-sparing NSAIDs

The advances that have been made in understanding the pathogenesis of NSAID-enteropathy provide important clues for designing novel NSAIDs that will not damage in the small intestine (or the stomach). Several approaches have been taken that show promise, mainly using the “co-drug” model of drug design^[142]. Co-drugs are somewhat like pro-drugs, with the key difference being that the promoiety is not inert; rather, it exerts important pharmacological effects^[129]. Two such classes of co-drugs are the nitric oxide (NO)-releasing NSAIDs and the hydrogen sulfide (H₂S)-releasing NSAIDs^[143-145]. In each case, the NSAID portion of the co-drug behaves the same as expected (inhibition of COX-1 and COX-2, leading to anti-inflammatory and analgesic effects), while the gaseous mediator portion of the co-drug exerts mucosal protective effects, very similar to the effects of endogenous prostaglandins^[146,147]. Both of these gaseous mediators are vasodilators and can inhibit leukocyte adherence to the vascular endothelium^[148,149]. Suppression of mucosal synthesis of NO or H₂S reduces the resistance of the stomach to the damaging effects of NSAIDs and other irritants, and impairs the healing of pre-existing damage^[148,150-156]. NO and H₂S donors can increase the resistance of the gastric mucosa to injury induced by NSAIDs and other noxious substances^[148,151,156,157] and can accelerate healing of ulcers in rodent models^[37,150,153,154,158]. Some of the other actions of NO-NSAIDs and H₂S-NSAIDs and their underlying mechanisms have been reviewed previously^[143,145,152].

NO-NSAIDs were shown to cause significantly less intestinal damage than the parent drugs^[159,160], and to be well tolerated in rats with pre-existing colitis^[159]. In a small, short-term clinical trial, an NO-NSAID produced significantly less of an increase in small intestinal permeability than was produced by an equivalent dose of the parent drug (naproxen)^[161]. Despite very promising results from clinical trials that demonstrated efficacy and safety

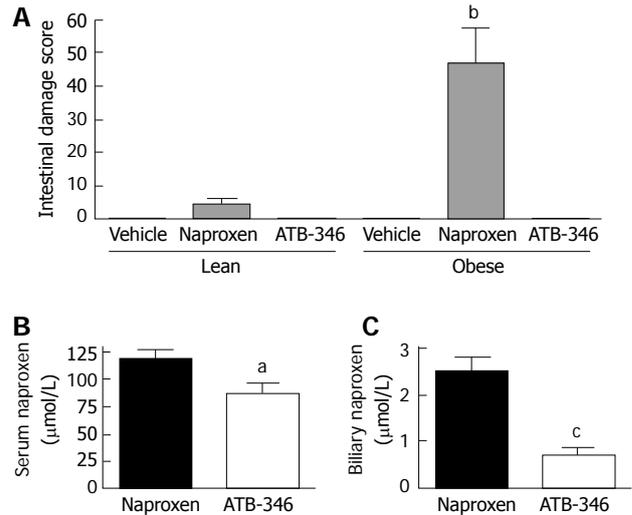


Figure 4 Intestinal safety and altered biliary excretion of ATB-346. A: ATB-346 is a hydrogen sulfide-releasing derivative of naproxen^[38]. When administered to obese Zucker rats, twice-daily for 4.5 d at 10 mg/kg, naproxen induced small intestinal damage that was significantly more severe in the obese rats ($P < 0.01$). However, at an equimolar dose, ATB-346 did not induce intestinal damage in lean or obese rats^[175]; B: The serum levels of naproxen in normal rats after 4.5 d of twice-daily administration of ATB-346 were marginally, but significantly ($P < 0.05$) lower than those in rats treated with an equimolar dose of naproxen; C: Biliary levels of naproxen in rats treated with ATB-346 (as above) were markedly reduced compared to those in rats treated with an equimolar dose of naproxen ($P < 0.001$). The data shown in this graph are from Blackler *et al*^[175].

in osteoarthritis^[162-166], NO-NSAIDs have not obtained regulatory approval because the safety advantages over the parent drug (naproxen) have not been sufficiently demonstrated. One key GI safety clinical trial fell just short of showing a significant benefit as compared to naproxen ($P = 0.066$)^[167].

H₂S-releasing NSAIDs exhibit enhanced anti-inflammatory activity relative to the parent drugs^[37,151,155,168,169], presumably attributable to the anti-inflammatory and pro-resolution effects of the H₂S released from these drugs^[149,158,170-174]. In addition to sparing the gastric mucosa of damage in several circumstances of impaired mucosal defence^[38,175], H₂S-releasing NSAIDs have been shown not to cause damage in the small intestine of rats^[38,155,175]. Moreover, when tested in co-morbidity and polypharmacy models, with repeated administration over several days, an H₂S-releasing derivative of naproxen (ATB-346) did not cause small intestinal damage^[175] (Figures 2 and 4). For example, obese rats that exhibited markedly greater naproxen-induced enteropathy than was observed in lean rats, but ATB-346 did not elicit damage in lean or obese rats^[175]. Interestingly, Zucker obese rats have a microbiota distinct from that of their lean littermates, with a marked reduction in intestinal levels of *Bifidobacteria*^[176]. Recall that we observed that PPIs increased the severity of NSAID-enteropathy in rats, and found that this was largely attributable to a decrease in intestinal *Bifidobacteria* levels^[37]. ATB-346 retained its favourable profile in the intestine even when co-administered with a PPI and/or low-dose aspirin^[175] (Figure 2).

A particularly important feature of ATB-346 that may be very important in terms of its lack of damaging effects in the small intestine is that, though metabolized to release naproxen, there are relatively low levels of naproxen in bile after administration of this compound^[175] (Figure 4). Moreover, the biliary levels of naproxen-glucuronide were reduced by 72% in the ATB-346 group as compared to the naproxen group^[175]. These altered pharmacokinetics of ATB-346 *vs* naproxen did not alter the anti-inflammatory activity of the drug^[37], but could contribute significantly to the intestinal-sparing properties of ATB-346.

Recently, a class of drugs was described that consists of an NSAID attached to moieties releasing both NO and H₂S^[177]. These compounds show comparable actions as the parent drugs in terms of inhibiting COX activity, but there are no available data on their GI toxicity.

NSAIDs pre-associated with phospholipids are a unique type of “co-drug”.

Surface-active phospholipids have been proposed to constitute an important component of the epithelial “barrier” to acid back-diffusion, and NSAIDs can to disrupt this barrier^[178,179]. Lichtenberger *et al*^[180] demonstrated that pre-associating an NSAID with a zwitterionic phospholipid prevents the NSAID from disrupting the barrier function of the epithelium. Thus, covalently linking phosphatidylcholine to aspirin, ibuprofen and other NSAIDs results in compounds with equivalent anti-inflammatory properties to the parent drug, but with markedly reduced gastric toxicity^[181]. This has been demonstrated in endoscopic clinical trials for an aspirin derivative^[181], and also with an ibuprofen derivative^[182], though in the latter trial, statistical significance was only seen in an older subset of the patients studied. Recently, Lichtenberger *et al*^[78] demonstrated that pre-associating aspirin with phosphatidylcholine greatly reduced the small intestinal damage produced by intraduodenal administration of this compound, as compared to aspirin alone.

CONCLUSION

NSAID-enteropathy has largely been ignored for decades as a result of the focus on NSAID-gastropathy, driven largely by the development of several commercially successful drugs targeting that disorder (H₂RA, PPIs, selective COX-2 inhibitors). Moreover, the difficulty in detecting NSAID-enteropathy and the lack of any proven-effective preventative or treatment options has contributed to an under-appreciation of the magnitude of this significant adverse reaction to a very widely used class of drugs. With the development of video capsule endoscopy, the frequency and severity of NSAID-enteropathy has become more evident. Techniques such as VCE also permit more conclusive studies of the safety of novel NSAIDs and of potential prevention or treatment strategies.

The animal models for NSAID-enteropathy are very good, and they have provided a great deal of information on the pathogenesis of this disorder. Moreover, the ani-

mal studies have given some direction as to viable strategies for preventing NSAID-enteropathy, and the models are useful for testing novel therapeutics agents.

As is the case with NSAID-induced injury in the upper GI tract, it is important that studies of NSAID-enteropathy focus on animal models that are most similar to the patients that use these drugs and most often develop serious adverse effects. Thus, future studies should focus on the use of animal models with relevant co-morbidities that display increased susceptibility to NSAID-enteropathy, and on patients most at risk of developing intestinal damage and bleeding.

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Role of T cell death in maintaining immune tolerance during persistent viral hepatitis

Juan Ramón Larrubia, Megha Uttam Lokhande, Silvia García-Garzón, Joaquín Miquel, Dolores Subirá, Eduardo Sanz-de-Villalobos

Juan Ramón Larrubia, Megha Uttam Lokhande, Silvia García-Garzón, Joaquín Miquel, Dolores Subirá, Eduardo Sanz-de-Villalobos, Translational Hepatology Unit, Guadalajara University Hospital, University of Alcalá, 19002 Guadalajara, Spain
Author contributions: Larrubia JR and Lokhande MU contributed equally towards the conception and design of the review; Larrubia JR and Lokhande MU co-wrote and Larrubia JR revised the manuscript; García-Garzón S, Miquel J, Subirá D and Sanz-de-Villalobos E contributed equally to the supportive work and supervision.

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Correspondence to: Megha Uttam Lokhande, MSc, Translational Hepatology Unit, Guadalajara University Hospital, University of Alcalá, Donante de Sangre st., 19002 Guadalajara, Spain. lokhandemu@gmail.com
Telephone: +34-949-209200 Fax: +34-949-209259
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Abstract

Virus-specific T cells play an important role in the resolution of hepatic infection. However, during chronic hepatitis infection these cells lack their effector functions and fail to control the virus. Hepatitis B virus and hepatitis C virus have developed several mechanisms to generate immune tolerance. One of these strategies is the depletion of virus-specific T cells by apoptosis. The immunotolerogenic liver has unique property to retain and activate naïve T cell to avoid the over reactivation of immune response against antigens which is exploited by hepatotropic viruses to persist. The deletion of the virus-specific T cells occurs by intrinsic (passive) apoptotic mechanism. The pro-apoptotic molecule Bcl-2 interacting mediator (Bim) has at-

tracted increasing attention as a pivotal involvement in apoptosis, as a regulator of tissue homeostasis and an enhancer for the viral persistence. Here, we reviewed our current knowledge on the evidence showing critical role of Bim in viral-specific T cell death by apoptotic pathways and helps in the immune tolerance.

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Key words: T cell death; Specific cytotoxic T lymphocytes; Hepatitis C virus immune tolerance; Apoptosis; Bcl-2 interacting mediator; Liver tolerance; Apoptotic pathways; Viral hepatitis

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INTRODUCTION

Hepatotropic, non-cytopathic viruses such as hepatitis B virus (HBV) and hepatitis C virus (HCV) behave as intracellular parasites. The activation of cellular immune response by priming of naïve specific CD4⁺ and CD8⁺ T cells in the lymph nodes is very important to control viral infection. However, the unique ability of the liver to retain and activate naïve CD8⁺ T cells leads to liver tolerance, by-passing normal activation in the lymph nodes. The continuous triggering of antigen presenting cells (APCs) in the sinusoids by the antigen-rich blood leads to peripheral tolerance to protect the liver tissue. This physiological feature can be used by hepatotropic viruses as a persistence mechanism. The depletion of liver activated CD8⁺ T cells is the critical part of the peripheral tolerance in HBV/HCV infection. The anticipated mecha-

nisms for immune tolerance in liver specific pathogens are linked to virus-specific T cells death. The vital role of pro-apoptotic molecule, Bcl-2 interacting mediator (Bim) in the death of the virus-specific T cells has been shown after intrahepatic T cell activation by hepatocytes^[1], in chronic HBV and HCV infection^[2,3]. Therefore, this review provides glimpse of the recent advances to understand the cellular and molecular mechanism involved on “T cell death” during viral hepatitis as a viral escape mechanism through the induction of a specific-immunotolerant status on the host.

VIRAL HEPATITIS

HBV and HCV viruses are two hepatotropic non-cytopathic, human blood-borne viruses. HBV is a small, enveloped DNA virus that undergoes a pro-viral state to persist in the host. HCV is an enveloped virus with a plus-strand RNA genome. It has been estimated that more than 350 million for HBV and 170 million people for HCV are infected. Approximately 80% of infections in HCV and > 90% of infected neonates, 5%-10% of infected adults in HBV succeed in establishing a chronic infection, with the potential for developing severe liver diseases such as cirrhosis and hepatocellular carcinoma^[4,5].

Highly productive and replicative viruses such as HBV and HCV are associated with ineffective antiviral immunity during persistent viral infections. The complex ineffective immunity involves the functional deterioration of antiviral T cell responses and contraction of the size of this response. In persistent HBV/HCV infections, T cells are continuously challenged by high levels of viral antigens that eventually result in limiting the antiviral T cell response and ultimately leading to T cell exhaustion. This is a progressive process, starting with the deficiency in cytokine production, proliferation and survival^[6], to end with physical deletion of specific antiviral T-cell populations^[7].

Meticulously, cytotoxic T lymphocytes (CTLs) play a vital role in viral eradication^[8] and in the pathogenesis of hepatitis^[9-11]. A strong, multi-specific and long-lasting T-cell immune response emerges to be important for control of viral infection^[12-14]. Appropriate, polyclonal, vigorous and multi-specific CTL responses can facilitate complete viral clearance, in which long-lasting protective T cell response is observed. However, specific CTL responses are usually not strong enough to eradicate the virus, hence contributing to persistent infection^[15,16].

HBV and HCV are hepatotropic viruses that replicate in the liver. This organ features a unique immune tissue, where the deletion of antiviral T cell populations has been shown, being involved in local and systemic immune tolerance.

LIVER AS A FOUNDATION OF IMMUNE TOLERANCE

Liver situates at a hemodynamic convergence, receiving

the splanchnic stream, which means an intense contact with exogenous antigens. This fact leads to the development of tolerance mechanisms to avoid inappropriate immune system activation, but it also allows antigen presentation by resident cells. Therefore, the liver is progressively more being recognized as an immune organ^[17]. Liver sinusoids, hepatic arteries and portal venous carry blood containing digested nutrients and micro antigens from intestine, and as a primary metabolic organ, the liver produces multiple neo-antigens. All these molecules pass through sinusoids and finally are taken up and metabolized by different hepatic resident cells. The liver has acquired specialized mechanisms of immune tolerance to avoid the over reactivation of immune response against antigens that are metabolized in the liver. In fact, this process may be beneficial for inducing tolerance to liver grafts but also to the liver specific pathogens. Therefore, hepatotropic viruses exploit these immunotolerogenic liver features to persist. It is important to remind that the liver has the ability to retain and activate naïve CD8+ T cells ineffectively, in contrast to other lymphoid tissues. This fact may allow pathogens to escape from T cell mediated immunity and establish a persistent hepatic infection due to immune tolerance induction. This immunotolerant state can be reached by the development of T cell anergy but also by specific T cell deletion.

Uniqueness of the liver

The unique character of the hepatic tissue to tolerate liver allograft across major histocompatibility complex (MHC) mismatch in the pig without immunosuppression was described by first time in 1969^[17]. Later studies confirmed that this occurred because of the induction of immunological tolerance in the liver^[18]. Initially, “graveyard theory” suggested that the exclusive ability of the liver to get rid of activated T cells, programmed to undergo apoptosis, was the root of the hepatic tolerance effect^[19]. This theory proposed two functions of the liver as a T cell graveyard: (1) passive killer of the liver cells after their life cycle; and (2) efficient killer of the activated antigen specific T cells. According to this theory, T cell receptor (TCR) triggering by cognate antigen on TCR transgenic T cells leads to activation and accumulation of those cells in the liver and undergoes depletion of mature T cells^[20].

The theory was again proved by Mehal *et al.*^[21] by indicating that the normal liver is a “sink” for activated T cells. The liver was perfused by T cells showing retention of activated, but neither resting nor apoptotic T cells^[21]. Liver as a graveyard for activated T cells theory forced to believe that all the immune response in the liver would be silent; in spite of this, the presence of an effective virus specific T cells in patients controlling hepatic viral infections^[22,23] could challenge this theory. Nonetheless, the removal of activated T cells by the liver cannot be excluded, as evidenced by the ability of liver allograft to rescue rejecting skin grafts^[21], in which lately tolerising capacity of the liver for activated allo-specific T cells occurs. In some cases, the limited capacity of the liver to induce tolerance

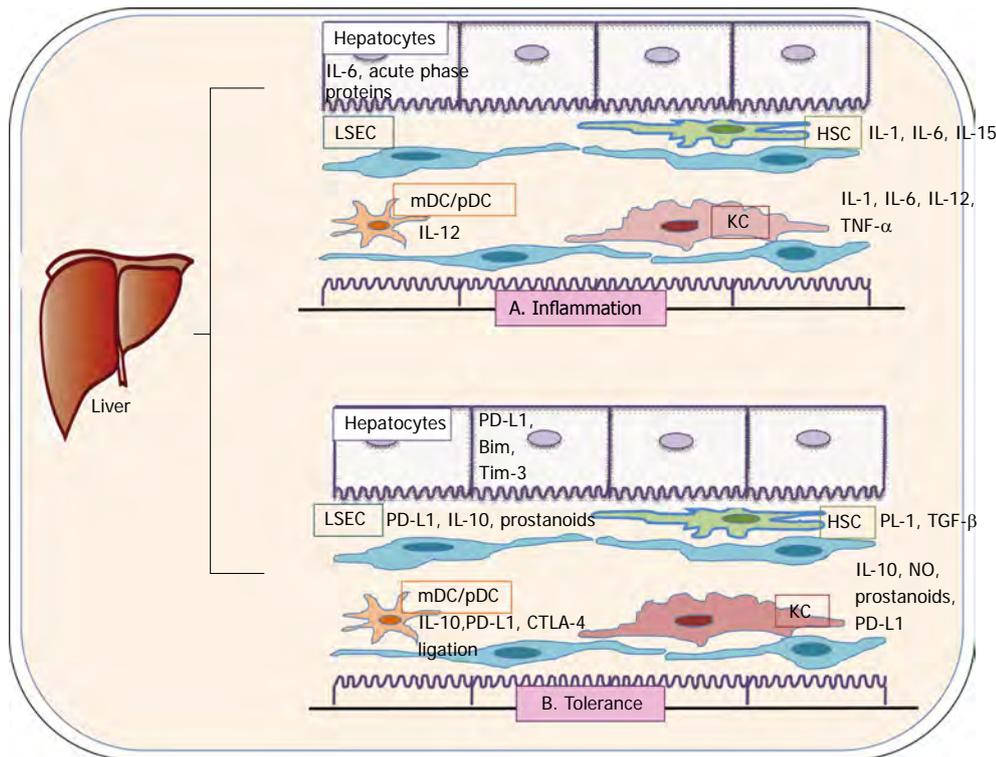


Figure 1 Collective illustration of the hepatic cells with inflammatory and tolerance activities by stimulation of different molecules or receptors. LSEC: Liver sinusoidal endothelial cells; KC: Kuffer cells; DC: Dendritic cells; HSC: Hepatic stellate cells; TNF: Tumor necrosis factor; IL: Interleukin; mDC: Myeloid dendritic cell; pDC: Plasmacytoid dendritic cell; PD-L1: Programmed death ligand-1; Bim: BCL-2 interacting mediator; Tim-3: T cell immunoglobulin mucin-3; CTLA-4: Cytotoxic T-lymphocyte antigen 4; TGF: Transforming growth factor; NO: Nitric oxide.

could be due to large number of activated T cells^[24].

Naïve T cells activation in the liver

The site of T cell activation is a determinant of the outcome of an immune response in the liver^[22]. Tolerance will occur when T cells are activated in the liver. On the other hand, an effective immune response will be generated, when T cells are activated in the lymph nodes. This model put forward the theory that tolerance during viral hepatitis could be the result of early deletion of antigen-specific T cells from the T cell repertoire in the liver^[22]. Usually, naïve T cells are activated in secondary lymphoid organs with consequent up regulation of adhesion molecules and integrins expression, which can bind to endothelial layer of the target organ and ultimately direct T cells to the parenchyma^[25]. Moreover, T cells are not able to interact with parenchymal cells easily and thus they are not usually activated in the solid organs. In spite of this, the situation in the liver is slightly different. Fenestrated endothelial layer in the liver makes available interactions between naïve T cells and liver cells^[26]. It has been showed by MHC class I -restricted, hepatitis B surface Ag-specific CD8+ polyclonal CTL adoptively transferred into wide-spread antigen expressing transgenic mouse model, leading to retention of those cells within the liver^[26]. Moreover, it has been shown that primary antigen-specific T cell can be activated in the liver independently of lymphoid tissues^[27].

Liver APCs in tolerance

Retention, activation and tolerance of naïve T cells in the liver is the result of the action of resident liver cells, including liver sinusoidal endothelial cells (LSEC), Kuffer

cells (KC), liver dendritic cells (DC), hepatic stellate cells (HSC) and hepatocytes. Their collective function in induction of inflammatory response and tolerance has been illustrated in the Figure 1.

Endocytosis specialist-LSEC can express MHC class I and II, accessory CD80, CD86 and CD40 molecules. These features enable those cells to behave as potent APCs with the ability to activate both naïve CD4 and CD8 T cells as well as to cross-present exogenous antigen towards CD8 T cells^[28]. However, LSEC primed naïve CD4+ T cells produce cytokines typical from Th0 rather than Th1 cells^[29]. In addition, LSECs constitutively expressed ICAM-1, which helped in trapping of specific CD8+ T cells in the liver, resulting this process in activated T cell apoptosis^[21]. Furthermore, the cross presentation of antigen by LSEC mainly leads to CD8+ T cells tolerance rather than immunity, demonstrating that LSEC-induced tolerance is an active and dynamic process^[30].

Bone marrow derived and largest group of liver resident macrophages-KC mediate host resistance to infection. Interleukin (IL)-1, IL-6, IL-12 and tumor necrosis factor- α (TNF- α) pro-inflammatory cytokines released by KC^[31] are involved in the inflammatory activities, whereas the nitric oxide, prostaglandin and IL-10 released by KC^[29,32] down-regulate the production of pro-inflammatory cytokines and thereby may contribute to induction of hepatic tolerance. Furthermore, DC-induced antigen-specific T cell activation can be inhibited by KCs^[29], which could also favor tolerance development. In addition, as in LSECs, KCs expressed ICAM-1 mediated trapping of specific CD8+ T cells in the liver resulting in activated T cell death^[21].

Liver DCs are primarily located within periportal areas

and around central veins, which exert tolerogenic properties due to “immature” phenotype. The production of PD-1 and cytotoxic T lymphocyte antigen-4 (CTLA-4) by resting DCs, which are crucial negative co-stimulatory molecules, helps in inducing peripheral CD8+ T cell tolerance by inhibiting proliferation and cytokine production of liver infiltrating effector T cells^[33]. In addition, liver generated DCs are more tolerogenic than DC in lymphatic tissue^[34].

The role of HSCs in hepatic fibrosis includes stimulation of CD4, CD8+ T cells and NKT cells^[35,36]. However, function of HSCs involves not only the inflammatory response^[36], but also a tolerogenic role^[37,38], which is the result of induction of T cell death^[38] by intrinsic mechanism of immune inhibition. The HSCs regulate immune modulation by inducible B7-H1 expression, an inhibitor molecule of B7 family, resulting in T cell apoptosis induction.

Hepatocytes are also capable of activating naïve CD8+ T cells^[39,40] and their interactions with CD8+ T cells may occur through LSEC fenestrations^[38]. However, hepatocytes fail to promote activated CD8+ T cells survival, leading to an impaired T cell activation^[39]. In addition, hepatocyte-activated T cells *in vitro* acquired activity and secrete cytokines but both levels are not constant and T cells consequently appeared to die by passive mechanisms^[41]. Furthermore, infiltrating CD4+ T cells differentiate into a less inflammatory phenotype due to the interaction with MHC II-expressing hepatocytes, which also helps to abrogate antiviral CD8+ T-cell response and viral clearance^[42], which conclude in the tolerance during infection. It has been already proved that T cells activated by hepatocytes undergo premature death^[43], whereas naïve CD8+ T cells priming by DC in the lymph nodes acquired effector functions in the liver.

The site of primary T cell activation could also induce emperipolesis of CD8+ T cells in the liver^[43], which leads to non-apoptotic, destruction of these CD8+ T cells after degradation by lysosomal proteolytic enzymes. This distinct form of emperipolesis has been termed as “suicidal emperipolesis” (SE)^[44]. Benseler *et al*^[44] suggested that SE is a significant mechanism by which death of activated naïve CD8+ T cells occur in the liver within the first few hours before T cells are able to divide and expand. It is also involved in maintenance of tolerance, which is reinforced by break of tolerance in immune-mediated liver damage by treatment of wortmannin^[44], inhibitor of phosphoinositide 3-kinases that blocks emperipolesis. Therefore, SE is an extremely efficient mechanism, able to rapidly delete T cells.

T cell stimulation in the liver encourages tolerance by using mechanisms such as, immune divergence^[45], generation of regulatory T cells^[46], T cell anergy^[47] and T cell death^[1]. Undeniably, hepatic tolerance can explain the elevated frequency of viral persistence during hepatotropic virus infections^[1]. Although there are evidences showing that most infectious microorganisms are promptly removed from the liver, a favorable situation for evading immune responses occurs in some viruses, leading to the

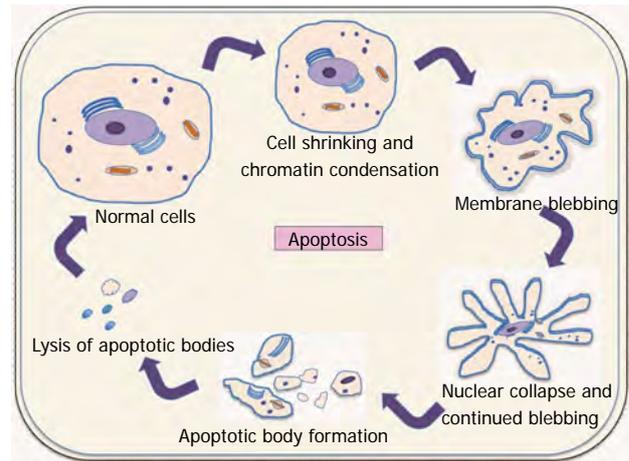


Figure 2 Apoptosis-programmed cell death.

triumph of certain pathogens such as HBV and HCV. Till date, there are two main mechanisms by which HBV and HCV could successfully escape from CTL action: escape mutant generation, and immunosuppressive effects exertion (effector T cell exhaustion and T cell death by apoptosis)^[2,48-50]. Among these mechanisms involved in viral hepatitis persistence, new advances on the role of T cell death induction have been obtained recently and our review in the apoptosis role, paying special attention to the last new insights in this issue will be discussed in the following pages.

APOPTOSIS

A normal cellular process involving physiologically relevant cell death and deletion of unwanted cells is called apoptosis. Apoptosis is essential for cell selection, tissue homeostasis, morphogenesis, and host defense in multicellular organisms. A cell that undergoes apoptosis dies neatly, without damaging its neighbors. The apoptotic signals give rise to activate various proteins and follow a specific classical caspase chain reaction set activation^[51]. Quickly and neatly dismantlement process includes membrane blebbing with shrinking of the cytoplasm and condensation of the nucleus. Phagocytic cells begin to pick up the apoptotic bodies, preventing the release of cellular content and ultimately avoiding inflammation^[52] (Figure 2). Apoptosis occurs by two mechanisms: active and passive mechanism. No presence of antigen gives a signal for termination of immune response by passive apoptotic mechanism (intrinsic pathway). On the other hand, the ligation of Fas (CD95) and TNF receptors-“death receptors” triggered apoptosis lead to active mechanism of apoptosis (extrinsic pathway). Briefly, apoptosis mechanisms involve a family of cysteine proteases, called caspases. These molecules are synthesized in the cell as inactive precursors, or pro-caspases for self-protection against accidental death, which are usually activated after receiving proper trigger by cleavage (Figure 3). Structurally, pro-caspases contain three domains: N terminal prodomain, a large subunit

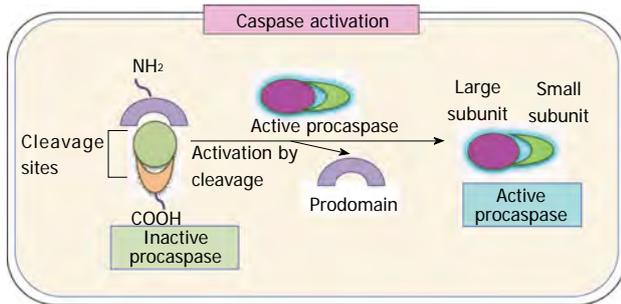


Figure 3 Caspase activation: Inactive proenzyme (procaspase) activated by proteolytic cleavage by another member of caspase family and cleaved two fragments associate to form the active site of the caspase.

and a small subunit. After activation, the active caspase enzyme is formed by heterodimerization of small and large subunits^[43]. Moreover, active caspase molecules are ready to cleave target proteins such as structural or signaling proteins and other effector caspases, preventing other proteins cleavage randomly^[52].

Extrinsic pathway

The extrinsic pathway initiates from outside the cell through triggering the activation of transmembrane “death receptors” that are members of the TNF receptor gene superfamily. Members of this receptor family bind to extrinsic ligands known as pro-apoptotic ligands^[53] and transduce intracellular signals that ultimately result in the destruction of the cell^[54,55]. To date the most well characterized ligands of these receptors are FasL, TNF- α , Apo3L and Apo2L and corresponding receptors are FasR, TNFR1, DR3 and DR4/DR5, respectively^[55-57]. The signal transduction of active cell death process involves several caspases. Activated caspases have an effect on several cellular functions as part of the process that results in the death of the cells^[53].

The signal transduction of mitochondrial-independent active cell death process involves binding of a pro-apoptotic ligand (such as FasL) with its receptors (Fas) on the surface of a target cell. The cytosolic tail of receptors contains a death domain, which when activated, binds to an adaptor protein, which in turn recruits the specific procaspase-8 and -10 and activates them by proteolytic cleavage^[58] that finally initiates the proteolytic caspase cascade leading to apoptosis. Activated caspase 8 triggers the caspase cascade *via* two different pathways, leading to cell death. In type 1 apoptosis, such as in lymphocytes, caspase 8 activates caspase 3 whereas in type 2 apoptosis, like in hepatocytes and pancreatic cells, caspase 8 activate the pro-apoptotic molecule Bid and go ahead for apoptosis *via* the disruption of mitochondrial membrane and cytochrome C release^[59] (Figure 4). The T cell death by type 1 and type 2 Fas induced apoptosis fate is decided by the ratio between proteolytically activated effector caspases, X-chromosome linked inhibitor of apoptosis protein and proto-typical effector caspase substrate inhibitor of caspase-activated DNase. Interestingly, HCV specific in-

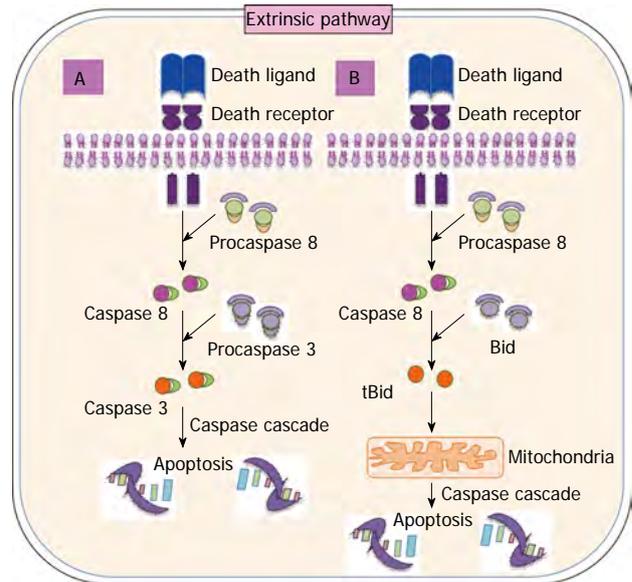


Figure 4 Extrinsic pathway. A: Mitochondria-independent extrinsic pathway: Fas-FasL ligation strikes to recruit pro-caspase 8 activation and induction of caspase cascade by caspase 3 leading to apoptosis; B: Mitochondria-dependent extrinsic pathway: Fas-FasL ligation trigger to activate the pro-caspase 8, which cleave Bid (pro-apoptotic Bcl-2 family molecule) to form truncated Bid (tBid). Then, mitochondrial dependent cell death begins with tBid.

trahepatic lymphocytes contribute to bystander killing *via* Fas-FasL interaction^[60], which support the fact that the liver facilitates liver-trapped activated T cell apoptosis^[61].

Intrinsic pathway

The intrinsic or mitochondrial pathway is initiated within the cell, involving non-receptor-mediated intracellular signals and inducing activities in the mitochondria that initiate apoptosis. DNA damage, loss of cell-survival factors or other types of severe cell stress causes the induction signal for the intrinsic pathway. This passive death process pivots on the balance of activity between pro- and anti-apoptotic signals of the B cell lymphoma 2 (Bcl-2) family proteins^[62]. This balance is maintained by regulation of the permeability of the mitochondrial membrane and by the pro- or anti-apoptotic signal that will be released inside the cell^[63]. Following mitochondrial permeabilization, the intrinsic pathway divides into two pathways: Apoptosis protease-activating factor-1 (Apaf-1) dependent and Apaf-1 independent pathway. In Apaf-1 dependent pathway, release of cytochrome c from mitochondria, by triggering the pro-apoptotic Bcl-2 family member^[64], and ATP activate monomer inactive Apaf-1 proteins by a conformational change, leading to form a heptamer of Apaf-1 molecules called apoptosome^[65]. Apoptosome allows activation of pro-caspase 9, which consequently triggers the caspase cascade^[66]. On the other hand, in Apaf-1 independent pathway, permeabilization of mitochondrial membrane release DIABLO like proteins, which activates effector caspases by provoking inhibitors of apoptosis proteins^[67] and triggers caspase cascade^[68] (Figure 5).

The balance of pro- and anti-apoptotic proteins main-

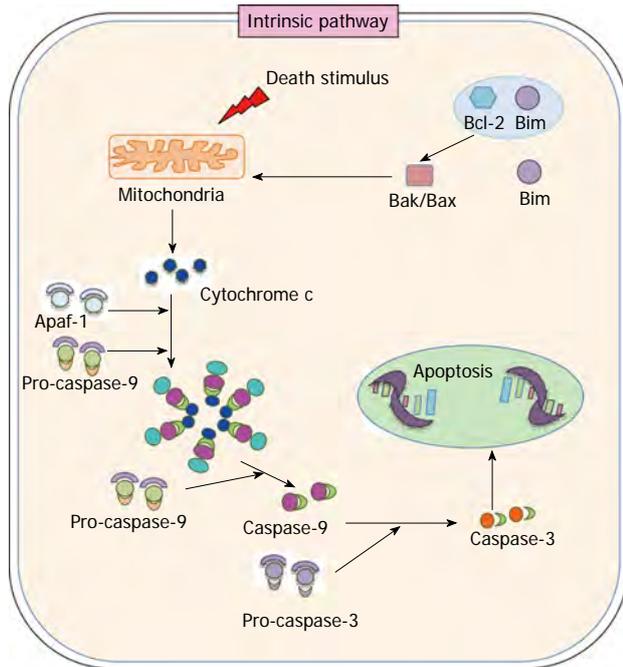


Figure 5 Intrinsic pathway. Death stimulation up regulates Bcl-2 interacting mediator leading to the separation from Bcl-2, favoring the activation of Bax, Bak, which form pores in the mitochondrial membrane leading to release of cytochrome c. Cytochrome c with Apaf-1 and procaspase 9 participate in the formation of apoptosome, which activate caspase 9. Caspase-9 activates caspase 3 after cleavage of pro-caspase-3. That caspase-3 triggers to induction of caspase cascade and cell death. Apaf-1: Apoptosis protease-activating factor-1. Bim: Bcl-2 interacting mediator.

tains the apoptotic activity^[69]. The Bcl-2 family members regulate mostly neglect or intrinsic pathway. This family is subdivided into three groups of proteins on the basis of their functions and the number of Bcl-2 homology (BH) motifs included in their primary structure; first group: “anti-apoptotic multidomain” members, such as Bcl-xL, have four BH domains (BH1 to BH4) which inhibits apoptotic process. Other two groups of “pro-apoptotic multidomain” members, which are Bax-like proteins and “BH3-only” proteins^[70]. Bax-like proteins possess three BH domain (BH1 to BH3), including Bax, Bak, and Bok, which are referred as death effector members. BH3-only members contain BH3 domain, including Bim, Bad, Bik, Puma, Noxa and Bid and are known as messengers of death. In addition, C-terminal transmembrane (TM) fragment is thought to confer anchorage to mitochondrial membranes, which is also possessed by most multi-BH members and several BH3-only proteins.

Three models (Figure 6) have been postulated by which the BH3 family promotes passive cell death in which Bax and Bak bind directly or indirectly with cell death sensitizer (*e.g.*, Bad, Bik) and activators of cell death (*e.g.*, Bim, tBid). The direct activation model proposes that sensitizer BH3-only proteins displace the activator BH3-only proteins from the anti-apoptotic proteins to promote apoptosis. Anti-apoptotic proteins inhibit the activator BH3-only proteins but not Bax and Bak to suppress apoptosis. In the displacement model, Bax and Bak

are sequestered by anti-apoptotic proteins for cell survival and constitutively active in cells. BH3-only proteins play the sensitizer role and inhibit their respective anti-apoptotic proteins to promote apoptosis. The third model, called embedded together model, highlights the interactions occurring in and on membranes, which were not explained by direct activation and displacement model. In embedded together model, Bcl-2 family proteins insert into and change their conformations according to their functions in membrane^[71]. The predominantly studied messenger death molecule, Bcl-2 interacting protein (Bim) will be focused further.

BIM

Bim/Bod is a pro-apoptotic protein belonging to the BH3-only group of Bcl-2 family members and is being called the “ghost” molecule or “suicide” molecule, which enables cells to expire gracefully. Two independent studies discovered Bim as a Bcl-2 binding protein and Mcl1-binding protein in 1998^[72,73]. Bim induces apoptosis by binding to and antagonizing anti-apoptotic members of the Bcl-2 family. The Bim interactions have been observed with Bcl-2 family members, such as Bcl-2, Bcl-xL, Mcl-1, Bcl-w, *etc*^[72,73].

Bim is a well-known pivotal initiator of apoptosis in thymocyte-negative selection^[74]. Bim has 19 Bim isoforms including three major isoforms, which have distinct sizes and pro-apoptotic activities in the mammals, caused by alternative splicing: BimEL (extra long), BimL (long) and BimS (small)^[73]. The shortest form, BimS, is the most potent and is generally only transiently expressed during apoptosis^[75]. The other two isoforms are sequestered to the dynein motor complex, and apoptotic activity of these longer isoforms is regulated by phosphorylation^[75,76], which is triggered by environmental stress, resulting in its dissociation from the dynein complex and increasing apoptotic activity.

Expression of Bim is up regulated in human T cells in response to TCR-triggering by protein kinase C and calcineurin pathways^[77]. Nevertheless, there are other mechanisms involved in Bim up-regulation during chronic infection, such as the effect of certain cytokines. In fact, in a persistent viral infection animal model, Bim-mediated apoptosis correlates with low IL-7 receptor expression on specific T cells^[78].

The regulation of Bim expression at transcriptional level in growth factor deprivation and in endoplasmic reticulum stress has observed by the class O fork-head box transcription factor (FOXO3A) and transcriptional factor CEPB- α respectively^[79,80]. Post-transcriptional phosphorylation of Bim can also regulate its function. Phosphorylated Bim is targeted for proteasomal degradation and avoid its interaction with Bax, thus maintaining cell existence^[81,82]. The signaling adaptor TNFR-associated factor 1 (TRAF1) negatively correlates with Bim and it contributes to CD8 T cell-mediated control of chronic viral infections. In addition, linking between survival

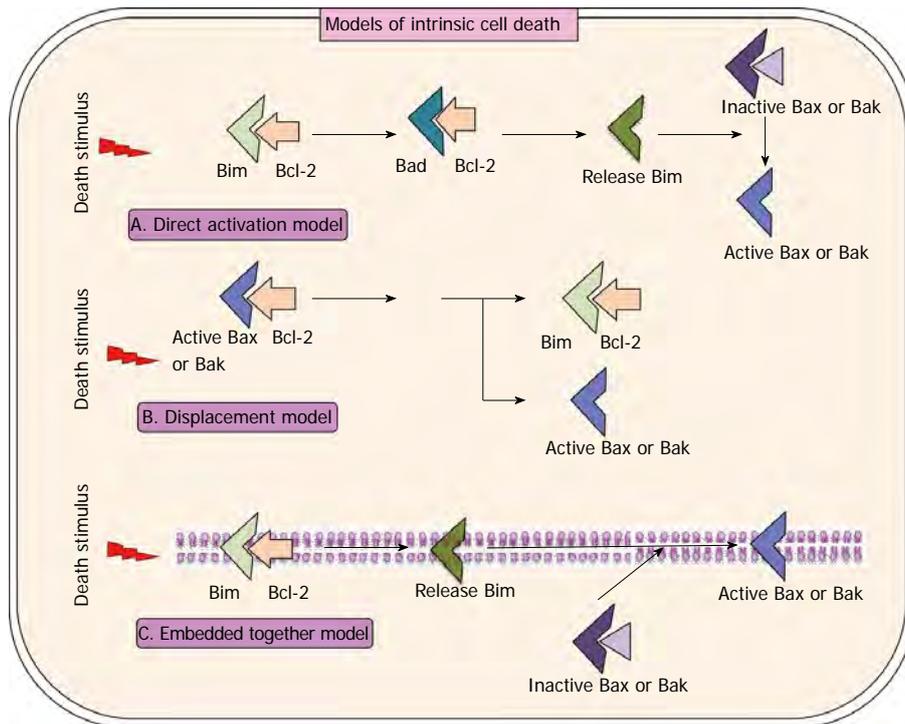


Figure 6 Models for intrinsic cell death. A: Direct activation model postulates Bcl-2 interacting mediator (Bim) is required for activating Bax and Bak. Anti-apoptotic proteins inhibit BH3-only proteins to suppress apoptosis, but not Bax or Bak. Replacement of Bim to sensitizer BH3-proteins from the anti-apoptotic proteins occurs to promote apoptosis; B: The displacement model proposes that anti-apoptotic proteins for cell survival must sequester constitutively active Bax and Bak in cells. Bim inhibits their respective anti-apoptotic proteins by playing sensitizer role to promote apoptosis; C: Embedded together model highlights the active role of the membrane, which is not defined in direct activation model and displacement model. Bcl-2 family proteins insert into and change their conformations that dictate their functions at the membrane. Sensitizer BH3-only proteins relocate the activator BH3-only proteins and Bax/Bak from the anti-apoptotic proteins to endorse apoptosis. Activator BH3-only proteins recruit Bax to the membrane to induce mitochondrial outer membrane permeabilization and apoptosis. These reversible interactions are directed by equilibrium constants that are depended on the concentrations and interactions of the proteins with each other and with membranes.

effects of TRAF1 and TRAF1-dependent Bim down-modulation has been shown in CD8 T cells^[83-85]. TRAF1 is particularly vanished from virus-specific CD8 T cells during the chronic human immunodeficiency virus and lymphocytic chorio-meningitis virus (LCMV) infection^[86].

Bim plays a vital role in the immune system, in bone biology and in tumor-genesis by inducing apoptosis^[87]. Bim in T cells, B cells, neurons and many other cell types can trigger apoptosis^[87]. Gene targeting in mice for the important region for apoptosis, BH3 region, uncovered the important physiological role in Bim^[88]. In fact, in the absence of Bim leukocytes in blood as well as in LNs, thymus, spleen were high in number^[88]. The role of Bim in apoptosis has been revealed in Bim^{-/-} thymocytes, which were more resistant to apoptosis after different apoptotic treatment such as ionomycin, taxol, γ irradiation^[88].

DEATH OF ACTIVATED T CELLS BY BIM

The liver is having a property that might explain its role in inducing tolerance due to its recognition as an alternative primary activation of CD8 T cells site. The phenotype of activated CD8 cells in the liver was the same as in lymph nodes. However, liver-activated CD8 T cells displayed poor effector functions and a unique CD25^{low} CD54^{low} phenotype, which was associated with increased expression of the Bim and caspase-3, demonstrating that these cells are programmed to apoptosis following intrahepatic activation. Strikingly, Bim deficient T cells survived following intrahepatic activation^[1]. Therefore, the phenotype and fate of naïve CD8 T cells activated by hepatocytes *in vivo* could explain the death penalty role of Bim in chronic hepatotropic viral infection^[1]. The dis-

tinct phenotype can be due to the lack of co-stimulatory molecule expression on hepatocytes^[43]; however the treatment with IL-2 or anti-CD28 antibodies could rescue hepatocyte-activated cells from death^[44].

Lymphocyte fate deciding pathways synergize to kill activated T cells in chronic herpes simplex viral immune responses, whereas death of activated T cells in acute immune responses relies only on the mitochondrial pathway involved only Bim with no contribution by Fas, which showed critical overlapping roles for Fas and Bim in T cell death during immune response shutdown, leading to immune tolerance^[23].

BIM IN HEPATITIS

Bim has been shown to be important for CD8 T cell viability during chronic LCMV infection in mice^[89]. In this study, in Bim mutated mice, Bim mutation almost completely blocked the deletion of cognate antigen specific CD8 T cells in liver during chronic viral infection. Bim has a critical role in maintaining naïve and memory T cells in LCMV infection^[90]. In another study, it has been shown that a defect in apoptosis dramatically not only enhances the antigen-specific memory T cells but also increased the number of virus-specific CD4⁺ T cells in the lymph nodes following acute LCMV infection, compared to the parental genotypes or wild type mice^[91]. Therefore, the loss of both Bim and Fas caused the increase in memory T cells in acute LCMV infection^[91]. The Bim role has been demonstrated in the development of LCMV-induced, T cell-mediated hepatitis by controlling the apoptosis of both T cells and hepatocytes^[92].

Bim attrition of virus specific CTLs during HBV

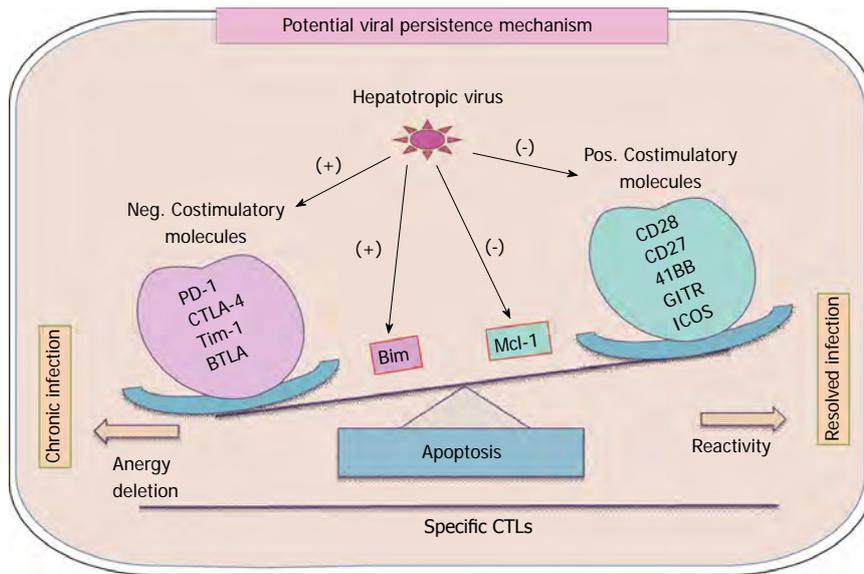


Figure 7 Balance between co-stimulatory/apoptotic molecules and viral-specific cytotoxic T lymphocytes reactivity according to infection outcome. Neg.: Negative; Pos.: Positive; CTLs: Cytotoxic T lymphocytes; (+): Possible molecules induced by viral infection; (-): Possible molecules down-regulated by viral infection; BIM: Bcl-2 interacting mediator; Mcl-1: Myeloid cell leukemia sequence-1.

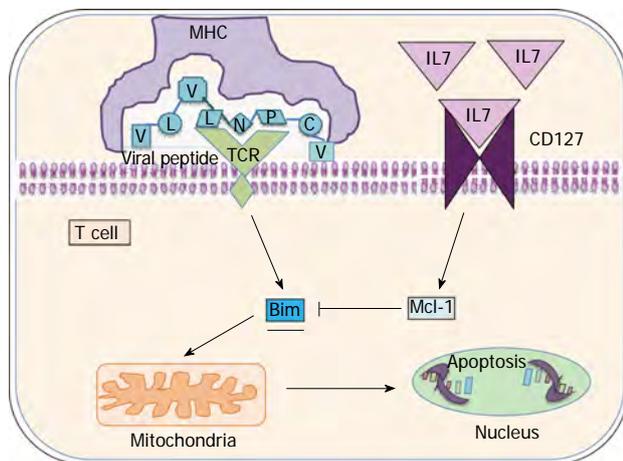


Figure 8 Cell survival marker CD127 modulates Bim and myeloid cell leukemia sequence-1 expression on hepatitis C virus-specific cytotoxic T lymphocytes after cognate antigen stimulation. Misbalance of Mcl-1/Bcl-2 interacting mediator (Bim) triggers to apoptosis of hepatitis C virus specific cytotoxic T lymphocytes. TCR: T cell receptor; Mcl-1: Myeloid cell leukemia sequence-1.

infection has also been confirmed^[3,93]. The gene expression profile in HBV infection showed different patterns of gene expression on HBV-specific CD8+ T cells according to viral control. Bim was one of the up-regulated genes in HBV-specific CD8+ T cells from patients with chronic HBV infection. Blocking Bim-mediated apoptosis improved recovery of HBV-specific CD8+ T cell function^[3]. Furthermore, the elevated apoptosis has been observed not only with Bim tolerogenic phenotype, but also with co-inhibitory signals through CTLA-4^[93] or T cell-intrinsic transforming growth factor- β ^[94].

As discussed earlier, robust CD8 responses are essential to control HCV infection. However, in HCV chronic infection, HCV specific CD8 are depleted by Bim mediated attrition, and remaining cells are functionally exhausted. The cell survival factor CD127 counteracts the induction of apoptosis after antigen encounter through myeloid cell leukemia sequence-1 (Mcl-1) expression and

Bim down-regulation^[95] after the cognate antigen recognition by TCR. Similarly, our group has shown in previous work, HCV-specific CTLs displayed a high Bim expression in persistent infection respect to resolved infection patients^[2], suggesting a similar apoptotic mechanism to the one described in chronic HBV infection.

The procedure of T cell death during chronic viral infection is determined by a carefully balanced and complex group of pro- and anti-apoptotic proteins of the Bcl-2 family, such as Bim and Mcl-1^[96] (Figure 7). Interestingly, persistent hepatotropic viral infection is characterized by continuous TCR triggering and CD127 down-regulation on viral-specific CTLs^[97], which could favor Bim up-regulation. In addition, it is well known that Bim is clearly involved in intrahepatic specific-CTL apoptosis in animal models^[1]. Furthermore, Bim pro-apoptotic effect is blocked by the action of Bcl-2 family anti-apoptotic proteins such as Mcl-1 and Bcl-2^[78,98], clearly pointing out that T cell death also depends on the anti-apoptotic protein expression. Bearing in mind all these facts, recently our group has suggested a model to explain specific CTL deletion during persistent hepatotropic viral infection (Figure 8). This model shows that CD127 phenotype modulates Bim and Mcl-1 expression on virus-specific CTLs, leading to Mcl-1/Bim imbalance during persistent infection, which impairs T cell reactivity and suggesting that restoration of T cell function could occur by correcting the levels of Mcl-1 and Bim expression.

In our work, Bim up-regulation has been observed on CD127^{low}-expressing HCV-specific CTLs but not on CD127^{high} cells after antigen encounter, suggesting that TCR triggering can only lead to Bim up-regulation in absence of IL-7 stimulation on HCV-specific CTLs. Nevertheless, Bim level is not enough to lead to T cell apoptosis. Our data also showed the Mcl-1/Bim ratio could decide the fate of the activated T cells by sequestration of experienced CD127^{low}/Mcl-1^{low}-expressing T cells to the liver and subsequent Bim up-regulation after antigen encounter due to the absence of IL-7 stimulus^[99]. Finally, Bim

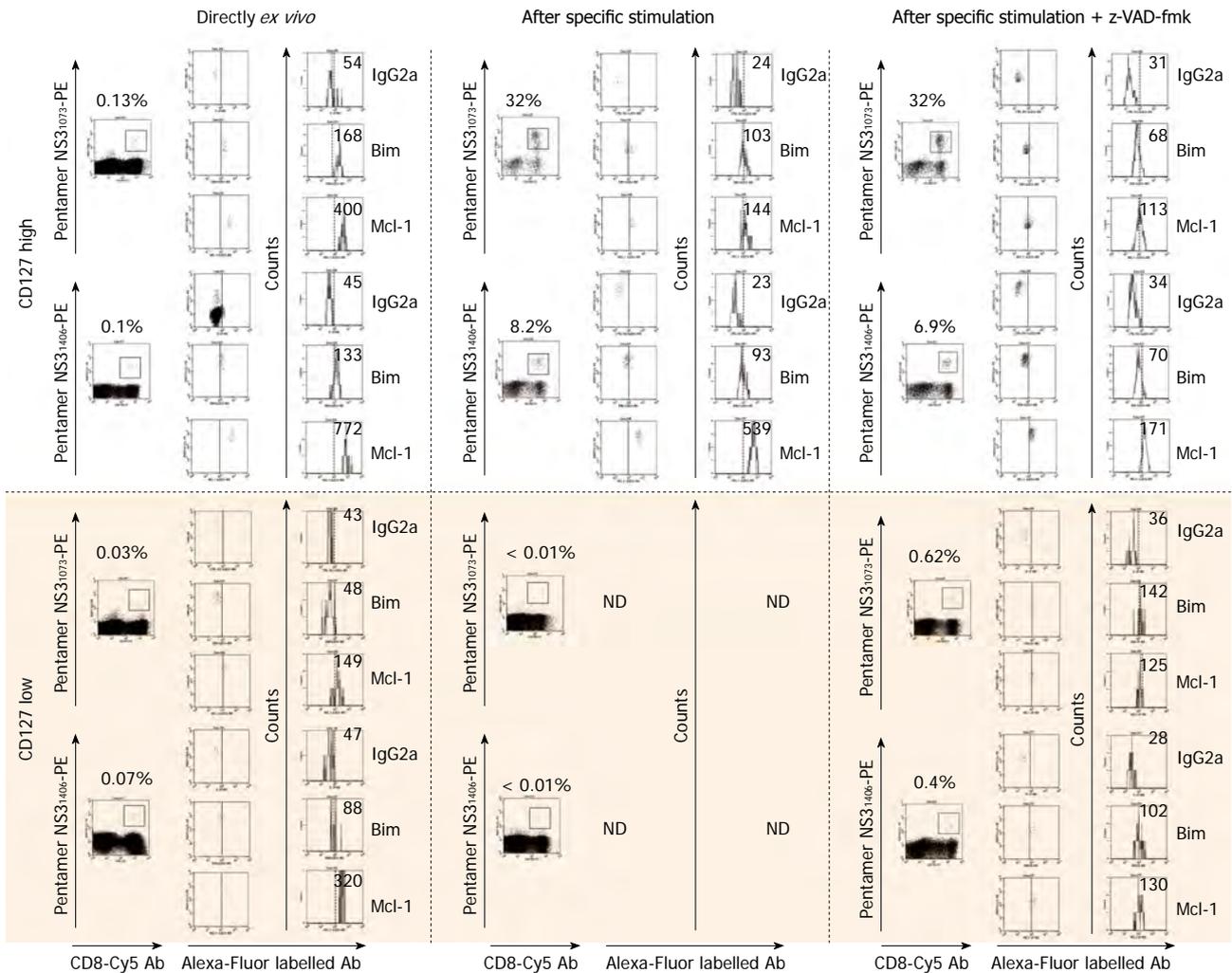


Figure 9 Figure illustrated the FACS[®] dot plots and histograms from peripheral blood lymphocytes from two hepatitis C virus patients with different CD127 expression on hepatitis C virus-specific cytotoxic T lymphocytes (CD8⁺/Pentamer⁺ cells). The different plots show the Bcl-2 interacting mediator (Bim) and myeloid cell leukemia sequence-1 (Mcl-1) expression directly *ex vivo* and after specific stimulation on peripheral CD8⁺/pentamer⁺ cells according to CD127 level. The figure on the top of the dot-plots represents the frequency of pentamer⁺ cells out of total CD8⁺ cells. The figure in the upper right corner in the histogram plot represents the MFI for Bim and Mcl-1 staining. The continuous and dashed line in the dot-plots and histograms represents the cut-off point to consider a staining positive according to the negative control. ND: Not done due to lack of pentamer⁺/CD8⁺ proliferation after specific stimulation.

would be released freely to activate Bax, due to the low level of the anti-apoptotic protein Mcl-1 during chronic HCV infection. Consequently, CD127 level play a central role in hepatotropic virus-specific CD8⁺ T cell apoptosis by regulation of Mcl-1 expression *in vivo* and by Bim modulation after antigen encounter, which is checked by T cell reactivity restoration and Mcl-1/Bim phenotype on CD127^{low} specific CTLs after apoptosis blockade (Figure 9), that suggested a link between apoptosis after TCR triggering and low CD127 expression on experienced specific CTLs during persistent infection that could be related to Mcl-1/Bim imbalance.

Therefore, CD127 phenotype modulates Bim and Mcl-1 expression on specific CTLs and this affect to T cell reactivity through apoptosis regulation. Specifically, during chronic infection, Mcl-1/Bim imbalance could be involved on CD127^{low} specific CTL hyporeactivity, but it could be overcome by blocking apoptosis.

For control of hepatotropic viral infection is essential

to develop a robust viral-specific cellular response. However, during chronic infection this response is altered, showing a pro-apoptotic phenotype due to the deprivation of IL-7 secondary to the low expression of CD127. Recently, it has been investigated that TRAF1 is a signal adapter for positive co-stimulatory receptors whose level depends on the action of IL-7 and inhibits the expression of the pro-apoptotic molecule Bim^[86]. Therefore, in situations of deprivation of IL-7, action of TRAF1 could be impaired, favoring an imbalance between anti- and pro-apoptotic molecules. On the other hand, in an experimental model, IL-7 deprivation during stressing conditions leads to Mcl-1 down-regulation on T cells, conducting to T cell death that could be avoided by IL-7 treatment^[100]. Consequently, strategies directed to block the pro-apoptotic effect of IL-7 deprivation should be designed to increase the effectiveness of CTL response restoration, by enhancing the TRAF1 and Mcl-1 expression level that could restore Bim/Mcl-1 balance. On of those strategies

could be short-term use of cyclosporine-A or FK506 could block the induction of the pro-apoptotic molecule Bim on CD127⁺ cells^[77]. This strategy could favor specific-CTL restoration during anti-viral treatments in combination with the standard of care. Another possible strategy to restore hepatotropic virus-specific CTL reactivity during chronic infection could be the administration of IL-7, in order to increase the stimulation of the reduced number of IL-7R molecules on specific CTLs, to modulate the balance between Bim and Mcl-1. In fact, in an animal model of cytotoxic T cell exhaustion, IL-7 treatment resulted in amplified cytokine production, increased T cell effector function, and viral clearance^[101].

CONCLUSION

The deletion of hepatitis virus-specific CD8⁺ T cells is likely to represent the deregulation of the Bim pro-apoptotic pathway. The balance between pro- and anti-apoptotic molecules is critical for cell survival. The unavailability of appropriate survival marker modulates Bim and Mcl-1 expression on virus hepatitis-specific CTLs and this affect to T cell reactivity through apoptosis regulation. The level of those molecules is regulated by CD127 (IL-7R) expression, which is down-modulated during persistent infection. Consequently, Mcl-1/Bim imbalance could be the reason for the deletion of virus hepatitis-specific T cells, but it could be overcome by interruption of apoptosis. The interruption of this tolerizing mechanism may provide a new strategy to restore the balance between apoptotic molecules in order to achieve viral specific T cell immunity, as a future treatment strategy of chronic viral hepatitis.

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Clinical application of liver stiffness measurement using transient elastography in chronic liver disease from longitudinal perspectives

Beom Kyung Kim, James Fung, Man-Fung Yuen, Seung Up Kim

Beom Kyung Kim, Seung Up Kim, Department of Internal Medicine, Institute of Gastroenterology, Yonsei University College of Medicine, Seoul 120-740, South Korea

Beom Kyung Kim, Seung Up Kim, Liver Cirrhosis Clinical Research Center, Seoul 120-740, South Korea

James Fung, Man-Fung Yuen, Department of Medicine, State Key Laboratory for Liver Research, the University of Hong Kong, Hong Kong, China

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Correspondence to: Seung Up Kim, MD, Department of Internal Medicine, Yonsei University College of Medicine, 250 Seongsanno, Seodaemun-gu, Seoul 120-752, South Korea. ksukorea@yuhs.ac

Telephone: +82-2-22281982 Fax: +82-2-3936884

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Abstract

Accurate determination of the presence and degree of fibrosis in liver is of great importance, because the prognosis and management strategies for chronic liver disease depend mainly on these factors. To date, liver biopsy (LB) remains the "gold standard" for assessing the severity of liver fibrosis; however, LB is often limited by its invasiveness, sampling error, and intra/inter-observer variability in histological interpretation. Furthermore, repeated LB examinations within a short time interval are indeed ineligible in a real clinical practice. Thus, due to the pressing need for non-invasive surrogates for liver fibrosis, transient elastography (TE),

as a novel ultrasound based technology, has allowed a noninvasive measurement of liver stiffness and has gained in popularity over recent years. In the past few years, additional roles for transient TE beyond the initial purpose of a non-invasive surrogate for LB have included the prediction of the most two critical consequences of fibrosis progression: the development of portal hypertension-related complications and hepatocellular carcinoma. This indicates that the role of transient TE is not merely limited to reducing the need for LB, but transient TE can enable the establishment of tailored management strategies by providing more detailed prognostic information. In particular, under the concept in which the clinical course of liver fibrosis is dynamic and bidirectional, especially when appropriate intervention is commenced, transient TE can be used to track the dynamic changes in fibrotic burden during antiviral or antifibrotic treatment. This review discussed extended applications of transient TE in prediction of the development of real clinical endpoints from a longitudinal perspective.

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Key words: Liver stiffness; Transient elastography; Fibroscan; Fibrosis; Longitudinal; Outcome

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INTRODUCTION

The prognosis and management of chronic liver disease (CLD) depend mainly on the amount and progression

of liver fibrosis, which is defined as the excessive accumulation of extracellular matrix proteins, resulting from chronic liver insults^[1,2]. The initiation of its deposition is an important phase of CLD. As liver fibrosis eventually progresses without appropriate intervention, this process will lead to architectural change of the liver, followed by deterioration of liver function and hemodynamics, complications due to portal hypertension, and an increased tendency for hepatocarcinogenesis^[3].

Thus, accurate determination of the presence and degree of liver fibrosis is of paramount importance in choosing treatment strategies, evaluating responses to treatment and the risks of developing liver-related complications, and predicting prognosis in patients with CLD. To assess the severity of liver fibrosis, liver biopsy (LB) remains the “gold standard”. However, LB is often limited by its invasiveness and rare, but serious, complications, including bleeding, pneumothorax, and procedure-related death^[4,5]. Moreover, repeated LB examinations within a short time interval are impractical. Additionally, concerning the reliability of pathological examinations, not only sampling error inherent in the percutaneous approach, but also intra- and inter-observer variability in histological interpretation may still occur^[6]. Even if the LB is performed by an experienced physician and interpreted by an expert pathologist, it has an up to 20% error rate in disease staging^[7,8].

Ideally, a method of evaluating liver fibrosis should accurately determine the presence of significant fibrosis, and be readily available, highly reproducible, and widely applicable to liver diseases of various etiologies. Although LB does not fulfil all these criteria, it has remained the gold standard, likely due to the absence of a better alternative. Recently, liver stiffness measurement using transient elastography (TE) was introduced as a promising non-invasive method for assessment of liver fibrosis^[9-15]. In many studies, TE proved to be a reliable and accurate surrogate for LB in terms of prediction of significant fibrosis or cirrhosis^[8,16-19]. In a large-scale meta-analysis including 50 studies, the mean areas under the receiver operating characteristic curves (AUROCs) for the diagnosis of significant fibrosis and cirrhosis were 0.84 and 0.94, respectively, with optimal cutoff values of 7.6 and 13.1 kPa, respectively^[20].

Most studies to date have focused on assessing the performance of TE, reflected by AUROC, from a cross-sectional perspective, with reference to histological fibrosis. However, because LB as a reference standard is imperfect, it may have only limited clinical implications in terms of increasing the AUROC of TE to 1 (*i.e.*, perfect concordance with LB). Thus, additional roles for TE, namely prediction of long-term prognosis of the disease and monitoring clinical courses, have recently begun to attract attention. This indicates that the role of TE is not merely limited to lessening the frequency of unnecessary LB, but TE can also enable establishment of tailored management strategies by providing more detailed prognostic information^[21]. In this regard, the “classical” end-points of “static” liver fibrosis in recent cross-sectional

studies on TE are shifting to the “real and solid” end-points of the development of clinical events related to liver fibrosis progression, including hepatic decompensation, hepatocellular carcinoma (HCC), or liver-related death in a longitudinal study from a prospective cohort with long-term follow-up. Additionally, the performance of non-invasive methods is being judged and compared from this viewpoint.

In this article, we reviewed recent studies that focused on the prognostic value of TE for prediction of clinical end-points related to liver fibrosis progression, such as decompensation events, HCC development, or liver-related death, from a longitudinal perspective.

PREDICTION OF THE DEVELOPMENT OF LIVER-RELATED COMPLICATIONS

Portal hypertension-related complications

The development of portal hypertension is a common consequence of fibrosis progression, leading to the formation of esophageal and gastric varices responsible for variceal bleeding, and other severe complications, such as portosystemic encephalopathy, spontaneous bacterial peritonitis and sepsis^[22-24]. Measurement of the hepatic venous pressure gradient (HVPG) is the gold standard for portal hypertension assessment in patients with cirrhosis; however, it is invasive and is routinely available only in experienced centers^[25-29]. Although TE was initially proposed for assessment of liver fibrosis, a good correlation between TE values and HVPG has been reported, as well as the presence of esophageal varices, suggesting that it may be a valuable tool for the non-invasive evaluation of portal hypertension^[30-32]. Subsequent studies have investigated correlations between TE values and the hepatic decompensation due to increased portal hypertension. A significant correlation between TE values and portal hypertension, expressed as the HVPG, was reported by Vizzutti *et al*^[33] suggesting that TE may reflect a progressive rise in portal pressure due primarily to increased hepatic vascular resistance, caused by fibrillar extracellular matrix accumulation. Based on this concept, Foucher *et al*^[34] first reported that cutoff values of 27.5, 37.5, 49.1, 53.7 and 62.7 kPa had > 90% negative predictive values for the presence of large esophageal varices (stage 2/3), Child-Pugh score B or C, past history of ascites, HCC and esophageal bleeding, respectively.

As variceal bleeding is a life-threatening complication of portal hypertension, the relationship between TE values and the presence of esophageal varices has been investigated in several studies^[35-40]. All demonstrated a significant correlation between TE values and the presence of esophageal varices and that TE values could predict the presence of large varices (more than grade 2)^[38,40]. Table 1 summarizes reports of the relationship between TE values and esophageal varices^[33,38,40-44].

Although TE can predict the presence of esophageal varices and consequently assist in selection of candidates for endoscopic screening or prophylactic treatment, sever-

Table 1 Diagnostic performance of transient elastography for prediction of esophageal varices or large esophageal varices

| Ref. | No. of patients (etiology) | Endpoints | AUROC | Cutoffs (kPa) | Sensitivity | Specificity | PPV | NPV |
|--|----------------------------|-----------|-------|---------------|-------------|-------------|-----|-----|
| Vizzutti <i>et al</i> ^[33] | 47 (HCV) | EV | 0.76 | 17.6 | 90% | 43% | 77% | 66% |
| Castéra <i>et al</i> ^[38] | 70 (HCV) | EV | 0.84 | 21.5 | 76% | 78% | 68% | 84% |
| Kazemi <i>et al</i> ^[40] | 165 (CLD) | Large EV | 0.87 | 30.5 | 77% | 85% | 56% | 94% |
| | | EV | 0.83 | 13.9 | 95% | 43% | 57% | 91% |
| Bureau <i>et al</i> ^[41] | 89 (CLD) | Large EV | 0.84 | 19.0 | 91% | 60% | 48% | 95% |
| | | EV | 0.85 | 21.1 | 84% | 71% | NA | NA |
| Pritchett <i>et al</i> ^[42] | 211 (CLD) | Large EV | 0.76 | 29.3 | 81% | 61% | NA | NA |
| | | EV | 0.74 | 19.5 | 76% | 66% | 56% | 82% |
| Nguyen-Khac <i>et al</i> ^[43] | 183 (CLD) | Large EV | 0.76 | 19.8 | 91% | 56% | 91% | 55% |
| | 58 (HCV/HBV) | Large EV | 0.76 | 48.0 | 73% | 73% | 44% | 90% |
| | 103 (alcohol) | Large EV | 0.73 | 19.8 | 89% | 55% | 27% | 97% |
| Malik <i>et al</i> ^[44] | 124 (CLD) | Large EV | 0.77 | 47.2 | 85% | 64% | 44% | 93% |
| | | EV | 0.85 | 20.0 | NA | NA | 80% | 75% |

AUROC: Area under the receiver operating characteristic curve; PPV: Positive predictive value; NPV: Negative predictive value; CLD: Chronic liver disease; EV: Esophageal varix; HCV: Hepatitis C virus; HBV: Hepatitis B virus; NA: Not available.

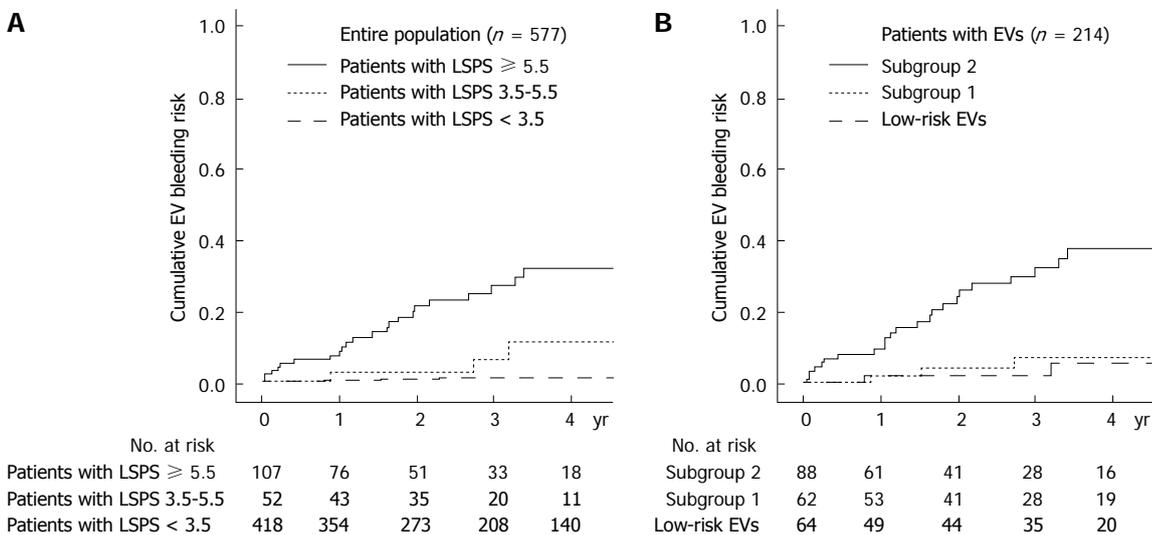


Figure 1 Cumulative incidences of variceal bleeding based on liver stiffness-spleen diameter to platelet ratio score values. A: The incidence of variceal bleeding increased significantly in association with higher liver stiffness-spleen diameter to platelet ratio score (LSPS) values (long-rank test, $P < 0.001$); B: In particular, among patients with high risk esophageal varices (EV), the incidence of variceal bleeding was significantly higher in patient with LSPS ≥ 6.5 (subgroup 2) than those with LSPS < 6.5 (subgroup 1).

al issues remain unresolved. First, the cutoff values (range, 13.9-21.5 kPa) and performance of TE varied (AUROC range, 0.76-0.85) among studies^[38-40]. Second, from data currently available, diagnostic performances of TE are acceptable for the prediction of esophageal varices, but far from satisfactory for screening cirrhotic patients without endoscopy confidently. Thus, Kim *et al*^[45] recently proposed a novel prediction model [liver stiffness-spleen diameter to platelet ratio score (LSPS)] to address this issue, achieving higher accuracy using TE values and other parameters simultaneously that reflect portal hypertension as constituent variables. Overall, this model had excellent diagnostic accuracy for the prediction of high-risk esophageal varices (HEV, AUROC = 0.953; negative predictive value 94.7%, positive predictive value 93.3%).

Beyond this cross-sectional analysis, a subsequent study by the same group recently showed that LSPS can be a reliable predictor of the development of variceal

bleeding^[20]. In this prospective, longitudinal study analyzing 577 patients with hepatitis B virus-related cirrhosis, those with LSPS ≥ 5.5 had higher cumulative incidences of esophageal variceal bleeding during the follow-up period and LSPS score ≥ 6.5 was an independent risk factor of variceal bleeding among those with HEV, indicating that further prophylactic treatment such as endoscopic ligation in addition to a non-selective beta-blocker should be considered in these high-risk patients (Figure 1). In a similar context, Kim *et al*^[46] stratified the risk of hepatic decompensation, such as ascites, hepatic encephalopathy, variceal hemorrhage, and deterioration of liver function to Child-Pugh class B or C, based upon three classes of TE values (TE value < 13 , 13-18 and ≥ 18 kPa) in histologically proven hepatitis B virus-related cirrhosis with well-preserved liver function and no history of decompensation. In a multivariate analysis, patients with a TE value of 13-18 kPa [hazard ratio (HR), 4.547; $P = 0.044$] and ≥ 18

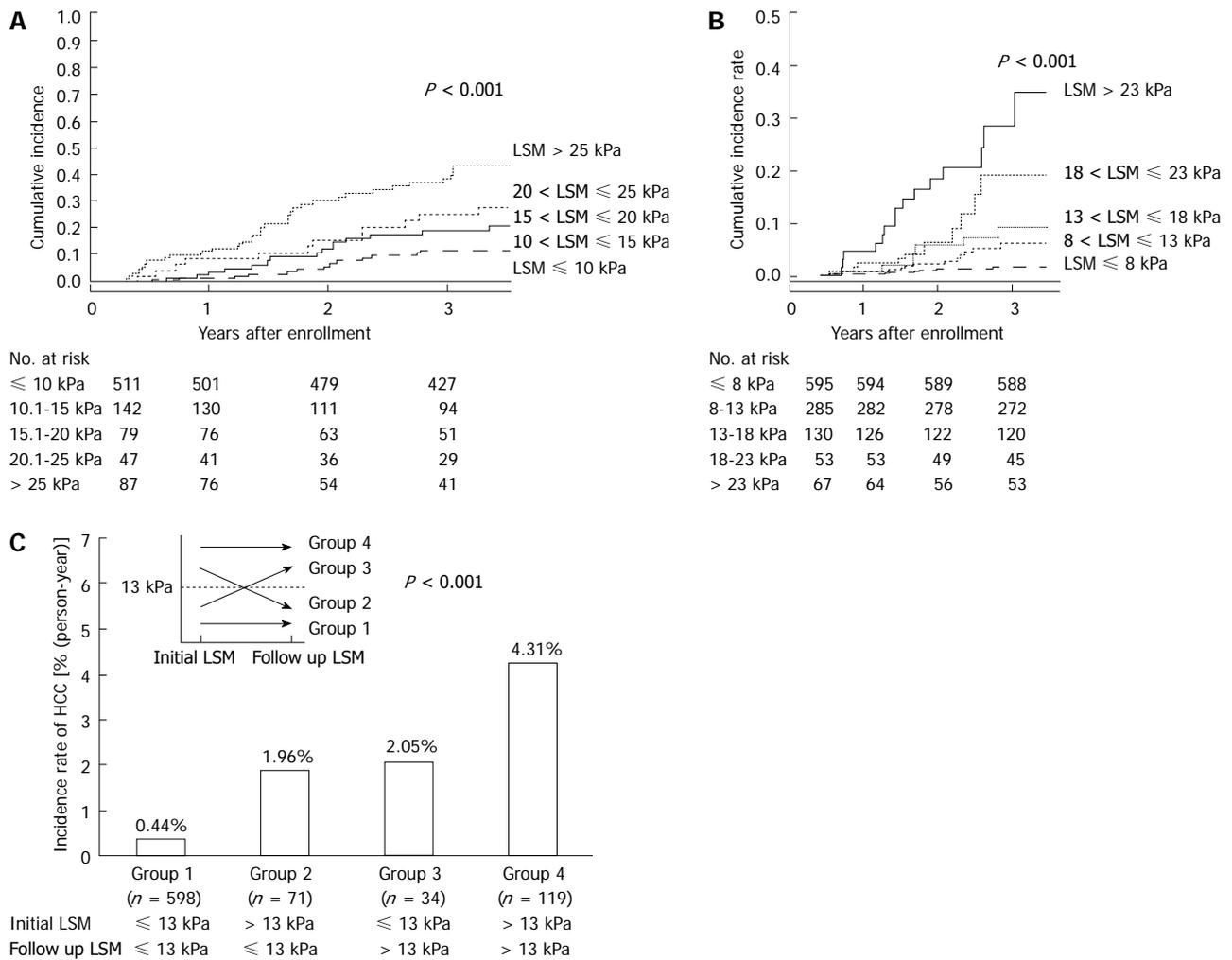


Figure 2 Cumulative incidence of hepatocellular carcinoma development based on stratified transient elastography values in patients with chronic hepatitis C (A, $n = 866$) and those with chronic hepatitis B (B, $n = 1130$). The cumulative incidences increased significantly in association with higher TE values (log-rank test, all $P < 0.001$). In particular, the overall incidence of HCC differed significantly among the four groups (C) (both initial and follow-up TE values ≤ 13 kPa (group 1), initial TE value > 13 kPa and follow-up TE value ≤ 13 kPa (group 2), initial TE value ≤ 13 kPa and follow-up TE value > 13 kPa (group 3), and both initial and follow-up TE values > 13 kPa (group 4) according to changing patterns of TE value during follow-up ($P < 0.001$; Figure 2C). A: Cited from Masuzaki *et al*^[52]; B and C: Cited from Jung *et al*^[53]. HCC: Hepatocellular carcinoma; TE: Transient elastography; LSM: Liver stiffness measurement.

kPa (HR, 12.446; $P < 0.001$) showed independently higher risks than patients with TE value < 13 kPa.

HCC

Another promising area for the application of TE, other than portal hypertension-related decompensation events, is the prediction of HCC development. Unless HCC is diagnosed at an early stage, a poor prognosis is expected due to the limited treatment options^[47-51]. Thus, early prediction of HCC development is of great importance, especially in high-risk patients. Among traditional risk factors, advanced liver fibrosis and cirrhosis is known to have a close association with risk of HCC development^[47]. Thus, assessment of the severity of liver fibrosis at a given time point with subsequent monitoring of liver fibrosis progression by serial check-up is essential for effective and optimized surveillance strategies for the early detection of HCC^[3].

Recently, several Asian studies have investigated the

clinical role of TE in the noninvasive prediction of HCC development^[52-56]. The first large prospective cohort study of 866 Japanese patients with chronic hepatitis C (CHC) tested whether TE can predict the future development of HCC^[52]. During a mean follow-up of 3 years, 77 patients developed HCC. By multivariate analysis, together with age, male gender, and clinical cirrhosis, stratified TE value was identified as an independent risk factor for HCC development, with relative risks of 16.7, 20.0, 25.6 and 45.5 for TE values of 10-15, 15-20, 20-25 and > 25 kPa, respectively, *vs* an TE value of < 10 kPa as the reference and the cumulative incidence of HCC showed a step-wise increase according to stratified TE value (Figure 2A). Despite there being no histological analysis in relation to TE values and inclusion of patients with high alanine aminotransferase (ALT) levels [$> 5 \times$ upper limit of normal (ULN)] both of which can attenuate the accuracy of TE, this study confirmed that severity of liver fibrosis, reflected by higher TE values, was closely associated with higher

risk of HCC development and suggested a clinical role for TE in a longitudinal setting using HCC development as a solid clinical endpoint. Interestingly, in this study, even patients with not so high level of TE (10-15 kPa) were still more subject to HCC development with an adjusted HR of 16.7, compared to those with a TE value < 10 kPa.

Another large Korean cohort study with 1130 patients with chronic hepatitis B (CHB) also confirmed the longitudinal role of TE on HCC development^[53]. Together with age, male gender, heavy alcohol consumption, lower serum albumin, and HBeAg positivity, stratified TE value was identified as an independent risk factor for HCC development, with relative risks of 3.07, 4.68, 5.55 and 6.60 for liver stiffness measurement (LSM) values of 8-13, 13-18, 18-23 and > 23 kPa, respectively, when compared with a LSM value of < 8 kPa as a reference (Figure 2B). In contrast to the Japanese study^[52], several additional issues were further analyzed in this Korean study. First, when the diagnosis of cirrhosis showed discordant results between TE-based and clinical-based criteria, patients with cirrhosis based on TE were at a higher risk of HCC development than those with cirrhosis based on clinical criteria, indicating the superiority of TE for diagnosis of compensated liver cirrhosis. Second, patients with TE values below the cutoff level for cirrhosis, 8-13 kPa, had a higher relative risk of HCC development than those with LSM values < 8 kPa. Although this finding should be validated in large prospective studies, the issue of expansion of the high-risk group for HCC surveillance to include those with significant fibrosis was raised by this study. Furthermore, when patients with available follow-up TE values were analyzed, the risk of HCC development changed according to the pattern of the changes in TE values, suggesting a potential role for serial measurements of TE as a dynamic monitoring tool for risk estimation of HCC development (Figure 2C). However, other confounding factors including lack of histological information, insensitive HBV DNA tests, and heterogeneity in antiviral treatment should be noted when interpreting these results. Recently, Chon *et al.*^[56] compared the performance of various noninvasive fibrosis prediction methods [aspartate aminotransferase-to-platelet ratio index (ARRI), age-spleen-to-platelet ratio index (ASPRI), TE, LSPS, P2/MS and FIB-4] for prediction of HCC development in patients with CHB and concluded that TE and LSPS showed the best performance (AUROC = 0.789 and 0.788, respectively). Using multivariate analyses, TE and LSPS were identified as independent predictors of HCC development.

In another study^[54] from Hong Kong, which followed up 528 patients with HBeAg negative CHB for a median length of 35 mo and identified seven patients with HCC development, the cumulative incidence of HCC was higher in patients with TE values \geq 10 kPa than those with TE values < 10 kPa (9% *vs* 0%, respectively; $P < 0.001$), and the cumulative liver-related mortality was also higher in patients with TE values < 10 kPa compared with those with TE values \geq 10 kPa (4% *vs* 0%, respec-

tively; $P < 0.001$). By multivariate analysis, only TE value was significantly associated with HCC development and liver-related mortality.

Similarly, Kim *et al.*^[55] investigated the prognostic role of TE in predicting the development of overall liver-related events (LREs), defined as development of HCC, hepatic decompensation, or liver-related mortality, among 128 patients with CHB showing histologically advanced liver fibrosis (\geq F3) and high viral loads (HBV DNA \geq 2000 IU/mL) before starting nucleos(t)ide analogs. When the study population was stratified into two groups using the optimal cutoff value (19 kPa), patients with TE values > 19 kPa were at significantly greater risk for LRE development than those with TE values \leq 19 kPa (HR, 7.176; $P = 0.001$). Moreover, the incidence of LREs was similar in patients with F3 and F4 (22.2% *vs* 13.6%; $P = 0.472$); however, it differed significantly between patients with TE values \leq 19 kPa and those with TE values > 19 kPa (6.9% *vs* 44.4%; $P < 0.001$), indicating the superior performance of TE to that of histology in prediction of LRE development.

Apart from predicting HCC development, the application of TE was validated in a study by Vergniol *et al.*^[57], in which 1457 patients with CHC were followed up; 5-year survival outcomes worsened as TE values increased. The prognostic values of TE were demonstrated to be statistically significant ($P < 0.0001$) after adjustment for other important factors, including treatment response, patient age, and estimates of necroinflammatory grade. For example, the 5-year overall survival was 96% in patients with TE value < 9.5 kPa, and 47% in patients with TE value > 40 kPa.

Overall, TE has shown the potential for a clinical role in predicting the development of portal hypertension-related hepatic decompensation and/or HCC and, in part, demonstrated superior performance to histology and other noninvasive tools^[41,58-63]. This is most likely due to the wider dynamic range of TE values in the evaluation of liver cirrhosis. In fact, as the stage of "cirrhosis" has to date been defined by histopathological evidence of one or two qualitative categories (METAVIR stage F4 or ISHAK S5-S6), or more generally by the presence of so-called "regenerative" or "cirrhotic nodules", an interval scale cannot be used in this setting^[64-66]. However, the degree of liver fibrosis may vary widely among patients in this category, and the risk of hepatic decompensation and HCC may not be uniform. Thus, in this regard, because TE value, expressed in kPa as a continuous variable, has a wide dynamic range within the cirrhotic stage from the cutoff level from non-cirrhosis (15-17 kPa) to the upper measurement limit of present devices (75 kPa), it would seem to be a more reasonable tool for detailed prognostication.

UTILITY OF TE IN THE SURGICAL SETTING

Because TE values show significant correlations with portal hypertension and HCC development, prediction

of postoperative short-term outcomes, such as hepatic insufficiency, and long-term outcomes, such as recurrence or liver-related death using TE has been tested in several pilot studies^[67-69]. Although further studies are required to validate these results, TE may facilitate stratification of patients undergoing curative resection according to different prognoses.

In the first place, Kim *et al.*^[67] investigated whether preoperative TE values could predict the development of postoperative hepatic insufficiency after curative resection of HCC. In this study, multivariate analyses revealed that a TE value > 25.6 kPa was the only predictor of postoperative insufficiency. The AUROC of 25.6 kPa was higher than that of indocyanine green R15, which is a popular method for assessment of preoperative functional reserve liver function (0.824 *vs* 0.620, respectively). Similar results were obtained in a subsequent investigation by the same group^[68]. In this study, the performance of TE was superior to that of diffusion-weighted magnetic resonance imaging, which has also been shown to be a noninvasive fibrosis prediction tool for the assessment of liver fibrosis and the prediction of postoperative hepatic insufficiency.

Another issue is prediction of HCC recurrence after curative resection, that is, *de novo* recurrence in the background liver with fibrotic burden, using preoperative TE. In an analysis of 133 patients who underwent preoperative TE and curative resection (HCC recurred in 62 patients), TE was selected as an independent predictor of recurrence, whereas histological fibrosis status was not^[69]. In the study, patients with preoperative TE values > 13.4 kPa were at a greater risk of recurrence, with an HR of 1.925 ($P = 0.010$). More specifically, when recurrence was stratified into early (< 2 years) and late (≥ 2 years), TE values were significantly related to late recurrence, thus supporting the hypothesis. These results suggest that preoperative TE could reveal the potential influence of liver fibrosis on recurrence and explain multicentric carcinogenesis in a fibrotic liver. However, more data are needed to clarify this issue.

ROLE OF TE IN MONITORING FIBROTIC BURDEN DURING ANTIVIRAL THERAPY

Recently, the concept of “cirrhosis” has changed from static and uncompromisingly progressive to rather dynamic and bidirectional, especially when treatment against the causative agent of tissue damage (*i.e.*, antiviral agents against CHB or CHC and antifibrotic agents) can be introduced successfully at this stage of the disease. The ideal approach to evaluate histological outcomes during antiviral therapy, such as fibrosis regression and necroinflammation stabilization, is serial LB examinations. However, this is impractical, primarily due to the inherent invasiveness of LB. Instead, because of the ease, safety, and rapidity of TE, it may be useful for monitoring the dynamic changes in liver fibrosis during antiviral or antifibrotic treatment. Indeed, several studies have reported

the clinical usefulness of TE for monitoring potential fibrosis regression during antiviral treatment in patients with CHC and CHB^[57,70-77].

Kim *et al.*^[71] analyzed 41 patients with CHB who received antiviral treatment using nucleos(t)ide analogs. To prevent the confounding effect of high ALT, patients with high ALT levels more than 2× ULN, were excluded. Although ALT levels did not show a statistically significant change during the first 12 or 24 mo of antiviral treatment, TE values decreased significantly, indicating potential fibrosis regression due to prolonged antiviral treatment. Indeed, fibrosis regression and stabilization of necroinflammation was noted in two patients with available paired LBs. Enomoto *et al.*^[70] reported the changes in LSM values during the first 12 mo of entecavir treatment in 20 patients. Median TE values decreased significantly from 11.2 to 7.8 kPa after 12 mo of treatment, and serum fibrosis markers, such as PIIINP and type IV collagen 7S domain, also decreased significantly. In one patient with available paired LBs, histological fibrosis regression and stabilization of necroinflammation were noted. Although these studies suggest a role for TE for monitoring fibrosis regression due to prolonged antiviral treatment, the short duration of observation and small sample sizes with paired biopsies are major limitations of these studies.

Recently, data regarding a longer antiviral treatment duration (more than 3 years) have become available^[72-74,78]. Fung *et al.*^[72] reported a significant decline in TE values from baseline after subsequent ALT normalization with 3-year treatment ($n = 110$, 7.8 to 6.1 kPa; $P = 0.002$). In this study, independent factors associated with a significant decline in TE value of ≥ 1 kPa included antiviral therapy and ALT levels at the follow-up time point. Another study by Andersen *et al.*^[78] also noted significant declines in TE values after a median antiviral treatment duration of 50.5 mo ($n = 66$), and concluded that prolonged antiviral treatment in patients with CHB resulted in significant declines in TE values, suggesting regression of fibrosis in a majority of patients with advanced fibrosis or cirrhosis.

Likewise, for patients with CHC, changes in TE values during antiviral treatment have been investigated in several studies. Two prospective studies by Vergniol *et al.*^[57] and Ogawa *et al.*^[75] demonstrated that patients with CHC showing sustained virological responses to pegylated interferon-ribavirin combination therapy had significantly reduced TE values at the end of follow-up. Moreover, Ogawa *et al.*^[75] reported that patients with non-sustained virological responses, but with a biochemical response, showed a greater reduction in TE values than did those with a non-biochemical response. Subsequent studies reported similar results, suggesting that changes in TE values during antiviral treatment in patients with CHC may represent alterations in the severity of liver fibrosis^[76,77]. However, it should be further confirmed whether the favorable changes in LSM values during or after antiviral treatment does have a significant influence

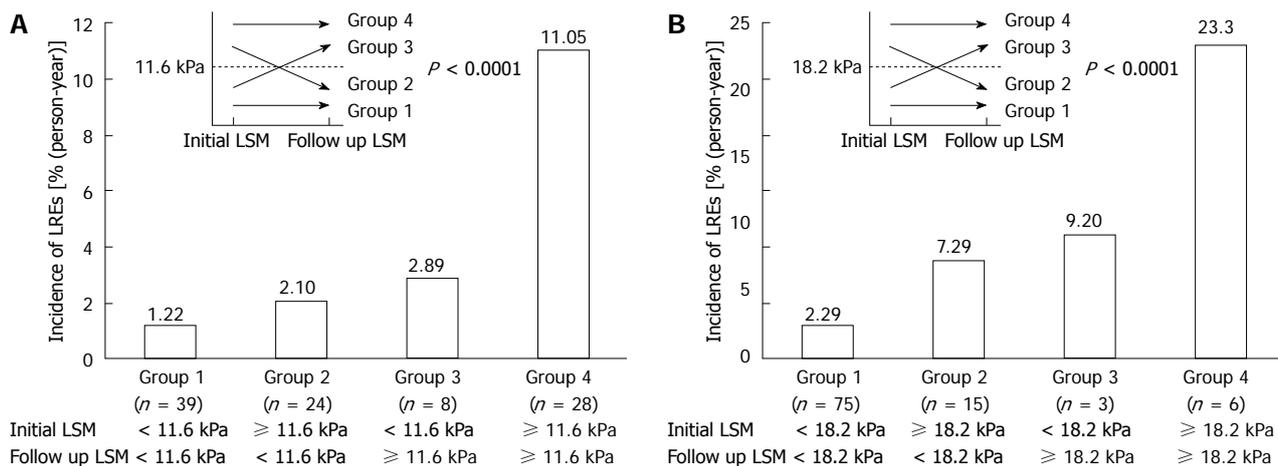


Figure 3 Incidence of liver-related events according to changes in transient elastography values after 6 mo of antiviral therapy. The overall incidence of liver-related events differed significantly among the four groups using TE value cutoffs of 11.6 kPa (A) and 18.2 kPa (B) (both $P < 0.0001$). Adapted from Kim *et al.*^[79]. TE: Transient elastography; LSM: Liver stiffness measurement; LREs: Liver-related events.

on the long-term prognosis such as disease-specific survival in patients with CHC.

Taken together, TE value seems to decrease during and after antiviral therapy. However, without paired histological results through repeated LB, whether the reduction in TE values is closely correlated with regression of liver fibrosis or improvement in necroinflammatory scores remains unclear. To clarify this, Lim *et al.*^[73] investigated patterns of TE values among patients who were treated with entecavir. In all subjects, the median TE value at baseline was 15.1 (range, 5.6-75.0) kPa and decreased significantly, to 8.8 (range, 3.0-33.8) kPa after 12 mo of therapy, and a decrease in TE values correlated significantly with increase in albumin, decrease in bilirubin, decrease in ALT level, and decrease in aspartate aminotransferase levels (all $P < 0.05$). However, among 15 patients with available paired LBs, decreases in TE values were correlated significantly with improved necroinflammatory scores, but not with fibrosis regression. Similarly, Wong *et al.*^[74] insisted that the decline in absolute TE values during antiviral treatment did not reflect the change in histologically assessed liver fibrosis, probably due to the confounding influence of ALT reduction caused by antiviral treatment.

However, regardless of whether TE values during antiviral treatment are due to fibrosis regression, activity stabilization, or both, changes in TE value during antiviral treatment can be translated into the overall response of chronically diseased liver to antiviral treatment from the viewpoint of its long-term clinical implications. Thus, it is more logical to investigate whether the decline in TE value can be used as a favorable predictor of long-term prognosis. Encouraging results were recently published by Jung *et al.*^[53] suggesting that the change in TE values in patients with CHB showed a significant correlation with differential future risk of HCC development. Additionally, Kim *et al.*^[79] insisted that changes in TE values were significantly associated with the difference risk of liver-related event occurrence, such as hepatic decompensation, HCC

development, and LREs (Figure 3). This would suggest that the assessment of overall background liver status using TE may be an important end-point in the management of CHB and prediction of long-term outcomes. Further research is needed to evaluate the reproducibility of such findings in independent populations.

LIMITATIONS OF TE

Although TE has demonstrated reliable diagnostic accuracy with excellent inter-observer and intra-observer agreement, additional space-occupying tissue abnormalities, such as edema and inflammation, cholestasis, and congestion may interfere with TE, regardless of the degree of liver fibrosis, because the liver is wrapped in a distensible, but non-elastic, envelope (Glisson's capsule)^[80].

First, the extent of histological necroinflammatory activity has been shown to influence TE results in patients with viral hepatitis, resulting in an overestimation of TE values that increases in parallel with the degree of necroinflammatory score^[81-85]. Consistent with these results, a risk of overestimation of TE values has been reported in cases of ALT flares in patients with acute viral hepatitis or CHB^[86-92]. Thus, in such subjects, TE examinations should be delayed until ALT levels have stabilized. In this regard, several studies have investigated the optimal period (3 to 6 mo) for restoration of the reliability of TE values in patients with acute flares^[88,91,93,94]. Furthermore, even mild to moderate elevation in ALT can be associated with higher liver stiffness values, and may cause discrepancies between TE results and the actual underlying fibrosis. Apart from necroinflammation, extrahepatic cholestasis^[95] and congestive heart failure^[96-98] may also contribute to the overestimation of TE.

Additionally, the performance of TE may be limited in patients with a high body mass index (BMI), narrow intercostal space, or ascites^[9]. Although TE reproducibility has been shown to be excellent in terms of inter-observer and intra-observer agreement, a high BMI (> 28

Table 2 Proposal of application of transient elastography in each clinical setting

| Clinical setting | Role of TE |
|--|--|
| Prediction of portal hypertension | TE with platelet counts and spleen size complementary to HVPG |
| Prediction of esophageal varices | TE with platelet counts and spleen size complementary to endoscopy |
| Prediction of developing esophageal variceal bleeding | TE with platelet counts and spleen size |
| Prediction of developing portal hypertension-related complications | TE with platelet counts and spleen size |
| Prediction of developing hepatocellular carcinoma | TE |
| Prediction of developing postoperative hepatic insufficiency after surgical resection | TE |
| Prediction of developing recurrence of hepatocellular carcinoma after curative resection | TE |
| Monitoring of fibrotic burden during antiviral treatment | TE |

TE: Transient elastography; HVPG: Hepatic venous pressure gradient.

kg/m²) and waist circumference were significantly associated with TE failure^[99]. These results emphasize the need for adequate operator training and for technological improvements in specific patient populations, such as those with non-alcoholic fatty liver disease. For this, a new TE probe (the XL probe) was recently introduced to lessen the TE failure rate in obese patients; however, its efficacy should be further validated^[100].

CONCLUSION

Over the past decade, significant progress has been made in the non-invasive assessment of liver fibrosis in patients with CLD. Of the methods now available, TE appears to be an excellent tool for assessing liver fibrosis, particularly for diagnosis of cirrhosis, and also has prognostic value from a longitudinal perspective. Although TE cannot completely obviate the need for invasive tests, such as LB, endoscopic examination for identification of varices, or HVPG, it represents an important non-invasive tool, enabling more efficient and tailored management strategies for patients with CLD (Table 2). We hope that other researchers will evaluate the usefulness of other similar techniques such as the measurement of spleen stiffness in comparison or in combination with TE in the future.

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Promising effect of Magliasa, a traditional Iranian formula, on experimental colitis on the basis of biochemical and cellular findings

Roja Rahimi, Amir Baghaei, Maryam Baeeri, Gholamreza Amin, Mohammad Reza Shams-Ardekani, Mahnaz Khanavi, Mohammad Abdollahi

Roja Rahimi, Gholamreza Amin, Mohammad Reza Shams-Ardekani, Mahnaz Khanavi, Department of Traditional Pharmacy, Tehran University of Medical Sciences, Tehran 1417653761, Iran

Roja Rahimi, Amir Baghaei, Maryam Baeeri, Mohammad Abdollahi, Pharmaceutical Sciences Research Center, Tehran University of Medical Sciences, Tehran 1417614411, Iran

Amir Baghaei, Mohammad Abdollahi, Department of Pharmacology and Toxicology, Tehran University of Medical Sciences, Tehran 1417614411, Iran

Gholamreza Amin, Mohammad Reza Shams-Ardekani, Mahnaz Khanavi, Department of Pharmacognosy, Tehran University of Medical Sciences, Tehran 1417614411, Iran

Author contributions: Rahimi R designed and performed research, and drafted the paper; Baghaei A contributed the animal studies; Baeeri M contributed the biochemical tests; Amin G contributed botanical authentication and the herbal studies; Khanavi M contributed the chemical drug analysis; Shams-Ardekani MR contributed in finding the appropriate prescription and to the preparation of the formula; Abdollahi M conceived the study and edited the paper.

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Correspondence to: Mohammad Abdollahi, Professor, Department of Pharmacology and Toxicology, Tehran University of Medical Sciences, Tehran 1417614411, Iran. mohammad.abdollahi@utoronto.ca

Telephone: +98-21-88611663 Fax: +98-21-88611663

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Abstract

AIM: To investigate the efficacy of Magliasa, a traditional Iranian formula, on experimental colitis.

METHODS: After botanical authentication of herbal

ingredients, formulation of Magliasa, quantitative determination of total glucosinolates and total phenolic content, and analysis of the thin layer chromatography profile were performed. Colitis was then induced in male rats by instillation of 2,4,6-trinitrobenzenesulfonic acid (TNBS) in all groups, aside from the Sham group. The experimental groups consisted of: the Sham group that received only normal saline; the Mag-50, Mag-100 and Mag-200 groups, which received 50, 100 and 200 mg/kg per day of Magliasa, respectively; the control group, which received vehicle water orally; the infliximab group, which received infliximab (5 mg/kg per day, subcutaneously); and the Dexa group, which received dexamethasone (1 mg/kg per day, orally). After completing the treatment period (2 wk), the rats were sacrificed, the colon was removed, its macroscopic and microscopic changes were recorded, and tumor necrosis factor-alpha (TNF- α), interleukin-1 beta (IL-1 β), total antioxidant capacity, myeloperoxidase (MPO), and lipid peroxidation (LPO) were assessed in colon homogenate.

RESULTS: The mean value of total glucosinolates in one gram of Magliasa was $19 \pm 1 \mu\text{mol}$. The mean value of the total phenolic content was $293.8 \pm 17.6 \text{ mg gallic acid equivalents per } 100 \text{ gram of Magliasa}$. Macroscopic scores were significantly decreased in Mag-100 (1.80 ± 0.58 , $P = 0.019$) and Mag-200 (1.20 ± 0.20 , $P = 0.001$) compared to the control group (3.40 ± 0.24), although some inflammation and hyperemia were evident. Treatment of rats by dexamethasone (0.33 ± 0.21 , $P < 0.001$) and infliximab (0.83 ± 0.31 , $P < 0.001$) remarkably attenuated scores where mild hyperemia was observed macroscopically. In comparison to the control group (4.00 ± 0.32), only Mag-200 (1.60 ± 0.40) showed a significant decrease in colonic histopathological scores ($P = 0.005$). Minimal mucosal inflammation was observed in the Dexa group (0.67

± 0.21 , $P < 0.001$). The levels of TNF- α , IL-1 β and MPO were significantly lower in all groups compared to the controls ($P < 0.05$). A significant decrease in LPO was seen in the Mag-200 (3.27 ± 0.77 , $P = 0.01$) and Dexa (3.44 ± 0.22 , $P = 0.011$) groups in comparison to the control group (6.43 ± 0.61). Only dexamethasone caused a significant increase in antioxidant power in comparison to the control group (346.73 ± 9.9 vs 228.33 ± 2.75 , $P < 0.001$). Infliximab and different doses of Magliasa did not show any remarkable increase in antioxidant capacity ($P > 0.05$). The effect of Magliasa in all of mentioned parameters, except antioxidant capacity, was dose dependent.

CONCLUSION: The effects of Magliasa in TNBS-induced colitis are encouraging and warrant clinical trials for further confirmation.

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Key words: Magliasa; Traditional Iranian medicine; Colitis; Neutrophil infiltration; Inflammatory cytokines; Oxidative stress

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INTRODUCTION

Inflammatory bowel disease (IBD) includes two main types (Crohn's disease and ulcerative colitis), and is categorized as one of the chronic disorders of the gastrointestinal tract with an unclear etiology. Related to the involvement of possible pathological factors such as immunological abnormalities^[1], oxidative stress^[2], gut microflora^[3], abnormal epithelial barrier^[4], and inflammatory factors^[5-9], various drugs are used for the management of IBD, including anti-tumor necrosis factor-alpha (TNF- α) drugs^[10-12], immunosuppressants^[13,14], antibiotics^[15,16], probiotics^[17,18], corticosteroids^[19], aminosalicylates^[20,21], selective cyclooxygenase-2 inhibitors^[9], nicotine preparations^[22], potassium channel openers^[23], adenosine triphosphate donors^[24], and phosphodiesterase inhibitors^[25-27]. It cannot be ignored that most of the conventional treatments for the management of IBD have serious adverse effects that reduce compliance in patients^[28-30], and therefore has led researchers to work on complementary and alternative medicines that can induce remission in disease activity with better safety and tolerability^[31-33]. As recently reviewed by Rahimi *et al.*^[32], there are many plants in traditional Iranian medicine (TIM) that were historically used for the management of IBD. Magliasa is a TIM herbal prescription that has been used to treat tenesmus and di-

arrhea mixed with blood and mucus for a long time^[34]. It consists of 6 components: the seeds of *Lepidium sativum*, *Linum usitatissimum*, and *Allium ampeloprasum* cv. Porrum, the fruit of *Bunium persicum* and *Terminalia chebula*, and the gum resin of *Pistacia lentiscus* (Table 1). Different mechanisms have been described in TIM for the usefulness of these plants in the treatment of colitis, including anti-inflammatory, antiulcer, wound healing, and anti-diarrheal effects^[35,36]. Regarding the aforementioned knowledge, the present study was planned to investigate the effect of Magliasa in an experimental model of colitis to determine the involved mechanisms.

MATERIALS AND METHODS

Materials

Plant materials (seeds of *Lepidium sativum*, *Linum usitatissimum*, and *Allium ampeloprasum* cv. Porrum, fruit of *Bunium persicum* and *Terminalia chebula*, and gum resin of *Pistacia lentiscus*), were obtained from the local market at the Tehran bazaar in 2010. After confirmation by a botanist, voucher samples were deposited at the herbarium of the Faculty of Pharmacy, Tehran University of Medical Sciences (Tehran, Iran). 2,4,6-trinitrobenzenesulfonic acid (TNBS, Sigma-Aldrich), ethanol, methanol, ethyl acetate, n-hexane, thiobarbituric acid, trichloroacetic acid, n-butanol, hexadecyl-trimethyl-ammonium bromide, 2,4,6-tri(2-pyridyl)-S-triazine (TPTZ), hydrochloric acid, anisaldehyde, malondialdehyde, ethylenediaminetetraacetic acid, Folin-Ciocalteu reagent, toluene, dichloromethane, o-dianisidine hydrochloride, hydrogen peroxide, acetic acid, sodium acetate, Coomassie reagent, bovine serum albumin (BSA), FeCl₃-6H₂O, Na₂SO₄, H₂SO₄, H₃PO₄, KH₂PO₄, K₂HPO₄, H₂O₂, Na₂CO₃, NaHCO₃, Na-K-tartrate, CuSO₄-5H₂O, Silica gel 60F254 (Merck, Germany), glucose kit (ZistChem, Iran), and rat-specific TNF- α and interleukin-1 β (IL-1 β) enzyme-linked immunosorbent assay (ELISA) kits (Bender Med Systems, Austria) were used in this study.

Botanical authentication

All 6 herbal ingredients were authenticated macroscopically and microscopically. Macroscopic examinations included measurements of appearance, size, shape, color, texture, odor, taste, fracture, and other characteristics according to pharmacopoeias^[36,37]. Microscopic examinations determined the characteristic elements of each ingredient in powder form. For this purpose, each herbal material was mounted on a microscope slide after tissue disintegration with potassium hydroxide and cleared with sodium hypochlorite. The examination protocols followed the World Health Organization's quality control methods for medicinal plant materials^[38]. Characteristic elements were photographed *via* a Leitz optical microscope.

Preparation of Magliasa

Bunium persicum fruit (22%), *Linum usitatissimum* seeds (8%), *Allium ampeloprasum* cv. Porrum seeds (8%), *Terminalia che-*

Table 1 Magliasa powder ingredient characteristics

| Scientific name | Iranian name | Part | Major constituents | Pharmacological activities | Herbarium No. |
|---------------------------------------|--------------|-----------|--|--|---------------|
| <i>Lepidium sativum</i> | Taretizak | Seed | Glucosinolates, imidazole alkaloids, fatty acids, and sterols ^[60-63] | ↓IL-2, TNF- α , leukotriene B4 and nitric oxide in immune cells; anti-inflammatory and analgesic in rats; prokinetic, and laxatives in mice; anti-diarrheal and spasmolytic in rats; anticholinergic and phosphodiesterase inhibitor ^[64-68] | PMP-716 |
| <i>Bunium persicum</i> | Zire kermani | Fruit | Flavonoids, essential oils, and tannins ^[69,70] | Antioxidant and radical scavenger; antinociceptive and anti-inflammatory in rats ^[70-73] | PMP-627 |
| <i>Linum usitatissimum</i> | Katan | Seed | Mucilage, cyanogenic glycoside, lignans, fatty acids, and phenylpropan derivatives ^[74] | Antiulcer, antioxidant, and protective against intestinal tumors in mice ^[75-77] | PMP-717 |
| <i>Allium ampeloprasum</i> cv. Porrum | Tare | Seed | Saponins, flavonoids, carotenoids, and sulfur-containing compounds ^[78-80] | Antioxidant ^[78] | PMP-718 |
| <i>Terminalia chebula</i> | Halile siah | Fruit | Tannins, anthraquinones, triterpene glycosides, and beta-Sitosterol ^[81,82] | Antioxidant, anti NF- κ B, antiulcer, ↓TNF- α , IL-1 β and IL-6, and antibacterial against intestinal bacteria ^[83-87] | PMP-606 |
| <i>Pistacia lentiscus</i> | Mastaki | Gum resin | Triterpene acids and alcohols, and essential oils ^[81] | Antioxidant, ↓NO, prostaglandin E2, iNOS, and Cox-2 delayed the onset and progression of colitis and prevented weight loss in mice; ↓Intensity of gastric mucosal damage, ↓TNF- α , CD activity index, plasma IL-6, and ↑total antioxidant potential in CD patients; ulcer healing in patients with duodenal ulcer ^[88-93] | PMP-811 |

Cox-2: Cyclooxygenase-2; iNOS: Inducible nitric oxide synthase; CD: Crohn's disease; NO: Nitric oxide; TNF- α : Tumor necrosis factor- α ; IL: interleukin; NF- κ B: Nuclear factor κ B.

bula fruit (8%), and *Pistacia lentiscus* gum resin (4%) were individually powdered by milling, and then mixed. Intact non-milled seed of *Lepidium sativum* (50%, w/w) was added to the powdered material and again mixed.

Quantitative determination of total glucosinolates and total phenols Magliasa

The amount of total glucosinolates as major constituents of *Lepidium sativum* and the amount of total phenolic compounds as major constituents of *Bunium persicum*, *Allium ampeloprasum* cv. Porrum, and *Terminalia chebula* were measured in Magliasa.

Total glucosinolates were determined by the measurement of enzymatically-released glucose^[59]. For this purpose, four accurately weighed 1 g samples of Magliasa were transferred into separate loaded ball-mill cups. To three cups 1 mL of water was added (samples), while the last cup had 1 mL of acidified 40% v/v methanol/water added instead (sample blank). All cups were milled side by side for 2 min, allowed to stand for 5 min, and then had 19 mL of acidified 40% v/v methanol added to each cup. After recapping and shaking vigorously, the cup contents were filtered through charcoal-coated papers. Immediately prior to colorimetric assay, each of the filtrates was diluted ten-fold with water, and then 0.2 mL was poured into separate 10 mL tubes. About 0.2 mL of water was added into a fifth tube (water blank), with 0.2 mL of standard glucose solution (1 mg/mL) (ZistChem, Iran) added into a sixth tube. Five mL of buffer/enzyme/chromogen reagent (ZistChem, Iran) was added to all tubes, mixed, and then placed in a water bath at 37 °C and read within 10-15 min. The absorbance of each solution against the water blank was measured at 610 nm.

The total phenolic contents in the medicinal plants

were determined spectrophotometrically according to the Folin-Ciocalteu method^[40]. Gallic acid was used to set up the standard curve. The phenolic compound content of the samples was expressed as gallic acid equivalents (GAE) in mg per 100 g of Magliasa. All the samples were analyzed in triplicate.

Thin layer chromatography profile of Magliasa

Thin layer chromatography (TLC) was performed to obtain preliminary data from essential oils and lipophilic substances. For this purpose, 1 g of Magliasa was extracted by shaking for 15 min in 10 mL of dichloromethane at room temperature. The suspension was filtered, and the clear filtrate evaporated to dryness. The residue was dissolved in 1 mL of toluene. Samples were then applied to the plates, which were developed at room temperature in glass chambers previously saturated for 1 h. The development distance was 5 cm. The mobile phase was n-hexane-ethyl acetate 5:4 (v/v). The spray reagent was anisaldehyde- sulfuric acid^[41].

Animals

Male Wistar-albino rats, weighing between 220 and 230 g, were maintained under standard conditions of temperature (23 °C \pm 1 °C), relative humidity (55% \pm 10%), a 12-h dark and light period, and fed with a standard pellet diet and water *ad libitum*. All ethical themes of the animal studies were considered carefully, and the experimental protocol was approved by the ethical committee of Tehran University of Medical Sciences (code number of 88-04-33-10094).

Interventions

Rats were randomly divided into seven groups containing six individuals in each group. Colitis was induced

by the instillation of TNBS in all groups except group 1. The groups were: (1) Sham which received normal saline; (2) Mag-50 which received 50 mg/kg per day of Magliasa; (3) Mag-100 which received 10 mg/kg per day of Magliasa; (4) Mag-200 which received 200 mg/kg per day of Magliasa; (5) control which received vehicle water orally; (6) Infliximab which received infliximab (5 mg/kg per day, subcutaneously); and (7) Dexa which received dexamethasone (1 mg/kg per day, orally). Magliasa was dissolved in water and administered by gavage. The doses for Magliasa were selected after a pilot study. The effective doses of infliximab and dexamethasone were selected from our previous studies^[42].

Induction of colitis

For induction of colitis, 36 h fasted rats were anesthetized with an intraperitoneal administration of 50 mg/kg of pentobarbital sodium, positioned on their right side, and then had 0.3 mL of a mixture containing six volumes of TNBS 5% w/v in H₂O (equal to 15 mg TNBS) plus four volumes of ethanol (99%) instilled *via* the rectum using a rubber cannula (8 cm long)^[43]. Following instillation of TNBS, rats were maintained in a supine Trendelenburg position in order to prevent anal leakage of TNBS. Medications were then administered to the animals for 14 d as described above. On the 15th day, the animals were sacrificed by an overdose of ether inhalation. The abdomen was rapidly dissected open and the colon was removed. The colon was cut open in an ice bath, cleansed gently using normal saline, observed normally for macroscopic changes, and scored in a manner described later. Samples were then cut into two pieces; one piece for histopathology assessment (fixed in 5 mL formalin 10%) and one piece for measuring biomarkers weighed and maintained in -20 °C for 24 h. The colonic samples were then homogenized in 10 volume ice-cold potassium phosphate buffer (50 mmol/L, pH 7.4), sonicated, and centrifuged for 30 min at 3500 × *g*. The supernatants were transformed into several microtubes for separate biochemical assays, and all were kept at -80 °C until analyses^[44].

Macroscopic and microscopic assessment of colonic damage

The macroscopic damage was assessed and scored according to criteria as described in our previous work^[45,46]. For microscopic analysis, the fixed segments in formalin 10% were embedded in paraffin and stained with hematoxylin and eosin. The scoring was performed by one who was blind to the treated groups.

Determination of TNF- α and IL-1 β

Quantitative detection of TNF- α and IL-1 β levels in colon tissues were performed using an ELISA kit. The absorbance of the final colored product was measured in 450 nm as the primary wavelength and 620 nm as the reference wavelength. TNF- α and IL-1 β levels were expressed as pg/mg protein of tissue, as described in our previous work^[44].

Total ferric reducing antioxidant power assay

Total antioxidant power of the colon was evaluated by measuring the ability to reduce Fe³⁺ to Fe²⁺. Interaction of TPTZ with Fe²⁺ results in the formation of a blue color, with a maximum absorbance at 593 nm. Data were expressed as mmol/L ferric ions reduced to ferrous per mg of protein, as described in our previous work^[47].

Myeloperoxidase activity measurement

In this test, supernatant was combined with o-dianisidine and 0.0005% H₂O₂ that resulted in an absorbance at 460 nm that was measured for 3 min. One unit of myeloperoxidase (MPO) activity is described as the change in absorbance per min at room temperature in the final reaction. Details of the procedure have been described in our previous work^[48].

Thiobarbituric acid-reactive substances assay

Levels of lipid peroxidation were assessed in colon tissue using thiobarbituric acid reactive substances (TBARS) assay as described in our previous work^[49]. Data were reported as μ g/mg of protein.

Total protein of colon homogenate

Total protein of the tissue was measured according to the Bradford method, using BSA as the standard^[50]. Results were reported as mg of protein per mL of homogenized tissue.

Determination of LD50

In order to determine the acute toxicity (LD50) of Magliasa, doses of 5, 50, 300 and 2000 mg/kg per day were gavaged to rats. The animals were observed for 1 wk and any mortality was recorded at the end of this period^[51].

Statistical analysis

Data were analyzed by StatsDirect ver. 2.7.8. One-way analysis of variance followed by a Newman-Keuls *post hoc* test for multiple comparisons were used. *P* values less than 0.05 were considered significant. Results are expressed as mean \pm SE.

RESULTS

Botanical authentication

Microscopic characteristics of different herbal components of Magliasa are shown in Figure 1.

Quantitative determination of total glucosinolates and total phenols in Magliasa

The mean value of total glucosinolates in one gram of Magliasa was 19 \pm 1 μ mol. The mean value of total phenolic content was 293.8 \pm 17.6 mg GAE per 100 g of Magliasa.

TLC analysis

Table 2 summarizes the retention value of spots visible in the TLC profile of Magliasa.

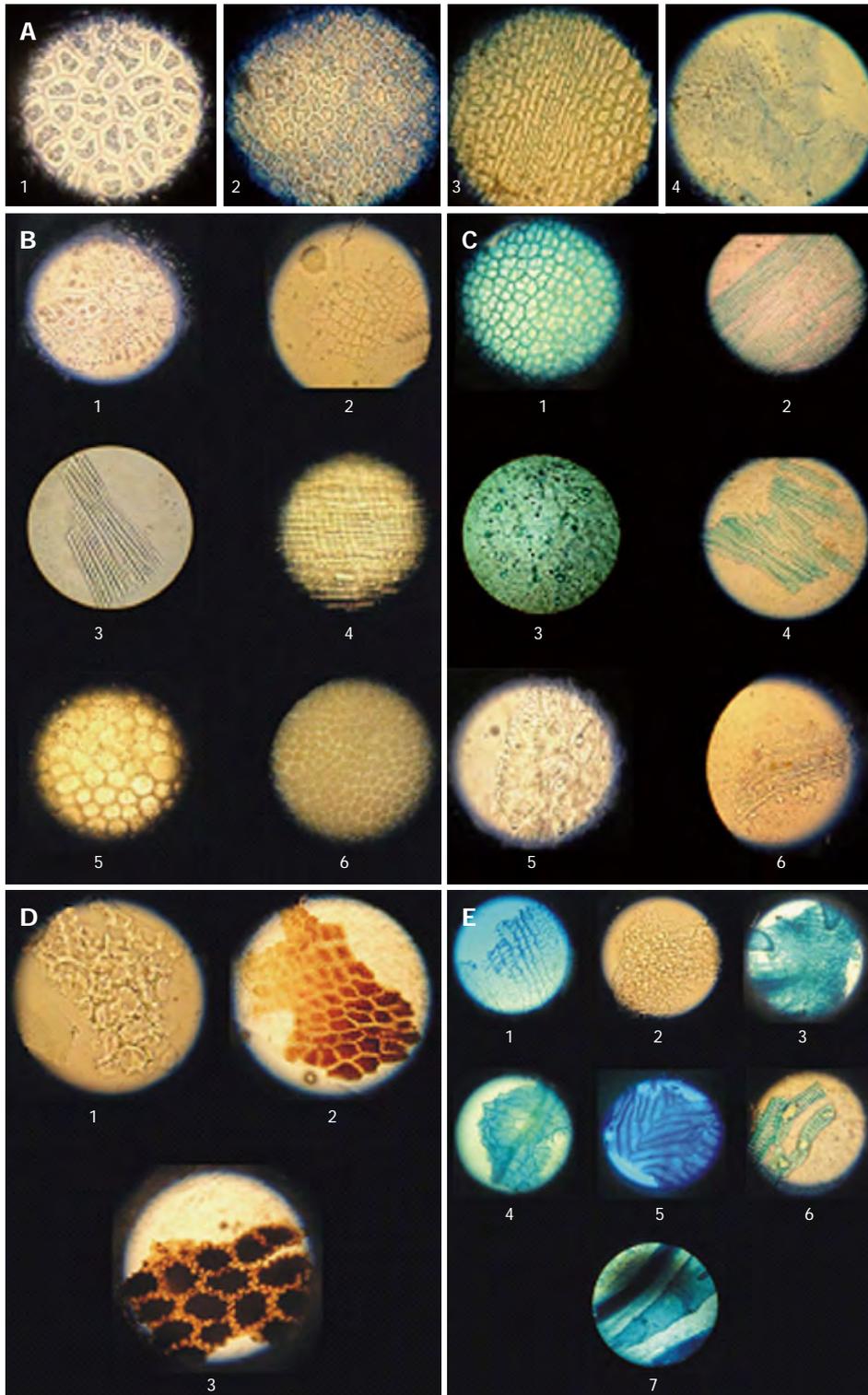


Figure 1 Microscopic characteristics of the herbal ingredients of Maglisa. A: *Lepidium sativum* seed. A1: Pericarp; A2 and A3: Sclereids of the mesocarp; A4: Parenchyma of the endosperm; B: *Linum usitatissimum* seed. B1: Endosperm; B2: Epidermis; B3: Fiber; B4: Sclerenchyma; B5: Parenchyma of the testa; B6: Pigment layer of testa; C: *Terminalia chebula* fruit. C1: Epidermis; C2: Fiber; C3: Parenchyma of the mesocarp; C4 and C5: Sclereids; C6: Vessels; D: *Allium ampeloprasum* cv. Porrus seed. D1: Endosperm; D2: Mesoderm; D3: Epidermis of the testa; E: *Bunium persicum* fruit. E1: Endocarp; E2 and E3: Endosperm; E4 and E5: Sclereids of the mesocarp; E6: Vessels; E7: Vittae. Magnification of all images was 40.

Macroscopic and microscopic assessment of colonic damage

Data are shown in Table 3. The colons of the Sham group appeared normal. In contrast, intracolonic administration of TNBS led to mucosal ulceration, inflammation, adhesion, and wall thickening in the control group. Treatment with Mag-50 did not significantly reduce macroscopic scores where linear ulceration and mesenteric inflammation were observed in some samples. Administration of Maglisa re-

duced the macroscopic score in a dose-dependent manner, and a significant effect was observed in the Mag-100 and Mag-200 groups, although some inflammation and hyperemia were evident. The median effective dose (ED50) value was 104.78 mg/kg. Treatment of rats by dexamethasone and infliximab remarkably attenuated scores where mild hyperemia was observed macroscopically.

Histopathological examination of the control group showed extensive severe transmural inflammation, dif-

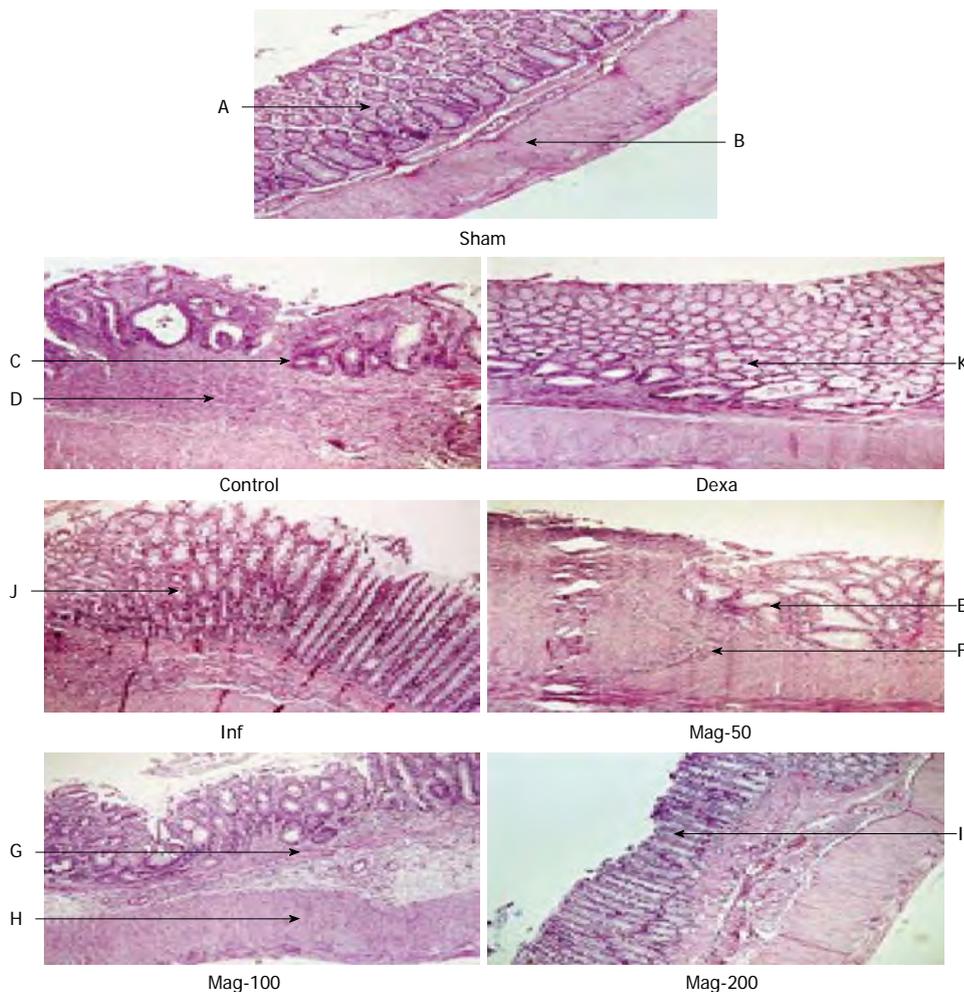


Figure 2 Histological images of colon samples. In the Sham group, colons were within normal limits including crypts (A) and submucosal tissue (B), but intense transmural inflammation (C), and severe crypt destruction (D) were observed in the control group. Moderate crypt distortion (E and G) and inflammatory cell infiltration (F and H) was seen in the Mag-5 and Mag-100 groups. Mild focal inflammation and crypt abscess were observed in the Mag-20 and infliximab groups (I and J). Mild focal inflammation was observed in the Dexa group (K). Magnification of all images was 100. Dexa: Dexamethasone; Inf: Infliximab; Mag-50: Magliasia at a dose of 50 mg/kg; Mag-100: Magliasia at a dose of 100 mg/kg; Mag-200: Magliasia at a dose of 200 mg/kg.

Table 2 Thin layer chromatography analysis of Magliasia

| Type of extract | Solvent system | RF values | Intensity of spot |
|-----------------|---------------------------------|-----------|--------------------|
| Dichloromethane | Hexane: ethyl acetate (5:4 v/v) | 0.048 | Moderately intense |
| | | 0.181 | Faint |
| | | 0.238 | Intense |
| | | 0.286 | Faint |
| | | 0.333 | Intense |
| | | 0.380 | Intense |
| | | 0.430 | Faint |
| | | 0.476 | Faint |
| | | 0.524 | Moderately intense |
| | | 0.571 | Faint |
| | | 0.619 | Faint |
| | | 0.670 | Faint |
| | | 0.714 | Faint |
| | | 0.762 | Intense |
| | | 0.838 | Faint |
| | | 0.876 | Moderately intense |

RF: Retention factor.

fused necrosis, mucosal and submucosal polymorphonuclear (PMN) leukocyte infiltration, and crypt destruction, whereas microscopic evaluation of the Sham group showed a normal situation. In the Mag-50 group, microscopic evaluation revealed moderate mucosal and submucosal inflammation, PMN infiltration, and extensive crypt

Table 3 Macroscopic and microscopic scores as criteria for assessing colonic damage

| Group | Macroscopic score | | Microscopic score | |
|---------|--------------------------------|----------------|------------------------------|----------------|
| | mean ± SE | Median (range) | mean ± SE | Median (range) |
| Sham | 0.00 ± 0.00 | 0 (0-0) | 0.00 ± 0.00 | 0 (0-0) |
| Control | 3.40 ± 0.24 ^a | 3 (3-4) | 4.00 ± 0.32 ^a | 4 (3-5) |
| Dexa | 0.33 ± 0.21 ^c | 0 (0-1) | 0.67 ± 0.21 ^c | 1 (0-1) |
| Inf | 0.83 ± 0.31 ^c | 1 (0-2) | 1.50 ± 0.34 ^c | 1 (1-3) |
| Mag-50 | 2.20 ± 0.37 ^{a,b,e,g} | 2 (1-3) | 2.60 ± 0.51 ^{a,b,e} | 3 (1-4) |
| Mag-100 | 1.80 ± 0.58 ^{a,c,e} | 1 (1-4) | 2.40 ± 0.75 ^a | 2 (1-5) |
| Mag-200 | 1.20 ± 0.20 ^c | 1 (1-2) | 1.60 ± 0.40 ^c | 1 (1-3) |

^a*P* < 0.05 vs Sham group; ^c*P* < 0.05 vs the control group; ^a*P* < 0.05 vs the dexamethasone group; ^b*P* < 0.05 vs the infliximab group. Dexa: Dexamethasone; Inf: Infliximab; Mag-50: Magliasia at a dose of 50 mg/kg; Mag-100: Magliasia at a dose of 100 mg/kg; Mag-200: Magliasia at a dose of 200 mg/kg.

distortion. In the Mag-100 group, moderate inflammation of the mucosa and submucosa, inflammatory cell infiltration, and some crypt abscess and destruction were observed. In the Mag-200 and infliximab groups, mild focal non-hemorrhagic edema and focal submucosal PMN infiltration were observed. Minimal mucosal inflammation was observed in the Dexa group (Figure 2). Administration of Magliasia reduced the microscopic score in a dose-dependent manner, and a significant effect was observed

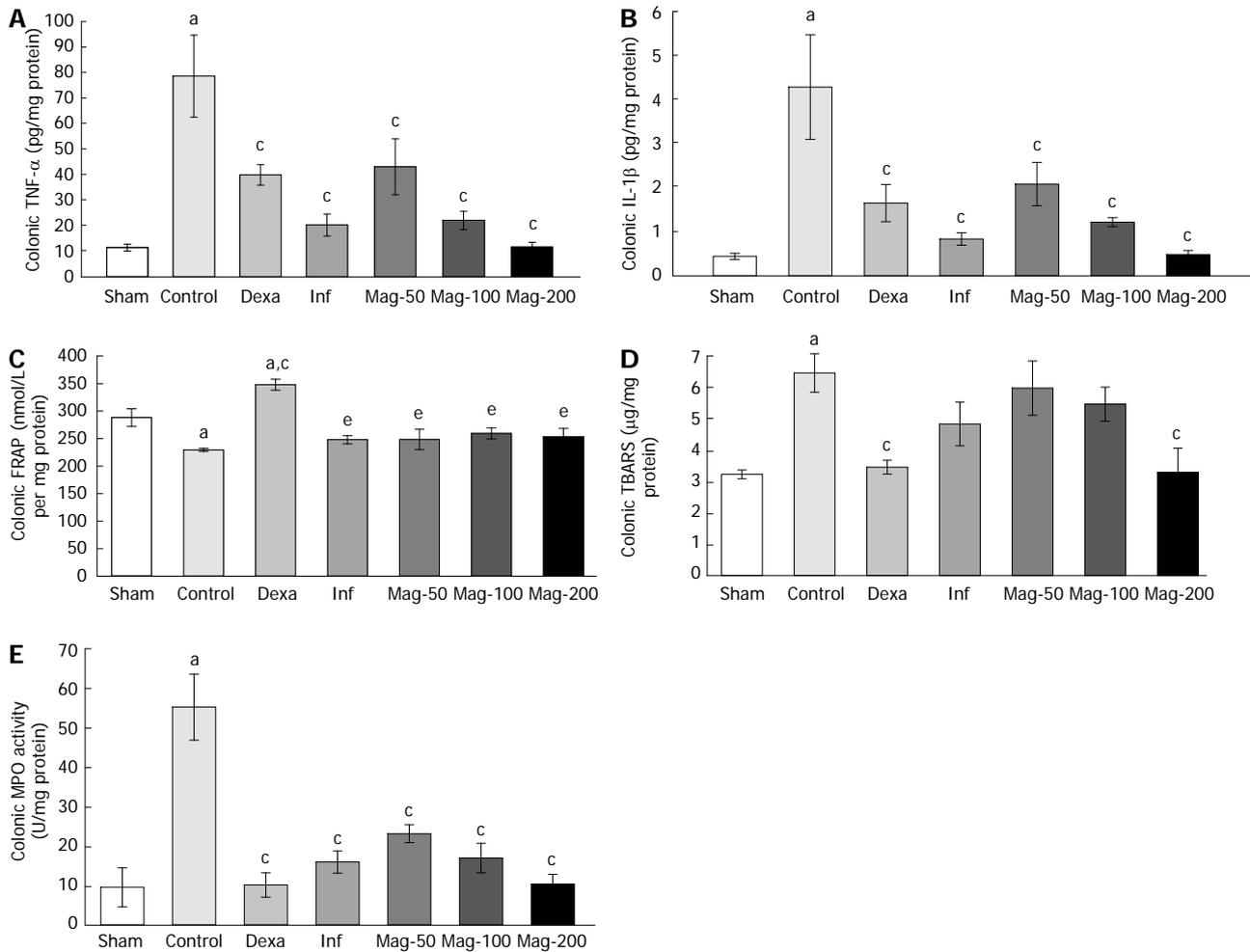


Figure 3 Levels of different biochemical parameters in the colon of rats after 2 wk of treatment. A: Tumor necrosis factor-alpha (TNF- α); B: Interleukin-1 beta (IL-1 β); C: Total antioxidant capacity as a ferric reducing antioxidant power (FRAP) level; D: Lipid peroxidation as a thiobarbituric acid reactive substances (TBARS) level; E: Neutrophil infiltration as myeloperoxidase (MPO) activity. Values are mean \pm SE. ^a $P < 0.05$ vs the Sham group; ^c $P < 0.05$ vs the control group; ^e $P < 0.05$ vs the dexamethasone group. Dexa: Dexamethasone; Inf: Infliximab; Mag-50: Magliasa at a dose of 50 mg/kg; Mag-100: Magliasa at a dose of 100 mg/kg; Mag-200: Magliasa at a dose of 200 mg/kg.

in the Mag-200 group, with an ED50 of 132.29 mg/kg.

Colonic TNF- α

A significant difference was seen in TNF- α between the control and Sham groups ($P = 0.000$). TNF- α was significantly lower in all groups compared to the control, with an ED50 of 55.36 mg/kg. The level of TNF- α in the Mag-100 group (21.99 ± 3.54) was near to that of the infliximab group (20.18 ± 4.29), and both were lower than that of the Mag-50 (42.85 ± 10.87) and Dexa (39.72 ± 3.97) groups. TNF- α in the Mag-200 group (11.60 ± 1.83) was lower than that of the infliximab group, but the difference was not significant ($P = 0.98$, Figure 3A).

Colonic IL-1 β levels

IL-1 β was higher in the control group compared to the Sham ($P = 0.000$). IL-1 β in all groups was lower than that of the control, with an ED50 of 48.78 mg/kg. IL-1 β in the Mag-100 group (1.21 ± 0.10) was near to that of the Dexa group (1.64 ± 0.42), and both were higher than that of the infliximab (0.83 ± 0.14) and Mag-200 (0.48 ± 0.09)

groups. IL-1 β in the Mag-200 group was near to that of the Sham (0.44 ± 0.07), and was lower than infliximab, but the difference was not significant ($P = 0.998$, Figure 3B).

Colonic total antioxidant power as ferric reducing/antioxidant power

The ferric reducing/antioxidant power (FRAP) value was significantly lower in the control compared to the Sham ($P = 0.008$). Among interventions, only dexamethasone caused a significant increase in FRAP when compared to the control ($P = 0.000$). None of the Mag-50, Mag-100, Mag-200 or infliximab groups showed a significant difference to the control in FRAP (Figure 3C). The effect of Magliasa in FRAP was not dose dependent.

Colonic lipid peroxidation level as TBARS

The TBARS value was significantly higher in the control compared to the Sham ($P = 0.005$), while TBARS in the Mag-200 (3.27 ± 0.77 , $P = 0.010$) and Dexa (3.44 ± 0.22 , $P = 0.011$) groups was significantly lower than that of the control (6.43 ± 0.61). Other groups did not show a signif-

icant difference from the control in TBARS (Figure 3D). Administration of Magliasia reduced TBARS in a dose-dependent manner, with an ED50 value of 216.4 mg/kg.

Colonic MPO activity

MPO in the control was significantly higher than that of the Sham ($P < 0.002$). Treatment with Magliasia in all groups significantly decreased MPO activity compared to the control. MPO in the Mag-200 group (10.65 ± 2.53) was lowest amongst the Mag groups, and close to the Dexa group (10.42 ± 3.18). MPO in the Mag-200 group was lower than that of the infliximab group (16.41 ± 2.89) (Figure 3E). The ED50 value was 34.38 mg/kg.

LD50

The acute toxicity test (LD50) demonstrated that Magliasia is not lethal up to a dose of 2000 mg/kg after oral administration. In the treated groups, no sign of toxicity was observed. It can therefore be considered as practically non-toxic.

DISCUSSION

There is a strong potential in the traditional and folkloric medicines of various countries, including Iran, for developing new and efficacious drugs for diseases that have a challenging treatment. One such disease is IBD. In this paper, Magliasia, one of the remedies recommended for colitis in TIM, was prepared, and its efficacy and possible mechanisms of action in different doses were evaluated in TNBS-induced colitis and compared with standard drugs. Macroscopic and microscopic scores, as criteria for colonic damage, improved by doses of 100 and 200 mg/kg per day with Magliasia. The microscopic score reduced only in the Mag-200 group, while the Mag-50 group showed no significant benefit against colonic damage. Colonic TNF- α , IL-1 β and MPO activities were significantly decreased by all doses of Magliasia. TNF- α and IL-1 β have been described as important mediators that contribute to intestinal inflammation in IBD patients^[52-54]. Increased TNF- α has been found in the serum and mucosa of patients with IBD^[55,56]. Moreover, inhibition of TNF- α by anti-TNF- α drugs, such as infliximab, has been an efficacious strategy in the management of IBD^[10,57]. MPO is located in the granules of neutrophils and released upon stimulation by free radicals. The activity of MPO has been known as a marker of neutrophil penetration to the site of inflammation^[58,59]. Magliasia did not affect oxidative stress as a factor involved in the pathophysiology of IBD^[2]. Lipid peroxidation in the colon decreased only with a high dose of Magliasia (Mag-200). The effects of Magliasia in all investigated parameters were dose-dependent, except in total antioxidant power.

The total phenolic content of Magliasia was determined because phenolic compounds have pharmacological activities (antioxidant, anti-inflammatory, anti-diarrheal, and antimicrobial) that are all useful for the management of IBD, considering its pathogenesis. There is concern

about the content uniformity of Magliasia, as intact non-milled seed of *Lepidium sativum* comprises 50% of the product. In addition to a reference marker for the standardization of Magliasia, total glucosinolates can be used for evaluating the content uniformity of the product.

There are some reports on the herbal ingredients of Magliasia that confirm their efficacy in IBD^[32]. These reports are summarized in Table 1. Anti-inflammatory, antioxidant, analgesic, spasmolytic, antiulcer, ulcer healing, immunomodulatory, antibacterial, and anti-diarrheal activity are among the pharmacological properties of these ingredients that make them useful for IBD. It seems that the efficacy of Magliasia in IBD is due to the combination of the mentioned activities.

Overall, the results obtained from the efficacy of Magliasia on TNBS-induced colitis of rats are encouraging, although clinical trials are required for confirmation of these results.

COMMENTS

Background

Conventional treatments for the management of inflammatory bowel disease (IBD) have serious adverse effects that reduce patient compliance, and therefore investigators are trying to find useful compounds from complementary and alternative medicines with better safety and tolerability. There are many herbal preparations in traditional Iranian medicine (TIM) that were used for the management of IBD. Magliasia is one of them, and contains 6 components: seeds of *Lepidium sativum*, *Linum usitatissimum*, and *Allium ampeloprasum* cv. Porrum, fruit of *Bunium persicum* and *Terminalia chebula*, and gum resin of *Pistacia lentiscus*. Although, the efficacy of some herbal components of Magliasia in IBD have been confirmed by previous studies, no other study to date has investigated the beneficial effects of this preparation.

Research frontiers

In the present study, after formulation and explanation of the quality control methods of Magliasia, its effects were investigated in trinitrobenzenesulfonic acid-induced colitis of rats to determine the involved mechanisms.

Innovations and breakthroughs

Magliasia demonstrated a significant reduction in macroscopic colonic damage, tumor necrosis factor-alpha, interleukin-1 beta, and neutrophil infiltration. Determination of total glucosinolates and total phenolic contents, as well as performing thin layer chromatography, can be used successfully for quality control of this herbal preparation.

Applications

Since the effects of Magliasia in the experimental model of colitis were encouraging, it could potentially be used as an effective medicine for IBD after confirmation of obtained results by clinical trials. Moreover, this study is a step toward strengthening TIM evidence.

Peer review

Hopefully reviewers are positive to this article and believe that this TIM formula has enough support to go forward future clinical trials.

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Expression and clinical significance of CD73 and hypoxia-inducible factor-1 α in gastric carcinoma

Xiao-Xia Lu, Yi-Tian Chen, Bing Feng, Xiao-Bei Mao, Bo Yu, Xiao-Yuan Chu

Xiao-Xia Lu, Yi-Tian Chen, Bing Feng, Xiao-Bei Mao, Xiao-Yuan Chu, Department of Medical Oncology, Nanjing General Hospital of Nanjing Military Command, Medical School of Nanjing University, Nanjing 210002, Jiangsu Province, China

Bo Yu, Department of Pathology, Nanjing General Hospital of Nanjing Military Command, Nanjing 210002, Jiangsu Province, China

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Correspondence to: Xiao-Yuan Chu, Associate Professor, Department of Medical Oncology, Nanjing General Hospital of Nanjing Military Command, Medical School of Nanjing University, No. 305 Zhongshan Road, Nanjing 210002, Jiangsu Province, China. chuxiaoyuan6906@gmail.com

Telephone: +86-25-80860072 Fax: +86-25-80860072

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Abstract

AIM: To investigate the expression of CD73 and hypoxia-inducible factor-1 α (HIF-1 α) in human gastric carcinoma, and explore their clinical significance and prognostic value.

METHODS: CD73 and HIF-1 α expressions were detected by immunohistochemistry in consecutive sections of tissue samples from 68 gastric carcinoma patients. The peritumor tissues 2 cm away from the tumor were obtained and served as controls. The presence of CD73 and HIF-1 α was analyzed by immunohistochemistry using the Envision technique.

RESULTS: CD73 and HIF-1 α expressions in gastric carcinoma were significantly higher than those in gas-

tric mucosal tissues as control ($P < 0.001$) and showed a close correlation (Spearman $r = 0.390$, $P = 0.001$). Overexpression of CD73 was positively correlated with differentiation of tumor ($P = 0.000$), histopathology ($P = 0.041$), depth of invasion ($P < 0.001$), nodal status ($P = 0.003$), metastasis ($P = 0.013$), and the American Joint Committee on Cancer (AJCC) stage ($P < 0.001$). High expression of HIF-1 α was positively correlated with tumor diameter ($P = 0.031$), depth of invasion ($P = 0.022$), and AJCC stage ($P = 0.035$). The overall survival rate was low in the patients with high expression of CD73 ($P < 0.001$). Moreover, CD73+/HIF-1 α + patients had the worst prognosis ($P < 0.001$). CD73 expression was proven to be an independent predictor for patients with gastric carcinoma by both multivariate Cox regression analysis ($P = 0.021$) and receiver operating characteristic curves ($P = 0.001$).

CONCLUSION: CD73 expression correlates closely with HIF-1 α expression in gastric carcinoma. CD73 could be an independent prognostic indicator for gastric carcinoma.

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Key words: CD73; Hypoxia-inducible factor-1 α ; Gastric carcinoma; Immunohistochemistry; Prognosis

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INTRODUCTION

Gastric carcinoma has been the fourth most common cancer in the world since the latter half of the 20th century^[1,2]. In spite of the recent advances in diagnostic techniques

for early detection and the improvement in surgical treatment, gastric carcinoma remains the second leading cause of cancer-related deaths^[3]. In China, Japan and Korea, the incidence of gastric carcinoma now has reached up to 80 new cases per 100 000 population annually^[4]. Changes observed in expression of tumor specific biomarkers in gastric carcinomas may be helpful to understand the transformation of histological heterogeneity and the underlying molecular mechanisms. Searching for specific biomarkers which determine the biological nature and behavior of gastric carcinoma would be of utmost importance to optimize individualized therapy.

Ecto-5'-nucleotidase/CD73 is a homodimer linked to the plasma membrane through a glycosylphosphatidylinositol lipid anchor, which was found in most tissues^[5]. It is a part of extracellular ATP metabolism, which dephosphorylates AMP into adenosine rapidly after CD39 catalyzes ATP, ADP and AMP^[6]. Recent studies have demonstrated that CD73 could participate in a variety of physiological responses including ischemic preconditioning, platelet function, hypoxia, vascular leak and tissue injury^[7,8]. CD73 was up-regulated in various human cancers, including those of lung, colon, breast, pancreas and ovary^[9,10]. Importantly, the high expression of CD73 was correlated with tumor neovascularization, invasiveness, metastasis, as well as shorter patient survival^[9-13]. These results suggested that CD73 might play a significant role in controlling tumor progression.

Oxygen is only able to diffuse 100-180 μ m from a capillary to cells, which makes hypoxia a common feature of rapidly growing solid tumors^[14]. Hypoxia-inducible factor-1 (HIF-1) is a heterodimeric basic helix-loop-helix transcription factor composed of HIF-1 α and HIF-1 β subunits; and HIF-1 α determines HIF-1 activity^[15]. It is found that HIF-1 α is widely expressed in various types of carcinomas, such as those of brain, breast, lung and colon^[16-18]. These results revealed that HIF-1 α was correlated with tumor progression, aggressive behavior, and patient prognosis. As is known, pathophysiologic conditions of hypoxia can cause adenine nucleotide metabolic changes. A recent study found that CD73 is transcriptionally regulated by ambient hypoxia and is one of the mechanisms involved HIF-1^[19].

The purpose of this study was to ascertain the correlation between CD73 and HIF-1 α expressions and their clinicopathological significance in gastric carcinoma, including patient survival. We hypothesized that CD73 expression would be correlated with clinicopathological factors and HIF-1 α expression, and the combination of the two molecules would predict recurrence and overall survival.

MATERIALS AND METHODS

Patients

Samples of gastric carcinoma were collected from the resected stomach of 68 patients who were diagnosed histologically as gastric carcinoma and underwent gastrec-

tomy at the Nanjing General Hospital of Nanjing Military Command. None of the patients had previously received radiotherapy, chemotherapy or other medical interventions before surgery. Among them, 43 (63%) patients were male and 25 (37%) were female, with a mean age of 49.86 years. All patients were followed up from the date of surgery until either the date of death or December 2011. For analysis of patient survival, the patients who were lost to follow-up or those who died from causes other than gastric carcinoma were regarded as censored data.

This study was approved by the Institutional Review Board, and informed consent was obtained from each patient.

Immunohistochemistry

The peritumor tissues 2 cm away from the tumor were collected and served as healthy controls. Tumor specimens and healthy control gastric mucosal tissues, which were fixed in 10% buffered formalin and embedded in paraffin, were cut into 4- μ m sections and placed on polylysine-coated slides. The staining was conducted by the avidin-biotin-peroxidase complex method. Each paraffin section was deparaffinized and rehydrated through graded alcohols, followed by antigen retrieval with epitope retrieval solution (10 mmol citrate buffer, pH 6.0) in a pre-heated water bath at 98 $^{\circ}$ C for 10 min. Endogenous peroxidase was blocked using 3% hydrogen peroxide. Subsequently, sections were incubated with the primary mouse monoclonal CD73 antibody (1:100, ab71322 Abcam) and mouse monoclonal HIF-1 α antibody (1:100, MAB1935 R and D) overnight at 4 $^{\circ}$ C, and then were stained with secondary antibody for 30 min. The sections were finally counterstained with haematoxylin (Zymed Laboratories Inc, San Francisco, CA, United States). Negative control was performed by replacing the primary antibody with a normal murine immunoglobulin G. Known immunostaining-positive sections were used as positive controls.

Evaluation of immunohistochemical analysis

We used semi-quantitative method. Five different perspectives were randomly selected under ordinary optical microscope at a magnification of 400. The percentage of positive cells was scored 0 for staining of < 1%, 1 for staining of 2%-25%, 2 for staining of 26%-50%, 3 for staining of 51%-75%, and 4 for staining > 75% of the cells examined. Staining intensity was calculated, no coloring, slightly yellow, brown yellow and tan stains were marked as 0, 1, 2 and 3. Finally, we calculated the product of staining intensity and positive cell percentage: ≤ 5 was defined as negative and ≥ 6 as positive. Two pathologists blinded to the clinical details reviewed the pathological films and staining points.

Statistical analysis

Categorical data were analyzed using the χ^2 or nonparametric test, while measurement data were evaluated with Student's *t* or one-way analysis of variance test. Correlation coefficient between expression of CD73 and HIF-1 α was estimated by the Spearman correlation method.

Table 1 Correlation of CD73 and hypoxia-inducible factor-1 α expression with clinicopathological characteristics of gastric carcinoma

| Clinicopathological data | CD73 expression | | <i>P</i> value | HIF-1 α expression | | <i>P</i> value |
|--------------------------------------|-----------------|-----|----------------|---------------------------|-----|----------------|
| | High | Low | | High | Low | |
| Gender | | | 0.144 | | | 0.136 |
| Male | 18 | 25 | | 21 | 22 | |
| Female | 13 | 12 | | 15 | 10 | |
| Age (yr) | | | 0.157 | | | 0.107 |
| < 49.82 | 10 | 15 | | 11 | 14 | |
| \geq 49.82 | 21 | 22 | | 25 | 18 | |
| Tumor diameter (cm) | | | 0.127 | | | 0.031 |
| \leq 5 | 7 | 17 | | 9 | 15 | |
| 5-10 | 10 | 5 | | 8 | 7 | |
| > 10 | 14 | 15 | | 19 | 10 | |
| Differentiation | | | 0.000 | | | 0.445 |
| Well | 1 | 4 | | 4 | 1 | |
| Moderate | 6 | 22 | | 13 | 15 | |
| Poor | 24 | 11 | | 19 | 16 | |
| Histopathology | | | 0.041 | | | 0.168 |
| Tubular adenocarcinoma | 3 | 16 | | 7 | 12 | |
| Poorly differentiated adenocarcinoma | 21 | 11 | | 21 | 11 | |
| Signet-ring cell carcinoma | 3 | 3 | | 2 | 4 | |
| Mucinous adenocarcinoma | 4 | 7 | | 6 | 5 | |
| Borrmann type | | | 0.140 | | | 0.430 |
| I | 5 | 19 | | 9 | 16 | |
| II | 9 | 12 | | 9 | 12 | |
| III | 15 | 13 | | 17 | 11 | |
| IV | 2 | 2 | | 1 | 3 | |
| Depth of invasion | | | 0.000 | | | 0.036 |
| T1-T2 | 1 | 21 | | 8 | 14 | |
| T3-T4 | 30 | 16 | | 28 | 18 | |
| Nodal status | | | 0.003 | | | 0.113 |
| N0 | 4 | 17 | | 9 | 12 | |
| N1/N2 | 27 | 20 | | 27 | 20 | |
| Metastasis | | | 0.013 | | | 0.192 |
| M0 | 20 | 33 | | 27 | 26 | |
| M1 | 11 | 4 | | 9 | 6 | |
| AJCC stage | | | 0.000 | | | 0.035 |
| I / II | 2 | 24 | | 10 | 16 | |
| III / IV | 27 | 15 | | 26 | 16 | |

HIF-1 α : Hypoxia-inducible factor-1 α ; AJCC: American Joint Committee on Cancer.

The Kaplan-Meier method was used to estimate the overall survival and the log-rank test was used to analyze the differences between the curves. Multivariate Cox proportional hazard regression model and receiver operating characteristic (ROC) curve analysis were established to assess the prognostic values of protein expression. All statistical analysis were performed using the SPSS software version 16.0 (SPSS, Chicago, IL, United States) and $P < 0.05$ was considered statistically significant.

RESULTS

Patient characteristics

The demographic and clinicopathological variables of

the 68 patients are shown in Table 1. The ages of the patients ranged from 24 to 59 years with the mean age 49.82 years. Based on the American Joint Committee on Cancer (AJCC) classification, there were 26 stage I / II patients and 42 stage III / IV patients. The patients were followed up for a period of 1-84 mo. Two were lost to follow-up and 37 patients died during the follow-up.

Immunohistochemical analysis of CD73 and HIF-1 α expression

CD73 and HIF-1 α were detected on consecutive sections and were found to be mainly expressed in the cytoplasm of gastric carcinoma (Figure 1). We repeated the experiment twice to exclude false positive results. Immunohistochemical analysis showed that in gastric carcinoma, 31 (45.6%) of 68 samples were CD73-positive and 36 (52.9%) of 68 were HIF-1 α -positive, while in healthy control gastric mucosa, 8 (11.8%) of 68 were CD73-positive and 12 (17.6%) of 68 were HIF-1 α -positive. CD73 ($P < 0.001$) and HIF-1 α ($P < 0.001$) expression was significantly higher than in healthy controls. CD73 expression was concordant with HIF-1 α expression in 69.1% (47 of 68) of gastric carcinoma cases (Spearman $r = 0.390$, $P = 0.001$). CD73 and HIF-1 α were found double-positive in 23 cases, double-negative in 24 cases, and either CD73-positive or HIF-1 α -positive only in 21 cases. The Spearman's rank correlation method was used to estimate the expression correlation coefficient ($r = 0.390$, $P = 0.001$), indicating a close correlation between CD73 and HIF-1 α expression in gastric carcinomas.

Correlation of CD73 and HIF-1 α expression with clinicopathological variables

Chi-squared test was used to investigate the correlation of CD73 and HIF-1 α expression with clinicopathological variables. Statistically, CD73 overexpression was significantly correlated with tumor differentiation ($P = 0.000$), histopathology ($P = 0.041$), depth of invasion ($P = 0.000$), nodal status ($P = 0.003$), metastasis ($P = 0.013$), and AJCC stage ($P = 0.000$) (Table 1). In contrast, there was no correlation between the expression of CD73 and age, gender, tumor size, or Borrmann type ($P > 0.05$, Table 1). In addition, HIF-1 α expression was significantly correlated with tumor size ($P = 0.031$), depth of invasion ($P = 0.036$) and AJCC stage ($P = 0.035$) (Table 1), but not with age, gender, tumor differentiation, histopathology, depth of invasion, nodal status, metastasis, or Borrmann type ($P > 0.05$, Table 1).

Survival analysis

Further Kaplan-Meier analysis demonstrated that high expression of CD73 (log-rank, $P < 0.001$) had a statistically significant correlation with a poor overall survival (Figure 2). But there was no significant correlation between overexpression of HIF-1 α and survival time (log-rank, $P = 0.103$). Moreover, we classified the patients into four groups stratified according to CD73/HIF-1 α expression, and a significant difference was observed

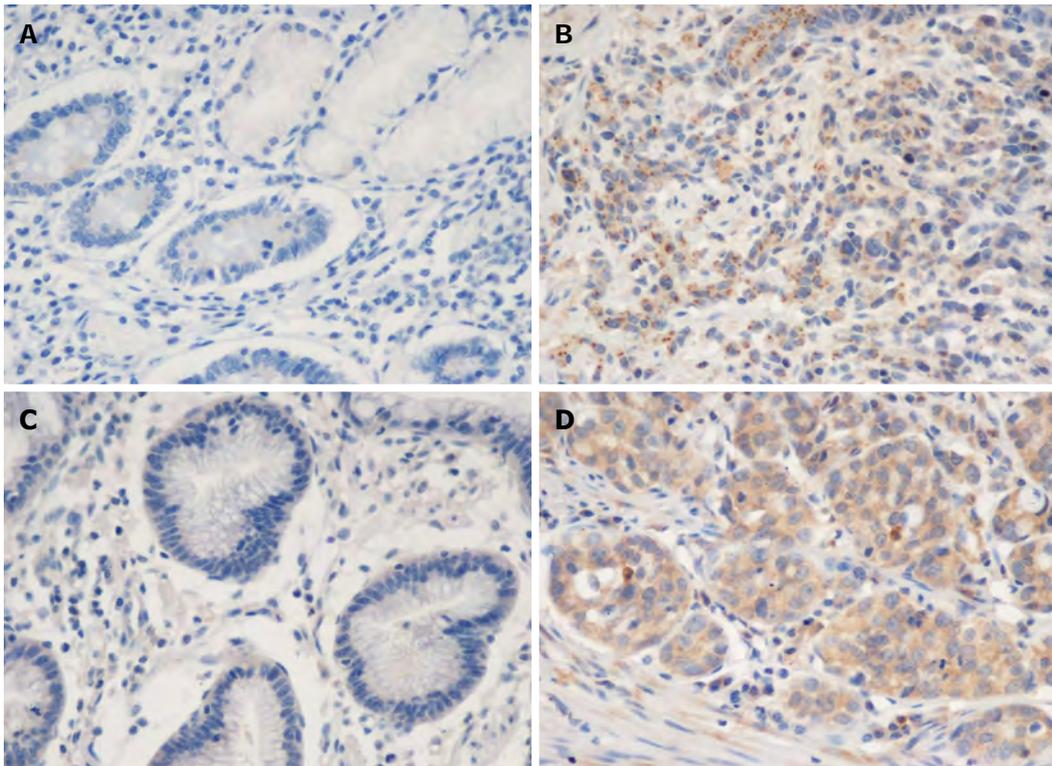


Figure 1 Expression of CD73 and hypoxia-inducible factor-1 α in gastric carcinoma (immunohistochemical stain, $\times 400$). A, C: Negative staining for CD73 (A) and hypoxia-inducible factor-1 α (HIF-1 α) (C) in healthy control gastric mucosa; B, D: Positive staining for CD73 (B) and HIF-1 α (D) in gastric carcinoma.

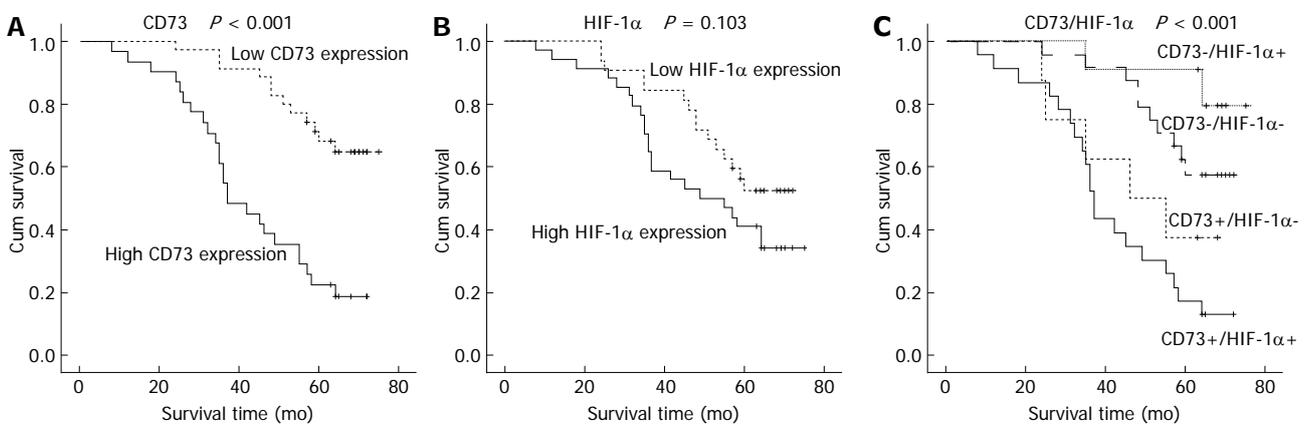


Figure 2 Kaplan-Meier curves for postoperative survival. A: The median survival time of patients with positive CD73 was shorter than that of patients with negative CD73 (log-rank test: $P < 0.001$); B: Hypoxia-inducible factor-1 α (HIF-1 α) expression had no correlation with the survival time of patients (log-rank test: $P = 0.103$); C: There was a significant difference among groups stratified according to CD73/HIF-1 α expression ($P < 0.001$). Patients with CD73+/HIF-1 α + had the worst prognosis.

among the groups (log-rank, $P < 0.001$). The patients with CD73+/HIF-1 α + carcinomas had the worst prognosis. The independent effects of all the significant factors were tested using the Cox proportional hazards model. The exploratory multivariate analyses demonstrated that CD73 [$P = 0.021$, hazard ratio (HR) = 0.385, 95%CI: 0.171-0.865] and AJCC stage ($P = 0.035$, HR = 1.585, 95%CI: 1.032-2.433) were independent prognostic factors, while HIF-1 α and others were not independent predictors. ROC curve analysis was also performed to further evaluate the prognostic value of CD73 and HIF-1 α expression, which revealed that CD73 expression was

encouragingly useful in predicting the overall survival of gastric carcinoma patients (area under the curve = 0.850, $P < 0.001$, Figure 3).

DISCUSSION

Gastric carcinoma is diagnosed with a high frequency throughout the world. In spite of improved diagnostic techniques and therapeutic methods, gastric carcinoma remains a major public health problem. Recently, some investigations showed that biomarkers might be promising predicting factors, and some of them were found

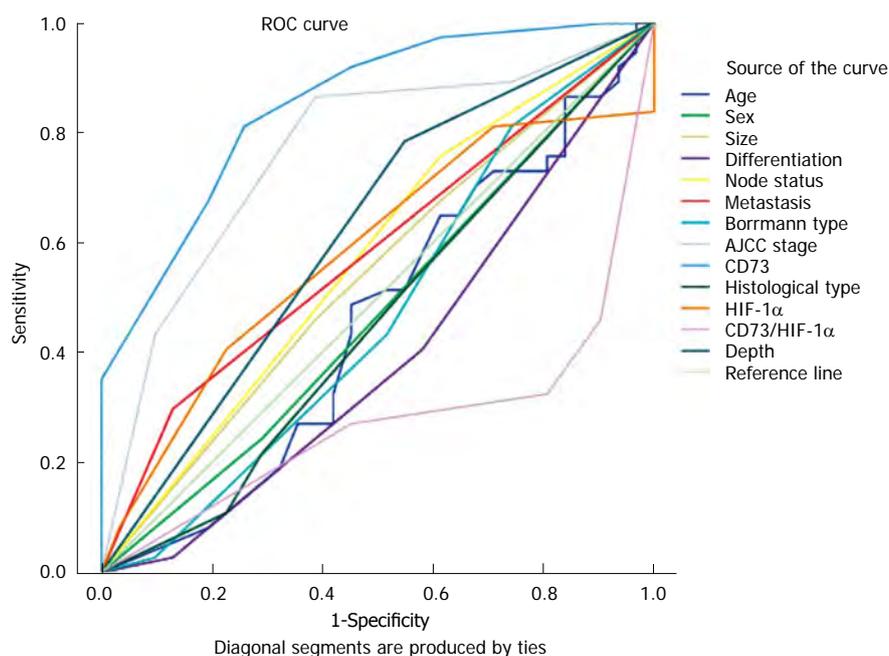


Figure 3 Receiver operating characteristic curves of clinicopathological variables, CD73 expression and hypoxia-inducible factor-1 α expression based on outcomes of gastric carcinoma patients. CD73 expression (AUC = 0.850; $P < 0.001$), hypoxia-inducible factor-1 α (HIF-1 α) (AUC = 0.582; $P = 0.247$), AJCC stage (AUC = 0.765; $P < 0.001$), CD73/HIF-1 α (AUC = 0.275; $P = 0.001$), Borrmann type (AUC = 0.472; $P = 0.689$), metastasis (AUC = 0.584; $P = 0.235$), nodal status (AUC = 0.572; $P = 0.310$), differentiation (AUC = 0.394; $P = 0.135$), histopathology (AUC = 0.459; $P = 0.559$), tumor diameter (AUC = 0.541; $P = 0.559$), gender (AUC = 0.476; $P = 0.740$), and age (AUC = 0.456; $P = 0.534$). ROC: Receiver operating characteristic; AJCC: American Joint Committee on Cancer; AUC: Area under the curve.

even superior to the AJCC staging system^[20,21]. Increasingly more researches have been conducted to discover the specific biomarkers although some of the results remained conflicting and inconsistent.

CD73, known as a purine salvage enzyme, might play a regulatory role in the immune system response^[22,23]. Jin *et al*^[22] found that the adenosine generated by tumor-derived CD73 could inhibit both the activation phase and effector phase of the antitumor T cell response and promote T cell apoptosis. Besides, some studies have indicated that CD73 could promote invasion, migration and adhesion of cancer cells^[24]. Moreover, the tumor-inhibiting effects of CD73 using siRNA or anti-CD73 antibody could restore efficacy of adoptive T cell therapy, leading to a long-term tumor-free survival^[22,25,26]. Host-derived CD73 was also observed in recent years, which provided evidence that CD73 knockdown could significantly delay tumor growth by regulating host immune system^[27-29].

The prognostic significance of CD73 has been studied in several cancers such as papillary thyroid carcinoma and breast cancer^[9-13]. It was suggested that high expression of CD73 in papillary thyroid carcinomas could be a useful indicator in the differential diagnosis of thyroid tumors. Moreover, strong expression of CD73 was found to be associated with invasiveness, metastasis, and shorter clinical survival in breast cancer. However, few studies have investigated the correlation between CD73 and gastric carcinoma.

CD73 expression of tumor cells may be induced by the selective pressure of the host immune system. Among other influencing factors in tumor microenvironment, hypoxia is the one which has been clearly defined^[19]. Hypoxia could induce upregulation of CD73 expression in brain microvessel endothelial cells, which will be reversed by reoxygenation of a short duration^[30]. Synnestvedt *et al*^[19] reported that hypoxia induced CD73 mRNA, increased protein expression levels and enhanced the CD73 activity

in intestinal epithelial cells (T84 cells) and this involved direct binding of HIF-1 to the *Nf5e* gene.

In tumor cells, adaptations to hypoxia are regulated by the activation of specific genes through HIF. And the transcription factor HIF-1 α which determines HIF activity is regarded as a hypoxia marker^[31]. Overexpression of HIF-1 α has been observed in various cancers, such as brain, bladder, lung, breast, esophagus, pancreas, colon, ovary, kidney, and prostate^[16-18,32-35]. Furthermore, it was reported that HIF-1 α overexpression was significantly correlated with highly aggressive disease, resistance to radiotherapy and chemotherapy, and poor prognosis in various carcinomas^[36,37]. Dellas *et al*^[38] found that high expression of HIF-1 α was associated with tumor progression and metastasis in advanced cervical cancer. Lu *et al*^[37] reported that elevated HIF-1 α expression was significantly correlated with poor prognosis of rectal adenocarcinoma patients.

In this study, we investigated the relationship between CD73, HIF-1 α , clinicopathological significance, and clinical prognosis in gastric carcinoma. We found that the expression of CD73 was significantly higher in gastric carcinoma than that in normal gastric mucosa, indicating the important role of CD73 in carcinogenesis. Furthermore, CD73 expression was closely correlated with differentiation, histopathology, depth of invasion, nodal status, metastasis, and AJCC stage, but not associated with age, gender, tumor diameter, or Borrmann type. Overexpression of HIF-1 α was found to be associated with tumor size, depth of invasion, and AJCC stage. The Spearman's rank correlation analyses indicated a close correlation between CD73 and HIF-1 α expressions in gastric carcinoma.

Our data also demonstrated that the overall survival curves in the CD73-negative group were significantly higher than in the CD73-positive group. However, there was no significant correlation between HIF-1 α overexpression and the poor overall survival. We classified the

patients into four groups stratified according to CD73/HIF-1 α expression, and a significant difference was observed among the groups. The patients with CD73+/HIF-1 α + carcinomas had the worst prognosis. Multivariate analyses showed that only CD73 expression was a prognostic factor independent of certain well-established clinicopathological parameters.

In conclusion, CD73 was correlated with the clinicopathological features in gastric carcinoma. High expression of CD73 was an indicator of poor clinical prognosis in patients with gastric carcinoma. Moreover, immunoreactivity of the combined CD73 and HIF-1 α could be a useful prognostic marker of gastric carcinoma.

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COMMENTS

Background

Gastric carcinoma has been the fourth most common cancer in the world since the latter half of the 20th century. Changes observed in expression of tumor specific biomarkers in gastric carcinomas may be helpful to understand the transformation of histological heterogeneity and the underlying molecular mechanisms. Searching for specific biomarkers which determine the biological nature and behavior of gastric carcinoma would be of utmost importance to optimize individualized therapy.

Research frontiers

Overexpression of CD73 has been observed in various cancers. However, few studies have investigated the correlation between CD73 and gastric carcinoma. Previous studies indicated that hypoxia could induce up-regulation of CD73 expression in different cells, but the correlation between CD73 expression and hypoxia-inducible factor-1 α (HIF-1 α) expression has not been observed. In this study, the authors demonstrate that CD73 expression is up-regulated in gastric carcinoma and shows close correlation with HIF-1 α expression.

Innovations and breakthroughs

In this paper, data firstly shows that there are close correlation between the two biomarkers in gastric carcinoma and the combination of CD73 and HIF-1 α could be a useful marker of the prognosis of gastric carcinoma. Moreover, high expression of CD73 was found to be an indicator of poor clinical prognosis in patients with gastric carcinoma.

Applications

Examination of CD73 and HIF-1 α expression by immunohistochemistry (IHC) analysis could be used as an additional effective way to identify patients at high risk of tumor progression, thus to optimize individual treatment of patients with gastric carcinoma.

Terminology

Ecto-5'-nucleotidase/CD73 is a homodimer linked to the plasma membrane through a glycosylphosphatidylinositol lipid anchor, which was found in most tissues. It is a part of extracellular ATP metabolism, which dephosphorylates AMP into adenosine rapidly after CD39 catalyzes ATP, ADP and AMP; HIF-1 is a heterodimeric basic helix-loop-helix transcription factor composed of HIF-1 α and HIF-1 β subunits; and HIF-1 α determines HIF-1 activity

Peer review

This manuscript investigate the expression of CD73 and HIF-1 α in human gastric carcinoma by IHC. The results showed that CD73 and HIF-1 α were higher expressions in gastric carcinoma than that of control and showed close correlation. They concluded that the combination of the two molecules and CD73 expression in gastric cancer tissue is associated with prognosis. The results are interesting and may represent an additional effective way to identify patients at high risk of tumor progression.

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Immunohistochemical study of the digestive tract of *Oligosarcus hepsetus*

Danielle A Vieira-Lopes, Nadja L Pinheiro, Armando Sales, Adriana Ventura, Francisco G Araújo, Iracema D Gomes, Aparecida A Nascimento

Danielle A Vieira-Lopes, Nadja L Pinheiro, Armando Sales, Adriana Ventura, Francisco G Araújo, Iracema D Gomes, Aparecida A Nascimento, Departamento de Biologia Animal, Instituto de Biologia, Universidade Federal Rural do Rio de Janeiro, Seropédica, Rio de Janeiro 23890-000, Brazil

Author contributions: Vieira-Lopes DA, Pinheiro NL, Sales A, Ventura A, Araújo FG, Gomes ID and Nascimento AA designed the research; Vieira-Lopes DA, Ventura A, Gomes ID and Nascimento AA performed the research; Vieira-Lopes DA, Pinheiro NL, Sales A, Araújo FG and Nascimento AA analyzed the data; Vieira-Lopes DA and Nascimento AA wrote the paper.

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Correspondence to: Dr. Danielle Vieira-Lopes, Departamento de Biologia Animal, Instituto de Biologia, Universidade Federal Rural do Rio de Janeiro, Seropédica, Rio de Janeiro 23890-000, Brazil. dmjvieira@hotmail.com

Telephone: +55-21-26821711 Fax: +55-21-26821711

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Abstract

AIM: To describe the histology of the digestive tract and to investigate the occurrence of endocrine cells in *Oligosarcus hepsetus* (*O. hepsetus*).

METHODS: The digestive tract (DT) of *O. hepsetus* was divided into esophagus, two stomach regions (glandular and non-glandular) and two intestinal regions (anterior and posterior). These specimens were processed by routine histological techniques and stained with hematoxylin-eosin, Gomori's trichrome, periodic acid Schiff (PAS) and Alcian blue (AB). An immunohistochemical method using avidin-biotin-peroxidase was employed.

RESULTS: The esophagus is lined with a non-keratin-

ized stratified squamous epithelium that is reactive to PAS and AB. The stomach has a mucosa lined with a simple columnar epithelium with mucus-secreting cells that are reactive only to PAS. The intestine has a simple columnar epithelium with a brush border and goblet cells that are reactive to PAS and AB. Somatostatin, serotonin and cholecystokinin immunoreactive cells were identified throughout the DT.

CONCLUSION: This study revealed adaptations for the species' diet and showed that the distribution and relative frequency of immunoreactive cells are similar to those of other fish.

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Key words: Fish; Esophagus; Stomach; Intestine; Endocrine cells

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INTRODUCTION

The literature stresses the importance of knowledge of the anatomy of the digestive tract (DT) of fishes, because this structure is highly variable, related to the diversity of feeding habits, type of food and lifestyles^[1-3].

The histological architecture of the DT includes a layer of mucus-secreting cells, observed by histochemical techniques in various studies of teleosts. The secretions vary among different fish species and also according to the location in the DT within the same species^[4]. These secretions play an important role in lubricating the or-

gan and protecting against proteolytic degeneration and pathogenic microorganisms^[5].

Besides this, to control the functions of the different DT segments, endocrine cells compose a complex system disseminated among the epithelial components, with the ability to secrete physiologically active polypeptide hormones and amines^[6]. According to Deveney *et al.*^[7], hormones have important functions in the overall regulation of the digestive process, such as nutrient absorption, the secretion of intestinal and associated glands, gut motility and intestinal blood flow

Oligosarcus hepsetus (*O. hepsetus*), known commonly as the thin dogfish, belongs to the Characidae family. It is carnivorous, with a diet basically composed of small fish. It mainly lives in rivers and reservoirs, generally at the middle to the bottom of the water column. It has a slightly rounded body with small fins and is considered a good swimmer^[8]. Many articles have been published about this species' distribution, ecology, feeding habits and diet, but no study has been published reporting the microscopic analysis of the organs of the DT of *O. hepsetus*. Although it does not have economic importance, it is one of the most abundant species in the reservoirs of Rio de Janeiro^[9] and the Paraíba do Sul River^[10], with distribution in the coastal region of south to south-eastern Brazil between Santa Catarina and Rio de Janeiro^[11].

Studies involving microscopic anatomy and histochemistry provide information to characterize the organs of the digestive system, facilitating understanding of the physiology of the DT and the feeding habits of the species under investigation^[12]. These studies are essential for efforts to restock native species to improve the condition of ecosystems^[13].

The purpose of the present work was to describe histological and histochemical aspects of the DT of *O. hepsetus* and to investigate immunohistochemically the occurrence of the endocrine cells secreting somatostatin (SOM), serotonin (5-hydroxytryptamine, 5-HT), cholecystokinin (CCK), gastrin (GAS), glucagon (GLUC) and insulin (INS) in the DT of this fish, seeking to relate them to its feeding habits, in order to provide data for future studies.

MATERIALS AND METHODS

Collection area

The specimens were collected from two reservoirs: Funil (22°30'S, 44°45'W), and Ribeirão das Lajes (22°43'S, 44°46'W), as well as two points of the Paraíba do Sul River: Ilha dos Pombos (21°84'S, 42°58'W) and Santa Cecília (22°48'S; 43°84'W), all located in Rio de Janeiro state, Brazil.

Tissue processing

Fourteen specimens were used in this study without sexual distinction, four collected in Funil Reservoir, four in Ribeirão das Lajes Reservoir, and three each from the Ilha dos Pombos and Santa Cecília sites of the Paraíba do

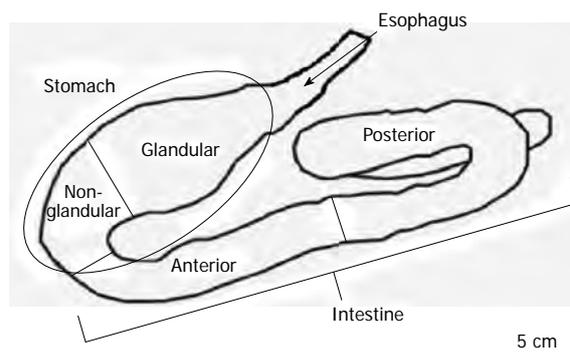


Figure 1 Anatomical diagram of the digestive tract of *Oligosarcus hepsetus*.

Sul River. The fish were dissected in the field, after being anesthetized with benzocaine hydrochloride (50 mg/L) and killed rapidly by hypothermia for immediate removal of the DT. Fragments of the DT were fixed for 8 h in Bouin's fluid and then placed in 70% alcohol. The esophagus, two stomach regions (glandular and non-glandular) and two intestinal regions (anterior and posterior) were obtained from each specimen (Figure 1). These materials were taken to the Histology and Embryology Laboratory of Rio de Janeiro Federal Rural University where they were processed by routine histological techniques, consisting of: dehydration (in a rising ethanol series - 70° GL to 100° GL), diaphanization in xylol and embedding in Histosec-Paraffin, to obtain 5 µm histological sections.

Histological and histochemical analysis

The sections obtained from the DT of *O. hepsetus* were stained with hematoxylin-eosin for analysis of the tissue architecture and with Gomori's trichrome for differential visualization of the connective tissue and collagen fibers.

The histochemical analysis involved periodic acid Schiff (PAS) and Alcian blue (AB) pH 2.5 staining to reveal the neutral and acid glycoconjugates (GCs), respectively. Five slides from each specimen were prepared for each protocol, one from each of the five sectioned regions.

Immunohistochemistry

For the immunohistochemical procedure, 5 µm-thick sections were cut by microtome and mounted on glass slides precoated with 0.1% poly-L-lysine, after being dewaxed and dehydrated by the routine protocol. They were incubated in citrate buffer (pH 6.0-0.01 M) and placed in a microwave oven for 15 min to recover the antigen, then they were incubated with a solution of 3% H₂O₂ in methanol for 15 min to block any endogenous peroxidase. Subsequently, the sections were incubated at room temperature in a humid chamber with a 1:100 µL dilution of bovine serum albumin in phosphate buffered saline (PBS) solution for 30 min. The sections were first incubated overnight at 4 °C with the primary antisera against the individual gastrointestinal hormones (Table 1). The sections were then incubated with biotinylated "Universal" secondary antibody diluted to 1:200 µL for 30 min at room temperature, then with avidin-biotin-peroxidase complex,

Table 1 Details of primary antisera used in this study

| Primary antisera | Donor | Code No. | Working dilution | Source |
|------------------|--------|----------|------------------|------------------------------------|
| Somatostatin | Rabbit | A566 | 1:300 | Dako Corp., CA, United States |
| Serotonin | Rabbit | S5545 | 1:8.000 | Sigma-Aldrich, Inc., United States |
| Cholecystokinin | Rabbit | C2581 | 1:8.000 | Sigma-Aldrich, Inc., United States |
| Gastrin | Rabbit | G0785 | 1:1.000 | Sigma-Aldrich, Inc., United States |
| Glucagon | Mouse | G2654 | 1:2.000 | Sigma-Aldrich, Inc., United States |
| Insulin | Mouse | I2018 | 1:1.000 | Sigma-Aldrich, Inc., United States |

Table 2 Regional distribution and intensity of immunoreaction in endocrine cells in the digestive tract of *Oligosarcus hepsetus*

| Antisera | Segment of the esophagus | Segments of the stomach | | Segments of the gut | |
|-----------------|--------------------------|-------------------------|-----|---------------------|------|
| | | GI | NGI | ANT | POST |
| Somatostatin | +++ | ++ | + | - | - |
| Serotonin | - | +++ | ++ | - | - |
| Cholecystokinin | - | - | - | - | ++ |
| Glucagon | - | - | - | - | - |
| Gastrin | - | - | - | - | - |
| Insulin | - | - | - | - | - |

Intensity of immunoreactions: (-), absent; (+), low; (++) , medium; (+++) , strong. GI: Glandular; NGI: Non-glandular; ANT: Anterior; POST: Posterior.

diluted at 1:200 µL for 30 min at room temperature. Subsequently, the peroxidase label was revealed by reaction with Stable DAB/Plus, prepared according to the kit's instructions. All dilutions and thorough washes between stages were performed using PBS (pH 7.4). The sections were counterstained with Harris hematoxylin, rinsed with deionized water, dehydrated through a series of ethanol and methylcyclohexane solutions and mounted using Entellan. To investigate the specificity of the reactions, negative and positive controls were used. The negative control was prepared by replacement of the primary antibody with non-immune serum and PBS (pH 7.4). Positive controls were produced using tissue sections for each respective antiserum, as indicated in the product data sheet.

Observation and photomicrography

Photomicrographs of all samples from each of the fourteen specimens were obtained with a digital camera Nikon Coolpix 4300 attached to a microscope Olympus BX41. The number of immunoreactive endocrine cells to each antiserum per analyzed segment was recorded and the intensity of immunoreaction was classified: absent (-) or low (+), medium (++) and strong (+++) immunoreactivity (Table 2).

RESULTS

Histological and histochemical study of digestive tract

The following layers were observed in the DT of *O.*

hepsetus: mucosa, submucosa, muscular and adventitia or serosa. The muscularis mucosae is absent in this species.

Esophagus

Histological examination revealed that the mucosa of the esophagus of *O. hepsetus* has many longitudinal folds and is lined with a stratified epithelium with non-keratinized squamous surface cells. The majority of the mucus-secreting cells are interspersed with a smaller number of non-secretory cells (Figure 2A).

The secretory cells reacted positively to PAS and AB staining, indicating the presence of neutral and acid GCs, respectively. The lamina propria is composed of connective tissue and does not have glands. The muscular layer is formed by two sub-layers of striated skeletal muscle, with an internal longitudinal and an external circular layer. Externally, the esophagus is enveloped by an adventitia, composed of connective tissue with some nerve fibers and blood vessels (Figure 2).

Stomach

In the stomach of *O. hepsetus*, the mucosa is lined with a simple epithelium layer composed of columnar mucus-secreting cells with basal nuclei. These were reactive to PAS but not to AB, revealing the presence of only neutral GCs. The stomach epithelium forms crypts along the gastric mucosa. The mucosa layer projects toward the organ's lumen, forming various gastric folds, arranged longitudinally. In the non-glandular region, the submucosa and muscular layers accompany the mucosa in forming these folds, making the lumen very small (Figure 3).

The division of the stomach observed in the present work is in accordance with the structural characteristics of the two regions. The glandular region is characterized by having well-developed tubular gastric glands, composed of oxynticopeptic cells, occupying the entire lamina propria (Figure 3B). These are smaller and less numerous in the initial portion and increase in number and size in the direction of the non-glandular region. The non-glandular region has a well-developed muscular layer (Figure 3D and E). The submucosa layer is composed of connective tissue and blood vessels.

In the stomach, the muscular layer is composed of smooth muscle fibers arranged in two directions: internal circular and external longitudinal (Figure 3E). The glandular region contains myenteric plexuses arranged in sparse groups, composing the enteric nervous system and located between the muscular sub-layers, and there is a serosa layer which surrounds these structures.

Intestine

Just as for the stomach, the adopted division of the intestine follows specific structural patterns in relation to the mucosa layer. The histological analysis of the intestine of *O. hepsetus* revealed that the pattern of folds varies, characterizing two distinct regions: anterior and posterior. The anterior region has numerous thin and elongated folds with villi (Figure 4A). In contrast, in the posterior

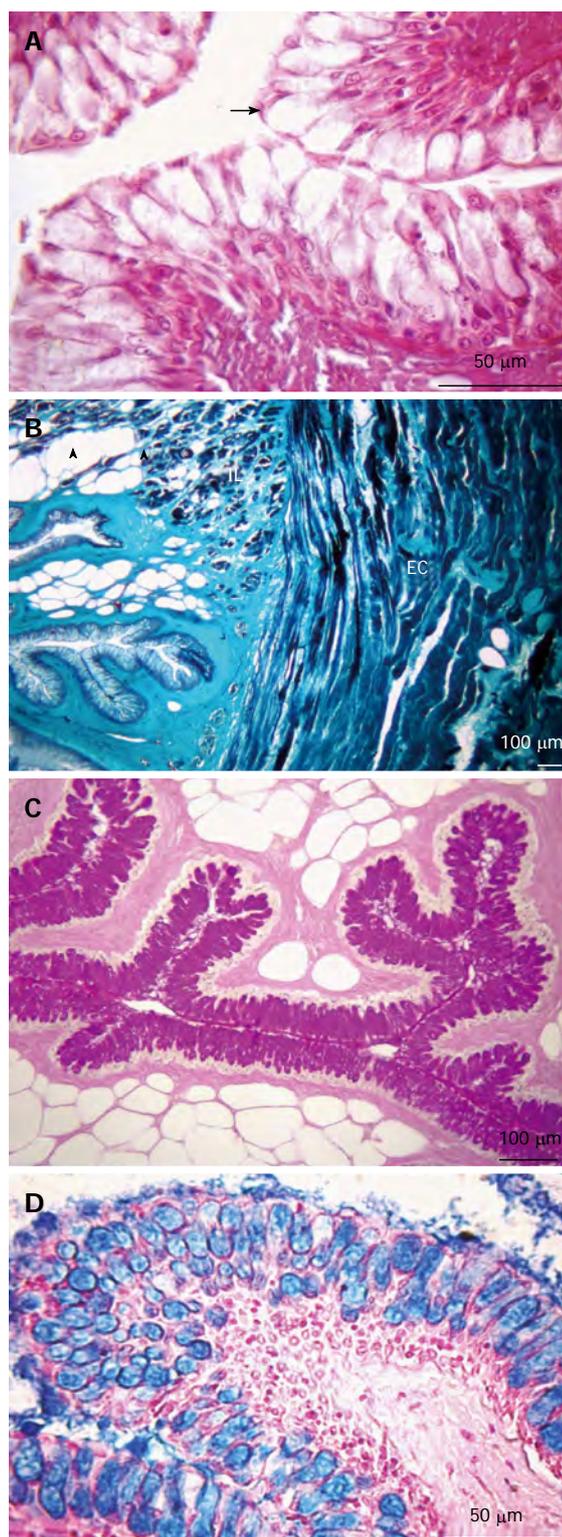


Figure 2 Transversal sections of the esophagus. A: Non-keratinized stratified squamous epithelium (arrow). Hematoxylin and eosin stain; B: Presence of adipose tissue (arrowheads) in the submucosa layer. Muscular layer formed by two sub-layers, internal longitudinal (IL) and external circular (EC). Gomori's trichrome stain; C: Presence of neutral glycoconjugates (GCs). Periodic acid-Schiff stain; D: Acid GCs. Alcian blue stain.

part the folds are less sinuous due to the absence of villi and are thicker, with some being leaf-shaped, and also have a greater number of goblet cells (Figure 4B).

The intestinal mucosa is lined by a simple columnar epithelium with a striated border and goblet cells, which were positive to PAS and AB stainings, with pink (PAS) and blue (AB) coloration (Figure 4), indicating the presence of neutral and acid GCs, respectively.

The limits between the lamina propria and the submucosa layer are not evident, with connective tissue and blood vessels present in both these regions. In both parts of the intestine, the muscular layer has the same organization as in the stomach, with an internal circular layer and an external longitudinal one, observed in the cross-sections, both composed of smooth muscle cells (Figure 4A). The posterior part of the intestine contains a continuous layer of nervous tissue and a myenteric plexus between the muscular sub-layers (Figure 4E). Externally there is a serosa layer.

Immunohistochemical study of digestive tract

SOM-, 5-HT- and CCK-immunoreactive (IR) cells were identified in the DT of *O. hepsetus*, but GAS-, GLUC- and INS-IR cells were not present (Table 2).

Esophagus

Somatostatin immunoreactivity: In the esophagus, SOM-IR cells were detected in the basal layer of the stratified squamous epithelium (Figure 5). Morphologically, these cells were totally colored by chromogen, making it impossible to visualize a nuclear halo. The nucleus of these cells is very small, occupying a tiny area inside them.

Stomach

Serotonin and somatostatin immunoreactivity: Serotonin (5-HT)-IR cells (Figure 6) and SOM-IR cells (Figure 7) were observed in the lining epithelium and glandular epithelium of the stomach. Regarding the morphology of immunoreactive cells, two types were found in this portion, namely closed-type cells and open-type cells.

Intestine

Cholecystokinin immunoreactivity: CCK-IR cells were only observed in the lining epithelium of the posterior part of the intestine of *O. hepsetus* (Figure 8). Closed-type and open-type immunoreactive cells were found.

DISCUSSION

The stratification of the wall of the DT of *O. hepsetus* has the same organization observed in the majority of other teleosts, with some modifications associated with the species' feeding habits. We observed four layers: mucosa, submucosa, muscular and serosa. The muscularis mucosae is not present in the examined areas of the DT, unlike that observed in *Pimelodus maculatus* (*P. maculatus*)^[14] and *Semaprochilodus insignis*^[15]. These authors assumed that the existence of muscular tissue between the lamina propria and submucosa aids in the elimination of the substances produced by the glands.

The very large longitudinal folds of the mucosa layer

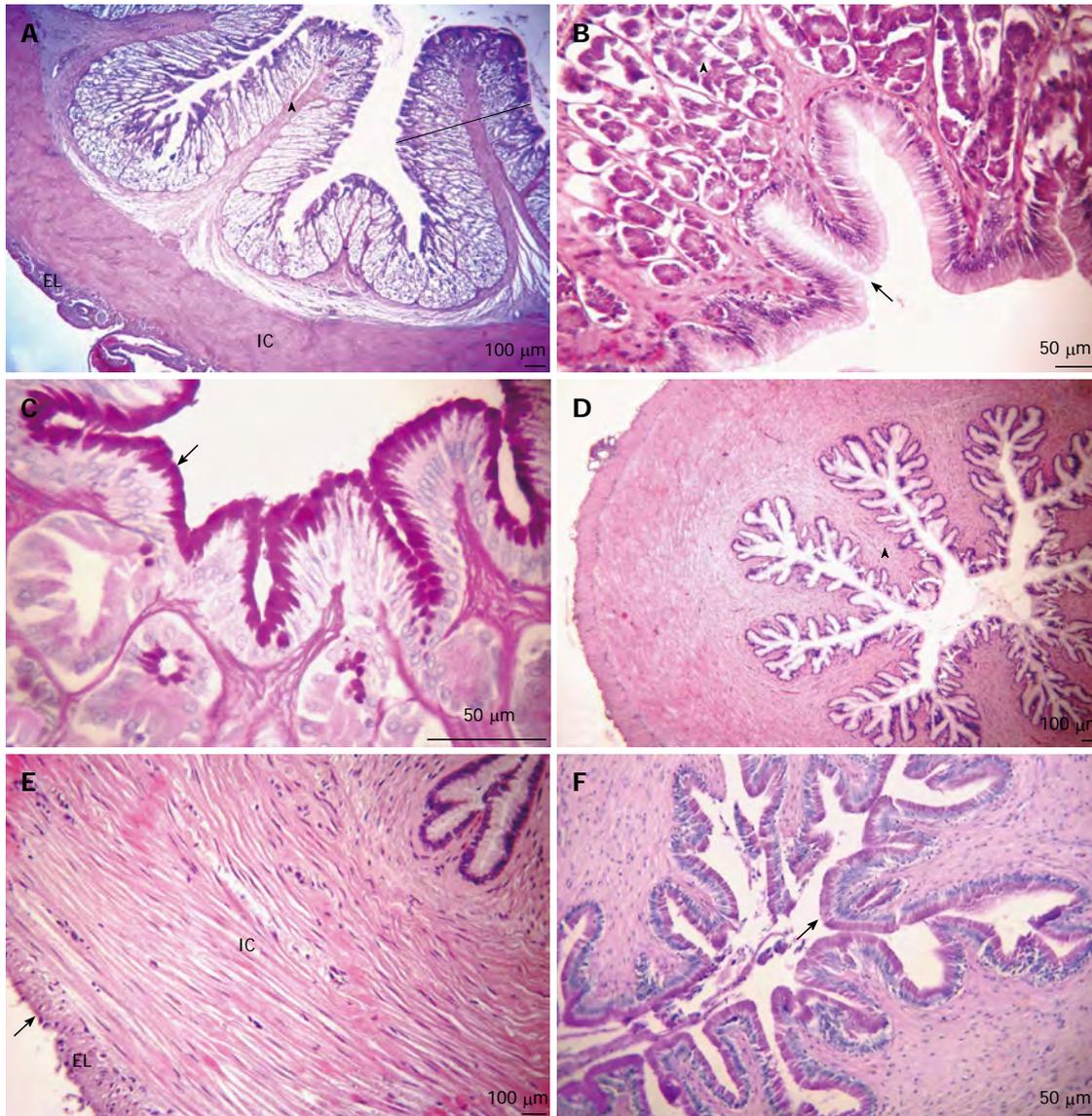


Figure 3 Transversal sections of the stomach. **A:** Mucosa layer with many high gastric folds (arrowhead), thickening of the lamina propria caused by a large number of tubular gastric glands (outline). Organization of the internal circular (IC) and external longitudinal (EL) muscular layers. Hematoxylin and eosin (HE) stain; **B:** The simple columnar epithelium with mucus-secreting cells forming faveola gastrica (arrow) and fundic glands composed of oxynticopeptic cells (arrowhead). HE stain; **C:** Presence of neutral glycoconjugates (GCs) (arrow). Periodic acid Schiff (PAS) stain; **D:** Mucosa layer, together with the sub-mucosa and internal muscular layers, forming large longitudinal folds (arrowhead). HE stain; **E:** The muscular layer of smooth muscle fibers, composed of IC and EL sub-layers and serosa (arrow). HE stain; **F:** Neutral GCs (arrow). PAS stain. (A-C: Glandular region; D-F: Non-glandular region).

of the esophagus of *O. hepsetus* substantially increases the organ's capacity for distension, an effect described by other researchers^[16,17]. The digestive capacity is related to the volume of the folds in the mucosa, with a greater number of folds implying more efficient digestion.

The lining of the mucosa by a stratified squamous epithelium, according to Hunbert *et al.*^[18], acts to protect the fish against mechanical aggression and invasive bacteria. The same pattern was found in *Prionotus carolinus* by Blake^[19], but was not observed in *Salmo trutta* by Burnstock^[20] or in *P. maculatus* by Santos *et al.*^[14]. The submucosa contains bundles of adipose tissue and blood vessels; the same was found in *Dentex dentex* (*D. dentex*)^[21].

The positive reaction of the epithelium to PAS and AB staining revealed the production of neutral and acid

GCs, respectively, in the esophagus. The first type of mucus has low viscosity and is important to assure laminar flow during the lubrication and treatment of particles, to enable digestion to be conducted by the esophagus until the upper region of the stomach. In turn, acid GCs have high viscosity and are fundamental to trap particles^[22]. The presence of these GCs was also reported in *Anguilla anguilla*^[23], *D. dentex*^[21], *P. maculatus*^[14], *Cynoscion guatucupa* (*C. guatucupa*)^[24], *Pelteobagrus fulvidraco* (*P. fulvidraco*)^[25] and *Hyphessobrycon anisitsi* (*H. anisitsi*)^[26].

The transition from the esophagus to the stomach is characterized by an abrupt change in the lining epithelium, which starts to present a single layer of columnar cells secreting mucus. This type of stomach lining epithelium has been observed in the majority of other teleosts as

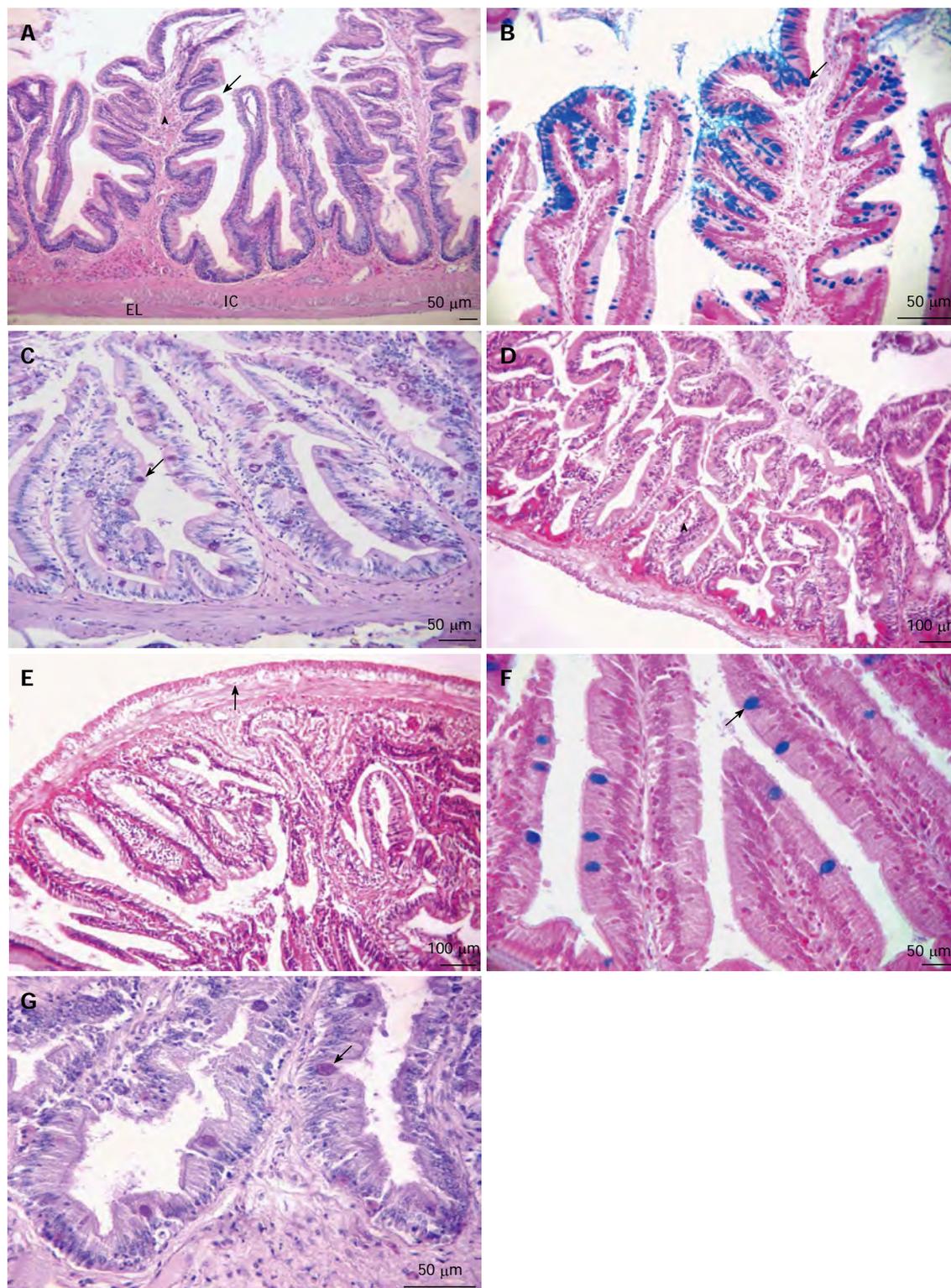


Figure 4 Longitudinal sections of the intestine. A-C: Anterior portion; D-F: Posterior portion; A: Overview of the anterior intestine, showing the arrangement of small folds (arrowhead) presenting villi (arrow). Organization of the internal circular (IC) and external longitudinal (EL) muscular layers. Hematoxylin and eosin (HE) stain; B: Simple columnar epithelium with brush border and goblet cells, indicating the presence of acid glycoconjugates (GCs) (arrow). Alcian blue (AB) stain; C: Neutral GCs (arrows). Periodic acid Schiff (PAS) stain; D: Mucosa layer with thick folds (arrowhead). HE stain; E: Myenteric plexus (arrow). HE stain; F: Simple columnar epithelium with striated border and goblet cells with acid GCs (arrow). AB stain; G: Neutral GCs (arrow). PAS stain.

well^[4,14,21,23,24], but in *Plecostomus plecostomus*^[27] and *Epinephelus marginatus*^[28], the epithelium described at the beginning of the stomach was of the squamous and cubic type, respectively, becoming simple columnar in the posterior regions.

The mucus-secreting cells were only reactive to PAS in the two stomach regions; the same was found in *C. guatuncupa*^[24], *P. fulvidraco*^[25] and *H. anisitsi*^[26], but was not like that observed in *Anguilla anguilla* (*A. anguilla*)^[23] and *Chanos cha-*

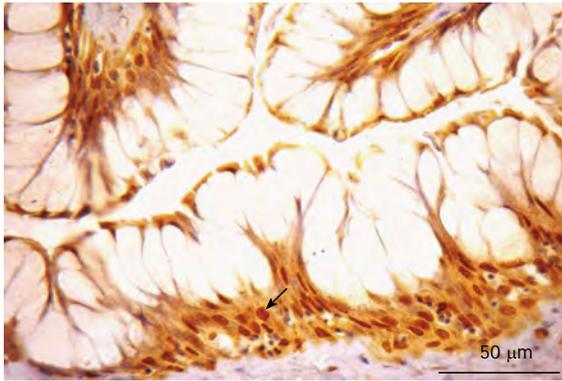


Figure 5 Somatostatin-immunoreactive cells in the esophagus. Somatostatin-immunoreactive cell were detected in the basal layer of the stratified squamous epithelium (arrow).

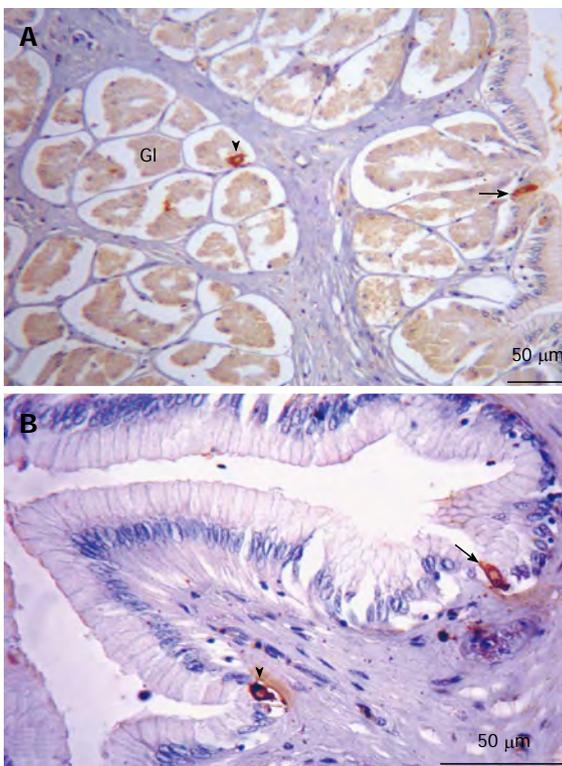


Figure 7 Somatostatin-immunoreactive cells in the stomach. A: Glandular region. Immunoreactive (IR) cells along the entire region, of the open (arrow) and closed (arrowhead) types, present both in the lining epithelium and the glandular epithelium; B: Non-glandular region. Indications of IR cells of the open (arrow) and closed (arrowhead) types. Gl: Glandular.

nos^[29]. As mentioned, the probable function of this mucus is to promote a flow able to carry the food bolus to the intestine. Besides this, since cells producing hydrochloric acid (HCL), essential for digestion, were identified in the stomach region, this mucus also functions as a layer to protect the epithelial cells.

The invaginations formed by the lining epithelium are called gastric crypts, which in the glandular region communicate with well-developed and branched tubular glands, as also observed by Díaz *et al*^[24] in *C. guatucupa* and by Domeneghini *et al*^[23] in *A. anguilla*. These are common

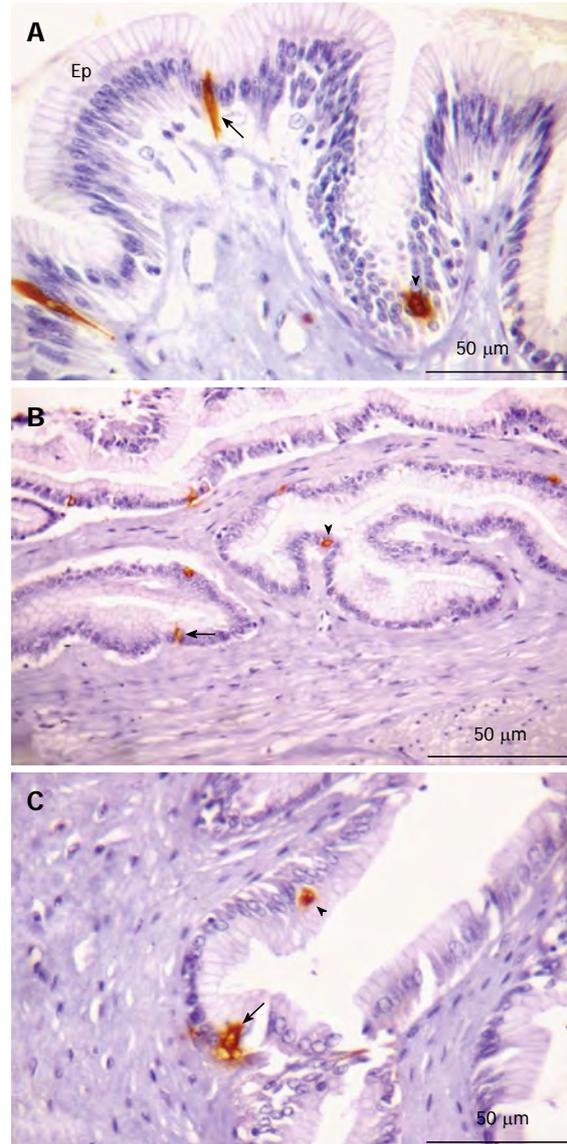


Figure 6 Serotonin-immunoreactive cells in the stomach. A-B: Glandular region; C: Non-glandular region. A: Presence of open (arrow) and closed (arrowhead) type cells interspersed in the epithelium (Ep); B: Highlight of open (arrow) and closed (arrowhead) type immunoreactive (IR) cells; C: Indications of open (arrow) and closed (arrowhead) IR cells.

characteristics of carnivorous fish species^[30]. The gastric glands are composed of oxynticopeptic cells, which play a role similar to that of the principal and parietal cells in mammals, by synthesizing HCL and pepsinogen. In this case, we believe the glandular region has digestive functions while the non-glandular region only acts to carry the food to the gut with the epithelial secretions, with the help of the muscular layer, which in this region is thicker^[14]. As seen in the esophagus, the stomach regions also contain well-developed longitudinal folds, whose function is to allow expansion of the organ's diameter to store a large volume of food^[16,17], another common characteristic of carnivorous fish species^[30].

The intestine of *O. hepsetus* has two distinct parts, the same as in *Tilapia spp.*^[31]. The anterior part is characterized by a larger number of thin and elongated longitudinal folds,

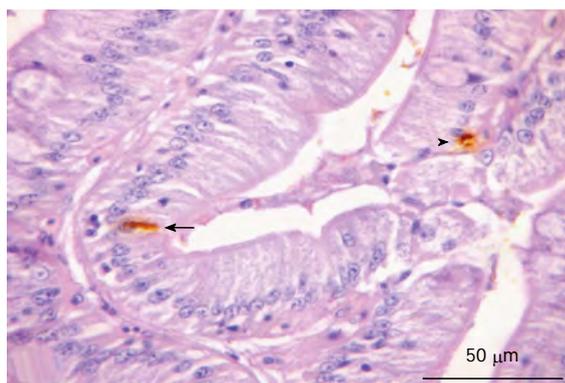


Figure 8 Cholecystikinin-immunoreactive cells in the posterior region of the intestine. Epithelial marking of open (arrow) and closed (arrowhead) immunoreactive cells.

which branch out to form villi, making the organ's lumen very small. The posterior region, endowed with thicker and less sinuous longitudinal folds, contains a larger number of goblet cells, similar to the pattern observed in the large intestine of mammals. This pattern of ample folds of the mucosa is common in carnivores, acting to expand the surface absorption area, since carnivores have a relatively short intestine compared to animals with other feeding habits^[30]. Sections of the lining epithelium of both regions, when submitted to the PAS and AB histochemical protocols, reacted positively, showing cells with pink (PAS) and blue (AB) coloring, indicating the presence of neutral and acid GCs, respectively, as also observed by Cinar *et al.*^[32] in *Pseudophoxinus antalyae*, by Carrasón *et al.*^[21] in *D. dentex* and by Leknes^[26] in *H. anisitsi*, but unlike that observed in *P. fulvidraco*^[25] and *Chanos chanos*^[29].

This allows the hindgut to lubricate the tube and to trap particles to be eliminated, permitting the food bolus to reach this region in dehydrated form^[33].

The organization of the muscular layer along the entire DT of *O. hepsetus* is the same as observed in *P. maculatus*^[14] and *P. fulvidraco*^[25], except in the esophagus, where the pattern resembled that found in mammals. The function of this layer is to promote motility in the DT, carrying and mixing food with the digestive secretions. This motility and also the release of these secretions are favored by the existence of a myenteric plexus between the muscular tissue sub-layers, observed in the glandular region of the stomach as well as in the posterior intestine. Unlike in mammals, the myenteric plexus does not have the form of ganglia, but rather appears in sparse form or in continuous layers, as seen in *Pimelodus maculatus*^[34], but unlike that observed in *Salmo trutta*^[20] and *P. fulvidraco*^[25].

The results obtained in this study demonstrate that the DT of *O. hepsetus* has three types (5-HT-, SOM- and CCK) of endocrine cells, but three other types (GAS-, GLUC- and INS-IR cells) were not present. However, these cells have been observed in other fish, such as: GLU-IR cells in the gastric mucosa of cartilaginous fishes^[35]; GAS-IR cells in the stomach pyloric region of *Oncorhynchus mykiss*; and INS-IR cells in the stomach py-

loric region of *Monopterus albus* (*M. albus*) and the stomach cardiac and pyloric regions of *Pelteobagrus fulvidraco*^[36]. The reason for the absence of these endocrine cells in the DT of *O. hepsetus* may be related to its digestive histophysiology, but further studies should be conducted to confirm this relationship.

The peptide SOM is a component responsible for inhibiting many substances, such as GAS, CCK, GLUC, INS, secretin, motilin and gastric acid^[37]. In mammals, it also controls the absorption of amino acids and glucose^[38]. It is thus essential in the DT, since it participates in basic mechanisms for efficient food processing. Ku *et al.*^[39] identified the production of this hormone along the DT of the reptile *Trachemys scripta elegans*, including in the esophagus, where we found SOM-IR cells in *O. hepsetus*. Other studies investigating the presence of this hormone in the DT have been performed by Lee *et al.*^[40] and Pan *et al.*^[36], the latter analyzing the presence of endocrine cells in eight fish species: *P. fulvidraco*, *M. albus*, *Siniperca chuatsi* (*S. chuatsi*), *Colossoma brachypomum* and *Tilapia nilotica*, all of which presented SOM-IR cells in the gastric mucosa, as observed in *O. hepsetus*. Although we did not visualize this hormone in the intestinal parts of the thin dogfish, it was reported in the gut of the spiny dogfish *Squalus acanthias*^[41], *Oncorhynchus mykiss*^[42], *P. fulvidraco*, *M. albus*, *S. chuatsi*^[36], *Zacco platypus*^[43] and *Coreoperca herzi* (*C. herzi*)^[44].

5-HT-IR cells have been detected in the DT of various vertebrates: fish^[44], amphibians^[45], reptiles^[46], birds^[47] and mammals^[48,49]. Researchers state that all vertebrates have this type of endocrine cell in the DT, assuming that these cells' location is based on the evolution of these higher life forms^[50]. It is known that in fish, serotonin promotes gastrointestinal motility^[51] and blood flow, in the latter case by triggering vasoconstriction^[52,53]. In *O. hepsetus*, 5-HT-IR cells were observed only in the regions of the stomach, both in the lining epithelium and the glandular epithelium, as also observed in *C. herzi* by Lee *et al.*^[44].

As was described for the fish species *Oncorhynchus mykiss*^[54], *Salmo trutta*^[55], *Odontesthes bonariensis*^[56] and *Rhamdia quelen*^[57], in *O. hepsetus* we observed CCK-IR cells in the intestine; in this case only in the posterior part, while in *O. bonariensis*^[56] these cells were observed throughout the gut, but with greater concentration in the hindgut. There were no CCK-IR cells in the other regions of the DT of *O. hepsetus*. This hormone controls intestinal motility, by stimulating the release of pancreatic juice and inhibiting gastric emptying^[58,59]. The existence of these cells has been verified in fish^[35,42], reptiles^[39], birds^[60] and mammals^[61].

In conclusion, our histological and histochemical study of the DT of *O. hepsetus* revealed adaptation for the species' feeding habits, to protect the tract and increase the absorptive processes. The immunohistochemical study showed that the DT of this fish species contains different types of endocrine cells similar to those found in other vertebrate species. This study will help comprehension of the digestive physiology of this species and provide a basis for diagnosing diseases that affect the digestive tract of carnivorous teleosts.

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COMMENTS

Background

Studies indicate the importance of knowledge of the morphology of the digestive tract (DT) because of the different physiological conditions in which animals live and their varied eating habits, which are manifested by adaptations and modifications in the digestive system. Information about the morphological aspects of the DT provides support for research on physiology and nutrition, aiming to improve the diet and management of livestock and support activities for restocking and restoration of natural ecosystems, besides being important for various research areas, including biological systems and conservation.

Research frontiers

The integration of the motor, secretory and absorptive phenomena of the DT is fundamental for the reduction of food into simpler particles that can be absorbed. This association is achieved by actions and interactions of the nervous and endocrine systems, which in the digestive system are represented by the endocrine cells. The diffuse neuroendocrine system (DNS) acts to control the motility and transit speed of the ingesta, the various secretions of the DT, the absorption of nutrients and the blood flow, to assure the activation and action of enzymes at the proper moment. Therefore, the products of the digestive system can be absorbed by the organism and reach the blood and lymphatic circulation systems.

Innovations and breakthroughs

The avidin-biotin-peroxidase complex method was applied to study the endocrine cells in the DT of *Oligosarcus hepsetus* (*O. hepsetus*). This method involves the use of three reagents: the primary antibody, which binds to the receptor of the specific hormone of interest; the secondary antibody, which is produced linked to a molecule of the vitamin biotin (C) and binds to receptors of the primary antibody; and the glycoprotein-avidin complex, produced from biotin and peroxidase, with joins with the previous reagent, the secondary antibody.

Applications

The immunohistochemical study showed that the DT of *O. hepsetus* contains different types of endocrine cells similar to those found in other vertebrate species. This study will help comprehension of the digestive physiology of this species and provide a basis for diagnosing diseases that affect the digestive tube of carnivorous teleosts.

Terminology

The gastrointestinal epithelium is permeated by a set of cells, originating from the DNS, called endocrine cells. The secretions of these cells control the digestion of food to ensure it is efficient, by regulating the digestive processes. Besides controlling the absorption of nutrients, they play an important role in determining secretions from the gut and associated glands and in regulating the intestinal blood flow.

Peer review

In this study, the authors describe the microscopic anatomy of the DT of a carnivorous fish species and analyze the functional components that aid the digestion of food. The results are relevant by enabling comparison with other fish species, contributing to phylogenetic studies.

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Decreased prevalence of celiac disease among Brazilian elderly

Lucas Malta Almeida, Luiz Claudio Castro, Rosa Harumi Uenishi, Fernanda Coutinho de Almeida, Patricia Maria Fritsch, Lenora Gandolfi, Riccardo Pratesi, Yanna Karla de Medeiros Nóbrega

Lucas Malta Almeida, Fernanda Coutinho de Almeida, Patricia Maria Fritsch, Graduate Program in Medical Sciences, University of Brasilia School of Medicine, Brasilia DF 70910900, Brazil
Lucas Malta Almeida, Fernanda Coutinho de Almeida, Patricia Maria Fritsch, Doctoral fellow of Coordenação de Aperfeiçoamento de Pessoal de Nível Superior, Brasilia DF 70910900, Brazil
Luiz Claudio Castro, Rosa Harumi Uenishi, Lenora Gandolfi, Riccardo Pratesi, Graduate Program in Health Sciences, University of Brasilia School of Health Sciences, Brasilia DF 70910900, Brazil
Luiz Claudio Castro, Rosa Harumi Uenishi, Lenora Gandolfi, Riccardo Pratesi, Research Center for Celiac disease, University of Brasilia School of Medicine, Brasilia DF 70910900, Brazil
Yanna Karla de Medeiros Nóbrega, Department of Pharmaceutical Sciences, University of Brasilia School of Health Sciences, Brasilia DF 70910900, Brazil
Yanna Karla de Medeiros Nóbrega, Research Center for Celiac Disease, University of Brasilia School of Medicine, Brasilia DF 70910900, Brazil

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Correspondence to: Yanna Karla de Medeiros Nóbrega, PhD, Professor, Department of Pharmaceutical Sciences, University of Brasilia School of Health Sciences, SQN 314 Bloco E Apt 501 Asa Norte, Brasilia DF 70910900, Brazil. yannanobrega@gmail.com

Telephone: +55-61-31071991 Fax: +55-61-31071991

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compare it with the previously known prevalence in a pediatric group living in the same geographical area.

METHODS: The research protocol was approved by the Ethics Committee of the University of Brasilia School of Medicine, Brasilia, Brazil. Blood samples from 946 individuals (295 male and 651 female) aged 60 years or older were collected between May 2010 and July 2011. The study subjects' mean and median ages were 68.1 and 67 years, respectively, ranging from 60 to 92 years. That age distribution closely corresponded to the age distribution of the Brazilian population according to the Brazilian 2010 census. The participants were consecutive and unselected outpatients undergoing blood tests at the University of Brasilia Hospital's Clinical Pathology Laboratory. All sera were tested for immunoglobulin A anti-transglutaminase antibodies (IgA-tTG) by enzyme-linked immunosorbent assay, and those that were positive were further tested for immunoglobulin A anti-endomysium antibodies (IgA-EMA). Human leukocyte antigen (HLA) genotyping was performed for all individuals who exhibited positive serologic results for IgA-tTG and/or IgA-EMA.

RESULTS: Out of the 946 studied patients, only one previously diagnosed case of biopsy-proven celiac disease was detected. For the remaining subjects, nine serum samples tested positive for IgA-tTG antibodies; however, none of them tested positive for IgA-EMA antibodies. The HLA genotyping of those nine subjects revealed that one was carrying DQA1*0501 and two were carrying DQB1*0201 alleles. These data showed that, among those 946 elderly individuals, the prevalence of celiac disease (CD) was 0.1% (95%CI: 0.00-0.59). The prevalence of CD for the elderly group was compared with that observed for the group of 2034 children younger than 15 years (age range, 1-14 years; mean age, 8 years) who took part in our previous CD prevalence screening study. All the children came from the same geographical region and shared a similar ethnic and low-income background. As in the elderly group in

Abstract

AIM: To evaluate the prevalence of celiac disease in a group of Brazilian individuals over 60 years of age and

the current study, the younger group was made up of consecutive outpatients who underwent blood evaluation at the University of Brasilia Hospital's Clinical Laboratory. The prevalence of biopsy-proven CD among those children was 0.54% (95%CI: 0.27-0.57). The comparative analysis between the two groups resulted in the following values: odds ratio = 0.19 (95%CI: 0.01-1.45) Fisher test $P = 0.06$.

CONCLUSION: The prevalence of CD among the children of our previous study was 5.4 times higher than that found in the present elderly group.

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Key words: Celiac disease; Gluten-sensitive enteropathy; Epidemiology; Elderly; Mortality

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INTRODUCTION

Celiac disease (CD) is a chronic autoimmune-mediated disease with both intestinal and systemic manifestations that are induced by the ingestion of gluten in genetically predisposed individuals. CD-related intestinal abnormalities are mainly characterized by villous atrophy, crypt hyperplasia, and lymphocyte infiltration of the small mucosa caused by T-cell responses to the enzyme transglutaminase 2^[1] and gluten-derived gliadin peptides^[2]. CD is a lifelong disease that can start at any age. As it involves multiple organs or systems, it may manifest in a wide range of clinical pictures. The only effective therapy for CD is strict dietary abstinence from gluten-containing foods.

During the last few decades, the advent of reliable serologic tests has greatly facilitated in the diagnosis of CD, allowing large-scale screening studies to be performed. Worldwide prevalence rates, determined by a similar sequential testing paradigm [*i.e.*, immunoglobulin A anti-transglutaminase (IgA-tTG) antibodies and/or anti-endomysium antibodies (IgA-EMA) tests] averaged 1:160 in the Western world^[3]. Recent epidemiological data showed that CD is also a common disease in developing countries (Middle East, South Asia, Africa, and South America), with a prevalence similar to that in Western countries^[4]. The prevalence of CD in Brazil has shown significant variation, probably due to the different degrees of miscegenation of the ethnic groups that make up the Brazilian population, especially Amerindians, Afro-descendants, and Europeans. Screening studies performed during the last decade in distinct Brazilian regions showed prevalence rates varying from 1:214 to 1:681 in presumably healthy blood donors^[5-8], and from 1:119 to 1:417 in the

general population^[9,10]. The geographical variation in the prevalence rates may also be due to differences in genetic background, age-related differences of exposure to gluten, and/or to changes in environmental risk factors. Due to the widely variable pattern of its clinical spectrum, the confirmatory diagnosis of CD can be delayed, after being left unrecognized for many years^[11,12].

Traditionally, CD has been regarded as a disease of childhood and early adulthood that rarely develops in older people. Nevertheless, during the last few decades, diagnosis of CD among adults and the elderly has increased, including patients 70 years of age and older. CD is considered a lifelong disease and consequently a progressive increase in its prevalence would be expected in older age groups.

Nevertheless, studies focusing on this topic are rather controversial. While some of the studies pointed to a high prevalence of CD in older age groups^[13,14], others showed a higher prevalence in children and adolescents^[10,15,16].

In a previous study, we found an increased prevalence of CD in children compared with adults and elderly individuals^[10]. Following the same line of research, in this study we aimed to determine the prevalence of CD in elderly Brazilians over 60 years of age, all of them living in the same geographic region and belonging to similar socioeconomic and cultural backgrounds as the children evaluated in our previous study.

MATERIALS AND METHODS

The research protocol was approved by the Ethics Committee of the University of Brasilia School of Medicine. All individuals included in the protocol were informed about the objectives, related risks, and benefits of this study, and agreed with the use of the collected specimens for research. Between May 2010 and July 2011, a total of 946 outpatients (295 male and 651 female) aged 60 years or older had a blood sample collected and stored at -20 °C until their use. The subjects' mean and median ages were 68.1 and 67 years, respectively, ranging from 60 to 92 years. That age distribution closely corresponded to the age distribution of the Brazilian population according to the 2010 census^[17].

The participants were consecutive and unselected outpatients undergoing blood tests at the Clinical Pathology Laboratory of the University of Brasilia Hospital. The most frequent reasons for blood testing were routine health check-up, suspected or recurrent infections, chronic ailments, metabolic disorders, and pre-operative check-up. Patients from the gastroenterology outpatient clinic were excluded to avoid a selection bias. No other exclusion criteria were applied, regardless of the possible existence of symptoms or conditions commonly associated with CD.

The University of Brasilia Hospital is a public reference hospital that predominately serves a low-income population from the city of Brasilia and the surrounding area in the midwest region of Brazil. Such individuals usually depend on the Governmental National Health

System. They exhibit mixed ancestry, with a considerable contribution of European intermixed with variable parcels of other races, mainly Afro-descendants and Amerindians.

The serum samples from the patients were tested for IgA-human anti-tissue-transglutaminase-IgA (htTG) antibodies using an IgA-htTG enzyme-linked immunosorbent assay Kit (QUANTA Lite® h-tTG IgA Inova Diagnostic, Inc. San Diego, CA, United States). The limit of positivity was set at 20 arbitrary units, in accordance with the manufacturer's instructions. As a second step, all IgA-htTG positive samples were further tested for the presence of IgA-EMA antibodies using indirect immunofluorescence on primate distal esophagus cryostatic sections (Inova Diagnostic, Inc. San Diego, CA, United States).

All individuals who exhibited positive serologic results for IgA-tTG and/or IgA-EMA antibodies underwent HLA genotyping. Genomic DNA was extracted from peripheral venous blood samples using the Illustra™ blood genomicPrep. Mini Spin Kit (GE Healthcare, Buckinghamshire, United Kingdom). HLA-DQA1*0501 (DQ2 α chain), HLA-DQB1*02 (DQ2 β chain), HLA-DQA1*0301 (DQ8 α chain), and DQB1*0302 (DQ8 β chain) genotyping was performed by polymerase chain reaction amplification using sequence-specific primers (PCR-SSP). For internal positive amplification control, each PCR reaction included a primer pair for a conserved region of the *DRB1* gene. The amplified products were separated using 2% agarose gel, stained with ethidium bromide and then visualized under an ultraviolet transilluminator.

RESULTS

Out of the 946 subjects, only a single previously diagnosed case of biopsy-proven CD in a 66-year-old woman was detected. Among the remaining subjects, nine serum samples tested positive for IgA-tTG antibodies. None of the patients tested positive for IgA-EMA antibodies. HLA genotyping disclosed the presence of one CD predisposing allele in three of the IgA-tTG positive elderly. The clinical and laboratory data of the nine patients who tested positive for IgA-tTG antibodies are depicted in Table 1. These data showed that among those 946 elderly individuals, the prevalence of CD ($n = 1$) was 0.1% (95%CI: 0.00-0.59).

The prevalence of CD for the elderly group was compared with that observed for the group of 2034 children younger than 15 years (age range, 1-14 years; mean age, 8 years) who took part in our previous CD prevalence screening study^[10]. All the children came from the same geographical region and presented a similar ethnic and low-income background. As in the elderly group in the current study, the younger group was made up of consecutive outpatients who underwent blood evaluation at the University of Brasilia Hospital's Clinical Laboratory. The prevalence of biopsy-proven CD ($n = 11$) among

those 2034 children was 0.54% (95%CI: 0.27-0.57).

DISCUSSION

Out of the 946 elderly individuals tested in this study, only a single case of previously-detected CD was found. Although nine individuals showed moderately increased levels of anti-tTG antibodies ranging from 30.6 to 52.3, no subjects tested positive for IgA-EMA antibodies. Although IgA-tTG is an effective screening test for CD, occasional anti-tTG false positive results cannot be excluded, especially in the presence of other autoimmune diseases^[18,19]. The clinical effectiveness of the IgA-tTG test is improved if its positive results are confirmed with the IgA-EMA test^[20] and by the presence of predisposing alleles on HLA PCR-SSP typing. Typing for HLA-DQ2 and HLA-DQ8 is a useful tool for either excluding CD or making its diagnosis unlikely in the case of a negative test result for both markers^[21,22]. Predisposing HLA alleles were present in only three of the subjects who tested positive for IgA-tTG antibodies. A jejunal biopsy was suggested and refused by both patients carrying the higher degree of risk allele DQB1*0201, although they agreed in being followed with periodical clinical evaluations and serological testing.

The results of this current screening are in agreement with the result obtained in our previous study, in which an unanticipated variation in the prevalence of CD was found^[10]. In this study performed in the same geographical area with a similar population group, most cases of CD were clustered in the younger age group, with the prevalence of CD in children aged 1 to 14 years being 2.6 times higher than the one found for adults and elderly individuals (5.44 *vs* 2.11 per 1000, respectively). This variation in the prevalence of CD between different age groups was actually unexpected, considering that intestinal sensitivity to gluten is a permanent condition. Aside from gluten ingestion, CD might be triggered at any stage of life by additional environmental factors that remain largely unknown. Thus, a progressive increase in prevalence rates towards advanced ages or, at least, a similar rate throughout life would be expected. Recent studies in Finland by Vilppula *et al*^[13,14] showed an increase in the prevalence of CD among individuals over 52 years of age compared with the general prevalence in Finnish children (2.13% *vs* 1%). Furthermore, the authors also demonstrated an increasing prevalence throughout a three-year period for the same group, going from 2.13% to 2.34%, and resulting in an annual CD incidence of 0.08% in that population. However, several other studies report contradictory results by showing a higher prevalence of CD in younger populations^[15,16,23,24].

Several hypotheses have been suggested to explain this discordance in the prevalence rates among different age groups, although none have been definitely proven. One hypothesis offered to justify a higher prevalence in the younger age group is that the incidence of CD, similarly to other autoimmune diseases, is progressively

Table 1 Clinical and laboratory data of patients who tested positive for immunoglobulin A anti-transglutaminase antibodies by enzyme-linked immunosorbent assay

| Patient | Sex | Age (yr) | tTG | EMA | HLA | Symptomatology and associated disorders |
|---------|-----|----------|------|-----|-----------|---|
| 1 | M | 63 | 39.9 | Neg | Negative | Anemia, arthritis |
| 2 | M | 71 | 34.5 | Neg | DQB1*0201 | Hyperthyroidism |
| 3 | M | 81 | 31.3 | Neg | Negative | No complaints |
| 4 | F | 60 | 42.9 | Neg | DQB1*0201 | No complaints |
| 5 | F | 60 | 30.6 | Neg | Negative | Osteopenia, arthritis, recurrent abdominal pain, flatulence |
| 6 | F | 63 | 52.3 | Neg | Negative | Arthritis, osteoporosis, hyperthyroidism |
| 7 | F | 65 | 34.0 | Neg | Negative | No complaints |
| 8 | F | 68 | 45.3 | Neg | DQA1*0501 | Osteoporosis, weight loss, flatulence |
| 9 | F | 72 | 45.2 | Neg | Negative | Osteoporosis |

M: Male; F: Female; Neg: Negative; tTG: Transglutaminase; EMA: Endomysium; HLA: Human leukocyte antigen.

increasing worldwide. CD was considered a rare disease until the late 1970s, and its prevalence was estimated to be as low as 0.03%^[25]. With the advent of highly sensitive and specific serological tests, a dramatic rise in its prevalence was observed during the following decades. However, this increase would not be solely due to better diagnostic methods that enabled extensive screening studies and diagnosis of atypical forms of the disease; consistent with other autoimmune disorders that have shown rising incidence rates over the last few decades^[26], CD would also have shown a significant increase in its prevalence, consequently justifying the increased number of cases found among younger age groups^[27,28]. Although the causes underlying this increased age-related prevalence remain unknown, likely explanations include environmental influences (such as changes in quantity and quality of cereal processing), changed patterns of early childhood exposure to infectious agents that impair the natural development of the immune system (hygiene hypothesis)^[29], and changes in infant dietetic habits^[30].

Another possible cause for an increased CD prevalence in the younger population was suggested by Mariné *et al*^[15], who proposed that a significant number of CD cases that appear during childhood progress to a latent form or into total gluten tolerance. Those patients would therefore exhibit negative serologic results as they got older. Several other studies support this hypothesis^[31-33], although the number of described spontaneous recoveries of normal villous architecture in CD patients on a gluten-containing diet is generally small^[34] and it is uncertain as to whether these clinically silent periods accompanied by negative serologic tests can be considered only temporary remissions or actual definitive recoveries. Extended follow-up is therefore required for these patients^[32,35].

A third explanation is the postulated existence of an increased mortality rate among CD patients. Publications addressing this issue are numerous, but conflicting^[28,36,37]. The reported overall mortality rates among CD patients vary from 1.26%^[29] to 3.9%^[19] in studies focusing on undiagnosed CD in adults. Despite the differing results, at least two publications point to undiagnosed CD as a major cause of increased morbidity and mortality among individuals with the disease^[28,38].

These three hypotheses are not mutually exclusive, and it is both possible and probable that each of these factors contribute, to a greater or lesser degree, to the observed variation in the prevalence of CD in elderly individuals, depending on the different environmental conditions found in distinct regions or countries.

In Brasilia, many of the low-income adult population came from poor regions of the country, where they have had little or no access to medical care during childhood, and awareness of CD was somehow deficient among healthcare professionals. In these regions, CD knowledge several decades ago was not much different from that before effective treatment for CD was established. Even with the current facilities for the proper diagnosis and compliance of CD patients, diet behaviors among individuals of economically disadvantaged backgrounds in Brazil is still far from ideal.

In Finland, the higher prevalence of CD in patients aged over 52 years and the lower CD mortality rate^[13,14] are noteworthy. Comparisons between Finland and Brazil are contentious at best, since they occupy different positions in the World Health Organization's health system ranking of countries^[39] (31st and 125th, respectively). If this same survey was again performed in Brasilia, but instead focused on a higher socioeconomic group with a higher quality of life, the outcomes would probably have been different.

Our study has potential limitations that should be noted. The screening of the elderly group was conducted in a single geographic setting and the participants were unselected outpatients undergoing routine blood tests, although both groups, children and elderly, were similar with regard to their ethnicity and socioeconomic level. In addition, the number of elderly screened was insufficient to reach statistical significance ($P = 0.06$). In spite of these drawbacks, this study supports our previous findings of an age-related variation in the prevalence rate of CD. Only a single biopsy-proven previously diagnosed case of CD was identified among the elderly group, showing that the prevalence of CD among the children of our previous study was 5.4 times higher than that found in the present elderly group. These findings reinforce that, for the low socioeconomic populations

of our region, prevalence of CD is unexpectedly higher in children compared to older individuals, and that this discrepancy increases towards older ages. We hypothesize that among the plausible explanations for the discrepancy between the CD prevalence rates among children and elderly, the most likely culprit would be an increased mortality rate among undiagnosed celiac disease patients.

COMMENTS

Background

Epidemiological studies during the last few decades have shown an increasing prevalence of celiac disease over time, not only in Europe and in people of European ancestry, but also in developing countries. Socioeconomically disadvantaged population groups in developing countries additionally suffer from a low level of awareness, clinical suspicion of this disorder among physicians, and difficult access by the patient to diagnostic methods, which result in unrecognized or delayed diagnosis of celiac disease (CD). Additionally, patients in whom an appropriate diagnosis is reached have their treatment hampered by an impossibility to use commercial gluten-free products, which are too expensive for these populations, as well as the lack of patients' awareness and information regarding their diet gluten content.

Research frontiers

Overcoming the difficulties of treating CD in the context of developing countries implies the introduction of CD diagnostic laboratory tests as routine in the State Health System, funding research of cheap sources of gluten free foods, and an increased recognition of CD and of its symptoms.

Applications

The results obtained in this study may provide support for future epidemiological studies to map the onset of CD in different age groups in at-risk populations in developing countries. It can additionally contribute to the adoption of public health policies that will allow the socioeconomically disadvantaged access to health services for medical consultations, laboratory tests and, after diagnosis, financial support for a lifelong gluten-free diet.

Peer review

In this interesting survey, the authors report their results on the prevalence of celiac disease in a typical Brazilian population. The manuscript is very well written. The abstract is appropriate in length and content. The results are reported clearly. The discussion is exhaustive and provides an interesting view of this controversial topic.

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Conventional endoscopic retrograde cholangiopancreatography vs the Olympus V-scope system

Martin Raithel, Andreas Nägel, Jürgen Maiss, Dane Wildner, Alexander Fritzkarl Hagel, Sandra Braun, Hiwot Diebel, Eckhart Georg Hahn

Martin Raithel, Andreas Nägel, Jürgen Maiss, Dane Wildner, Alexander Fritzkarl Hagel, Sandra Braun, Hiwot Diebel, Eckhart Georg Hahn, Department of Medicine 1, Gastroenterology, Endoscopy, University Erlangen-Nuremberg, 91054 Erlangen, Germany

Author contributions: Raithel M, Hagel AF and Hahn EG designed the study; Raithel M, Nägel A, Maiss J, Wildner D, Braun S and Diebel H performed the examinations; Hagel AF, Wildner D and Nägel A analysed the data; Raithel M, Maiss J and Hahn EG wrote the paper.

Correspondence to: Martin Raithel, MD, Professor of Medicine, Department of Medicine 1, Gastroenterology, Functional Tissue Diagnostics, University Erlangen-Nuremberg, Ulmenweg 18, 91054 Erlangen, Germany. martin.raithel@uk-erlangen.de
Telephone: +49-9131-8535151 Fax: +49-9131-8535152

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Abstract

AIM: To compare the new Olympus V-scope (VS) to conventional endoscopic retrograde cholangiopancreatography (ERCP).

METHODS: Forty-nine patients with previous endoscopic papillotomy who were admitted for interventional ERCP for one of several reasons were included in this single-centre, prospective randomized study. Consecutive patients were randomized to either the VS group or to the conventional ERCP group. ERCP-naïve patients who had not undergone papillotomy were excluded. The main study parameters were interventional examination time, X-ray time and dose, and premedication dose (all given below as the median, range) and were investigated in addition to each patient's clinical outcome and complications. Subjective scores to assess each procedure were also provided by the physicians and endoscopy assistants who carried out the procedures. A statistical analysis was carried out using the Wilcoxon rank-sum test.

RESULTS: Twenty-five patients with 50 interventions were examined with the VS ERCP technique, and 24 patients with 47 interventions were examined using the conventional ERCP technique. There were no significant differences between the two groups regarding the age, sex, indications, degree of ERCP difficulty, or interventions performed. The main study parameters in the VS group showed a nonsignificant trend towards a shorter interventional examination time (29 min, 5-50 min vs 31 min, 7-90 min, $P = 0.28$), shorter X-ray time (5.8 min, 0.6-14.1 min vs 6.1 min, 1.6-18.8 min, $P = 0.48$), and lower X-ray dose (1351 cGy/m², 159-5039 cGy/m² vs 1296 cGy/m², 202.2-6421 cGy/m², $P = 0.34$). A nonsignificant trend towards fewer adverse events occurred in the VS group as compared with the conventional ERCP group (cholangitis: 12% vs 16%, $P = 0.12$; pain: 4% vs 12.5%, $P = 0.33$; post-ERCP pancreatitis: 4% vs 12.5%, $P = 0.14$). In addition, there were no statistically significant differences in assessment by the physicians and endoscopy assistants using subjective questionnaires.

CONCLUSION: ERCP using the short-guidewire V-system did not significantly improve ERCP performance or patient outcomes, but it may reduce and simplify the ERCP procedure in difficult settings.

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Key words: Endoscopic retrograde cholangiopancreatography; Short guidewire endoscopic retrograde cholangiopancreatography system; X-ray protection; V-scope; Bile duct stenosis

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INTRODUCTION

Endoscopic retrograde cholangiopancreatography (ERCP) is a complex diagnostic and therapeutic approach that is used to identify and treat various hepatobiliary and pancreatic diseases. ERCP is a time-consuming, expensive, and laborious method that requires the patient to be exposed to substantial doses of premedication, contrast medium, and X-rays, especially when difficult cannulation of the papilla, biliary, or pancreatic ducts or strictures occurs and/or difficult interventions are performed^[1-5]. Thus, future advances to simplify the technical process of ERCP, reduce known ERCP risks for the patient, and reduce the time and effort of the physician for this procedure, as well as attempts to reduce costs, are important issues given the restricted financial resources of hospitals regarding radiation protection, hygiene, and the need for greater patient safety^[5-10].

With the release of a specialised side-viewing endoscope from Olympus [V-scope (VS)], which contains a specialised elevator lever with a V-groove in combination with the use of a specialised short guidewire system and a V-holder, further optimization of the entire ERCP process appears possible. The VS, including the V-groove of the elevator lever and the V-holder and its dedicated guidewires, was constructed to help the endoscopist secure the guidewire at a particular visible length during accessory exchange. This allows the physician to perform a guidewire or accessory exchange by him/herself and may thus lead to quicker instrumentation when working with or without assistance^[11-14]. In addition, the availability of a specialised short guidewire (2.6 m in length) and corresponding accessories promises to improve guidewire handling during ERCP and to increase hygienic aspects of ERCP, and it may also reduce the efforts of physicians and assistants^[12-14].

To analyse this procedure systematically, we performed a randomized prospective pilot trial to explore and document whether clinical, practical, and subjective improvements occur in daily ERCP practice when using the VS and its dedicated guidewire system as compared with conventional ERCP (without a VS, usually using long guidewires of 4.0-4.5 m in length). The main objectives were to evaluate the parameters of interventional examination time, X-ray time and dose, premedication dose, and interventions. In addition, subjective scores from physicians and endoscopy assistants were obtained to provide information about practical handling, hygienic aspects, and the convenience of this new ERCP method at a tertiary care university medical centre.

MATERIALS AND METHODS

Patient population

From June to October, 2007, 49 consecutive patients who were admitted to the Department of Medicine 1 of the University Erlangen-Nuremberg for interventional ERCP were included in this ERCP pilot trial if they

Table 1 Patient characteristics and indications for endoscopic retrograde cholangiopancreatography

| | ERCP with V-scope/V-system | Conventional ERCP |
|---------------------------------------|----------------------------|-------------------|
| Examinations (n) | 25 | 24 |
| Age, median (range), yr | 57 (33-83) | 57 (19-96) |
| Sex (male/female) | 8/17 | 8/16 |
| Hepaticolithiasis | 4 | 5 |
| Biliary strictures (benign/malignant) | 7 (4/3) | 5 (4/1) |
| Chronic pancreatitis | 12 | 12 |
| Pancreatic tumour | 2 | 2 |

ERCP: Endoscopic retrograde cholangiopancreatography.

had a previous endoscopic papillotomy and had one or more of the following conditions choledocholithiasis or hepaticolithiasis, malignant or benign bile duct stenosis, and chronic pancreatitis or pancreatic tumour. In addition, patients agreed to participate in this pilot trial with randomization to one ERCP technique, the collection of prospective scientific documentation, and the evaluation of their clinical and ERCP findings. Patient characteristics and indications for interventional ERCP are given in Table 1. All patients gave informed consent to participate and agreed to the collection of scientific documentation of the examination results. This clinical study was carried out in accordance with the Helsinki declaration.

Because cannulation of native papilla, papillotomy, and potential treatment of its associated risks may require a substantial amount of time, ERCP-naïve patients without papillotomy were excluded from this study, as were patients with coagulation disorders, septic cholangitis, or severe cardiovascular or pulmonary disease or who were pregnant^[5,9,15,16].

Informed consent to participate in this practice study was obtained the evening before the scheduled ERCP. Thirty minutes before the scheduled ERCP procedure, patients were randomized either to ERCP using the VS and its dedicated (short) guidewire system (VS group) or to conventional ERCP.

ERCP and interventions

ERCP in the VS group was performed with the commercially available Olympus side-viewing VS (TJF160 VR; Olympus, Hamburg, Germany), which contains a modified elevator lever with a V-groove and a specialised fixable guidewire system. The V-groove of the elevator lever induces an increased angle of articulation in the VS and allows complete locking of a specialised guidewire for use with the VS^[13,14,17]. This guidewire consists of a linear guide and a flexible hydrophilic tip (5 cm in length) combined with a long, stiff nitinol wire (0.35 mm in diameter, 2.6 or 4.2 m in length); both the linear guide and the nitinol wire have endoscopically visible markings at 5 cm (Olympus). In addition, a V-holder, which attaches to the working channel of the VS, allows the physician, in conjunction with securing the guidewire with the newly constructed V-elevator, to perform changes of instru-

ments and accessories (*e.g.*, appropriate baskets, balloons, papillotomes, *etc.*; Olympus) with or without assistance. This procedure accelerates instrumentation and may reduce X-ray time because of endoscopically visible control of the fixed guidewire^[13,14,17].

ERCP in the conventional ERCP group was performed with a side-viewing duodenoscope (Olympus TJF160) and typical 4.0- to 4.8-m-long guidewires (Terumo radifocus guidewire, flexible hydrophilic; Terumo Corporation, Leuven, Belgium; straight green guidewire, Teflon coated; Dispomedica, Hamburg, Germany; tracer metro wire guide; Aqua Coat Tip, Cook Ireland Ltd., Limerick, Ireland) and corresponding accessories (catheters, bougies, baskets, balloons, *etc.*)^[1,2,18-20]. Exchange of instruments was performed conventionally, and only if necessary, using fluoroscopy with the endoscopist pressing down the elevator lever and carefully withdrawing the instrument, while the assistant tried to retain the position of the guidewire in the cannulated area.

In brief, interventional ERCP in both groups was performed using the following steps: first prosthesis extraction (if necessary for exchange), cannulation of the papilla, visualization of the biliary system or pancreatic ducts with contrast medium (Peritrac 300/60%; Dr. Köhler Chemie GmbH, Alsbach Hähnlein, Germany), radiological documentation of pathological findings in two radiological axes, selective cannulation of pathologically changed biliary or pancreatic ducts using appropriate guidewires, performance of one or more interventions (*e.g.*, bougienage, concrement extraction, prosthesis insertion, *etc.*), and radiological and endoscopic documentation of results.

Of note, the interventional examination time for ERCP did not include insertion of the side-viewing duodenoscope down to the papilla or extraction of the endoprosthesis. To appropriately determine the effects of VS-guided ERCP, the interventional examination time was defined as the start of the ERCP once the side-viewing endoscope had been appropriately positioned in front of the papilla. Timing was started with a stopwatch once the cannulation catheter had been introduced to the working channel of the endoscope for the first time. The interventional examination time ended when the last intervention (endoprosthesis insertion, stone extraction, *etc.*) was completed and the final endoscopic photograph for documentation had been taken. Withdrawal of the endoscope from the patient was not included in the interventional examination time.

X-ray time and dose of each ERCP procedure from the first cannulation until the entire procedure and the final intervention had been finished were automatically registered and documented with the multifunctional digital Axiom artis fluoroscope (AXIOM Artis MP, Siemens, Munich, Germany) for each patient.

ERCPs were performed by two experienced investigators with > 10 years experience in gastrointestinal endoscopy, each of whom had performed more than 1500 ERCPs. Before the start of the trial, both investigators and the endoscopy assistants underwent 2 mo of

learning and training to become familiar with the VS and its fixable guidewire system. During this learning phase, each investigator performed more than 20 VS ERCPs. From this training phase, it became clear that handling, time requirements, radiological or endoscopic control of intrahepatically or intrapancreatically placed guidewires, and the method of performing instrument exchange had to be learned and require repeated training to improve skills and perhaps to reduce intervention times. From the team of endoscopy assistants, four individuals, each with experience of > 1000 ERCPs, were involved in this prospective randomised study.

Physicians and assistants completed the study documents immediately after the end of the ERCP and independently gave subjective scores (0-10, best to worst) in terms of “overall performance of ERCP”, “difficulty of ERCP” and “hygienic performance”. “Overall performance of ERCP” concerned global assessment of the course of the entire ERCP process. “Difficulty of ERCP” was described as the degree of interventional technical difficulty of the ERCP. “Hygienic performance” concerned whether the guidewire or accessories exchange was perfectly hygienic and whether guidewires contacted the patient’s face or head, were ever outside the covered sterile working area, *etc.*

Premedication was achieved in most patients with midazolam/pethidine and in younger patients with high levels of anxiety or in patients with high consumption of alcohol with propofol/pethidine. Conscious sedation was administered and monitored by a second physician who was responsible for analgo-sedation and documentation of all findings relevant to the study. All patients received continuous measurement of cutaneous oxygen saturation, pulse, blood pressure, and adequate oxygen supply during ERCP, which was performed in the prone position.

Cost analysis was also performed for each ERCP case for all consumables used during the study. The institutional costs for the side-viewing endoscopes and personnel costs for training purposes were not included in this analysis.

Statistical analysis

Statistical analysis was done using SPSS (SPSS for Windows Version 16.0.2, Ehningen, Germany) with descriptive statistics (median and range) for all parameters and performance of the Wilcoxon rank-sum test (*U* test). The statistical hypothesis was that use of the Olympus VS and its fixable guidewire system in the VS group would make ERCP faster (interventional examination time), reduce the X-ray time and dose, and reduce the premedication dose. Additional statistical descriptions are provided for subjective scores describing the convenience and performance of each ERCP procedure given by the endoscopists and the assistants.

RESULTS

Table 2 list all objective and subjective parameters used to

Table 2 Objective and subjective score results from comparison of endoscopic retrograde cholangiopancreatography by using the V-scope with conventional endoscopic retrograde cholangiopancreatography

| | ERCP with V-scope/V-system | Conventional ERCP | P value |
|---|----------------------------|-------------------|---------|
| Objective results | | | |
| Total interventions (n) | 50 | 47 | |
| Bougienage bile ducts | 7 | 6 | |
| Bougienage pancreas | 2 | 8 | |
| Endoprosthesis insertion | 24 | 16 | |
| Extraction of biliary concretions | 10 | 9 | |
| Extraction of pancreatic concretions | 3 | 3 | |
| Bile duct biopsy | 1 | 0 | |
| Nasobiliary catheter | 1 | 2 | |
| Partial guidewire dislocation | 1 | 3 | |
| Loss of guidewire | 1 | 0 | |
| Examination time (min), median (range) | 29 (5–50) | 31 (7–90) | 0.28 |
| X-ray time (min), median (range) | 5.87 (0.6–14.15) | 6.12 (1.67–18.85) | 0.48 |
| X-ray dose (cGy/m ²), median (range) | 1351 (159–5039.2) | 1296 (202.3–6421) | 0.34 |
| Premedication dose (mg), median (range) | | | |
| Midazolam | 7 (0–11.5) | 6.75 (0–11.5) | 0.33 |
| Pethidine | 100 (0–200) | 100 (0–200) | 0.48 |
| Propofol | 0 (0–720) | 0 (0–490) | 0.42 |
| Diazepam | 0 (0–10) | 0 (0–15) | 0.33 |
| Adverse events (n patients, % of each group) | | | |
| Abdominal pain >24 h without inflammation | 1 (4) | 3 (12.5) | 0.59 |
| Cholangitis ¹ | 3 (12) | 4 (16.7) | 0.77 |
| Post-ERCP pancreatitis ² | 1 (4) | 3 (12.5) | 0.59 |
| Perforation | 0 | 0 | |
| Subjective score results | | | |
| Endoscopy assistants (n = 4), median (range) | | | |
| Overall performance of ERCP | 3 (1–8) | 2 (1–7) | 0.51 |
| Hygienic aspects of ERCP | 3 (1–6) | 3 (1–7) | 0.33 |
| Endoscopists (n = 2), median (range) | | | |
| Overall performance of ERCP | 3 (1–8) | 3 (1–7) | 0.47 |
| Position to papilla | 3 (1–7) | 4 (1–7) | 0.29 |
| Difficulty of ERCP, median (range) | 2 (1–2) | 2 (1–3) | 0.49 |

¹Cholangitis was diagnosed by post-procedural elevation of inflammatory markers in conjunction with an intermittent increase in cholestatic enzymes and/or bilirubin, or subfebrile/febrile temperatures after endoscopic retrograde cholangiopancreatography (ERCP). Cholangitis was mild, and cases resolved within a median of 8 d (range, 2–10 d); ²Post-ERCP pancreatitis was diagnosed by elevation of lipase (more than twofold of the upper normal value) and the presence of abdominal pain after ERCP. These patients had mild pancreatitis, and cases resolved after a median of 4 d (range, 2–7 d).

compare ERCP in the VS group and in the conventional group.

Age, indications, and the number and difficulty of interventions were not different between the VS group and the control group. Although the median interventional examination time was 2 min shorter in the VS group (29 min *vs* 31 min), the difference was not statistically signifi-

cant. Similarly, the median X-ray time and dose, as well as the premedication dose, were nearly the same in both groups. Interestingly, fewer adverse events were seen in the VS group, but this difference was also not statistically significant, perhaps because of the low number of cases (Table 2).

Subjective assessment scores by physicians and endoscopy assistants concerning “overall performance of ERCP” also did not reveal any significant differences between the VS group and the conventional ERCP group. Scores were also the same for “hygienic aspects of the ERCP” as assessed by the assistants.

Individual cost analysis of the ERCP materials and accessories used during the ERCP study revealed no significant difference in consumables used. Accessories used in the VS group amounted to 349 EUR (range, 44–673 EUR), and costs in the conventional ERCP group were 335 EUR (range, 135–604 EUR).

DISCUSSION

ERCP is a resource-intensive, complex, interventional, multi-step endoscopic-radiologic procedure for the treatment of various biliary and pancreatic diseases^[1–5,19,22]. However, performance of ERCP, whether it results in therapeutic success or technical failure, harbours a known risk of side effects for the patient (*e.g.*, cholangitis, post-ERCP pancreatitis, analgo-sedation-induced complications, *etc.*), including a radiation risk, which the endoscopy team also experiences. ERCP requires the substantial use of fluoroscopy and expensive materials (balloons, guidewires, *etc.*), and technical success is often accompanied by substantial time and physical efforts on the part of the endoscopist and his/her team of assistants. Thus, further innovations are currently being studied to make ERCP safer for patients, to reduce X-ray dose and premedication, and to simplify the technical ERCP process^[2,6,9,17,21].

One possible future approach for a more convenient and perhaps safer, faster, and easier ERCP procedure may be the use of specialized fixable (short) guidewire systems as compared with the use of conventional long guidewires (4.0–4.5 m), which require longer exchange times. Several studies have been published using prototype VSs and prototype linear guidewires that demonstrated shorter accessory exchange times and a reduced need for guidewire adjustments^[13,14,17,23]. However, the benefit of the use of this Olympus V-system with respect to the overall ERCP outcome, interventional examination time, and fluoroscopy requirements has not been completely evaluated in daily ERCP practice. Thus, in an effort to optimize ERCP quality and hospital costs in a high-volume ERCP centre, an investigator-driven, prospective, randomized pilot trial was performed to explore whether the use of the Olympus VS and its dedicated guidewire system significantly improves the outcome of patients undergoing ERCP or reduces intervention time, fluoroscopy, or the endoscopists’ and assistants’ work, handling, and efforts.

This prospective, single-centre study did not, how-

ever, reveal any statistically significant differences between ERCP using the V-system and conventionally performed ERCP in terms of interventional examination time, fluoroscopy, analgo-sedation requirements, or subjective assessments obtained from the endoscopists and the endoscopy assistants. Interestingly, interventional examination time was somewhat shorter in the VS group (29 min) than in the control group (31 min), despite the need to perform additional endoprosthesis insertions in the VS group (Table 2), raising the question of whether the study population was too small to observe significant differences. Alternatively, this result may merely reflect the high quality and training status of the individuals who carry out conventional ERCP at a high-volume tertiary ERCP centre (> 1000 ERCPs per year).

Joyce *et al*^[17], in a previous multicentre comparative trial, also did not demonstrate any significant effect of the V-system on ERCP examination time or on fluoroscopy time, although they did demonstrate significant benefits of the V-system when particular individual ERCP working steps were analysed, such as the median exchange time of accessories or the need for guidewire repositioning. Combined with our findings, these data show that a possible improvement in one single working step in ERCP (*e.g.*, exchange of accessories) is not necessarily coupled with an improvement or reduction in the entire examination or fluoroscopy time, especially when varying degrees of case difficulties are being treated by various experienced endoscopists^[5,7,11-14,17,24]. However, the real benefit of V-system-guided ERCP may become relevant when the high interindividual variation in ERCP complexity and the different experiences of endoscopists are compensated for in an appropriate follow-up study protocol in which one patient is examined by the same investigator during follow-up interventions, such as endoprosthesis replacement, stone extraction, *etc.*, using both ERCP techniques. Such a stratified study protocol that would compare both ERCP techniques performed by one endoscopist on the same patient with the same therapeutic indication promises to better demonstrate whether a real benefit exists concerning interventional examination time and possibly other ERCP parameters including quality or outcome findings when using the V-system.

This small, prospective, randomized, single-centre pilot ERCP study in routine patients showed that several other uncontrolled factors during ERCP intervention influenced the examination time and radiation requirements more than the proposed time saving that is attributed to the V-system^[11-14,17-19,21,25,26]. Thus, from the experience gained during the use of the VS and its short guidewire system, it became apparent that the V-system may be helpful in individual cases with repetitive interventions and several instrument changes (*e.g.*, multiple stenting, numerous stone extractions), but these impressions have not yet been objectively proven with a corresponding interventional ERCP study.

Although previous studies dealing with the use of the V-system have focused primarily on technical aspects and

time requirements of single ERCP working steps^[14,15,17], this prospective pilot trial also documented all adverse events and analgo-sedation requirements in each group. Interestingly, in the VS group, the frequency of adverse events in terms of abdominal pain lasting longer than 24 h, cholangitis, and post-ERCP pancreatitis was not significantly different as compared with that of the conventional ERCP group. However, the tendency of fewer instances of post-ERCP pancreatitis in the VS group raises the question of whether the completely fixed guidewire within the pancreatic duct reduces mechanical irritation of the pancreatic tissue, which may be a cause of an inflammatory response during conventional ERCP. This unexpected observation warrants further prospective studies, because post-ERCP pancreatitis is still a major adverse event following ERCP procedures^[4,16,18,19].

Cost evaluation of the consumables used in each case revealed that both ERCP techniques have nearly the same cost to the hospital according to Germany University prices. This analysis does not favour the exclusive use of only one ERCP technique.

In addition, evaluation of the subjective assessment scores from endoscopists and the endoscopy assistants also demonstrated no advantage of the V-system as compared with the conventional ERCP technique. The overall performance of ERCP and the hygienic aspects of ERCP were similar in the VS group and in the conventional group, although working with shorter guidewires may be more convenient for the personnel than longer guidewires^[12-15,17]. However, as discussed above, these results may be related to the high training status of the personnel at our high-volume ERCP centre and to the fact that performing a safe and effective bougienage within the biliary system or pancreas requires the use of the long linear guidewire (4.2 m), which may have influenced the judgement of the personnel. Subjective assessment may vary according to the training status of the endoscopy team, which would preclude the translation of these results to low-volume endoscopy hospitals.

In conclusion, this prospective ERCP trial using the VS and V-system did not show a significant advantage of this dedicated short guidewire system at a high-volume ERCP centre as compared with the conventional ERCP technique. The real value of this V-system in ERCP practice requires further investigation in follow-up interventional studies that compensate for interindividual variation in both patients and endoscopists. Studies should be performed in low-volume endoscopy centres as well.

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COMMENTS

Background

Endoscopic retrograde cholangiopancreatography (ERCP) is a complex and cost-intensive diagnostic and therapeutic approach for the identification and

treatment of hepatobiliary and pancreatic disorders. To simplify this method, many new endoscopic techniques and instruments are currently being developed. In this pilot study, authors compared the new Olympus V-Scope (VS) system with the normal Olympus duodenoscope in patients admitted for interventional ERCP.

Research frontiers

The new VS system with its specialized fixable guidewire system (Olympus TJF160 VR) was evaluated and compared to the conventional ERCP technique (Olympus TJF160) with respect to the interventional examination time, X-ray time and dose, premedication dose, frequency of adverse events, handling in daily routine, and cost effectiveness.

Innovations and breakthroughs

Although detailed parameters of the ERCP technique and the outcomes were carefully assessed, no statistically significant differences were found between ERCPs performed with the VS and V-system and those performed using the conventional ERCP technique. Objective parameters such as interventional examination time and X-ray dose, outcome parameters such as adverse events, and subjective assessment scores by the endoscopy personnel were all similar in both ERCP technique groups. However, the study group was small, various levels of ERCP difficulty were included, and the results may have been influenced by the high-level training status of the personnel at our high-volume ERCP centre.

Applications

Regarding the fairly small number of patients, this study was designed as a pilot study to provide preliminary results concerning future study parameters for sample size estimations, outcome parameters and cost assessments. In further studies with a larger number of patients, the potential benefits of the Olympus V-system may be better evaluated when including only one or two defined ERCP indications and by including endoscopists who examine the same patient during follow-up and use both ERCP techniques.

Terminology

ERCP: A diagnostic and therapeutic tool for selective radiographic illustration of the biliary tract and pancreatic ducts that enables important interventions such as endoprosthesis insertion for drainage, stone extraction, or tumour palliation *via* short or long guidewire techniques. The Olympus side-viewing VS contains a modified elevator lever with a V-groove, which allows the use of short guidewires that can be fixed. However, these potential advantages have not yet been shown to improve the technical aspects of ERCP instrumentation, patient outcomes, or adverse events.

Peer review

This pilot study carefully assessed several outcomes and technical and subjective parameters of both ERCP techniques. Perhaps because of the small number of patients, no significant differences were demonstrated between ERCP using the V-system technique and conventional ERCP. The results obtained may be used for sample size calculation for a larger, more definitive study with more homogeneous ERCP indications.

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Factors associated with early virological response to peginterferon- α -2a/ribavirin in chronic hepatitis C

Javier García-Samaniego, Miriam Romero, Rafael Granados, Remedios Alemán, Miguel Jorge Juan, Dolores Suárez, Ramón Pérez, Gregorio Castellano, Carlos González-Portela

Javier García-Samaniego, Miriam Romero, Liver Unit, Hospital Carlos III, Centro de Investigación Biomedica en Red de Enfermedades Hepáticas y Digestivas, 28029 Madrid, Spain
Rafael Granados, Department of Gastroenterology, Hospital Universitario de Gran Canaria Dr. Negrín, 35010 Las Palmas de Gran Canaria, Spain

Remedios Alemán, Gastroenterology Service, Hospital Universitario de Canarias, 38320 Tenerife, Spain

Miguel Jorge Juan, Department of Gastroenterology, Hospital Universitario Insular de Gran Canaria, 35016 Las Palmas de Gran Canaria, Spain

Dolores Suárez, Internal Medicine Service, Hospital Arquitecto Marcide, 15405 Ferrol, Spain

Ramón Pérez, Gastroenterology Service, Hospital Central de Asturias, 33006 Oviedo, Spain

Gregorio Castellano, Gastroenterology Service, Hospital 12 de Octubre, 28041 Madrid, Spain

Carlos González-Portela, Gastroenterology Service, Hospital Policlínico de Vigo, 36211 Vigo, Spain

Author contributions: García-Samaniego J and Romero M designed the study and contributed to the interpretation of data; all authors collected data, revised the article critically and approved the final version of the article.

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Correspondence to: Javier García-Samaniego, MD, Liver Unit, Hospital Carlos III, Centro de Investigación Biomedica en Red de Enfermedades Hepáticas y Digestivas, C/ Sinesio Delgado 10, 28029 Madrid, Spain. javiersamaniego@telefonica.net
Telephone: +34-91-4532510 Fax: +34-91-7336614

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Abstract

AIM: To evaluate the impact of sociodemographic/clinical factors on early virological response (EVR) to peginterferon/ribavirin for chronic hepatitis C (CHC) in clinical practice.

METHODS: We conducted a multicenter, cross-sectional, observational study in Hepatology Units of 91 Spanish hospitals. CHC patients treated with peginterferon α -2a plus ribavirin were included. EVR was defined as undetectable hepatitis C virus (HCV)-ribonucleic acid (RNA) or ≥ 2 log HCV-RNA decrease after 12 wk of treatment. A bivariate analysis of sociodemographic and clinical variables associated with EVR was carried out. Independent factors associated with an EVR were analyzed using a multiple regression analysis that included the following baseline demographic and clinical variables: age (≤ 40 years *vs* > 40 years), gender, race, educational level, marital status and family status, weight, alcohol and tobacco consumption, source of HCV infection, alanine aminotransferase (ALT) and aspartate aminotransferase (AST) levels, and gamma glutamyl transpeptidase (GGT) (≤ 85 IU/mL *vs* > 85 IU/mL), serum ferritin, serum HCV-RNA concentration ($< 400\,000$ *vs* $\geq 400\,000$), genotype (1/4 *vs* 3/4), cirrhotic status and ribavirin dose (800/1000/1200 mg/d).

RESULTS: A total of 1014 patients were included in the study. Mean age of the patients was 44.3 ± 9.8 years, 70% were male, and 97% were Caucasian. The main sources of HCV infection were intravenous drug abuse (25%) and blood transfusion (23%). Seventy-eight percent were infected with HCV genotype 1/4 (68% had genotype 1) and 22% with genotypes 2/3. The HCV-RNA level was $> 400\,000$ IU/mL in 74% of patients. The mean ALT and AST levels were 88.4 ± 69.7 IU/mL and 73.9 ± 64.4 IU/mL, respectively, and mean GGT level was 82 ± 91.6 IU/mL. The mean ferritin level was 266 ± 284.8 μ g/L. Only 6.2% of patients presented with cirrhosis. All patients received 180 mg of peginterferon α -2a. The most frequently used ribavirin doses were 1000 mg/d (41%) and 1200 mg/d (41%). The planned treatment duration was 48 wk for 92% of patients with genotype 2/3 and 24 wk for 97% of those with genotype 1/4 ($P < 0.001$). Seven percent of patients experienced at least one reduction in ribavi-

rin or peginterferon α -2a dose, respectively. Only 2% of patients required a dose reduction of both drugs. Treatment was continued until week 12 in 99% of patients. Treatment compliance was $\geq 80\%$ in 98% of patients. EVR was achieved in 87% of cases (96% *vs* 83% of patients with genotype 2/3 and 1/4, respectively; $P < 0.001$). The bivariate analysis showed that patients who failed to achieve EVR were older ($P < 0.005$), had higher ALT ($P < 0.05$), AST ($P < 0.05$), GGT ($P < 0.001$) and ferritin levels ($P < 0.001$), a diagnosis of cirrhosis ($P < 0.001$), and a higher baseline viral load ($P < 0.05$) than patients reaching an EVR. Age < 40 years [odds ratios (OR): 0.543, 95%CI: 0.373-0.790, $P < 0.01$], GGT < 85 IU/mL (OR: 3.301, 95%CI: 0.192-0.471, $P < 0.001$), low ferritin levels (OR: 0.999, 95%CI: 0.998-0.999, $P < 0.01$) and genotype other than 1/4 (OR: 4.716, 95%CI: 2.010-11.063, $P < 0.001$) were identified as independent predictors for EVR in the multivariate analysis.

CONCLUSION: CHC patients treated with peginterferon- α -2a/ribavirin in clinical practice show high EVR. Older age, genotype 1/4, and high GGT were associated with lack of EVR.

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Key words: Antiviral therapy; Baseline factors; Early virological response; Peginterferon α -2a; Ribavirin

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INTRODUCTION

Until recent approval of the first direct acting antivirals (DAAs) against hepatitis C virus (HCV)^[1-4], the combination of pegylated interferon and ribavirin was the standard of care (SOC) for chronic hepatitis C (CHC)^[5-8] with the goal of achieving a sustained viral response (SVR) [undetectable hepatitis C virus ribonucleic acid (HCV-RNA) at week 24]. However, the overall SVR rate with standard peginterferon and ribavirin combination does not exceed 56%-63%^[5,9,10], and is even lower in some patient subgroups^[10]. Indeed, pivotal studies showed that HCV genotype 1 patients achieve a SVR rate of 41%-56%, whereas in those infected with HCV genotype 2/3, a SVR is obtained in 74%-80%^[5,9,11]. Variability in virological response depends on diverse patient factors as well as virological and histological factors. Genotype other than 1, low baseline viral load, age less than 40 years, body weight ≤ 75 kg and absence of advanced fibrosis and/or cirrhosis have been identified as predictive factors of SVR in the studies evaluating peginterferon α -2a plus

ribavirin combination^[9,12].

Pivotal studies have shown that early virological response (EVR) is highly predictive of SVR^[9,13]. Accordingly, patients who do not achieve an EVR have an almost null probability of achieving a SVR^[9,13-15]. The study conducted by Fried *et al*^[9] showed that only 3% of patients who did not obtain an EVR with peginterferon α -2a plus ribavirin achieved a SVR. The high negative predictive value (PPV) of EVR has great clinical value as it allows us to decide whether to continue or discontinue treatment at week 12, therefore preventing or minimizing the adverse effects related to treatment continuation. Current treatment guidelines for hepatitis C include this decision criteria at week 12^[5,7,8,16] and recommend discontinuation of treatment in patients who fail to achieve an EVR. In addition, the clinical utility of EVR has been demonstrated in the routine clinical practice setting, particularly in genotype 1-infected patients^[17].

Limited data have been reported regarding the EVR predictive factors in patients receiving peginterferon α -2a plus ribavirin combination therapy. Correct identification of these factors could be a useful strategy to optimize treatment in CHC and improve SVR rates, particularly in patients with genotype 1. On the other hand, treatment adherence is key to achieve successful treatment. The occurrence of adverse effects associated with peginterferon and/or ribavirin is the main reason for dose reduction or treatment discontinuation. As a result, 15%-20% of patients participating in clinical trials and approximately 25% of those in routine clinical practice discontinue treatment^[18]. Lack of adherence during the first 12 wk of treatment has been shown to have a particularly negative impact on EVR^[13,19]. Thus, the PPV of EVR associated with good treatment compliance is very high, achieving a SVR in 75% of cases with EVR and good treatment adherence^[9].

Given that sociodemographic and clinical factors associated with EVR are not well known and considering that treatment adherence is a variable closely related to EVR, characterization of viral and patient factors associated with treatment compliance, and hence EVR, has important clinical implications. The present study was designed to analyze baseline sociodemographic and clinical characteristics associated with EVR and antiviral treatment compliance in CHC patients treated with peginterferon α -2a in the routine clinical practice setting in Spain.

MATERIALS AND METHODS

Study design

This was a national, multicenter, cross-sectional, observational study. The study was carried out in the Hepatology Units of 91 Spanish hospitals. All participating patients gave their written informed consent, and the study was approved by the Clinical Research Ethics Committee of the Hospital Carlos III of Madrid. The study was conducted in accordance with the Declaration of Helsinki and Good Clinical Practice Guidelines and their amendments.

The study included adult patients (over 18 years of age) diagnosed with CHC and treated with peginterferon α -2a plus ribavirin under routine clinical practice conditions. Patients with any contraindication to hepatitis C treatment according to the prescribing information and those with HBV and/or human immunodeficiency virus coinfection were excluded.

The main purpose of the study was to analyze EVR in relation to sociodemographic and clinical characteristics. For this purpose, the following variables were recorded at the start of treatment: age, sex, race, nationality, educational level, marital status, occupation and family status, weight, cigarette smoking, alcohol consumption, source of HCV infection, methadone replacement therapy, alanine aminotransferase (ALT), aspartate aminotransferase (AST), gamma glutamyl transpeptidase (GGT) and ferritin levels, cirrhotic status, viremia and HCV genotype. In addition, ribavirin dose and scheduled duration of treatment, dose reductions of peginterferon α -2a and/or ribavirin up to week 12, data on premature treatment discontinuation (week and reason for discontinuation) were also collected. Furthermore, data on HCV-RNA concentrations at week 12 were recorded and EVR was analyzed (complete/partial). HCV-RNA levels were measured using quantitative polymerase chain reaction assays, mostly the Amplicor HCV Monitor (Roche, Kenilworth NJ, United States), although other commercial tests were used in some centers. A lower limit of detection of 50 IU/mL was considered in all participating hospitals. The secondary objective of the study was to evaluate treatment adherence at week 12.

Virological response criteria

EVR was defined as undetectable levels of HCV-RNA at week 12 (complete EVR) or ≥ 2 log reduction in HCV viral load from baseline (partial EVR).

Treatment adherence criteria

To evaluate treatment adherence, compliance was recorded according to the 80/80/80 rule^[20] and modified according to the study design. Treatment compliant or adherent patients were those receiving 80% or more of the total dose of peginterferon α -2a plus ribavirin during 80% of the time until week 12. Likewise, noncompliant patients included those who received $< 80\%$ of the prescribed dose of one or both drugs during $< 80\%$ of the expected duration (12 wk).

Statistical analysis

A descriptive statistical analysis was performed on the sociodemographic and clinical variables collected from the medical records at the start of treatment with peginterferon α -2a plus ribavirin. Quantitative variables were described using measures of central tendency and dispersion (mean, median, SD, minimum, maximum, first quartile and third quartile) and the results are expressed as mean \pm SD or median (range). Qualitative variables are presented as absolute and relative frequencies. To characterize the population based on patient sex and the

influence of sociodemographic and clinical characteristic on EVR, a bivariate analysis was carried out using Student's *t* test for quantitative variables and the chi-square test for the remaining sociodemographic and clinical qualitative independent variables. Similarly, a bivariate analysis of sociodemographic and clinical variables associated with EVR was carried out based on patient race. Variables with statistical significance or with $P < 0.10$ in the bivariate model were analyzed in a multivariate logistic regression model. Some factors that were not statistically significant were retained in the model based on previous clinical evidence. Odds ratios (OR) and 95%CI were calculated for the independent predictive factors of EVR. In the multivariate logistic regression analysis, only patients with available data for all the variables taken into account for the analysis were included. Significance level was set at $P < 0.05$. The statistical analysis was performed using the Statistical Package for the Social Sciences (SPSS) version 17.0 (SPSS Inc, Chicago, IL, United States).

RESULTS

Baseline patient characteristics

Baseline patient characteristics are shown in Table 1. A total of 1202 patients were included in the study. Thirty-six were excluded as they met at least one of the following criteria: peginterferon dose other than 180 mg (30 patients), negative HCV-RNA at baseline visit (1 patient), HCV-RNA level not available at week 12 when withdrawal had not occurred (8 patients). The number of evaluable patients was 1166, of which 1014 were analyzed and 152 were excluded for having a detectable viral load at week 12 without specifying the value and/or detection level.

Sociodemographic characteristics

Seven hundred and twelve (70%) patients were male. The vast majority of patients were Spanish (91%) and Caucasian (97%). Fifty percent of patients had completed compulsory education. Of the total number of patients, 580 (57%) were married and 898 (89%) patients lived with another person (Table 1).

The mean age was 43.3 ± 9.0 years in men and 46.6 ± 11.4 years in women ($P < 0.001$). No differences were found in race, educational level or family status based on gender. No significant differences were found in baseline sociodemographic characteristics based on race. The only differences noted were a greater proportion of women over 40 who were Caucasian ($P < 0.005$) and a higher educational level among Caucasian patients ($P < 0.01$).

Clinical characteristics

Alcohol consumption was reported in 154 (15%) patients and 514 (51%) were smokers. The most common source of HCV infection was intravenous drug use (25%) followed by transfusion (23%). Mean ALT and AST levels

Table 1 Patient baseline characteristics *n* (%)

| Characteristics | |
|---------------------------------------|-------------------|
| Patient sociodemographics | |
| Sex | |
| Male | 712 (70) |
| Female | 302 (30) |
| Age (yr), mean \pm SD | 44.3 \pm 9.8 |
| Nationality | |
| Spanish | 919 (91) |
| Other | 93 (9) |
| Race | |
| Caucasian | 980 (97) |
| Other | 27 (3) |
| Educational level | |
| Did not complete compulsory education | 125 (12) |
| Compulsory education | 506 (50) |
| Professional training | 243 (24) |
| University | 131 (13) |
| Postgraduate/Master/PhD | 7 (1) |
| Marital status | |
| Single | 280 (28) |
| Married | 580 (58) |
| Separated | 138 (14) |
| Widowed | 11 (1) |
| Family status | |
| Lives with another person | 898 (89) |
| Lives alone | 97 (10) |
| Prison inmate | 16 (2) |
| Clinical characteristics | |
| Weight (kg), mean \pm SD | 75.7 \pm 13.4 |
| Alcohol consumption | 154 (15) |
| Tobacco consumption | 514 (51) |
| Source of HCV infection ¹ | |
| IVDU | 256 (25) |
| Transfusion | 234 (23) |
| Other | 55 (6) |
| Unknown | 462 (46) |
| Methadone replacement therapy | 65 (7) |
| ALT (IU/mL), mean \pm SD | 88.4 \pm 69.7 |
| AST (IU/mL), mean \pm SD | 73.9 \pm 64.4 |
| GGT (IU/mL), mean \pm SD | 82.0 \pm 91.6 |
| Ferritin (μ g/L), mean \pm SD | 266.0 \pm 284.8 |
| Cirrhosis | |
| HCV genotype | 62 (6.2) |
| 1/4 | 784 (78) |
| 2/3 | 223 (22) |
| HCV-RNA | |
| < 400 000 IU/mL | 264 (26) |
| \geq 400 000 IU/mL | 744 (73) |

¹One patient could have more than one presumed source of infection. Data are presented as number (percentage) of patients unless otherwise indicated. Proportions of patients are presented as valid percentages. Percentages may not add up to 100 due to rounding error. Values for age, weight and serum levels of alanine aminotransferase (ALT), aspartate aminotransferase (AST), gamma glutamyl transferase (GGT) and ferritin are expressed as mean \pm SD. IVDUs: Intravenous drug users; HCV RNA: Hepatitis C virus ribonucleic acid.

were 88.4 \pm 69.7 and 73.9 \pm 64.4 IU/mL, respectively, and the mean GGT level was 82 \pm 91.6 IU/mL. The mean ferritin level was 266 \pm 284.8 μ g/L. Only 62 (6.2%) patients presented with cirrhosis.

Of the total number of patients, 784 (78%) had genotype 1/4, of which 694 (68%) had genotype 1. The HCV-RNA level was greater than 400 000 IU/mL in 744 (73.8%) patients (Table 1). The proportion of patients

Table 2 Treatment *n* (%)

| Treatment data | |
|---|---------------------|
| Peginterferon α -2a | |
| Dose: 180 mg | 1014 (100) |
| At least one dose reduction of peginterferon | 66 (7) ¹ |
| At least one discontinuation of peginterferon | 46 (81) |
| Ribavirin | |
| Dose | |
| 800 mg/d | 183 (18) |
| 1000 mg/d | 413 (41) |
| 1200 mg/d | 416 (41) |
| 1400 mg/d | 1 (0.1) |
| At least one dose reduction of ribavirin | 66 (7) ¹ |
| At least one discontinuation of ribavirin | 56 (89) |
| Scheduled treatment duration | |
| 24 wk | 234 (23) |
| 48 wk | 773 (77) |

¹The percentage of patients with dose reduction of peginterferon α -2a and the frequency of patients with dose reduction of ribavirin coincide, but are not exactly the same patients. The frequencies presented correspond to valid percentages. Percentages may not add up to 100 due to rounding error.

with tobacco and alcohol consumption habit was greater in men ($P < 0.001$). The diagnosis of cirrhosis was also more common in men ($P < 0.05$). Intravenous drug abuse as the source of HCV infection was more frequent in men and transfusion was more frequent in women ($P < 0.001$). Among the clinical variables analyzed, there were no differences in baseline viral load level or HCV genotype according to gender, but men had higher ALT, AST and GGT values ($P < 0.001$) and higher levels of ferritin ($P < 0.001$). With regard to ribavirin dose, 800 mg/d and 1000 mg/d were the most common doses given to women, whereas men received the 1200 mg/d dose more frequently ($P < 0.001$).

Peginterferon α -2a plus ribavirin treatment

All patients received 180 μ g of peginterferon α -2a. The most frequently used ribavirin doses were 1000 mg/d (41%) and 1200 mg/d (41%) (Table 2). In accordance with current treatment recommendations, the 1000 mg/d and 1200 mg/d doses of ribavirin were used more often for patients with genotype 1/4 (49% and 45% of patients received doses of 1200 and 1000 mg/d, respectively), whereas more than half of those with genotype 2/3 (58%) received 800 mg/d of ribavirin ($P < 0.001$).

In more than three-quarters of patients, the scheduled duration of treatment was 48 wk (Table 2). For 97% of patients with genotype 1/4, the planned treatment duration was 48 wk and in 92% of those with genotype 2/3 the scheduled duration was 24 wk ($P < 0.001$).

Treatment discontinuation and dose reduction

Sixty-six (7%) patients had their peginterferon α -2a dose reduced on at least one occasion. Of these, 46 (81%) patients had only one dose reduction. Similarly, 66 (7%) patients experienced at least one dose reduction of ribavirin. Of these, 56 (89%) patients had at least one dose

Table 3 Sociodemographics, clinical and pathological characteristics of patients with early virological response and non-early virological response by bivariate analysis n (%)

| Factors | EVR | Non-EVR | P value ¹ |
|---------------------------------|---------------------------|---------------------------|----------------------|
| Patient sociodemographics | | | |
| Age (yr), mean ± SD | 43.9 ± 9.7 | 47.0 ± 10.0 | < 0.005 |
| Sex | | | |
| Male | 602 (86) | 101 (14) | NS |
| Female | 264 (89) | 33 (11) | |
| Origin | | | |
| Developed country | 64 (93) | 5 (7) | NS |
| Developing country | 801 (86) | 129 (14) | |
| Race | | | |
| Caucasian | 836 (87) | 130 (14) | NS |
| Other | 24 (89) | 3 (11) | |
| Educational level | | | |
| Equivalent to or less than high | 537 (86) | 85 (14) | NS |
| Professional training | 209 (87) | 31 (13) | |
| University or higher | 119 (88) | 17 (13) | |
| Marital status | | | |
| Single | 235 (85) | 41 (15) | NS |
| Married | 491 (86) | 81 (14) | |
| Separated/divorced/widowed | 135 (92) | 12 (8) | |
| Family status | | | |
| Lives alone | 82 (85) | 15 (16) | NS |
| Lives with another person | 769 (87) | 117 (13) | |
| Clinical characteristics | | | |
| Weight (kg), mean ± SD | 75.6 ± 13.4 | 77.2 ± 13.5 | NS |
| Alcohol consumption | | | |
| No | 731 (86) | 115 (14) | NS |
| Yes | 133 (88) | 19 (13) | |
| Tobacco consumption | | | |
| No | 414 (85) | 75 (15) | NS |
| Yes | 450 (88) | 59 (12) | |
| Source of HCV infection | | | |
| Injection drug use | 221 (90) | 26 (11) | NS |
| Transfusion | 126 (89) | 16 (11) | |
| IV route | 79 (87) | 12 (13) | |
| Other | 435 (85) | 78 (15) | |
| Methadone replacement therapy | | | |
| No | 803 (86) | 126 (14) | NS |
| Yes | 57 (89) | 7 (11) | |
| ALT (IU/mL), mean ± SD | 86.3 ± 69.4 | 101.7 ± 71.5 | < 0.05 |
| AST (IU/mL), mean ± SD | 72.1 ± 65.3 | 84.8 ± 58.5 | < 0.05 |
| GGT (IU/mL), mean ± SD | 73.6 ± 85.2 | 134.0 ± 114.4 | < 0.001 |
| Ferritin (µg/L), mean ± SD | 248.0 ± 268.8 | 388.8 ± 357.3 | < 0.001 |
| Cirrhosis | | | |
| No | 817 (88) | 112 (12) | < 0.001 |
| Yes | 41 (67) | 20 (33) | |
| HCV genotype | | | |
| 1/4 | 645 (84) | 126 (16) | < 0.001 |
| 2/3 | 214 (96) | 8 (4) | |
| HCV-RNA (IU/mL), mean ± SD | 3 354 135.6 ± 5 978 359.9 | 3 781 940.0 ± 4 780 000.6 | NS |
| Baseline viral load | | | |
| < 400 000 IU/mL | 237 (91) | 25 (10) | < 0.05 |
| ≥ 400 000 IU/mL | 623 (85) | 109 (15) | |

Data are presented as number (percentage) of patients unless otherwise indicated. Proportions of patients are presented as valid percentages. Values for age, weight, and alanine aminotransferase/aspartate aminotransferase (ALT/AST), gamma glutamyl transferase (GGT) and ferritin are expressed as mean ± SD. ¹P value of bivariate analysis. EVR: early virological response; HCV-RNA: Hepatitis C virus ribonucleic acid; IV route: Intravenous route; NS: Not significant.

reduction before week 12. Only 15 (2%) patients required a dose reduction of both drugs (Table 2).

Early virological response

Of 1014 patients included in the analysis, 866 (87%) achieved an EVR. Of these patients, 699 (70%) had a complete EVR and 176 (18%) achieved a partial EVR. The results showed significant differences in EVR depending on genotype, and the percentage of patients with EVR at week 12 was higher in the group of patients with genotype 2/3 (96% *vs* 83% of patients with genotype 2/3 and genotype 1/4, respectively; *P* < 0.001).

Predictive factors of early virological response

Table 3 shows the sociodemographic and clinical characteristics of the early responders (EVR) and non-responders (non-EVR). According to the results obtained from the bivariate analysis, the only sociodemographic variable associated with EVR was age (*P* < 0.005). The clinical variables associated with EVR were ALT (*P* < 0.005), AST (*P* < 0.05) and GGT values (*P* < 0.001), ferritin levels (*P* < 0.001), presence of cirrhosis (*P* < 0.001), viral genotype (*P* < 0.001), and baseline viral load (*P* < 0.05).

In the multivariate analysis, the only sociodemographic factor identified as a predictor of EVR was age (OR: 0.543, 95%CI: 0.373-0.790, *P* < 0.01) and the clinical factors predictive of EVR were GGT level (OR: 3.301, 95%CI: 0.192-0.471, *P* < 0.001), ferritin level (OR: 0.999, 95%CI: 0.998-0.999, *P* < 0.01) and genotype (OR: 4.716, 95%CI: 2.010-11.063, *P* < 0.001). Age ≤ 40 years, GGT level ≤ 85 IU/mL, low ferritin levels and HCV genotype other than 1/4 were independent predictors of EVR (Figure 1).

Adherence to treatment and predictive factors associated with compliance

Treatment compliance was greater than 80% in 971 (97.8%) patients. No significant differences were found between patients with treatment adherence greater than 80% and those whose compliance was less than 80% with regard to their sociodemographic and clinical characteristics (data not shown).

DISCUSSION

The response to treatment with peginterferon plus ribavirin is heterogeneous and non-optimal in several HCV patients, as occurs in those infected with genotype 1, high viral load, advanced fibrosis, metabolic syndrome or non-CC polymorphisms of the interleukin 28b gene (*IL28b*)^[12,13,15,21,22].

EVR is highly predictive of SVR^[9,23] and provides hepatologists with a valuable tool to decide on continuation and duration of treatment, as well as providing patients with an additional motivation to adhere to treatment. The predictive value of EVR in patients infected with genotype 1 in the clinical practice setting in Spain^[17] was previously shown to be comparable to that obtained in pivotal trials^[9,23]. However, although identification of both viral and host factors associated with EVR may be very useful to predict SVR and therefore guide thera-

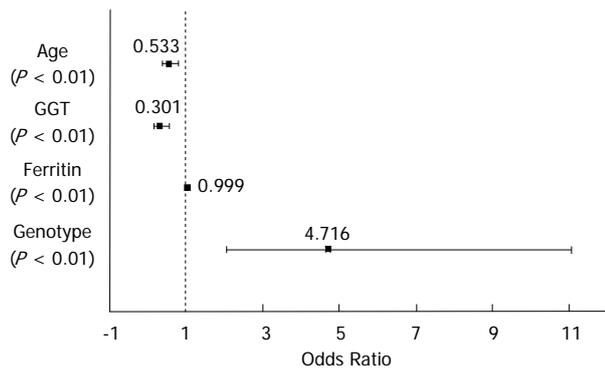


Figure 1 Independent factors associated with an early virologic response according to multiple logistic regression analysis. Baseline demographic factors used in the multiple regression analysis included age (≤ 40 years vs > 40 years), gender, race, educational level, marital status and family status. Clinical baseline factors included in the multiple regression analysis were weight, alcohol and tobacco consumption, source of hepatitis C virus (HCV) infection, alanine aminotransferase, aspartate aminotransferase and gamma glutamyl transpeptidase (GGT) levels (≤ 85 vs > 85), serum ferritin, serum HCV-RNA concentration ($< 400\,000$ vs $\geq 400\,000$), genotype (1/4 vs 3/4), cirrhotic status and ribavirin dose (800/1000/1200 mg/d). An odds ratio equal to 1 (dashed lines) indicates no difference between the subgroups defined according to the given factors. Bars indicate 95%CI. The results demonstrated that age ≤ 40 years ($P < 0.01$), low levels of GGT (≤ 85 IU/L) ($P < 0.001$), low ferritin ($P < 0.01$) and genotype 1/4 ($P < 0.001$) were independent predictors of early virological response.

py^[13], to date, there are limited data available addressing this issue.

To our knowledge, the present study is the largest series from a clinical setting to analyze factors associated with EVR in CHC patients treated with peginterferon plus ribavirin under routine clinical practice conditions. The data analysis showed that 87% of patients achieved EVR. This response rate is comparable or even higher than that reported in international pivotal trials with peginterferon α -2a plus ribavirin^[9,23] and confirm the results reported in previous studies conducted in Spain, also under routine clinical practice conditions, but with a considerably lower number of patients^[17,24]. A complete EVR was observed in 70% of our patients and again this rate was higher than that reported in other previous studies, which ranged from 34% to 64%^[9,10,12,25,26].

As expected, the percentage of patients with EVR was higher in the group of patients with genotype 2/3 than in those with genotype 1/4 according to previous studies^[9,10,27-29]. However, despite including a high percentage of patients with genotype 1 (the most difficult to cure), our study achieved response rates as high as those in the pivotal trials.

Patients who failed to achieve EVR were older, had higher ALT, AST, GGT and ferritin levels, a more frequent diagnosis of cirrhosis and a higher baseline viral load than patients achieving EVR. Most of the limited available studies on factors associated with EVR have shown that low baseline viral load, younger age, absence of overweight/obesity and lack of cirrhosis are independent factors associated with EVR^[30-33]. In our study, age < 40 years, GGT levels < 85 IU/mL, low ferritin levels and

genotype non-1/4 were identified as independent predictive factors of EVR in the multivariate analysis.

Interestingly, a recent study has identified high GGT as a predictor of nonresponse to treatment with peginterferon in patients infected with genotype 1^[34]. The precise mechanism whereby increased GGT levels may affect treatment response in CHC remains a matter of debate, although it may be related either to a more intense degree of necroinflammatory activity, more advanced fibrosis, or to hepatic steatosis^[35,36]. In this regard, a positive correlation between serum GGT levels and the hepatic expression of proinflammatory tumor necrosis factor- α (TNF- α) mRNA in CHC has been suggested^[37]. Indeed, hepatic levels of TNF- α mRNA have been found to be significantly higher in nonresponders to IFN- α -based therapy than in those with a SVR. On the other hand, although the multivariate analysis identified high levels of ferritin as an independent predictor of EVR, this association was clearly irrelevant (OR = 0.999). Therefore, ferritin level may lack clinical validity as a predictive factor of EVR in this setting.

Age was found to be an independent factor for EVR in our study, in agreement with previous series where it was shown that age greater than 40 years is an independent predictor of nonresponse to treatment^[12,29,34]. Furthermore, older patients have been suggested to be more resistant to peginterferon-based therapies since they are more frequently infected by HCV genotype 1b, have a longer disease duration and exhibit greater liver damage than younger patients.

Consistent with most published studies^[10,12,17,38], our findings show that the proportion of women with CHC treated with peginterferon plus ribavirin in routine practice in Spain is much lower in comparison to the proportion of men, in the absence of demographic variables justifying this difference, or known barriers of access to treatment for women. In addition, women are treated at an older age than men. We can speculate that the lower fibrosis severity as well as the higher rate of normal ALT levels in women with CHC can explain the relatively low proportion of female patients treated in our series. Although in some studies women have shown higher response rates than men^[31,38], our data did not show differences in EVR based on gender despite the high proportion of male patients. In light of these results, new epidemiological studies on the prevalence of HCV infection, as well as clinical studies including a larger number of women are needed to determine if there is gender inequality in the prescription of antiviral therapy.

Baseline HCV viral load is a well-known independent predictive factor of treatment response^[12,29-31,33]. In agreement with previous evidence, the patients in our study who achieved EVR were those who had lower baseline viral loads ($< 400\,000$ IU/mL), although viral load did not constitute a predictive factor for EVR in the multiple logistic regression analysis.

Race has been identified as a predictive factor of EVR in previous studies^[12]. The data from our study, despite its large sample size, does not allow us to conclude

that race plays a role in EVR because the vast majority of patients included in the study were Caucasian (97.3%). Furthermore, when the study was designed, genetic analysis of *IL28b* gene polymorphisms, which are related to race^[21], was not available. Indeed, one limitation of our study is the lack of information on IL-28B genotype as it has been described as a relevant predictor of treatment response^[39,40]. However, the impact of the imbalance of this genetic polymorphism between groups on treatment response was described just after the data from this study were collected.

Our study also revealed that Spanish CHC patients treated in routine clinical practice receive the correct doses of each drug according to current treatment guidelines, and this is critical since both dose optimization and treatment duration are essential to maximize response^[17]. The treatment regimen is individualized, according to current guideline recommendations, based on the viral genotype, so that patients with genotype 1/4 are treated with ribavirin in weight-adjusted doses of 1000-1200 mg/d for 48 wk, whereas patients carrying genotypes 2/3 are treated in most cases with ribavirin at a dose of 800 mg/d for 24 wk.

Combination therapy frequently causes adverse effects. Most are mild and controlled by reducing the dose of each drug or with additional treatments including growth factors, but in some cases they require discontinuation of treatment. Various studies have shown that dose reductions and particularly treatment discontinuation are associated with a marked reduction in the EVR rate^[20]. Other reports have noted that dose reductions of peginterferon and/or ribavirin are quite frequent during the first 12 wk of treatment^[13]. However, in the current analysis, only 7% of patients required dose reduction of peginterferon or ribavirin. Moreover, it should be noted that only 7 patients of the total population analyzed discontinued treatment due to serious adverse effects in the first 12 wk. This percentage (less than 1%) is markedly lower than that reported in other studies^[11,41].

Treatment compliance is a variable closely related to EVR^[13,19,20]. It has been shown that lack of adherence during the first 12 wk of treatment has a negative impact on EVR. Previous studies have demonstrated that patients who met the 80/80/80 rule have a greater response than those receiving lower doses or for less time^[20]. In our series, 98% of patients met the 80/80/80 rule up to week 12. Good treatment compliance is one of the factors explaining the high rate of EVR seen in our study. Treatment modifications and the motivation of patients may have had a significant impact on adherence to treatment. The low dose reductions required for both drugs could have encouraged patients to complete the prescribed course of treatment. Additionally, determination of EVR provides patients and physicians with an early goal and motivates them to adhere to treatment recommendations. Moreover, it is noteworthy that patients were treated by hepatologists belonging to units with wide experience in the care of CHC patients.

The main limitations of this study arise from the oc-

currence of the major advance in CHC in the last years, the development of DAAs and the recent approval of the triple therapy as the new SOC for CHC. Nevertheless, despite the obvious change in treatment paradigm, therapy based on peginterferon plus ribavirin will continue to play an important role as SOC, especially for non-1 HCV patients, considering that protease inhibitors must be combined with peginterferon plus ribavirin in genotype 1 patients. In addition, while remarkably effective, the recently approved protease inhibitors are also accompanied by frequent serious toxicities and considerable costs. Therefore, some patients who cannot tolerate protease inhibitors will need to be treated with dual combination given that triple combination regimens have a higher side effect burden^[3,42,43]. Additionally, the high cost of the DAAs will probably preclude the use of triple-combination therapies in health care systems constrained by rising costs and economically disadvantaged regions. It is well known that patients with rapid virological response to dual combination achieve SVR in higher rates, close to 90%, and therefore, despite the above limitations, our findings may be relevant and applicable at the onset of the DAAs era. Furthermore, our findings provide a meaningful assessment of factors associated with EVR regarding applicability to guide therapy of real-world patients given that our study population comprises a larger, more representative cohort of patients than those included in clinical trials evaluating predictive factors of EVR. However, further studies will be needed to validate whether the predictive factors of EVR to dual therapy remain a predictive tool in the context of new DAA agents in the routine clinical practice setting.

In summary, CHC patients treated with peginterferon α -2a plus ribavirin in routine clinical practice in Spain have high EVR rates, similar to those obtained in pivotal studies, and a high level of treatment compliance. Age > 40 years, genotype 1/4 and GGT \geq 85 IU/mL were independent predictive factors of lack of EVR. In the new era of hepatitis C treatment where standard treatment is incorporated with DAAs, identification of predictive factors such as the new definitions of extended rapid viral response will be an essential tool to achieve maximum response rates.

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Hospital Virgen de las Nieves, Granada, Spain; Sansó A, Hospital de Manacor, Mallorca, Spain; Montoliú S, Hospital Joan XXIII, Tarragona, Spain; Pardo A, Hospital Joan XXIII, Tarragona, Spain; Sánchez J, Hospital Río Carrión, Palencia, Spain; Amine S, Hospital General de Lanzarote, Las Palmas de Gran Canaria, Spain; Alonso MM, Hospital Universitario de Canarias, Tenerife, Spain; Eraña L, Hospital Santiago Apóstol, Vitoria, Spain; Guerrero J, Hospital Cruz Roja del INGESA, Ceuta, Spain; García E, Hospital General de Fuerteventura, Las Palmas de Gran Canaria, Spain; Sánchez JJ, Virgen de la Salud, Toledo, Spain; Calvo S, Hospital Comarcal de El Escorial, Madrid, Spain; Vázquez E, Complejo Hospitalario Pontevedra, Pontevedra, Spain; Moreno D, Hospital de Móstoles, Madrid, Spain; González R, Hospital de Móstoles, Madrid, Spain; Morán S, Hospital Santa María del Rosell, Cartagena, Spain; Torrente V, Hospital Sant Joan de Reus, Tarragona, Spain; Jiménez E, Hospital Universitario Insular de Gran Canaria, Las Palmas de Gran Canaria, Spain; Lee C, Hospital Dr. Negrín, Las Palmas de Gran Canaria, Spain; Hervías D, Hospital Virgen de Altagracia-Manzanares, Ciudad Real, Madrid, Spain; Rincón JP, Santa María del Rosell, Cartagena, Spain; Gimenez A, Hospital General de Granollers, Barcelona, Spain; Rodríguez R, Complejo Hospitalario Santa María Madre, Orense, Spain; Moreno M, Hospital General de Fuerteventura, Las Palmas de Gran Canaria, Spain; Casanova C, Hospital Universitario de Canarias, Tenerife, Spain; Sánchez H, Hospital La Inmaculada, Almería, Spain; Juan Carlos Penalva, Hospital de Torrevieja, Alicante, Spain; Compañy L, Hospital General de Alicante, Alicante, Spain; Sáez J, Hospital de Elche, Alicante, Spain.

COMMENTS

Background

Peginterferon plus ribavirin is the standard therapy for chronic hepatitis C (CHC) world-wide. Early virological response (EVR) predicts sustained virological response (SVR) in CHC. Although identification of both viral and host factors associated with EVR may be very useful to predict SVR and therefore guide therapy, to date, there are limited data available addressing this issue in clinical practice.

Research frontiers

The high negative predictive value of EVR has remarkable value in clinical practice since it allows to decide whether to continue or discontinue treatment at week 12, therefore preventing or minimizing the adverse effects related to treatment continuation. Indeed, current treatment guidelines for hepatitis CHC include this decision criterion and recommend discontinuing treatment in patients who fail to achieve an EVR.

Innovations and breakthroughs

The present study is the largest series from a clinical setting to analyze predictive factors of EVR in CHC patients treated with peginterferon α -2a plus ribavirin in routine clinical practice. The remarkably high EVR rates obtained in our study were similar to those reported with this dual therapy in pivotal studies, and confirm the data from previous studies in clinical practice in Spain which included a significantly lower number of patients. Age > 40 years, genotype 1/4 and gamma glutamyl transpeptidase (GGT) \geq 85 IU/mL were identified as independent predictive factors of lack of EVR.

Applications

The results from this study may be useful in guiding treatment decision making. In particular, early prediction of virological response to dual therapy can help to

identify candidates who are unlikely to have a SVR before treatment initiation or in the early treatment phase.

Terminology

EVR indicates EVR which is defined as undetectable hepatitis C virus ribonucleic acid (HCV-RNA) or \geq 2 log decrease in HCV-RNA after 12 wk of treatment.

Peer review

This study is well constructed and it is based in a big CHC patient cohort coming from 91 hospitals through all Spanish territory. This is an interesting and relevant study which confirms the results of previous studies with a notably lower number of Spanish cases. Moreover, this study consolidates some basal factors such a GGT levels, age or viral genotype as predictive factors of EVR. This study also highlights the higher rate of ERV in Spain compared with other regions.

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Endoscopic submucosal dissection for the treatment of neoplastic lesions in the gastrointestinal tract

Andrzej Białek, Anna Wiechowska-Kozłowska, Jan Pertkiewicz, Katarzyna Karpińska, Wojciech Marlicz, Piotr Milkiewicz, Teresa Starzyńska

Andrzej Białek, Wojciech Marlicz, Teresa Starzyńska, Gastroenterology Department, Pomeranian Medical University, 71-242 Szczecin, Poland

Anna Wiechowska-Kozłowska, Piotr Milkiewicz, Department of Endoscopy, Ministry of Internal Affairs Hospital, 70-382 Szczecin, Poland

Jan Pertkiewicz, Endotherapy Ltd, 02-091 Warsaw, Poland

Katarzyna Karpińska, Cell Pathology Department, Pomeranian Medical University, 71-242 Szczecin, Poland

Piotr Milkiewicz, Liver Unit, Pomeranian Medical University, 71-242 Szczecin, Poland

Author contributions: Białek A, Wiechowska-Kozłowska A, Pertkiewicz J, Milkiewicz P and Starzyńska T designed research; Białek A, Wiechowska-Kozłowska A, Pertkiewicz J, Karpińska K, Milkiewicz P and Starzyńska T performed research; Białek A, Wiechowska-Kozłowska A, Marlicz W and Milkiewicz P analyzed data; Białek A, Wiechowska-Kozłowska A, Milkiewicz P and Starzyńska T wrote the paper.

Correspondence to: Andrzej Białek, MD, Gastroenterology Department, Pomeranian Medical University, ul. Unii Lubelskiej 1, 71-242 Szczecin, Poland. bialeka@pam.szczecin.pl

Telephone: +48-91-4253211 Fax: +48-91-4253211

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Abstract

AIM: To investigate the indications, resection rate, and safety of endoscopic submucosal dissection (ESD) for neoplastic lesions in the gastrointestinal tract at a European referral center.

METHODS: We carried out a retrospective analysis of the ESD procedures performed in our center for mucosal neoplastic and submucosal lesions of the gastrointestinal tract. The duration of the procedure, *en bloc* and complete (R0) resection rates, and complication rates were evaluated. Variables were reported as mean \pm SD or simple proportions. Univariate analysis and

comparisons of procedure times and resection rates were performed using Mann-Whitney *U* tests, or χ^2 tests for dichotomous variables.

RESULTS: Between 2007 and 2011, ESD was performed in a total of 103 patients (46.7% male, mean age 64.0 ± 12.7 years). The indications for the procedure were epithelial tumor ($n = 54$), submucosal tumor ($n = 42$), or other ($n = 7$). The total *en bloc* resection rate was 90.3% (93/103) and R0 resection rate 80.6% (83/103). The median speed of the procedure was 15.0 min/cm². The complete resection rate was lower for submucosal tumors arising from the muscle layer (68%, 15/22, $P < 0.05$). Resection speed was quicker for submucosal tumors localized in the submucosal layer than for lesions arising from the muscularis propria layer (8.1 min/cm² vs 17.9 min/cm², $P < 0.05$). The R0 resection rate and speed were better in the last 24 mo (90.1%, 49/54 and 15.3 min/cm²) compared to the first 3 years of treatment (73.5%, 36/49, $P < 0.05$ and 22.0 min/cm², $P < 0.05$). Complications occurred in 14.6% ($n = 15$) of patients, including perforation in 5.8% ($n = 6$), pneumoperitoneum in 3.9% ($n = 4$), delayed bleeding in 1.9% ($n = 2$), and other in 2.9% ($n = 3$). Only one patient with delayed perforation required surgical treatment. During the mean follow-up of 26 ± 15.3 mo, among patients with R0 resection, recurrence occurred in one patient (1.2%).

CONCLUSION: ESD is an effective and safe method for resection of neoplastic lesions with low recurrence. Speed and the R0 resection rate increased after 50 procedures.

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Key words: Endoscopic submucosal dissection; Gastrointestinal neoplasms; Gastrointestinal stromal tumors; Treatment

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INTRODUCTION

In Japan and South Korea, endoscopic submucosal dissection (ESD) is a commonly accepted method for the resection of early neoplastic lesions in the upper and lower gastrointestinal tract. Good results from and the safety of procedures performed in the last few years have resulted in an increase in the number of procedures. Publications of the results of treatment with this method include more than 1000 patients. Although the dynamic development of this method is visible in Far East countries, this method is still in development in Europe and has not gained in popularity. A few publications describe the results of treatment in more than 50 patients, and some small series of patients or case reports have been published, but there is an overall lack of new European data.

ESD involves the removal of both benign neoplastic (pre-malignant) and malignant non-invasive lesions, aiming for the highest R0 resection rate (83%-98%) and lowest rate of local recurrence (0%-3%) of all endoscopic techniques^[1]. The removal of submucosal lesions is also possible, including those growing out of the proper muscle layer^[2].

The papers from Japan and Asia focus on confirming that ESD is a safe method with few complications and a mortality rate of 0%. The time needed to perform the procedure significantly decreases with the number of procedures performed, but it is still longer than the time needed for mucosectomy.

The present paper is one of the few European reports showing the results of treating gastrointestinal malignancies with ESD in a referral center. We present the indications, results, and complications with regard to different parts of the gastrointestinal tract.

MATERIALS AND METHODS

Patients

The procedures were performed between April 2007 and December 2011. Before qualifying for the procedure, patients received both oral and written explanations of the endoscopic examination and possible treatment options, and they signed an informed written consent form.

For each patient with pathology of the upper gastrointestinal tract, endoscopic ultrasound (EUS) was performed before admission for ESD. The sector-scanning echoendoscope (Olympus GF-UM130 or GF-UE160 Olympus Medical Systems Co., Tokyo, Japan) or linear echoendoscope (Olympus GF-UCT140) were used to

examine lesion size, EUS layer from which it derives (submucosal tumors, SMTs) or infiltrated layers (other lesions), SMT growth type (inside or outside the walls of the gastrointestinal tract), and lymph node diameter.

Neoplastic lesions of the upper gastrointestinal tract

Neoplastic mucosal tumors were included in the study if > 10 mm with a low risk of lymph node metastases. Lesions in the stomach were included based on the expanded criteria from the Japanese Gastric Cancer Association (JGCA)^[3]: well-differentiated carcinoma without ulceration, irrespective of size; well-differentiated carcinoma with ulceration (type III) ≤ 30 mm; or well-differentiated carcinoma with submucosal invasion and no more than 500 μm in size.

Exclusion criteria were lack of consent from the patient for the endoscopic procedure, massive infiltration of the submucosal layer or infiltration of muscle layers assessed in EUS, or enlarged local lymph nodes or metastases found in imaging studies (*i.e.*, EUS, ultrasound, and computed tomography).

Neoplastic lesions of the lower gastrointestinal tract

Patients were qualified for ESD if any of the following were in the previous endoscopic examination: large sessile polyp (LST) (type I s) that could not be removed in one piece, granular type (LST-G) flat polyps with a dominant nodule greater than 2 cm, non-granular type (LST-NG) flat polyps of any size, or scars from previous non-therapeutic tumor resections. Exclusion criteria were a lack of consent from the patient for the endoscopic procedure or deep ulceration apparent in the lesion, suggesting massive submucosal invasion.

Submucosal tumors

Resection of SMT by ESD was performed if the tumor was 1 to 8 cm in size with no growth outside the gastrointestinal tract on EUS. Patients who did not meet these criteria were referred to follow-up (submucosal lesions < 1 cm), mucosectomy (smaller or pedunculated lesions), or surgical treatment (submucosal lesions > 8 cm, epithelial tumors). All cases but one lipoma (bleeding gastric tumor) were excluded from endoscopic treatment.

Procedural technique

Before the treatment of epithelial malignancies by ESD, indigo carmine chromoendoscopy, magnifying endoscopy, NBI, or a combination of these techniques was performed to qualify the patients for the procedure based on the classification of Paris^[4] and properly assess the lateral margins of the lesion. ESD in the upper gastrointestinal tract was performed under general anesthesia, whereas analgesedation with midazolam (2.5-5 mg *iv*) and fentanyl (50-100 μg *iv*) was used for procedures in the lower gastrointestinal tract. An Olympus endoscope GIF-T140 with a transparent cap (Olympus D-201-12 704) was used, allowing a better view of the submucosal layer during the procedure. For submucosal injection, 0.9% NaCl solution

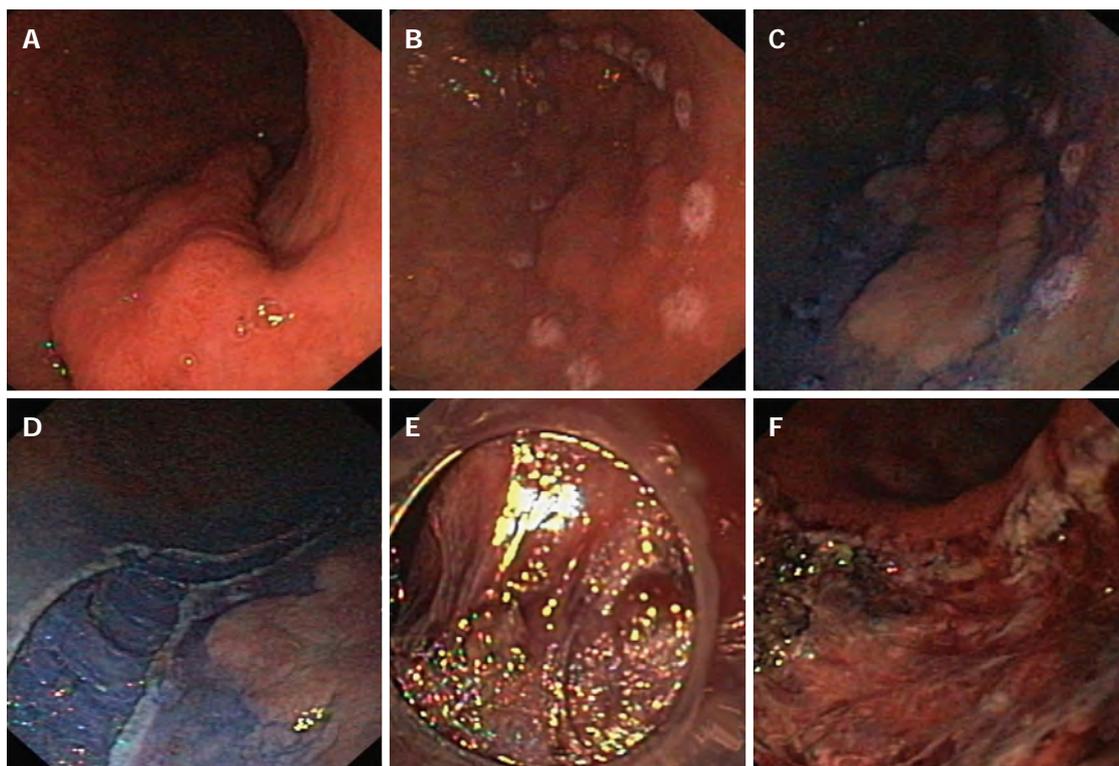


Figure 1 Endoscopic submucosal dissection of gastric neoplastic lesion. A: Lesion type II a+c localized in the antrum; B: Margins of the lesion border were marked with the needle knife; C: Solution of indigo carmine in saline was injected into the submucosal space; D: An incision was made in the mucosa and submucosa around the lesion with normal mucosal margin; E: Submucosal dissection performed directly under vision control; F: Mucosal defect after the completed procedure.

with epinephrine (1 mg/250 mL NaCl) and indigo carmine dye was used. In some cases in which the elevation of the mucosa after the injection of the solution was too short, a solution of hyaluronic acid was used (Sigmavisc, Hyaltech Ltd, Livingston, United Kingdom).

The resection was started by marking the lesion borders with the needle knife (Olympus KD-441Q) or dual knife (Olympus KD-650L) using forced coagulation of 35 W (Erbe ICC 200, Erbe Elektromedizin GmbH, Tübingen, Germany). The solution was injected submucosally next to the markers. The initial incision, approximately 3-5 mm in length, was made with a needle knife, and then a circular incision was made around the lesion using the isolated-tip knife IT or IT-2 (Olympus KD-611L, EndoCut mode, effect 3, 100 W) or a dual knife (EndoCut mode, effect 3, 35-50 W). The next step was the injection of the same solution directly into the submucosal layer under the lesion. The blue color of the indigo carmine allowed this layer to be distinguished from the proper muscle and the lesion itself. Dissection of the submucosal layer and the tumor was performed using the IT knife, hook-knife (Olympus KD-620LR, EndoCut mode, effect 2-3, 50-90 W), flex-knife (Olympus KD-630L), or dual knife (Figures 1 and 2). Muscular SMTs attached to the muscle layer by muscle fibers or muscle pedicle were cut away from the muscle layer of the wall (Figure 3). Tumors that were fused tightly with muscle and over a larger area were cut away to obtain enough material for pathological examination. Scar tissue was cut using a

needle knife along the muscle layer. Lesions located in the cardia and just behind the anal canal were removed mostly in retroflexion. During the procedure and immediately after the removal of a tumor, all visible blood vessels in the submucosal and muscle layer were coagulated using a coagrasper (Olympus FD-410LR, soft coagulation 35 W) or argon coagulation (Erbe APC 300, 35 W, flow 1.6 L/min), or hemoclips (Olympus HX-610-135) applied. The removed lesion was affixed to a polystyrene substrate, fixed in formalin, and examined morphologically.

Bleeding during the procedure that did not cause hemodynamic disorders or anemia was not considered a complication. All bleeding was treated endoscopically with coagrasper hemostatic forceps, argon beamer coagulation, or hemoclip application. Perforations noticed during the procedure were treated endoscopically by closing the wall defect using hemoclips. After the procedure, antibiotics were administered intravenously (amoxicillin 2 × 1000 mg) for 5 d, as well as proton pump inhibitors if the lesion was resected in the upper gastrointestinal tract (omeprazol 8 mg/h *iv* for 2 d, then 2 × 20 mg orally for 8 wk). After ESD in the lower gastrointestinal tract, patients received ciprofloxacin (2 × 200 mg *in*) and metronidazol (3 × 500 mg *in*). On the day of the procedure (day zero) the patients fasted. The first day after the procedure the patients received neutral fluids orally, and normal diet the next day. Colon patients received normal diet from the first day after the procedure. The patients were discharged from the hospital on day 3-5 after the procedure.

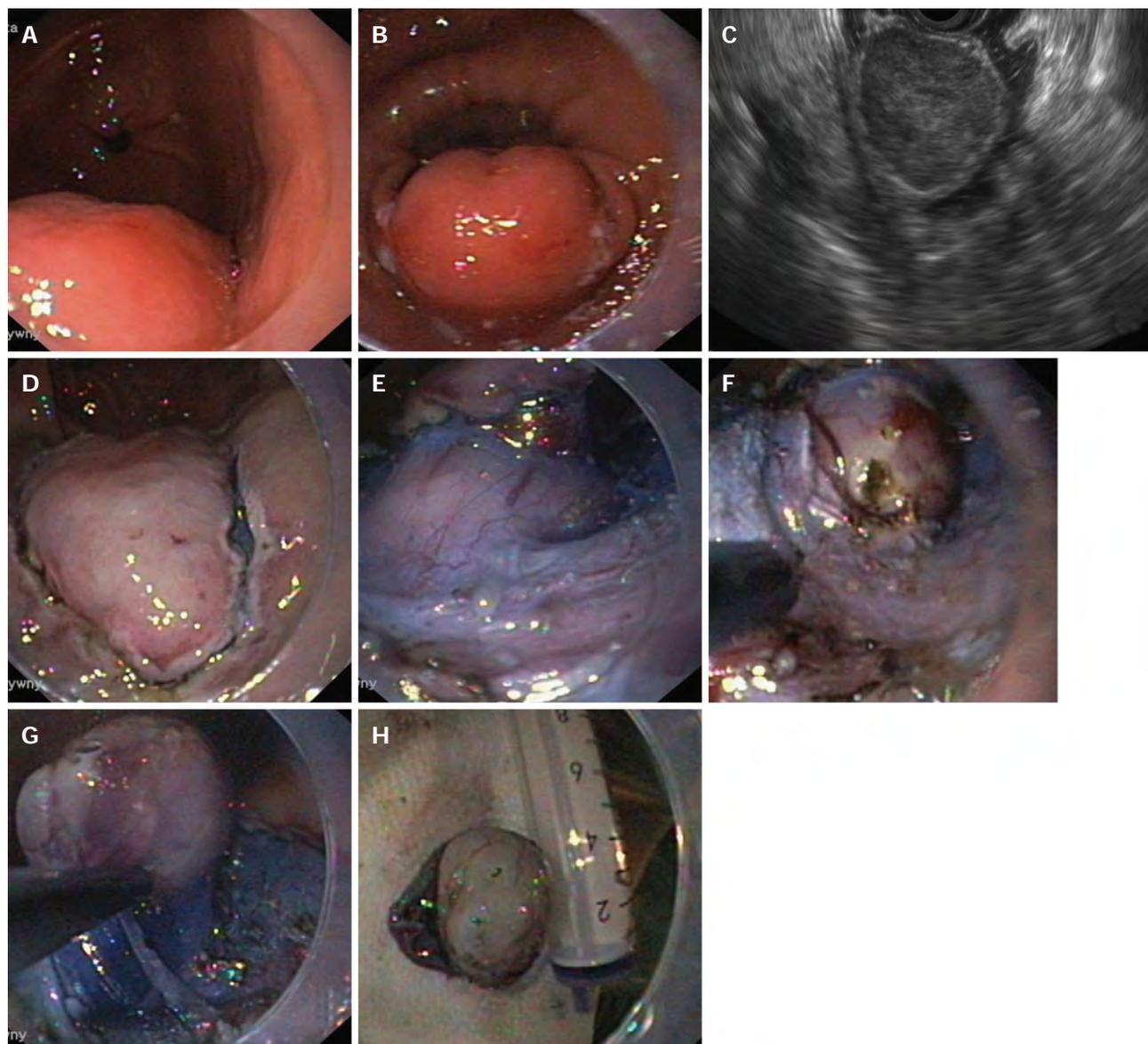


Figure 2 Endoscopic submucosal dissection of gastric submucosal tumor. A, B: Submucosal tumor in the stomach; C: Endosonographic view of the tumor, which is not connected to the muscle layer of the gastric wall; D: An incision was made in the mucosa and submucosa after indigo carmine solution injection into the submucosal layer around the lesion; E-G: Submucosal dissection of the tumor, exposing the tumor in the submucosa and carefully cutting after injecting the solution; H: Resected tumor (GIST, 25 mm, MI 2/50 HPF). GIST: Gastrointestinal stromal tumor; MI: Mitotic index; HPF: High power field.

Pathological examination

The resected specimens were pinned to a mounting board with clearly marked oral and anal orientations and routine formalin fixation performed. The borders of each specimen were colored with ink and sections taken every 2 mm. The pathological reports of the resected specimens included the macroscopic appearance, size, histological type (the most important Lauren classification of gastric cancer), and extent of the tumor depth. The presence of ulceration and lymphovascular involvement, as well as the status of the vertical and lateral resection margins, were reported in detail.

In addition, the SMT preparations were labeled with DAKO antibodies (Dako Polska Sp.z o.o.). Gastrointestinal stromal tumors (GISTs) were characterized by a positive reaction to c-KIT (CD 117) or DOG-1 and CD34

antibodies. Leiomyomas were diagnosed when the mesenchymal tumors had a positive reaction for smooth muscle actin and desmin. A positive reaction for S-100 protein and negative reaction for muscle markers and CD117 indicated nerve tumors. The neoplastic potential of the stromal tumors was determined on the basis of their size and mitotic index (MI, number of mitoses counted in 50 large fields) according to the classification of Miettinen *et al*^[5].

The criteria for curative resection of cancer lesions were: the depth of neoplasm infiltration limited to the mucosal or superficial submucosal layer (up to 500 μm in the cardia and stomach and up to 1000 μm in the large intestine as measured from the lower border of the muscle layer of the mucosa), no infiltration or congestion of carcinoma cells in blood vessels and lymph vessels (angioinvasion), lateral and bottom margins free of neoplasm, and

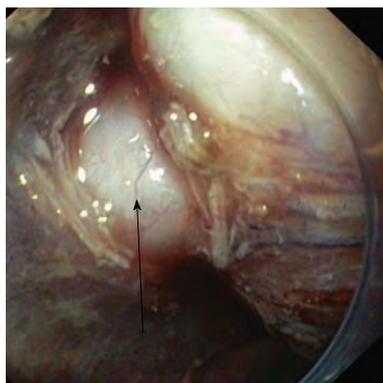


Figure 3 Submucosal tumor (leiomyoma) resected by endoscopic submucosal dissection and connected to the gastric wall with the muscle peduncle (arrow).

well or medium-differentiated cancer. For GIST, the only criterion was confirmed tissue-free margins.

Follow-up

All side effects of the dissection were recorded according to standard procedures^[6,7]. The first follow-up visit was 2-3 wk after ESD. The first follow-up endoscopy was performed 3 or 12 mo after surgery using a standard endoscope or EUS. Patients with incompletely resected neoplastic changes were referred for surgery. A control endoscopy after 3 mo was performed in the case of piecemeal resection of the tumor or uncertain histopathological confirmation of the complete tumor resection (Rx). The following cases were qualified for endoscopy after 12 mo: removal of a tumor that fulfilled the criteria for R0, resection of non-neoplastic lesions, and incomplete SMT resection of non-stromal tumors.

Statistical analysis

Variables were reported as mean \pm SD or simple proportions. Univariate analysis and comparisons of procedure times and resection rates were performed using Mann-Whitney *U* tests, or χ^2 tests for dichotomous variables. Statistica 9.1 software was used for all data analyses (StatSoft, Inc. 2010; Statistica).

RESULTS

Over a period of 57 mo, ESD was performed in 103 patients (46 males, 44.66%). The mean patient age was 64.0 ± 12.7 years. A total of 69 procedures were performed in the upper gastrointestinal tract, 34 in the colon. The indications for resection were epithelial tumors ($n = 54$), SMT ($n = 42$), scars from previous non-therapeutic tumor resection ($n = 2$), and others ($n = 5$). All procedures were performed by two physicians (Bialek A, Pertkiewicz J).

The total *en bloc* and R0 resection rates were 90.3% (93/103) and 80.6% (83/103), respectively. The rate of *en bloc* and R0 resection for epithelial lesions reached 85.3% (52/61), for overall SMT the rates were 97.6% (41/42) and 73.8% (31/42), respectively, but did not differ significantly. The complete resection rate for SMTs arising from the muscle layer was 68% (15/22), which is significantly

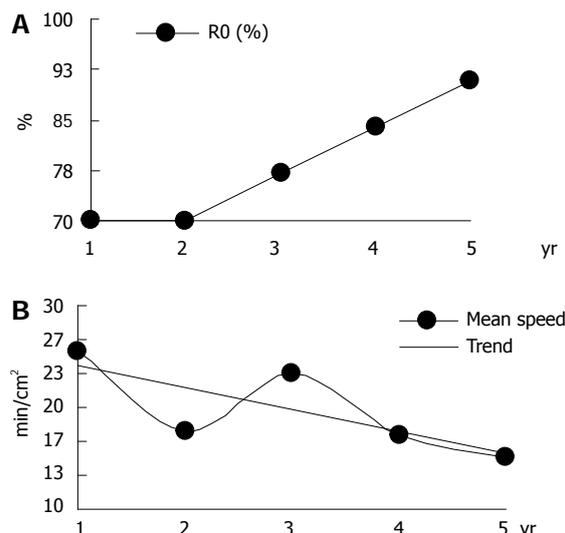


Figure 4 R0 resection rate and mean speed of the procedure in the years following endoscopic submucosal dissection. A: R0 resection rate; B: Mean speed of the procedure.

lower than that of epithelial lesions ($P < 0.05$).

The mean speed of performing the procedure was 19.0 ± 14.6 min/cm². The overall resection speed for SMTs was 15.85 ± 10.99 and 21.17 ± 16.41 min/cm² for mucosal lesions, but this difference was not significant. The resection speed was faster for SMTs localized in the submucosal layer compared to lesions arising from the muscularis propria layer (8.1 min/cm² *vs* 17.9 min/cm², $P < 0.05$).

During the first 3 years following ESD, 49 procedures were performed. Thirty-six of the procedures (73.5%) were R0 resections, and the procedure speed was 22.02 ± 15.33 min/cm². In the last 24 mo, the R0 resection rate increased to 90.1% (49/54, $P < 0.05$, Table 1; Figure 4) with a mean speed of 15.3 ± 13.25 min/cm² ($P < 0.05$, Table 1; Figure 4). The complication rates did not differ significantly. During a mean follow-up of 26 ± 15.3 mo, recurrence was detected in only one patient who underwent R0 resection (1.2%, 1/83), a 54-year-old female with intestinal type gastric cancer (G1, M2), and recurrence occurred within 6 mo of the procedure. The patient was sent for surgery and middle grade dysplasia diagnosed in the resected specimen.

Esophagus and cardia

ESD procedures were performed in the lower esophagus and cardia in 14 patients. The indications for the procedure were SMTs suspected to be GISTs in 5 patients and mucosal tumors in 9 patients.

Morphologically, tumors were type I -1, II a-2, II a+c-3, II b+c-2 and II b-1 according to the Paris classification. The mean tumor size was 2.37 ± 0.95 cm for all lesions, 2.36 ± 1.0 cm for mucosal lesions, and 2.4 ± 0.96 cm for SMTs. The *en bloc* and R0 resection rates as well as the histological diagnoses of resected tumors are shown in Table 2.

The mean procedure duration was 99 ± 77.2 min and significantly shorter for the resection of SMTs (38

Table 1 Mean speed of endoscopic submucosal dissection and R0 resection rate related to time

| Performing ESD (yr) | Procedures (n) | Speed (min/cm ²) (mean ± SD) | R0 |
|---------------------|----------------|--|-------|
| 1 | 10 | 25.5 ± 25.54 | 70.0% |
| 2 | 17 | 17.6 ± 25.54 | 70.0% |
| 3 | 22 | 23.4 ± 11.87 | 77.0% |
| 4 | 32 | 17.4 ± 15.33 | 84.0% |
| 5 | 22 | 15.1 ± 12.94 | 90.9% |

ESD: Endoscopic submucosal dissection.

± 13.03 min) than for mucosal lesions (128 ± 80.06 min, *P* < 0.05). Overall, the speed of resection was 24.96 ± 22.71 min/cm² and significantly slower for the resection of mucosal lesions (33.29 ± 24.4 min/cm²) than for SMTs (9.96 ± 6.9 min/cm², *P* < 0.05). The overall rate of *en bloc* and R0 resection was 100% (14/14) and 64.3% (9/14), respectively, and it did not differ significantly between mucosal lesions and SMTs.

Both of the incompletely removed SMTs were leiomyomas. The three neoplastic lesions were adenocarcinomas; cancerous infiltration was present in the lower margin (SM3) in two cases and in the lateral margin in one case. One of the patients was treated surgically; the adenocarcinoma was found in neither the cardia nor the lymph nodes of the surgical specimen. The other two patients were not qualified for surgery because of a lack of consent and high risk of surgery due to serious comorbidities.

Stomach

ESD was performed in the stomach in 54 patients, among which 35 were suspected to have GIST (mean size 2.9 ± 1.2 cm) and 19 mucosal neoplastic lesions (mean size 2.6 ± 1.1 cm). According to the Paris classification, the mucosal lesions were type I s-2, II a+c-11, II a-4, II b-1 and II b+c-1. Histological diagnoses and the rates of *en bloc* and R0 resection are shown in Table 2. Four lesions in the stomach qualified according to JGCA classic criteria^[7] were removed completely (R0 resection rate 100%). Out of 12 lesions, only 9 (75.0%) reached R0 resection according to expanded criteria.

In two cases of incomplete resection of SMT, GIST was diagnosed. In one of these patients, the tumor was 4 cm, MI = 6 (risk of progression: moderate), and the patient referred for surgery; in the other, the tumor was 1.9 cm, MI = 3 (risk of progression: low), and the patient referred for follow-up examination. In two cases of incomplete resection of adenocarcinoma, one had a lower margin positive for cancer (sm3) and the other a lateral margin positive for cancer (piecemeal resection). The first patient qualified for surgical resection, but the other did not give consent for surgical treatment; in 33 mo of follow-up no tumor recurrence was found.

The mean duration of the endoscopic procedure was 103.8 ± 77.3 min for all lesions, 108.1 ± 88.0 min for epithelial lesions, and 101.4 ± 72.1 min for SMTs. The

Table 2 Endoscopic submucosal dissection resection rate according to histology in esophagus and cardia, stomach and colon n (%)

| | Diagnosis | Patients | <i>En bloc</i> | R0 |
|-----------|-------------------------|----------|------------------------|-----------|
| Esophagus | SMT | 5 | 5 (100.0) | 3 (60.0) |
| | GIST | 1 | 1 (100.0) | 1 (100.0) |
| | Leiomyoma | 4 | 4 (100.0) | 2 (50.0) |
| | Non-SMT | 9 | 9 (100.0) | 6 (66.7) |
| | Early cancer/HGD | 6 | 6 (100.0) | 3 (50.0) |
| | Neoplasia grade min/med | 3 | 3 (100.0) | 3 (100.0) |
| Stomach | SMT | 35 | 34 (97.1) ^a | 27 (77.1) |
| | GIST | 18 | 18 (100.0) | 16 (88.9) |
| | Leiomyoma | 6 | 5 (83.3) | 3 (50.0) |
| | Other | 11 | 11 (100.0) | 8 (72.7) |
| | Non-SMT | 19 | 14 (73.7) ^a | 16 (84.2) |
| | Early cancer/HGD | 12 | 8 (66.7) | 10 (83.3) |
| | Neoplasia grade min/med | 4 | 3 (75.0) | 3 (75.0) |
| | Other | 3 | 3 (100.0) | 3 (100.0) |
| Colon | SMT | 2 | 2 (100.0) | 2 (100.0) |
| | Fibroepithelioma | 1 | 1 (100.0) | 1 (100.0) |
| | Leiomyoma | 1 | 1 (100.0) | 1 (100.0) |
| | Non-SMT | 32 | 28 (87.5) | 29 (90.6) |
| | Early cancer/HGD | 19 | 17 (89.5) | 17 (89.5) |
| | Neoplasia grade min/med | 11 | 10 (90.9) | 10 (90.9) |
| | Other | 2 | 1 (50.0) | 2 (100.0) |

^a*P* < 0.05 vs non-submucosal tumor (SMT). GIST: Gastrointestinal stromal tumor; HGD: High grade dysplasia.

mean speed of dissection was 18.05 ± 12.1 min/cm² for all lesions, 20.1 ± 13.4 min/cm² for epithelial lesions, and 16.9 ± 11.4 min/cm² for SMTs. The speed of the resection of SMTs connected to the muscle layer (*n* = 19) was 19.7 ± 8.5 min/cm² and 14.2 ± 15.2 min/cm² for tumors without such a connection (*n* = 12).

Colon

In the large intestine, ESD was performed in 34 patients. The main indications were laterally spreading tumor (LST) type II a-11 or II a+c-9 and polyps type I s-10. In two cases, ESD was performed for the radicalization of previously incomplete polypectomy procedures and in two cases due to symptomatic SMTs of the rectum. Low or middle grade dysplasia was diagnosed in 11 cases, and high-grade dysplasia or adenocarcinoma in 21 cases (Table 2). Two SMTs were diagnosed as fibroepithelioma and leiomyoma. The majority of lesions were located in the rectum (*n* = 28), followed by the sigmoid colon (*n* = 5) and ascending colon (*n* = 1). The rate of *en bloc* and R0 resection was 87.5% (28/32) and 90.6% (29/32), respectively. Among the lesions with incomplete resection, two adenocarcinomas with infiltration of the lower cut border (sm3) and tubular adenoma with low-grade dysplasia were diagnosed. In both cases of adenocarcinoma, the patients underwent surgical treatment, but cancerous tissue was not found in the surgical specimen. The patient with non-radically resected adenoma was administered follow-up examinations (20 mo) with no recurrence.

The average overall time and speed of treatment was 82.0 ± 56.6 min and 17.9 ± 14.2 min/cm², respectively,

Table 3 Complications after endoscopic submucosal dissection according to location

| | Patients | Delayed bleeding | Perforation | Other (n) | |
|-------------|----------|------------------|-------------|---|---|
| Cardia | 14 | 0 | 1 | Pneumoperitoneum | 2 |
| Stomach | 54 | 2 | 5 | | |
| Upper part | 21 | 1 | 4 | Mucosal tear of lower pharyngeal sphincter region, pneumoperitoneum | 3 |
| Middle part | 7 | 0 | 1 | | |
| Lower part | 26 | 1 | 0 | Stenosis of the pylorus | 2 |
| Colon | 34 | 0 | 0 | | 0 |
| Duodenum | 1 | 0 | 0 | | 0 |
| All | 103 | 2 | 6 | | 7 |

85.1 ± 56.9 min and 18.2 ± 14.5 min/cm², respectively, for epithelial neoplastic lesions, and 32.5 ± 3.5 min/cm² and 11.9 ± 9.3 min/cm², respectively, for SMTs.

Complications after ESD

Severe complications occurred in two patients in the form of perforations, resulting in prolonged hospitalization for more than 10 d, including surgical treatment in one case. Mild or moderate complications occurred in 13 patients: 2 patients with delayed bleeding treated by transfusion, 1 patient with a mucosal pharyngeal sphincter tear who required prolonged hospitalization for less than 3 d, 4 with pneumoperitoneum who required prolonged hospitalization for less than 3 d, 4 patients with perforation treated conservatively and requiring prolonged hospitalization for 4-10 d, and 2 patients with pyloric stenosis requiring additional endoscopy. The complication rate according to localization is presented in Table 3.

In six patients with perforation, endoscopic closure of the defect was performed using hemoclips, decompression of the peritoneum by puncture, and conservative treatment with fasting, antibiotic therapy, and active suction with a nasogastric tube. In one of the patients, delayed perforation occurred on the fourth day when trying to implement oral feeding.

Bleeding in two patients was controlled by hemoclip application, and patients required the transfusion of two units of blood. In two patients, stenosis occurred within 4 wk after dissection of neoplastic lesions in the pylorus. Both patients were successfully treated with 20-mm balloon dilatation in one and two sessions. In one patient a mucosal tear in the throat sphincter occurred when removing a large, > 3 cm resected SMT. The patient had no symptoms and did not require additional treatment.

DISCUSSION

ESD is a technique aimed at resecting early neoplastic lesions in the gastrointestinal tract without compromising the integrity of the wall. The technique allows R0 resection to be achieved, even for large mucosal and submucosal lesions, by removing them in one piece (*en bloc*), which

allows proper pathological evaluation of specimens. The method is the most effective of the endoscopic resection techniques and used by many centers in Japan and the Far East. Many papers, including the multicenter studies and those presenting the results of treatment in over 1000 patients, confirm the technique's efficacy in both the upper and lower parts of the gastrointestinal tract^[8-12]. The percentage of *en bloc* resection is estimated to range from 92%-100% in the upper gastrointestinal tract and 81.6%-92.7% in the lower gastrointestinal tract, and the rate of R0 resection is estimated to be 73.6%-94.7% in the upper gastrointestinal tract and 69.7%-89% in the lower gastrointestinal tract, which is significantly higher than that of mucosectomy techniques, in which R0 resection is estimated to be 33%-56%^[1,9,13], especially for the resection of large tumors (> 2 cm in size). ESD also allows resection of SMTs, even those growing from the muscularis propria^[2,10-18], and allows surgeons to save the organ via a minimally invasive resection of the lesion itself.

Despite such good results of treatment, the method is still rarely used in Europe^[2,19-22]. To the best of our knowledge, only two European papers from Augsburg, Germany, present the results of treating more than 100 patients in one center^[19,20]. One of the reasons for this low usage could be the time it takes to perform the procedure, especially at the beginning of the learning curve. In the Japanese centers, which have published the results of treating more than 1000 patients, the average time for ESD in the stomach is approximately 37 min^[19]. In the present study, both time and speed were worse than in the Japanese studies due to the relatively low volume at the center. The number of ESD procedures necessary to master the method is thought to be approximately 50. In the present study, the speed of performing the procedure increased approximately 30% after the first 49 procedures over 3 years. Similarly, Probst *et al.*^[20] and Japanese authors noted a significant increase in the speed of the resection after 40-50 procedures^[13,19].

The location of the lesions in the upper part of the stomach, their size, and the presence of submucosal fibrosis are associated with longer procedure duration, a lower resection rate, and a higher rate of complications^[23-25]. In the present study, the percentage of resection related to lesion localization and timing in the stomach did not differ significantly, probably due to a heterogeneous patient group and a small number of procedures in different locations.

The other factor responsible for the small number of ESD procedures in Europe may be the greater number of complications compared to mucosectomy. The most serious complication of ESD is perforation, which occurs in 1.2%-9.7% of cases, and bleeding, which occurs in 0.1%-15.6% of procedures^[1,8,13,20]. In the present study, the percentage of complications was similar, 4.9% for perforation and 1.9% for delayed bleeding, and surgical treatment was required in only one case. Importantly, the mortality rate after endoscopic treatment is 0% in both the present study and most published papers. Factors associated with a higher incidence of complications have

been identified, including location in the upper and middle part of the stomach, lesion size, and the number of procedures performed when less than 50^[11-12,25,26]. Also, in the present study, lesions located in the upper and middle third of the stomach were associated with a higher incidence of perforation, as five of six perforations occurred in these locations.

In the present study, the rate of *en bloc* resection and R0 resection was 90.3% and 80.6%, respectively, which is comparable with other European studies but lower than that of studies from Japan and South Korea. This difference is due to the lesser experience of authors in the first years of implementing the procedure. In the last two years, the rate of R0 resection increased from 73.5% to 90.1%, which approached the level of Japanese authors (Table 1; Figure 2). This finding confirms a long learning curve for this technique, which was nearly three years in our study, with 49 treatments completed. A similar tendency has been noticed by other authors, with the rate of total *en bloc* resection beginning at 50%-65.7% and increasing to 72.2%-100%^[11,19]. An important factor in achieving R0 resection was a larger proportion of eligible patients fulfilling extended ($n = 12$), compared to classical ($n = 4$), indications for endoscopic resection of gastric cancer. The rate of R0 resection for classical indications in the present study was 100% (4/4), which was higher than the rate with the extended criteria (9/12, 75%). A similar tendency was observed by Probst *et al.*^[20], who reported 90% (9/10) and 68.6% (35/51), respectively, and Japanese authors, who reported 97.1% and 91.1%, respectively^[19,27,28].

In the present study, the significant effect on the lower total resection rate comprised a relatively large proportion of patients with SMTs, including tumors arising from the muscularis propria layer, for which the R0 resection rate was significantly lower. Among the patients with confirmed complete R0 resection, only one recurrence occurred within 6 mo of the procedure (1.2%). In the publications from the last few years, the rate of recurrence after R0 resection was 0%-5.1%^[29-31].

A limitation of this study is the relatively small number of procedures performed in the reference center and retrospective type of analysis.

In summary, ESD allows endoscopic resection of tumors with high efficacy and low complication rates, even in a low-volume center. Most complications, including perforation, are mild or moderate in severity and can be treated endoscopically or conservatively. Both speed and complete resection rate improved after approximately 50 procedures.

COMMENTS

Background

In Japan and South Korea, endoscopic submucosal dissection (ESD) is a commonly accepted method for the resection of early neoplastic lesions in the upper and lower gastrointestinal tract. Although the dynamic development of this method is visible in Far East countries, ESD is still in development in Europe and has not gained in popularity. A few publications have described the results of treatment in more than 50 patients, and some small series of patients or case reports have been published, but new European data is lacking overall. The

present paper is one of the few European reports showing the results of treating gastrointestinal malignancies with ESD in a referral center. Authors present the indications, results, and complications with regard to different parts of the gastrointestinal tract.

Research frontiers

ESD is a technique aimed at resecting early neoplastic lesions in the gastrointestinal tract without compromising the integrity of the wall. The technique allows R0 resection to be achieved, even for large mucosal and submucosal lesions, by removing them in one piece (*en bloc*), which allows for proper pathological evaluation of specimens. The method is the most effective endoscopic resection technique to date and is used by many centers in Japan and the Far East.

Innovations and breakthroughs

One of the reasons for the lack of usage of ESD could be the time required to perform the procedure, especially at the beginning of the learning curve. In the present study, the speed of performing the procedure and R0 resection rate increased approximately 30% after the first 49 procedures over 3 years. Other authors have also noted a significant increase in the speed and R0 resection rate after 40 to 50 procedures. Another factor potentially responsible for the small number of ESD procedures in Europe may be the greater number of complications associated with ESD compared to mucosectomy. The most serious complications of ESD are perforation, which occurs in 1.2%-9.7% of cases, and bleeding, which occurs in 0.1%-15.6% of cases. In this study, the percentage of complications was similar: 4.9% for perforation and 1.9% for delayed bleeding, irrespective of the center experience. Surgical treatment was required in only one case. The significant effect on the total resection rate included a relatively large proportion of patients with submucosal tumors, including tumors arising from the muscularis propria layer, for which the R0 resection rate was significantly lower. Among the patients with confirmed complete R0 resection, only one recurrence occurred within 6 mo of the procedure (1.2%). In publications from the last few years, the rate of recurrence after R0 resection has been 0%-5.1%.

Applications

ESD allows endoscopic resection of tumors with high efficacy and low complication rates, even in a low-volume center. Most complications, including perforations, are mild or moderate in severity and can be treated endoscopically or conservatively. Both speed and the complete resection rate improved after approximately 50 procedures.

Terminology

R0 resection: Complete resection of the lesion confirmed by a pathologist by microscopic examination of the tumor-free borders of resected species.

Peer review

The authors report European experience for ESD. Though the number of cases is small and some points need to be clear, this is valuable study as they mentioned that is European own experience.

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Curcumin attenuated paracetamol overdose induced hepatitis

Kanjana Somanawat, Duangporn Thong-Ngam, Naruemon Klaikeaw

Kanjana Somanawat, Duangporn Thong-Ngam, Department of Physiology, Faculty of Medicine, Chulalongkorn University, Bangkok 10330, Thailand

Naruemon Klaikeaw, Department of Pathology, Faculty of Medicine, Chulalongkorn University, Bangkok 10330, Thailand

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Correspondence to: Duangporn Thong-Ngam, MD, Associate Professor, Department of Physiology, Faculty of Medicine, Chulalongkorn University, Phayathai Road, Bangkok 10330, Thailand. dr.duangporn@gmail.com

Telephone: +662-256-4267 Fax: +662-256-4267

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(serum glutamic oxaloacetic transaminase and serum glutamic pyruvic transaminase) and inflammatory cytokines [interleukin (IL)-12 and IL-18] levels by enzyme linked immunosorbent assay.

RESULTS: Serum transaminase, hepatic MDA, and inflammatory cytokines increased significantly in the APAP compared with the control group. Curcumin supplementation in APAP + CUR 200 and APAP + CUR 600 groups significantly decreased these parameters compared with the APAP group. The level of GSH decreased significantly in the APAP compared with the control group. Curcumin supplementation in APAP + CUR 200 and APAP + CUR 600 groups significantly increased these parameters compared with the APAP group. The histological appearance of the liver in the control group showed normal. In the APAP-treated group, the liver showed extensive hemorrhagic hepatic necrosis at all zones. Curcumin supplementation in APAP + CUR 200 and APAP + CUR 600 groups, caused the liver histopathology to improve. In the APAP + CUR 200 group, the liver showed focal necrosis and but the normal architecture was well preserved in APAP + CUR 600 group.

CONCLUSION: APAP overdose can cause liver injury. Results indicate that curcumin prevents APAP-induced hepatitis through the improvement of liver histopathology by decreased oxidative stress, reduced liver inflammation, and restoration of GSH.

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Key words: N-acetyl-P-aminophenol; Curcumin; Oxidative stress; Hepatitis; Interleukin-12; Interleukin-18

Abstract

AIM: To investigate whether curcumin could attenuate hepatitis in mice with paracetamol overdose.

METHODS: Male mice were divided into four groups. Group 1 (control, $n = 8$); was fed with distilled water; Group 2 [N-acetyl-P-aminophenol (APAP), $n = 8$]; was fed with a single dose of 400 mg/kg APAP dissolved in distilled water; Group 3 [APAP + curcumin (CUR) 200, $n = 8$], was fed with a single dose of 400 mg/kg APAP and 200 mg/kg CUR; Group 4 (APAP + CUR 600, $n = 8$), was fed with a single dose of 400 mg/kg APAP and 600 mg/kg CUR. Twenty-four hours later, the liver was removed to examine hepatic glutathione (GSH), hepatic malondialdehyde (MDA), and histopathologically. Then whole blood was withdrawn from heart to determine transaminase

Somanawat K, Thong-Ngam D, Klaikeaw N. Curcumin attenuated paracetamol overdose induced hepatitis. *World J Gastroenterol* 2013; 19(12): 1962-1967 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v19/i12/1962.htm> DOI: <http://dx.doi.org/10.3748/wjg.v19.i12.1962>

INTRODUCTION

In therapeutic doses, N-acetyl-P-aminophenol (APAP) or paracetamol is mainly metabolized *via* glucuronidation and sulfation pathway and in conjugated forms is excreted from the cells. Besides, APAP is partly metabolized by cytochrome P450 (CYP 2E1), to produce toxic metabolites such as N-acetyl-p-benzoquinone imine. These metabolites are produced in the liver and detoxified by reduced glutathione (GSH) and then removed from cells. In APAP overdose, it is mainly metabolized by CYP 2E1 and causes increase of toxic metabolites and GSH depletion. These metabolites interact with biomolecules such as protein, lipid, and nucleic acid *via* covalent binding, which disrupts hepatocytes function causing hepatic necrosis and liver injury^[1-6].

A previous study demonstrated that APAP overdose treatment showed significantly increase in serum transaminase, hepatic malondialdehyde (MDA), and decreased hepatic GSH. Histological examination showed a severe centrilobular hepatic necrosis with fatty changes^[7-9].

Curcumin (diferuloylmethane), a polyphenol, is an active ingredient of turmeric (*Curcuma longa*) and is pharmacologically safe for humans and animals. Curcumin has many biological activities, including anti-inflammatory, antioxidant, anti-carcinogenic, anti-mutagenic, and anti-diabetic activities^[10-13]. The hepatoprotection of curcumin has been widely acknowledged and used in traditional medicines for treatment of inflammatory conditions such as hepatitis^[14].

A previous study demonstrated that curcumin treatment showed significantly decrease in serum transaminase, hepatic MDA, increase hepatic GSH, and caused improvement of liver histopathology^[7-9].

However, it is still unclear whether curcumin has any effect in APAP-induced hepatotoxicity. Therefore, the present study aims to examine the protective effect of curcumin on hepatitis in mice with APAP overdose.

MATERIALS AND METHODS

Animal preparation

Male mice (4-5 wk), weighing 25-30 g, were purchased from the National Laboratory Animal Center, Mahidol University (Bangkok, Thailand). They were acclimatized at least 1 wk in a climate-controlled room on a 12-h light-dark cycle and were fed *ad libitum*. The experimental protocol was approved by the Ethical Committee of Faculty of Medicine, Chulalongkorn University, Thailand.

Paracetamol and curcumin preparation

A single dose of 400 mg/kg of APAP (Tylenol®) was dissolved in distilled water that was freshly prepared for the experiment. A single dose of 200 and 600 mg/kg of curcumin (95% purified curcumin, Cayman Chemical Company, Ann Arbor, MI, United States) were dissolved in corn oil that was freshly prepared for the experiment.

Experimental protocol

All mice were fasted, with free access to water *ad libitum*,

for 18 h before the experiment. They were randomly divided into four experimental groups.

Group 1 (control, $n = 8$); mice were fed distilled water orally *via* an intragastric tube; Group 2 (APAP, $n = 8$); mice were fed a single dose of 400 mg/kg of APAP orally *via* an intragastric tube; Group 3 [APAP + curcumin (CUR) 200, $n = 8$]; mice were fed a single dose of 400 mg/kg of APAP with a single dose of 200 mg/kg of curcumin orally *via* an intragastric tube; Group 4 (APAP + CUR 600, $n = 8$); mice were fed a single dose of 400 mg/kg of APAP with a single dose of 600 mg/kg of curcumin orally *via* an intragastric tube.

Twenty-four hours later, the mice were anesthetized with intraperitoneal injection of thiopental (50 mg/kg body weight). The abdominal wall was incised and liver was removed and washed with cold normal saline (4 °C -8 °C). The liver was chopped into small pieces, frozen in liquid nitrogen, and stored at -80 °C to examine hepatic MDA and hepatic GSH. The hepatic MDA was quantified by thiobarbituric acid reaction as described by Ohkawa *et al.*^[15]. The hepatic GSH was quantified by GSH Assay Kit (Cayman Chemical Company, United States). The remaining liver was fixed in 10% formalin solution to examine histologically. Then whole blood was withdrawn from heart. The blood was allowed to coagulate at room temperature (2 h) and centrifuged for 20 min at 3000 × *g* to obtain serum. The serum was collected to examine transaminase (serum glutamic oxaloacetic transaminase, serum glutamic oxaloacetic transaminase (SGOT) and serum glutamic pyruvic transaminase, SGPT) and inflammatory cytokines interleukin (IL)-12 (R and D Systems, Inc., United States) and IL-18 (Medical and Biological Laboratory Co., Ltd, Japan) by enzyme-linked immunosorbent assay method.

Histopathology

Samples of the liver were excised and transferred to formalin and later processed by routine techniques prior to embedding in paraffin. Sections were cut at the thickness of 5 μm and stained with hematoxylin and eosin (HE). An experienced pathologist blinded to the experiment evaluated all samples. All histopathological changes were observed under light microscope. Hepatic necroinflammation score in each section was graded according to the criteria described by Brunt *et al.*^[16] from 0 to 3 as follow; Score 0 = No hepatocyte injury/inflammation; Score 1 = Sparse or mild focal zone 3 hepatocyte injury/inflammation; Score 2 = Noticeable zone 3 hepatocyte injury/inflammation; Score 3 = Severe zone 3 hepatocyte injury/inflammation.

Statistical analysis

The data were expressed as mean ± SD. For comparison among all groups of animals, one-way analysis of variance (one-way ANOVA) and Tukey PostHoc comparisons were employed. *P*-value at less than 0.05 was considered statistically significant. The data were analyzed using the SPSS software version 17.0 for windows.

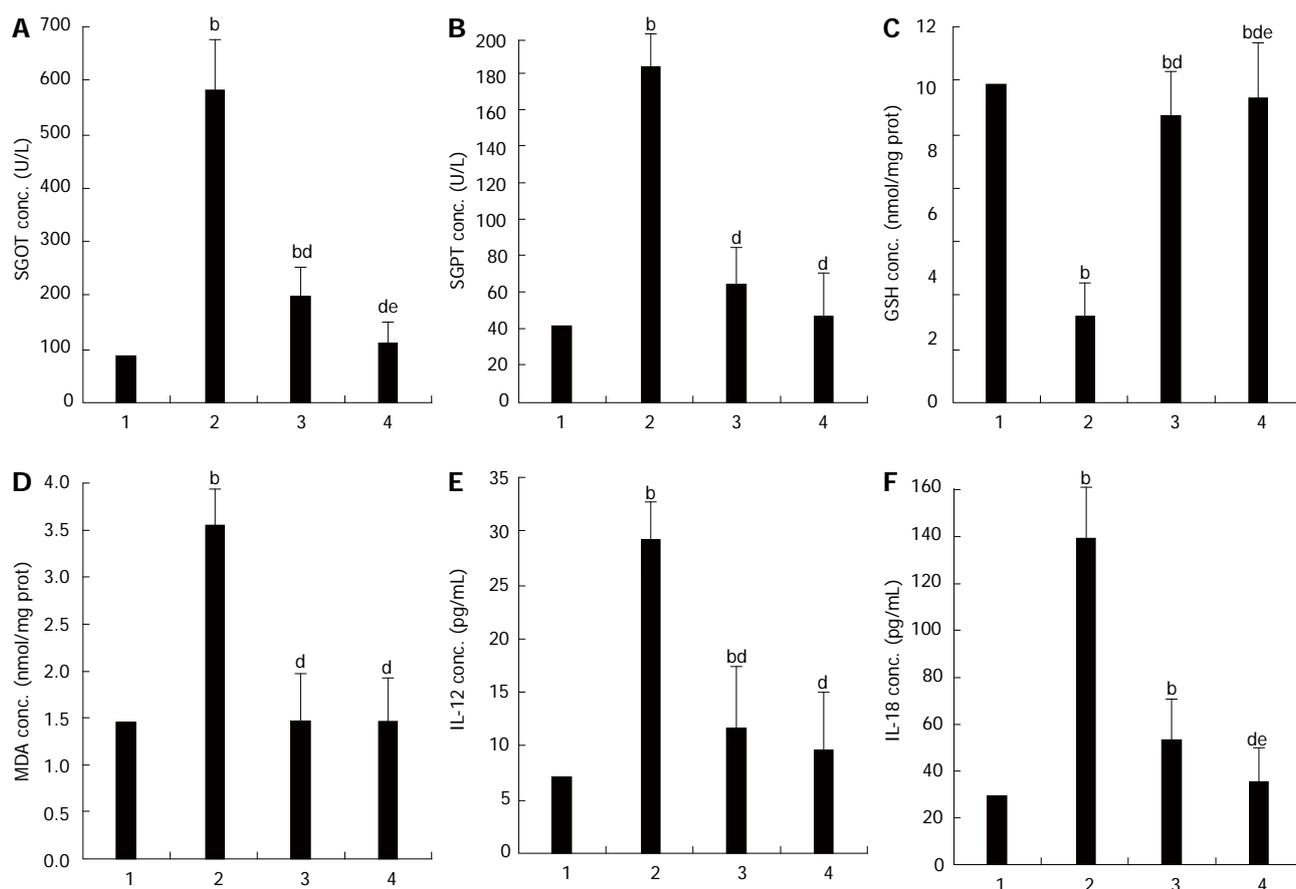


Figure 1 Effects of curcumin on serum glutamic oxaloacetic transaminase, serum glutamic pyruvic transaminase, hepatic glutathione, hepatic malondialdehyde, interleukin-12, interleukin-18 in mice with paracetamol overdose. A: Serum glutamic oxaloacetic transaminase (SGOT); B: Serum glutamic pyruvic transaminase (SGPT); C: Hepatic glutathione (GSH); D: Hepatic malondialdehyde (MDA); E: Interleukin (IL)-12; F: IL-18. ^b $P < 0.01$ vs control group; ^d $P < 0.01$ vs N-acetyl-P-aminophenol (APAP) group; ^e $P < 0.05$ vs APAP + curcumin (CUR) 200 group. 1: Control; 2: APAP; 3: APAP + CUR 200; 4: APAP + CUR 600.

RESULTS

Effects of curcumin on serum transaminase in mice with paracetamol overdose

Serum SGOT increased significantly in the APAP when compared to the control group (SGOT, 583.25 ± 118.30 U/L *vs* 86.13 ± 6.90 U/L, $P < 0.001$). These were significantly lower in the APAP + CUR 200 and APAP + CUR 600 groups than that in the APAP group (197.38 ± 14.39 U/L *vs* 583.25 ± 118.30 U/L and 111.38 ± 8.33 U/L *vs* 583.25 ± 118.30 U/L, $P < 0.001$, respectively). There was statistically significant difference in serum SGOT in APAP + CUR 200 and APAP + CUR 600 groups (197.38 ± 14.39 U/L *vs* 111.38 ± 8.33 U/L, $P < 0.05$) (Figure 1A).

Serum SGPT increased significantly in the APAP group when compared to the control group (186.00 ± 43.73 U/L *vs* 42.63 ± 6.95 U/L, $P < 0.001$). They were significantly lower in the APAP + CUR 200 and APAP + CUR 600 groups than in the APAP group (65.25 ± 3.11 U/L *vs* 186.00 ± 43.73 U/L and 47.50 ± 4.72 U/L *vs* 186.00 ± 43.73 U/L, $P < 0.001$, respectively). There was no statistically significant difference in serum SGPT in APAP + CUR 200 and APAP + CUR 600 groups (65.25 ± 3.11 U/L *vs* 47.50 ± 4.72 U/L, $P > 0.05$) (Figure 1B).

Effects of curcumin on hepatic GSH in mice with paracetamol overdose

Hepatic GSH were significantly lower in the APAP group compared to the control group (2.75 ± 0.16 nmol/mg protein *vs* 10.17 ± 0.11 nmol/mg protein, $P < 0.001$). Hepatic GSH in APAP + CUR 200 and APAP + CUR 600 groups were significantly higher than in the APAP group (9.16 ± 0.49 nmol/mg protein *vs* 2.75 ± 0.16 nmol/mg protein and 9.72 ± 0.22 nmol/mg protein *vs* 2.75 ± 0.16 nmol/mg protein, $P < 0.001$, respectively). There was statistically significant difference in hepatic GSH in APAP + CUR 200 and APAP + CUR 600 groups (9.16 ± 0.49 nmol/mg protein *vs* 9.72 ± 0.22 nmol/mg protein, $P < 0.05$) (Figure 1C).

Effects of curcumin on hepatic MDA in mice with paracetamol overdose

Hepatic MDA was elevated significantly in the APAP group when compared to the control group (3.55 ± 0.05 nmol/mg protein *vs* 1.45 ± 0.01 nmol/mg protein, $P < 0.001$). Hepatic MDA in APAP + CUR 200 and APAP + CUR 600 groups were significantly lower than in the APAP group (1.47 ± 0.01 nmol/mg protein *vs* 3.55 ± 0.05 nmol/mg protein and 1.46 ± 0.01 nmol/mg protein *vs* 3.55 ± 0.05 nmol/mg protein, $P < 0.001$, respectively). There

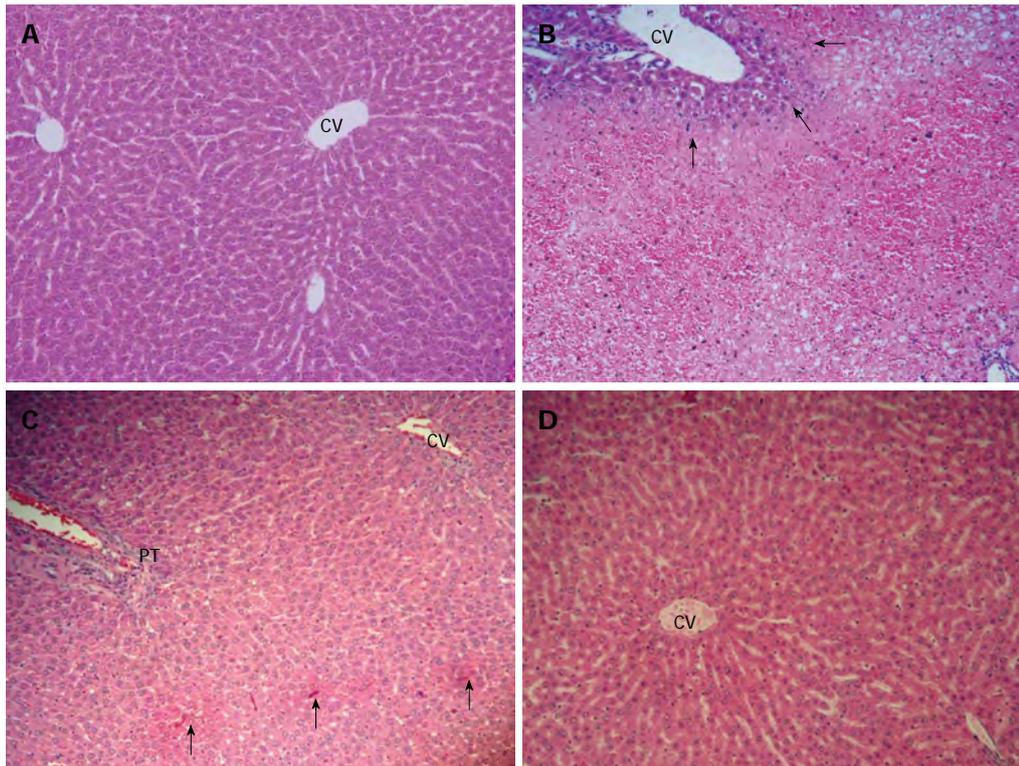


Figure 2 Effects of curcumin improved liver histopathology (hematoxylin and eosin stain, $\times 10$). A: Control group showed normal; B: N-acetyl-P-aminophenol (APAP) group showed extensive hemorrhagic hepatic necrosis at all zones; C: APAP + curcumin (CUR) 200 group showed mild focal necrosis and hepatic lobule was preserved; D: APAP + CUR 600 group showed the hepatic lobule was preserved with limited hepatic change. Arrows indicate hepatic necrosis, CV indicate central vein, and PT indicate portal system.

was no statistically significant difference in hepatic MDA in APAP + CUR 200 and APAP + CUR 600 groups (1.47 ± 0.01 nmol/mg protein *vs* 1.46 ± 0.01 nmol/mg protein, $P > 0.05$) (Figure 1D).

Effects of curcumin on serum IL-12 and IL-18 in mice with paracetamol overdose

The level of serum IL-12 increased significantly in the APAP group compared with the control group (29.16 ± 3.34 pg/mL *vs* 7.08 ± 1.40 pg/mL, $P < 0.001$). Serum IL-12 significantly decreased in the APAP + CUR 200 and APAP + CUR 600 groups compared with the APAP group (11.60 ± 1.68 pg/mL *vs* 29.16 ± 3.34 pg/mL and 9.63 ± 1.38 pg/mL *vs* 29.16 ± 3.34 pg/mL, $P < 0.001$, respectively). There was no statistically significant difference in serum IL-12 in APAP + CUR 200 and APAP + CUR 600 groups (11.60 ± 1.68 pg/mL *vs* 9.63 ± 1.38 pg/mL, $P > 0.05$) (Figure 1E).

The level of serum IL-18 increased significantly in the APAP group compared with the control group (139.52 ± 15.59 pg/mL *vs* 29.17 ± 3.72 pg/mL, $P < 0.001$). Serum IL-18 significantly decreased in the APAP + CUR 200 and APAP + CUR 600 groups compared with the APAP group (53.48 ± 18.19 pg/mL *vs* 139.52 ± 15.59 pg/mL and 35.21 ± 2.18 pg/mL *vs* 139.52 ± 15.59 pg/mL, $P < 0.001$, respectively). There was statistically significant difference in serum IL-18 in APAP + CUR 200 and APAP + CUR 600 groups (53.48 ± 18.19 pg/mL *vs* 35.21 ± 2.18 pg/mL, $P < 0.05$) (Figure 1F).

Effects of curcumin on histopathology in mice with paracetamol overdose

The histological appearance of the liver in the control

group showed normal (Figure 2A). In the APAP group, the liver showed extensive hemorrhagic hepatic necrosis of all zones (Figure 2B). In APAP + CUR 200 and APAP + CUR 600 groups, the liver histopathology improved. The APAP + CUR 200 group showed focal necrosis (Figure 2C), whereas the majority of hepatic lobules were preserved as normal architecture with limited hepatic change in the APAP + CUR 600 groups (Figure 2D). The summary of hepatic necroinflammation score in the control and experimental groups are shown in Table 1.

DISCUSSION

This study demonstrated that treatment of APAP overdose induced hepatitis in mice and could be attenuated by treatment with curcumin. This result corresponds to previous observations studied in mice and rat models^[7-9].

Chemoprevention is promising as a preventive approach for hepatitis. Curcumin (diferuloylmethane), a polyphenol compound, is an active ingredient of turmeric (*Curcuma longa*)^[10]. The phenolic and methoxy groups on the benzene rings of curcumin are important structural features that contribute to its anti-oxidant properties and its ability to reduce the amount of free radicals^[17-20]. Curcumin shows beneficial effects in inflammatory conditions including hepatitis^[14].

To assess lipid peroxidation we used MDA as a marker^[21] and reduced GSH as indicator of hepatoprotectivity for cells. In this study the oxidative damage caused by APAP overdose was significantly attenuated by administering curcumin. It was conceivable that curcumin could protect against free radical mediated oxidative stress by scavenging for free radicals that limits lipid peroxidation

Table 1 Summary of the hepatic necroinflammation score in all groups

| Group | n | Hepatic necroinflammation scores | | | |
|----------------|---|----------------------------------|---|---|---|
| | | 0 | 1 | 2 | 3 |
| Control | 8 | 8 | - | - | - |
| APAP | 8 | - | 1 | 1 | 6 |
| APAP + CUR 200 | 8 | 5 | 2 | 1 | - |
| APAP + CUR 600 | 8 | 5 | 3 | - | - |

Data are expressed as the number of mice exhibiting each score of hepatic necroinflammation ($n = 8$). Hepatic necroinflammation score in each section was graded according to the criteria described by Brunt *et al*^[16] from 0 to 3 as follow; Score 0: No hepatocyte injury/inflammation; Score 1: Sparse or mild focal zone 3 hepatocyte injury/inflammation; Score 2: Noticeable zone 3 hepatocyte injury/inflammation; Score 3: Severe zone 3 hepatocyte injury/inflammation. 0 = None; 1 = Mild; 2 = Moderate; 3 = Severe; APAP: N-acetyl-P-aminophenol; CUR: Curcumin.

involved in membrane damage. In this study the GSH depletion caused by APAP overdose was significantly attenuated by administering curcumin. The protective action of curcumin can be explained by GSH inducer through induction of glutathione reductase enzyme system.

Serum transaminase, SGOT and SGPT, are often used as markers as their increase indicates liver damage^[22,23]. There is a significant increase of both SGOT and SGPT in APAP overdose. We demonstrated that this increase was reduced by the administration of curcumin.

It has been reported that IL-12 is produced by dendritic cells, monocytes, Langerhans cells, neutrophils, and keratinocytes^[24]. IL-18 is produced by activated macrophages, keratinocytes, intestinal epithelial cells, oestoblasts, adrenal cortex cells, and the murine diencephalon. IL-18 is synthesized as a precursor 24 kilodalton molecule without a signal peptide and must be cleaved to produce an active molecule. IL-1 converting enzyme (or caspase-1) cleaves pro-IL-18, producing the mature, bioactive peptide that is readily released from cells^[25-29]. Importantly, both of these cytokines are produced from Kupffer cells or hepatic macrophages in the liver. It may be possible that inflammation is due to hepatocytes releasing cytokines into the blood stream. Furthermore, IL-12, in combination with IL-18, causes inflammation *via* the activity of interferon-gamma, which is produced from T-lymphocyte and NK cells.

Curcumin is modulates the inflammatory response by down-regulating the activity of COX-2, inducible NO synthase, tumor necrosis factor- α , IL-1, IL-2, IL-6 and IL-8^[30]. This shows that curcumin is an anti-inflammatory substance. These cytokines are required for the expression of many cells linked with the inflammatory response. However, there are no experiments which study the effect of curcumin in preventing hepatitis resulting from APAP overdose and influence the levels of cytokines IL-12 and IL-18. Therefore, we studied the effect of curcumin on decreasing hepatitis resulting from APAP overdose. We showed that curcumin could prevent hepatitis resulting from APAP overdose and cause decrease in IL-12 and

IL-18 levels. It may be possible that curcumin can inhibit caspase-1 enzyme, which is cleaved pro-IL-18 into bioactive peptide or active-IL-18^[26]. This could explain that another pathway may also reduce liver inflammation that is indirectly mediated by oxidative stress inhibition.

In the present study, APAP overdose caused extensively hepatic necrosis. In curcumin supplementation, liver histopathology improved and showed only a focal hepatic necrosis of lobules.

In conclusion, APAP overdose can cause liver injury. Our results show curcumin could attenuate APAP-induced liver injury by decrease oxidative stress, reduce liver inflammation, restore hepatic GSH, and improve liver histopathology. In addition, curcumin at the dose of 600 mg/kg tends to be more potent than 200 mg/kg in preventing the effects of APAP hepatotoxicity. Hence, curcumin might be a novel therapeutic strategy against hepatitis caused by APAP overdose.

COMMENTS

Background

N-acetyl-P-aminophenol (APAP) overdose induced liver damage is one of the most widespread drug-induced side-effects. Although the exact mechanism of APAP remains largely unknown, it appears to involve two pathways: direct hepatotoxicity and adverse immune reactions. This impairment of liver functions can culminate in cell death, leading to a variety of pathological conditions, including acute hepatitis. Curcumin has been shown to possess a wide spectrum of biological actions. These include anti-inflammatory and anti-oxidant activities. Authors postulated that curcumin, acting through the oxidative stress inhibition, could reduce the production of inflammatory cytokines; thus resulting in the attenuation of liver injury in APAP-induced hepatitis in mice.

Research frontiers

Curcumin (diferuloylmethane) is the main yellow bioactive component of turmeric (*Curcuma longa*). It has been shown to possess a wide spectrum of biological actions by the inhibition of oxidative stress and reduction of inflammatory cytokines. APAP can cause liver injury through the increase in oxidative stress and release of inflammatory cytokines leading to liver injury. The hallmark of this study was that we showed an attenuation of liver damage and decrease in oxidative stress, reduced liver inflammation, restoration of hepatic glutathione (GSH), and improved liver histopathology after curcumin administration in APAP-treated animals.

Innovations and breakthroughs

The previous study showed that curcumin is an anti-inflammatory and anti-oxidant agent in an *in vivo* study. However, it is unclear whether curcumin has any effect in APAP-induced hepatitis *in vivo*. Therefore, in this study, authors examined the protective effects of curcumin in APAP-induced liver damage in mice and authors found that curcumin could attenuate APAP-induced liver injury through the decrease in oxidative stress, reduce liver inflammation, restoration of hepatic GSH, and improve liver histopathology.

Applications

Curcumin might be a novel therapeutic strategy against hepatitis caused by APAP overdose.

Peer review

This is an interesting study of the effects of curcumin on APAP-induced hepatitis in mice. This study showed the effects of curcumin in attenuation of APAP-induced hepatitis reflected in attenuated levels of hepatic malondialdehyde, transaminase, inflammatory cytokines, hepatic GSH, and improved liver histopathology.

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Loss of BRCA1 expression leads to worse survival in patients with gastric carcinoma

Zi-Zhen Zhang, Yuan Jie Charles Liu, Xiao-Lu Yin, Ping Zhan, Yi Gu, Xing-Zhi Ni

Zi-Zhen Zhang, Xing-Zhi Ni, Department of General Surgery, Renji Hospital, School of Medicine, Shanghai Jiaotong University, Shanghai 200127, China

Yuan Jie Charles Liu, Xiao-Lu Yin, Ping Zhan, Yi Gu, AstraZeneca, Innovation Center China, Shanghai 201203, China

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Correspondence to: Xing-Zhi Ni, MD, Professor of Medicine, Department of General Surgery, Renji Hospital, School of Medicine, Shanghai Jiaotong University, Shanghai 200127, China. niyin@yahoo.com

Telephone: +86-21-61092234 Fax: +86-21-61097711

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Abstract

AIM: To investigate the expression deficiency of key molecular markers in the homologous recombination pathway.

METHODS: Expression loss of breast cancer type 1 susceptibility protein (BRCA1), ataxia telangiectasia mutated (ATM), ATM-Rad3-related (ATR), mediator of DNA damage checkpoint protein 1 (MDC1) and meiotic recombination 11 (Mre11) were correlated with their clinicopathological parameters in gastric cancer (GC). One hundred and twenty treatment-naïve GC samples were formalin-fixed and paraffin-embedded into tissue blocks. Two representative cores from each block were extracted and constructed into tissue microarrays. Expression levels of BRCA1, ATM, ATR, MDC1 and Mre11 were determined using immunohistochemical analysis, and correlated with clinical parameters, including age, gender, Lauren subtype, tumor grades, clinical stage and overall survival.

RESULTS: Expression loss of BRCA1, ATM, ATR, MDC1, and Mre11 was found in 21.4%, 20.2%, 21.0%, 11.1% and 4.6%, respectively, of interpretable cases. BRCA1 loss was significantly associated with patients of diffused subtype (intestinal *vs* diffused, 8.2% *vs* 31.7%, $P = 0.001$), higher tumor grade (I/II *vs* III, 10.7% *vs* 20.5%; I/II *vs* IV, 10.7% *vs* 54.5%, $P = 0.047$) and advanced clinical stage (I/II *vs* III, 12.9% *vs* 16.9%; I/II *vs* IV, 12.9% *vs* 45.5%, $P = 0.006$). MDC1 loss was significantly associated with patients of diffused subtype (intestinal *vs* diffused, 0% *vs* 19.7%, $P = 0.001$) and higher tumor grade (I/II *vs* III, 0% *vs* 12%; I/II *vs* IV, 0% *vs* 30.8%, $P = 0.012$). In addition, the survival time of the patients with expression loss of BRCA1 was significantly shorter than those with positive expression of BRCA1 (2-year survival rate, 32.4% *vs* 62.8%, $P = 0.015$). No correlations were found between clinicopathological parameters and expression loss of ATM, ATR and Mre11.

CONCLUSION: Our results support the hypothesis that homologous recombination deficiency plays an important role in the progression of gastric carcinoma. Loss of expression of BRCA1 and MDC1 may serve as predictive factors in tumor development or progression in GC patients.

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Key words: Homologous recombination deficiency; Gastric cancer; Breast cancer type 1 susceptibility protein; Mediator of DNA damage checkpoint protein 1; Ataxia telangiectasia mutated; Ataxia telangiectasia mutated-Rad3-related

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INTRODUCTION

DNA lesions constantly threaten the integrity of our genome. Of the major DNA lesions, double-strand DNA breaks (DSBs) pose the most dangerous threat^[1]. DSBs occur when both complementary strands of DNA break simultaneously, and failure to repair these DSBs can result in chromosomal aberrations including mutations, deletions, amplifications, translocations, all of which can lead to cancer predispositions. Cells employ two major pathways to repair DSBs: homologous recombination (HR) and non-homologous end joining (NHEJ). HR and NHEJ differ mainly in two aspects. First, they differ in the frequency of errors that occur during DSB repairs. NHEJ employs a direct ligation mechanism that is highly error-prone, while HR utilizes the genomic information stored in homologous strands to proof-read the repair process and thus is essentially error-free. Second, the two pathways differ in the cell cycles in which they are primarily involved. NHEJ is most commonly found in G0 and G1 phases; meanwhile HR predominates in S and G2 phases, which are two critical stages that require high-fidelity transmission of genetic information. Attributed to its error-free mechanism and deployment in key cell-cycle phases, HR plays a central role in the protection against DSBs and hence is crucial in maintaining the genomic stability of the cells^[2].

A complex and hierarchical network of proteins is implicated in the HR pathway to detect, signal and repair DSBs. In this network, breast cancer type 1 susceptibility protein (BRCA1), ataxia telangiectasia mutated (ATM), ATM-Rad3-related (ATR), mediator of DNA damage checkpoint protein 1 (MDC1) and meiotic recombination 11 (Mre11) are most important functionally. In brief, ATM/ATR located at the top of the signaling cascades act as the core sensors of DSBs^[3] by collaborating with other sensor molecules, including MDC1^[4] and the complex of MRE11-Rad50-NBS1^[5]. Downstream substrates that are involved in checkpoint activation, among them BRCA1/2^[6], are then phosphorylated by ATM and ATR, causing cell cycle arrest until DSBs are repaired^[7].

Defects in the HR pathway or homologous recombination deficiency (HRD) directly compromise the genomic stability and predispose to cancer formation^[8]. The relationship between HRD and development of many cancer types has been well established^[9]. Genetic aberrations of BRCA1/2, the most widely studied markers in the HR pathway, have been found to promote both tumor initiation and progression^[10,11]. These genetic abnormalities, together with BRCA1/2 protein loss, were reported in many carcinomas^[12], especially in breast cancer (BC) and ovarian cancer (OC). In BC, *BRC1/BRC2* mutations are responsible for 3%-8% of all cases and 30%-40% of familial cases. Ten percent of patients with OC have a genetic predisposition. About 80% of families with a history of OC have *BRC1* mutations, while 15% have *BRC2* mutations^[13]. Aberrations in ATM function are linked with head and neck squamous cell carcinoma^[14], chronic lymphocytic leukemia^[15], colorectal can-

cer^[16], BC and OC^[17]. Genetic alterations of ATR were frequently reported in BC and OC^[18-20]. Dysfunctional MDC1 was implicated in BC development^[21,22] among other cancer types^[23,24]. Abnormal Mre11 signaling is strongly linked with BC, with mutations and protein loss found to be associated with BC pathogenesis^[25-28].

These HRD tumors also demonstrated enhanced sensitivity toward DNA-damaging agents, through the so-called "synthetic lethality"^[29]. These specific populations of tumor cells, under DNA-damaging agents, such as poly (ADP-ribose) polymerase (PARP) inhibitors^[30], are unable to recruit the necessary cellular machinery for the repair of DSBs and will undergo apoptosis. Pre-clinical studies of PARP inhibitors had raised the expectations for this highly selective therapeutic approach in HRD patients although these hypotheses need to be further validated in the clinical studies^[31,32]. Therefore, it is useful to understand the status of HRD-specific markers in different tumor types, such as GC.

GC is the second leading cause of cancer-related death worldwide and is particularly prevalent in Asia. Previous reports suggested that HRD could play a role in the carcinogenesis of the stomach^[33-37]. Yet, HRD's prognostic perspective in GC has not been fully explored and this study aims to address these questions. In order to assess the involvement of HRD in gastric tumorigenesis, we have analyzed the immunohistochemical expression of BRCA1, ATM, ATR, MDC1 and Mre11 in 120 GC samples and correlated them with clinicopathological parameters.

MATERIALS AND METHODS

Clinical samples and patient information

One hundred and twenty formalin-fixed and paraffin-embedded (FFPE) tissue samples were collected from Shanghai Renji Hospital for the study. All patients underwent radical resection between 2007 and 2010. The median age of the patients (82 males and 38 females) was 61.3 years (range: 22-87 years). All tumor tissues were diagnosed with gastric adenocarcinomas by two qualified pathologists.

Immunohistochemistry

GC tumor tissue and adjacent non-tumor tissue samples were collected after surgery following standard FFPE procedure. Tissue microarray (TMA) was then made with 2 representative cores withdrawn from FFPE block for each case. Four μm -thick tissue sections were cut from TMA for immunohistochemical (IHC) study. The slides were baked at 56 °C for 1 h, then de-paraffinized in xylene for 20 min and rehydrated through a graded series of ethanol concentrations (5 min in 100% ethanol first, followed by 5 min in 70% ethanol). Antigen retrieval was done in pressure cooker for 5 min using Target Retrieval Solution (Dako, Copenhagen, Denmark). Endogenous peroxidase activity was blocked by Peroxidase Blocking Reagent (Dako, Copenhagen, Denmark) for 5 min. Primary antibodies (ATM, 1:50, Epitomics, cat. No. 1549-1; ATR,

Table 1 Association between expression loss of homologous recombination markers and clinicopathological parameters in gastric cancer patients *n* (%)

| | BRCA1 expression (<i>n</i> = 112) | | ATM expression (<i>n</i> = 114) | | ATR expression (<i>n</i> = 86) | | MDC1 expression (<i>n</i> = 117) | | Mre11 expression (<i>n</i> = 86) | |
|------------------|---------------------------------------|----------------|-------------------------------------|----------------|------------------------------------|----------------|--------------------------------------|----------------|--------------------------------------|----------------|
| | BRCA1-negative/ total cases | <i>P</i> value | ATM-negative/ total cases | <i>P</i> value | ATR-negative/ total cases | <i>P</i> value | MDC1-negative/ total cases | <i>P</i> value | Mre11-negative/ total cases | <i>P</i> value |
| Age, yr (median) | | | | | | | | | | |
| < 61.3 | 15 (25.0) | 0.043 | 9 (16.1) | 0.133 | 8 (19.5) | 0.193 | 8 (14.3) | 0.625 | 2 (4.9) | 0.119 |
| ≥ 61.3 | 9 (17.3) | | 14 (24.1) | | 10 (22.2) | | 5 (8.2) | | 2 (4.4) | |
| Gender | | | | | | | | | | |
| Male | 15 (19.7) | 0.284 | 15 (19.5) | 0.715 | 12 (21.1) | 0.969 | 10 (12.7) | 0.430 | 3 (5.0) | 0.333 |
| Female | 9 (25.0) | | 8 (21.6) | | 6 (20.7) | | 3 (7.9) | | 1 (3.4) | |
| Lauren type | | | | | | | | | | |
| Intestinal | 4 (8.2) | 0.001 | 12 (24.0) | 0.846 | 8 (21.6) | 0.891 | 0 (0.0) | 0.001 | 2 (5.0) | 0.303 |
| Diffused | 20 (31.7) | | 11 (17.2) | | 10 (20.4) | | 13 (19.7) | | 2 (4.3) | |
| Tumor grade | | | | | | | | | | |
| I / II | 3 (10.7) | 0.047 | 7 (24.1) | 0.513 | 5 (29.4) | 0.327 | 0 (0.0) | 0.012 | 1 (4.2) | 0.742 |
| III | 15 (20.5) | | 15 (20.5) | | 10 (16.7) | | 9 (12.0) | | 3 (5.8) | |
| IV | 6 (54.5) | | 1 (8.3) | | 3 (33.3) | | 4 (30.8) | | 0 (0.0) | |
| Clinical stage | | | | | | | | | | |
| I / II | 4 (12.9) | 0.006 | 6 (19.4) | 0.560 | 6 (23.1) | 0.593 | 3 (9.4) | 0.092 | 2 (7.7) | 0.562 |
| III | 10 (16.9) | | 11 (17.7) | | 7 (16.7) | | 5 (7.7) | | 2 (4.2) | |
| IV | 10 (45.5) | | 6 (28.6) | | 5 (27.8) | | 5 (25.0) | | 0 (0.0) | |

BRCA1: Breast cancer type 1 susceptibility protein; ATM: Ataxia telangiectasia mutated; ATR: ATM-Rad3-related; MDC1: Mediator of DNA damage checkpoint protein 1; Mre11: Meiotic recombination 11.

1:100, Santa-Cruz Technology, cat. No. sc-1887; BRCA1, 1:100, Merck, cat. No. OP92; MDC1, 1:500, Sigma, cat. No. M2444; Mre11, 1:200, Abcam, cat. No. ab214) were then applied to cover the specimen for 1 h at room temperature, followed by incubation with labeled polymer-HRP anti-rabbit or anti-mouse secondary antibody (Dako, Copenhagen, Denmark) for 30 min at room temperature. Thorough rinsing with TBST was done after incubation with each reagent. The slides were visualized using DAB substrate-chromagen (Dako, Copenhagen, Denmark) and washed with deionized water before counterstaining with haematoxylin. The slides were then dehydrated through a graded series of ethanol concentrations, cleared in xylene and coverslipped in DPX mounting medium.

Immunohistochemical scoring

The intensity of the staining in the nuclear of tumor cells was recorded. Scoring was established as follows: 0, if absence of staining was observed; 1+, if the tumor cells had weak staining; 2+, if tumor cells had moderate staining; and 3+, if tumor cells had strong staining. Tumors with 1+, 2+ and 3+ expression were interpreted as positive and tumors with no expression (0 score) were interpreted as expression loss. Given the heterogeneity of protein expression in tumor cells, the highest scoring from either one of TMA cores was counted as the final result.

Statistical analysis

The analysis was conducted with SPSS 16.0 software. Characteristics of the two groups were compared using the χ^2 likelihood ratio test. Logistic regression model was applied to interrogate association of IHC data and individual clinical parameter. The Kaplan-Meier method was used to estimate the survival distributions. The log-rank test was

used to compare the survival distributions. Two-sided *P* values < 0.05 were considered statistically significant.

RESULTS

Among the 120 cases, 69.2% of tumors (83/120) involved the ventricular sinuses, 14.1% (17/120) involved the ventricle corpora and 16.7% (20/120) involved the cardia in the stomach. All tumor samples were diagnosed with adenocarcinoma with different tumor grade and Lauren subtypes.

The overall follow-up rate is 87% with a median follow-up time of 32 mo. At the time of analysis, 49.2% (49/120) patients were alive and 50.8% (61/120) patients died. The overall 2-year survival rate was 54.2%. Loss of BRCA1 expression was observed in 21.4% (24/112), ATM in 20.2% (23/114), ATR in 20.9% (18/86), MDC1 in 11.1% (13/117), and Mre11 in 4.7% (4/86) of the GC patients (Figure 1). Clinicopathological parameters and expression of HRD biomarkers in the samples are displayed in Table 1.

Expression loss of each marker and its correlation with clinicopathological parameters

The clinicopathological parameters of patients in the study included age, gender, Lauren type, tumor grade and clinical stage according to 2010 World Health Organization tumor-node-metastasis classification. Statistical analysis of IHC data and clinicopathological parameters are shown in Table 1. Loss of ATM, ATR and Mre11 expression was not associated with gender or clinical stage. BRCA1 loss was significantly associated with patients of diffused subtype (*P* = 0.001), higher tumor grade (*P* = 0.047) and advanced clinical stage (*P* = 0.006). MDC1

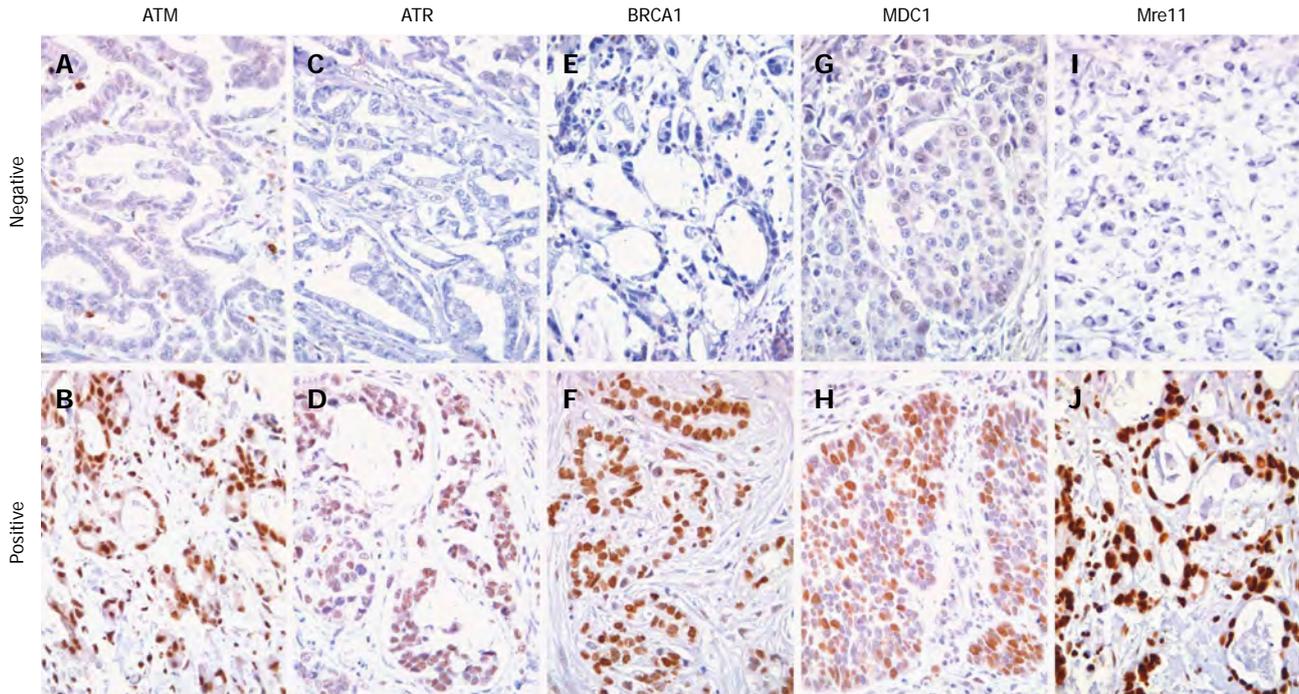


Figure 1 Immunohistochemical expression of ataxia telangiectasia mutated, ataxia telangiectasia mutated-Rad3-related, breast cancer type 1 susceptibility protein, mediator of DNA damage checkpoint protein 1 and meiotic recombination 11 in gastric cancer tissues. ATM: Ataxia telangiectasia mutated; ATR: Ataxia telangiectasia mutated-Rad3-related; BRCA1: Breast cancer type 1 susceptibility protein 1; MDC1: Mediator of DNA damage checkpoint protein 1; Mre11: Meiotic recombination 11. 3,3'-Diaminobenzidine staining, $\times 200$.

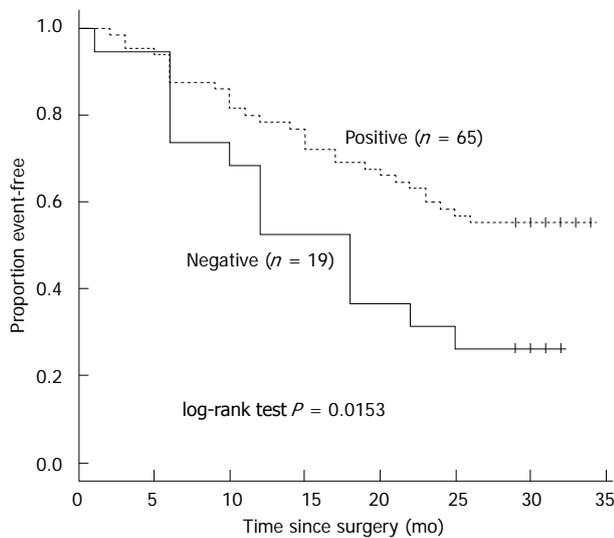


Figure 2 Negative effect of breast cancer type 1 susceptibility protein loss on patient overall survival. The survival time of the patients with positive expression of breast cancer type 1 susceptibility protein (BRCA1) was significantly longer than those with negative expression of BRCA1.

loss was significantly associated with patients of diffused subtype ($P = 0.001$) and higher tumor grade ($P = 0.012$).

Correlation between BRCA1 expression and survival

Expression loss of BRCA1 was significantly associated with the progression of the GC patients. The survival time of the patients with BRCA1 expression loss was

significantly shorter than BRCA1-positive patients (2-year survival rate, 32.4% *vs* 62.8%, $P = 0.015$; Figure 2). Expression of the other four markers was not significantly associated with survival ($P > 0.05$).

Combined biomarker analysis

Twenty-seven (51.9%, 27/52) cases had positive expression of all 5 protein kinases (HR+ group) and 25 (48.1%, 25/52) cases had expression loss of at least one protein kinase (HRD group). Significant difference of tumor grade was observed between the two groups, with the HRD group showing significant association with higher tumor grades ($P = 0.013$). But there was no significant difference in gender, Lauren type or clinical stage. Survival analysis also showed no significant difference between the two groups.

DISCUSSION

Gastric cancer is one of the leading causes of cancer-related death worldwide, and although the incidence has decreased in Western countries, Asia remains the specific high-risk area. Various reports have suggested that HRD could play a role in gastric tumorigenesis. However, a systematic analysis of the key markers in the HR pathway is largely missing. In the present study, the expression losses of the five key markers, namely BRCA1, ATM, ATR, MDC1 and Mre11 were correlated with the clinicopathological parameters in a cohort of Chinese GC patients.

Recent studies of the relationship between BRCA1

and tumors mainly focused on BC and OC. The frequency of BRCA1 mutations among breast cancer patients is less than 5%^[38], while the loss of BRCA1 protein expression is higher at around 20%^[39]. However, BRCA1 expression in gastric cancer has rarely been studied. Our data showed that BRCA1 expression deficiency occurred in 24/112 (21.4%) GC patients. BRCA1 deficiency was significantly associated with patients of diffused Lauren type, higher tumor grades and advanced clinical stage. Patients with BRCA1 deficiency lived significantly shorter ($P = 0.015$) than those patients with positive expression of BRCA1, indicating that loss of BRCA1 can serve as a prognostic marker. Mutations of *BRCA1* in gastric cancer were not found commonly^[40]. Rather, microsatellite instability and loss-of-heterozygosity of *BRCA1* gene at locus D17S855 were shown to be the predominant genetic abnormalities found in GC^[41]. Both of these genetic instabilities may lead to the reduction or loss of the functional BRCA1 protein. Recently, a high frequency of hypermethylation on the BRCA1 promoter was found in tumor tissues and these epigenetic changes correlated with the loss or reduction of protein expression^[42]. These reports together with our data, suggest that BRCA1 protein loss may be a suitable indicator of cancer development in GC.

Lack of reports on *MDC1* mutations suggests that down-regulation of the marker at the protein level may serve as a better prognostic marker. MDC1 protein loss/reduction was previously described^[22], although its correlation with survival was not assessed. Patel *et al.*^[21] addressed this question by profiling MDC1 in subsets of early-stage BC patients who underwent breast-conserving surgery and radiation therapy and found that decreased MDC1 was not related to overall survival. However, they found that MDC1 reduction correlated with nodal failure and concluded the role of MDC1 in early cancer development. To our knowledge, our study is the first to assess MDC1 expression in GCs. The strong association between MDC1 deficiency and diffused subtype indicates that MDC1 plays a major role in this subtype's development. In addition, the association between MDC1 and higher tumor grade also suggests that MDC1 deficiency is implicated in GC pathogenesis. Although MDC1 loss failed to establish a significant correlation with survival, the strong linkage of MDC1 loss with diffused type and higher tumor grades warrants further research into this marker.

Our data suggested ATM, ATR and Mre11 deficiencies were commonly found in GC patients. But there was no significant difference in clinicopathological features between the patients with negative and positive expression for each marker. Mutations of *ATM* have been suggested to play a possible role in the carcinogenesis of other cancer types. The rate of *ATM* mutations in advanced GC has been previously studied and although several variants were found, there were no hot spots. In the same study, decreased level of phosphorylated ATM at Ser1981 significantly correlated with poor differentiation, lymph node metastasis and poor 5-year survival^[43]. Mutation of *ATR* was previously reported in BC^[19], OC^[18] and colon

cancers^[44], but has never been found in GC. In addition, protein loss of ATR has never been studied in GC and we report here for the first time that protein loss of ATR is a common feature in GC. We investigated whether Mre11 mutation could play a role in GC. In a previous study^[45] that correlated MRE11 poly(I)11 mutations with clinicopathological features, a significant association was found only in patients with a family history of GC. In addition, the authors demonstrated that this *MRE11* mutation was associated with absent or strongly reduced Mre11 immunostaining, indicating that protein loss of Mre11 may be a suitable surrogate for the detection of Mre11-related HRD in GC. In our study, the same antibody (Clone 12D7) for the detection of Mre11 was used and the results agreed with those from the previous studies.

In the combined biomarker analysis, we found significant difference in tumor grade between the HR+ and HRD groups, under the assumption that loss of one protein kinase is sufficient to cause a non-functional HR pathway. Our data suggested that HR deficiency played an important role in the GC pathogenesis but is not necessarily crucial in gastric tumor maintenance. Further work will be done to address whether significant association would appear when a larger patient population and a longer follow-up time are available.

These results have made possible the clinical use of DNA-damaging agents in HRD GCs, although finding markers that could predict response is still a daunting challenge^[46]. While most of the PARP inhibitors in BC and OC employed *BRCA1/2*-mutation as the patient selection criteria^[47,48], this may not be the best strategy in GC, as protein loss is evidently the driver. For the other HRD biomarkers, their prognostic and predictive values need to be further investigated. In our opinion, unless they are validated in both pre-clinical and clinical settings, BRCA1 remains the strongest predictor of response to compounds that are exploiting the HRD pathway.

COMMENTS

Background

DNA lesions constantly threaten the integrity of our genome. Of the major DNA lesions, double-strand DNA breaks (DSBs) pose the most dangerous threat. DSBs occur when both complementary strands of DNA break simultaneously, and failure to repair these DSBs can result in chromosomal aberrations, including mutations, deletions, amplifications and translocations, all of which can lead to cancer predispositions.

Research frontiers

Various reports have suggested that homologous recombination deficiency (HRD) could play a role in gastric tumorigenesis. However, a systematic analysis of the key markers in the homologous recombination pathway is largely missing. In the present study, the expression losses of the five key markers, namely breast cancer type 1 susceptibility protein (BRCA1), ataxia telangiectasia mutated, ataxia telangiectasia mutated-Rad3-related, mediator of DNA damage checkpoint protein 1 and meiotic recombination 11, were correlated with the clinicopathological parameters in a cohort of Chinese gastric carcinoma (GC) patients.

Innovations and breakthroughs

The results have made possible the clinical use of DNA-damaging agents in HRD GCs, although finding markers that could predict response is still a daunting challenge.

Applications

For the other HRD biomarkers, their prognostic and predictive values need to be further investigated. In author's opinion, unless they are validated in both pre-clinical and clinical settings, BRCA1 remains the strongest predictor of response to compounds that are exploiting the HRD pathway.

Peer review

This is an interesting study in which authors investigated the expression deficiency of key molecular markers in the HR pathway. The results are interesting and suggest that homologous recombination deficiency plays an important role in the progression of GC.

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Prognostic assessment of different metastatic lymph node staging methods for gastric cancer after D2 resection

Jia Xu, Yu-Hai Bian, Xin Jin, Hui Cao

Jia Xu, Yu-Hai Bian, Xin Jin, Hui Cao, Department of General Surgery, Ren Ji Hospital, Shanghai Jiao Tong University School of Medicine, Shanghai 200127, China

Author contributions: Xu J performed the majority of the research and wrote the first draft; Bian YH and Jin X contributed to the follow-up of patients and were also involved in editing the manuscript; Cao H was the guarantor and designed the study.

Correspondence to: Dr. Hui Cao, Department of General Surgery, Ren Ji Hospital, Shanghai Jiao Tong University School of Medicine, Dongfang Road No. 1630, Shanghai 200127, China. caohuishcn@hotmail.com

Telephone: +86-21-68383751 Fax: +86-21-58394262

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Abstract

AIM: To compare the prognostic assessment of lymph node ratio and absolute number based staging system for gastric cancer after D2 resection.

METHODS: The clinical, pathologic, and long-term follow-up data of 427 patients with gastric cancer that underwent D2 curative gastrectomy were retrospectively analyzed. The relationships between the metastatic lymph node ratio (MLR), log odds of positive lymph nodes (LODDS), and positive lymph nodes (pN) staging methods and the long-term prognoses of the patients were compared. In addition, the survival curves, accuracy, and homogeneity were compared with stratification to evaluate the prognostic assessment of the 3 methods when the number of tested lymph nodes was insufficient (< 10 and 10-15).

RESULTS: MLR [hazard ratio (HR) = 1.401, $P = 0.012$], LODDS (HR = 1.012, $P = 0.034$), and pN (HR = 1.376, $P = 0.005$) were independent risk factors for gastric cancer patients. The receiver operating characteristic (ROC) curves showed that the prognostic accuracy of the 3 methods was comparable ($P > 0.05$). Spearman

correlation analysis confirmed that MLR, LODDS, and pN were all positively correlated with the total number of tested lymph nodes. When the number of tested lymph node was < 10, the value of survival curves staged by MLR and LODDS was superior to those of pN staging. However, the difference in survival curves between adjacent stages was not significant. In addition, the survival rate of stage 4 patients using the MLR and LODDS staging methods was 26.7% and 27.3% with < 10 lymph node, respectively which were significantly higher than the survival rate of patients with > 15 tested lymph nodes (< 4%). The ROC curve showed that the accuracy of the prognostic assessment of MLR, LODDS, and pN staging methods was comparable ($P > 0.05$), and the area under the ROC curve of all 3 methods were increased progressively with the enhanced levels of examined lymph nodes. In addition, the homogeneity of the 3 methods in patients with ≤ 15 tested lymph nodes also showed no significant difference.

CONCLUSION: Neither MLR or LODDS could reduce the staging bias. A sufficient number of tested lymph nodes is key to ensure an accurate prognosis for patients underwent D2 radical gastrectomy.

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Key words: Gastric cancer; Metastatic lymph node ratio; Lymph node metastasis; Prognosis

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INTRODUCTION

Gastric carcinoma is one of the most common cancers in

many Asian countries including South Korea and Japan, and the second most common cause of cancer-related death worldwide^[1]. There are nearly 470 000 newly diagnosed cases every year in China. Of these cases, approximately 75% of the patients will die, making gastric cancer the third leading cause of cancer deaths^[2]. Because of its long-term efficacy, D2 radical gastrectomy has been accepted in most countries, including those in the Europe and the United States, as the standard surgery for gastric cancer^[3-5]. The pathological staging of gastric cancer after D2 radical gastrectomy is not only closely related to the long-term survival of patients but is also the main basis to guide subsequent adjuvant therapy. In the currently accepted criteria of postoperative tumor-node-metastasis (TNM) staging of gastric cancer, the staging of regional lymph node metastasis (N) is of great significance. This staging is currently controversial and changes frequently. In both the latest 7th edition of the American Joint Cancer Committee (AJCC)^[6] and the 14th edition of the Statute of Gastric Cancer Treatment in Japan^[7] in 2010, the absolute number of positive lymph nodes (pN) in the perigastric region was used as the staging basis, and the staging criteria for each stage were unified. Meanwhile, many studies have supported the N staging by computing the metastatic lymph node ratio. Currently, there are 2 main methods in the staging of relative number of positive lymph nodes, the metastatic lymph node ratio (MLR)^[8] and the log odds of positive lymph nodes (LODDS)^[9]. The former calculates the ratio of the number of pN over the total number of the tested lymph nodes, while the latter calculates the log value, $\log[(\text{pnod} + 0.5)/(\text{tnod} - \text{pnod} + 0.5)]$, of the ratio between positive and negative lymph nodes. Previous studies have shown that, especially when the number of the tested lymph nodes was insufficient, the staging of MLR and LODDS could more accurately assess the prognosis of patients with gastric cancer than staging using the absolute value (pN)^[9-13]. However, a unified standard of specific staging for relative number of positive lymph nodes is not currently available, and whether this ratio is superior to the pN staging is also unknown^[14,15]. Therefore, the clinical data and long-term follow-up results of the gastric cancer patients that received D2 radical gastrectomy were retrospectively analyzed in this study, and the values of the above staging methods for regional lymph node metastasis in assessing patient prognosis were compared.

MATERIALS AND METHODS

Clinical data

The clinical data of 427 gastric cancer patients who were admitted and underwent standard D2 radical gastrectomy at Affiliated Renji Hospital, Shanghai Jiaotong University School of Medicine from June 2005 to December 2008 and had complete follow-up data were collected. All patients underwent either distal partial gastrectomy, proximal partial gastrectomy or total gastrectomy with regional lymph nodes dissection to D2 with curative intent

by the same gastrointestinal professional operation team. However, due to the defects of pathological examination, the number of examined lymph nodes of most patients (65.1%) failed to reach the 7th edition of AJCC requirement, which recommended at least 16 lymph nodes should be retrieved for adequate staging. The clinical and pathological data are shown in Table 1. All surviving cases were followed for 39-81 mo with a median follow-up time of 55 mo. The last follow-up was on March 11, 2012. The overall survival rate was 52.5% for all patients. The survival rate was 38.9% for the patients with lymph node metastasis and 80.6% for the patients without lymph node metastasis. The overall median survival time was 44 mo.

Lymph node staging

Of the 427 patients, those without lymph node metastasis were staged as MLR 0. For the remaining patients, the ratio of the number of pN over the number of tested lymph nodes was calculated, and 20 layers were established from 0 to 1 in 5% intervals. The log-rank test was used to compare differences in the survival curves of 2 adjacent layers. The layers with no differences were merged. Finally, based on prognosis, all patients with lymph node metastases were staged MLR 1-4. Similarly, the patients were staged LODDS 0-4 by the log-rank survival test. The pN staging criteria was defined in accordance to the 2010 AJCC/UICC 7th edition TNM staging criteria. The staging criteria and the number of cases for each group are shown in Table 2.

Statistical analysis

The cumulative survival rate was obtained using a Kaplan-Meier curve, and the differences in cumulative survival rates were compared by the log-rank test. The multivariate prognostic analysis was conducted with the Cox proportional risk regression model. The correlation between MLR, LODDS, and pN, as well as the total number of the tested lymph nodes, was analyzed with the Spearman correlation analysis. The accuracy of the prognosis assessment of each staging method was compared using the receiver operating characteristic curve (ROC) and the area under the curve (AUC). The group in each pN stage was re-grouped in accordance with MLR and LODDS, and the overall survival differences within groups and between groups were analyzed using the log-rank survival test to compare the homogeneity of the 3 staging methods. All statistical analyses were completed with SPSS 17.0 software; $P < 0.05$ was considered significant.

RESULTS

Correlation between MLR, LODDS, and pN and the prognosis of patients with gastric cancer

The results of univariate analysis of the correlation between various prognostic factors related to lymph node status and the prognosis of gastric cancer patients after D2 radical gastrectomy showed that the total number of

Table 1 Clinical and histopathological characteristics of the patients

| Factors | n |
|--|--------------|
| Gender (male/female) | 281/146 |
| Age (≤ 60 yr/> 60 yr) | 200/227 |
| Site (antrum/body/fundus/others) | 234/163/22/8 |
| Size (< 3 cm/3-6 cm/≥ 6 cm) | 36/216/175 |
| Histological grade (well/moderately/poorly) | 11/313/103 |
| Depth of invasion (T1/T2/≥ T3) | 4/79/344 |
| Lymphatic/venous invasion (absence/presence) | 359/68 |
| Perineural invasion (absence/presence) | 396/31 |
| Examined lymph nodes (< 10/10-15/> 15) | 126/152/149 |

the tested lymph nodes, MLR, LODDS, and pN staging all had an impact on the patient prognosis (Table 3). When the above factors were individually fitted into the Cox proportional risk model, the results showed that MLR [hazard ratio (HR) = 1.401, *P* = 0.012], LODDS (HR = 1.012, *P* = 0.034), and pN (HR = 1.376, *P* = 0.005) were independent risk factors for the prognosis of patients with gastric cancer.

Comparison between MLR, LODDS, and pN staging methods in the prognostic assessment of gastric cancer patients

The 5-year survival of the 427 patients after surgery was used as the gold standard to draw the ROC curve to compare the accuracy of the 3 staging methods in the prognostic assessment of gastric cancer patients. In the groups with no staging, the corresponding area under the curve for MLR, LODDS, and pN was 0.784 ± 0.022 , 0.790 ± 0.022 , and 0.765 ± 0.023 respectively (Figure 1A), with no significant differences (*P* > 0.05). In the groups with staging, the corresponding areas under the curve for MLR, LODDS, and pN were 0.775 ± 0.023 , 0.767 ± 0.023 , and 0.765 ± 0.023 , respectively (Figure 1B), with no significant differences.

Correlation between the MLR, LODDS, and pN staging methods and the total number of the tested lymph nodes

The results of Spearman correlation analysis showed that MLR, LODDS, and pN staging were all positively correlated with the total number of the tested lymph nodes, with a correlation coefficient of 0.177, 0.053, and 0.410, respectively, and all *P* values were < 0.01, which suggested that all of the 3 staging methods were more or less affected by the total number of tested lymph nodes. pN was positively correlated with MLR and LODDS with a correlation coefficient of 0.919 and 0.871, respectively, and the *P* values were both < 0.001.

Assessment value of the MLR, LODDS, and pN staging methods in patients with an insufficient number of tested lymph nodes

Some previous studies have suggested that, for the patients with an insufficient number of tested lymph nodes,

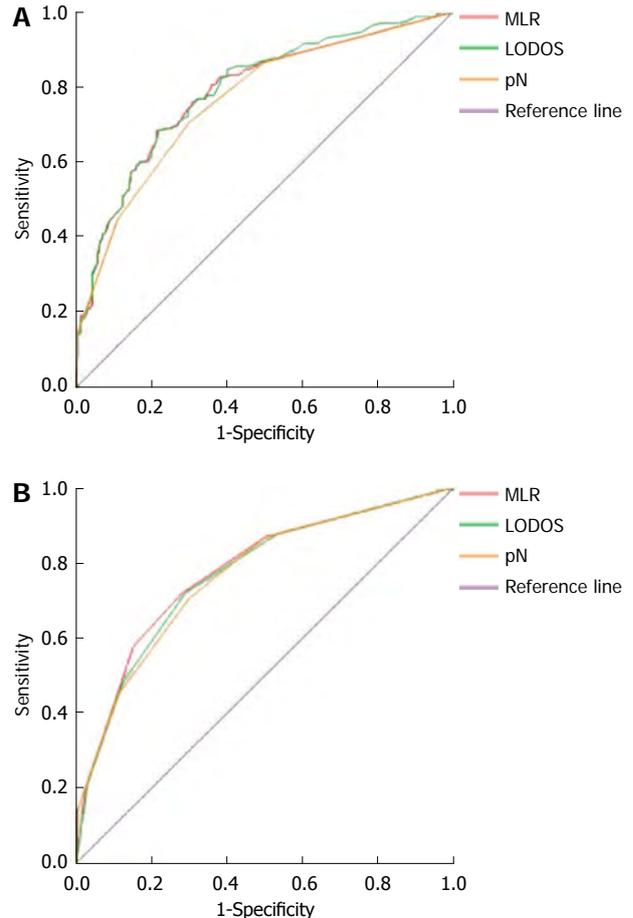


Figure 1 Comparison of receiver operating characteristic curves with metastatic lymph node ratio, log odds of positive lymph nodes, and positive lymph nodes staging methods. A: Receiver operating characteristic (ROC) curves with no staging; B: ROC curves with staging. MLR: Metastatic lymph node ratio; LODDS: Log odds of positive lymph nodes; pN: Positive lymph nodes.

the prognosis-assessment value of MLR staging was superior to that of the staging based on absolute number of positive lymph nodes^[9-12]. Therefore, all patients were divided into 3 subgroups according to the total number of tested lymph nodes: the number of the tested lymph nodes was < 10 (*n* = 126), 10-15 (*n* = 152) or > 15 (*n* = 149). A comparison was performed to compare the differences in the postoperative survival curve, the prognostic accuracy, and the homogeneity of the 3 staging methods in the patients with < 15 tested lymph nodes.

Comparison of survival curves

For the patient group with < 10 tested lymph nodes, the 5-year survival rate of patients exhibited a downward trend with the enhanced levels of MLR and LODDS staging. A log-rank test was conducted to compare the difference between adjacent stages, and the results showed that only the difference in survival curves between stage MLR 0 and MLR 1, and stage LODDS 2 and LODDS 3 was significant, with the *P* values of 0.026 and 0.028 respectively; The difference of remaining survival curves between adjacent stages was not significant. The value of

Table 2 Staging criteria of positive lymph nodes, metastatic lymph node ratio and log odds of positive lymph nodes classifications *n* (%)

| Grade | MLR | LODDS | pN |
|-------|---------------------------|--------------------------|----------------|
| 0 | Nr = 0 139 (32.6) | Nr < -1 129 (30.2) | 0 139 (32.6) |
| 1 | 0 < Nr ≤ 0.2 79 (18.5) | -1 ≤ Nr < -0.5 87 (20.4) | 1-2 78 (18.3) |
| 2 | 0.2 < Nr ≤ 0.4 58 (13.6) | -0.5 ≤ Nr < 0 85 (19.9) | 3-6 94 (22.0) |
| 3/3a | 0.4 < Nr ≤ 0.7 104 (24.4) | 0 ≤ Nr < 0.5 76 (17.8) | 7-15 86 (20.1) |
| 4/3b | 0.7 < Nr ≤ 1 47 (11.0) | Nr ≥ 0.5 50 (11.7) | > 15 30 (7.0) |

MLR: Metastatic lymph node ratio; LODDS: Log odds of positive lymph nodes; pN: Positive lymph nodes.

prognostic assessment based on pN staging system was not satisfactory, and the 5-year survival rate for each pN stage was 76.3% for pN 0, 44.4% for pN 1, 34.5% for pN 2, and 45.5% for pN 3a.

For the patient group with 10-15 tested lymph nodes, no significant difference in survival curves between any adjacent stages was found in the subgroup of MLR. Similar with MLR staging, the difference in survival curves also was not significant between any adjacent stages of LODDS.

For the group of patients with > 15 tested lymph nodes, the 5-year survival rates for each stage between MLR, LODDS and were comparable. However, the survival curves of pN staging appeared to better assess prognosis than the ratio-based staging methods, with significant difference in survival curves between any various stages (*P* < 0.05; Figure 2).

Comparison of the accuracy of prognostic assessment

The ROC curves showed that, regardless of staging, the corresponding areas under the curves of the MLR, LODDS, and pN staging methods were all increased progressively with the enhanced levels of examined lymph nodes. the AUC using the MLR, LODDS and pN staging methods increased from 0.716 ± 0.047, 0.718 ± 0.046 and 0.688 ± 0.048 with < 10 lymph node to 0.843 ± 0.031, 0.818 ± 0.034 and 0.836 ± 0.032 with > 15 tested lymph nodes, which were significantly larger than former groups. However, the AUC was not significantly different between the 3 methods within groups.

Comparison of the homogeneity of prognostic assessment

The various pN groups in which patients had < 10 or 10-15 tested lymph nodes were re-grouped according to MLR staging, and the results were shown in Table 4. When the numbers of retrieved lymph nodes were less than 10, only for patients in stage pN 1, the difference in the 5-year survival rate among different MLR stages was significant (*P* < 0.05). Furthermore, there was no significant difference in the 5-year survival rate among the different pN groups within the one MLR group. In 10-15 retrieved-node group, there was no significant difference of 5-year survival rates between the different MLR groups in one pN group. In addition, the difference of

Table 3 Univariate analysis of various prognostic factors correlated to retrieved lymph nodes *n* (%)

| Variable | 5-yr survival rate | Log rank χ^2 value | <i>P</i> value |
|----------------------|--------------------|-------------------------|----------------|
| Examined lymph nodes | | | |
| < 10 | 126 (29.5) | 57.1% | 4.256 |
| 10-15 | 152 (35.6) | 55.9% | |
| > 15 | 149 (34.9) | 45.0% | |
| pN | | | |
| 0 | 139 (32.6) | 80.6% | 97.014 |
| 1-2 | 78 (18.3) | 57.7% | |
| 3-6 | 94 (22.0) | 44.7% | |
| 7-15 | 86 (20.1) | 27.9% | |
| > 15 | 30 (7.0) | 3.3% | |
| MLR | | | |
| Nr = 0 | 139 (32.6) | 80.6% | 103.984 |
| 0 < Nr ≤ 0.2 | 79 (18.5) | 62.0% | |
| 0.2 < Nr ≤ 0.4 | 58 (13.6) | 50.0% | |
| 0.4 < Nr ≤ 0.7 | 104 (24.4) | 26.9% | |
| 0.7 < Nr ≤ 1 | 47 (11.0) | 12.8% | |
| LODDS | | | |
| Nr < -1 | 129 (30.2) | 80.6% | 96.214 |
| -1 ≤ Nr < -0.5 | 87 (20.4) | 63.2% | |
| -0.5 ≤ Nr < 0 | 85 (19.9) | 43.5% | |
| 0 ≤ Nr < 0.5 | 76 (17.8) | 27.6% | |
| Nr ≥ 0.5 | 50 (11.7) | 14.0% | |

MLR: Metastatic lymph node ratio; LODDS: Log odds of positive lymph nodes; pN: Positive lymph nodes.

5-year survival rates between different pN groups in one MLR group was also not significant.

Because the staging of the patients in MLR 0 (no pN detected) was the same as in pN 0, some studies have stated that the prognostic assessment of LODDS staging was more accurate for these patients^[9,16]. Therefore, when comparing the homogeneity of pN staging and LODDS staging, according to different numbers of the tested lymph nodes, the stage pN 0 was divided into two layers. The results showed that the pN0 patients with < 10 tested lymph nodes could be further staged LODDS 0-2, and the 5-year survival rate for the 3 stages was 81.8%, 70.0%, and 66.7%, respectively (Figure 3). However, the log-rank survival test showed that the differences between the 3 stages were not significant (*P* = 0.476). Furthermore, the pN 0 patients with 10-15 tested lymph nodes had the same LODDS stage. Generally, the difference in the 5-year survival rates between the different LODDS groups in one pN group was not significant. In addition, the difference in the 5-year survival rates between the different pN groups in one LODDS group was also not significant (Table 5).

DISCUSSION

Due to the decent long-term survival rate, surgical resection, represented by standard D2 radical gastrectomy, is currently the preferred treatment for gastric cancer. However, in recent years, with the rise of the concept of individualized treatment and the application of new adjunct treatment in clinical practice, an accurate prognostic assessment of patients with gastric cancer after surgery

Table 4 Five-year overall survival of patients with ≤ 15 tested lymph nodes based on positive lymph nodes and metastatic lymph node ratio staging system

| | | MLR 0 | | MLR 1 | | MLR 2 | | MLR 3 | | MLR 4 | | χ^2 | P value |
|----------|----------|-------|--------|-------|--------|-------|--------|-------|--------|-------|--------|----------|---------|
| | | n | 5-YSR | | |
| < 10 LN | pN 0 | 59 | 76.30% | - | - | - | - | - | - | - | - | - | - |
| | pN 1 | - | - | 8 | 50% | 14 | 57.10% | 2 | 0% | 3 | 0% | 22.293 | 0 |
| | pN 2 | - | - | - | - | 4 | 50% | 22 | 31.80% | 3 | 33.30% | 0.51 | 0.775 |
| | pN 3a | - | - | - | - | - | - | 3 | 66.70% | 8 | 37.50% | 0.78 | 0.377 |
| | χ^2 | - | - | - | - | - | 0.258 | - | 9.278 | - | 1.658 | - | - |
| | P | - | - | - | - | - | 0.611 | - | 0.098 | - | 0.437 | - | - |
| 10-15 LN | pN 0 | 56 | 80.40% | - | - | - | - | - | - | - | - | - | - |
| | pN 1 | - | - | 24 | 62.50% | - | - | - | - | - | - | - | - |
| | pN 2 | - | - | 1 | 100% | 20 | 50% | 18 | 44.40% | - | - | 0.755 | 0.686 |
| | pN 3a | - | - | - | - | - | - | 23 | 17.40% | 10 | 20% | 0.068 | 0.794 |
| | χ^2 | - | - | 0.836 | - | - | - | - | 3.613 | - | - | - | - |
| | P | - | - | 0.658 | - | - | - | - | 0.057 | - | - | - | - |

MLR: Metastatic lymph node ratio; 5-YSR: 5-year survival rate; LN: Examined lymph nodes; pN: Positive lymph nodes.

Table 5 Five-year overall survival of patients with ≤ 15 tested lymph nodes based on positive lymph nodes and log odds of positive lymph nodes staging system

| | | LODDS 0 | | LODDS 1 | | LODDS 2 | | LODDS 3 | | LODDS 4 | | χ^2 | P value |
|----------|----------|---------|--------|---------|--------|---------|--------|---------|--------|---------|-------|----------|---------|
| | | n | 5-YSR | |
| < 10 LN | pN 0 | 33 | 81.80% | 20 | 70% | 6 | 66.70% | - | - | - | - | 1.486 | 0.476 |
| | pN 1 | - | - | 10 | 50% | 12 | 58.30% | 4 | 0 | 1 | 0 | 22.349 | 0 |
| | pN 2 | - | - | - | - | 14 | 42.90% | 13 | 30.80% | 2 | 0 | 4.202 | 0.122 |
| | pN 3a | - | - | - | - | - | - | 3 | 66.70% | 8 | 37.5% | 0.78 | 0.377 |
| | χ^2 | - | - | 1.44 | - | 0.969 | - | 5.689 | - | 1.083 | - | - | - |
| | P | - | - | 0.23 | - | 0.619 | - | 0.128 | - | 0.582 | - | - | - |
| 10-15 LN | pN 0 | 56 | 80.40% | - | - | - | - | - | - | - | - | - | - |
| | pN 1 | - | - | 24 | 62.50% | - | - | - | - | - | - | - | - |
| | pN 2 | - | - | 4 | 75% | 23 | 43.50% | 12 | 50% | - | - | 1.241 | 0.538 |
| | pN 3a | - | - | - | - | 2 | 0% | 19 | 15.80% | 12 | 25% | 3.413 | 0.182 |
| | χ^2 | - | - | 0.222 | - | 6.785 | - | 3.614 | - | - | - | - | - |
| | P | - | - | 0.638 | - | 0 | - | 0.057 | - | - | - | - | - |

LODDS: Log odds of positive lymph nodes; 5-YSR: 5-year survival rate; LN: Examined lymph nodes; pN: Positive lymph nodes.

is essential to the development of relevant follow-up treatment strategies^[17,18]. Currently, the postoperative pathological TNM staging is accepted and widely applied as the prognostic evaluation indicator in clinical practice. With regard to the N portion of the TNM staging, there has been considerable controversy ranging from earlier staging based on anatomical sites of metastatic lymph nodes^[19] to the specific staging criteria based on the number of regional metastatic lymph nodes^[20,21]. The N staging criteria were not unified until the 7th edition of the AJCC^[4] and the 14th edition of the Statute of Gastric Cancer Treatment in Japan^[5] unified the criteria for the first time in 2010. However, many researchers still believe that when the staging is based on the absolute number of metastatic lymph nodes, the number of pN is easily influenced by the numbers of removed and tested lymph nodes. When the number of tested lymph nodes is insufficient, staging bias may occur, affecting the accuracy of the prognostic assessment^[22,23]. The N staging based on MLR can overcome the above shortcomings^[24,25]. Therefore, when comparing the prognostic assessment of dif-

ferent lymph node metastasis staging methods in gastric cancer patients after D2 radical gastrectomy, this study focused on the impact of the 3 staging methods on long-term survival rate when the number of pathologically tested lymph nodes after surgery was insufficient.

To date, neither MLR nor LODDS staging has accurate and widely accepted criteria; therefore, the log-rank survival test was first conducted to verify the staging criteria of MLR and LODDS (Table 2). The 5-year survival rates of various stages according to the above criteria were similar to those of the corresponding pN stages (TNM staging criteria in the 7th edition of AJCC/UICC). The correlation analysis of the 3 staging methods also showed that MLR and LODDS were significantly positively correlated with pN. The ROC curves also showed that the accuracy of prognosis assessment of the 3 staging methods in gastric cancer patients was not significantly different. The subsequent univariate and multivariate analyses both showed that the MLR, LODDS, and pN staging methods were all closely related to patient prognosis-they were all independent risk factors for the

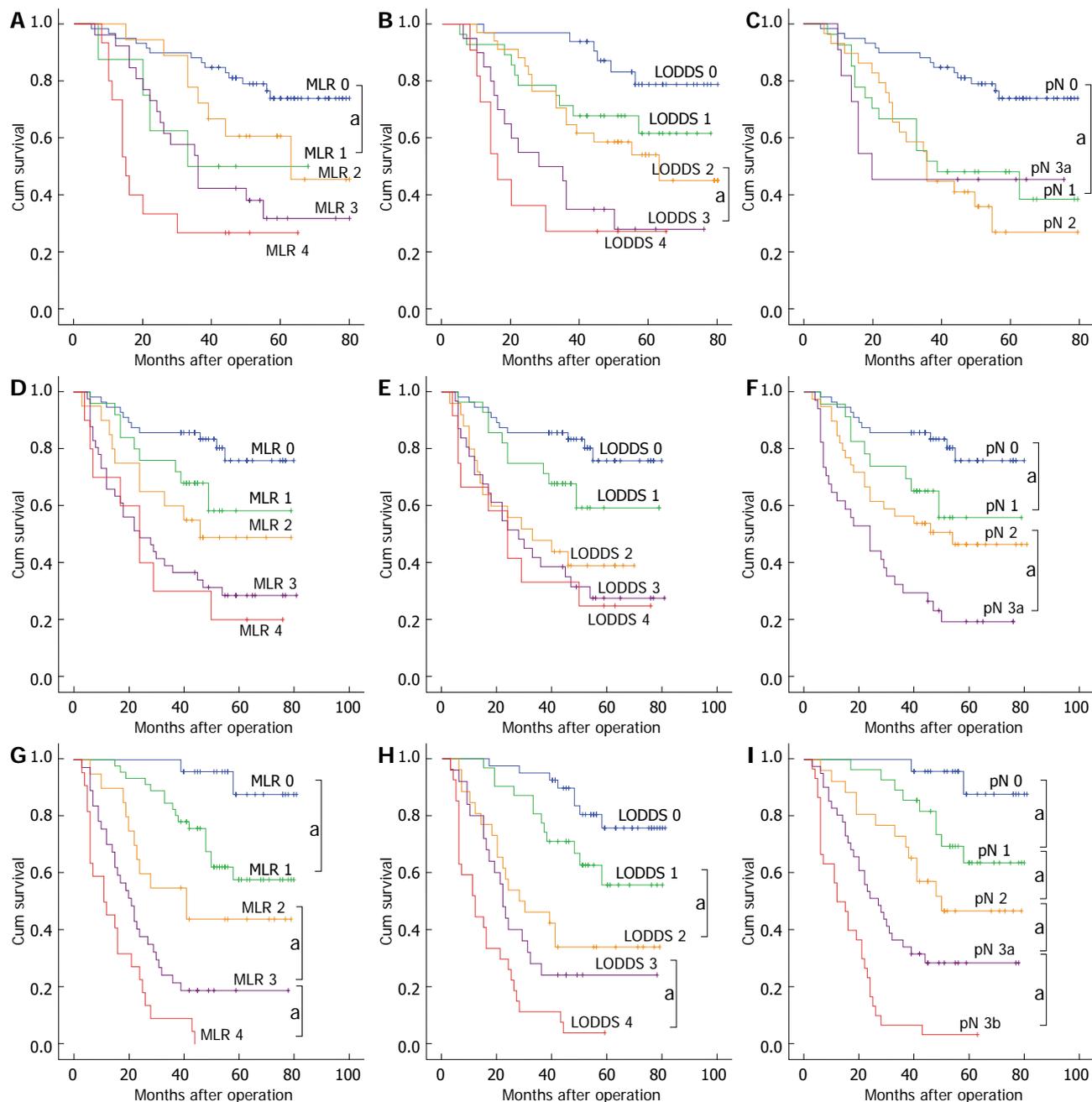


Figure 2 Comparison of survival curves in 3 staging system according to the number of lymph nodes retrieved. ^a $P < 0.05$ between adjacent stages. A: MLR, LN < 10; B: LODDS, LN < 10; C: pN, LN < 10; D: MLR, $10 \leq \text{LN} \leq 15$; E: LODDS, $10 \leq \text{LN} \leq 15$; F: pN, $10 \leq \text{LN} \leq 15$; G: MLR, LN > 15; H: LODDS, LN > 15; I: pN, LN > 15. MLR: Metastatic lymph node ratio; LODDS: Log odds of positive lymph nodes; pN: Positive lymph nodes; LN: Examined lymph nodes.

prognoses of gastric cancer patients. The above results suggest that MLR, LODDS, and pN staging methods can all be used for the prognostic assessment of gastric cancer, and the assessment efficacies of the 3 methods were similar.

Although the total number of tested lymph nodes in the Cox proportional risk regression model was not a significant independent risk factor for patient prognosis, univariate analysis showed that as the number of tested lymph nodes increased, the 5-year survival rate of patients exhibited a downward trend ($P = 0.039$); moreover, a correlation analysis showed that MLR,

LODDS, and pN were all positively correlated with the number of tested lymph nodes. When only the correlation coefficient of the number of tested lymph nodes was considered ($pN > \text{MLR} > \text{LODDS}$), the impact of the number of tested lymph nodes on the MLR and LODDS was smaller than that of the absolute number of pN, which suggests that compared with pN staging system, the MLR and LODDS were less affected by the total number of tested lymph nodes. The subsequent results of the survival curve of patients with insufficient tested lymph nodes also showed that, when the number of tested lymph node was < 10 , the MLR and LODDS

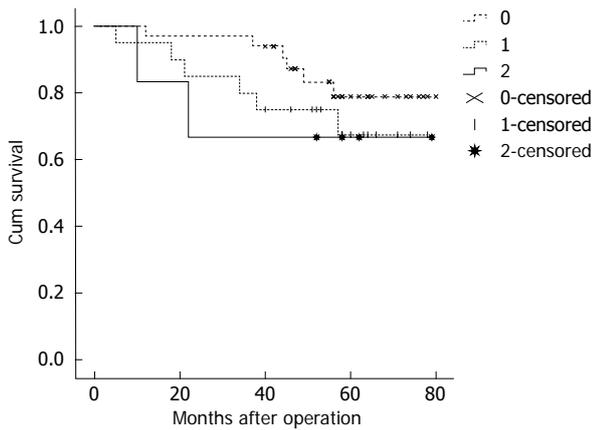


Figure 3 Survival curve of no positive lymph nodes patients with < 10 tested lymph nodes re-staged with the log odds of positive lymph nodes staging method.

staging methods appeared to better assess prognosis than pN staging. However, as shown in Figure 2, although the MLR and LODDS staging methods could more accurately assess the 5-year postoperative survival rate of gastric cancer patients at the early-middle stages (stage 0-2), the difference in the prognostic assessment of the patients at middle-late stages (stage 3 and 4) was not significant. The main reason was that, although the ratio could reduce the impact of sampling error compared with the absolute number, as the number of pN increased, the impact of the sampling error due to the insufficient number of tested lymph nodes also increased. Therefore, the difference in the prognoses of patients in the middle-late stages was not significant. In addition, for the patients in the middle-late stages of MLR and LODDS, especially those in stage 4, the 5-year survival rates were all $\geq 20\%$, which was significantly higher than that of the patients with > 15 ($< 4\%$) tested lymph nodes. The reason for this result may be that when the total number of tested lymph nodes was insufficient, the sampling sites were too concentrated near the lesion; therefore, the ratio of pN was higher, resulting in an overestimation of the actual pathological staging of patients. Moreover, the comparison of the 5-year survival rate of the 3 methods in patients with ≤ 15 tested lymph nodes also confirmed that the accuracy and homogeneity of the staging methods based on the MLR, LODDS or the absolute number of pN were similar, with no significant difference. At the same time, the comparison of the survival curve and ROC curve of the 3 staging methods in the < 10 , $10-15$ and > 15 group showed that the difference in the 5-year survival rate between stages and the assessment accuracy of survival rate were all increased progressively with the enhanced levels of examined lymph nodes, and the 3 staging methods exhibited no significant difference. The above results all confirmed that, regardless of the staging method, a sufficient number of tested lymph nodes was the key factor. When the number of tested lymph nodes was ≤ 15 , the staging based on MLR or LODDS could not compensate for the inadequacy of pN staging, and thus could not ac-

curately assess patient prognosis.

Although the number of cases did not affect the results of the statistical analysis significantly, it could be observed from the survival curve that the LODDS staging method appeared to better assess prognosis for patients at MLR and pN stage 0 with an insufficient number of tested lymph nodes. However, the advantage of the LODDS staging method was only apparent when the number of tested lymph nodes was < 10 . Additionally, because the 5-year survival rate for patients in stage pN0 was relatively high, the survival rates of patients in various LODDS stages were not significantly different after re-staging. Finally, the calculation method of LODDS was complicated. All of the above factors limited the practical application of LODDS staging method, and the practical value was low.

Some studies have found that the staging methods based on the MLR and LODDS could more accurately predict the prognosis of gastric cancer patients than the staging method based on the absolute number (pN), especially when the number of tested lymph nodes was insufficient^[9-13]. The above conclusions in this study appeared to be inconsistent with those previous findings. Different surgical methods may be the main cause of the contradictory findings^[26]. In a study recently published in *Annals of Surgery* in 2012^[13], the postoperative clinical, pathologic and follow-up data of 18 043 gastric cancer patients retrieved from the Surveillance, Epidemiology, and End Results database of United States were retrospectively analyzed, and the results showed that when the number of tested lymph nodes was insufficient, the MLR staging method could more accurately assess the patient prognosis than the pN staging method. However, only 10% of the patients in this study underwent D2 radical gastrectomy, and the scope of lymph node removal in the remaining patients was D1 or below. The insufficient number of tested lymph nodes was mainly limited by the scope of lymph node removal. Some studies have confirmed that the average number of removed lymph nodes during D2 radical gastrectomy could reach 32^[27]. The smaller the number of tested lymph nodes, the greater the sampling error. A sufficient number of tested lymph nodes is key to reducing sampling error. Therefore, to accurately assess the prognosis of patients after D2 radical gastrectomy, no staging method can replace a sufficient number of tested lymph nodes.

In summary, the MLR, LODDS and pN are all independent risk factors for the long-term postoperative survival of gastric cancer patients. The accuracy of the prognostic assessment of the MLR and LODDS staging methods is comparable to that of the pN staging method in gastric cancer patients. However, for the patients that undergo a D2 radical gastrectomy, when the number of tested lymph nodes is insufficient (≤ 15), neither the staging method based on metastatic lymph node ratio nor the pN staging method can avoid staging bias. Therefore, as D2 radical gastrectomy is increasingly accepted, a sufficient number of tested lymph nodes is the only key to

ensure an accurate prediction of gastric cancer patient prognosis.

COMMENTS

Background

Gastric cancer is one of the leading fatal malignancies worldwide. D2 radical gastrectomy has been accepted in most countries as the standard surgery for gastric cancer. In the currently widely applied criteria of postoperative tumor-node-metastasis staging system, the staging of regional lymph node metastasis (N) is of great significance for accurate prognosis-assessment.

Research frontiers

Currently, the metastatic lymph node ratio has been considered as an alternative to the absolute number of positive lymph nodes. Although the 7th edition of the American Joint Cancer Committee and the 14th edition of the Statute of Gastric Cancer Treatment in Japan unified the pN staging criteria for the first time in 2010, many researchers still believe that the prognostic assessment of ratio staging was superior to that of the staging based on absolute number of positive lymph nodes, which demands the examination of at least 15 lymph nodes.

Innovations and breakthroughs

The clinical, pathologic, and long-term follow-up data of 427 patients underwent D2 radical gastrectomy were stratified and compared to evaluate the prognostic assessment of the 3 metastatic lymph node staging methods. The findings from this study suggested that neither metastatic lymph node ratio nor log odds of positive lymph nodes could avoid the staging bias due to the insufficient number of tested lymph nodes for patients underwent D2 radical gastrectomy.

Applications

This study compared three lymph node based N staging systems for gastric cancer patients with radical resection and D2 lymphadenectomy, and then demonstrated that a sufficient number of tested lymph nodes was key to ensure an accurate prognosis-assessment. The results are clinical significance in the prognostic assessment of patients with gastric cancer after surgery.

Peer review

This is a retrospective research of 427 gastric cancer patients undergoing radical resection plus D2 lymphadenectomy, with a median follow-up of 55 mo. The authors have analyzed patient outcomes in considerable depth, their data is well characterized. They provide an in depth analysis of factors contributing to survival and have utilized multivariate analysis in doing this. The information in the manuscript is highly relevant and useful.

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Effect evaluation of interleukin-1 receptor antagonist nanoparticles for mesenchymal stem cell transplantation

Xiao-Lei Shi, Wei Zhu, Jia-Jun Tan, Jiang-Qiang Xiao, Lin Zhang, Qian Xu, Zheng-Liang Ma, Yi-Tao Ding

Xiao-Lei Shi, Jia-Jun Tan, Jiang-Qiang Xiao, Yi-Tao Ding, Department of Hepatobiliary Surgery, the Affiliated Drum Tower Hospital of Nanjing University Medical School, Nanjing 210008, Jiangsu Province, China

Xiao-Lei Shi, Yi-Tao Ding, Key Medical Center of Jiangsu Province for Hepatobiliary Diseases, Nanjing 210008, Jiangsu Province, China

Wei Zhu, Gulou Clinical Medical College of Combined TCM and Western Medicine, Nanjing University of Chinese Medicine, Nanjing 210008, Jiangsu Province, China

Wei Zhu, Zheng-Liang Ma, Department of Anesthesiology, the Affiliated Drum Tower Hospital of Nanjing University Medical School, Nanjing 210008, Jiangsu Province, China

Lin Zhang, Qian Xu, School of Public Health, Southeast University, Nanjing 210009, Jiangsu Province, China

Author contributions: Shi XL and Zhu W contributed equally to this work; Shi XL, Zhu W, Ma ZL and Ding YT designed the research; Zhu W and Tan JJ wrote the paper; Xiao JQ, Zhang L and Xu Q performed the research; Tan JJ and Xu Q did data analysis.

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Correspondence to: Yi-tao Ding, Professor, Department of Hepatobiliary Surgery, the Affiliated Drum Tower Hospital of Nanjing University Medical School, No. 321 Zhongshan Road, Nanjing 210008, China. yitaoding@hotmail.com

Telephone: +86-25-83105502 Fax: +86-25-83107080

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Abstract

AIM: To study the efficacy of marrow mesenchymal stem cells (MSCs) transplantation combined with interleukin-1 receptor antagonist (IL-1Ra) for acute liver failure (ALF).

METHODS: Chinese experimental miniature swine were randomly divided into four groups ($n = 7$), and all animals were given D-galactosamine (D-gal) to induce ALF. Group A animals were then injected with 40 mL saline *via* the portal vein 24 h after D-gal induction;

Group B animals were injected with 2 mg/kg IL-1Ra *via* the ear vein 18 h, 2 d and 4 d after D-gal induction; Group C received approximately 1×10^8 green fluorescence protein (GFP)-labeled MSCs (GFP-MSCs) suspended in 40 mL normal saline *via* the portal vein 24 h after D-gal induction; Group D animals were injected with 2 mg/kg IL-1Ra *via* the ear vein 18 h after D-gal induction, MSCs transplantation was then carried out at 24 h after D-gal induction, and finally 2 mg/kg IL-1Ra was injected *via* the ear vein 1 d and 3 d after surgery as before. Liver function, serum inflammatory parameters and pathological changes were measured and the fate of MSCs was determined.

RESULTS: The optimal efficiency of transfection (97%) was achieved at a multiplicity of infection of 80, as observed by fluorescence microscopy and flow cytometry (FCM). Over 90% of GFP-MSCs were identified as CD44+ CD90+ CD45- MSCs by FCM, which indicated that most GFP-MSCs retained MSCs characteristics. Biochemical assays, the levels of serum inflammatory parameters and histological results in Group D all showed a significant improvement in liver injury compared with the other groups ($P < 0.05$). The number of GFP-MSCs in Group D was also greater than that in Group B, and the long-term cell proliferation rate was also better in Group D than in the other groups.

CONCLUSION: MSCs transplantation is useful in ALF, IL-1Ra plays an important role in alleviating the inflammatory condition, and combination therapy with MSCs transplantation and IL-1Ra is a promising treatment for ALF.

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Key words: Interleukin-1 receptor antagonist; Mesenchymal stem cells; Cell transplantation; Acute liver failure; Inflammatory environment

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INTRODUCTION

Acute liver failure (ALF) is defined by the development of coagulopathy and encephalopathy within a short period of time in patients with no previous history of liver disease^[1]. The essence of ALF is severe inflammation leading to cell necrosis in a large number of liver cells caused by paracetamol, idiosyncratic drug reactions, hepatitis B, or seronegative hepatitis^[2,3]. The key to the treatment of ALF is the reduction of liver cell necrosis and the stimulation of liver cell regeneration.

Liver transplantation is the only efficient treatment for ALF; however, difficulties including severe donor shortage, numerous complications, immunological rejection and high medical costs limit its use^[1,4]. Mesenchymal stem cells (MSCs) due to their sufficient source, low immunogenicity and the potential ability for differentiation into hepatocyte-like cells make MSCs transplantation a promising treatment for ALF^[5-7]. In order to achieve better results, we transplanted MSCs into a pig model of acute liver failure in addition to interleukin-1 receptor antagonist (IL-1Ra) injection which is used to improve liver inflammation^[8,9]. IL-1 is primarily a proinflammatory cytokine due to its ability to stimulate the expression of a number of inflammation-associated genes through the IL-1 signaling cascade^[10-12]. IL-1Ra can bind to the IL-1 receptor and blocks IL-1 action through competitive inhibition, but will not initiate the IL-1 signaling cascade due to its IL-1-like structure which will not induce a signal at all^[8]. In this study, we evaluated the efficiency of combination therapy with IL-1Ra and MSCs transplantation for the treatment of ALF in swine.

MATERIALS AND METHODS

Animals

Chinese experimental miniature swine (10 ± 3 kg, aged approximately 5 to 8 mo) were obtained from the Laboratory Animal Centre of the Affiliated Drum Tower Hospital of Nanjing University Medical School. All experiments were approved by the Institutional Animal Care and Use Committee.

In vitro experiment

MSCs were isolated by density gradient centrifugation from pig bone marrow and cultured in L-Dulbecco's modified Eagle's medium supplemented with 10% foetal bovine serum, 100 U/mL penicillin, and 100 µg/mL streptomycin (all from Gibco BRL, Grand Island, NY, United States). The cells were then transfected with a lentiviral vector carrying the gene encoding green fluorescence protein (GFP), and the multiplicity of infection

(MOI) of transfection was determined by fluorescent inverted phase contrast microscopy and flow cytometry (FCM). The surface markers (CD44, CD45, and CD90) of GFP-labeled MSCs (GFP-MSCs) were identified by FCM for their MSCs characteristics.

ALF model

The animals received a single intravenous injection of D-galactosamine (D-gal) 0.3 g/kg to induce experimental hepatic injury.

Experimental groups and treatments

Twenty-eight pigs were randomly divided into four groups, Group A (control, $n = 7$), Group B (IL-1Ra, $n = 7$), Group C (MSCs transplantation, $n = 7$), Group D (combined therapy, $n = 7$). Group A received 40 mL normal saline *via* the portal vein 24 h after D-gal induction; Group B received 2 mg/kg IL-1Ra (Institute of Process Engineering, Chinese Academy of Sciences, China) *via* the ear vein 18 h, 2 d and 4 d after D-gal induction; Group C received approximately 1×10^8 GFP-MSCs suspended in 40 mL normal saline *via* the portal vein 24 h after D-gal induction; Group D received 2 mg/kg IL-1Ra *via* the ear vein 18 h after D-gal induction, MSCs transplantation was carried out 24 h after D-gal induction, and finally 2 mg/kg IL-1Ra was injected *via* the ear vein 1 d and 3 d after surgery as before. Liver function, and inflammatory cytokines IL-1β, IL-2 and tumor necrosis factor α (TNF-α) were measured using enzyme-linked immuno sorbent assay Kits (Corbett Life Science, Australia) pre-operatively, intra-operatively, 1-6 d and 1-4 wk after surgery.

Histological analysis

Swine were humanely killed for histological examination at 3 d and every week after surgery. Liver tissues were immersion fixed, embedded in paraffin and sectioned at 5 µm, and the slices were submitted for hematoxylin and eosin (HE) and anti-Ki67 (Abcam Ltd., United Kingdom) staining. To determine liver cell proliferation, six high-powered fields of vision were obtained for each slice, and the average number of Ki67⁺ cells was used for statistical analysis. To trace transplanted MSCs, cells expressing GFP were analyzed by fluorescent microscopy and conventional immunohistochemistry using anti-GFP (Abcam Ltd., United Kingdom).

Statistical analysis

All statistical analysis were performed using SPSS 16.0. The data are reported as mean ± SD. The statistical significance was analyzed by two-way analysis of variance. $P < 0.05$ was considered to denote statistical significance.

RESULTS

In vitro experiment

The optimal efficiency of transfection (97%) was achieved at an MOI of 80, as observed by fluorescence microscopy (Figure 1A and B) and FCM (Figure 1C). Over 90% of

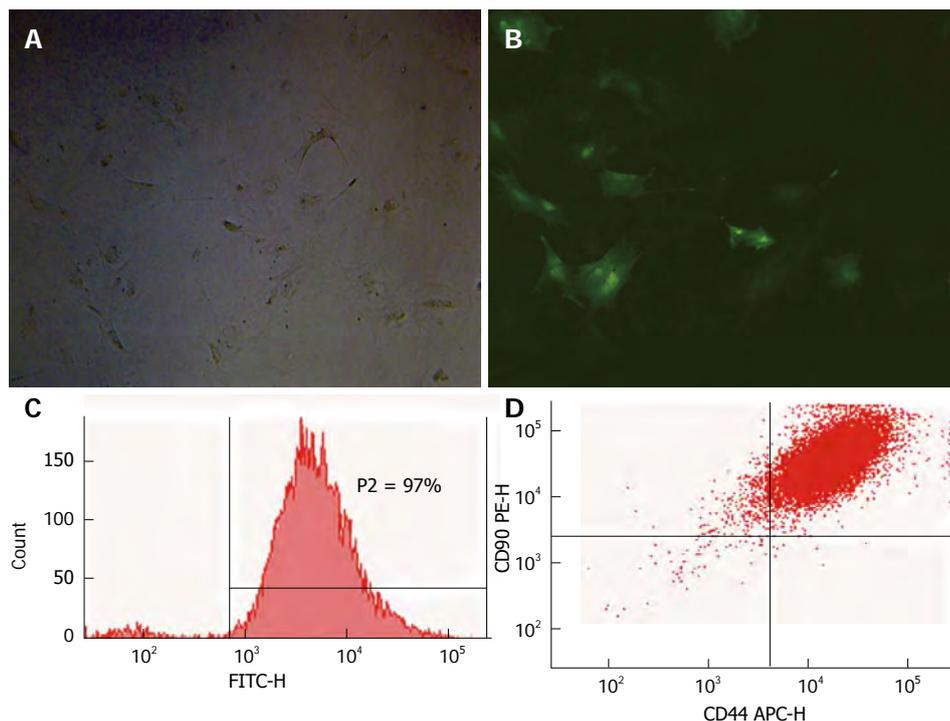


Figure 1 Mesenchymal stem cells transfected with a lentiviral vector carrying the gene encoding green fluorescence protein *in vitro*. A: GFP-MSCs cultivated for 3 d and observed by light microscopy ($\times 400$); B: GFP-MSCs with green fluorescence ($\times 400$); C: Over 97% of GFP-MSCs successfully expressed GFP after propagation; D: Most GFP-MSCs were identified as CD44⁺ CD90⁺. GFP: Green fluorescence protein; MSCs: Mesenchymal stem cells; GFP-MSCs: GFP-labeled MSCs.

GFP-MSCs were identified as CD44⁺ CD90⁺ CD45⁻ MSCs (Figure 1D) by FCM, which indicated that most GFP-MSCs retained MSCs characteristics.

Liver function

Dramatic changes in alanine aminotransferase (ALT), aspartate aminotransferase (AST), total bilirubin (TB), ammonia and prothrombin time (PT) demonstrated that acute liver injury was successfully achieved by D-gal induction in all four groups. Highly significant increases in ALT, AST, TB, ammonia and PT were found in all groups within 48 h after D-gal injection, and then gradually declined. The most significant improvements were found in Group D following combination therapy compared with the other groups; ALT and ammonia in Group D were significantly lower than those in Group A ($P < 0.05$) within 1 to 4 d after combination therapy, and the low level of TB in Group D lasted longer than that in Group A ($P < 0.05$) and Group B ($P < 0.05$) from the first day to 1 wk, the improvement in ALB in Group D appeared the second day after treatment; Group B showed a significant reduction in ALT level within 1 to 3 d after IL-1Ra injection compared with Group A ($P < 0.05$); MSCs transplantation showed a slight effect on reducing TB and ammonia level in Group C (Table 1).

Serum inflammatory cytokines

Following D-gal induction, the levels of inflammatory cytokines, IL-1, IL-2 and TNF- α , in all groups increased significantly and reached a peak within 3 d, which lasted for several days before gradually declining. Group B and Group D had a faster improvement in IL-1 and TNF- α than the other groups, Group D had the lowest inflammatory level of all, with IL-1 and TNF- α levels signifi-

cantly lower than Group A ($P < 0.05$) from the first injection of 2 mg/kg IL-1Ra; serum inflammatory cytokine levels were better in Group C than in Group A, however, no statistically significant difference between the groups was observed (Figure 2).

Histological analysis

D-gal-induced liver damage was observed by HE staining in all groups, however, less inflammatory cell infiltration and relatively complete lobular architecture were found in Group D, and liver damage was worst in Group A (Figure 3). The number of Ki-67⁺ cells increased quickly after D-gal induction and reached a peak within 1 wk in all groups, and then a sharp decline was observed in Group A to a normal proliferation level at the end of 3 wk after operation, however, the Ki-67 positive cell index in Group D was maintained at a high level ($P < 0.05$); the performance of Group B and Group C were better than Group A, but there were no significant differences between the groups (Figure 4). Fluorescent microscopy revealed that there were more GFP-MSCs in Group D than in Group B at 1 wk after treatment, and most of these were distributed in the hepatic lobule along the central vein. Similar results were obtained by immunohistochemistry using anti-GFP (Figure 5).

DISCUSSION

ALF is a severe liver disease with large quantities of liver cell necrosis; liver transplantation is the only effective treatment, but has a number of difficulties. MSCs can be easily obtained from bone marrow and have multilineage potential. Petersen *et al*^[13] and Schwartz *et al*^[5] showed that MSCs possess the potential ability for hepatocyte differ-

Table 1 Biochemical parameters of Group A (control), Group B (interleukin-1 receptor antagonist), Group C (mesenchymal stem cells transplantation) and Group D (combination therapy) (mean \pm SD)

| | Group | Pre-operation | Intra-operation | Time after operation | | | | | | | |
|--------------------|-------|-----------------|-------------------|-----------------------------------|---------------------------------|--------------------------------|---------------------------------|---------------------------------|--------------------------------|---------------------------------|-----------------|
| | | | | D1 | D2 | D3 | D4 | D5 | W1 | W2 | W4 |
| ALT (U/L) | A | 54.7 \pm 22.7 | 111.4 \pm 44.1 | 167.25 \pm 25.8 | 108.8 \pm 25.2 | 89.3 \pm 32.8 | 63.7 \pm 24.6 | 54.1 \pm 13.7 | 48.3 \pm 17.1 | 38.7 \pm 16.3 | 32.4 \pm 4.2 |
| | B | 48.9 \pm 17.3 | 127.5 \pm 20.8 | 108.5 \pm 21.8 ^a | 77.6 \pm 18.9 ^a | 78.5 \pm 16.6 ^a | 68.7 \pm 10.8 | 55.6 \pm 12.9 | 51.6 \pm 10.7 | 42.8 \pm 11.6 | 45.6 \pm 6.9 |
| | C | 52.7 \pm 14.7 | 113.1 \pm 23.5 | 157.8 \pm 31.3 | 118.6 \pm 16.7 | 83.6 \pm 19.3 | 70.5 \pm 11.2 | 58.8 \pm 14.9 | 50.6 \pm 7.3 | 51.7 \pm 9.1 | 44.5 \pm 6.9 |
| | D | 45.2 \pm 7.7 | 112.6 \pm 22.9 | 75.4 \pm 10.1 ^{a,c} | 66.1 \pm 16.7 ^{a,c} | 48.2 \pm 11.9 ^{a,c} | 55.7 \pm 9.3 | 51.9 \pm 10.8 | 44.7 \pm 6.1 | 35.9 \pm 2.6 | 33.6 \pm 4.2 |
| TB (μ mol/L) | A | 2.9 \pm 1.3 | 24.0 \pm 8.5 | 32.3 \pm 7.4 | 26.2 \pm 8.8 | 17.5 \pm 5.1 | 12.9 \pm 3.8 | 7.9 \pm 1.9 | 5.8 \pm 1.9 | 3.1 \pm 1.2 | 1.9 \pm 0.5 |
| | B | 2.3 \pm 1.5 | 20.8 \pm 7.6 | 28.3 \pm 7.2 | 19.4 \pm 5.7 | 9.7 \pm 2.8 | 9.2 \pm 1.8 | 8.6 \pm 2.2 | 6.5 \pm 0.8 | 3.6 \pm 0.9 | 2.1 \pm 0.5 |
| | C | 1.3 \pm 0.9 | 18.9 \pm 5.7 | 27.5 \pm 5.3 | 15.8 \pm 4.1 ^a | 10.7 \pm 2.6 | 7.3 \pm 0.8 | 5.1 \pm 0.8 | 1.9 \pm 0.3 ^a | 1.7 \pm 0.6 | 0.98 \pm 0.1 |
| | D | 1.52 \pm 0.73 | 24.2 \pm 4.4 | 17.1 \pm 2.7 ^{a,c,c} | 8.9 \pm 3.51 ^{a,c} | 5.11 \pm 3.3 ^a | 2.68 \pm 2.03 ^{a,c} | 1.96 \pm 1.51 ^{a,c} | 1.47 \pm 0.75 ^{a,c} | 1.25 \pm 0.7 ^a | 0.43 \pm 0.05 |
| NH3 (μ mol/L) | A | 33 \pm 5.2 | 344.5 \pm 102.1 | 267.5 \pm 134.6 | 179 \pm 33.6 | 159.2 \pm 41.3 | 111.7 \pm 32.6 | 88.5 \pm 30.7 | 64.8 \pm 7.3 | 99.3 \pm 16.4 | 69 \pm 24.2 |
| | B | 39 \pm 10.4 | 318 \pm 67.8 | 206.5 \pm 22.1 | 160.6 \pm 18.2 | 128.8 \pm 18.5 | 92.5 \pm 11.3 | 114.2 \pm 25.6 | 88.5 \pm 19.7 | 73 \pm 16 | 55.6 \pm 11.8 |
| | C | 59 \pm 12.4 | 335.8 \pm 81.5 | 210.1 \pm 21.7 | 163.5 \pm 18.2 | 92.8 \pm 23.3 ^a | 121.4 \pm 24.5 | 110.8 \pm 53.2 | 97.2 \pm 26.8 | 48.9 \pm 10.4 ^a | 57.2 \pm 10.5 |
| | D | 38 \pm 13.5 | 369.3 \pm 104.2 | 148.7 \pm 39.4 ^{a,c,c} | 106.1 \pm 23.8 ^{a,c} | 81.3 \pm 24.2 ^{a,c} | 84.6 \pm 17.5 ^a | 87.7 \pm 23.9 | 62 \pm 17.8 | 42.7 \pm 13.3 ^{a,c} | 41.7 \pm 11.3 |
| ALB (g/L) | A | 30.5 \pm 4.3 | 27.7 \pm 2.4 | 26.2 \pm 3.0 | 25.1 \pm 2.1 | 24.4 \pm 1.6 | 25.2 \pm 1.9 | 25.6 \pm 1.4 | 26.3 \pm 1.2 | 26.7 \pm 1.7 | 28.5 \pm 2.5 |
| | B | 31.4 \pm 3.3 | 28.7 \pm 2.6 | 27.1 \pm 1.8 | 25.7 \pm 2.4 | 25.9 \pm 2.7 | 25.6 \pm 1.5 | 26.9 \pm 0.9 | 26.5 \pm 3.2 | 26.8 \pm 3.7 | 28.8 \pm 2.3 |
| | C | 28.8 \pm 2.8 | 27.2 \pm 2.2 | 26.6 \pm 3.3 | 24.9 \pm 1.8 | 25.1 \pm 1.1 | 25.8 \pm 1.6 | 26.3 \pm 0.8 | 27.1 \pm 4.2 | 27.9 \pm 1.5 | 30.5 \pm 2.1 |
| | D | 30.6 \pm 3.5 | 26.7 \pm 1.2 | 25.1 \pm 2.2 | 26.2 \pm 2.1 | 27.7 \pm 1.5 | 30.1 \pm 0.9 ^{a,c,e} | 30.7 \pm 1.4 ^{a,c,e} | 31.7 \pm 2.6 | 32.2 \pm 2.1 ^{a,c,e} | 33.5 \pm 2.5 |

^a $P < 0.05$ vs Group A; ^c $P < 0.05$ vs Group B; ^e $P < 0.05$ vs Group C. ALT and ammonia in Group D were significantly lower than those in Group A ($P < 0.05$) within 1 to 4 d after combination therapy, and the low level of TB in Group D lasted longer than that in Group A ($P < 0.05$) and Group B ($P < 0.05$) from the 1 d to 1 wk. The improvement in ALB in Group D was seen on the second d after treatment; Group B showed a significant reduction in ALT level within 1 to 3 d after interleukin-1 receptor antagonist injection compared with Group A ($P < 0.05$); MSCs transplantation displayed a slight effect on reducing TB and ammonia level in Group C. MSCs: Mesenchymal stem cells; ALT: Alanine aminotransferase; ALB: Albumin; TB: Total bilirubin.

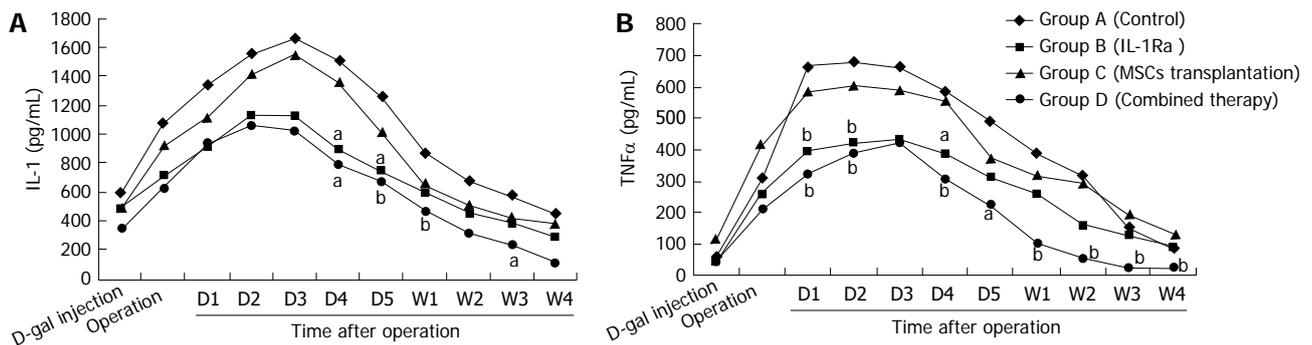


Figure 2 Changes in inflammatory cytokines (interleukin-1 and tumor necrosis factor α) levels. Interleukin-1 (IL-1) and tumor necrosis factor α (TNF α) levels in all groups increased after D-galactosamine (D-gal) injection, and then declined slowly. Group D showed a faster reduction in these cytokines following IL-1 receptor antagonist (IL-1Ra) injection than the other groups. ^a $P < 0.05$, ^b $P < 0.01$ vs control group. MSCs: Mesenchymal stem cells.

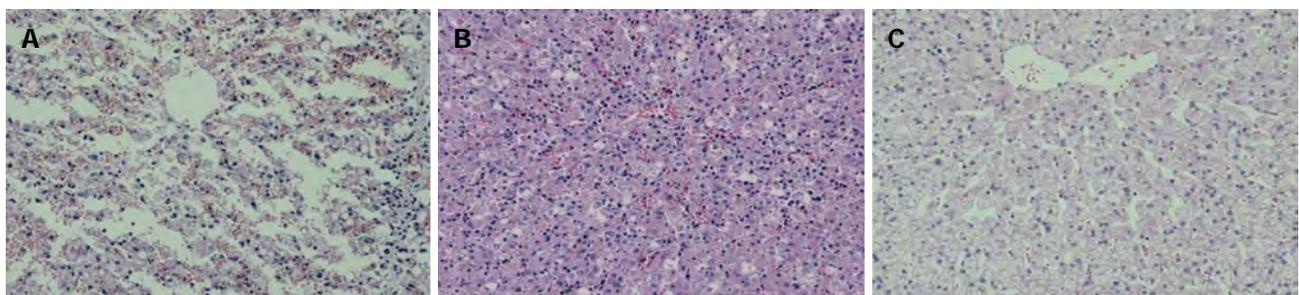


Figure 3 Hematoxylin and eosin staining of liver tissues 3 d after surgery. A: Extensive neutrophil infiltration and lobular architecture collapse was seen in Group A (control group); B: Lobular architecture can be seen in Group B (interleukin-1 receptor antagonist injection group), however, hepatic lobules were filled with cell necrosis and inflammatory cells; C: The lobular architecture in Group C (mesenchymal stem cells transplantation group) was destroyed but can still be recognized; D: Group D had clear hepatic lobular architecture and slight inflammatory cell infiltration. Magnification, $\times 200$.

entiation *in vitro* and *in vivo*. Sakaida *et al*^[14] confirmed that MSCs were involved in both liver repair and reconstruc-

tion. In their research, MSCs transplantation was used to reduce CCl₄-induced liver fibrosis in mice, and their

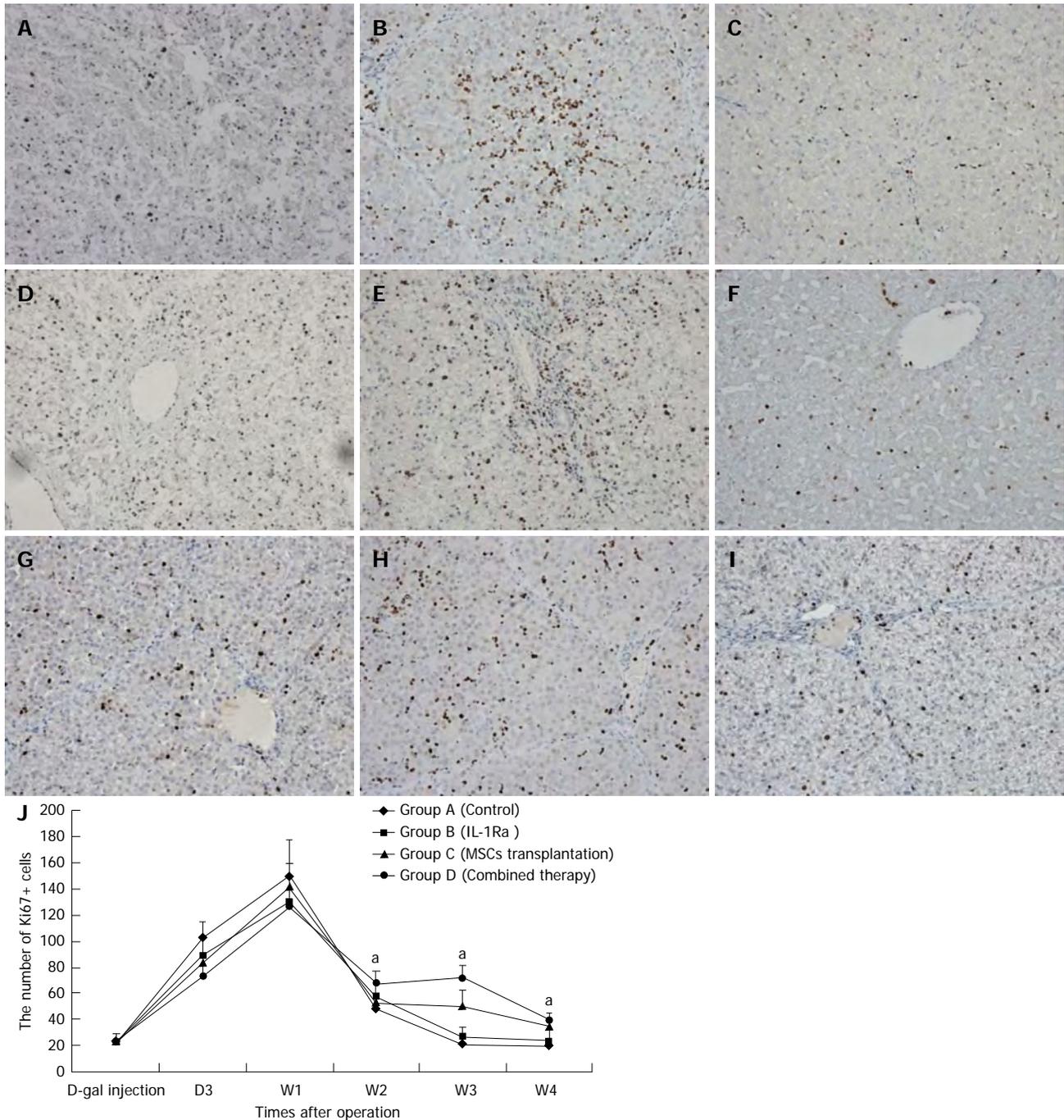


Figure 4 Changes in liver cell proliferation in all four groups. A, B and C showed the anti-Ki67 stain of Group A at the time point of D3, W1 and W4, respectively ($\times 200$); D, E, F and G, H, I showed anti-Ki67 stain of Group B and Group C, respectively, just as Group A did ($\times 200$); J: Changes in the number of Ki67+ cells demonstrated that Group D had better long-term proliferation. ^a $P < 0.05$ vs control group. IL-1Ra: Interleukin-1 receptor antagonist; MSCs: Mesenchymal stem cells; D-gal: D-galactosamine.

findings showed that MSCs transplantation was an ideal candidate treatment for liver disease. What will happen if MSCs transplantation is used for the treatment of ALF? di Bonzo *et al*^[15] xenografted human MSCs into acute liver injured NOD/SCID mice and CCl₄-induced liver injury in mice and demonstrated that the number of original human MSCs in acute liver injured mice was less than in chronically injured livers, and the number of hepatocytes undergoing differentiation was even less. In our study, there was no obvious improvement in liver function in the MSCs transplantation group (Group C), few GFP-

MSCs were observed on fluorescent microscopy, and little differentiation was seen. Therefore, we concluded that MSCs transplantation for the treatment of ALF is largely limited by its low implantation and differentiation rate in acute liver injured patients.

In recent years, experimental studies have demonstrated that microcirculatory dysfunction and an inflammatory environment are determinants of ALF, and pro-inflammatory mediators such as IL-1, IL-2 and TNF- α are the key players^[9,12,16-18]. Vodovotz also proved that the levels of these cytokines in ALF patients were significant-

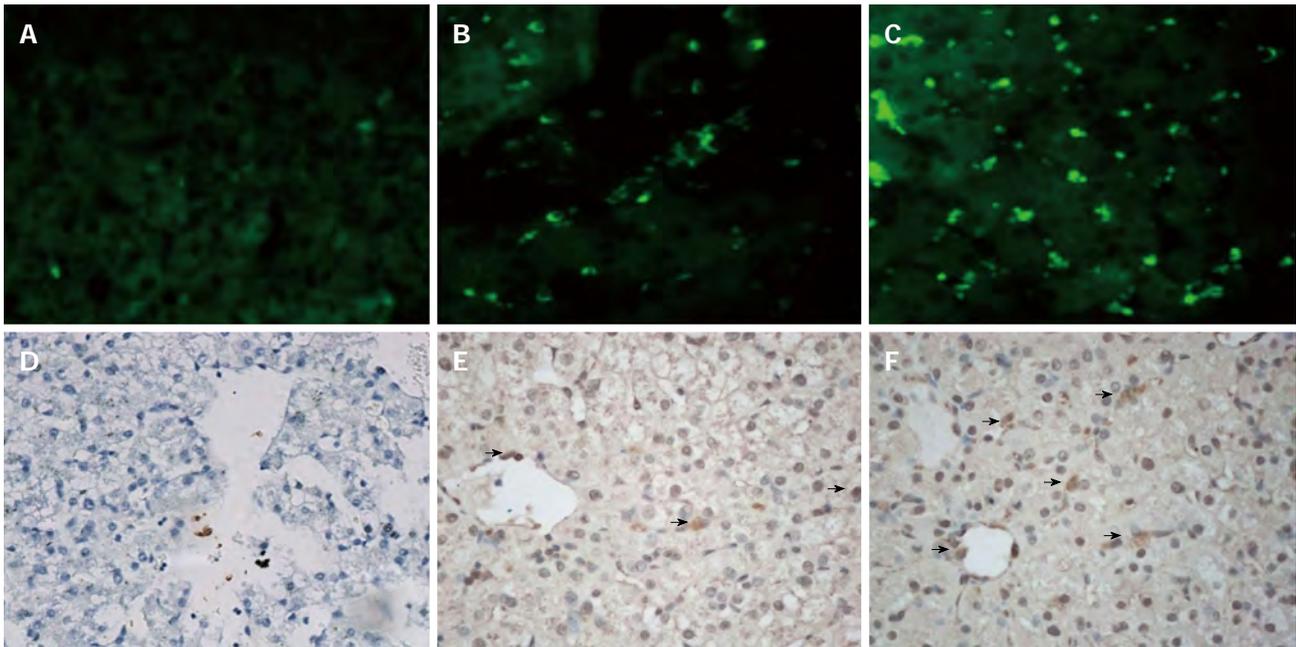


Figure 5 Fluorescence and anti-green fluorescence protein stained images of liver tissue 2 wk after surgery. A, B and C are fluorescence images of Group B, Group C and Group D, respectively; D, E and F are anti-green fluorescence protein immunohistochemical images of Group B, Group C and Group D, respectively. More cells expressing green fluorescence protein were seen in Group D (arrow). Magnification, $\times 400$.

ly higher than in normal and chronic hepatitis patients; IL-1 may be a main driver of late inflammation which leads to further injury^[3,9,19]. Given this, we presume that the inflammatory environment in ALF patients may be largely related to the low efficiency of MSCs transplantation, that is the inflammatory environment caused by proinflammatory mediators leads to the low survival and differentiation rate of transplanted MSCs. Our pre-experiment results proved this assumption: animals with lower levels of inflammatory cytokines had a higher MSCs implantation rate and differentiation rate. Therefore, we realized that reducing the inflammation level in the acutely injured liver may be a way of improving the efficacy of MSCs transplantation in ALF patients. IL-1Ra is a natural IL-1 antagonist, and can block the inflammatory process by competitively binding to the IL-1 receptor with an avidity equal to that of IL-1, but fails to stimulate downstream signals, thereby reducing the inflammation level^[8,20]. An imbalance between IL-1 and IL-1Ra can be observed in a variety of inflammatory diseases including ALF^[3,8,12,20]. IL-1Ra is significantly associated with the level of liver inflammation and is an independent marker unaffected by obesity, alcohol consumption, and insulin resistance^[21], and can inhibit the process of hepatocellular apoptosis in mice with acetaminophen-induced ALF significantly improving their survival rate^[22]. Therefore, will MSCs transplantation become an efficient treatment for ALF when it is combined with IL-1Ra used to relieve liver inflammation?

In this research, the combined therapy of IL-1Ra with MSCs transplantation was administered for acute liver injury, and the results obtained were very promising. Increased levels of proinflammatory cytokines such as

IL-1, IL-2 and TNF- α were seen in all animals injected with D-gal, a slight improvement was observed when MSCs were transplanted in Group B animals, and better results were achieved in Group C and Group D animals following IL-1Ra injection. Thus, exogenous IL-1Ra had an enormous effect in reducing some proinflammatory mediators, and improving the inflammatory environment. This effect may last for at least a month as the animals in Group C and Group D showed a continuous reduction in these proinflammatory mediators compared with the other two groups in later experiments, which was thought to be mainly related to the damaged inflammatory cycle caused by IL-1Ra in the very early phase. Improved liver inflammation was then observed following MSCs transplantation. As shown in the results section, Group D treated with combination therapy had the highest GFP-MSCs implantation rate and the best liver function. In addition, the trend in proliferation level in the four groups was different, although the level in all groups peaked at a similar time point after surgery, Group D had a higher proliferation level than the other groups at the end of 2 wk of combination therapy and this difference was maintained for at least 2 wk; which was thought to be caused by both IL-1Ra and MSCs transplantation. IL-1Ra improved the liver inflammatory environment then increased liver cell proliferation rate and MSCs transplantation efficiency, and high MSCs transplantation efficiency may directly lead to a higher hepatocyte differentiation rate, proliferation level and better liver function.

Thus, IL-1Ra can improve liver inflammation and then enhance the effect of MSCs transplantation. Combination therapy with IL-1Ra and MSCs transplantation can promote the restoration and reconstruction of acute

liver injury in swine, and is a promising future treatment for patients with ALF.

COMMENTS

Background

Cell transplantation is an effective therapy for acute liver failure; however, the activity and function of transplanted cells are largely limited by the inflammatory environment of acute liver failure (ALF) liver. Interleukin-1 (IL-1) is primarily a proinflammatory cytokine and IL-1 receptor antagonist (IL-1Ra) is the most effective antagonist. The combination therapy with IL-1Ra and mesenchymal stem cell (MSC) transplantation for the treatment of ALF is an interesting way and responded well.

Research frontiers

The essence of ALF is severe inflammation leading to cell necrosis in a large number of liver cells. Microcirculatory dysfunction and an inflammatory environment are determinants of ALF, and proinflammatory mediators such as IL-1, IL-2 and tumor necrosis factor α are the primary players. The key to the treatment of ALF is the reduction of liver cell necrosis and the stimulation of liver cell regeneration.

Innovations and breakthroughs

Recent reports have highlighted the effect of IL-1Ra injection on ALF models. In this study, the authors investigated the effect of bone marrow MSCs transplantation combined with IL-1Ra injection on ALF swine. Based on the results of the study, the authors concluded that MSCs transplantation is somewhat useful for ALF swine and that the combined therapy of IL-1Ra with MSCs transplantation is a promising treatment for ALF.

Applications

According to this article, it may represent a future strategy for therapeutic intervention in the treatment of patients with ALF.

Terminology

IL-1Ra is the interleukin-1 receptor antagonist, which can bind to the IL-1 receptor and blocks IL-1 action through competitive inhibition, but will not initiate the IL-1 signaling cascade due to its IL-1-like structure which will not induce a signal at all.

Peer review

The authors evaluated the effect of bone marrow mesenchymal stem cell transplantation combined with IL-1Ra injection on ALF swine. Group D (combine therapy of MSC transplantation + IL-1Ra) showed significantly improvement of biochemical assay, serum inflammation level and histological results compared with other groups ($P < 0.05$), labeled MSCs of Group D were more than Group B, and the long-term cell proliferation rate was the best in all groups too. They concluded that MSC transplantation is somewhat useful for ALF swine, IL-1Ra plays an important role in alleviating inflammatory condition, and the combined therapy is a promising treatment for ALF. These results are interesting and important in the therapy for ALF.

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Usefulness of positron emission tomography in primary intestinal follicular lymphoma

Akira Tari, Hideki Asaoku, Masaki Kunihiro, Shinji Tanaka, Tadashi Yoshino

Akira Tari, Masaki Kunihiro, Division of Gastroenterology, Department of Internal Medicine, Hiroshima Red Cross Hospital and Atomic-Bomb Survivors Hospital, Hiroshima 734-8551, Japan

Hideki Asaoku, Department of Clinical Laboratory, Hiroshima Red Cross Hospital and Atomic-Bomb Survivors Hospital, Hiroshima 734-8551, Japan

Shinji Tanaka, Department of Endoscopy, Hiroshima University Hospital, Hiroshima 734-8551, Japan

Tadashi Yoshino, Department of Pathology, Okayama University Graduate School of Medicine, Dentistry and Pharmaceutical Sciences, Okayama 700-8530, Japan

Author contributions: Tari A performed the endoscopic examination, diagnosed and decided treatment policy, analyzed the data and wrote the paper; Asaoku H performed the hematological examination, diagnosed and decided treatment policy; Kunihiro M performed the endoscopic examination and diagnosis; Tanaka S performed the double-balloon enteroscopy and diagnosis; and Yoshino T performed the pathological diagnosis.

Correspondence to: Dr. Akira Tari, Division of Gastroenterology, Department of Internal Medicine, Hiroshima Red Cross Hospital and Atomic-Bomb Survivors Hospital, 1-9-6 Sendamachi, Naka-ku, Hiroshima 734-8551, Japan. stomach2@hiroshima-med.jrc.or.jp

Telephone: +81-82-2413111 Fax: +81-82-2460676

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deoxyglucose positron emission tomography combined with computed tomography (PET-CT). The endoscopic findings of these 4 cases included lesions with wall thickening, which comprised macroscopically clusters of nodules, dense clusters of whitish granules or small nodules, fold thickening and ulcers with irregular margins that occupied the whole lumen with edematous mucosa. All patients fulfilled the World Health Organization grade 1 criteria. ¹⁸F-fluorodeoxyglucose PET-CT can help predict the risks that may result from certain endoscopic examinations, such as DBE and video capsule endoscopy.

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Key words: Capsule endoscopy; Double-balloon enteroscopy; Follicular lymphoma; Positron-emission tomography; Computed tomography; Small intestine

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Abstract

Double-balloon enteroscopy (DBE) and video capsule endoscopy are useful for the diagnosis of lymphoma in the small intestine. However, DBE cannot be safely performed in cases with passage disturbance due to wall thickening and stenosis. Additionally, video capsule endoscopy cannot be performed in such cases because of the risk of retention. Here, we report 4 cases of primary follicular lymphoma of the gastrointestinal tract that could be detected using ¹⁸F-fluoro-

INTRODUCTION

Primary follicular lymphoma of the gastrointestinal tract (FL-GI) is often diagnosed by initially detecting duodenal lesions using esophago-gastro-duodenoscopy (EGD)^[1,2]. FL-GI lesions can exist in broad areas, ranging from the descending portion of the duodenum to the ileum, and may include lymph node involvement^[1,2]. The correct diagnosis of the locations of lesions is vital to decisions regarding therapeutic plans^[3]. Double-balloon enteroscopy (DBE) and video capsule endoscopy (VCE) are useful for the diagnosis of lesions in the small intestine^[4].

However, DBE cannot be safely performed in cases with passage disturbance due to wall thickening and stenosis, and VCE cannot be performed in these cases because of the risk of retention^[4]. Conversely, ¹⁸F-fluorodeoxyglucose (FDG) positron emission tomography combined with computed tomography (PET-CT) has been reported to be useful for detecting lymph node involvement in the diagnosis of the clinical stages of follicular lymphomas^[5]. This study reports the cases of 4 patients for whom PET-CT was useful in the detection of FL-GI in the digestive tract, the location of which ranged from the duodenum to the ileocecal valve.

CASE REPORT

Twenty FL-GI patients (male/female 9/11, age 46-82 years, mean 58 years) consulted the Division of Gastroenterology, Department of Internal Medicine, Hiroshima Red Cross Hospital and Atomic-Bomb Survivors Hospital from December 2004 to May 2010. In 2 of the 18 patients with duodenal lesions, FL-GI lesions were detected using PET-CT. One of the 14 patients with jejunal lesions and 2 of the 12 patients with ileal lesions had abnormal accumulations according to the PET-CT results.

Table 1 shows the 4 FL-GI patients with abnormal accumulations by PET-CT who had gastrointestinal lesions detected from the duodenum to the ileocecal valve. Case 1 had duodenal lesion, case 2 had jejunal lesions, case 3 had lesions from the terminal ileum to the ileocecal valve and case 4 had lesions in broad areas from the duodenum to the ileum. All lesions were diagnosed by endoscopic examination with biopsy.

The mandatory examinations included palpation of the superficial lymph nodes, blood tests, urinalysis, chest radiography, abdominal ultrasonography, contrast-enhanced computed tomography (CT) scan (General Electric, Fairfield, CT, United States) (CT/CE+) of the neck, chest, abdomen and pelvis, PET-CT (General Electric, Fairfield, Connecticut, United States), bone marrow aspiration, endoscopy with biopsies [EGD (Olympus, Tokyo, Japan), colonoscopy (Fujinon, Tokyo, Japan), DBE (Fujinon, Tokyo, Japan)] and VCE (Given Imaging, Yoqneam, Israel). Each patient was classified by the location of the lesions, clinical stage (Lugano International classification^[6]), FL histological grade (World Health Organization grade)^[7] and follicular lymphoma international prognosis index^[8]. The macroscopic findings of FL-GI were classified by endoscopy into the following 6 types: whitish granules, multiple small nodules, fold swelling and thickening, mass forming, ulcers with irregular margins and rough mucosa^[1].

Case 1 is a 52-year-old man who had no symptoms but was diagnosed to have abnormalities in the descending portion of the duodenum by EGD for gastric cancer screening (Table 1). This patient had wall thickening with abnormal accumulation at the descending portion of the duodenum and at the duodenojejunal flexure [maximum

standardized uptake value (SUVmax) 6.6 and 5.5, respectively] (Figure 1A) by PET-CT. However, there were no abnormal findings in the small intestine by PET-CT. EGD showed lesions with dense clusters of whitish granules, making it difficult to see the folds in the descending and horizontal portions of duodenum (Figure 1B). The jejunum showed only rough clusters of whitish granules. The terminal ileum of this patient showed mucosa with normal lymphoid tissues.

Case 2 is a 62-year-old woman who presented with swelling of the mesenteric lymph nodes (max, 1.5 cm in diameter) by abdominal ultrasonography and a mild accumulation of FDG (SUVmax, 3.0) by PET-CT. A laparotomic lymph node biopsy led to the diagnosis of FL. Regarding the intestinal lesions, localized accumulation was demonstrated by PET-CT (SUVmax, 3.9) performed in the jejunum 25 mo later. The EGD showed swelling and thickening of the folds that occupied half of the lumen (Table 1).

Case 3 is a 66-year-old woman who underwent EGD because of a 1-wk history of postprandial epigastric discomfort and dyspepsia. There were abnormal findings in the papillae of Vater (Table 1). This case showed intestinal wall thickening with abnormal accumulation from the terminal ileum to the cecum by PET-CT (SUVmax, 6.73) (Figure 2A and C). The endoscopic findings in the duodenum included eruptions with mild swelling and erosion at the papillae of Vater. The jejunum and the ileum had sparse clusters of small nodules. Portions from the terminal ileum (Figure 2B) to the ileocecal valve (Figure 2D) had dense clusters of granules and nodules.

Case 4 is a 61-year-old woman undergoing examinations for leukocytosis (white blood cells, 29 740) (Table 1). The PET-CT of this patient showed abnormal accumulations (SUVmax, 5.6) in broad areas from the descending portion of the duodenum through the jejunum to the ileum (Figure 3A). The color of the duodenal bulb was normal, and the macroscopic finding was rough mucosa. The mucosa of the descending and horizontal portions of the duodenum showed clusters of numerous whitish granules. The jejunum and the ileum had multiple ulcers with irregular margins that occupied the whole lumen, with edematous change in broad areas of the mucosa (Figure 3B).

DISCUSSION

FL-GI patients frequently have duodenal and small intestinal lesions, as has been already reported^[2]. The macroscopic findings of patient endoscopies that were also detected using PET-CT were clusters of numerous whitish granules spreading over the mucosa, concealing the folds, obvious swelling and thickening of folds and dense clusters of granules and nodules (Cases 1-3). PET-CT could detect lesions of ulcers with irregular margins that occupied the whole lumen with edematous mucosa as well as lesions with clusters of numerous whitish granules

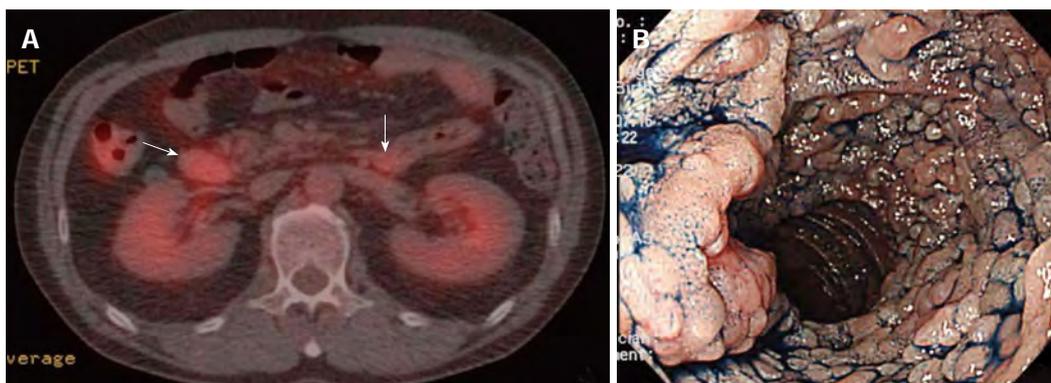


Figure 1 A 52-year-old man with follicular lymphoma of the gastrointestinal tract. A: ^{18}F -fluorodeoxyglucose positron emission tomography combined with computed tomography in transaxial images showed focal hypermetabolic activities (maximum standardized uptake value, 6.6 and 5.5, respectively) in the lesions of the descending portion of the duodenum and the duodenojejunal flexure (arrows), respectively; B: The esophago-gastro-duodenoscopic view with indigo carmine dye-spray of the descending portion of the duodenum. Numerous whitish granules densely clustered together.

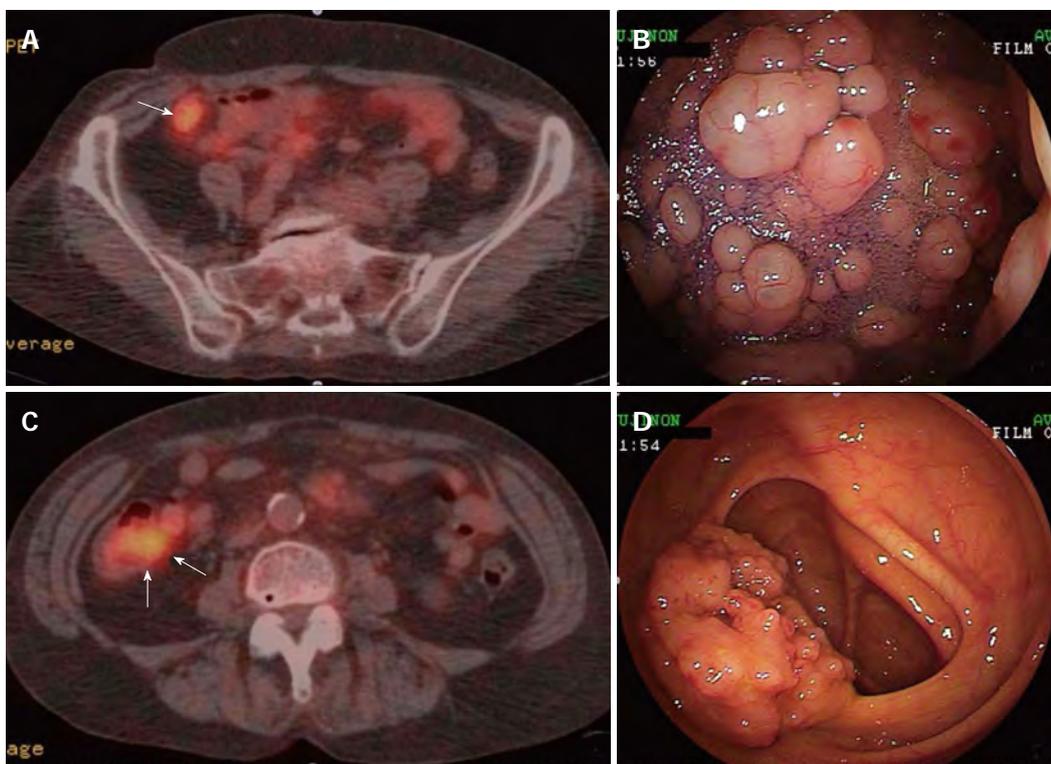


Figure 2 A 66-year-old woman with follicular lymphoma of the gastrointestinal tract. A, C: ^{18}F -fluorodeoxyglucose positron emission tomography combined with computed tomography in transaxial images showed focal hypermetabolic activities (maximum standardized uptake value, 6.73) in the lesion of the terminal ileum (A) (arrow) and the cecum (C) (arrows); B: The colonoscopic view with indigo carmine dye-spray of the terminal ileum. Numerous polypoid lesions of varying sizes (granules to small nodules) were densely clustered; D: The colonoscopic view of the ileocecal valve. Numerous polypoid lesions of varying sizes (granules to small nodules) were densely clustered.

(Case 4). Lesions with a scattered distribution of whitish granules and small nodules were not shown as abnormal accumulations by PET-CT (Cases 1 and 3). It is assumed that a greater wall thickness of a GI lesion results in a greater possibility of abnormal accumulation according to the PET-CT results.

There were 2 FL-GI patients with small intestinal tumors whose initial symptom was ileus (4.3% of FL-GI at our division in April 2012). PET-CT is a valuable tool for the detection of the lesions in these patients (PET-

CT findings not shown). The detection rate of the GI lesions of FL by PET-CT is generally reported to be rather low^[9,10]. However, among the gastrointestinal lesions that were detected by PET-CT were cases with wall thickening and macroscopic clusters of nodules, dense clusters of whitish granules or small nodules, fold thickening or ulcers that showed irregular margins and occupied the whole lumen with edematous mucosa. Therefore, PET-CT is useful in cases with lesions that are difficult to approach using DBE and in cases that have a risk of

Table 1 Patients detected to have primary follicular lymphoma of gastrointestinal tract by ¹⁸F-fluorodeoxyglucose positron emission tomography combined with computed tomography

| Case No. | Age (yr) | Sex | Diagnosis (WHO grade) | Clinical stage (Lugano) | FLIPI | Locations in GI tract | Endoscopic appearances | PET-CT |
|----------|----------|-----|-----------------------|-------------------------|--------------|---|--|---|
| 1 | 52 | M | FL (grade 1) | I | Low | Duodenum descending portion-duodenojejunal flexure Jejunum Terminal ileum | Dense cluster of whitish granules Cluster of whitish granules Normal lymph follicles | (+) descending portion and duodenojejunal flexure of duodenum (-) (-) |
| 2 | 62 | F | FL (grade 1) | II 2 | Intermediate | Jejunum Ileum | Swelling and thickening of folds Normal lymph follicles | (+) jejunum (-) |
| 3 | 66 | F | FL (grade 1) | II 2 | Intermediate | Papilla vater Jejunum Ileum Terminal ileum-ileocecal valve | Mild swelling and erosion Sparse cluster of small nodules Sparse cluster of small nodules Dense cluster of granules and nodules | (-) (-) (-) (+) ileocecal valve |
| 4 | 61 | F | FL (grade 1) | IV | High | Duodenal bulb Duodenum descending portion-jejunum Ileum | Rough mucosa Cluster of numerous whitish granules Multiple ulcers with irregular margin | (-) (+) duodenum-jejunum (+) ileum |

M: Male; F: Female; FL: Follicular lymphoma; WHO: World Health Organization; FLIPI: Follicular lymphoma international prognostic index; PET-CT: Positron emission tomography combined with computed tomography; GI: Gastrointestinal.

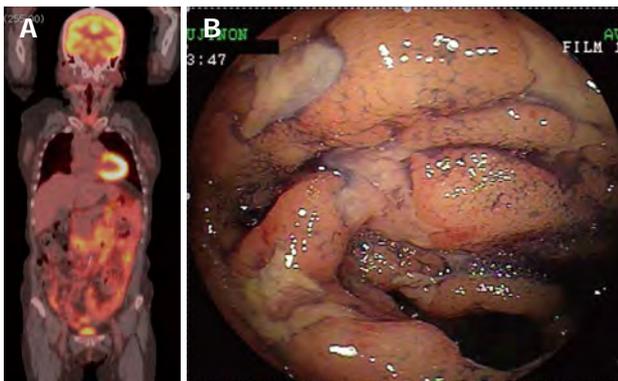


Figure 3 A 61-year-old woman with follicular lymphoma of the gastrointestinal tract. A: ¹⁸F-fluorodeoxyglucose positron emission tomography combined with computed tomography in a projected image showed hypermetabolic foci in broad areas of the gastrointestinal tract from the 2nd portion of the duodenum to the terminal ileum (maximum standardized uptake value, 5.6); B: The colonoscopic view with indigo carmine dye-spray of the terminal ileum. Multiple ulcers with irregular margins were observed.

retention with VCE because of stenosis due to tumors or wall thickening of the deep portion of small intestine. PET-CT is a useful tool for detecting the involvement of lymph nodes and other organs in follicular lymphoma^[3]. It is not only useful for deciding whether VCE should be performed, but also for deciding the method by which lesions in the small intestine are approached using DBE, which is necessary for the pathological diagnosis of the biopsy.

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Mizutani T, Honda K, Taki K, Murao H, Ogino H, Kanayama K, Akiho H, Goto A, Segawa Y, Yao T, Takayanagi R. Impact of double-balloon endoscopy on the diagnosis of jejunoileal involvement in primary intestinal follicular lymphomas: a case series. *Endoscopy* 2009; **41**: 175-178 [PMID: 19214900 DOI: 10.1055/s-0028-1119467]

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Transmesocolic hernia with strangulation in a patient without surgical history: Case report

Peel Jung, Min Dae Kim, Tae Hyun Ryu, Sung Ho Choi, Han Se Kim, Kang Hun Lee, Jhong Hyun Park

Peel Jung, Tae Hyun Ryu, Sung Ho Choi, Han Se Kim, Kang Hun Lee, Department of Internal Medicine, Bongseng Memorial Hospital, Busan 601-723, South Korea

Min Dae Kim, Department of Gastroenterology, Bongseng Memorial Hospital, Busan 601-723, South Korea

Jhong Hyun Park, Department of General Surgery, Bongseng Memorial Hospital, Busan 601-723, South Korea

Author contributions: Jung P drafted and edited the manuscript; Ryu TH and Choi SH treated the patient; Kim HS and Lee KH contributed to the literature review; Park JH performed the operation; Kim MD contributed to the final approval.

Correspondence to: Min Dae Kim, MD, Department of Gastroenterology, Bongseng Memorial Hospital, 401 Jwacheon 1-dong, Dong-gu, Busan 601-723, South Korea. mdmdk69@hanmail.net

Telephone: +82-51-6644000 Fax: +82-51-6644059

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Abstract

Transmesenteric hernias have bimodal distribution and occur in both pediatric and adult patients. In the adult population, the cause is iatrogenic, traumatic, or inflammatory. We report a case of transmesocolic hernia in an elderly person without any preoperative history. An 84-year-old Korean female was admitted with mid-abdominal pain and distension for 1 d. On abdominal computed tomography, we diagnosed transmesocolic hernia with strangulated small bowel obstruction, and performed emergency surgery. The postoperative period was uneventful and she was discharged 11 d after surgery. Hence, it is important to consider the possibility of transmesocolic hernia in elderly patients with signs and symptoms of intestinal obstruction, even in cases with no previous surgery.

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Key words: Transmesocolic hernia; Strangulation; Op-

eration; Abdominal computed tomography; Small bowel obstruction; Internal hernia

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INTRODUCTION

The incidence of internal hernia is less than 1%^[1], and transmesocolic hernia is a particularly rare type of internal hernia. The overall mortality is more than 50% in cases with strangulated small bowel obstruction^[2]. In adults, transmesocolic hernias are most often caused by previous surgical procedures, abdominal trauma or intraperitoneal inflammation, and transmesocolic hernia in a person without a surgical history is extremely rare. We report such a case of transmesocolic hernia with strangulated intestinal obstruction.

CASE REPORT

An 84-year-old Korean female, with no past history of surgery, was admitted with mid-abdominal pain and distension for 1 d. Upon admission, her blood pressure was 120/80 mmHg, heart rate 72 beats/min, and body temperature 36.6 °C. On physical examination of the area of concern, mid-abdominal tenderness was observed. On admission, laboratory assessments were as follows: white blood cell count 14 500/mm³ (segmented neutrophil 91.4%), hemoglobin concentration 12.8 g/dL, platelet count 272 000/mm³, sodium 135 mmol/L, potassium 4.5 mmol/L, blood urea nitrogen 21.5 mg/dL, creatinine 0.9 mg/dL, aspartate aminotransferase 18 IU/L, alanine aminotransferase 10 IU/L, alkaline phosphatase 256 IU/L,



Figure 1 Abdominal computed tomography findings. Crowded small bowel loop (from distal jejunum to proximal ileum) with circumferential wall thickening and decreased enhancement in the middle and lower abdomen, stretched mesenteric vessels with mesenteric edema. A: Sagittal view; B: Transverse view.



Figure 2 Intraoperative findings. Herniated small intestine with strangulation by perforated omentum (arrow) below the transverse colon.

lactate dehydrogenase 415 IU/L, γ -glutamyltransferase 17 IU/L, and C-reactive protein 1.04 mg/dL. A simple abdominal X-ray showed distended small bowel loops. Abdominal computed tomography (CT) revealed crowded small bowel loops (from distal jejunum to proximal ileum), with circumferential wall thickening and decreased enhancement in the middle and lower abdomen, and stretched mesenteric vessels with mesenteric edema (Figure 1). We diagnosed transmesocolic hernia with intestinal obstruction and performed emergency surgery. During the operation, a herniated small intestine with strangulation by perforated omentum was noted below the transverse colon (Figure 2). Strangulated herniation was seen 190 cm from the ligament of Treitz, for a length of 160 cm. The strangulated small intestine herniation was resected and the omentum defect was closed. The postoperative course was uneventful and the patient was discharged on postoperative day 11, with a favorable follow-up as an outpatient for 1 mo.

DISCUSSION

An internal hernia is the protrusion of an abdominal organ through a normal or abnormal mesenteric or peritoneal aperture^[3]. An internal hernia can be acquired as a result of trauma or a surgical procedure, or may be

constitutional and related to congenital peritoneal defects. In the broad category of internal hernias, there are several main types based on their location, as traditionally described by Meyers. These consist of paraduodenal (53%), pericecal (13%), foramen of Winslow-related (8%), transmesenteric and transmesocolic (8%), intersigmoid (6%), and retroanastomotic (5%), with the overall incidence of internal hernia being 0.2%-0.9%^[2]. The transmesenteric hernia has three main types: transmesocolic, through a small-bowel mesenteric defect, and Peterson's hernia^[1]. Most transmesocolic hernias in children result from a congenital defect in the small bowel mesentery close to the ileocecal region. Congenital defects occur following the embryonic formation of an intestinal loop in thin avascular areas of the mesentery (*e.g.*, the mesenteries of the lower ileum, the sigmoid mesocolon, and the transverse mesocolon). As a consequence, there are multiple theories of congenital causes of such mesenteric defects^[4]. It is likely that the congenital condition is associated with prenatal intestinal ischemic accidents due to the observed frequently in infants with atretic bowel segments. In adults, transmesocolic hernias are most often caused by previous surgical procedures, abdominal trauma or intraperitoneal inflammation. When the small bowel is herniated through a defect in the mesentery or omentum, the herniated bowel is compressed against the abdominal wall. In this case, the herniated bowel is clustered and lies outside the colon which is displaced centrally^[5].

Transmesocolic hernias are more likely than other subtypes to develop volvulus and strangulation, or ischemia, the reported incidence of which are as high as 30% and 40%, respectively, with mortality rates of 50% for treated groups and 100% for non-treated subgroups^[2,6]. Clinically, internal hernias can be asymptomatic, or can cause discomfort ranging from constant vague epigastric pain to intermittent colicky periumbilical pain, while additional symptoms include nausea and vomiting^[1].

An internal hernia is difficult to diagnosis by physical examination, and the most important diagnostic method is abdominal CT. It has been suggested that the two findings of a peripherally located small bowel, and lack

of omental fat between the loops and the anterior abdominal wall, might be the most helpful CT signs, with an overall sensitivity of 85% and 92% for each respective finding^[6,7]. Observation of the clustering of small bowel loops and an abnormality in the mesenteric vessels are helpful findings on abdominal CT.

In adults, a previous surgical procedure, as well as trauma or inflammation are the most common causes of transmesocolic hernia. Our case was a rare presentation in an elderly person without a history of trauma, and without previous surgery^[8-12]. The patient had non-specific symptoms and signs on plain abdominal X-ray, but also non-specific abdominal distension upon physical examination. In the case of internal hernia, these defects may be idiopathic, but we can speculate that a small congenital defect existed without any hernia, and enlarged due to the aging. A transmesocolic hernia is difficult to diagnosis preoperatively despite the array of diagnostic techniques currently available. In patients suspected of having an internal hernia, early surgical intervention may be advisable due to the high morbidity and mortality rates. Therefore, it is important to consider the possibility of a transmesocolic hernia when patients have signs and symptoms of intestinal obstruction, even in cases of elderly patients with no previous surgical history.

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Rare case of *Helicobacter pylori*-related gastric ulcer: Malignancy or pseudomorphism?

Ting-Ting Li, Feng Qiu, Zhi-Qiang Wang, Lu Sun, Jun Wan

Ting-Ting Li, Jun Wan, Department of Geriatric Gastroenterology, Chinese PLA General Hospital, Beijing 100853, China

Feng Qiu, Department of Neurology, Chinese Navy General Hospital, Beijing 100037, China

Zhi-Qiang Wang, Department of Geriatric Gastrointestinal Endoscopy, Chinese PLA General Hospital, Beijing 100853, China

Lu Sun, Department of Pathology, Chinese PLA General Hospital, Beijing 100853, China

Author contributions: Li TT and Qiu F substantially contributed to conception and design, acquisition of data, analysis and interpretation of data; Wang ZQ and Sun L drafted the article and revised it critically for important intellectual content; Wan J approved the final version to be published.

Correspondence to: Dr. Jun Wan, Department of Geriatric Gastroenterology, Chinese PLA General Hospital, Beijing 100853, China. wanjun301@126.com

Telephone: +86-10-66876266 Fax: +86-10-66876266

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Abstract

Helicobacter pylori (*H. pylori*) is a pathogen and the most frequent cause of gastric ulcers. There is also a close correlation between the prevalence of *H. pylori* infection and the incidence of gastric cancer. We present the case of a 38-year-old woman referred by her primary care physician for screening positron emission tomography-computed tomography (PET-CT), which showed a nodular strong accumulation point with standardized uptake value 5.6 in the gastric fundus. Gastroscopy was then performed, and a single arched ulcer, 12 mm in size, was found in the gastric fundus. Histopathological examination of the lesion revealed chronic mucosal inflammation with acute inflammation and *H. pylori* infection. There was an obvious mitotic phase with widespread lymphoma. Formal anti-*H. pylori* treatment was carried out. One month later, a gastroscopy showed a single arched ulcer, measuring 10 mm in size in the gastric fundus. Histopathological ex-

amination revealed chronic mucosal inflammation with acute inflammation and a very small amount of *H. pylori* infection. The mitotic phase was 4/10 high power field, with some heterotypes and an obvious nucleolus. Follow-up gastroscopy 2 mo later showed the gastric ulcer in stage S2. The mucosal swelling had markedly improved. The patient remained asymptomatic, and a follow-up PET-CT was performed 6 mo later. The nodular strong accumulation point had disappeared. Follow-up gastroscopy showed no evidence of malignant cancer. *H. pylori*-associated severe inflammation can lead to neoplastic changes in histiocytes. This underscores the importance of eradicating *H. pylori*, especially in those with mucosal lesions, and ensuring proper follow-up to prevent or even reverse early gastric cancer.

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Key words: *Helicobacter pylori*; Gastric ulcer; Gastric cancer; Positron emission tomography-computed tomography; Gastroscopy

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INTRODUCTION

Helicobacter pylori (*H. pylori*) is a Gram-negative microaerophilic bacterium that colonizes the stomach of approximately two-thirds of the human population and is involved in the pathogenesis of various gastroenterological diseases including gastric ulcer and gastric cancer. *H. pylori*'s interaction with the host has an impact on the severity of these diseases and their clinical outcome^[1,2].

The mechanisms of *H. pylori*-related colonization are not fully understood. However, different types of *H. py-*

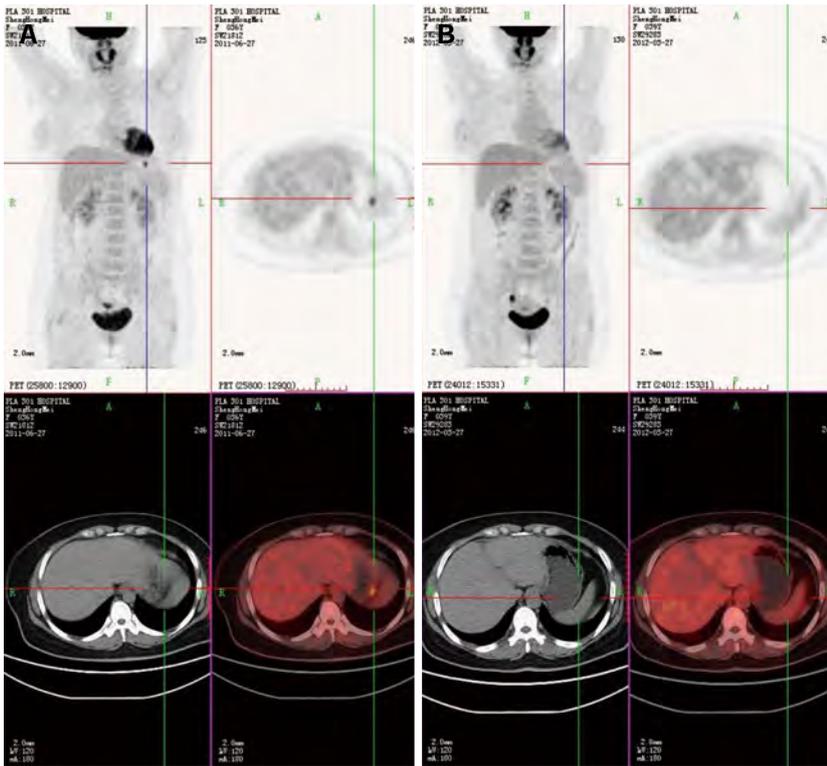


Figure 1 Positron emission tomography-computed tomography. A: Positron emission tomography-computed tomography showed a nodular strong accumulation point with standardized uptake value 5.6 in the gastric fundus; B: After treatment, the nodular strong accumulation point in the gastric fundus had disappeared.

lori virulence factors, especially cytotoxin-associated gene A (*CagA*), vacuolating cytotoxin A (*VacA*), outer inflammation protein A and so on are reported to be correlated with *H. pylori*-related diseases. One of the major bacterial virulence factors, the *VacA*, seems to be involved in the physiologic mechanism. The *VacA* protein encoded by the polymorphic *H. pylori VacA* gene, is produced and secreted by all bacterium strains and induces the formation of intracellular vacuoles in epithelial cell lines *in vitro*. Environmental and demographic data also interfere with the pathophysiology of *H. pylori*-associated gastric diseases^[3].

CASE REPORT

We present a case of a 38-year-old woman with a history of thyroid cancer who was referred by her primary care physician for a screening positron emission tomography-computed tomography (PET-CT). She was essentially asymptomatic and did not report any abdominal pain, dysphagia, nausea, or vomiting. Findings of a physical examination were unremarkable.

PET-CT showed a nodular strong accumulation point with standardized uptake value (SUV) 5.6 in the gastric fundus (Figure 1A). Gastroscopy was then performed, and demonstrated a single arched ulcer, measuring 12 mm in size, in the gastric fundus (Figure 2A and B). Histopathological examination revealed that the lesion had chronic mucosal inflammation with acute inflammation and *H. pylori* infection. There was an obvious mitotic phase with widespread lymphoma immunohistochemical staining was positive for CD4 (T cell), CD3 (T cell), CD20, Ki-67 (+25%), CD79a (+++), PAX-5, CD45RO and negative for CD56, TIA-1, TIF-1, Bcl-6, CD10,

CD30, CD34, CD117, CK, MUM-1, MPO (Figure 3A).

The patient was given *H. pylori* eradication therapy, based on proton pump inhibitor-clarithromycin-amoxicillin-mucosal protective agent treatment, the so-called quadruple 14 d therapy. One month later, gastroscopy was performed and showed a single arched ulcer, measuring 10 mm in size in the gastric fundus (Figure 2C). Histopathological examination revealed that the lesion had chronic mucosal inflammation with acute inflammation and a small amount of *H. pylori* infection (Figure 3B). The mitotic phase was 4/10 high power field with some heterotypes and an obvious nucleolus. Immunohistochemical staining showed tissue cell-like cells positive for S-100, vimentin, CD68, Ki-67 (30%) and negative for CD1a, CD21, Bcl-2, CD3, CD20, CD30, CD45RO, CD117 and PAX-5 (Figure 3C). For further examination, immunohistochemical staining was repeated by the Beijing Cancer Hospital and showed that staining for CD1a was positive for focal lesions (Figure 3D). The shape and immunophenotype indicated Langerhans histiocytosis. Because of the active growth of cancer cells, the patient was referred for medical oncology evaluation for this unusual pathologic finding with malignant potential.

Follow-up gastroscopy 2 mo later showed that the gastric ulcer was in stage S2 (Figure 2D). The mucosal swelling was markedly reduced. Endoscopic ultrasonography showed that the local echo was normal and each layer was clearly divided (Figure 2E). Histopathological examination showed chronic mucosal inflammation with lymphoid tissue hyperplasia in the lamina propria (Figure 3E).

The patient remained asymptomatic, and a follow-up PET-CT was performed 6 mo later. The nodular strong accumulation point with SUV 5.6 in the gastric fundus

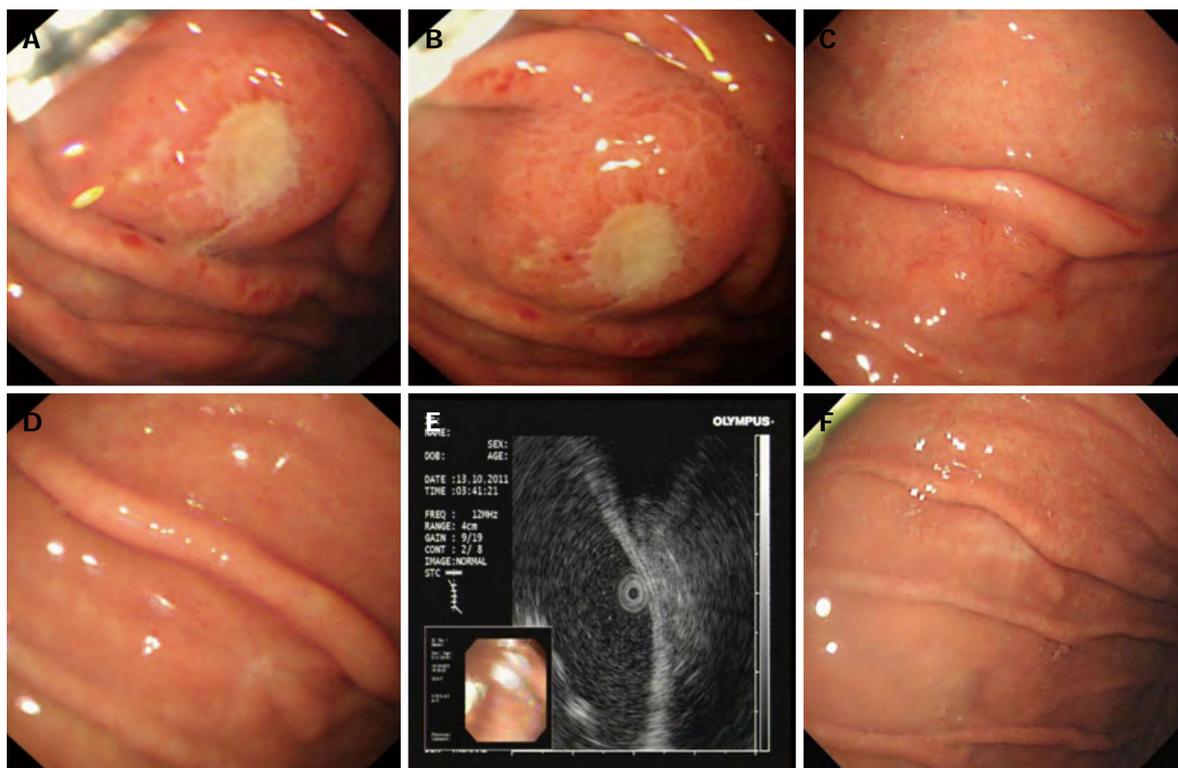


Figure 2 Gastroscopy showed a single arched ulcer and changes after treatment. A, B: A single arched ulcer, measuring 12 mm in size, was found in the gastric fundus (July 1, 2011); C: A single arched ulcer, measuring 10 mm in size, was found in the gastric fundus (August 16, 2011); D: The gastric ulcer was in stage S2 (October 13, 2011); E: Endoscopic ultrasonography showed that the local echo was normal and each layer was clearly divided (October 13, 2011); F: The gastric ulcer was in stage S2 (April 25, 2012).

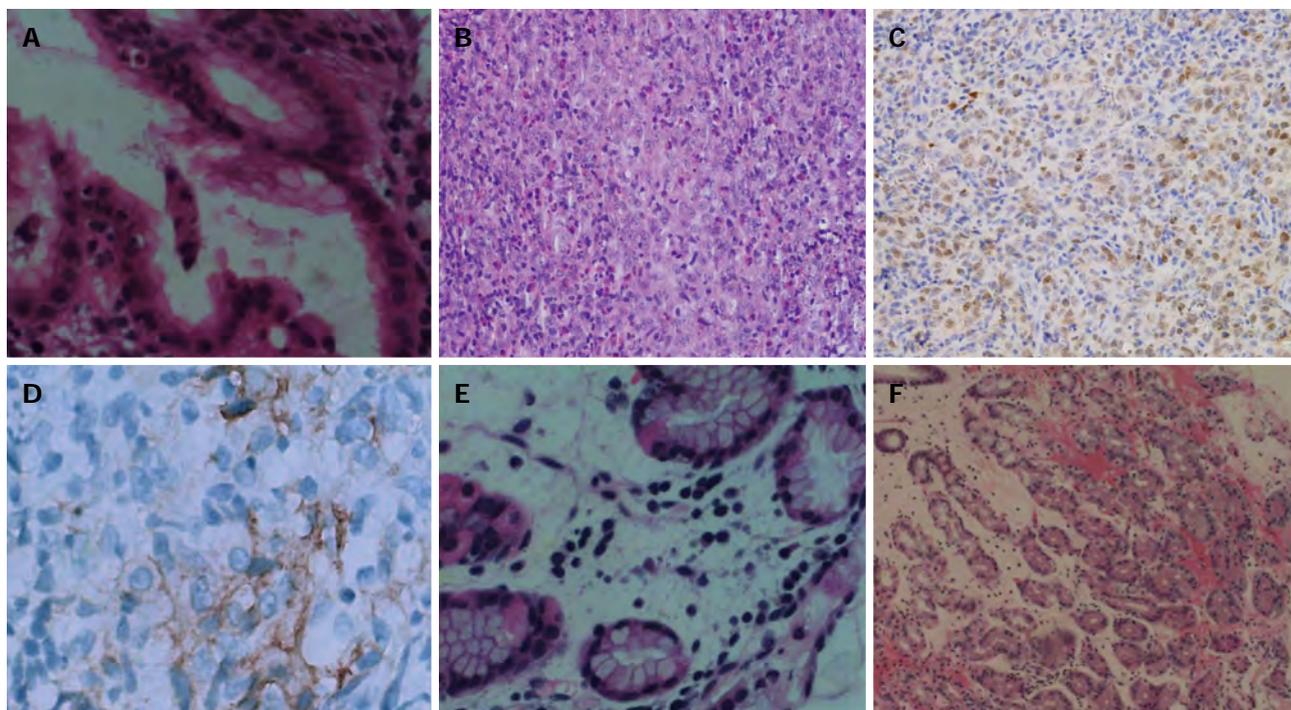


Figure 3 Histopathology changes after treatment. A: Histopathological examination revealed that the lesion had chronic mucosal inflammation with acute inflammation and a small amount of *Helicobacter pylori* (*H. pylori*) infection. The mitotic phase was obvious and lymphoma was widespread [hematoxylin and eosin (HE), $\times 400$, July 2, 2011]; B: Histopathological examination revealed chronic mucosal inflammation with acute inflammation and a only a small amount of *H. pylori* infection. The mitotic phase was 4/10 high power field (HE, $\times 400$, August 17, 2011); C: Immunohistochemical staining showed tissue cell-like cells with S-100 ($\times 400$, August 17, 2011); D: Immunohistochemical staining was repeated by the Beijing Cancer Hospital and showed that staining for CD1a was positive for focal lesions ($\times 400$, August 25, 2011); E: Histopathological examination showed chronic mucosal inflammation with lymphoid tissue hyperplasia in the lamina propria (HE, $\times 400$, October 14, 2011); F: Histopathological examination revealed that chronic mucosal inflammation with acute inflammation (HE, $\times 10$, May 8, 2012).

had disappeared (Figure 1B). Follow-up gastroscopy at the same time showed that the gastric ulcer was in stage S2 (Figure 2F). Histopathological examination revealed that the lesion had chronic mucosal inflammation with acute inflammation (Figure 3F). Therefore, we found no evidence of malignant cancer.

DISCUSSION

H. pylori infection is a worldwide disease, with about half of the world's population harboring this bacterium in their stomach. The infection is asymptomatic in most individuals. However, it is the leading cause of non-ulcer dyspepsia, peptic ulcers and gastric tumors^[4].

H. pylori is able to survive in the gastric acidic environment because of its ability to synthesize urease, an enzyme which can neutralize the stomach acidic pH. It seems to play a role in the mechanisms which lead to gastric cancer by inducing methylation in different genes, interfering with apoptotic pathways and by causing inflammatory events leading to gastritis, then to atrophic gastritis and possibly to gastric cancer^[5]. It may affect the acid secretion of the parietal cells by causing mucosal inflammation. Gastric acid secretion depends on the localization and the degree of the inflammation. Acute infection with *H. pylori* results in hypochlorhydria, whereas chronic infection can cause either hypo- or hyper-chlorhydria, depending on the distribution of the infection and the degree of corpus gastritis^[6]. *H. pylori* is a powerful carcinogen, since it is able to induce genetic changes, such as hypermethylation events, contributing to cell transformation^[5].

H. pylori is well recognized as a class I carcinogen because long-term colonization by this organism can provoke chronic inflammation and atrophy, which can further lead to malignant transformation^[7]. Chronic inflammation plays important roles in the development of various cancers, particularly in digestive organs, including *H. pylori*-associated gastric cancer^[8]. During chronic inflammation, *H. pylori* can induce genetic and epigenetic changes, including point mutations, deletions, duplications, recombinations, and methylation of various tumor-related genes through various mechanisms, which act in concert to alter important pathways involved in normal cellular function, and hence accelerate inflammation-associated cancer development^[9]. Alfizah *et al*^[10] reported that variant of *H. pylori* CagA proteins induce different magnitudes of morphological changes in gastric epithelial cells. In his study, the CagA protein was injected into gastric epithelial cells and supposedly induced morphological changes termed the "hummingbird phenotype", which is associated with scattering and increased cell motility. The molecular mechanisms leading to the CagA-dependent morphological changes are only partially known^[11,12]. The activity of different CagA variants in the induction of the hummingbird phenotype in gastric epithelial cells depends at least in part on EPIYA motif variability. The difference in CagA genotypes might influence the potential of individual CagAs to cause morphological changes

in host cells. Depending on the relative exposure of cells to CagA genotypes, this may contribute to the various disease outcomes caused by *H. pylori* infection in different individuals^[10].

Epidemiologic studies have demonstrated that *H. pylori* infection is associated with increased risk of the development of gastric cancer^[13-15]. Animal studies have also shown that *H. pylori* infection leads to gastric carcinogenesis, especially intestinal phenotypes. Yu *et al*^[16] carried out an *in vitro* study of cell transformation induced by *H. pylori* and showed that *H. pylori* induced morphologic changes in GES-1 cells and significantly increased the proliferation of GES-1 cells. Because the transition from inflamed mucosa to atrophic change is a common route to carcinogenesis, the effect of *H. pylori* eradication on the incidence of this early precursor lesion is of interest^[17]. Since *H. pylori* infection is associated with gastric carcinoma, therapy is warranted for its eradication^[5,18,19].

This was a rare case of a *H. pylori*-related gastric ulcer that resembled gastric cancer. PET-CT SUV was high, and gastroscopy showed a large ulcer with malignant-like histopathological features. However, after *H. pylori* eradication treatment, the lesion recovered quickly and follow-up examination showed no evidence of malignant cancer.

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Coexistence of gastrointestinal stromal tumor, esophageal and gastric cardia carcinomas

Yong Zhou, Xu-Dong Wu, Quan Shi, Jing Jia

Yong Zhou, Department of General Surgery, Yancheng City No.1 People's Hospital, Yancheng 224005, Jiangsu Province, China

Xu-Dong Wu, Department of Gastroenterology, Yancheng City No.1 People's Hospital, Yancheng 224005, Jiangsu Province, China

Quan Shi, Department of Radiology, Yancheng City No.1 People's Hospital, Yancheng 224005, Jiangsu Province, China
Jing Jia, Department of Nephrology, Yancheng City No.1 People's Hospital, Yancheng 224005, Jiangsu Province, China

Author contributions: Zhou Y, Wu XD, Shi Q and Jia J contributed to the manuscript writing and revision.

Correspondence to: Xu-Dong Wu, PhD, Department of Gastroenterology, Yancheng City No.1 People's Hospital, 16 Yuehe Road, Yancheng 224005, Jiangsu Province, China. hnjsycwxd@163.com

Telephone: +86-510-88508910 Fax: +86-510-88508910

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Abstract

Gastric gastrointestinal stromal tumor (GIST), esophageal squamous cell carcinoma and gastric cardia adenocarcinoma are distinct neoplasms originating from different cell layers; therefore, simultaneous development of such carcinomas is relatively rare. Auxiliary examinations revealed coexistence of esophageal and gastric cardia carcinoma with lymph node metastasis in a 77-year-old man. Intraoperatively, an extraluminal tumor (about 6.0 cm × 5.0 cm × 6.0 cm) at the posterior wall of the gastric body, a tumor (about 2.5 cm × 2.0 cm) in the lower esophagus, and an infiltrative and stenosing tumor (about 1.0 cm × 2.0 cm) in the gastric cardia were detected. Wedge resection for extraluminal gastric tumor, radical esophagectomy for lower esophageal tumor, and cardiac resection with gastroesophageal (supra-aortic arch anastomoses) were performed. Postoperative histological examination showed synchronous occurrence of gastric GIST, esophageal squamous cell carcinoma, and gastric car-

dia adenocarcinoma. Furthermore, immunohistochemistry indicated strong staining for c-Kit/CD117, Dog-1, Ki-67 and smooth muscle, while expression of S-100 and CD34 was negative.

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Key words: Gastrointestinal stromal tumor; Esophageal squamous cell carcinoma; Gastric cardia adenocarcinoma

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INTRODUCTION

Recently, cases of synchronous development of a gastrointestinal stromal tumor (GIST) and another neoplasm with different incidence, etiology, evolution and prognosis have been reported more frequently^[1-3]. Although squamous cell carcinoma and adenocarcinoma constitute the most common type of esophageal and gastric cardia tumor, respectively, simultaneous development of a GIST is relatively rare. Here, we report a case of synchronous occurrence of gastric GIST, esophageal squamous cell carcinoma, and gastric cardia adenocarcinoma.

CASE REPORT

A 77-year-old man presented with dysphagia for 2 mo. Upper gastrointestinal endoscopy was performed, which showed a tumor arising from the lower esophagus and extending into the lumen, and an ulcerated tumor located in the cardia, just below the gastroesophageal junction.



Figure 1 Esophagography showed a filling defect in the anterior wall of the lower esophagus.

He had no relevant past history or family history. Clinical examination did not find any palpable abdominal mass. Laboratory examination was normal. Esophagography showed a filling defect in the anterior wall of the lower esophagus (Figure 1). Computed tomography (CT) showed circumferential thickening of the lower esophageal wall with loss of the lumen. Scanning at a lower level displayed focal thickening of the gastric cardia wall with marked enhancement. Furthermore, scans obtained at lower levels displayed a large, heterogeneous, round mass close to the greater curvature of the stomach. The patient was diagnosed presumptively with synchronous esophageal and gastric cardia carcinoma with lymph node metastasis (Figure 2).

Intraoperatively, an extraluminal tumor (about 6.0 cm × 5.0 cm × 6.0 cm) at the posterior wall of the gastric body, a tumor (about 2.5 cm × 2.0 cm) in the lower esophagus, and an infiltrative and stenosing tumor (about 1.0 cm × 2.0 cm) in the gastric cardia was detected. Wedge resection for extraluminal gastric tumor, radical esophagectomy for lower esophageal tumor, and cardiac resection with gastroesophageal (supra-aortic arch anastomoses) were performed.

On histopathological examination, the gastric cardia tumor was a mid-differentiated gastric adenocarcinoma (pT_{1b}N₀M₀), and the lower esophageal tumor was a low-mid-differentiated squamous cell carcinoma (pT₃N₀M₀) (Figure 3). There was no vascular invasion and no lymph node metastasis.

Further histopathological examination of the extraluminal gastric tumor revealed GIST of the high-risk category, which showed a high mitotic index (> 10 mitoses/50 high-power fields). Immunohistochemistry indicated strong staining for c-Kit/CD117, Dog-1, Ki-67 and smooth muscle, while expression of S-100 and CD34 was negative (Figure 3). The patient was diagnosed with high-grade gastric GIST due to large tumor size (> 5 cm) and unfavorable histopathological features (high mitotic index and strong positivity for Ki-67).

DISCUSSION

GISTs are rare, accounting for only 0.1%-3% of all

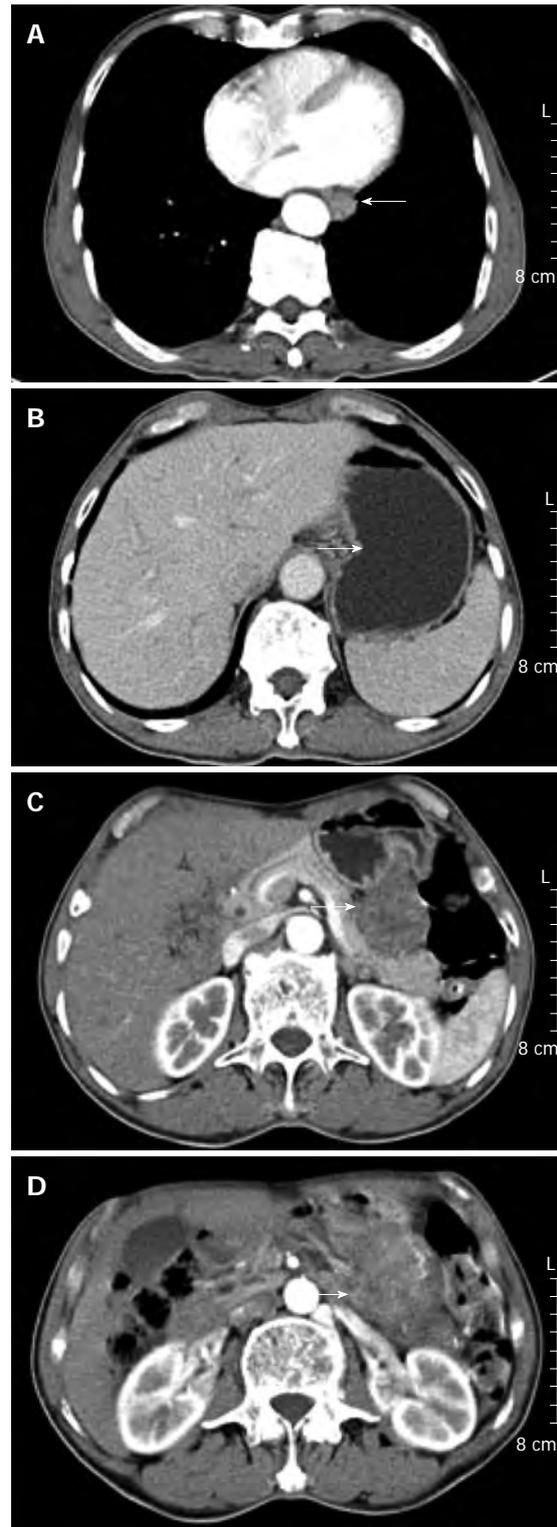


Figure 2 Computed tomography scan. A: Circumferential thickening (arrow) of the lower esophageal wall with loss of lumen; B: Lower level displayed focal thickening (arrow) of the gastric wall with marked enhancement; C and D: Lower levels displayed a large, heterogeneous, round mass close to the greater curvature of the stomach (arrows).

gastrointestinal malignancies. Primary GISTs arise most commonly in the stomach (50%-70%), followed by the small intestine (25%-35%), colon and rectum (5%-10%) and esophagus (< 5%)^[4]. These tumors are considered

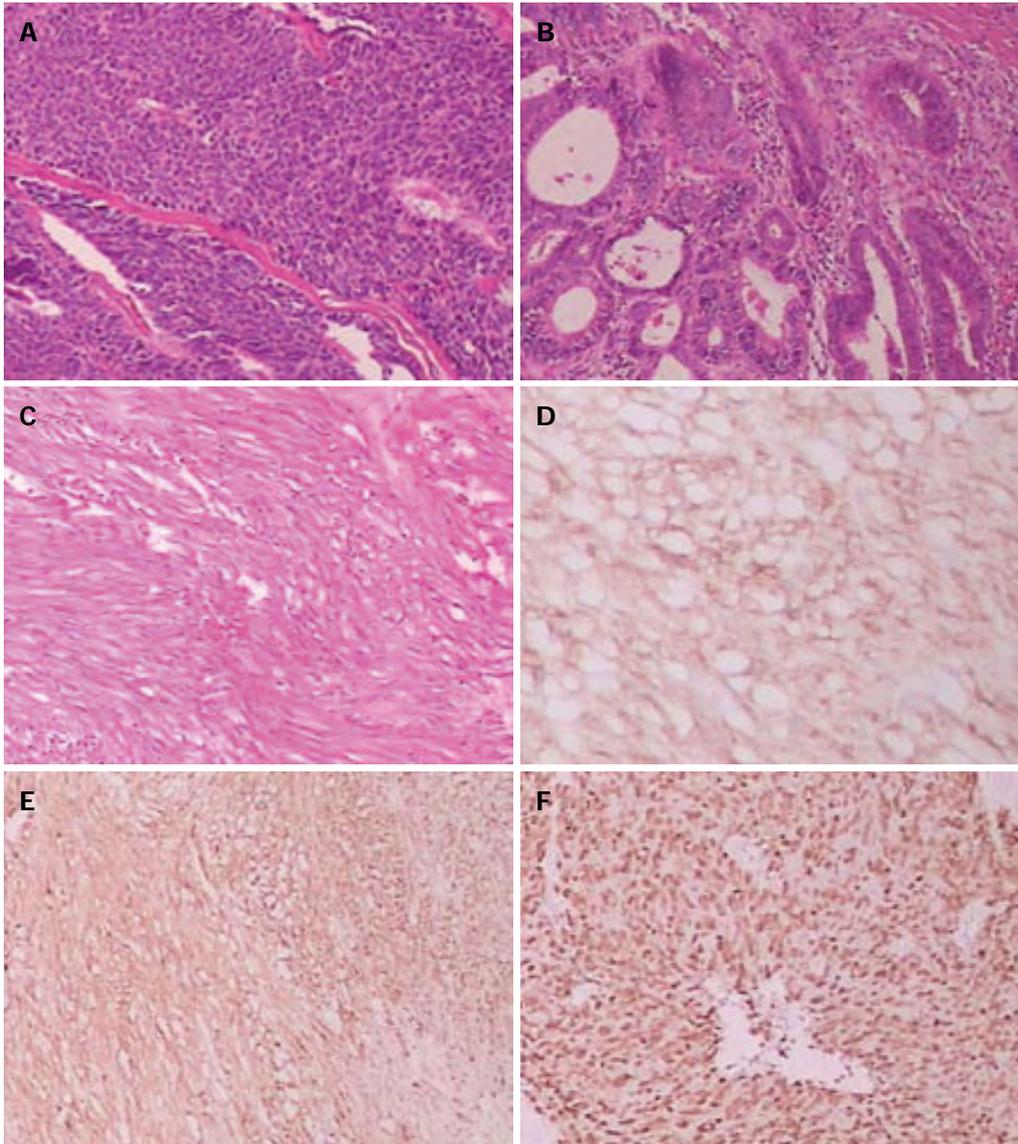


Figure 3 Microscopic images. A: Esophageal squamous cell carcinoma ($\times 10$); B: Gastric cardia adenocarcinoma ($\times 10$); C: Gastric gastrointestinal stromal tumor ($\times 10$); D-F: Immunohistochemistry indicated strong staining for Dog-1 (D, $\times 40$), c-Kit/CD117 (E, $\times 10$), Ki-67 (F, $\times 10$).

to originate from interstitial cells of Cajal or their precursors, because both strongly express the c-Kit protein (CD117; a type III tyrosine kinase receptor encoded by the c-Kit proto-oncogene)^[5].

Radical surgery is the main treatment in primary resectable GISTs. Recurrence, metastatic disease or unresectable tumors could be treated with imatinib (a small-molecule tyrosine-kinase inhibitor)^[6].

Adenocarcinoma of the stomach ranks as the second most common cancer worldwide, which comprises 80% of all stomach cancers. Squamous cell carcinoma mainly occurs in the mid to lower esophagus, and is not commonly accompanied by other cancerous lesions. Suzuki *et al*^[7] have reported that most lesions are in the stomach (59.6%), followed by the colon and rectum (12.3%). Various hypotheses, such as gene mutation, expression of metallothioneins, neighboring tissues being influenced by the same carcinogens, have been proposed regarding the simultaneous development of GIST and other

cancers^[8,9]. However, at present, no data are available to support such hypotheses. Furthermore, simultaneous occurrence of gastric GIST, esophageal squamous cell carcinoma, and gastric adenocarcinoma has not often been reported in the literature. Simple coincidence could be the most reasonable explanation.

For patients with primary GIST, surgical resection is the only chance for cure. Resection can usually be accomplished with only wedge resection of the stomach or segmental resection of the small bowel for small GISTs, whereas extensive surgery is occasionally required for larger or poorly positioned GISTs^[6].

The only curative treatment for esophageal or gastric cardia cancer is surgical resection. After esophagectomy, digestive tract reconstruction can be accomplished using the remaining stomach, depending on the location of the gastric tumor. The colon or jejunum is the frequent choice for esophageal substitution. In our opinion, digestive tract reconstruction with the remaining stomach

should be a reasonable choice for old people (age > 75 years). Although the GIST is large, only wedge resection of the stomach was performed to keep enough stomach for digestive tract reconstruction.

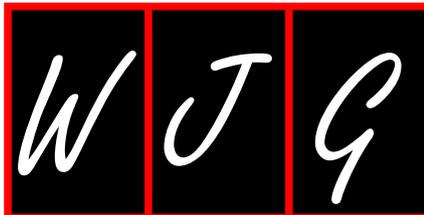
In our case, we considered gastric adenocarcinomas to be an early stage gastric cancer and esophageal squamous cell carcinoma to be a middle stage esophageal cancer. Meantime, the patient was diagnosed with high-grade gastric GIST due to large tumor size (> 5 cm) and unfavorable histopathological features (high mitotic index and strong positivity for Ki-67). Therefore, we suggested that the patient should undergo chemoradiation therapy and adjuvant imatinib treatment. However, the patient refused. A postoperative CT scan performed 3 mo later showed no evidence of tumor recurrence. The patient needs a long follow-up period.

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Tumor rupture during surgery for gastrointestinal stromal tumors: Pay attention!

Nadia Peparini, Piero Chirletti

Nadia Peparini, Azienda Sanitaria Locale Roma H, 00043 Ciampino, Italy

Piero Chirletti, Department of Surgical Sciences, Sapienza University of Rome, 00161 Rome, Italy

Author contributions: Peparini N conceived and drafted the manuscript, critically revised the manuscript and gave the final approval; Chirletti P critically revised the manuscript and gave its final approval.

Correspondence to: Nadia Peparini, MD, PhD, Azienda Sanitaria Locale Roma H, Via Mario Calò, 5-00043 Ciampino, Italy. nadiapeparini@yahoo.it

Telephone: +39-339-2203940 Fax: +39-76-488423

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Abstract

In a recently published letter to the editor, we debated the proposal by Coccolini *et al* to treat gastrointestinal stromal tumors (GISTs) of the esophagogastric junction with enucleation and, if indicated, adjuvant therapy. We highlighted that, because the prognostic impact of a T1 high-mitotic rate esophageal GIST is worse than that of a T1 high-mitotic rate gastric GIST, enucleation may not be adequate surgery for esophagogastric GISTs with a high mitotic rate. In rebuttal, Coccolini *et al* pointed out the possible bias in assessment of the mitotic rates due to the lack of standardized methods and underlined that the site and features of the tumor need to be carefully considered in evaluation of the risk-benefit balance. Here we confirm that, apart from the problematic issue of mitotic counting, enucleation should not be indicated for GISTs at any site to reduce the risk of tumor rupture, which has been recently considered to be an unfavorable prognostic factor, and to avoid microscopic residual tumor.

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Key words: Gastrointestinal stromal tumor; Esophago-

gastric junction; Surgery; Resection; Enucleation

Peparini N, Chirletti P. Tumor rupture during surgery for gastrointestinal stromal tumors: Pay attention! *World J Gastroenterol* 2013; 19(12): 2009-2010 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v19/i12/2009.htm> DOI: <http://dx.doi.org/10.3748/wjg.v19.i12.2009>

TO THE EDITOR

In a recent issue of *World Journal of Gastroenterology*, we debated^[1] the proposal by Coccolini *et al*^[2] to treat gastrointestinal stromal tumors (GISTs) of the esophagogastric junction with enucleation and, if indicated, adjuvant therapy. We highlighted that, because the prognostic impact of a T1 high-mitotic rate esophageal GIST is worse than that of a T1 high-mitotic rate gastric GIST, enucleation may not be adequate surgery for esophagogastric GISTs with a high mitotic rate. In rebuttal, Coccolini *et al*^[3] pointed out the possible bias in the assessment of the mitotic rate due to the lack of standardized methods and underlined that the site and features of the tumor need to be carefully considered in the evaluation of the risk-benefit balance.

Apart from the prognostic differences related to the anatomic localization of the gastric GISTs (gastroesophageal junction-body-distal antrum), problematic mitotic counting is a significant issue in the staging and therapy of GISTs. Controversies exist regarding how large the 50 high-power field areas should be^[4], varying from 5 mm² to 10 mm². The area recommended by the European Guideline represents half of the area recommended by TNM Classification of Malignant Tumors^[5,6].

However, tumor rupture is a highly unfavourable prognostic factor, which should be considered rather than the mitotic rate, tumor site and tumor size in planning an effective treatment for GISTs. According to the modified risk stratification proposed by Joensuu *et al*^[7] and Rutkowski *et al*^[8], patients with tumor rupture are in-

cluded in high-risk category GISTs.

On the other hand, according to updated National Comprehensive Cancer Network Guidelines^[9], Coccolini *et al.*^[2] pointed out the value of complete resection, leaving a negative margin and an intact pseudocapsule. GISTs may be soft and fragile because of intratumoral hemorrhage and/or necrosis; anyway they are surrounded by a pseudocapsule that should not be torn during surgery to avoid intra-abdominal seeding. From technical point of view, enucleation of GIST implies that the plane of dissection is conducted along the pseudocapsule with no distance margin on the entire surface of the tumor - *i.e.*, at best microscopic residual tumor (R1) surgery - or rather, enucleation maximizes the risks of R1 and tumor rupture.

We think that complete resection should remain the standard surgical treatment for localized GISTs at any site through wedge resection for small size favorably positioned GISTs and variably extended segmental organ resection depending on the size and site for large and/or unfavourably positioned GISTs. To reduce the risk of tumor rupture with consequent risk of tumor relapse and avoid microscopic residual tumor enucleation should not be indicated for any GISTs. For the risk of tumor rupture, laparoscopic surgery should be avoided with large GISTs^[5].

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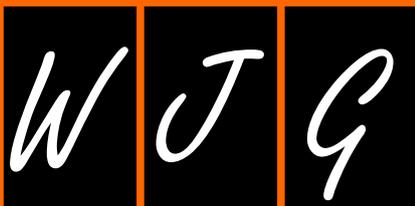
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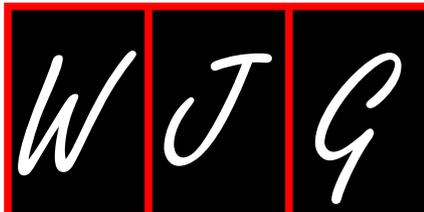
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Clinical relevance of cancer genome sequencing

Chee Seng Ku, David N Cooper, Dimitrios H Roukos

Chee Seng Ku, Department of Medical Epidemiology and Biostatistics, Karolinska Institute, SE-17177 Stockholm, Sweden
David N Cooper, Institute of Medical Genetics, School of Medicine, Cardiff University, Cardiff CF14 4YU, United Kingdom
Dimitrios H Roukos, Department of Surgery, Ioannina University School of Medicine, 45110 Ioannina, Greece
Dimitrios H Roukos, Centre for Biosystems and Synthetic Genomic Network Medicine, Centre for BioSystems and Genomic Network Medicine, Ioannina University, 45110 Ioannina, Greece
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Correspondence to: Dimitrios H Roukos, Associate Professor, Department of Surgery, Ioannina University School of Medicine, 45110 Ioannina, Greece. droukos@cc.uoi.gr
Telephone: +30-26510-07423 Fax: +30-26510-07094
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Abstract

The arrival of both high-throughput and bench-top next-generation sequencing technologies and sequence enrichment methods has revolutionized our approach to dissecting the genetic basis of cancer. These technologies have been almost invariably employed in whole-genome sequencing (WGS) and whole-exome sequencing (WES) studies. Both WGS and WES approaches have been widely applied to interrogate the somatic mutational landscape of sporadic cancers and identify novel germline mutations underlying familial cancer syndromes. The clinical implications of cancer genome sequencing have become increasingly clear, for example in diagnostics. In this editorial, we present these advances in the context of research discovery and discuss both the clinical relevance of cancer genome sequencing and the challenges associated with the adoption of these genomic technologies in a clinical setting.

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Key words: Next-generation sequencing; Exome; Can-

cer; Diagnostics; Familial cancer syndrome; Somatic mutation

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INTRODUCTION

Sporadic cancers are complex diseases that are caused by the accumulation of somatic mutations that are acquired by the genomes of the cells of the tissue in which the cancer originated^[1]. The importance of identifying the “driver” (causal) somatic mutations amongst the much more numerous “passenger” mutations has long been recognized. However, previous cancer genome sequencing studies have also been constrained by technological limitations. Although several early attempts were made to sequence the coding regions of the majority of the consensus coding sequence and/or RefSeq genes in several cancers (*e.g.*, breast and colorectal), these studies were conducted in “brute-force” mode employing traditional low-throughput polymerase chain reaction-Sanger sequencing methods^[2,3]. The advent of next-generation sequencing (NGS) technologies has revolutionized the sequencing of cancer genomes, the first example of which employed whole-genome sequencing (WGS) to characterize an acute myeloid leukemia (AML) genome thereby identifying numerous tumor-specific mutations^[4]. This study clearly demonstrated the technical feasibility of applying NGS to interrogate the genome-wide somatic mutational spectra of entire cancer genomes in tandem with paired constitutional DNA samples.

In parallel, the development of a variety of exome enrichment methods to selectively capture the entire collection of exons in the human genome has made whole-exome sequencing (WES) technically feasible^[5]. Coupling this development to the high-throughput NGS techniques has allowed the exome to be sequenced very

rapidly and in unprecedented detail. In comparison to WGS, WES is more affordable for larger sample sizes and is analytically less challenging since only 1%-2% of the entire genome needs to be sequenced^[6-8]. As a result, a larger number of cancer DNA samples have been sequenced by WES than WGS in attempts to identify recurrent mutations (*i.e.*, identical mutations that recur in different samples) and highly mutated genes (genes harboring multiple mutations in a significant proportion of the cancer samples)^[9-12]. The other reason for the more widespread adoption of WES has been that the mutations identified within protein coding regions are inherently easier to interpret than those in the non-coding regions, which still remain largely uncharacterized in functional terms. In addition to the advances being made in characterizing the somatic mutational landscape in various cancers, the applications of cancer genome sequencing in a clinical setting have also become increasingly numerous.

SOMATIC MUTATIONS IN SPORADIC CANCERS

WGS and WES have been commonly applied to study the patterns of somatic mutation in a range of different cancers^[13,14]. Collectively, these endeavors have generated new insights into the mutational landscape of various cancers, and have resulted in the identification of a large number of recurring mutations as well as many highly mutated genes. For example, in the context of gastrointestinal cancers, WES of 15 gastric adenocarcinomas and their matched normal DNAs succeeded in identifying several frequently mutated genes such as *TP53*, *PIK3CA* and *ARID1A*^[15]. In addition, it was found that cell adhesion was the most enriched biological pathway among the frequently mutated genes. More importantly, mutations in three chromatin remodeling genes (*ARID1A*, *MLL3* and *MLL*) were detected in almost half of the gastric cancers examined^[15]. In fact, an earlier study which performed WES in 22 gastric cancer samples also identified frequent inactivating mutations in *ARID1A*, which encodes a member of the switch/sucrose non-fermenting chromatin remodeling family. Further, the mutational spectrum for *ARID1A* was found to differ between molecular subtypes of gastric cancer^[7]. In similar vein, mutations in multiple chromatin regulator genes such as *ARID1A*, *ARID1B*, *ARID2*, *MLL* and *MLL3* were also found in about half of hepatocellular carcinomas through WGS^[16]. The consistent finding of mutations in chromatin remodeling genes in different cancers, which also included renal carcinoma and glioblastoma multiforme, further highlights a close inter-relationship (or possibly a “synergy” interaction effect) between somatic mutations and aberrant epigenetic regulation in the pathogenesis of cancers^[8,17-19].

In addition to individual studies, technological advances have made possible large-scale international projects such as the International Cancer Genome Consor-

tium which aims to interrogate the somatic mutational landscape of at least 50 different cancer types and subtypes in thousands of samples, with the eventual aim of integrating these genomic data with both transcriptomic and epigenomic data. NGS technologies are instrumental in generating these “omics datasets”^[20]. The concept of an integrative approach for a range of different omics data is not new, but in recent years it has resurfaced and become reinvigorated by technological advancement. The integrative analysis of different omics datasets (providing information in different dimensions, from DNA sequence to the transcriptional and translational levels) is expected to be more informative, and hence ought to provide new and more detailed biological insights, than would be possible using individual datasets^[21].

Although most of the cancer genome sequencing studies were not conducted with a view to investigating their applications in a direct clinical context *per se* (but rather to characterize the somatic mutational spectrum in order to understand better the genetic basis and biology of the cancer in question), the data generated are nevertheless important as a means to identify the drug targets as well as potential biomarkers (*e.g.*, single mutations or mutational patterns that could be used for diagnostic and prognostic applications). The potential of driver mutations to shape the future science of cancer taxonomy was recently outlined by Stratton (2011) *i.e.*, the drawing up of a system based on causal mutations rather than the conventional organ-based (*e.g.*, breast, lung or colorectum tissue) classification and TNM-staging system that are widely applied in the clinic^[22].

So far, what are the potential implications of cancer genome sequencing for the clinical setting? The application of cancer genome sequencing in diagnostics has been increasingly evident, as demonstrated by two recent studies using WGS^[23,24]. WGS has demonstrated both its discovery and confirmatory role in a specific patient characterized by an ambiguous diagnosis or clinical presentation. More specifically, it has been used to determine the genetic aberration in a patient with a diagnosis of AML of unclear subtype^[23]. The ambiguity came from the observation that the patient’s clinical presentation was consistent with acute promyelocytic leukemia (which is a subtype of AML with a favorable prognosis), but it was contradicted by cytogenetic analysis. The cytogenetic analysis revealed a different subtype associated with a poor prognosis for which bone marrow transplantation in first remission is recommended. The diagnostic and treatment uncertainty was resolved by WGS performed on the original leukemic bone marrow and from a skin biopsy. The WGS analysis detected a novel insertional translocation on chromosome 17 which generated a pathogenic *PML-RARA* gene fusion thereby confirming a diagnosis of acute promyelocytic leukemia. This type of complex rearrangement could not have been made by targeted sequencing (as the genetic etiology was unsuspected), further demonstrating that WGS represents both a discovery and a comprehensive

analytical tool for the entire genome. More importantly, the molecular confirmatory diagnosis carries important clinical implications for the treatment and management of the patient^[23]. Similarly, WGS was also employed to resolve the genetic basis of a suspected cancer susceptibility syndrome based upon the early onset of several primary tumors^[24]. Further, therapeutic prediction has also benefited from NGS as a powerful discovery tool. For example, a recent study employed a targeted NGS approach to sequence 138 cancer genes in melanomas derived from a patient (before and after relapse) and succeeded in identifying the underlying genetic mutation in the *MEK1* gene responsible for acquired resistance to PLX4032 (vemurafinib) after an initial dramatic response, revealing a novel mechanism of acquired drug resistance^[25].

The potential applications of cancer genome sequencing in the clinical arena are promising, but what are the challenges associated with their adoption? As WGS and WES are high-throughput methods which generate huge amounts of data, our ability to perform both the analysis and the interpretation of the data in a clinically relevant time-frame is critical. This challenge is being addressed in the context of a “comprehensive genomic approach” where WGS, WES and transcriptome sequencing are applied to cancer samples to evaluate their clinical utility and feasibility (in terms of technical, time and cost)^[26]. In particular, the time required, from biopsy sampling and wet-lab experiments to computational analysis and initial results, was streamlined to just 24 d with the cost of all the sequencing and analysis estimated to be only USD5400. An obvious advantage of this “integrative genomic approach” is that the findings can be cross-validated more efficiently. For example, both WGS and WES detected an amplification event on chromosome 13q spanning the *CDK8* gene in a metastatic colorectal carcinoma; the over-expression of *CDK8* was confirmed by transcriptome sequencing. Although this “comprehensive genomic approach” was shown to be both time- and cost-effective, the handling and interpretation of the huge amount of genomic data remains a key issue. To address this challenge, it was proposed that a multi-disciplinary “sequencing tumor board” (which included professionals from multiple disciplines such as clinicians, geneticists, pathologists, biologists, bioinformatic specialists and bioethicists) should take responsibility for the clinical interpretation of the sequencing data obtained from each patient^[26].

FAMILIAL CANCER SYNDROMES

In addition to the investigation of somatic mutations in sporadic cancers, cancer genome sequencing has also made significant advances in relation to the study of the germline mutations underlying familial cancer syndromes. The early successes in the identification of causal mutations and genes for familial cancer syndromes (e.g., *RB1* and *APC*) were achieved by painstaking family

linkage analysis and positional cloning. However, the genetic causes of many familial cancer syndromes have remained elusive. For example, *CDH1* was the first and only causal gene identified for hereditary diffuse gastric cancer through linkage analysis^[27], but germline mutations in this gene account for only a proportion of hereditary diffuse gastric cancer cases. Thus germline mutations in *CDH1* were found in 30%-40% of clinically defined families with hereditary diffuse gastric cancer from different ethnic backgrounds^[28,29]. This suggests that an as-yet-to-be identified gene(s) is likely to be responsible for the remaining cases unexplained by *CDH1*. On the other hand, whereas most Lynch syndrome cases can be accounted for by mutations in DNA mismatch repair genes, the genetic basis of familial colorectal cancer type X still remains elusive^[30,31]. Similarly, the genetic causes of other familial cancer syndromes, such as familial pancreatic cancer, still remain largely unknown^[32,33]. In a fashion similar to that noted with the identification of somatic mutations, cancer genome sequencing provides new opportunities to identify germline mutations for familial cancer syndromes. This is well exemplified by the case of hereditary pheochromocytoma, a rare neural crest cell tumor; by harnessing the latest technological advances, germline mutations in *MAX* were identified in three unrelated individuals with hereditary pheochromocytoma by WES^[34]. The segregation of two *MAX* gene variants with hereditary pheochromocytoma was observed in families from whom DNA from affected relatives was available. Further, additional data to support the causative role of the *MAX* variants came from their absence (or non-detection) in more than 750 population-matched control chromosomes. Additional screening for *MAX* mutations in 59 cases lacking germline mutations in known genes for hereditary pheochromocytoma then identified two additional truncating mutations and three missense variants in the gene^[34]. Following this discovery, a recent study found that germline mutations in *MAX* are responsible for 1.12% pheochromocytomas or paragangliomas (both are genetically heterogeneous neural crest-derived neoplasms) by sequencing *MAX* in 1694 patients^[35].

In addition to its role in research discovery, cancer genome sequencing has also been used as a diagnostic tool to detect known germline mutations for familial cancer syndromes. Indeed, by leveraging technological advances in genomic sequence enrichment methods and NGS technologies, studies have developed NGS-based diagnostic tests for breast and ovarian cancers and Lynch syndrome. For example, Walsh *et al.*^[36] designed custom oligonucleotides in solution to capture 21 genes responsible for hereditary breast and ovarian cancers, and the enriched genomic DNA was then subjected to sequencing using an NGS platform. This NGS-based test was evaluated in 20 women diagnosed with breast or ovarian cancer and with a known mutation in one of the genes responsible for inherited predisposition to these cancers. The results were very promising in that all the known

point mutations and small indel mutations (ranging from 1 bp to 19 bp), as well as large genomic duplications and deletions (ranging from 160 bp to 101 013bp), were detected in all the samples. The potential to detect different mutations of various sizes has further demonstrated the technical advantages of NGS-based tests over conventional PCR-Sanger sequencing methods. For example, two different tests were offered separately to detect point mutations and large deletions/amplifications for genetic testing of *BRCAl/2* genes, respectively^[36]. Similarly, attempts have also been made to incorporate custom genomic enrichment and NGS methods into the genetic diagnostic testing of Lynch syndrome by capturing every exon in a panel of 22 genes (most of which are known to be associated with hereditary colorectal cancer) followed by NGS^[37].

Technological advances have facilitated the accessibility of cancer genome sequencing in the clinical arena. In addition to the custom sequence enrichment methods (*i.e.*, either based on polymerase chain reaction amplification such as Fluidigm and RainDance technologies, or based on target-probe hybridization such as the Agilent and Nimblegen technologies) that allow one to selectively capture the genomic regions of interest, the arrival of several bench-top NGS instruments has not only made the sequencing of a panel of genes highly feasible technically but also cost-effective^[5,38,39]. This is an important step towards the development and adoption of NGS-based diagnostic tests in the clinic. The bench-top NGS instruments (Roche 454 Genome Sequencer Junior, Ion Torrent Personal Genome Machine Sequencer and IlluminaMiSeq) have a much lower throughput (ranging from > 10 Mb to > 1 Gb per run) than the conventional high-throughput NGS machines^[38,39]. The bench-top NGS instruments are therefore more suitable in terms of their throughput for sequencing panels of genes (as discussed earlier for the panels of genes for breast/ovarian cancers and Lynch syndrome) than WES or WGS. Further, sample indexing (or barcoding) is also available for the bench-top NGS instruments which can further optimize sample throughput and cost-effectiveness by multiplexing up to several tens of samples for sequencing. However, the level of multiplexing is dependent on the sizes of the regions to be sequenced and the throughputs of the instruments being used. Although it remains to be demonstrated in the context of cancer, WES has been widely assessed and shown as a promising diagnostic tool for various Mendelian disorders^[40-43]. In addition to diagnosis, WGS has also been applied to optimize patient treatment regimens, although not in the context of cancer. In the context of inherited disease, WGS has been applied to sequence a fraternal twin pair diagnosed with dopa (3,4-dihydroxyphenylalanine)-responsive dystonia (OMIM 128230); germline compound heterozygous mutations were identified in the *SPR* gene encoding sepiapterin reductase. As a result, supplementation of L-dopa therapy with 5-hydroxytryptophan has led to clinical improvements in both twins^[44].

PERSPECTIVES AND CONCLUSIONS

NGS technologies have already made a major contribution to characterizing somatic mutations in cancer genomes. This endeavor will be further accelerated by international initiatives such as the International Cancer Genome Consortium. Although the number of studies is currently still very limited, NGS should also be applied to identify germline mutations in those familial cancer syndromes whose genetic causes have not yet been fully characterized. On the other hand, the successful demonstration of the applications of NGS/WGS/WES in a clinical setting such as cancer diagnostics are likely to be just the first examples of how the new technologies will prove their worth; the numbers are expected to increase in the coming year.

So far, the applications of NGS in a clinical setting have been very promising. However, challenges ranging from technical, analytical and interpretational, to the need for a considerable number of well-trained professionals from a range of disciplines in these genomic technologies must be further addressed before the adoption of NGS-based tests in the clinic. The technical challenges include, for example, incomplete capture of the exons in WES and uneven sequencing across the genome which might result in poor sequence coverage in some of the regions and affect both the sensitivity and specificity of variant detection^[39]. Having specialists trained in genomic technologies is critical to (1) obtaining fully informed consent from patients in relation to the genomic tests; (2) ensuring accurate and reliable interpretation of the data for clinical decision-making; and (3) counselling the patients on the basis of the results obtained. It is also evident from the Global Cancer Genomics Consortium (GCGC) that the translation of emerging cancer genomics knowledge into clinical applications can only be achieved through the integration of multi-disciplinary expertise^[45]. The GCGC is an international collaborative platform that brings cancer biologists and cutting edge high-throughput genomics expertise together with medical oncologists and surgical oncologists to address the most important translational questions that are central to cancer research, diagnosis and treatment.

As to test affordability, although the total cost of sequencing for several genomic experiments was only a few thousands of USD per patient, it should be appreciated that this is unlikely to be the final chargeable cost to the patients. The cost of sequencing is currently plummeting and will become ever cheaper in the future with new developments. However, it should be appreciated that hidden costs are likely to be incurred for data storage, interpretation of results and subsequent clinical consultation.

Further, handling of the complex ethical issues such as revealing findings that might be considered incidental to the initial testing (WGS and WES) procedure must also be given serious consideration^[46]. Determining what to disclose and what not to disclose to the patients is

likely to be quite challenging *e.g.*, those results which are deemed clinically important *i.e.*, those which could have a direct impact on the patient's care or management, but which are irrelevant to the initial purpose of the diagnostic test (*i.e.*, incidental findings). Have the patients the right to be informed about those results which are/might be clinically important but not actionable *e.g.*, mutations that are considered likely to predispose to certain inherited diseases, although preventive treatments are not yet available? Adequate consultation must also be given to the reporting of results that are of unknown clinical importance. This raises concerns as to whether periodic re-analysis of the WGS/WES data might be needed, which in turn would lead to some practicality issues potentially incurring additional costs. Finally, any results from WGS- and WES-based tests that would affect clinical decision-making must be properly validated or the tests must be performed in a heavily regulated clinical setting according to the College of American Pathologists/Clinical Laboratory Improvement Amendments.

It is widely anticipated that cancer genome sequencing or the NGS-based tests will gradually become more accessible in clinical practice once the associated challenges and ethical issues have been adequately addressed. Irrespective of the challenges that still remain to be overcome, the application of NGS in the clinic appears inevitable.

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Clinical application of microRNA in gastric cancer in Eastern Asian area

Ming Gao, Hao Yin, Zhe-Wei Fei

Ming Gao, Zhe-Wei Fei, Department of Surgery, Xin Hua Hospital (Chongming), Shanghai Jiao Tong University School of Medicine, Shanghai 202150, China

Hao Yin, Transplant Section, Department of Surgery, Shanghai Changzheng Hospital, Shanghai 200003, China

Ming Gao, Hao Yin, Department of Surgery, the University of Chicago, Chicago, IL 606372, United States

Author contributions: Gao M, Yin H and Fei ZW contributed equally to this work.

Correspondence to: Zhe-Wei Fei, MD, Department of Surgery, Xin Hua Hospital (Chongming), Shanghai Jiao Tong University School of Medicine, Shanghai 202150, China. roytina0241032@hotmail.com

Telephone: +86-21-69692701 Fax: +86-21-69692703

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Abstract

Recent research has shown that microRNA (miRNA), which is involved in almost every step of gastric carcinogenesis, has broad prospective application in diagnosis and therapy of gastric carcinoma. Eastern Asia (South Korea, Japan and China) has the highest incidence of gastric cancer in the world. There were 988 000 new cases of gastric cancer worldwide and 736 000 deaths in 2008. Approximately 60% of the cases of gastric cancer are found in East Asia (mainly China). We herein provide a brief review of the clinical applications of miRNA, which include the following aspects: (1) miRNA may serve as a potential new generation of tumor markers; (2) a complete miRNA expression profile is highly specific, can reflect the evolutionary lineage and differentiation of tumors, and be used to carry out diversity analysis; (3) detecting specific miRNA expression in peripheral blood will become a new method for diagnosis of gastric cancer; (4) miRNA can predict prognosis of gastric cancer; (5) miRNA has predictive value in determining chemotherapy and radiotherapy resistance; and (6) miRNA could be a type

of innovative drug. Finally, we focus on assessing the value of miRNA from laboratory to clinical application and the challenges it faces in East Asia.

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Key words: microRNA; Prognosis; Clinical application; Gastric cancer; Eastern Asia

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INTRODUCTION

Gastric cancer is a leading disease in Eastern Asia (South Korea, Japan and China) (Figure 1). The incidence and mortality of gastric cancer in East Asian areas rank respectively the second and the third among the most common cancers worldwide^[1,2]. According to World Health Organization^[3] statistics, there were 988 000 new cases of gastric cancer worldwide and 736 000 deaths in 2008. Approximately 60% of the cases are found in East Asia (mainly China). In China, approximately two-thirds of patients develop advanced or metastatic disease, and more than half have recurrent disease after curative surgery. The median survival time for these patients is only 6-9 mo^[4-6]. Several reasons restrict the diagnosis and treatment of gastric cancer: (1) limited diagnostic measures for early detection; (2) weak prognostic value of outcome; (3) poor effect of surgery or cytotoxic cell treatment for advanced disease; and (4) lack of biomarkers for targeted therapy. The discovery of microRNA (miRNA) may change the above-mentioned difficulties, and improve the level of diagnosis and treatment of gastric cancer.

miRNAs include 20-24 nucleotides and are a class of noncoding small molecular single chain RNAs, and

have highly conservative, temporal and tissue-specific characteristics^[7-9]. Through complete or incomplete base pairing with target gene mRNA, RNA-induced silencing complex degrades mRNA or blocks its translation, and regulates target gene expression at the post-transcriptional level^[10]. They exist widely in eukaryotic organisms and regulate cell proliferation, differentiation and apoptosis. Although the tissues of the body appear malignant, specific miRNAs are overexpressed or underexpressed in different tumors and at different stages, which implies a correlation with occurrence and development of tumor and prognosis^[11-13]. Further study of the relation of miRNA and gastric cancer could provide new applications in early tumor detection, monitoring, prognosis, gene therapy, and resolving chemotherapy resistance.

miRNA AND CANCER DIAGNOSIS

Specific tumor markers are often ideal screening tools. Existing clinical tumor markers [such as carcinoembryonic antigen (CEA), cancer antigen (CA)19-9, and CA72-4] for gastric cancer lack specificity and sensitivity^[14]. miRNA may serve as a potential new generation of tumor markers for the following reasons^[15-17]: (1) good tissue specificity - Rosenfeld *et al*^[18] have detected unknown sources of miRNA in order to make clear its sources, and its specificity is 90%; (2) expression of miRNA in tumors differs significantly from that in normal tissues; (3) miRNA participates in tumor occurrence and development; (4) expression of miRNA has stage specificity - the same tumors at different stages have different expression profiles^[19,20]; and (5) miRNA in fresh tissues, paraffin-embedded tissues, and cells and peripheral blood shows good stability^[21].

Detection methods of miRNA and miRNA expression in gastric cancer: miRNA is a good tumor marker in clinical application^[22,23]. At present, the detection method for miRNA has become mature. By deep sequencing, we can discover unknown miRNAs, and miRNA chips can be used for identification of the differences in miRNAs between the study and control groups. Finally, they can be verified by real-time quantitative polymerase chain reaction (qPCR)^[24].

In diagnosis of gastric cancer, a single miRNA is often characterized by poor specificity or sensitivity, but a complete miRNA expression profile is highly specific, can reflect the evolutionary lineage and differentiation of tumors, and be used to carry out diversity analysis^[25,26]. Through horizontal comparison of gastric carcinoma with adjacent normal tissues, we have found specific expression of miRNAs in cancer tissues. Further longitudinal comparison at different tumor stages has enabled us to identify the different miRNAs at each stage and to complete final tumor diagnosis and staging^[27,28].

We acquired gastric cancer miRNA expression profiles from numerous Chinese and international study groups from 2008 to 2012^[29-36] (Table 1). These results differed considerably and lacked stability and consistency

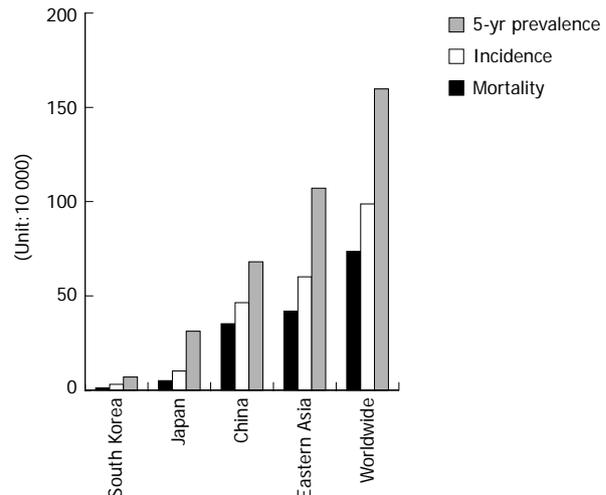


Figure 1 Gastric cancer incidence, mortality and prevalence in South Korea, Japan, China, Eastern Asia, and worldwide in 2008.

for the following reasons: (1) differences in miRNA chips and software; (2) individual differences between races and patients; (3) differences in collected specimen standards; (4) differences in sample size; and (5) miRNA expression profile differences for different cancer types and stages. According to the above expression profiling, we confirmed several reliable miRNAs in the multiple experiments which had 1.5 fold differential expressions between gastric cancer and normal gastric tissues. We are looking forward to having a large sample multi-center study or even international cooperation to compare complete miRNA expression profiling based on different pathological types and stages of gastric cancer. In particular, countries like China, Japan and South Korea should cooperate using the same platform in complete standard miRNA expression profiling of gastric cancer in East Asian populations.

Change in miRNA expression is an early event during the development of gastric tumor^[37,38]. Tracking the changes in miRNA expression profiling in the relevant gastric tissues might enable early tumor diagnosis. Traditional methods for the detection of gastric cancer are endoscopy and biopsy. A minimally invasive examination method would be helpful for screening and early detection of cancer in high-risk populations^[39]. Detection of tumor in the peripheral blood of patients with specific miRNA expression level has been a research hotspot in recent years, which will perhaps become a new method for diagnosis of gastric cancer^[40-42].

Researchers have shown that 90% of plasma miRNA is based on protein-miRNA complex formation. As tumor markers in peripheral blood, miRNAs have the following advantages: (1) miRNAs exist in great volume in peripheral blood^[43,44]; (2) miRNAs can resist enzymatic digestion^[45-47]; (3) miRNAs have strong resistance to the external environment; and (4) miRNAs show abnormal expression in tumor patients' serum^[48].

In the past two years, Japanese and Chinese research-

Table 1 microRNA expression in gastric cancer in 2008-2012

| Group | Method | Sample | Upregulated | Downregulated |
|--|------------------------------|---|--|--|
| Katada <i>et al</i> ^[29] | TaqManmiRNA assays + qRT-PCR | 42 undifferentiated gastric cancer and controls | miR-34b miR-34c miR-128a | miR-128b, miR-129, miR-148 |
| Guo <i>et al</i> ^[30] | Microarray | 3 gastric cancers and adjacent normal tissues | miR-20b, miR-20a, miR-17, miR-106a, miR-18a, miR-21, miR-106b, miR-18b, miR-421, miR-340, miR-19a, miR-658 | miR-768-3p, miR-378, miR-31, miR-139-5p, miR-195, miR-497, miR-133b, miR-638, miR-378 |
| Yao <i>et al</i> ^[31] | Microarray + qRT-PCR | 10 gastric cancers and adjacent normal tissue | miR-223, miR-106b, miR-147, miR-34a, miR-130b, miR-106a, miR-18a, miR-17, miR-98, miR-616, miR-181a-2, miR-185, miR-1259, miR-601, miR-196a, miR-221, miR-302f, miR-340, miR-337-3p, miR-520c-3p, miR-575 and miR-138 | |
| Luo <i>et al</i> ^[32] | Microarray + qRT-PCR | 24 gastric cancers | MiR-26b, miR-30a-5p, miR-212, miR-320, miR-379, miR-518b, miR-409-3b | MiR-9, miR-19b, miR-155, miR-188, miR-197, miR-338, miR-370, miR-383, miR-433, miR-490, miR-503, miR-545, miR-551a, miR-567, miR-575, miR-611, miR-630, miR-649, miR-652 |
| Ueda <i>et al</i> ^[33] | Microarray + qRT-PCR | 353 (184 gastric cancers 169 controls) | miR-181d, miR-181a-1, miR-181a-2, miR-181c, miR-181b-1, miR-181b-2, miR-21, miR-25, miR-92-1, miR-92-2, miR-93, miR-17-5p, miR-106a, miR-20b, miR-135a-1, miR-135a-2, miR-425-5p, miR-106b, miR-20a, miR-19b-1, miR-19b-2 | miR-148a, miR-148b, miR-375, miR-29b-1, miR-29b-2, miR-29c, miR-152, miR-218-2 miR-451, miR-30d |
| Tsukamoto <i>et al</i> ^[34] | Microarray (470) + qRT-PCR | 22 gastric cancers | miR-18a, miR-106a, miR-17, miR-146a, miR-93, miR-19a, miR-20a, miR-20b, miR-25, miR-15b, miR-425, miR-92a, miR-194, miR-10a, miR-222, miR-7, miR-106b, miR-320a, miR-21, miR-34a, miR-19b, miR-103, miR-215, miR-192, miR-429, miR-27a, miR-223, miR-23a, miR-107, miR-200b, miR-24, miR-15a, miR-16 miR-223, miR-21, miR-23b, miR-222, miR-25, miR-23a, miR-221, miR-107, miR-103, miR-99a, miR-100, miR-125b, miR-92, miR-146a, miR-214 and miR-191, | miR-375, miR-29c, miR-148a, miR-30a-5p, miR-30e-5p, miR-638 |
| Li <i>et al</i> ^[35] | TaqManmiRNA assays + qRT-PCR | 30 gastric cancer and controls | miR-107, miR-103, miR-99a, miR-100, miR-125b, miR-92, miR-146a, miR-214 and miR-191, | let-7a, miR-126, miR-210, miR-181b, miR-197, miR-30aa-5p |
| Carvalho <i>et al</i> ^[36] | Microarray + qRT-PCR | 76 gastric cancers | miR-582-5p, miR-151-5p, miR-296-5p, miR-30b, miR-513-5p, miR-335, miR-576-5p, miR-219-2-3p, miR-331-5p, miR-889, miR-152, miR-992, miR-93, miR-519c, miR-599, miR-520a-5p, miR-631, miR-550, miR-136, miR-22, miR-515-5p, miR-127-3p, miR-374a, miR-181a, miR-192, miR-532-3p, miR-30d, miR-640, miR-425, miR-92b, miR-501-5p, miR-514, miR-576-3p, miR-519e, miR-149, miR-219-1-3p, miR-424, miR-220, miR-96, miR-218-2, miR-649, miR-215, miR-182, miR-122, miR-524-3p, miR-187, miR-526b, miR-770-5p, miR-545, miR-200b, miR-9, miR-141, miR-579, miR-493, miR-137, miR-216a, miR-503, miR-126, miR-23b, miR-99b, miR-101, miR-323-3p, miR-25, miR-92a-1, miR-429 | miR-451, miR-502-3p, miR-101 miR-33a, miR-516a-3p/miR-516b |

qRT-PCR: Quantitative reverse transcriptase polymerase chain reaction.

ers have investigated miRNA in the peripheral blood of patients with gastric cancer^[49-55] (Table 2) and have obtained some positive results. For example, by comparing serum of 61 gastric cancer patients with that of 61 healthy persons, Liu *et al*^[49] found that the expression of miR-378 in the gastric cancer group was significantly higher than that in the healthy group. The area under the receiver-operating characteristic curve was 0.861 (95%CI: 0.766-0.928), and sensitivity/specificity was 87.5%/70.7%, respectively. Similarly, after investigating the peripheral serum in 69 gastric cancer patients and 30 healthy volunteers by qRT-PCR, Tsujiura *et al*^[55] found that the plasma concentrations of miRNAs (miR-106a and miR-106b) were significantly higher in the patients than in the controls, whereas let-7a concentration was lower in the patients, in which the area under the curve (AUC) for miR-106a and let-7a was 0.879, and sensitivity/specificity was 85.5%/80.0%, respectively. These miRNAs could become ideal tumor markers for gastric cancer. In addition, Liu *et*

al^[49] observed that the plasma miRNAs (miR-1, miR-20a, miR-27a, miR-34, and miR-423-5P) in the gastric cancer patients had significantly higher expression than in the control group (164 gastric cancer patients *vs* 127 healthy individuals). The AUC was 0.879 (95%CI: 0.822-0.936). It is interesting that, in the same sample, they also compared the AUC values of CEA and CA19-9 which were only 0.503 and 0.600, respectively. The results show that miRNA has some advantages as a tumor marker. We have found that miRNAs have good sensitivity and specificity for gastric cancer and are promising tumor markers. However, at present, some factors still limit their clinical diagnostic applications^[56]: (1) relative difficulty of detection (in quality and quantity); (2) lack of a unified testing platform and standardization; (3) plasma miRNA source and release mechanism are not clear; and (4) differences in expression of tissue and peripheral blood miRNA still exist^[57]. Thus, searching and identifying specific miRNAs for the diagnosis of gastric cancer is the first task that

Table 2 Expression of miRNA and area under curve, sensitivity and specificity in serum samples of patients with gastric cancer

| Group | Sample | MicroRNA | AUC | Method | Sensitivity/specificity (%) |
|--|--------------|--|-------|----------------------|-----------------------------|
| Liu <i>et al.</i> ^[49] | 61 GC/61 C | miR-378↑ | 0.861 | qRT-PCR | 87.5/70.7 |
| Liu <i>et al.</i> ^[50] | 164 GC/127 C | (miR-1, miR-20a, miR-27a, miR-34, miR-423-5P)↑ | 0.879 | Microarray + qRT-PCR | 79.3/86.5 |
| Konish <i>et al.</i> ^[51] | 56 GC/30 C | miR-451↑ | 0.96 | Microarray + qRT-PCR | 96.0/100 |
| | | miR-486↑ | 0.92 | | 86.0/97.0 |
| Song <i>et al.</i> ^[52] | 82 GC/82 C | miR-221, miR-744 and miR-376c↑ | NA | qRT-PCR | 82.4/58.8 |
| Zhou <i>et al.</i> ^[53] | 90 GC/27 C | miR-106a↑ | 0.684 | Microarray + qRT-PCR | 48.2/90.2 |
| | | miR-17↑ | 0.743 | | 51.9/92.7 |
| Wang <i>et al.</i> ^[54] | 174 GC/39 C | miR-21↑ | 0.81 | Microarray + qRT-PCR | 56.7/94.9 |
| Tsujiura <i>et al.</i> ^[55] | 69 GC/30 C | miR-106b↑ | 0.72 | Microarray + qRT-PCR | NA |
| | | miR-106a↑, let-7a↓ | 0.879 | | 85.5/80.0 |

qRT-PCR: Quantitative reverse transcriptase polymerase chain reaction; GC: Gastric cancer, C: Control; AUC: Area under curve; NA: Not available; ↑: Upregulated; ↓: Downregulated.

Table 3 Gastric cancer with potential predictive role of miRNAs

| | Potential predictive role of miRNA | |
|-------------------------|--|--|
| | MiRNAs of high expression | MiRNAs of low expression |
| Short survival time | miRNA-20b, miRNA-150 ^[29] , miRNA-142-5p ^[61] , miRNA-375, miRNA-214 ^[62] | miRNA-451, let7g ¹ , miRNA-433 ^[33] , miRNA-125-5p ^[63] |
| Lymph node metastasis | miRNA-27a ^[29] , miRNA-650 ^[64] | miRNA-126 ^[65] , miRNA-146a ^[66] , miRNA-148 ^[67] , miRNA-218 ^[68] , miRNA-335 ^[69] , miRNA-429 ^[70] |
| Relapse | miRNA-375 ^[61] , miRNA-451 ² , miRNA-199-3p ² , miRNA-195 ^[71] | miRNA-142-5p ^[61] |
| Advanced gastric cancer | miRNA-221 ^[72] | miRNA-126 ^[65] , miRNA-148a ^[67] , miRNA-218 ^[68] |
| Invasion, metastasis | miRNA-223 ^[35] , miRNA-148a ^[73] , miRNA-107 ^[74] | miRNA-610 ^[75] , miRNA-200b ^[576] , miRNA-7 ^[77] |

¹The two miRNAs also indicate short survival and lymph node metastasis, and deeper invasion; ²The three miRNAs also indicate short survival and recurrence, especially miRNA-451; ³The miRNA also indicates short survival and hepatic metastases, deeper invasion, and tumor enlargement; ⁴The miRNA also indicates lymph node metastasis and deeper invasion, and advanced gastric cancer; ⁵The miRNA also indicates lymph node metastasis and deeper invasion, and the tumor is enlarged.

must be undertaken. Establishment of a suitable standard testing system for clinical application, including quality control, and diagnostic threshold determination are still issues that require some work. There is a high incidence of gastric cancer in East Asian countries. High-risk populations could be screened by miRNAs, which should be able to increase the detection rate of early gastric cancer and improve the effects of treatment.

miRNA AND PROGNOSIS PREDICTION

Predicting patient survival time, disease progression, prognostic outcome or response to treatment is challenging. Because of the stability and specificity of expression in tissues and circulation, miRNA may be regarded as a forecasting tool for disease outcomes. A lot of the literature suggests that miRNAs have a close relationship with survival time of gastric cancer patients, disease stage, tumor recurrence, and lymph node metastasis. Li *et al.*^[58] have shown that a seven-miRNA signature (miR-10b, miR-21, miR-223, miR-338, let-7a, miR-30a-5p, and miR-126) is an independent predictor of overall survival [hazard ratio (HR) = 3.046; $P = 0.015$] and relapse-free survival (HR = 3.337; $P = 0.012$). It can predict the prognosis of the patient in relation to tumor stage, cytological

subtypes, and Lauren classification^[59,60]. In gastric cancer, many Chinese and other research teams have discovered a number of miRNAs that play a role as a predictor. For example, high expression of miRNA-20b, miRNA-150, miRNA-142-5p^[61], miRNA-375 and miRNA-214^[62] and low expression of miRNA-451, let7g, miRNA-433 and miRNA-125-5p^[63] are associated with short survival time. High levels of miRNA-27a and miRNA-650^[64] and low levels of miRNA-126^[65], miRNA-146a^[66], miRNA-148^[67], miRNA-218^[68], miRNA-335^[69] and miRNA-429^[70] indicate lymph node metastasis. Patients with overexpression of miRNA-375, miRNA-451, miRNA-199-3p and miRNA-195^[71] and decreased expression of miRNA-142-5p are more likely to relapse. High levels of miRNA-221^[72] and decreased levels of miRNA-126, miRNA-148a and miRNA-218 indicate advanced gastric cancer. High expression of miRNA-223, miRNA-148a^[73] and miRNA-107^[74] and reduced expression of miRNA-610^[75], miRNA-200b^[76] and miRNA-7^[77] were associated with invasion and metastasis (Table 3). Therefore, many potential predictors have proved useful for judging the prognosis of gastric cancer patients and are the basis for targeted therapy. However, researching miRNAs as prognostic factors involves small sample sets, high volume of work in validation, and research of independent cohorts,

Table 4 Expression of miRNA and prediction of the effect of chemotherapy and radiotherapy

| | Upregulated | Downregulated |
|------------------|--|--|
| Chemosensitivity | (let-7g, miR-342, miR-16, miR-181, miR-1, miR-34) ^[81] | |
| Chemoresistance | (miR-518f, miR-520a, miR-520d, miR-519e, miR-363, miR-517) ^[81] | (miR-196a, miR-200family, miR-338, miR-126, miR-31, miR-98, let-7g, miR-7) ^[82] miR-15b, miR-16 ^[83] |
| Radiosensitivity | miR-451 ^[85] | |
| Radioresistance | miR-221/222 ^[97] | |

¹Drugs were cisplatin and 5-fluorouracil; ²Drug was hydroxy camptothecin.

all of which are required before assays for miRNAs can be used clinically.

miRNAs AND CHEMOTHERAPY AND RADIOTHERAPY

Resistance to chemotherapy and radiotherapy is a major obstacle to improving the survival of the patients with gastric cancer^[78-80]. We can predict the occurrence of resistance to chemotherapy and radiotherapy^[81-83] (Table 4) through detecting the miRNA expression profile of the patients. Through investigating drug resistance to cisplatin and 5-fluorouracil in 90 patients with gastric cancer and comparing patients' miRNA expression before and after chemotherapy, Kim *et al.*^[81] found that high expression of let-7g, miR-342, miR-16, miR-181, miR-1 and miR-34 indicated sensitivity to chemotherapy, and high expression of miR-518f, miR-520a, miR-520d, miR-519e, miR-363 and miR-517 indicated resistance to chemotherapy. By predicting miRNAs, we used a new method for choosing chemotherapy regimen and monitoring its effects, and even reversing the chemotherapy resistance through transfecting specific pre-miRNA. miRNA-15b and miRNA-16 are downregulated severely in the multi-drug resistant gastric carcinoma cell line SGC7901/VCR. By improving miRNA-15b and miRNA-16 expression levels, sensitivity to vincristine was enhanced. Chen *et al.*^[84] transfected miRNA-200c into SGC7901/DDP gastric cancer cells, which increased sensitivity to DDP, 5-fluorouracil, paclitaxel and doxorubicin. The same situation occurred in radiotherapy on gastric cancer by transfection into AS-miRNA-221/222, which down-regulated the miRNA-221/222 expression in gastric cancer cell line SGC7901. Zhang *et al.* found that the survival rate of cancer cell was significantly lower than that in the control group. Radiosensitivity was promoted through 0-6 Gy irradiation. In addition, Bandres *et al.*^[85] transfected cancer cells with pre-miRNA-451, which improved expression of miRNA-451 in AGS gastric cancer cells. Under 0-4 Gy irradiation, the effect of treatment was significantly better than that in the control group.

miRNAs AND TREATMENT

miRNAs are regulatory factors for gene expression and act as a control center in the process of tumor development^[86,87]. miRNAs can modulate protein expression

and affect multiple information pathways^[88]. miRNAs will be more effective than coding genes as a biological treatment of tumor target molecules. The basic strategy of current treatment based on miRNAs is to adopt gene knockout to inhibit or downregulate the expression level of oncogene miRNAs. On the contrary, for anti-oncogenes, we used the method of gene knock-in to introduce foreign miRNAs, increase the expression level, and achieve the purpose of tumor treatment. The following strategies were used: administration of small molecule drugs for inhibiting miRNA, *e.g.*, anti-miRNA oligonucleotides (AMOs) following base pairing rules, competitively blocked the miRNA with target gene interaction^[89], such as locked nucleic acid^[90-91]; miRNA sponges, *e.g.*, the adsorption with miRNA could not combine with the natural target^[92]; and miR-Mask^[93], miRNA inhibitors and so on. miRNA expression is often increased by using viruses as a carrier to introduce a specific miRNA or miRNA mimics and finally upregulate miRNA and inhibit the tumor^[94].

A number of Eastern Asian researchers followed the above principle of miRNA-mediated treatment and achieved good results *in vitro* and in animal experiments. For example, miRNA-221/222 is upregulated in gastric cancer cell line SGC7901. By transfecting AS-miRNA-221/222 2000 into cancer cells with liposomes, miRNA-221/222 is knocked out. This inhibits gastric cancer cell growth and invasion. Their target molecule is PTEN^[95]. MiR-516-3p has been transfected into the gastric scirrhous carcinoma cell line 44AS3 with liposomes, was significantly overexpressed, and finally inhibited cancer cell growth, invasion and metastasis^[96]. Similar results were obtained in a nude mouse transplantation model of human gastric cancer. Zhang *et al.*^[97] through AMOs, knocked down the originally high expression of miRNA-21 and caused the proliferation of gastric cancer cells to slow and apoptosis to increase visibly. In addition, Ji *et al.*^[98] have added a miRNA-34 analog to *p53* mutated gastric cancer cell lines to restore its function and upregulate its expression, which inhibited cell growth and maintained them at phase G1.

Many studies based on treatment of gastric cancer by miRNA have shown good results. In particular, Chinese, Japanese and South Korean researchers have attempted this. However, we still have several major obstacles to overcome. First, the multi-targeting nature of miRNAs brings the risk of unconscious off-target effects. Second, the expression of target genes may often be regulated by

multiple miRNAs, which could greatly reduce the effect of treatment based on a specific miRNA. Finally, we still lack good specificity and an efficient miRNA delivery system for treatment^[99,100].

CONCLUSION

miRNAs are involved in almost all stages of gastric carcinogenesis, and may have broad applications in early diagnosis of gastric carcinoma, prognosis, detection of radiotherapy and chemotherapy efficacy, and be a new target for treatment. However, studies based on the clinical application of miRNAs for gastric cancer still lack reliable and exact data from large multi-center studies. In recent years, miRNAs have been a focus of biomedical research. New miRNAs have been discovered and research techniques constantly updated. It will be a great challenge to integrate new data and establish standard procedures. For diagnosis, we need unified standards and testing platforms. For treatment, we need better-designed small-molecule drugs based on a well detailed and more accurate medication carrier without toxic side effects. We look forward to further studies of miRNAs improving their clinical applications for the diagnosis and treatment of gastric cancer. East Asia, as an area with a high incidence of gastric cancer, should undertake more studies for the application of miRNAs in gastric cancer.

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Measurement of calprotectin in ascitic fluid to identify elevated polymorphonuclear cell count

Emanuel Burri, Felix Schulte, Jürgen Muser, Rémy Meier, Christoph Beglinger

Emanuel Burri, Felix Schulte, Christoph Beglinger, Gastroenterology and Hepatology, University Hospital Basel, 4031 Basel, Switzerland

Emanuel Burri, Rémy Meier, Gastroenterology, Hepatology and Clinical Nutrition, Cantonal Hospital, 4410 Liestal, Switzerland

Jürgen Muser, Central Laboratories, Cantonal Hospital, 4410 Liestal, Switzerland

Author contributions: Burri E and Beglinger C participated in study concept and design; all authors participated in data acquisition, analysis, interpretation, drafting and critical revision of the manuscript, all of them read and approved the final manuscript.

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Correspondence to: Dr. Emanuel Burri, MD, Gastroenterology and Hepatology, University Hospital Basel, Petersgraben 4, 4031 Basel, Switzerland. burrie@uhbs.ch

Telephone: +41-61-2652525 Fax: +41-61-2655352

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Abstract

AIM: To evaluate the diagnostic capability of calprotectin in ascitic fluid for detecting a polymorphonuclear (PMN) cell count > 250/μL ascites.

METHODS: In this prospective observational study, a total of 130 ascites samples were analysed from 71 consecutive patients referred for paracentesis. Total and differential leukocyte cell counts were determined manually with a Neubauer chamber and gentian-violet stain. Calprotectin was measured in 1 mL ascetic fluid by enzyme-linked immunosorbent assay (ELISA) and a point-of-care (POC) lateral flow assay with the Quantum Blue® Reader (Bühlmann Laboratories). All

measurements were carried out in a central laboratory by senior personnel blinded to patient history. A PMN count > 250/μL was the primary endpoint of the study. The diagnostic value of ascitic calprotectin measurement was assessed by comparing to the final diagnosis of each patient that had been adjudicated by investigators blinded to calprotectin values.

RESULTS: The PMN count was > 250/μL in 19 samples (14.6%) from 15 patients (21.1%) and varied widely among the study population (range 10-19 800/mL and 1-17 820/mL, respectively). Spontaneous bacterial peritonitis (SBP) was the final diagnosis in four patients (5.6%). All patients with PMN ≤ 250/μL had negative bacterial culture. PMN count was elevated in five patients with peritoneal carcinomatosis, three with lymphoma, one with neuroendocrine carcinoma, and two with secondary peritonitis due to abdominal perforation. PMN cell counts correlated with ascitic calprotectin values (Spearman's rho; $r = 0.457$ for ELISA, $r = 0.473$ for POC). A considerable range of ascitic calprotectin concentrations was detected by ELISA [median 0.43 μg/mL, interquartile range (IQR) 0.23-1.23 (range 0.10-14.93)] and POC [median 0.38 μg/mL, IQR 0.38-0.56 (range 0.38-13.31)]. Ascitic calprotectin levels were higher in samples with PMN > 250/μL, by both ELISA [median (IQR) 2.48 μg/mL (1.61-3.65) vs 0.10 μg/mL (0.10-0.36), $P < 0.001$] and POC [2.78 μg/mL (2.05-5.37) vs 0.38 μg/mL (0.38-0.41), $P < 0.001$]. The area under the receiver operating characteristics curve for identifying an elevated PMN count was 0.977 (95%CI: 0.933 to 0.995) for ELISA and 0.982 (95%CI: 0.942 to 0.997) for POC ($P = 0.246$ vs ELISA). Using the optimal cut-off value for ELISA (0.63 μg/mL), ascitic calprotectin had 94.8% sensitivity, 89.2% specificity, positive and negative likelihood ratios of 8.76 and 0.06 respectively, positive and negative predictive values of 60.0% and 99.0% respectively, and 90.0% overall accuracy. Using the optimal cut-off value for POC (0.51 μg/mL), the respective values were 100.0%, 84.7%, 6.53, 0.00, 52.8%, 100% and 87.7%. Correlation be-

tween ELISA and POC was excellent ($r = 0.873$, $P < 0.001$). The mean \pm SD of the difference was -0.11 ± 0.48 $\mu\text{g/mL}$ with limits of agreement of $+ 0.8$ $\mu\text{g/mL}$ (95%CI: 0.69 to 0.98) and -1.1 $\mu\text{g/mL}$ (95%CI: -1.19 to -0.91).

CONCLUSION: Ascitic calprotectin reliably predicts PMN count $> 250/\mu\text{L}$, which may prove useful in the diagnosis of SBP, especially with a readily available bedside testing device.

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Key words: Calprotectin; Ascites; Liver cirrhosis; Spontaneous bacterial peritonitis; Polymorphonuclear cells

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INTRODUCTION

Liver cirrhosis is the clinical end-stage of different entities of chronic liver disease when patients suffer from substantial mortality and morbidity, both of which are positively correlated with disease severity^[1,2]. Ascites is the most common complication, and around 60% of patients with compensated cirrhosis develop ascites within 10 years of disease onset^[3]. Spontaneous bacterial peritonitis (SBP) is an important cause of morbidity and mortality in cirrhotic patients with ascites. SBP is estimated to affect 10%-30% of cirrhotic patients hospitalised with ascites, and mortality in this group approaches 30%^[4,5]. Many of these patients are asymptomatic, and it is therefore recommended that all patients with ascites undergo paracentesis at the time of admission to confirm the SBP status^[5]. Although SBP is less prevalent in an outpatient setting, it is reasonable to also evaluate the ascitic fluid of outpatients because of the high mortality associated with SBP.

The diagnosis of SBP is based upon the polymorphonuclear (PMN) leukocyte cell count exceeding $250/\mu\text{L}$ in ascitic fluid^[6,7]. Currently, differential cell count is usually performed by a manual method using light microscopy and counting chambers. However, the diagnosis is often delayed when laboratory personnel are not readily available or in the private practice setting where specimens are sent to an offsite laboratory. This is a major drawback, as rapid diagnosis of SBP and immediate initiation of antibiotic treatment is of paramount importance. Alternative methods using automated PMN counting^[8,9], reagent strips (urine dipsticks)^[10-26], or ascitic lactoferrin^[27] have been developed; unfortunately, their diagnostic accuracies are limited and their use is dependent upon availability of laboratory personnel and reagents/components from

the commercial source. Therefore, an accurate and convenient method of rapid diagnosis of SBP remains an unmet clinical need.

Calprotectin, a calcium and zinc-binding protein, is detected almost exclusively in neutrophils^[28], and its presence in body fluids is proportional to the influx of neutrophils^[29-33]. However, only one study to date has investigated calprotectin levels in ascites and found higher concentrations in patients with malignant disease than in those with non-malignant disease^[34]. In contrast, faecal calprotectin is a well-established marker of inflammation and is used to monitor inflammatory bowel disease^[35]. A rapid bedside test has been developed to measure calprotectin in faeces; systematic comparison with the established enzyme-linked immunosorbent assay (ELISA) technique showed good correlation between the two tests' results^[36] and the rapid bedside test has been suggested as an equally valuable tool for diagnosing inflammatory bowel disease^[37]. It is possible that such a rapid bedside test may be useful for measuring calprotectin in ascitic fluid to indicate PNM levels and SBP status, however the diagnostic accuracy of such a measurement in ascitic fluid is unknown.

This study was designed to test our hypothesis that calprotectin in ascitic fluid could be useful as a surrogate PMN marker for identifying SBP patients ($> 250/\mu\text{L}$ PNM). To this end, we measured calprotectin in ascites of consecutive patients referred for paracentesis using a rapid bedside test and compared the results to those from the traditional ELISA.

MATERIALS AND METHODS

Setting and participants

In this prospective observational study, we recruited patients with ascites referred for paracentesis to the Department of Gastroenterology and Hepatology at the University Hospital Basel, and to the Department of Gastroenterology, Hepatology and Clinical Nutrition at the Cantonal Hospital Liestal in Switzerland. All patients with ascites were eligible for study enrolment, irrespective of the aetiology of ascites. The decision to perform paracentesis was based on clinical findings evaluated by the referring physician who was otherwise not involved in the study. Exclusion criteria were age < 18 years and recent abdominal surgery (< 3 mo). Standardised patient history, clinical symptoms, and demographic data were obtained from all participants. The study was carried out in accordance with the principles of the Declaration of Helsinki and with pre-approval from the local Ethic Committees of both study sites. All patients provided written informed consent prior to participation in any protocol-specific procedures.

Endpoint

The diagnostic value of ascitic calprotectin measurement was assessed in comparison to the adjudicated final diagnosis. A PMN count $> 250/\mu\text{L}$ was the primary endpoint of the study.

Adjudication of the final diagnosis

One month after study participation, the final diagnosis (SBP) and the aetiology of ascites were independently adjudicated in a blinded fashion by two board-certified gastroenterologists not involved in the patients' care. Their final assessment was based upon available medical records, including PMN count and the results of all diagnostic investigations, as well as the patient's response to treatment. Current recommendations were followed^{16,71}. The two physicians designated the aetiology of ascites by choosing one or more of the following diagnoses from a standardized list: liver cirrhosis (alcoholic, chronic hepatitis, non-alcoholic steatohepatitis, hemochromatosis, primary biliary cirrhosis, others to be specified), hepatocellular carcinoma, cholangiocellular carcinoma, liver metastasis, peritoneal carcinomatosis, right heart failure, nephrotic syndrome, and others to be specified. If more than one cause of ascites was identified, the leading disorder responsible for the current episode was established and recorded. Any disagreements in the final diagnosis of a given study participant were resolved by consensus with a third clinician who was considered an expert in the field and recruited to independently review and adjudicate the cases.

Paracentesis

Paracentesis was performed under aseptic conditions with the patient in the supine position and the puncture site in the left or right lower quadrant. Prior to needle insertion, ultrasound was performed to visualise the intra-abdominal structures. No study participant suffered complications related to the abdominal puncture procedure. All samples for diagnostic testing were immediately collected at the bedside and processed by laboratory personnel without further delay. Specifically, aliquots of approximately 1 mL ascites were centrifuged for 15 min at $500 \times g$. The supernatant phase was transferred to a fresh tube and stored at -20°C until analysis by ELISA or POC, which occurred within 72 h.

Blood samples were also obtained at this time. The ascites samples were used to measure total cell count, PMN count, calprotectin, albumin, total protein, glucose, and lactate dehydrogenase. In addition, two 10 mL aliquots of ascites were subjected to bacterial culture (bottle method) respectively. The serum-ascites albumin gradient (SAAG) was calculated as the difference of albumin in serum and albumin in ascites.

Differential cell count and cytopathology

All laboratory analyses were performed in the Central Laboratories BL (Schönenbuch, Switzerland) by senior laboratory personnel blinded to patient history and calprotectin levels. Total and differential leukocyte cell counts were determined by the manual method using a Neubauer chamber and gentian-violet staining (Leukotic®; bioanalytic GmbH, Freiburg, Germany). The Central Laboratories BL is accredited according to ISO/IEC 17 025 and ISO 15 189 standards. For all study participants who underwent repeated paracentesis, the cytopathological analysis was performed at least once.

Laboratory-based quantitative calprotectin measurement

Ascitic calprotectin in ascites was assayed using a commercially-available ELISA (Bühlmann Laboratories AG, Schönenbuch, Switzerland) and following the manufacturer's instructions. Briefly, 10 μL aliquots of the supernatant samples were diluted 1:50 in incubation buffer and 100 μL was applied to a microtiter plate coated with a monoclonal capture antibody highly specific for the calprotectin heterodimeric and polymeric complexes. After incubation, washing and further incubation with a detection antibody conjugated to horseradish peroxidase, the tetramethylbenzidine chromogenic substrate was added. The reaction was terminated by a stop solution and the absorbance (optical density at 450 nm) was measured by spectrophotometry. The measuring range of the test was 0.2–12 μg calprotectin/mL ascites with an intra- and inter-assay coefficient of 4.7% and 11.3%, respectively.

Point-of-care quantitative calprotectin measurement

The Quantum Blue® quantitative calprotectin lateral flow assay (Bühlmann Laboratories AG) was used for the point-of-care (POC) measurement of ascitic calprotectin. The Quantum Blue® reader is currently marketed for 2500 USD (\$), and the test cartridges cost 20 USD per sample and analysis. Aliquots of 60 μL 1:10 diluted ascites samples (20 μL ascites in 180 μL extraction buffer) were pipetted respectively onto the sample loading port of the test cartridge. After a 12 min incubation, the test cartridge was quantitatively read by the Quantum Blue® Reader. The measurement range of the lateral flow test was 0.38–3.8 μg calprotectin/mL ascites, with an inter-assay coefficient of 15.6%. Specimens with concentrations above this measurement range were further diluted with extraction buffer.

In addition, a random subgroup of samples ($n = 17$) was immediately measured by POC, without first performing the centrifugation step of processing. These results were compared to the results from the POC measurements obtained in the laboratory setting after processing and storage.

Statistical analysis

All statistical analyses were performed using the SPSS software package, version 19.0 (SPSS Inc., Chicago, IL, United States). A P -value of less than 0.05 indicated statistical significance. Intergroup comparisons were made using the Mann-Whitney U test and the χ^2 test where appropriate. Correlations between numerical data were determined with the Spearman's rank correlation coefficient. All hypothesis testing was two-tailed. The Bland-Altman plot was used to assess agreement between ELISA test results and POC test results, in which the differences between the results of the two tests for each individual patient were plotted against the corresponding mean of the two readings. The mean and SD of the differences and the limits of agreement, defined as the mean \pm 2 SD of the difference (95%CI), were calculated. Analysis of the receiver operator characteristics (ROC) and calculation of the area under the curve (AUC) were used to evaluate the capability of calprotectin to identify a PMN count >

Table 1 Baseline characteristics of patients with liver cirrhosis ($n = 54$)

| | |
|-------------------------------|------------------|
| Aetiology of liver cirrhosis | |
| Alcoholic | 36 (66.6) |
| Viral hepatitis | 7 (13.0) |
| Alcoholic + viral hepatitis | 5 (9.3) |
| Non alcoholic steatohepatitis | 1 (1.9) |
| Others | 5 (9.3) |
| Child-turcotte-pugh class | |
| Child A | 0 |
| Child B | 29 (53.7) |
| Child C | 25 (46.3) |
| MELD score | 12.2 (10.0-16.0) |

Others included 2 patients with autoimmune hepatitis, 2 patients with primary biliary cirrhosis and 1 patient with primary sclerosing cholangitis. The child-turcotte-pugh classification contains five variables, including serum levels of bilirubin and albumin, prothrombin time, ascites, and encephalopathy. Child A: 5-6 points; Child B: 7-9 points; Child C: 10-12 points. The model for end-stage liver disease (MELD) score: $9.57 \ln$ [serum level of creatinine (mg/dL)] + $3.78 \ln$ [serum bilirubin (mg/dL)] + $11.2 \ln$ (international normalised ratio for prothrombin time) + 6.43. Data are presented as number of patients (%) or medians (25th to 75th percentiles).

250/ μ L. The ROC analysis identified the cut-off points for maximal diagnostic capability. The test characteristics of sensitivity, specificity, positive and negative likelihood ratios (LR+ and LR-), and positive and negative predictive values (NPV) were determined. Overall accuracy of the test was calculated according to the following formula: [(true positive test results + true negative test results)/total population]. As this study was exploratory in design, no formal power calculations were carried out.

RESULTS

Patient characteristics

A total of 136 samples from 75 patients were prospectively collected from October 2010 to January 2012. Among these, 130 samples were included in the final analysis, representing 71 patients (94.7% of the total; 40 males and 31 females) with a median age of 64 years (IQR 55-71 years). Sixty-three of the patients (88.7%) had been referred for diagnostic paracentesis. Twenty-four of the patients (33.8%) underwent the procedure more than once (median 3, range 2-12).

The majority of patients (54, 76.1%) suffered from liver cirrhosis (Table 1). A total of 11 patients (15.5%) had malignant ascites, which included three ovarian, two lymphomas, two breast, one stomach, one colorectal, one pancreatic cancer, and one neuroendocrine carcinoma. Of those 11 patients, two also had liver cirrhosis. Additionally, three patients with ascites also had heart failure and five patients with ascites also had portal hypertension from metastatic liver disease (but no malignant cells were present in the ascites). No intervention-related complications occurred after paracentesis.

Ascitic fluid cell count

Total cell count and PMN cell count at presentation varied widely among the study population (range 10-19 800/

Table 2 Ascitic fluid analysis

| Variable | PMN count > 250/ μ L ($n = 19$) | PMN count \leq 250/ μ L ($n = 111$) |
|------------------|---------------------------------------|---|
| Total cell count | 1300.0 (350.0-19 800.0) | 250.0 (10.0-1970.0) |
| PMN count | 553.0 (277.0-17 820.0) | 21.0 (1.0-212.0) |
| Albumin, g/L | 13.0 (4.8-16.8) | 7.0 (3.0-10.0) |
| Protein, g/L | 22.0 (9.3-37.5) | 12.0 (8.0-20.0) |
| LDH, U/L | 117.0 (100.8-138.5) | 55.0 (42.0-81.0) |
| Glucose, mmol/L | 7.6 (6.2-9.7) | 7.0 (6.2-8.2) |

Values are given as medians (range) for total cell count and polymorphonuclear cell count (PMN) count and median (25th to 75th percentiles) for all other values. LDH: Lactate dehydrogenase.

mL and 1-17 820/mL, respectively). PMN count > 250/mL was detected in 19 samples (14.6%) from 15 patients (21.1%). Among the study population, SBP was the final diagnosis for four patients (5.6%) and only one of these four had positive ascitic bacterial cultures (*Streptococcus pneumoniae*). All bacterial cultures from patients with PMN \leq 250/mL were negative. Additionally, PMN count was elevated in five patients with peritoneal carcinomatosis (two with ovarian cancer, and one each with gastric, colorectal and pancreatic cancer), in three patients with lymphoma, in one patient with neuroendocrine carcinoma, and in two patients with secondary peritonitis due to an abdominal perforation. All patients with SBP received antibiotic treatment and recovered well. None of the patients died. Table 2 details the findings from ascitic fluid analysis.

Calprotectin measurement in ascitic fluid

The ascitic calprotectin concentrations ranged considerably in the ELISA [median 0.43 μ g/mL, IQR 0.23-1.23 (range 0.10-14.93)] and POC test [median 0.38 μ g/mL, IQR 0.38-0.562 (range 0.38-13.31)]. However, the calprotectin values measured by the laboratory-based ELISA and the POC test correlated well with the PMN count ($r = 0.476$, $P < 0.001$ and $r = 0.473$, $P < 0.001$, respectively), and the correlation between the two tests was excellent ($r = 0.873$, $P < 0.001$). The degree of agreement between the measurements of ascitic calprotectin from the ELISA and the POC test is illustrated in Figure 1. The mean \pm SD of the difference was -0.11 ± 0.48 μ g/mL, with limits of agreement of $+0.8$ μ g/mL (95%CI: 0.69 to 0.98) and -1.1 μ g/mL (95%CI: -1.19 to -0.91).

Comparative analysis of the POC detection of ascitic calprotectin levels in samples measured at the bedside (unprocessed and processed after centrifugation) and in the lab (after centrifugation) showed that the calprotectin measurements correlated well. For unprocessed samples, $r = 0.831$ ($P < 0.001$), and for processed samples, $r = 0.656$ ($P = 0.004$).

Diagnostic value of ascitic calprotectin

Ascitic calprotectin levels were higher in samples ($n = 19$) with PMN > 250/ μ L, both when measured by ELISA [median (IQR) 2.48 μ g/mL (1.61-3.65)] *vs* 0.10 μ g/mL (0.10-0.36), $P < 0.001$] and the POC test [median 2.78

Table 3 Test characteristics of ascitic calprotectin to identify > 250 polymorphonuclear leukocytes per mL ascites

| | AUC (95%CI) | Best cut-off ($\mu\text{g/mL}$) | Sens (%) | Spec (%) | LR+ | LR- | PPV (%) | NPV (%) | Accuracy (%) |
|-------|---------------------|-----------------------------------|----------|----------|------|------|---------|---------|--------------|
| ELISA | 0.977 (0.933-0.995) | 0.63 | 94.8 | 89.2 | 8.76 | 0.06 | 60.0 | 99.0 | 90.0 |
| POC | 0.982 (0.942-0.997) | 0.51 | 100 | 84.7 | 6.53 | 0.00 | 52.8 | 100 | 87.7 |

Area under the receiver operating characteristics curve (AUC) with corresponding sensitivity (Sens), specificity (Spec), positive and negative likelihood ratio (LR+, LR-), and negative and positive predictive values (NPV, PPV) for ascitic calprotectin to identify polymorphonuclear > 250/ μL . Overall accuracy was calculated using the following formula: (true positive test results + true negative test results)/total population. ELISA: Enzyme-linked immunosorbent; POC: Point-of-care.

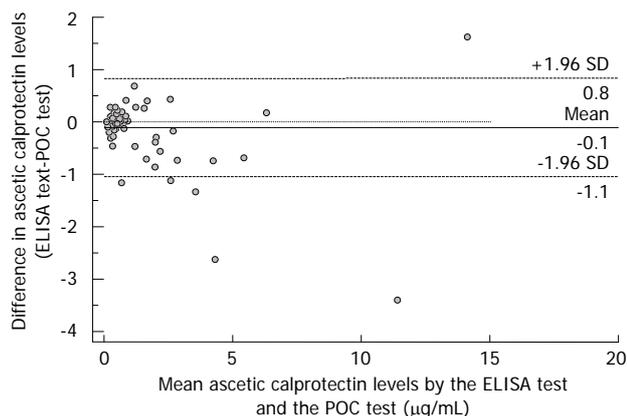


Figure 1 Measurement of ascitic calprotectin with the enzyme-linked immunosorbent test and the point-of-care test (Bland Altman plot). The differences between the results of the enzyme-linked immunosorbent (ELISA) and point-of-care (POC) tests in each patient are plotted against the mean of the two measurements, showing the limits of agreement, defined as the mean \pm 2 SD of the difference.

$\mu\text{g/mL}$ (2.05-5.37) *vs* 0.38 $\mu\text{g/mL}$ (0.38-0.41), $P < 0.001$) (Figure 2). Evaluation of the ascitic calprotectin measurement as a diagnostic test to identify patients with PMN count > 250/ μL yielded an AUC of 0.977 (95%CI: 0.933-0.995) for the ELISA and an AUC of 0.982 (95%CI: 0.942-0.997) for the POC test. Furthermore, the two tests did not show significantly different diagnostic capacity ($P = 0.246$ *vs* ELISA) (Figure 3).

Using the optimal cut-off value from the ROC of ELISA (0.63 $\mu\text{g/mL}$), ascitic calprotectin yielded a sensitivity of 95%, a specificity of 89.2%, and an accuracy of 90.0% (Table 3). To identify all patients with PMN count > 250/ μL and to obtain 100% test sensitivity, a slightly lower cut-off value (0.44 $\mu\text{g/mL}$) is necessary. However, use of this lower value is accompanied by lower specificity (82.9%) and lower LR+ (5.84).

Analysis of the POC test characteristics revealed a nearly identical profile to the ELISA characteristics (Table 3). The optimal cut-off value for POC (0.51 $\mu\text{g/mL}$) yielded a sensitivity of 100% and a specificity of 84.7%, with 6.53 LR+ and 0.0 LR-. The overall accuracy of the POC test was 87.7% (Figure 4).

Patients with false positive test results had PMN counts between 3 and 212 (median 70.0, IQR 35.0-127.5) for the ELISA, and between 3 and 197 (median 45.0, IQR 16.0-100.0) for the POC test.

The ELISA and POC test had similar diagnostic capability for identifying PMN > 250/ μL in the subgroup of

patients with liver cirrhosis (95 samples from 54 patients; ELISA AUC 0.987, and POC test AUC 0.982). In addition, when the ascites samples were analysed according to the SAAG > 11g/L (115 samples from 62 patients), the AUCs of ascitic calprotectin were 0.983 for the ELISA and 0.988 for the POC test (data not shown).

DISCUSSION

This prospective study evaluated the diagnostic utility of measuring calprotectin in ascites to identify ascitic PMN counts > 250/ μL in patients referred for paracentesis, and provides the following new information: Patients with an elevated PMN count (> 250/ μL) had higher ascitic calprotectin levels than those with normal cell counts; this finding indicates that ascitic calprotectin levels correlate well and reliably with PMN count. It is clinically significant that calprotectin levels in ascitic patients can identify elevated PMN counts using both laboratory-based ELISA and bedside POC testing. Indeed, ascitic calprotectin may serve as a surrogate marker for PMN count and would be amenable to routine SBP screening, especially when measured by a bedside test.

Ascites is commonly found in patients with liver cirrhosis and may promote bacterial translocation, enhancing the risk of SBP^[3]. SBP in outpatients is rare, but when it occurs it often requires hospitalisation to manage to disease course^[4,5]. In our study, four of 71 patients were diagnosed with SBP (5.6%). In general, SBP symptoms are nonspecific and current guidelines recommend paracentesis be performed in all patients with ascites to rule out abdominal infection^[6,7]. The diagnosis of SBP in patients with liver cirrhosis is based on a PMN count of > 250/ μL in ascitic fluid, with or without positive bacterial cultures^[5-7]. This cut-off is recognized as more sensitive than other criteria (PMN > 500/ μL ; white blood cell count > 500/ μL)^[38,39] for identifying SBP^[40]. SBP diagnosis based solely on bacterial culture is considered unreliable, since up to 60% of patients with increased PMN count are reported as culture-negative^[41,42]. In our study, all patients with culture-positive abdominal infection, including both SBP and secondary peritonitis patients, had elevated PMN counts. In the four SBP patients, the bacterial cultures were positive for only one (25.0%).

Our study measured calprotectin in ascitic fluid in 130 unselected samples from 71 consecutive patients. Ascitic calprotectin levels correlated well and reliably with PMN counts, and the samples with PMN > 250/ μL also had higher ascitic calprotectin levels than the samples with

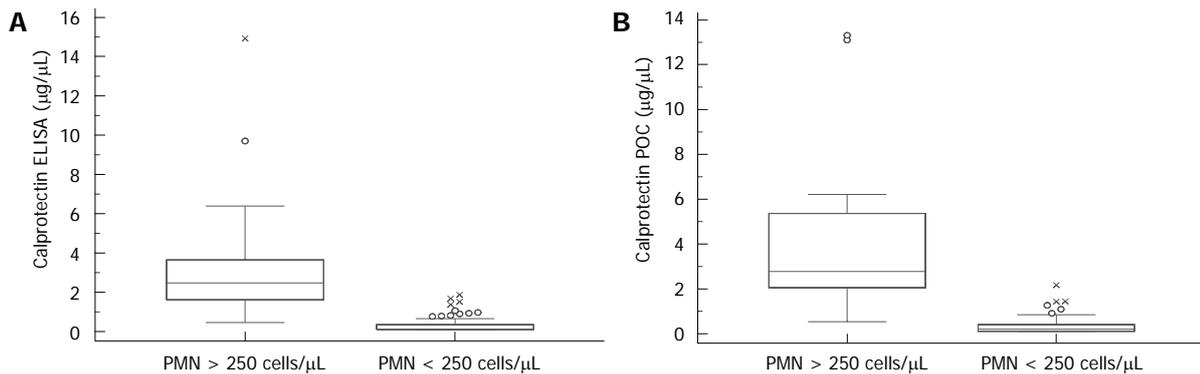


Figure 2 Ascitic calprotectin values in patients with normal and elevated ascitic polymorphonuclear cell count. Box-and-whisker plot representing the median, 25th to 75th percentiles, minimum/maximum values, and outliers outside 1.5 times (circle) and 3 times (cross) the interquartile range of ascitic calprotectin values, measured with the enzyme-linked immunosorbent (ELISA) test (A) and the point-of-care (POC) test (B). PMN: Polymorphonuclear.

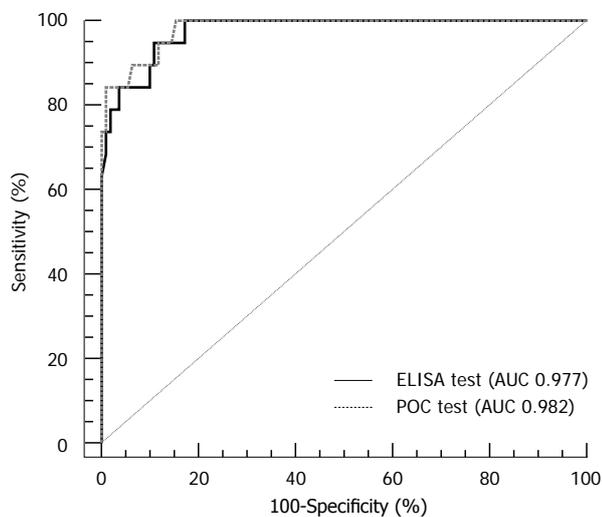


Figure 3 Receiver operating characteristics analysis of the enzyme-linked immunosorbent test and the point-of-care test to identify elevated ascitic polymorphonuclear cell count. The area under the receiver operating characteristics curve (AUC) for ascitic calprotectin for identifying a polymorphonuclear count > 250/ μ L. No differences between the two tests ($P = 0.155$) were detected. The diagonal line represents no discrimination. POC: Point-of-care; ELISA: Enzyme-linked immunosorbent.

PMN $\leq 250/\mu\text{L}$. Both the ELISA and the POC test accurately measured ascitic calprotectin, and the correlation between the two tests was excellent with high sensitivity (95% and 100%, respectively) and high specificity (89% and 85%, respectively) at the optimal cut-off points (from ROC analysis). In a diagnostic test that is used to screen for a specific disease, it is preferred to test all patients at risk, especially when potentially life-threatening complications may occur. In screening tests, high sensitivity is therefore favoured over high specificity. In our study, the NPVs of calprotectin testing in ascites were excellent (99% for the ELISA and 100% for the POC test). Notably, these results suggest that no patient with elevated PMN count would have been missed by the bedside test.

In daily clinical practice, PMN count is often not readily available and clinicians frequently rely on total cell count when initiating empiric antibiotic treatment^[43]. It has been suggested that a total cell count < 1000/ μL

(obtained from automated cell counting procedures) is unlikely to signify SBP, having a NPV of 96%^[44]. In our study, using such a criteria would have misclassified five patients (26.3%) with elevated PMN counts. Moreover, the use of total cell count in combination with ascitic calprotectin measurement did not increase the diagnostic accuracy of calprotectin testing, as calculated by ROC analysis (data not shown).

To avoid diagnostic delay, it has been proposed that automated PMN counting should replace the laborious and time-consuming manual cell counting technique^[8,9]. Studies have demonstrated that automated blood cell counts correlate well with manual ascitic leukocyte differential counts^[45]. However, despite the potential benefit of automated cell counters in clinical practice, widespread use of this technology is limited by the cost of the sophisticated laboratory equipment and requirement for trained operators; this is a particular challenge for practitioners' office settings and small clinics without in-house laboratories.

The use of reagent strips (urine dipsticks) for PNM counting (by colorimetric detection of leukocyte esterase activity) has also been evaluated as a rapid SBP diagnostic tool^[45,46]. A number of these studies have reported sensitivities between 85% and 100% and specificities between 90% and 100%^[10-25]. However, these results came from mostly single-centre studies with small numbers of SBP cases. The only large, multicentre study reported in the literature produced very different results; in particular, using 2123 paracenteses, the sensitivity was only 45% for identifying PMN > 250/ μL in cirrhotic patients^[26]. Although specificity was still high, it was concluded that urinary dipstick testing lacks sufficient accuracy for diagnosing SBP. The risk of false negative results seemed to be especially problematic in patients with lower PMN counts^[46]. These results dampened the initial enthusiasm for reagent strips, and currently this method is not recommended for rapid diagnosis of SBP^[45].

Only one study in the literature, to date, has provided data on calprotectin measurement in ascites^[35]. In that study, Homann *et al*^[34] compared ascites from patients with malignant disease to ascites from patients with non-

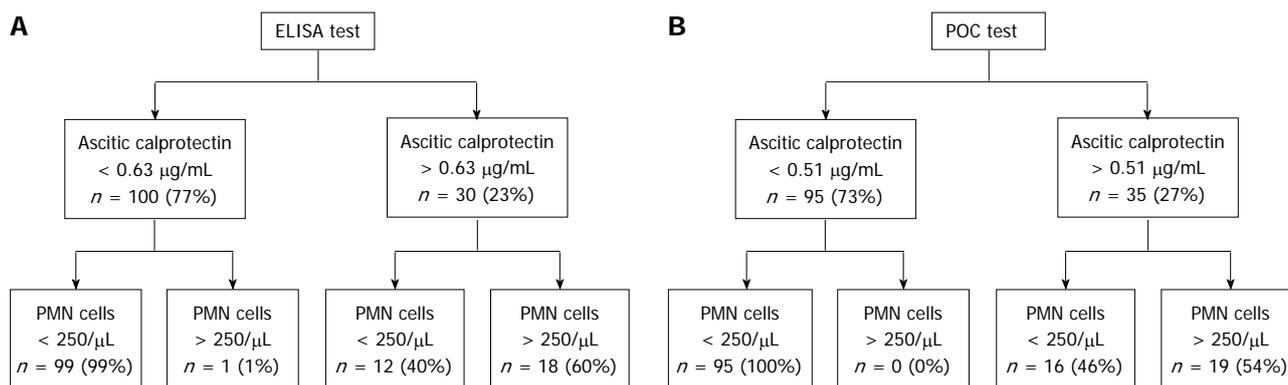


Figure 4 Diagnostic accuracy. For the enzyme-linked immunosorbent (ELISA) test, the overall test accuracy of ascitic calprotectin was 87% when 0.63 µg/mL was used as the best cut-off value (A) and was 84% for the point-of-care (POC) test when 0.51 µg/mL was used as best cut-off (B). PMN: Polymorphonuclear.

malignant disease. Higher ascitic calprotectin levels were found in the malignant patients and shown to correlate with increased mortality in patients with decompensated liver cirrhosis. However, the authors did not investigate the diagnostic potential of ascitic calprotectin. More recently, Parsi *et al*²⁷¹ measured ascitic lactoferrin (an iron-binding protein also found in PMNs) in cirrhotic patients with ascites and investigated its potential for identifying SBP. The lactoferrin measurements correctly identified PMN counts > 250/µL in 22 of 218 samples (10.1%), yielding a sensitivity of 95.5% and specificity of 97.0%. However, the quantitative assay (ELISA) used in that study is not commercially available, and to date no bedside test, qualitative or quantitative, exists for lactoferrin.

The results from our current study confirm the findings reported by Parsi *et al*²⁷¹. Specifically, we show that measurement of calprotectin, a leukocyte-specific protein, may serve as a surrogate marker for the PMN count in ascitic fluid. A particular strength of our study is the quantitative measurement of calprotectin by two methods, a laboratory-based ELISA and a commercially available bedside test. Rapid bedside measurement is advantageous for hospitalised patients, as it supports early antibiotic intervention and limits unnecessary treatments. It will also benefit the outpatient setting by providing on-site testing, since samples are otherwise required to be transported to an offsite laboratory. The POC test that we used can accomplish quantitative measurement of ascitic calprotectin within minutes, and this feature is expected to minimize the problems associated with diagnostic delay that clinicians currently face. Additionally, the cost of POC testing may be less than the other methods, such as contracting with the offsite laboratories.

There are several limitations to the current study that merit consideration. First, the prevalence of SBP in our study cohort was lower than expected from the literature. Second, we included all patients with ascites, irrespective of the aetiology, and it may be that our results cannot be generalised to all patients with liver cirrhosis. Third, this was an exploratory study that aimed to establish the concept of measuring a PNM-related inflammatory protein, rather than PNM cells themselves, as an indicator of elevated cell count in ascites; therefore, no formal sample

size calculation was performed. Finally, our sample size was small and larger studies are needed to evaluate this test in different clinical settings and to establish a reliable cut-off for ascitic calprotectin for optimal identification of PMN counts > 250/µL.

In conclusion, we have demonstrated that measurement of calprotectin in ascitic fluid correlates well with the PMN count and reliably predicts levels > 250/µL. Additionally, we showed that an elevated PMN count could easily be measured by a POC test device which may enable a treating physician to obtain useful bedside measurements, especially those practicing in settings with limited equipment and/or technical personnel. Further studies are warranted to define a clinically useful cut-off for the diagnosis of SBP in cirrhotic patients with ascites.

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COMMENTS

Background

Ascites is the most common complication of patients with cirrhosis, and around 60% of patients will develop ascites within 10 years of disease commencement. Spontaneous bacterial peritonitis (SBP) is an important cause of morbidity and mortality in these patients. SBP is estimated to affect 10%-30% of hospitalised patients with ascites, and it is recommended that all patients with ascites undergo paracentesis at the time of admission to assess SBP status and initiate timely therapy.

Research frontiers

The diagnosis of SBP is based on a polymorphonuclear leukocyte (PMN) cell count > 250/µL in the ascitic fluid. Currently, differential cell count is usually performed manually using light microscopy and counting chambers. This procedure is time consuming, and diagnosis may be further delayed when laboratory personnel are not readily available. Several other methods to diagnose SBP (automated cell counting and urine dipstick-based screening for leukocytes) have proven unreliable in clinical practice and are inferior to the manual method. In this study, authors investigated the potential of calprotectin, a neutrophilic

protein and established marker of intestinal inflammation, to screen for SBP when measured in ascites.

Innovations and breakthroughs

To date, only one study in the public literature has measured calprotectin in ascites, and the conclusion was that higher concentrations of calprotectin exist in malignant disease conditions as compared to non-malignant conditions. However, diagnostic accuracy was not assessed and calprotectin was measured using a laboratory-dependent enzyme-linked immunosorbent assay (ELISA). In this study, authors have demonstrated that measurement of calprotectin in ascitic fluid correlates well with PMN count and reliably indicates PNM levels > 250/ μ L. Additionally, we showed that an elevated PMN count could be easily measured within minutes using a point-of-care (POC) bedside test, suggesting its potential as a rapid diagnostic approach for SBP.

Applications

The rapid diagnosis of SBP and immediate start of antibiotic treatment is of paramount importance as mortality estimates approach 30%. A particular strength of this study is the quantitative measurement of ascitic calprotectin by two methods: a laboratory-based ELISA and a commercially available POC test device. Rapid bedside measurement is advantageous for hospitalised patients, since it facilitates timely antibiotic therapy and minimizes unnecessary treatments. However, it may be especially beneficial to an outpatient setting, where samples are otherwise required to be transported to an offsite laboratory for testing. The POC test device that we used allows quantitative measurement of ascitic calprotectin within minutes and is likely to minimize the diagnostic delay that clinicians currently face.

Terminology

Calprotectin, a calcium and zinc-binding protein, is found almost exclusively in neutrophils. The presence of calprotectin in body fluids is proportional to the influx of neutrophils during inflammation.

Peer review

This article discusses a new method for the diagnosis of SBP. This is a well-designed and methodologically correct exploratory study.

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Efficacy of cap-assisted endoscopy for routine examining the ampulla of Vater

Young Rak Choi, Joung-Ho Han, Young Shim Cho, Hye-Suk Han, Hee Bok Chae, Seon Mee Park, Sei Jin Youn

Young Rak Choi, Joung-Ho Han, Young Shim Cho, Hye-Suk Han, Hee Bok Chae, Seon Mee Park, Sei Jin Youn, Department of Internal Medicine, College of Medicine, Chungbuk National University, Cheongju 361-711, South Korea

Author contributions: Choi YR and Han JH designed, conceptualized the study and performed the procedures; Youn SJ, Park SM provided clinical advice, assessed the results from the picture archiving, communication system images and video recordings; Han HS, Cho YS, Chae HB were involved in final editing and writing of the manuscript.

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Correspondence to: Joung-Ho Han, MD, Department of Internal Medicine, College of Medicine, Chungbuk National University, 410 SungBong-Ro Heungdeok-Gu, Cheongju-Si Chungbuk, Cheongju 361-711, South Korea. joungcho@cbnu.ac.kr
Telephone: +82-43-2696802 Fax: +82-43-2733252

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Abstract

AIM: To determine the efficacy of a cap-assisted endoscopy (CAE) to completely visualize the ampulla of Vater (AV) in patients failed by conventional endoscopy.

METHODS: A prospective study was conducted on 120 patients > 20 years of ages who visited the Health Promotion Center of Chungbuk National University Hospital for conscious sedation esophagogastroduodenoscopy (EGD) as a screening test from July to October, 2011. First, forward-viewing endoscopy was performed with reasonable effort using a push and pull method. We considered complete visualization of the AV when we could observe the entire AV including the orifice clearly, and reported the observation as complete or incomplete (partial or not found at all). Second, in cases of complete failure of the observation, an additional AV examination was conducted by attaching a short cap

(D-201-10704, Olympus Medical Systems, Tokyo, Japan) to the tip of a forward-viewing endoscope. Third, if the second method failed, we replaced the short cap with a long cap (MH-593, Olympus Medical Systems) and performed a re-examination of the AV.

RESULTS: Conventional endoscopy achieved complete visualization of the AV in 97 of the 120 patients (80.8%) but was not achieved in 23 patients (19.2%). Age (mean \pm SD) and gender [male (%)] were not significantly different between the complete observation and the incomplete observation groups. Additional short CAE was performed in patients in whom we could not completely visualize the AV. This group included 13 patients (10.9%) with partial observation of the AV and 10 (8.3%) in which the AV was not found. Short CAE permitted a complete observation of the AV in 21 of the 23 patients (91.3%). Patients in whom visualization of the AV failed with short CAE had satisfactory outcomes by replacing the short cap with a long cap. The additional time for CAE took an average of 141 ± 88 s. There were no complications and no significant mucosal trauma.

CONCLUSION: CAE is safe to use as a salvage method to achieve complete visualization of the AV when a regular EGD examination fails.

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Key words: Ampulla of Vater; Conventional endoscopy; Cap-assisted endoscopy; Screening test; Complete observation

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INTRODUCTION

It is recommended to visualize the second portion of the duodenum including the ampulla of Vater (AV) during a standard esophagogastroduodenoscopy (EGD) procedure^[1,2]. Adequate visualization of the AV is important for early detection of periampullary or pancreaticobiliary diseases^[3,4].

EGD textbooks and guidelines have emphasized complete visualization of the AV^[1,2], but at the same time indicated that complete visualization of the AV is difficult due to the anatomical characteristics of the second portion of the duodenum, including the tangential angle, the periampullary diverticulum, and loop formation in the scope^[5]. Thus, a side-viewing endoscope has been recommended for complete visualization of the AV in patients with a suspected AV lesion or in whom the AV cannot be observed completely. However, a side-viewing endoscope is not always available in an ordinary endoscopy suite^[6]. So clinicians should refer the patient for side-viewing endoscopy. In the case of South Korea, 4.2 million EGDs being performed as a screening test per year. Among them, 84% were performed in clinics that not equipped with side-viewing endoscope^[6]. And, although tertiary referral center were equipped with side-viewing endoscope, the Health Promotion Center for screening test were separated from Hospital Endoscopy Center for inpatient. So many endoscopists for screening test are not familiar with side-viewing endoscope for endoscopic retrograde cholangiopancreatography (ERCP). Therefore an additional examination using a side-viewing endoscope is expensive, time-consuming and difficult.

Cap-assisted endoscopy (CAE) has been used widely to facilitate detection of polyps^[7-10], improve the success rate of cecal intubation^[11,12], and to facilitate inspection of lesions situated in blind areas of the colon^[13-15]. We have found that the complete visualization rate of the AV in patients who are referred for incomplete visualization of the AV can be improved by attaching a transparent cap. Therefore, we conducted a prospective observational study to investigate the complete visualization rate of the AV during routine EGD and to evaluate the efficacy and safety of endoscopic examination using a transparent cap to completely visualize the AV in patients in whom this procedure failed with a conventional endoscope.

MATERIALS AND METHODS

Patients

A prospective study was conducted on 120 patients > 20 years of ages who visited the Health Promotion Center of Chungbuk National University Hospital for conscious sedation EGD as a screening test from July to October, 2011. The following patients were excluded from the study: (1) those with poor general condition who had an American Society of Anesthesiology classification ≥ 3 ; (2)

those who received previous upper gastrointestinal tract surgery or ERCP; and (3) patients with severe comorbidities. All patients provided written informed consent to participate in the study. This study was reviewed and approved by the Institutional Review Board of Chungbuk National University Hospital (D-2011-06-006).

Procedure

All examinations were conducted with the patient lying on their left side, and midazolam was administered as a sedative and pain medication. Approximately 30-40 mg of propofol was also administered depending on patient weight^[16]. Additional administration of propofol was used when deemed necessary, according to procedure time. Heart rate and oxygen saturation were checked in real time. Demographic data, procedure time, visualization of the AV, and complications were recorded. All examinations were carried out with a forward-viewing endoscope (GIF 230; Olympus Optical Co, Ltd, Tokyo, Japan) by a well-qualified endoscopist (Han JH), who has conducted more than 1500 EGDs including 400 ERCPs annually. Two types of transparent caps were used: disposable distal attachments; "soft and short" cap (D-201-10704, outer diameter: 11.35 mm, length from distal end of endoscope: 4 mm, Olympus Medical Systems) and a "hard and long" cap (MH-593, outer diameter: 12.9 mm, length from distal end of endoscope: 11 mm; Olympus Medical Systems) (Figure 1).

First, forward-viewing endoscopy was performed with reasonable effort using a push and pull method. We considered complete visualization of the AV when we could observe the entire AV including the orifice clearly, and reported the observation as complete or incomplete (partial or not found at all). Second, in cases of complete failure of the observation, an additional AV examination was conducted by attaching a short cap to the tip of a forward-viewing endoscope. Third, if the second method failed, we replaced the short cap with a long cap and performed a re-examination of the AV.

Statistical analysis

Descriptive statistical analyses were performed using the SPSS software, version 12.0 (SPSS Inc, Chicago, IL, United States), and frequency, percentage, mean, and range were used for descriptive analyses. A *P* value < 0.05 was considered statistically significant.

RESULTS

Patients' characteristics and outcomes of conventional endoscopy

A total of 181 patients were examined, 61 were excluded. 120 patients were enrolled and agreed to participate in the study by signing an informed consent (Figure 2). Conventional endoscopy achieved complete visualization of the AV in 97 of the 120 patients (80.8%) but was not achieved in 23 patients (19.2%) (Figure 3). The reasons

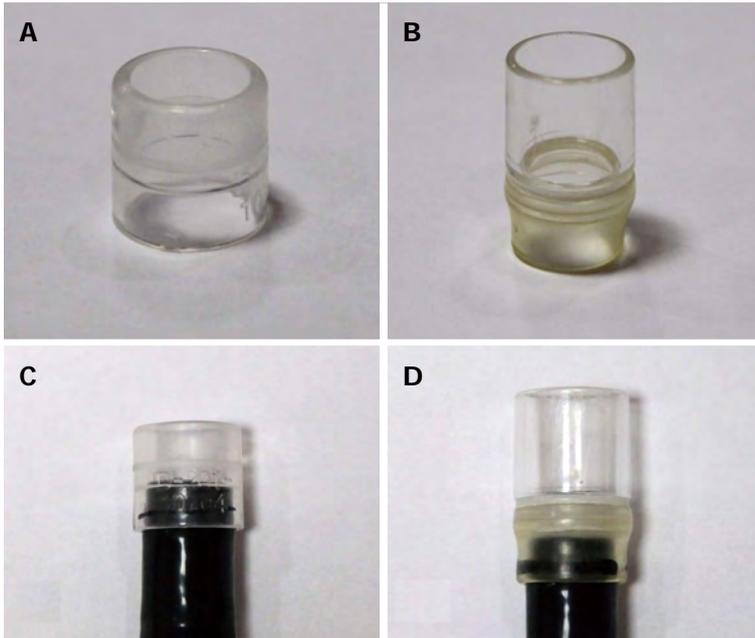


Figure 1 Endoscopic transparent cap. A: Short transparent cap (Olympus distal attachment D-201-10704, outer diameter: 11.35 mm, length from distal end of endoscope: 4 mm; Olympus Tokyo, Japan); B: Long transparent cap (Olympus distal attachment MH-593, outer diameter: 12.9 mm, length from distal end of endoscope: 11 mm; Olympus); C: Short cap attached to the tip of a forward-viewing endoscope; D: Long cap attached to the tip of a forward-viewing endoscope.

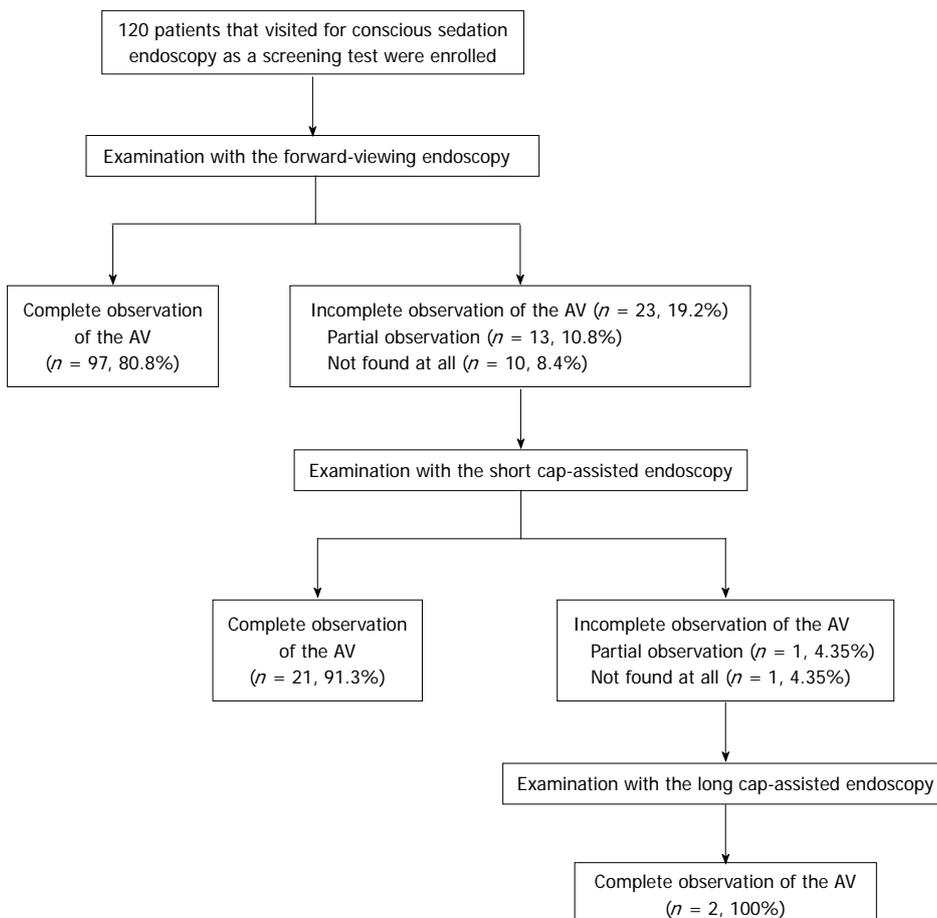


Figure 2 Flow diagram of patient enrollment and examinations. In total, 120 patients were examined by forward-viewing endoscopy. In cases when complete observations of the ampulla of Vater (AV) were unsuccessful, an additional examination for the AV was conducted by short cap-assisted endoscopy. If that examination was unsuccessful, the short cap was replaced with a long cap, and a re-examination was performed.

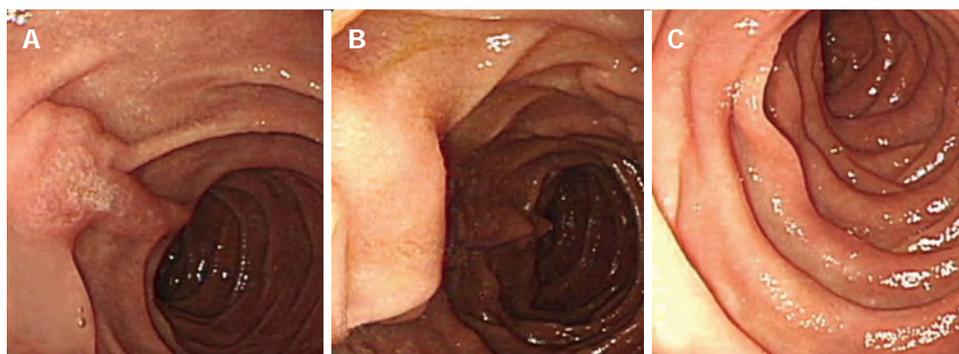


Figure 3 Outcomes of conventional endoscopy. A: Complete observation of the ampulla of Vater (AV), including the orifice, by forward-viewing endoscopy; B: Partial observation of the AV with a folded mucous membrane by forward-viewing endoscopy; C: The AV was not found during forward-viewing endoscopy.

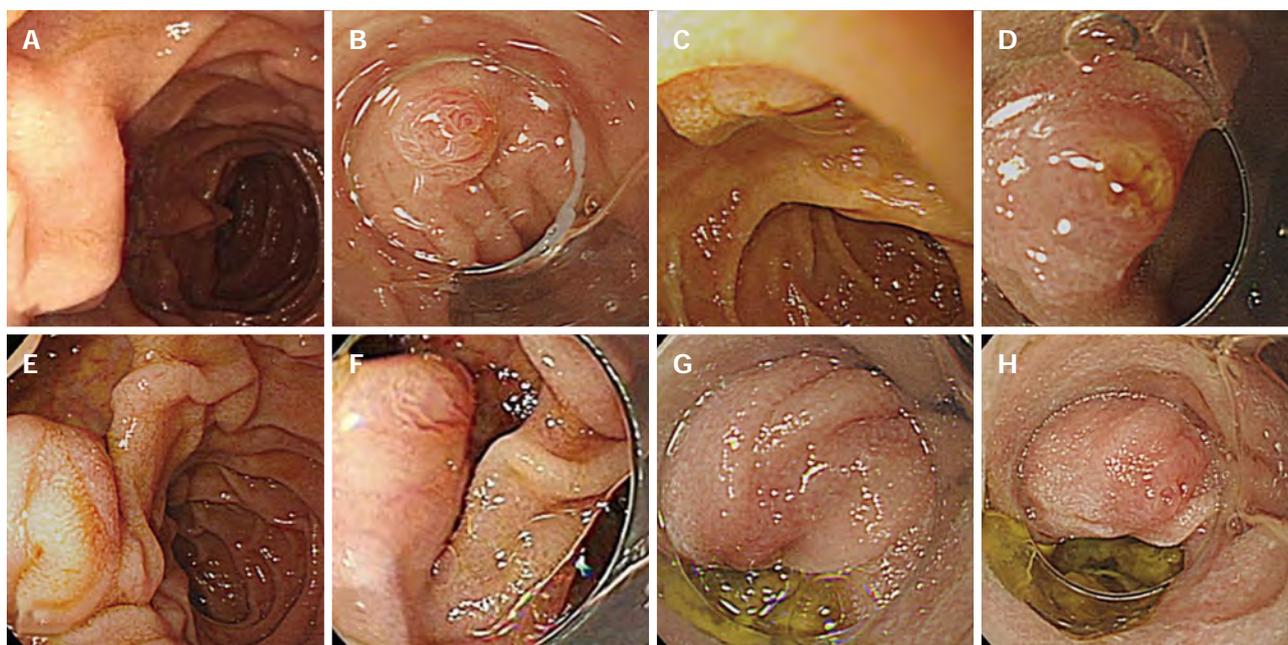


Figure 4 Incomplete observation of the ampulla of Vater by conventional endoscopy and outcomes of cap-assisted endoscopy. A: Incomplete observation of the ampulla of Vater (AV) by forward-viewing endoscopy due to a folded mucous membrane; C: Incomplete observation of the AV due to the close proximity of the endoscope tip to a superior ampulla lesion; E: Incomplete observations of the AV on the edge of diverticulum; B, D, F: Complete observation of the AV, including the orifice, by short cap-assisted endoscopy (A→B, C→D, E→F); G: Incomplete observation of the AV by short cap-assisted endoscopy due to a loop in the scope; H: Complete observation of the AV by long cap-assisted endoscopy (G→H).

for failure to visualize the ampulla were large fold in 16 patients, and diverticulum in 7 patients. Age (mean \pm SD) and gender [male (%)] were not significantly different between the complete observation and the incomplete observation groups [55.3 ± 12.6 years, 59 (60.8%); 55.2 ± 16.2 years, 12 (52.2%)], respectively.

Outcomes of CAE

Additional short CAE was performed in patients in whom we could not completely visualize the AV. This group included 13 patients (10.8%) with partial observation of the AV and 10 (8.4%) in which the AV was not found. Short CAE permitted a complete observation of the AV in 21 of the 23 patients (91.3%).

In cases of an incomplete visualization due to folded mucous membranes, complete observation of the orifice

areas was achieved by uncovering the fold with a short cap (Figure 4A and B). Due to a too close proximity of the endoscope tip to the superior ampulla lesion, the short cap provided a proper distance and the ability to straighten the mucosal fold by pressing the area surrounding the lesion (Figure 4C and D). The cap made it easier to access the AV at the edge of the diverticulum by directing the force vector along the tip of the endoscope (Figure 4E and F). Patients in whom visualization of the AV failed with short CAE had satisfactory outcomes by replacing the short cap with a long cap; one AV was observed only partially due to the deep location of the AV in the large diverticulum, whereas the other was not found due to a loop in the scope (Figure 4G and H). The time for CAE was 141 ± 88 s. No complications or significant mucosal trauma occurred. In one case of

suspected abnormal lesions, an additional side-viewing endoscopy with biopsy revealed a tubular adenoma, so an endoscopic ampullectomy was performed.

DISCUSSION

The transparent cap was first proposed in 1990 by Inoue *et al.*^[17] to improve the accessibility of the forward-viewing endoscope. Since then, transparent CAE has been used widely as a method to increase the success rate of the procedure^[18,19]. For example, CAE improves cecal intubation times and polyp detection rates during colonoscopy^[9,11], it achieves higher success rates of afferent loop intubation and bile-duct cannulation in patients with a Billroth II gastrectomy^[20-22], and it possible to clip a lesion too tangential to be clipped by routine endoscopy^[23-27].

Although there is a growing need to identify CAE as an effective approach to visualize the AV, no studies have investigated the AV observation rate during routine EGD or the efficacy of CAE for AV observation. Although small studies (preliminary data of Leal-Salazar *et al.*^[28] described the feasibility of visualizing the AV by attaching a long cap from a variceal band ligation set to a conventional endoscope) have been conducted, almost all cases (19 of 20) required emergency endoscopic hemostasis treatment, were not for screening test. That study reported conventional endoscopy permitted an inadequate observation of the AV in all cases (20 of 20), which is questionable, although they reported that observations of the AV using CAE were effective.

Although our cases had a wide variation in procedure duration, complete visualization of the AV was possible in up to 100% by attaching a cap to the tip of a conventional endoscope. The additional time for this procedure was short (average, 141 s). Moreover, the AV could be visualized completely in almost all patients using short CAE. Although a long cap has been used widely, the long cap is hard and difficult to pass over the larynx and pylorus, so it is easy to damage the mucosa, whereas the short cap is soft, safer, and relatively simple and easy to handle.

The transparent cap made the following multiple mechanisms possible: (1) The transparent cap provided a proper distance between the AV and the tip of the endoscope, which may prevent sticking of the endoscope to the duodenal lumen; (2) The cap allowed efficient manipulation of a tangentially placed AV to a more square approach; (3) The cap made it easier to access hidden areas by straightening the mucosal folds by pressing the surrounding lesion areas; and (4) the cap reduced loop formation by "hooking" the tip of the endoscope in the second portion of the duodenum and directing the force vector of the endoscope tip^[29-33]. Moreover, the long cap improved all of these functions; thus, it allowed an approach to a deeply AV located deep in the diverticulum^[34-36].

This study had some limitations. First, data collection was limited to screening tests in a healthy population that visited the Health Promotion Center; these patients can

have lower disease prevalence or anatomical variations in the AV than those in the general population. Thus, the success rate of CAE may be different in the general population. Second, because EGD was performed by one ERCP expert, the results cannot be generalized to a conventional endoscopist with less experience visualizing the AV.

In conclusion, CAE was an effective salvage technique when regular EGD was ineffective for visualizing the AV properly. CAE can increase the diagnostic accuracy of a forward-viewing endoscope and decrease the need for side-viewing endoscopy.

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COMMENTS

Background

Adequate visualization of the ampulla of Vater (AV) is important for early detection of periampullary or pancreaticobiliary diseases. But visualization of the AV with a forward-viewing endoscope is difficult due to the anatomical characteristics of the second portion of the duodenum. Thus, a side-viewing endoscope has been recommended for complete visualization of the AV. However, a side-viewing endoscope is not always available in an ordinary endoscopy suite.

Research frontiers

Cap-assisted endoscopy (CAE) has been used widely to facilitate detection of polyps, improve the success rate of cecal intubation, and to facilitate inspection of lesions situated in blind areas of the colon. The authors have found that the complete visualization rate of the AV in patients who are referred for incomplete visualization of the AV can be improved by attaching a transparent cap.

Innovations and breakthroughs

Although there is a growing need to identify CAE as an effective approach to visualize the AV, no studies have investigated the AV observation rate during routine esophagogastroduodenoscopy or the efficacy of CAE for AV observation. This is the first study to determine the efficacy and safety of an endoscopic examination using a transparent cap to completely visualize the AV in patients failed by conventional endoscopy.

Applications

CAE can increase the diagnostic accuracy of a forward-viewing endoscope and decrease the need for side-view endoscopy. This study may represent a future strategy for effective endoscopic examination as a screening test.

Terminology

Endoscopic caps are commonly used for both diagnosis and therapy during endoscopy. Transparent caps are attached to the distal end of the endoscope. Currently, many different sized caps are available. Cap-assisted endoscopic mucosal resection is the most common application of endoscopic caps. The appropriate selection of an endoscopic cap based on indication and location of the lesion is important for procedural success.

Peer review

The authors examined the efficacy of a CAE to completely visualize the AV in patients failed by conventional endoscopy. It revealed that CAE can increase the diagnostic accuracy of a forward-viewing endoscope and decrease the need for side-view endoscopy. The result are interesting and promising for endoscopic examination as a screening test.

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Effects of different resuscitation fluid on severe acute pancreatitis

Gang Zhao, Jun-Gang Zhang, He-Shui Wu, Jin Tao, Qi Qin, Shi-Chang Deng, Yang Liu, Lin Liu, Bo Wang, Kui Tian, Xiang Li, Shuai Zhu, Chun-You Wang

Gang Zhao, Jun-Gang Zhang, He-Shui Wu, Jin Tao, Qi Qin, Shi-Chang Deng, Yang Liu, Lin Liu, Bo Wang, Kui Tian, Xiang Li, Shuai Zhu, Chun-You Wang, Pancreatic Disease Institute, Union Hospital, Tongji Medical College, Huazhong University of Science and Technology, Wuhan 430022, Hubei Province, China

Chun-You Wang, Pancreatic Disease Institute, Union Hospital, Tongji Medical College, Huazhong University of Science and Technology, Wuhan 430022, Hubei Province, China

Author contributions: Zhao G and Wang CY designed the research; Zhang JG, Wu HS, Tao J, Qin Q, Deng SC, Liu Y, Liu L and Wang B performed the research; Tian K, Li X and Zhu S analyzed the data; Zhao G wrote the paper.

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Correspondence to: Chun-You Wang, Professor, Pancreatic Disease Institute, Union Hospital, Tongji Medical College, Huazhong University of Science and Technology, Wuhan 430022, Hubei Province, China. chunyouwang52@126.com

Telephone: +86-27-85351621 Fax: +86-2785351669

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Abstract

AIM: To compare effects of different resuscitation fluid on microcirculation, inflammation, intestinal barrier and clinical results in severe acute pancreatitis (SAP).

METHODS: One hundred and twenty patients with SAP were enrolled at the Pancreatic Disease Institute between January 2007 and March 2010. The patients were randomly treated with normal saline (NS group), combination of normal saline and hydroxyethyl starch (HES) (SH group), combination of normal saline, hydroxyethyl starch and glutamine (SHG group) in resuscitation. The ratio of normal saline to HES in the SH and SHG groups was 3:1. The glutamine (20% glutamine dipeptide, 100

mL/d) was supplemented into the resuscitation liquid in the SHG group. Complications and outcomes including respiratory and abdominal infection, sepsis, abdominal hemorrhage, intra-abdominal hypertension, abdominal compartment syndrome (ACS), renal failure, acute respiratory distress syndrome (ARDS), multiple organ dysfunction syndrome (MODS), operation intervention, length of intensive care unit stay, length of hospital stay, and mortality at 60 d were compared. Moreover, blood oxygen saturation (SpO₂), gastric intramucosal pH value (pHi), intra-abdominal pressure (IAP), inflammation cytokines, urine lactulose/mannitol (L/M) ratio, and serum endotoxin were investigated to evaluate the inflammatory reaction and gut barrier.

RESULTS: Compared to the NS group, patients in the SH and SHG groups accessed the endpoint more quickly (3.9 ± 0.23 d and 4.1 ± 0.21 d *vs* 5.8 ± 0.25 d, $P < 0.05$) with less fluid volume (67.26 ± 28.53 mL/kg/d, 61.79 ± 27.61 mL/kg per day *vs* 85.23 ± 21.27 mL/kg per day, $P < 0.05$). Compared to the NS group, incidence of renal dysfunction, ARDS, MODS and ACS in the SH and SHG groups was obviously lower. Furthermore, incidence of respiratory and abdominal infection was significantly decreased in the SH and SHG groups, while no significant difference in sepsis was seen. Moreover, less operation time was needed in the SH and SHG group than the NS group, but the difference was not significant. The mortality did not differ significantly among these groups. Blood SpO₂ and gastric mucosal pHi in the SH and SHG groups increased more quickly than in the NS group, while IAP was significantly decreased in the SH and SHG group. Moreover, the serum tumor necrosis factor- α , interleukin-8 and C-reactive protein levels in the SH and SHG groups were obviously lower than in the NS group at each time point. Furthermore, urine L/M ratio and serum endotoxin were significantly lower in the SH group and further decreased in the SHG group.

CONCLUSION: Results indicated that combination of normal saline, HES and glutamine are more efficient in resuscitation of SAP by relieving inflammation and sustaining the intestinal barrier.

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Key words: Microcirculation; Intestinal barrier; Inflammatory reaction; Intra-abdominal hypertension; Capillary leakage syndrome

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INTRODUCTION

Severe acute pancreatitis (SAP) has a mortality rate about 30% and is characterized by pancreatic necrosis, cytokine activation, systemic inflammatory response syndrome (SIRS), and multiple organ dysfunction syndrome (MODS)^[1,2]. Accumulative results have demonstrated that microcirculation perfusion and hypoxia have a significant impact on the early stages of disease and play an important role in the pathogenesis of necrosis^[3]. Different from normal hypovolemia caused by trauma or bleeding, microcirculatory disorder of SAP is caused by special SIRS. Overexpressed inflammatory media such as tumor necrosis factor (TNF)- α , interleukin (IL)-6 and IL-8 in SIRS will injury the microcirculation endothelium and then increase capillary permeability and fluid sequestration, leading to capillary leakage syndrome and MODS^[4].

Moreover, the microcirculatory disorder of the intestinal will lead to intestinal ischemia reperfusion injury and damage the intestinal barrier, which could facilitate translocation of intestinal bacteria and enhance leukocyte activation and inflammatory cytokine release^[5]. If the microcirculatory disorder cannot be blocked at the initial stage, it will be exaggerated and form positive feedback. Thus, the excess cytokine will further injury the distal organs and lead to irreversible multiorgan failure^[6,7]. Moreover, bacterial translocation from the gut will cause severe infection^[8]. Therefore, adequate prompt fluid resuscitation is crucial for prevention of systemic complications^[9-11]. Thus, the purpose of effective fluid resuscitation of SAP is not only to supply the deficiency of blood volume, but also especially to stabilize the capillary permeability, modulate the inflammation reaction, and sustain the intestinal barrier function.

Two types of fluids frequently used today for active resuscitation are colloid fluids with large molecules (hetastarch, dextran 40, and albumin) that keep fluid intravascular, and crystalloid fluids with added electrolytes (normal saline, Ringer's, and lactated Ringer's). Both crystalloid and colloid solutions are considered effective

for the resuscitation of a hypovolemic patient, because neither fluid provides a survival benefit that is superior to the other^[12]. When trying to augment cardiac output and blood pressure, colloids have an advantage over crystalloid solutions, because a larger percentage enters the intravascular space and remains there for a longer period of time. This is because colloids provide the greatest effect on intravascular volume expansion and improve flow secondary to their low viscosity, which is equal to that of water^[13].

In the septic shock model with concomitant capillary leakage in the presence of a marked albumin loss, the hydroxyethyl starch (HES) could preserve systemic oxygenation and hemodynamics^[14]. Increased capillary permeability leading to fluid loss from the intravascular space and fluid sequestration into the third space are hallmarks of SAP^[15]. Clinically, capillary leakage is reflected by intravascular fluid loss leading to hypovolemia [low central venous pressure (CVP)], hemoconcentration (high packed cell volume), and extravascular fluid sequestration in the retroperitoneal, lungs, pleural and abdominal cavities^[16]. Although colloids such as HES are supposed more suitable than crystalloids for volume resuscitation of hypovolemia, prospective clinic studies have seldom involved comparing effects of crystalloids and HES in volume resuscitation in SAP^[10,11,17].

Some research has shown that HES reduces intestinal permeability by modulating inflammatory response and has a promising effect on survival, together with antibiotics under septic conditions^[18]. Although glutamine has been proved to protect the intestinal barrier as a nutrient supplement in nutritional support of SAP, the effects of glutamine delivered as a supplement in resuscitation fluid in the early stage of SAP have not been defined^[19-21].

In the present study, we compared the clinical results of different resuscitation fluids including normal saline, combination of normal saline and HES, and combination of normal saline, HES and glutamine in resuscitation of SAP. Moreover, the effects on microcirculation, inflammation reaction and intestinal barrier were investigated.

MATERIALS AND METHODS

Patients

One hundred and twenty patients with SAP were enrolled at the Pancreatic Disease Institute, Union Hospital (Wuhan, China) between January 2007 and March 2010. All of the patients were diagnosed with SAP according to the Atlanta Classification System^[22], and were aged 18-60 years. Exclusion criteria were heart disease, severe renal and hepatic dysfunction, coagulation disturbances, and allergy to HES or glutamine. Those patients with manifestation more than 48 h or who received resuscitation from the other hospital were also excluded. Informed consent was obtained from the patients and approval was obtained from the Ethics Committee of Union Hospital, Huazhong University of Science and Technology. The demographic information of the patients is shown in

Table 1 Demographic information of patients with severe acute pancreatitis treated by different resuscitation fluid (mean \pm SD)

| | NS group (n = 40) | SH group (n = 40) | SHG group (n = 40) |
|-----------------|----------------------|----------------------|-----------------------|
| Age (yr) | 41.86 \pm 13.85 | 44.50 \pm 9.77 | 45.11 \pm 11.57 |
| Sex n (%) | | | |
| Male | 20 (50) | 22 (55) | 21 (52.5) |
| Female | 20 (50) | 18 (45) | 19 (47.5) |
| Height (cm) | 165.86 \pm 6.04 | 165.00 \pm 9.03 | 169.25 \pm 6.67 |
| Weight (kg) | 66.5 \pm 8.63 | 69.00 \pm 9.68 | 72.38 \pm 8.43 |
| APACHE II score | 11.2 \pm 0.7 | 10.9 \pm 0.6 | 11.3 \pm 0.4 |
| MAP (mmHg) | 62.3 \pm 9.3 | 64.8 \pm 9.2 | 63.6 \pm 8.9 |
| BUN/Cr ratio | 23.9 \pm 3.6 | 24.2 \pm 3.2 | 23.7 \pm 3.7 |

No significant differences were observed among the study groups regarding demographic data. APACHE: Acute physiology and chronic health evaluation; MAP: Mean arterial pressure; BUN/Cr: Blood urea nitrogen/creatinine; NS group: Normal saline; SH group: Combination of normal saline and hydroxyethyl starch; SHG group: Combination of normal saline, hydroxyethyl starch and glutamine.

Table 1. If the patient could not successfully achieve a balance of output and input within 7 d, we considered them to have failed resuscitation. However, these cases were not removed from the study and all the results were still included.

Management of resuscitation

The patients were randomly divided into three groups that were resuscitated with normal saline (NS group), combination of normal saline and hydroxyethyl starch (SH group; 130 kD, Sino-Swed Pharmaceutical Corp. Ltd.), or combination of normal saline, hydroxyethyl starch and glutamine (SHG group; glutamine dipeptide, Sino-Swed Pharmaceutical Corp. Ltd.). The ratio of normal saline to HES in the SH and SHG groups was 3:1. Glutamine (20% glutamine dipeptide, 100 mL/d) was supplemented into the resuscitation liquid in the SHG group.

All of the patients with suspected SAP were initially transferred to the pancreatic intensive care unit (PICU). The vital signs, oxygen saturation (SpO₂), gastric intramucosal PH value (pHi), arterial blood-gas analysis, mean arterial pressure (MAP) and intra-abdominal pressure (IAP) were monitored to guide the treatment. All the patients received a central venous catheter capable of measuring central venous oxygen saturation (ScvO₂) and CVP.

Meanwhile, the fluid resuscitation was promptly conducted as early as possible. Generally, we infused 1 L normal saline in the NS group or combination of 500 mL normal saline and 500 mL HES in the SH and SHG groups in the first 2 h (500 mL/h) to achieve a CVP of 8-12 mmHg. If the MAP was < 65 mmHg, vasopressors were given to maintain a MAP of at least 65 mmHg. If the MAP was > 90 mmHg, vasodilators were given until it was \leq 90 mmHg^[23]. If the urine output was < 0.5 mL/kg/h after the CVP and MAP were stabilized, 20 mg dihydrochlorothiazide bolus was infused and followed with 1-2 mL/h continuous infusion *via* syringe pump to maintain the urine output at > 0.5 mL/kg per hour. After that,

resuscitation fluid was continually infused at a speed of 150-300 mL/h (approximately 2-3 mL/kg per hour, ratio of normal saline to HES is 3:1) and modulated depending on the reaction of early resuscitation and parameters in the later course, which maintained the urine at 0.5-1 mL/kg per hour and prevented excess resuscitation.

Moreover, if the oliguria continued for > 2 d and the ratio of blood urea nitrogen/creatinine (BUN/Cr) significantly increased, continuous venovenous hemofiltration (CVVH) with ultrafiltration modulation was performed to extra the liquid sequestration in the "3rd space" and overexcited inflammatory mediators. Most of the patients were accompanied with respiratory dysfunction, and for those patients with ScvO₂ < 70% after stabilization of CVP and MAP, we did not perform blood transfusion but intensified oxygen supplementation through pressure oxygen mask or tracheal intubation with artificial respirator. Except for the CVP, MAP, urine and ScvO₂ adopted as early resuscitation parameters, IAP was also used to assess the microcirculation dysfunction. Even without significant abnormality in the other parameters, the infusion was carefully controlled and the CVVH applied to balance the output and input in those patients with significantly increased IAP. The input of resuscitating liquid depended on the output including ultrafiltration volume of CVVH, urine and non-dominant water loss. The ultimate endpoint of resuscitation was defined as the balance of input and output. Those patients who approached the endpoint in 7 d were considered to have successful resuscitation, otherwise it had failed.

Complications and clinical outcomes

Complications and outcomes were recorded throughout the whole course, including respiratory infection, abdominal infection, sepsis, abdominal hemorrhage, intra-abdominal hypertension (IAH), abdominal compartment syndrome (ACS), renal failure, acute respiratory distress syndrome (ARDS), MODS, operation intervention, length of intensive care unit stay, length of hospital stay and mortality at 60 d.

All the clinical parameters were recorded from day 1 to day 7 after administration. Resuscitation fluid input, abdominal drainage fluid and urine output were recorded every day. Oxygen supply and microcirculation perfusion was evaluated by pulse SpO₂ and pHi, which were detected by bedside monitor (Life Scope A, Nihon Kohden, Irvine, CA, United States) and gastric mucosal pH monitor (Tonocap, Datex-Ohmeda, Finland) respectively^[24]. Harvested heparinized blood was centrifuged and the plasma was removed and stored at -80 °C for later examination.

Measurements of IAP

IAP was measured according to the method of Oda *et al.*^[25]. In brief, an 18-gauge catheter was inserted into the culture aspiration port of the urine catheter, and a line filled with saline was connected to the pressure transducer. After urine had been completely drained from the bladder, the urine catheter line was clamped, and then 100 mL of saline was instilled in the bladder and the pres-

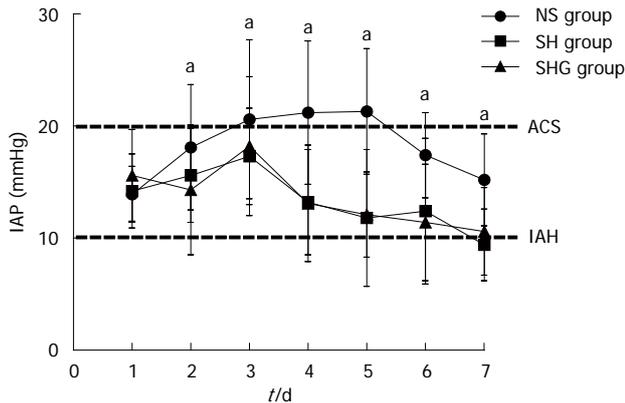


Figure 1 Effect of different resuscitation fluids on changes in intra-abdominal pressure in severe acute pancreatitis. Intra-abdominal pressure (IAP) was indirectly measured via a bladder catheter during 7 d and performed twice daily. All of the patients had intra-abdominal hypertension on d 1 (IAP > 10 mmHg). ^a $P < 0.05$ vs normal saline (NS group). SH group: Combination of normal saline and hydroxyethyl starch; SHG group: Combination of normal saline, hydroxyethyl starch and glutamine; ACS: Abdominal compartment syndrome; IAH: Intra-abdominal hypertension.

sure was measured at the end of expiration. IAP was measured first on admission to the PICU, and twice daily thereafter in the morning and evening, in principle. According to the consensus guidelines, IAH was defined as IAP ≥ 12 mmHg and ACS as IAP > 20 mmHg with evidence of organ dysfunction.

Plasma cytokines assay

Serum TNF- α , IL-8 and C-reactive protein (CRP) from day 1 to day 7 were evaluated by enzyme-linked immunosorbent assay (R-D Systems, Minneapolis, MN, United States) according to the manufacturer's instructions.

Urine lactulose/mannitol ratio assay

Urine lactulose/mannitol (L/M) ratio during 7 d was analyzed to evaluate intestinal permeability as described before^[21]. All the patients fasted for at least 6 h and their bladders were emptied before the test. The test solution consisted of 10 g lactulose and 5 g mannitol in a total volume of 50 mL through nasojunal tube. The urine volume was collected for the subsequent 6 h. The urine volume was recorded, and 10 mL was frozen and stored at -80°C . L/M ratio in urine was analyzed by Hi-Crush Partners LP.

Endotoxin assay

Serum concentration of endotoxin at 7 d was detected by quantitative chromogenic limulus amoebocyte lysate assay (QCL-1000; Whittaker MA Bioproducts, Walkersville, MD, United States) according to the manufacturer's instructions. Blood was drawn aseptically into lipopolysaccharide-free tubes. All samples were processed in a laminar flow hood. To minimize nonspecific plasma inhibitors, samples were diluted with pyrogen-free water and heat inactivated at 100°C for 10 min. *Escherichia coli* 055:B5 reference endotoxin (1 endotoxin unit = 0.6 ng/mL) was used for the standard curve (Whittaker MA

Bioproducts).

Statistical analysis

Statistical analyses were performed with SPSS version 12.0.2. Data are presented as mean \pm SD. χ^2 analysis and one-way repeated-measures analysis of variance were used for the analysis of differences. $P < 0.05$ was considered significant.

RESULTS

Demographic information

As shown in Table 1, no significant differences were observed between the study groups regarding demographic data including age, agenda, height, weight, Acute physiology and chronic health evaluation II score, MAP and BUN/Cr ratio at the initial time.

Fluid resuscitation and fluid balance

All patients in the three groups received resuscitation at an early stage after manifestation of SAP (12.8 ± 6.7 h, 13.1 ± 5.4 h, and 12.2 ± 6.3 h). The balance of input and output was considered as the ultimate endpoint of resuscitation. Compared to the NS group, it took a significantly shorter time to approach the resuscitation endpoint in the SH and SHG groups (5.8 ± 0.25 d vs 3.9 ± 0.23 d and 4.1 ± 0.21 d, $P < 0.05$). Nevertheless, the volumes of fluid administered in the SH and SHG groups was obviously lower than that in the NS group (67.26 ± 28.53 and 61.79 ± 27.61 vs 85.23 ± 21.27 mL/kg per day, $P < 0.05$), while the abdominal drainage fluid in the SH and SHG groups was significantly lower than that in the NS group (Table 2).

Complications and outcomes

When infection was suspected, ultrasound-guided percutaneous aspiration of pancreatic tissue, sputum and blood was performed with Gram's stain and culture. If ultrasound-guided percutaneous aspiration confirmed the infection, antibiotics were administered following the results of culture and antibiotic sensitivity. If the episode is not effectively relieved, the patient should undergo debridement. Compared to the NS group, the incidence of renal dysfunction, ARDS, MODS and ACS in the SH and SHG groups was obviously lower. Furthermore, the incidence of respiratory and abdominal infection was significantly decreased in the SH and SHG groups, while no significant difference in sepsis was seen. Moreover, less operation time was needed in the SH and SHG groups than in the NS group, but the difference was not significant (Table 3). IAP in the NS group was significantly higher than in the SH and SHG groups at each time point (Figure 1). Patients in the SH and SHG groups had a notably shorter length of stay in the intensive care unit (ICU) and hospital than the NS group had. Although mortality in the NS group was higher than in the SH and SHG groups, no significant difference was observed. No significant difference in any of the parameters was noted between the SH and SHG groups.

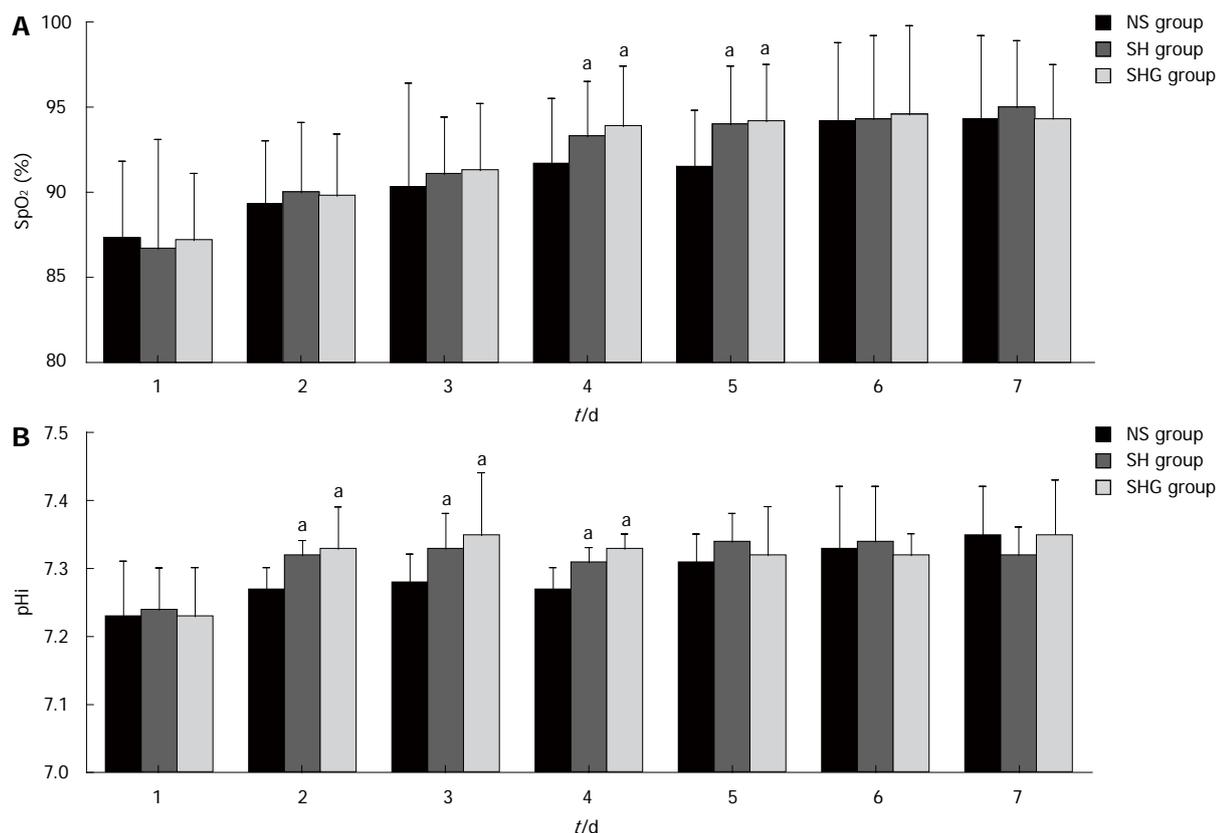


Figure 2 Effect of different resuscitation fluids on circulation oxygen supply and microcirculation perfusion. A: Effect of different fluids on circulation oxygen supply was evaluated with pulse oxygen saturation (SpO₂) by automatic monitoring; B: Microcirculation perfusion was assessed with gastric intramucosal pH value (pHi) by Tonocap monitor. ^aP < 0.05 vs normal saline (NS group). SH group: Combination of normal saline and hydroxyethyl starch; SHG group: Combination of normal saline, hydroxyethyl starch and glutamine.

Table 2 Window of manifestation to resuscitation, time of resuscitation endpoint, volume of resuscitation fluid, urine output, and abdominal drainage fluid in different groups

| | NS group | SH group | SHG group |
|--|--------------|----------------------------|---------------------------|
| Window to resuscitation (h) | 12.8 ± 6.7 | 13.1 ± 5.4 | 12.2 ± 6.3 |
| Time to endpoint (d) | 5.8 ± 0.25 | 3.9 ± 0.23 ¹ | 4.1 ± 0.21 ¹ |
| Resuscitation fluid (mL/kg per day) | 61.79 ± 7.61 | 46.93 ± 12.38 ¹ | 44.75 ± 8.53 ¹ |
| Urine output/d (mL/kg per day) | 31.3 ± 5.47 | 28.71 ± 11.62 | 27.94 ± 10.62 |
| Abdominal drainage fluid (mL/kg per day) | 11.32 ± 2.13 | 6.28 ± 3.26 ¹ | 6.35 ± 1.42 ¹ |

¹P < 0.05 vs normal saline (NS group). Statistic measure of variation is standard error. SH group: Combination of normal saline and hydroxyethyl starch; SHG group: Combination of normal saline, hydroxyethyl starch and glutamine.

SpO₂ and microcirculation perfusion

The SpO₂ of the SH and SHG groups increased more quickly than in the NS group and was significantly higher at day 4 and day 5. Similarly, pHi in the SH and SHG groups also increased faster than in the NS group from day 2 to day 4 (Figure 2).

Serum cytokine and CRP concentration

Serum TNF-α, IL-8 and CRP were detected daily from

Table 3 Complications and outcomes of patients treated by different resuscitation fluid

| | NS group | SH group | SHG group |
|---------------------|-----------|------------------------|-----------------------|
| Renal dysfunction | 11 | 31 | 41 |
| ARDS | 16 | 61 | 71 |
| MODS | 10 | 31 | 31 |
| ACS | 6 | 11 | 11 |
| Lung infection | 13 | 51 | 51 |
| Abdominal infection | 11 | 31 | 21 |
| Sepsis | 3 | 2 | 3 |
| Operation | 6 | 2 | 2 |
| LOIS | 14 ± 8.2 | 10 ± 9.4 ¹ | 11 ± 6.3 ¹ |
| LOHS | 31 ± 22.8 | 22 ± 18.9 ¹ | 21 ± 23.71 |
| Mortality | 5 | 2 | 3 |

¹P < 0.05 vs normal saline (NS group). SH group: Combination of normal saline and hydroxyethyl starch; SHG group: Combination of normal saline, hydroxyethyl starch and glutamine; ARDS: Acute respiratory distress syndrome; MODS: Multiple organ dysfunction syndrome; ACS: Abdominal compartment syndrome; LOIS: Length of intensive care unit stay; LOHS: Length of hospital stay.

day 1 to day 7. Although the TNF-α concentration in all three groups increased to a peak at d 3 and then gradually decreased, it was significantly higher in the NS group at days 3-5 (Figure 3A). Unlike the highest IL-8 concentration in the NS group at day 4, the SH and SHG groups had the highest IL-8 concentration at day 3 and then it

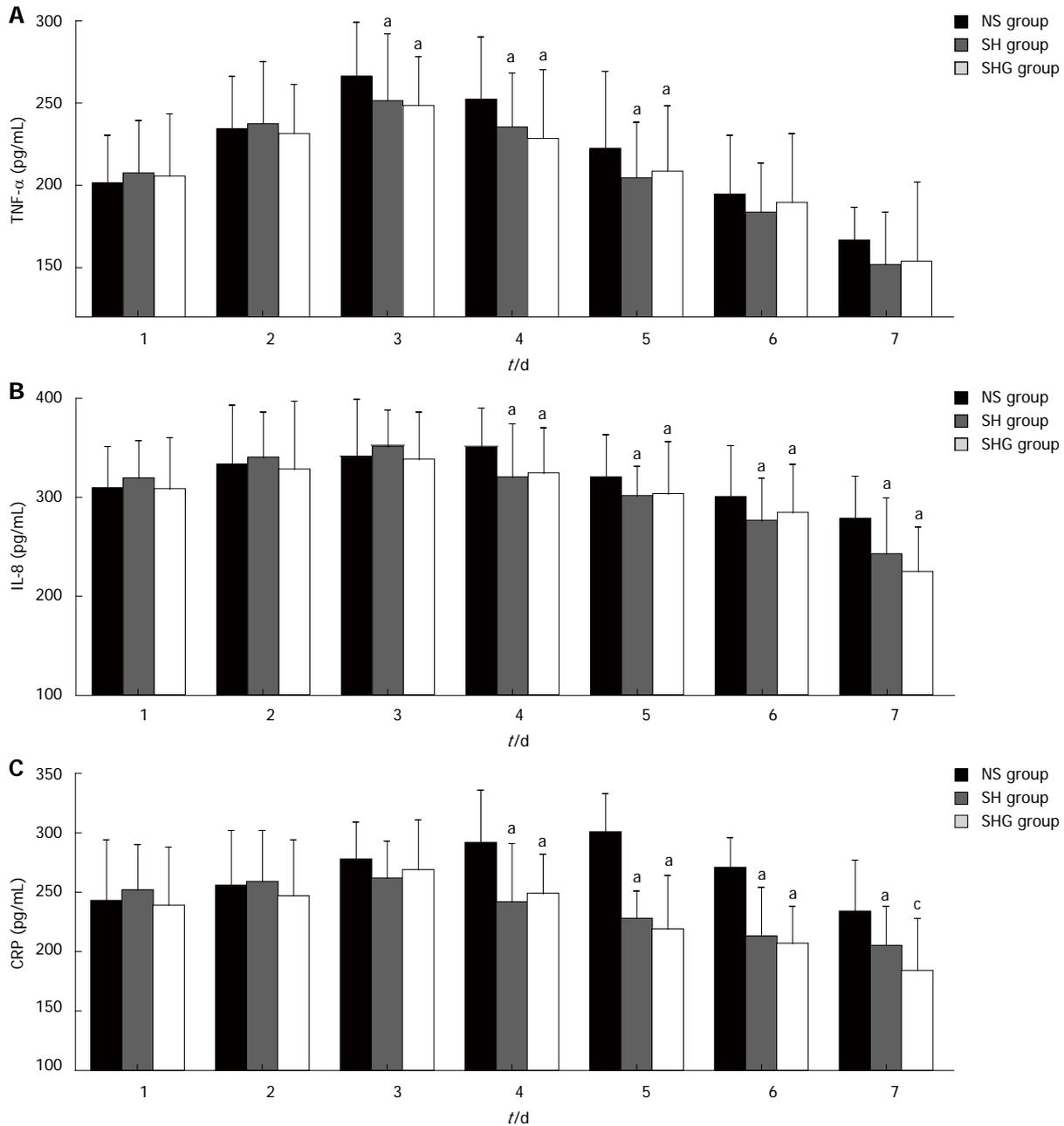


Figure 3 Effects of different resuscitation fluids on serum cytokine and C-reactive protein. Serum tumor necrosis factor (TNF)-α (A), interleukin (IL)-8 (B) and C-reactive protein (CRP) (C) concentration was evaluated by enzyme-linked immuno sorbent assay. ^a*P* < 0.05 vs normal saline (NS) group; ^c*P* < 0.05 vs combination of normal saline and hydroxyethyl starch (SH group). SHG group: Combination of normal saline, hydroxyethyl starch and glutamine.

significantly decreased. Furthermore, the IL-8 concentration in the SHG group was obviously lower than in the SH group at days 4-7 (Figure 3B). The CRP concentration in the NS group continually increased until day 5, while it was significantly decreased in the SH and SHG groups after day 3. Moreover, the CRP concentration in the SHG group was significantly lower than that in the SH group after day 7 (Figure 3C).

Intestinal permeability

Intestinal permeability was assessed by urine L/M ratio and serum endotoxin. Urine L/M ratio in all three groups increased after administration of SAP. Compared to the

NS group, urine L/M ratio in the SH and SHG groups was significantly lower after day 4. Moreover, compared to the SH group, it further decreased in the SHG group after day 5. Parallel to urine L/M ratio, serum endotoxin in the NS group was also significantly higher than that in the SH and SHG groups. Nevertheless, serum endotoxin in the SHG group was markedly lower than that in the SH group after day 6 (Figure 4).

DISCUSSION

Many studies have indicated that colloids such as HES are more suitable than crystalline solutions for volume

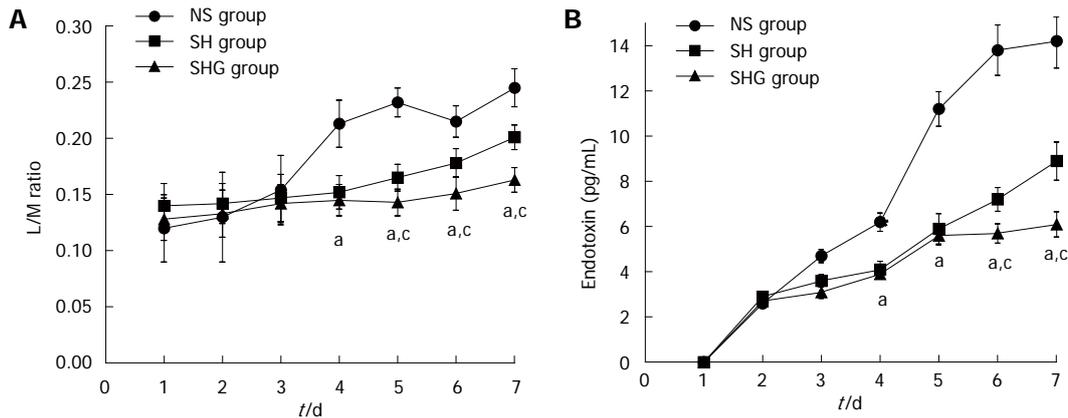


Figure 4 Effect of different fluids on intestinal mucosa barrier function. A: Lactulose/mannitol (L/M) ratio of urine in normal saline (NS group), combination of normal saline and hydroxyethyl starch (SH group) and combination of normal saline, hydroxyethyl starch and glutamine (SHG group) was measured by Hi-Crush Partners LP; B: Serum endotoxin in different groups was detected by quantitative chromogenic limulus amoebocyte lysate assay reagent. ^a*P* < 0.05 vs NS group; ^c*P* < 0.05 vs SH group.

resuscitation in hypovolemia of sepsis, of which microcirculation arrangement are same as SAP^[26-28]. Our present study demonstrated that less time and volume were needed to achieve the resuscitation endpoint in the SH and SHG groups. Moreover, higher urine output and lower abdominal drainage were seen in the SH and SHG groups. Furthermore, the incidence of MODS and ACS were significantly decreased in the SH and SHG groups.

This indicated that combination of HES and crystalloids might effectively attenuate the capillary leakage by maintaining colloid osmotic pressure and then decreasing extravascular fluid sequestration, such as pleural effusion and abdominal ascites. As an important clinical clue for extensive sequestration, the IAP and IAH in the SH and SHG groups were decreased significantly more than those in the NS group.

Although the mortality was not significantly decreased, the incidence of respiratory infection and abdominal infection was significantly reduced in the SH and SHG groups. This indicated that the decreased liquid sequestration in the abdomen and lungs significantly reduced the infection risk. Furthermore, less operation time was needed and a lower level of organ dysfunction occurred in the SH and SHG groups. Thus, shorter length of ICU and hospital stay was seen in the SH and SHG groups. These results further suggest that combination of HES and normal saline might be effective in resuscitation and effectively decrease infective complications and organ failure in SAP. Although the NS group had a higher incidence of respiratory and abdominal infection, incidence of sepsis and mortality in the NS did not differ significantly from that in the SH and SHG groups. This might be because the treatment of SAP is multimodal including resuscitation, organ support, nutrition support, operative intervention, and antibiotics and these different single resuscitation managements were not sufficient to affect mortality.

The target of effective resuscitation is not only maintaining the blood volume, but more importantly, to improve tissue oxygenation and microcirculation perfusion.

In our research, arterial SpO₂ and pHi were detected to evaluate the oxygenation and microcirculation perfusion. We showed that SpO₂ and pHi in the SH and SHG groups recovered more quickly than in the NS group. We showed that combination of HES and normal saline was effective in expanding the blood volume and improving the microcirculation and tissue oxygenation.

Our research demonstrated that inflammatory factors including TNF- α , IL-8 and CRP, in the SH and SHG groups were significantly lower and decreased earlier than those in the NS group, which indicates that HES might modulate the inflammatory reaction. Other research has also discovered that HES attenuates capillary leakage through modulation of the inflammation reaction in sepsis and abdominal surgery. It is speculated that HES may inhibit nuclear factor- κ B activation and neutrophil adhesion and migration^[29-31], but the exact mechanism has not been defined. Other research implies that HES prevents the inflammatory reaction through relieving ischemia-reperfusion injury in the intestine^[18,32]. The present study demonstrated that intestinal permeability significantly decreased in the SH and SHG groups, which also implied that the inflammation reaction was modulated by improvement of intestinal ischemia-reperfusion injury.

Flint *et al.*^[33] have shown that the severity of SAP can be exacerbated by intestinal ischemia-reperfusion injury. When the intestinal barrier is disrupted, the luminal content invades the portal venous and lymphatic systems. This translocation activates immune cells downstream from the intestinal mucosa to release inflammatory mediators that drive the onset of SIRS and MODS in SAP^[34-36]. Our present study demonstrated that urine L/M ratio and endotoxin were significantly decreased after treatment with HES and normal saline. It indicated that sustaining the intestinal barrier by HES is one of the important reasons for decreased mortality from infection and MODS.

Many studies have shown that nutrition support supplemented with specific immunonutrients such as glutamine may protect the intestinal barrier and modulate the acute phase responses, thereby potentially improving

outcome in SAP^[19-21]. We speculated that supplementation of glutamine in resuscitation could further sustain intestinal function and improve the clinical outcomes of SAP. Our results showed that the IL-8, CRP, urine L/M ratio and serum endotoxin were further decreased after supplementation with glutamine, while the clinical outcomes and complications had no significant change. This implies that ischemia-reperfusion injury is a major reason for intestinal dysfunction in the early stage of SAP.

In summary, the present study showed that combination of HES and normal saline was more efficient in fluid resuscitation of SAP by modulating inflammation and the intestinal barrier, which resulted in a lower level of infection and organ failure. Nevertheless, although supplementation with glutamine could further modulate inflammatory reaction and intestinal, while no significant changes were seen in clinical results.

COMMENTS

Background

Accumulative results demonstrated that microcirculation perfusion and hypoxia leading to capillary leakage syndrome (CLS) and multiple organ dysfunction syndrome (MODS) in severe acute pancreatitis (SAP). Although the colloid such as hydroxyethyl starch (HES) are supposed more suitable than crystalloid in volume resuscitation in hypovolemia, seldom prospective clinic researches have involved in SAP. Given to protect intestinal barrier as nutrient supplement in SAP, the effects of glutamine delivered as supplement of resuscitation fluid in early stage of SAP were not defined. Thus, the present in present set to compare clinical results of different resuscitation fluid including normal saline, combination of normal saline and HES as well as combination of normal saline, HES and glutamine in resuscitation of SAP, respectively.

Research frontiers

Overexpressed inflammatory cytokine of SAP such as tumor necrosis factor- α (TNF- α), interleukin (IL)-6, IL-8 will injury the capillary permeability and lead to CLS, which further damage the intestinal barrier and induce translocation of intestinal bacterial. Thus, the effective fluid resuscitation of SAP should be not only to sustain body volume but also improve capillary permeability and intestinal barrier function. Although the colloid such as HES are supposed more suitable than crystalloid in volume resuscitation in hypovolemia, seldom prospective clinic researches have involved in comparing effects of crystalloid and HES in volume resuscitation in SAP.

Innovations and breakthroughs

Many researches indicated that colloid such as HES are supposed more suitable than crystalline in volume resuscitation in hypovolemia of sepsis, but it is unknown whether the HES is also more efficient in SAP which microcirculation arrangement is similar to septic. The present results firstly demonstrated that less time and volume were needed to get resuscitation endpoint in combination of normal saline and hydroxyethyl starch (HS) group and HES and glutamine (SHG group). Moreover, higher urine output and lower abdominal drainage were showed in SH and SHG groups. Furthermore, the incidence of MODS and abdominal compartment syndrome (ACS), were significantly decreased in SH and SHG groups. The results displayed that oxygen saturation and intramuscular pH value of combination of normal saline and hydroxyethyl starch (SH group) and SHG group recovered more quickly than normal saline (NS) group which implied that combination of HES and normal saline is not only effective expanding blood volume but also improving the microcirculation and tissue oxygenation. This research demonstrated that the inflammatory factor including TNF- α , IL-8 and C-reactive protein of SH and SHG group were significantly lower and decreased earlier than those of NS group. Moreover, our present study demonstrated that urine lactulose/mannitol ration and endotoxin were significantly decreased after treated with HES and normal saline.

Applications

The present study showed that combination of HES and normal saline was more efficient in fluid resuscitation of SAP by modulate inflammation and intestinal barrier, which resulted in lower modality of infection and organ failure.

Moreover, the supplementary with glutamine could further modulate inflammatory reaction and intestinal. It implied that combination of normal saline, HES and glutamine might be an efficient resuscitation fluid in SAP which deserves to be investigated in further clinical study.

Terminology

CLS is a rare medical condition characterized by self-reversing episodes during which the endothelial cells which line the capillaries are thought to separate for a few days, allowing for a leakage of fluid from the circulatory system to the interstitial space, resulting in a dangerous hypotension (low blood pressure), hemoconcentration, and hypoalbuminemia. It is a life-threatening illness because each episode has the potential to cause damage to, or the failure of, vital organs due to limited perfusion. ACS occurs when the abdomen becomes subject to increased pressure. Specific cause of ACS is not known, although some causes can be sepsis and severe abdominal trauma. Increasing pressure reduces blood flow to abdominal organs and impairs pulmonary, cardiovascular, renal, and gastro-intestinal function, causing MODS and death.

Peer review

This is an interesting paper with potentially clinical use.

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Noninvasive methods for prediction of esophageal varices in pediatric patients with portal hypertension

Marina Rossato Adami, Cristina Targa Ferreira, Carlos Oscar Kieling, Vania Hirkata, Sandra Maria Gonçalves Vieira

Marina Rossato Adami, Cristina Targa Ferreira, Carlos Oscar Kieling, Vania Hirkata, Sandra Maria Gonçalves Vieira, Universidade Federal do Rio Grande do Sul, Post-Graduation in Gastroenterology and Hepatology, Hospital de Clínicas de Porto Alegre, Pediatric Gastroenterology Unit, Porto Alegre, Rio Grande do Sul 91350-170, Brazil

Author contributions: Adami MR, Ferreira CT, Kieling CO, Vieira SMG designed the research; Adami MR, Kieling CO performed the research; Adami MR, Kieling CO, Hirkata V analyzed the data; Adami MR, Ferreira CT, Kieling CO, Hirkata V, Vieira SMG wrote the paper.

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Correspondence to: Dr. Marina Rossato Adami, Universidade Federal do Rio Grande do Sul, Post-Graduation in Gastroenterology and Hepatology, Hospital de Clínicas de Porto Alegre, Pediatric Gastroenterology Unit, Porto Alegre, Rio Grande do Sul 91350-170, Brasil. marinaadami2008@gmail.com

Telephone: +55-51-99613460 Fax: +55-51-99613460

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Abstract

AIM: To evaluate clinical and laboratory parameters for prediction of bleeding from esophageal varices (EV) in children with portal hypertension.

METHODS: Retrospective study of 103 children (mean age: 10.1 ± 7.7 years), 95.1% with intrahepatic portal hypertension. All patients had no history of bleeding and underwent esophagogastroduodenoscopy for EV screening. We recorded variceal size (F1, F2 and F3), red-color signs and portal gastropathy, according to the Japanese Research Society for Portal Hypertension classification. Patients were classified into two groups: with and without EV. Seven noninvasive markers were evaluated as potential predictors of EV: (1) platelet count; (2) spleen size z score, expressed as a standard deviation score relative to normal values for age; (3)

platelet count to spleen size z score ratio; (4) platelets count to spleen size (cm) ratio; (5) the clinical prediction rule (CPR); (6) the aspartate aminotransferase to platelet ratio index (APRI); and (7) the risk score.

RESULTS: Seventy-one children had EV on first endoscopy. On univariate analysis, spleen size, platelets, CPR, risk score, APRI, and platelet count to spleen size z score ratio showed significant associations. The best noninvasive predictors of EV were platelet count [area under the receiver operating characteristic curve (AUROC) 0.82; 95%CI: 0.73-0.91], platelet: spleen size z score (AUROC 0.78; 95%CI: 0.67-0.88), CPR (AUROC 0.77; 95%CI: 0.64-0.89), and risk score (AUROC 0.77; 95%CI: 0.66-0.88). A logistic regression model was applied with EV as the dependent variable and corrected by albumin, bilirubin and spleen size z score. Children with a CPR < 114 were 20.7-fold more likely to have EV compared to children with CPR > 114. A risk score > -1.2 increased the likelihood of EV (odds ratio 7.47; 95%CI: 2.06-26.99).

CONCLUSION: Children with portal hypertension with a CPR below 114 and a risk score greater than -1.2 are more likely to have present EV. Therefore, these two tests can be helpful in selecting children for endoscopy.

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Key words: Portal hypertension; Clinical predictors; Pediatric patients; Esophageal varices

Core tip: Children with portal hypertension (PH) are at risk for variceal bleeding. The standard test for screening varices is endoscopy, an invasive method. We evaluated non-invasive markers for diagnosing esophageal varices (EV) in 103 children (95% intrahepatic PH). All patients had no bleeding history and underwent endoscopy for EV screening. Platelet count (< 115 000), clinical prediction rule (< 114) and risk score (cutoff > -1.2) were the best predictors of EV. Limitations are the retrospective

design and the small number of pre-hepatic PH patients. The strength is the paucity of pediatric studies related to this issue and the assessment of risk score in children.

Adami MR, Ferreira CT, Kieling CO, Hirakata V, Vieira SMG. Noninvasive methods for prediction of esophageal varices in pediatric patients with portal hypertension. *World J Gastroenterol* 2013; 19(13): 2053-2059 Available from: URL: <http://www.wjg-net.com/1007-9327/full/v19/i13/2053.htm> DOI: <http://dx.doi.org/10.3748/wjg.v19.i13.2053>

INTRODUCTION

Portal hypertension is the underlying pathophysiological process that leads to the formation of portosystemic collaterals and heralds the onset of a severe complication: variceal hemorrhage. It is estimated that approximately 50% of pediatric patients with chronic liver disease and 90% of those with extrahepatic portal vein obstruction (EHPVO) will experience gastrointestinal bleeding^[1,2]. Esophagogastroduodenoscopy (EGD) is considered the primary modality for detection and surveillance of esophageal varices (EV) and to determine the risk of bleeding.

Guidelines for adult cirrhotic patients recommend universal EV screening by EGD at the time of the diagnosis of cirrhosis^[3-7].

Many studies have sought to determine clinical, laboratory, or other noninvasive methods that could predict the presence of EV. Preliminary data suggests that laboratory tests such as platelet count, albumin and ultrasonographic parameters such as presence of splenomegaly, spleen size z score and platelet count to spleen size ratio and the clinical prediction rule (CPR; calculated from platelet count, spleen size z-score, and albumin concentration) developed by Gana may be useful as first-line tools for identification of adults and pediatric patients at risk of variceal development and thus reduce the number of unnecessary EGDs^[8-22].

The aim of this study was to analyze the following non-invasive methods for predicting EV in pediatric patients with portal hypertension submitted to EGD: platelet count, spleen size z score, platelet count to spleen size (cm) ratio, platelet count to spleen size z score ratio, CPR, risk score and the aspartate aminotransferase to platelet ratio index (APRI).

MATERIALS AND METHODS

We conducted a retrospective evaluation of patients aged < 18 years with a diagnosis of chronic liver disease or EHPVO who underwent EGD between the 2000 and 2011 at Hospital de Clinicas de Porto Alegre, a tertiary referral center in Southern Brazil. Portal hypertension was defined after the diagnosis of some conditions which natural progression occurs along with portal hypertension such as chronic liver disease and extra hepatic portal vein thrombosis. The following exclusion criteria were

applied: active or previous variceal bleeding, prior variceal treatment (any type) or variceal bleeding prophylaxis (including nonselective β -blocker use, endoscopic variceal ligation or sclerotherapy, surgical portosystemic shunt or transjugular intrahepatic portosystemic shunt insertion), liver transplantation, and malignancy.

The presence of EV on endoscopy was the primary endpoint. Clinical and demographic data, diagnoses, medication use, physical examination findings, and severity of liver disease, as assessed by pediatric end-stage liver disease and model for end-stage liver disease (for children > 12 years old) and the Child-Pugh classification, were reviewed. The results of laboratory tests and ultrasound scans were considered for analysis if performed within 3 mo of EGD.

Endoscopy was carried out as part of routine clinical care. Four different endoscopists, recorded variceal size (F1, F2 and F3), red-color signs, and portal gastropathy, according to Japanese Research Society for Portal Hypertension classification^[23], and gastric varices according to the Sarin classification^[24].

Three thousand EGDs were reviewed and 127 patients with chronic liver disease or EHPVO were identified. Twenty-four patients were excluded: eleven due to previous variceal bleeding, four due to non-selective β -blocker therapy, four due to liver transplantation, two with laboratory test performed over than 3 mo of EGD, one due to surgical shunting, one due to no etiologic confirmation and one due to band ligation.

Seven non-invasive markers, previously described in adults and pediatric patients with portal hypertension, were evaluated as potential predictors of EV: (1) platelet count; (2) spleen size z score, expressed as a standard deviation score relative to normal values for age^[25]; (3) platelet count to spleen size z score ratio; (4) platelet count to spleen size (cm) ratio; (5) the CPR, proposed by Gana *et al*^[22] which is calculated according to the following formula: $[(0.75 \times \text{platelets}) / (\text{spleen z score} + 5)] + (2.5 \times \text{albumin})$; (6) the APRI test; and (7) a risk score, calculated as follows: $[14.2 - 7.1 \times \log_{10} \text{platelets} (10^9/\text{L})] + [4.2 \times \log_{10} \text{bilirubin} (\text{mg/dL})]$ ^[21].

For statistical analyses, patients were classified into two groups: patients with EV and patients without EV.

Statistical analysis

Data are expressed as mean and standard deviation, median and interquartile range, and proportions and 95%CI as appropriate. A *P* value of < 0.05 was considered statistically significant in all analyses. Continuous variables (such as laboratory data, spleen size z score, CPR, risk score) were compared using the Student *t*-test or the Mann-Whitney *U* test. Categorical variables (such as ascites, encephalopathy, and splenomegaly) were compared by the chi-square test or Fisher's exact test.

To determinate test performance for prediction of EV, a receiver operator characteristic (ROC) curve was constructed and the area under the ROC curve (AUROC) was calculated. The cutoff value of the variables was determined at the point of highest sensitivity and specific-

Table 1 Univariate analysis for esophageal varices

| Variables | Varices (n = 71) | No varices (n = 32) | P value |
|------------------------------------|---------------------------|---------------------------|------------|
| Age (yr) | 9.1 ± 4.9 | 8.5 ± 4.4 | 0.530 |
| AST (U/L) | 87 (51-158) | 68 (36-178) | 0.417 |
| ALT (U/L) | 78 (40-141) | 54 (22-160) | 0.197 |
| INR | 1.2 (1.1-1.4) | 1.1 (1.1-1.3) | 0.066 |
| Bilirubin (mg/dL) | 1.4 (0.8-2.4) | 0.6 (0.4-2.2) | 0.016 |
| Albumin (g/dL) | 3.8 ± 0.6 | 4.1 ± 0.7 | 0.077 |
| Creatinine (mg/dL) | 0.5 ± 0.2 | 0.5 ± 0.2 | 0.686 |
| Splenomegaly | 64 (95.5%) | 22 (73.3%) | 0.001 |
| Spleen size (cm) | 14.6 ± 3.3 (n = 65) | 12.2 ± 2.7 (n = 24) | 0.001 |
| Spleen size z score | 6.3 ± 3.2 (n = 65) | 3.7 ± 2.6 (n = 24) | 0.000 |
| Platelets (10 ³ /μL) | 102 ± 50.8 | 195 ± 85.2 | 0.000 |
| Encephalopathy(1/2/3) ¹ | 31/0/0 | 68/3/0 | 0.245 |
| Ascites (1/2/3) ² | 31/0/1 | 59/9/3 | 0.100 |
| Model of end-stage liver disease | 6.6 ± 4.6 (n = 23) | 3.6 ± 7.1 (n = 8) | 0.290 |
| Pediatric end-stage liver disease | -1.3 ± 8.6 (n = 48) | -2.2 ± 10.2 (n = 24) | 0.708 |
| Child-Pugh A/B/C | 35/31/5 | 22/8/2 | 0.169 |
| Child score | 7.0 ± 1.4 | 6.6 ± 1.3 | 0.074 |
| Clinical prediction rule | 103.6 ± 17.5 (n = 65) | 121.1 ± 21.1 (n = 24) | 0.001 |
| Platelets/spleen size z score | 16.7 (7.9-31.1) | 47.1 (27.2-123.3) | 0.000 |
| APRI | 2.3 (1.0-3.7) | 1.0 (0.3-2.3) | 0.001 |
| Platelets/spleen size | 0.7 (0.4-1.1) (n = 65) | 1.3 (0.8-2.2) (n = 24) | 0.000 |
| Risk score | 1.2 ± 2.6 | -1.6 ± 2.9 | 0.000 |

¹Determined clinically or by electroencephalogram: 1 = none; 2 = grade 1 or 2; 3 = grade 3 or 4; ²Determined clinically or by ultrasound: 1 = no ascites; 2 = controlled or mild; 3 = moderate or tense. AST: Aspartate aminotransferase; ALT: Alanine aminotransferase; INR: International normalized ratio; APRI: Aspartate aminotransferase to platelet ratio index.

ity. Sensitivity, specificity, predictive values and likelihood ratios were calculated for these cutoff values. A logistic regression model was used to evaluate the variables that reached statistical significance on univariate analysis, with EV as the dependent variable. All statistical analyses were performed in the SPSS 18.0. This study was approved by the local Research Ethics Committee.

RESULTS

A hundred and three patients were included, with a mean age of 8.9 (± 4.7) years. Fifty-six (56/103; 54.3%) patients were female. Ninety-eight (98/103; 95%) patients had a diagnosis of chronic liver disease and five (5/103; 4.8%) had EHPVO. Seventy-one of the (71/103; 68.9%) patients had EV. Varices were classified as F2 and F3 in 35 patients, with red spots in 12 patients. Sixteen (16/71; 22.5%) patients presented both esophageal and gastric varices, and one had isolated gastric varices. Twenty (20/103; 19.4%) patients had portal hypertensive gastropathy.

Spleen size, platelet count, CPR, APRI, platelet count to spleen size ratio, platelet count to spleen size z score ratio, and the risk score were able to discriminate patients with and without varices (Table 1).

On ROC curve analysis, the best predictors of EV

Table 2 Odds ratios for esophageal varices

| Variables | OR ¹ | 95%CI | P value |
|------------------------------------|-----------------|-------------|---------|
| CPR < 115 | 7.99 | 1.45-43.82 | 0.017 |
| CPR < 114 | 20.74 | 2.66-161.50 | 0.004 |
| Platelets/spleen size z score < 25 | 4.27 | 0.90-20.26 | 0.067 |
| Platelets/spleen size < 1 | 2.20 | 0.65-7.43 | 0.203 |
| Platelets | 0.98 | 0.97-0.99 | 0.016 |
| Platelets < 115 | 3.10 | 0.97-9.88 | 0.056 |
| Risk score > -1.2 | 7.47 | 2.06-26.99 | 0.002 |
| APRI > 1.4 | 1.85 | 0.56-6.10 | 0.309 |

¹Odds ratio computed by multivariate logistic regression model. CPR: Clinical prediction rule; APRI: Aspartate aminotransferase to platelet ratio index.

were: platelet count; platelet count to spleen size z score ratio; CPR; risk score; platelet count to spleen size (cm) ratio; spleen size z score; and the APRI test (Figure 1). The cutoff points were established with the best relationship between sensitivity and specificity for each variable as follows: platelet count, 115 000; platelet count to spleen size z score ratio, 25; CPR, 114; risk score, -1.2; platelet count to spleen size ratio, 1.0; APRI test, 1.4.

A logistic regression model was applied with EV as the dependent variable, corrected by albumin, bilirubin and spleen size z score. Patients with a CPR < 114 were 20.74-fold more likely to have EV compared to children with CPR > 114. Risk score > -1.2 increased the likelihood of varices (odds ratio 7.47; 95%CI: 2.06-26.99) (Table 2). Sensitivity, specificity, positive predictive value, negative predictive value, positive likelihood ratio and negative likelihood ratio values for CPR, platelet count, platelet count to spleen size z score ratio, platelet count to spleen size (cm) ratio, risk score and APRI as EV predictors are presented on Table 3.

DISCUSSION

We evaluated seven non-invasive markers, two of which had never been tested in children, the platelet count to spleen size (cm) ratio and the risk score. We found that platelets, the platelet count to spleen size z score ratio, CPR, and the risk score were able to predict EV. The prevalence of EV observed in our sample was similar to those reported elsewhere^[22,26,27].

Thrombocytopenia is a common complication of chronic liver disease, affecting 76% of cirrhotic patients^[28]. Unlike in adults^[13], isolated platelet count has been described as a predictor of EV in four out of four studies of pediatric patients^[22,26,27,29]. Nevertheless, there is still no consensus as to the best cutoff points, ranging from 100 000 to 130 000^[22,26]. Gana *et al*^[27] demonstrated that platelet count (cutoff point = 115 000) was the best predictor of EV, with an area under the AUROC curve of 0.79 (95%CI: 0.69-0.90). In the present study, the cutoff of point was similar to that observed by Gana *et al*^[22] with an area under the ROC curve = 0.82 (95%CI: 0.73-0.91).

Splenomegaly is an important clinical sign of portal hypertension, especially in patients with chronic liver dis-

Table 3 Diagnostic performance of variables as esophageal varices predictors

| Variables | Sensitivity (95%CI) | Specificity (95%CI) | Positive predictive value (95%CI) | Negative predictive value (95%CI) | Positive likelihood ratio (95%CI) | Negative likelihood ratio (95%CI) |
|------------------------------------|---------------------|---------------------|-----------------------------------|-----------------------------------|-----------------------------------|-----------------------------------|
| Clinical prediction rule < 115 | 76.6 (64.0-85.8) | 70.8 (48.7-86.5) | 87.5 (75.3-94.4) | 53.1 (35.0-70.4) | 2.63 (1.38-4.96) | 0.33 (0.20-0.53) |
| Clinical prediction rule < 114 | 75.0 (62.3-84.6) | 79.2 (57.3-92.0) | 90.5 (78.5-96.5) | 54.2 (36.8-70.8) | 3.60 (1.63-7.95) | 0.32 (0.20-0.49) |
| Platelets < 115 | 67.6 (55.3-77.9) | 81.3 (62.9-92.1) | 88.8 (76.6-95.4) | 53.0 (38.4-67.2) | 3.61 (1.72-7.54) | 0.40 (0.28-0.56) |
| Platelets/spleen size z score < 25 | 68.8 (55.8-79.4) | 79.2 (57.3-92.0) | 89.8 (76.9-96.2) | 48.7 (32.7-64.9) | 3.30 (1.48-7.32) | 0.39 (0.27-0.58) |
| Platelets/spleen size < 1 | 72.3 (59.6-82.3) | 66.7 (44.6-83.6) | 85.4 (72.8-93.0) | 47.0 (30.1-64.6) | 2.17 (1.20-3.89) | 0.42 (0.27-0.64) |
| Risk score > -1.2 | 80.3 (67.2-89.3) | 70.9 (51.7-85.1) | 86.3 (75.2-93.2) | 61.1 (43.5-76.4) | 2.77 (1.57-4.85) | 0.28 (0.17-0.45) |
| APRI > 1.4 | 63.4 (51.0-74.2) | 65.6 (46.7-80.8) | 80.3 (67.2-89.3) | 44.7 (30.4-59.8) | 1.84 (1.10-3.07) | 0.56 (0.39-0.78) |

APRI: Aspartate aminotransferase to platelet ratio index.

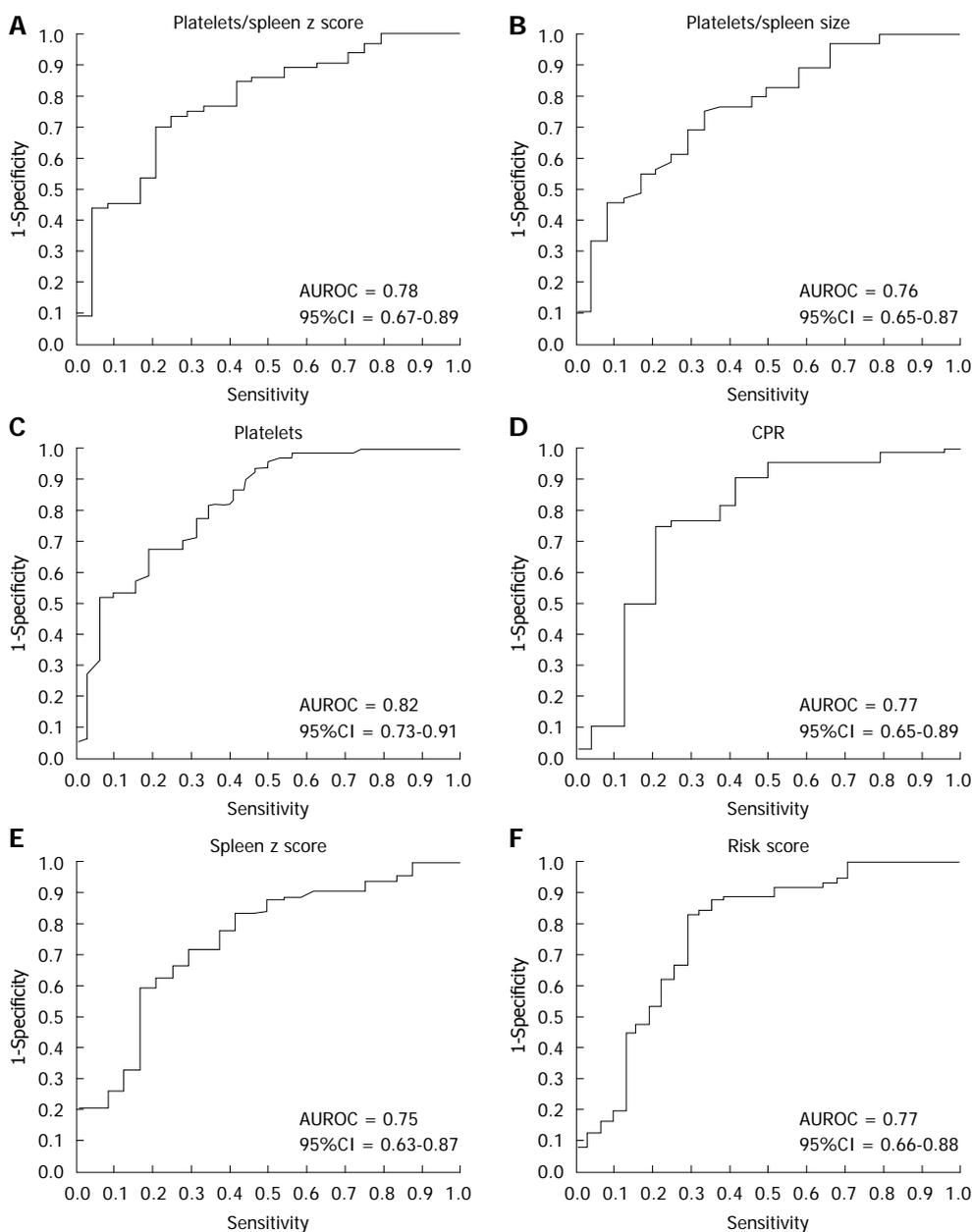


Figure 1 Receiver operator characteristic curves for presence of esophageal varices. AUROC: Area under the receiver operating characteristic curve.

ease^[30]. It has been used as such in several studies, both as an isolated parameter and as a component of scores or mathematical algorithms^[22,26,27,29]. In cirrhotic children

studied by Fagundes *et al*^[26] patients with splenomegaly were almost 15-fold more likely to have EV compared with those without splenomegaly. In our study, 83.5%

of patients had splenomegaly on physical examination, and, as observed by others, this variable discriminated patients with and without EV ($P = 0.001$).

Based on the premise that both thrombocytopenia and splenomegaly may depend on several factors related to chronic liver disease *per se*, Giannini *et al*^[8] proposed studying the platelet count to spleen diameter ratio as a noninvasive rule predicting EV. According to the authors, a ratio less than 909 was independently associated with the presence of EV, the negative predictive value found was reproducible even in patients with compensated disease, and it was cost effective^[8].

In our study, a platelet count to spleen size (cm) ratio < 1.0 was able to discriminate patients with and without EV ($P < 0.000$), but did not reach statistical significance on logistic regression (OR = 2.2; 95%CI: 0.65-7.43; $P = 0.203$). This could be explained by age and gender differences in spleen size. We tried to minimize the impact of this factor by using the spleen size z score, but this parameter was also not able to reach statistical significance on logistic regression (OR = 4.7; 95%CI: 0.90-20.26; $P = 0.067$). A disadvantage of considering platelet count and spleen size is that this evaluation needs to be synchronously due the great variability of both.

More recently, a systematic review and meta-analysis sought to determine the evidence on the diagnostic accuracy of platelet count to spleen diameter ratio < 909 as a noninvasive predictor of EV and concluded that the quality of evidence of these studies was low, raising questions about the reliability of the platelet count to spleen diameter ratio as a good predictor of EV. We agree with Chawla *et al*^[20] that the heterogeneity of patients studied may limit the value of this ratio as a noninvasive predictor of EV. The etiological diversity of patients in our sample may have played a role in our findings.

An interesting CPR was developed and validated by Gana *et al*^[22] in a retrospective study, using platelet count, spleen size z score and albumin as variables^[22]. In a prospective, multicenter clinical trial, a CPR ≤ 116 had a sensitivity of 81%, a specificity of 73% and an AUROC of 0.84. The authors suggested that CPR under 115 could screen patients for endoscopy^[27].

Apart from Gana *et al*^[22], we are the first group to test the CPR in children. To do so, we used two different cutoff points: 115 and 114. The best specificity was observed with a cutoff of 114 (79%). Others predictors identified by Gana *et al*^[22] were platelet count under 115 000 and serum albumin level. On multivariate analysis, CPR (OR 0.62; 95%CI: 0.45-0.84; $P = 0.002$) and albumin (OR 3.1; 95%CI: 1.5-6.7; $P = 0.004$) were independent predictors^[27]. In our study, logistic regression, adjusted for albumin, bilirubin and spleen size z score, had an OR of 7.79 (95%CI: 1.45-43.82) with a CPR cutoff of 115 and an OR of 20.74 (95%CI: 2.66-161.5; $P = 0.004$) with a CPR cutoff of 114. This mathematical algorithm is simple, composed by available and noninvasive variables, and our results suggest that it is reproducible.

The degree of fibrosis can determine significant changes in the hepatic venous pressure gradient, and seems related to complications such as the development of EV^[7]. Non-invasive markers for fibrosis have been tested in children with biliary atresia^[29,31]. There was good correlation between APRI and Metavir scores in patients studied by Kim *et al*^[31], suggesting that the APRI test could predict the appearance of fibrosis in those patients (a cutoff of 1.42 was correlated with grade 4 fibrosis).

The APRI was studied by Colecchia *et al*^[29] as a non-invasive marker of EV in pediatric patients with chronic liver disease. A cutoff > 0.96 had a total accuracy of 83%. These results were not confirmed on multivariate analysis^[29]. APRI, with a cutoff of > 1.4 , was also used as a variable in this study, and we did not find this parameter to be statistically significant for prediction of EV on logistic regression. We did not test other cutoffs. In fact, the exact thresholds of APRI for prediction of fibrosis constitute the main issue related to its diagnostic accuracy^[32].

The risk score was another clinical model tested for predicting EV in adults with advanced fibrosis and portal hypertension. The AUROC of the risk score for prediction of EV was 0.82. The -1.0 cutoff had a sensitivity of 82% and a specificity of 76%. The authors suggested that this score should be validated as a noninvasive test to detect the presence of EV^[29]. This was the first pediatric study to use the risk score. The cutoff of -1.2 had a sensitivity of 80.3%, a specificity of 70.9%, an AUROC of 0.77 (95%CI: 0.66-0.88), and an OR of 7.47 (95%CI: 2.06-26.99; $P = 0.002$). This method is also composed by simple and available variables that proved to be good noninvasive parameters for EV detection in our patients. Furthermore, this method avoids the frequent use of ultrasound. It is worth noting that this method has not been tested in patients with pre-hepatic portal hypertension, and may not be an effective method in such patients, whose bilirubin levels are usually normal.

We tried to apply all known non-invasive clinical methods for prediction of EV to the study population. According to other pediatric studies, we also found platelet count to be a good predictor of EV, with a cutoff of 115 000. Children with a CPR under 114, in a logistic regression model, were 20.7-fold more likely to have EV compared to children with CPR > 114 . More studies of this rule are required to find the optimal cutoff value.

The risk score, previously studied in adults, was a good and inexpensive predictor of EV in our patients. We believe it should be tested as a tool that can potentially limit the number of endoscopies in pediatric patients.

This study has some limitations. The retrospective design precludes blinding of the endoscopists or controlling for interobserver variability in ultrasound tests. The small number of patients with pre-hepatic portal hypertension could not be compared with those with intrahepatic portal hypertension.

In conclusion, the results of this study suggest that

platelet count, the CPR and risk score could be used to screen children with portal hypertension for endoscopy. Further studies with a prospective design are necessary to confirm these suggestions.

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COMMENTS

Background

Esophageal varices (EV) bleeding is a severe complication of portal hypertension. The standard diagnostic screening tool for EV is endoscopy, which is considered an invasive procedure in pediatric patients. Evaluate clinical and laboratory parameters for prediction of EV is very important to avoid unnecessary endoscopy, especially in children. Some studies have reported that platelet count and spleen size could be used to predict EV, but there is no agreement in the cut-off point.

Research frontiers

The development of mathematical models, such as clinical prediction rule (CPR) and risk score, that involves variables associated with intrahepatic portal hypertension seems to be promising. The research hotspot is to evaluate the parameters that could predict EV in children and reduce the indication of endoscopy.

Innovations and breakthroughs

Few previous studies in pediatric patients evaluated platelet count, CPR splenomegaly isolated in different population. The risk score was studied only in adults and aspartate aminotransferase to platelet ratio index was not used to predict EV in children. The risk score, that use platelet count and bilirubin as variables, should be used as a tool that can limit endoscopies indications in pediatric patients with the advantage of not using spleen size. The predictive value was similar to CPR and better than platelet count isolated.

Applications

The study suggests that both CPR and risk score could be used to screen children with portal hypertension for endoscopy.

Terminology

CPR is a clinical prediction rule, proposed by Gana *et al* that use as independent variables platelet count, spleen size z score (based on age and gender) and albumin. Risk score is a score proposed by Park *et al* to be used in patients with advanced fibrosis to determine clinically significant portal hypertension and was used to predict EV, using platelet count and bilirubin.

Peer review

This is a very interesting manuscript that further delineates clinical variables that are readily available and which can be used to increase the yield of endoscopy for identifying EV in children with portal hypertension. Though the variables studied have all been reported previously, the validation of these variables in children is an important extension of this work.

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Atrophic gastritis: Risk factor for esophageal squamous cell carcinoma in a Latin-American population

Emiliano de Carvalho Almodova, Walmar Kerche de Oliveira, Lucas Faria Abrahão Machado, Juliana Rigotto Grejo, Thiago Rabelo da Cunha, Wagner Colaiacovo, Erika Veruska Paiva Ortolan

Emiliano de Carvalho Almodova, Thiago Rabelo da Cunha, Wagner Colaiacovo, Endoscopy Department, Cancer Hospital of Barretos, Barretos, São Paulo 147830-066, Brazil

Emiliano de Carvalho Almodova, Surgery Post Graduate Course, Botucatu Medical School, State of Sao Paulo University, Botucatu, São Paulo 18600-000, Brazil

Walmar Kerche de Oliveira, Erika Veruska Paiva Ortolan, Surgery and Orthopedics Department, Botucatu Medical School, State of Sao Paulo University, Botucatu, São Paulo 18600-000, Brazil

Lucas Faria Abrahão Machado, Pathology Department, Cancer Hospital of Barretos, Barretos, São Paulo 147830-066, Brazil
Juliana Rigotto Grejo, Endoscopy Department, Botucatu Medical School, State of Sao Paulo University, Botucatu, São Paulo 18600-000, Brazil

Author contributions: Almodova EC, Oliveira WK and Ortolan EVP designed the research; Almodova EC, Grejo JR and Cunha TR performed the research; Almodova EC, Machado LFA, Colaiacovo W and Ortolan EVP analyzed the data; Almodova EC and Ortolan EVP wrote the paper; Colaiacovo W and Ortolan EVP revised the final version.

Correspondence to: Erika Veruska Paiva Ortolan, PhD, MD, Assistant Professor, Surgery and Orthopedics Department, Botucatu Medical School, State of Sao Paulo University, Botucatu, São Paulo 18600-000, Brazil. epaiva@fmb.unesp.br

Telephone: +55-14-38116269 Fax: +55-14-38157428

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Abstract

AIM: To study the association between atrophic gastritis (AG) and esophageal squamous cell carcinoma (ESCC) in a Latin-America population.

METHODS: A case-control study was performed at two reference Brazilian hospitals including patients diagnosed with advanced ESCC and dyspeptic patients who had been subjected to upper gastrointestinal endoscopy, with biopsies of the gastric antrum and body.

All cases with ESCC were reviewed by a single pathologist, who applied standard criteria for the diagnosis of mucosal atrophy, intestinal metaplasia, and dysplasia, all classified as AG. The data on the patients' age, sex, smoking status, and alcohol consumption were collected from clinical records, and any missing information was completed by telephone interview. The association between AG and ESCC was assessed by means of univariate and multiple conditional logistic regressions.

RESULTS: Most patients were male, and the median age was 59 years (range: 37-79 years) in both the ESCC and control groups. Univariate analysis showed that an intake of ethanol greater than 32 g/d was an independent risk factor that increased the odds of ESCC 7.57 times ($P = 0.014$); upon multiple analysis, alcohol intake of ethanol greater than 32 g/d exhibited a risk of 4.54 ($P = 0.081$), as adjusted for AG and smoking. Smoking was shown to be an independent risk factor that increased the odds of ESCC 14.55 times ($P = 0.011$) for individuals who smoked 0 to 51 packs/year and 21.40 times ($P = 0.006$) for those who smoked more than 51 packs/year. Upon multiple analyses, those who smoked up to 51 packs/year exhibited a risk of 7.85 ($P = 0.058$), and those who smoked more than 51 packs/year had a risk 11.57 times higher ($P = 0.04$), as adjusted for AG and alcohol consumption. AG proved to be a risk factor that increased the odds of ESCC 5.33 times (95%CI: 1.55-18.30, $P = 0.008$) according to the results of univariate conditional logistic regression.

CONCLUSION: There was an association by univariate conditional logistic regression between AG and ECSS in this sample of Latin-American population.

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Key words: Atrophic gastritis; Esophagus; Squamous cell carcinoma; Risk factor; Alcohol; Tobacco

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INTRODUCTION

Esophageal cancer (EC) is the eighth most common cancer worldwide with 481 000 new cases (3.8% of all cancers) estimated in 2008, and is the sixth most common cause of death from cancer with 406 000 deaths (5.4%)^[1]. The two main histological types of EC are adenocarcinoma and esophageal squamous cell carcinoma (ESCC), which differ regarding their risk factors and demographic distributions. Although adenocarcinoma has been the most frequent subtype among white males in the United States since the beginning of the 90s^[2], ESCC remains as the most frequent subtype worldwide, corresponding to more than 80% of all cases^[3]. The highest mortality rates of ESCC are found in East Asia, Southern and Eastern Africa^[1]. In Brazil, which is the largest Latin-American country, ESCC represents 96% of all cases^[4]. The main risk factors for ESCC in the West are alcohol consumption and smoking^[5,6]. An increased risk of ESCC was initially reported among patients with pernicious anemia^[7], and more recently, patients with atrophic gastritis (AG) were also found to be more susceptible^[8]. This hypothesis was strengthened by a Swedish study that assessed the association among *Helicobacter pylori* (*H. pylori*) infection, gastric mucosal atrophy, and ESCC. These researchers discovered that an infection by cytotoxin-associated gene A (CagA)-positive *H. pylori* was associated with a higher risk of ESCC, particularly among patients with gastric atrophy. When the correlation between gastric atrophy and ESCC was assessed independently from the *H. pylori* serotype, gastric atrophy exhibited a strong association with increased risk for ESCC. The authors suggest that gastric mucosal atrophy represents an intermediate step in the pathway from a CagA-positive *H. pylori* infection to ESCC^[9]. More recently, a meta-analysis^[10] selected and analyzed seven studies that investigated this association and concluded that AG increases the risk of ESCC^[3,9,11-15]. However, this meta-analysis did not address any studies that considered populations outside Northern Europe or Asia; therefore, the association has not been studied in Latin-American populations. Considering that the South American and Caribbean populations include 572 million people and represent 8.6% of the world population^[16], the present study aimed to investigate the correlation between gastric mucosal atrophy and ESCC in a sample from that unexamined population.

MATERIALS AND METHODS

All of the patients with ESCC who were treated at the

Cancer Hospital of Barretos between April 2011 and April 2012 were retrospectively analyzed. Exclusion criteria included the following: a diagnosis of obstructive ESCC, *i.e.*, where endoscopic access to the stomach was hindered; previous gastrointestinal (GI) tract surgery and age greater than 80 years. For each case, a gender- and age-matched control was randomly selected among patients who had been subjected to upper GI endoscopy due to dyspeptic complaints at the General Hospital of the Botucatu Medical School. The controls were chosen from patients with dyspeptic complaints, because in Brazil there is no protocol to screen asymptomatic individuals by means of upper GI endoscopy. Because the two centers that participated in the present study are referral centers for patients nationwide, demographic characteristics were not taken into account for matching.

Following routine protocols at both hospitals, all of the patients were subjected to endoscopic biopsies of the gastric antrum and body. The *H. pylori* infection was diagnosed by urease test (pink color after 30 min) and Warthin-Starry stain (Artisan-Dako, Denmark), was used for the visualization of *H. pylori* (with this method *H. pylori* is stained black while the background is stained golden yellow). The criteria for the diagnosis of ESCC were based on the endoscopy aspects and the final pathology report. The histopathologic features included true invasion of lamina propria, at least, by tumoral isolated cells or tumor clusters from the squamous esophagic epithelium. The cells were generally polygonal with pink cytoplasm and distinct cell borders and the nuclei were enlarged, hyperchromatic and pleomorphic. At high power intercellular bridges and keratinization within the cells were commonly seen, although they were absent in poorly differentiated tumors. The adjacent surface epithelium sometimes exhibited the same neoplastic cells, containing therefore, an intraepithelial (“*in situ*”) component. All cases with ESCC were reviewed by a single pathologist, who applied standard criteria for the diagnosis of mucosal atrophy, intestinal metaplasia, and dysplasia. All three conditions were classified as AG in the present study.

The data on the patients’ age, sex, smoking status, and alcohol consumption were collected from clinical records, and any missing information was completed by telephone interview. Alcohol consumption was calculated as grams of ethanol per day, and tobacco consumption was calculated as number of packs per year.

Statistical analysis

For statistical analysis, categorical values are given in frequencies and percentages. Means, medians, and standard deviations were calculated for numerical variables. To assess the risk factors of ESCC, univariate and multiple conditional logistic regressions were used. In addition, to assess the correlation between the use of alcohol or smoking and AG, non-conditional logistic regression was performed. The level of significance was established as 5%. Analyses were performed using software SPSS version 19 and Stata/SE. The research ethics committees of both participating centers approved the present study.

Table 1 Clinical, lifestyle, and diagnostic characteristics between cases and controls *n* (%)

| Characteristic | Category | Case | Control |
|----------------------------|-----------------|-----------|-----------|
| Gender | Female | 7 (14.3) | 7 (14.3) |
| | Male | 42 (85.7) | 42 (85.7) |
| Age group (yr) | 36-45 | 3 (6.1) | 3 (6.1) |
| | 46-55 | 14 (28.6) | 14 (28.6) |
| | 56-65 | 21 (42.9) | 21 (42.9) |
| | 66-75 | 8 (16.3) | 8 (16.3) |
| | 76-85 | 3 (6.1) | 3 (6.1) |
| Alcohol | Never drinker | 10 (20.4) | 16 (32.7) |
| | Current drinker | 39 (79.6) | 16 (32.7) |
| | Former drinker | 0 (0.0) | 17 (34.7) |
| Tobacco | No | 8 (16.3) | 24 (49.0) |
| | Yes | 41 (83.7) | 25 (51.0) |
| Hot drinks | No | 49 (100) | 49 (100) |
| Tylosis | No | 49 (100) | 49 (100) |
| Achalasia | No | 48 (98.0) | 49 (100) |
| | Yes | 1 (2.0) | 0 (0.0) |
| Caustic esophagitis | No | 49 (100) | 49 (100) |
| Plummer-Vinson | No | 49 (100) | 49 (100) |
| Head and neck SCC | No | 49 (100) | 49 (100) |
| Endoscopic gastric atrophy | No | 10 (20.4) | 42 (85.7) |
| | Yes | 39 (79.6) | 7 (14.3) |
| Gastric atrophy | No | 37 (75.5) | 45 (91.8) |
| | Yes | 12 (24.5) | 4 (8.2) |
| Intestinal metaplasia | No | 34 (69.4) | 41 (83.7) |
| | Yes | 15 (30.6) | 8 (16.3) |
| <i>Helicobacter pylori</i> | No | 25 (51.0) | 22 (44.9) |
| | Yes | 24 (49.0) | 27 (55.1) |
| Low-grade dysplasia | No | 47 (95.9) | 49 (100) |
| | Yes | 2 (4.1) | 0 (0.0) |
| High-grade dysplasia | No | 48 (98.0) | 49 (100) |
| | Yes | 1 (2.0) | 0 (0.0) |
| Total | | 49 (100) | 49 (100) |

SCC: Squamous cell carcinoma.

RESULTS

The comparison between both investigated groups is described in Table 1. Most patients were male, and the median age was 59 years (range: 37-79 years) in both the ESCC and control groups.

Only one individual in the ESCC group exhibited achalasia, and none of the patients exhibited any of the following classic risk factors: consumption of warm beverages, caustic esophagitis, squamous cell carcinoma of the head and neck, Plummer-Vinson syndrome or tylosis.

Table 2 describes the association between risk factors and ESCC. Univariate analysis showed AG to be an independent risk factor, which increased the odds of ESCC 5.332 times ($P = 0.008$). On multiple analysis, AG exhibited risk of 3.76 ($P = 0.063$), as adjusted for alcohol and smoking.

Univariate analysis showed that an intake of ethanol greater than 32 g/d was an independent risk factor that increased the odds of ESCC 7.57 times ($P = 0.014$); upon multiple analyses, alcohol intake of ethanol greater than 32 g/d exhibited a risk of 4.54 ($P = 0.081$), as adjusted for AG and smoking.

Smoking was shown to be an independent risk factor that increased the odds of ESCC 14.55 times ($P = 0.011$)

Table 2 Univariate and multiple logistic regression for case-control study according to gastric atrophy, alcohol intake, and tobacco consumption

| Variables | Univariate | | Multiple | |
|-----------------|------------------|----------------|-------------|----------------|
| | OR unadjusted | <i>P</i> value | OR adjusted | <i>P</i> value |
| Gastric atrophy | 5.332 | 0.008 | 3.76 | 0.063 |
| Alcohol | Non-drinker | 1 | 1 | |
| | 0-32 g ethanol/d | 1.29 | 0.736 | 0.98 |
| | > 32 g ethanol/d | 7.57 | 0.014 | 4.54 |
| Tobacco | No smoker | 1 | 1 | |
| | 51 pack/yr | 14.55 | 0.011 | 7.85 |
| | > 51 pack/yr | 21.40 | 0.006 | 11.57 |

OR: Odds ratio.

for individuals who smoked 0 to 51 packs/year and 21.40 times ($P = 0.006$) for those who smoked more than 51 packs/year. Upon multiple analyses, those who smoked up to 51 packs/year exhibited a risk of 7.85 ($P = 0.058$), and those who smoked more than 51 packs/year had a risk 11.57 times higher ($P = 0.04$), as adjusted for AG and alcohol consumption.

Non-conditional logistic regression assuming alcohol consumption and smoking as risk factors for AG did not find evidence for an association.

In the present study, *H. pylori* infection did not exhibit association with ESCC [odds ratio (OR) = 0.81, $P = 0.578$] and behaved as a protective factor against AG (OR = 0.3, $P = 0.009$).

DISCUSSION

Recent interest in the correlation between AG and ESCC led to a meta-analysis of seven studies from Asia and northern Europe that demonstrated an association between the conditions^[10].

The present case-control study also discovered significant statistical associations from a non-adjusted analysis between the following: AG and ESCC; heavy use of alcohol (more than 32 g of ethanol/d) and ESCC; and smoking and ESCC. Nevertheless, the use of adjusted models to investigate AG, alcohol, and smoking found a statistically significant association between ESCC and heavy smoking (more than 51 packs/d) alone. From the seven studies included in the aforementioned meta-analysis, three did not adjust their analysis for other risk factors besides AG^[12,13,15]. The fact that the associations discovered by the present study often lost statistical significance after adjustment could be explained by the small sample sizes ($n = 49$ cases, $n = 49$ controls). The loss of significance attributable to heavy alcohol consumption, in particular, corroborates this interpretation; alcohol consumption is a classic risk factor for ESCC in Western countries. de Vries *et al.*^[15] suggest that the association between AG and ESCC might be explained by confounding factors, such as smoking, after demonstrating an association between AG and small cell lung car-

cinoma. However, in the present study, univariate non-conditional logistic regression did not indicate an association between alcohol consumption or smoking with AG. It is worth noting that four other studies performed logistic regression adjusted for risk factors, and each study found a statistically significant association between AG and ESCC^[3,9,11,14].

The present study did not find an association between ESCC and *H. pylori*, which, in fact, proved to be protective against AG. Although seemingly paradoxical, this finding may be explained by the fact that *H. pylori* does not colonize atrophic mucosa nor areas with intestinal metaplasia.

This study, as well as other similar studies, encountered limitations regarding the selection of healthy controls. Although some Japanese studies, such as the one by Akiyama *et al.*^[3], have been able to use healthy controls undergoing screening upper GI endoscopy, these studies also report difficulty in selecting appropriate controls. As such screening programs do not exist in Brazil, the controls used in the present study were individuals subjected to endoscopy due to dyspeptic complaints; consequently, higher odds of exhibiting pathological findings in the GI tract may exist.

Although related literature shows an association between AG and ESCC, whether that relationship is causal is still unknown. A possible mechanism of this relationship is that achlorhydria in patients with gastric atrophy may generate an intragastric environment that favors bacterial overgrowth and n-nitrosation, thus resulting in increased exposure of the esophageal mucosa to carcinogenic endogenous nitrosamines^[17].

Other explanations for the positive association between gastric atrophy and ESCC are worth exploring. It is possible that both conditions share genetically determined pathogenetic mechanisms that facilitate a similar destructive process (*i.e.*, inflammatory response or defective DNA repair), damaging both gastric and esophageal epithelia^[15,18-20].

It is also possible that the observed association between ESCC and AG is due to the fact that patients with advanced ESCC can only ingest small amounts of food, owing to esophageal stenosis and lack of appetite, and this reduced ingestion may lead to disuse atrophy. That hypothesis, however, is contested in the study by Kamanagar *et al.*^[13], which found that a lower serum PGI/II ratio was linearly associated with higher risk of esophageal squamous dysplasia, a preneoplastic condition of ESCC. It is worth noting that patients with esophageal dysplasia do not exhibit dysphagia.

Despite the abovementioned limitations, the present study, as far as we know, is the first to identify a statistically significant association by univariate conditional logistic regression between AG and ESCC in the Latin-American population. In conclusion, in the present study the AG was an independent risk factor in this sample from Latin-America population, as has been demonstrated in studies of population samples from Asia and northern Europe. This fact highlights the importance of conducting pro-

spective, multicenter studies enrolling larger populations that represent different ethnic groups, to investigate the causal relationship between AG and ESCC.

COMMENTS

Background

Esophageal cancer (EC) is the eighth most common cancer worldwide with 481 000 new cases (3.8% of all cancers) estimated in 2008, and is the sixth most common cause of death from cancer with 406 000 deaths (5.4%). The highest mortality rates of esophageal squamous cell carcinoma (ESCC) are found in East Asia, Southern and Eastern Africa. In Brazil, which is the largest Latin-American country, ESCC represents 96% of all EC. The main risk factors for ESCC in the Western countries are alcohol consumption and smoking. More recently, a meta-analysis concluded that AG increases the risk of ESCC. However, this meta-analysis did not address any studies that considered populations outside of Northern Europe or Asia; therefore, the association has not been studied in Latin-American populations. Considering that the South American and Caribbean populations include 572 million people and represent 8.6% of the world population, the present study aimed to investigate the correlation between gastric mucosal atrophy and ESCC in a sample from that unexamined population.

Research frontiers

The better knowledge of risk factors and pathophysiological mechanisms of EC can lead to better preventive and therapeutic options in a cancer type that presents with low survival rates.

Innovations and breakthroughs

An increased risk of ESCC was initially reported among patients with pernicious anemia and more recently, patients with atrophic gastritis (AG) were also found to be more susceptible. This hypothesis was strengthened by a Swedish study that assessed the association among *Helicobacter pylori* (*H. pylori*) infection, gastric mucosal atrophy and ESCC. These researchers discovered that an infection by cytotoxin-associated gene A (CagA)-positive *H. pylori* was associated with a higher risk of ESCC, particularly among patients with gastric atrophy. When the correlation between gastric atrophy and ESCC was assessed independently from the *H. pylori* serotype, gastric atrophy exhibited a strong association with increased risk for ESCC. The authors suggest that gastric mucosal atrophy represents an intermediate step in the pathway from a CagA-positive *H. pylori* infection to ESCC.

Applications

The study results suggest an association between AG and ESCC.

Terminology

All three conditions (mucosal atrophy, intestinal metaplasia, and dysplasia) were classified as AG in the present study.

Peer review

This is a small but well conducted study that shows that also in South America AG seems to predispose to ESCC thus showing that the association can be found despite the obvious environmental differences between Asia, Europe and North America. The study thus further generalizes the hypothesis and should encourage research into the mechanism behind this.

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Increased endothelin receptor B and G protein coupled kinase-2 in the mesentery of portal hypertensive rats

Qing-Hong Du, Lin Han, Jun-Jie Jiang, Peng-Tao Li, Xin-Yue Wang, Xu Jia

Qing-Hong Du, Lin Han, Jun-Jie Jiang, Peng-Tao Li, Xin-Yue Wang, Xu Jia, Department of Pathology, School of Fundamental Medicine, Beijing University of Chinese Medicine, Beijing 100029, China

Author contributions: Li PT proposed the study; Li PT and Du QH performed research and wrote the first draft; Han L collected and analyzed the data; all authors contributed to the design and interpretation of the study and to further drafts; Li PT is the guarantor.

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Correspondence to: Dr. Peng-Tao Li, Department of Pathology, School of Fundamental Medicine, Beijing University of Chinese Medicine, 11 Bei San Huan Dong Lu, Beijing 100029, China. lipengtao0413@hotmail.com

Telephone: +86-10-64287015 Fax: +86-10-64287015

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Abstract

AIM: To elucidate the mechanisms of mesenteric vasodilation in portal hypertension (PHT), with a focus on endothelin signaling.

METHODS: PHT was induced in rats by common bile duct ligation (CBDL). Portal pressure (PP) was measured directly via catheters placed in the portal vein tract. The level of endothelin-1 (ET-1) in the mesenteric circulation was determined by radioimmunoassay, and the expression of the endothelin A receptor (ETAR) and endothelin B receptor (ETBR) was assessed by immunofluorescence and Western blot. Additionally, expression of G protein coupled kinase-2 (GRK2) and β -arrestin 2, which influence endothelin receptor sensitivity, were also studied by Western blot.

RESULTS: PP of CBDL rats increased significantly (11.89 ± 1.38 mmHg vs 16.34 ± 1.63 mmHg). ET-1 expression decreased in the mesenteric circulation 2 and

4 wk after CBDL. ET-1 levels in the systemic circulation of CBDL rats were increased at 2 wk and decreased at 4 wk. There was no change in ETAR expression in response to CBDL; however, increased expression of ETBR in the endothelial cells of mesenteric arterioles and capillaries was observed. In sham-operated rats, ETBR was mainly expressed in the CD31⁺ endothelial cells of the arterioles. With development of PHT, in addition to the endothelial cells, ETBR expression was noticeably detectable in the SMA⁺ smooth muscle cells of arterioles and in the CD31⁺ capillaries. Following CBDL, increased expression of GRK2 was also found in mesenteric tissue, though there was no change in the level of β -arrestin 2.

CONCLUSION: Decreased levels of ET-1 and increased ETBR expression in the mesenteric circulation following CBDL in rats may underlie mesenteric vasodilation in individuals with PHT. Mechanistically, increased GRK2 expression may lead to desensitization of ETAR, as well as other vasoconstrictors, promoting this vasodilatory effect.

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Key words: Portal hypertension; Mesentery; Endothelin; Endothelin B receptor; G protein coupled kinase-2

Core tip: Portal hypertension (PHT) is a life-threatening condition which frequently develops in patients with liver cirrhosis, and has limited treatment options. For many years, endothelin-1 (ET-1) has received considerable interest in the area of liver cirrhosis for its potential contribution to PHT. The aim of the present study was to directly examine the expression of ET-1 and its receptors in the mesentery of rats with PHT, and to clarify how the ET-1 signaling system changed with the development of PHT.

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dothelin receptor B and G protein coupled kinase-2 in the mesentery of portal hypertensive rats. *World J Gastroenterol* 2013; 19(13): 2065-2072 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v19/i13/2065.htm> DOI: <http://dx.doi.org/10.3748/wjg.v19.i13.2065>

INTRODUCTION

Portal hypertension (PHT) is one of the most significant complications associated with liver cirrhosis, which can give rise to many other severe and often lethal conditions, such as bleeding esophageal varices. Increased resistance to portal blood flow is the primary factor leading to PHT and is aggravated by a hyperdynamic, vasodilated, splanchnic circulation^[1]. Though the pathophysiology of PHT is becoming better understood, excepting β -receptor blockers, there is no effective treatment approach for PHT. One reason to explain this shortfall is that the mechanism of splanchnic vasodilation is unclear. The organs involved in splanchnic hyperdynamic circulation are those whose blood flows into the portal vein, including the intestine, mesentery, colon, spleen and stomach. Previous studies have indicated that vasodilation of the mesenteric vascular bed plays the greatest role in PHT, by increasing portal inflow^[2]. In previous work from our laboratory, we also observed vasodilation of the mesenteric vascular bed; thus in the present study we focused on this tissue to try to explain splanchnic vascular dilation and possibly to identify new therapeutic targets for treating PHT.

Splanchnic vasodilation is associated with the imbalance of vasoactive mediators^[3,4] and hyporeactivity to vasoconstrictors^[5]. Endothelin-1 (ET-1) is a potent endothelium derived vasoactive peptide. For many years, ET-1 has received considerable interest in the area of liver cirrhosis for its potential contribution to PHT^[6-9]. This has led to many studies being focused upon the effect of ET-1 and its receptors in the liver; however, there are only a few studies examining the possible mechanism of the endothelin signaling system in hyperdynamic circulation. It has been established that the divergent effect of ET-1 on blood vessels depends on the different expression of endothelin receptors on smooth muscle and endothelial cells^[10]. There are two known types of ET-1 receptor: the endothelin A receptor (ETAR) and the endothelin B receptor (ETBR)^[11]. ETBR has two recognized subtypes: ETB1 and ETB2^[10-13]. ETAR and ETB2 are predominantly expressed in vascular smooth muscle cells, whereas ETB1 is characteristic of endothelial cells. ET-1 binds to ETAR and ETB2 to induce vasoconstriction, while ET-1 binds to ETB1 to cause vascular relaxation^[14]. Both mixed ETAR-ETBR antagonists and selective ETBR antagonists have been proven to decrease portal pressure (PP) and increase mean arterial pressure (MAP), while ETAR antagonists have been shown to have no effect on MAP^[14,15]. Moreover, selective ETBR inhibition *in vivo* significantly ameliorated hepatopul-

monary syndrome (HPS)^[16-18], which is also known to be caused by dilation of the microcirculation, similar to PHT^[18]. Based on this literature, we speculate that ETBR may play a primary role in the hyperdynamic circulation associated with PHT.

In addition to the localization and expression level of ET-1, its signaling is known to be affected by other regulators. For example, it has been observed that in some instances of high splanchnic ET-1 expression, the vascular bed of this tissue was still dilated, leading the authors to speculate that the sensitivity of the ET-1 receptor(s) was decreased^[19]. Endothelin receptors may be desensitized by phosphorylation through G-protein-coupled receptor kinases (GRKs) and binding of β -arrestin 2^[20]. So far, seven kinds of GRKs have been cloned^[21]. GRK2 is the most likely of the GRKs to initiate human human endothelin A and B receptor desensitization^[22]. Endothelin signalling in arterial smooth muscle is tightly regulated by GRK2^[23]. As such, in this study we also focused on two known regulators, GRK2 and β -arrestin 2. The current literature has indicated a possible role for ET-1 signaling in splanchnic vasodilation, though there is a lack of experimental data to support this hypothesis. The aim of the present study was to directly examine the expression of ET-1 and its receptors in the mesentery of rats with PHT, and to clarify how the ET-1 signaling system changed with the development of PHT.

MATERIALS AND METHODS

Animal models

Male Sprague-Dawley rats (approximately 250 g; Vital River Laboratory Animal Technology Co. Ltd., Beijing, China) underwent sham surgery or common bile duct ligation (CBDL). In brief, the common bile ducts of rats were exposed after median laparotomy and ligated twice. In each animal, the segment between the 2 ligations was resected, and the animal's abdomen was sutured closed. Sham-operated rats served as controls. In these rats, the common bile duct was exposed, but no ligation or resection was performed. Seven animals were used in each group. The study was approved by the local committee for animal studies.

Measurement of portal blood pressure and ET-1

In brief, after 2 wk or 4 wk following CBDL, a PE-50 catheter was inserted into the portal vein to measure portal blood pressure. After stable recordings of portal venous pressure were obtained, blood was drawn from the superior mesenteric artery (SMA) for further analysis. The level of ET-1 was measured in the mesenteric circulation using a commercial RIA kit (PLA Institute of RIA, Beijing, China) according to the manufacturer's protocol.

Immunofluorescence

For immunofluorescence double staining of ETAR and ETBR, mesentery tissues were harvested at 2 and 4 wk

and fixed in 4% neutral paraffin. After antigen retrieval, all sections were incubated with PBS containing 1% bovine serum albumin (block buffer) for 60 min in a wet chamber at room temperature. Then, for ETAR, sections were incubated with primary anti-ETAR (polyclonal antibody; Santa Cruz Biotechnology, Santa Cruz, CA; 1:100 dilution) and anti-smooth muscle actin SMA (Santa Cruz Biotechnology, Santa Cruz, CA, United States; 1:200 dilution); for ETBR, slides were incubated with primary anti-ETBR (polyclonal antibody; Santa Cruz Biotechnology, Santa Cruz, CA, United States; 1:300 dilution) and anti-CD31 (polyclonal antibody; Santa Cruz Biotechnology, Santa Cruz, CA, United States; 1:100 dilution) antibodies at 4 °C overnight. Then all sections were washed with PBS. For the ETAR, the slides were incubated with goat anti-rabbit/mouse secondary antibodies at the same time. For the ETBR, the sections were incubated with rabbit anti-sheep and goat anti-rabbit antibodies at the same time (Invitrogen, San Diego, CA, United States) for 1 h at room temperature. Subsequently, sections were washed with phosphate-buffered saline (PBS). Control sections were incubated with secondary antibody in the absence of primary antibody. The results were analyzed using confocal laser scanning microscope.

Western blot analysis

For Western blot analysis, samples of rat mesentery were homogenized in radio immunoprecipitation assay (RIPA) lysis buffer containing 50 mmol/L Tris (pH 7.4), 150 mmol/L NaCl, 1% Triton X-100, 1% sodium deoxycholate, 0.1% sodium dodecyl sulfate (SDS), 5 mmol/L ethylenediamine tetraacetic acid, 1 mmol/L sodium orthovanadate, 20 mmol/L pepstatin A, 20 mmol/L leupeptin and 1 mmol/L phenylmethanesulfonyl fluoride. The protein content of the cleared homogenates was assessed with bicinchoninic acid assay kit (Applygen, Beijing, China). After boiling with SDS sample buffer (Applygen, Beijing, China), 50 µg of protein per lane of each sample was subjected to SDS-polyacrylamide gel electrophoresis (10% gels for GRK2, 12.5% gels for ETAR, ETBR and β-arrestin 2). After blotting on polyvinylidene difluoride membrane (Millipore, Bedford, MA, United States), the membranes were probed with primary antibodies diluted in TBS containing blocking protein and 0.1% Tween, and left to incubate overnight at 4 °C. The following primary antibodies in the indicated dilutions were used: mouse anti-GRK2, 1:500 (Abcam); rabbit anti-ETAR/ETBR and mouse β-arrestin 2, 1:200 (Santa Cruz Biotechnology, Santa Cruz, CA, United States). Thereafter, the membranes were washed and incubated with appropriate peroxidase-coupled secondary antibodies diluted 1:5000 in TBS containing blocking protein and 0.1% Tween for 45 min (goat anti-rabbit or goat anti-mouse; Jackson, West Grove, PA, United States). Detection was performed with enhanced chemiluminescence (Applygen, Beijing, China), and films were developed using Kodak film. Densitometric quantification was performed using Phoretix 1D gel image analysis software for free.

Statistical analysis

All data are presented as the mean ± SD; statistical comparisons were performed using one way analysis of variance. Physiological and biochemical findings represent averages of seven rats. Results of molecular assays represent averages of samples from at least five rats in each group. *P* values < 0.05 were considered statistically significant.

RESULTS

Increased PP following CBDL

PP was measured in sham and experimental rats 2 and 4 wk after CBDL; PP of CBDL rats increased significantly (16.34 ± 1.63 mmHg *vs* 11.89 ± 1.38 mmHg for sham-operated animals). The increase in PP in CBDL rats was statistically significant compared with sham-operated rats at the 2 and 4 wk timepoint (*P* < 0.005).

Concentration of ET-1 in the mesenteric and systemic circulation

The concentration of ET-1 was measured in the mesenteric and systemic circulation 2 and 4 wk after CBDL. At 2 and 4 wk, ET-1 levels in the mesenteric circulation of CBDL rats were 92.09 ± 13.26 pg/mL and 100.35 ± 16.36 pg/mL, which were significantly lower than that in sham-operated rats. Furthermore, in the systemic circulation, compared with sham-operated rats, ET-1 levels of CBDL rats were also significantly increased at 2 wk (142.77 ± 27.67 pg/mL); however, systemic ET-1 levels were decreased at 4 wk (88.62 ± 15.40 pg/mL).

ETAR and ETBR immunofluorescence

The expression pattern of ETAR and ETBR in mesenteric tissues was determined by immunofluorescence (Figures 1 and 2). From these pictures, the expression of ETAR was observed on SMA⁺ smooth muscle cells (Figure 1). In sham-operated rats, ETBR was mainly expressed in CD31⁺ endothelial cells of the vasculature, though the microcirculation also had weak immunostaining (Figure 2A). Our data indicates that with PHT development, in addition to endothelial cells, ETBR expression was noticeably detectable in the CD31⁺ capillaries (Figure 2B and C). We also noted increased vasodilation in the mesentery and formation of hyperdynamic circulation in CBDL rats, which was associated with increased angiogenesis (Figure 2B and C).

Quantification of ETAR, ETBR, GRK2 and β-arrestin 2 expression by Western blot

To confirm our immunofluorescence results, we investigated ETAR and ETBR expression by Western blot; we also used this method to assess GRK2 and β-arrestin 2 expression, as it relates to the sensitivity of the ET-1 receptors during the development of a hyperdynamic circulation (Figure 3). In agreement with our immunostaining, we found no statistically significant difference in ETAR expression between sham-operated and CBDL

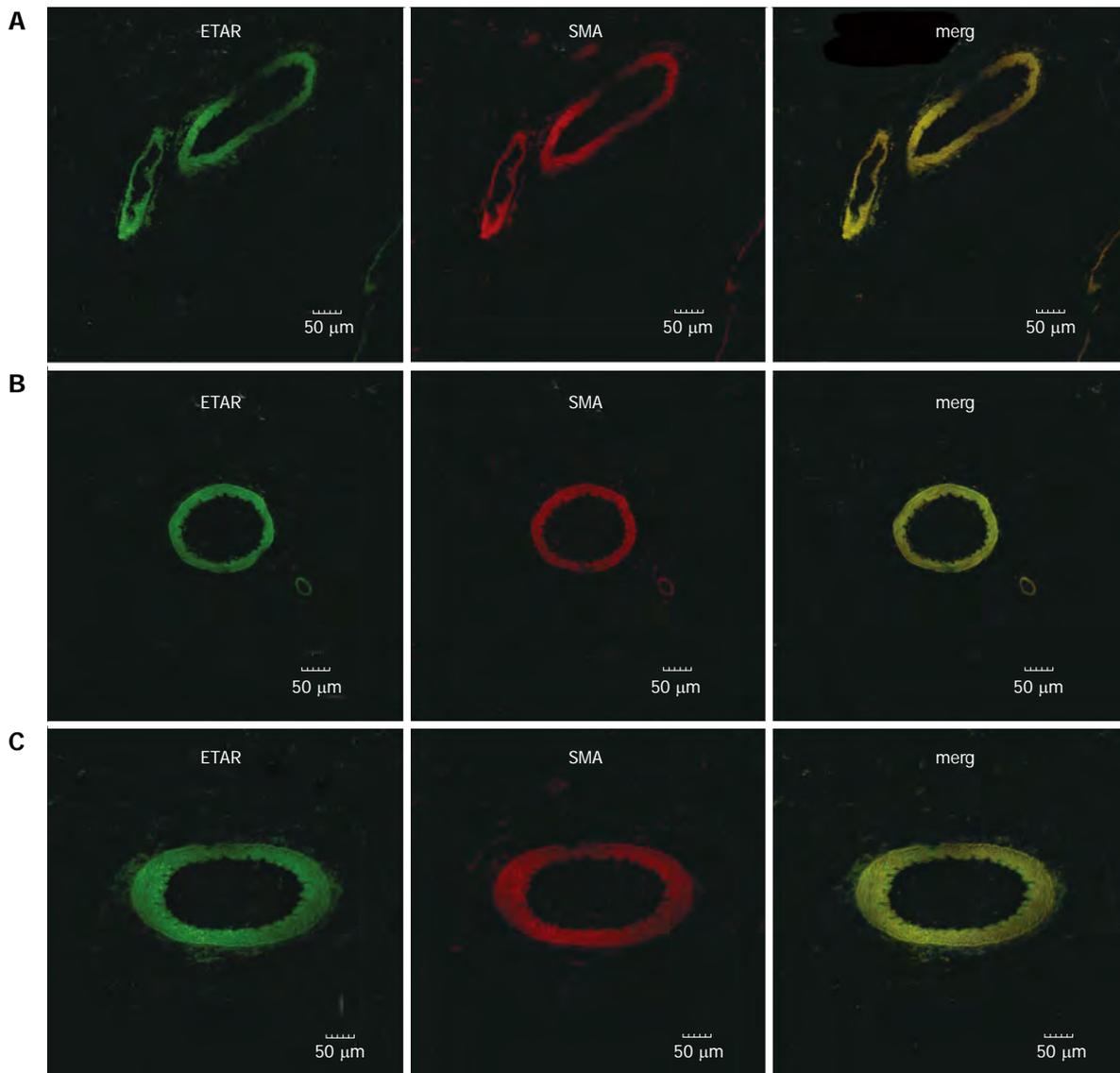


Figure 1 Expression of endothelin A receptor in rat mesentery. Expression of endothelin A receptor (ETAR, green) on smooth muscle cells (red) in mesentery of sham-operated rats (A), and common bile duct ligation rats at 2 wk (B) and 4 wk (C). ETAR was expressed extensively in smooth muscle cells of the vascular.

rats at the 2 and 4 wk timepoints (Figure 3A). ETBR was significantly upregulated in CBDL rats after 2 wk ($P < 0.01$) and 4 wk ($P < 0.01$), as compared to sham-operated rats (Figure 3B). Similar to previous research, which has shown that GRK2 levels are increased in the aortas of CBDL rats^[24], we also observed an upregulation of GRK2 protein levels in the mesentery of CBDL rats at both timepoints, which was statistically significant ($P < 0.005$; Figure 3C). We also observed no significant change in the protein expression level of β -arrestin 2 between sham-operated and CBDL rats after 2 or 4 wk ($P > 0.05$; Figure 3D).

DISCUSSION

In accordance with Ohm's law, PP depends on intrahepatic resistance and portal inflow. In cases of cirrhosis, both intrahepatic resistance and splanchnic blood flow are increased. The initiating factor is an increase in in-

trahepatic vascular resistance, whereas the increase in splanchnic blood flow is a secondary phenomenon that maintains or worsens the increased PP and gives rise to the hyperdynamic systemic state^[25]. In terms of the relevant literature^[16,17], it has been speculated that the ET-1 signaling system may play an important role in the hyperdynamic circulation, even though there is no direct experimental evidence. Moreover, there are also articles about endothelin receptor antagonism treatment in humans with PHT^[26,27]. In this regard, this is the first *in vivo* study examining the expression of the ET-1 signaling system in the mesentery of PHT rats.

We have found that the concentration of ET-1 in the mesenteric circulation decreases as the liver becomes fibrotic. We also found that ETBR expression, but not ETAR expression, increased in vascular smooth muscle cells and in the microcirculation of mesentery tissue, which may mean decreased vasoconstriction and increased vasodilatation induced by local ET-1 in the mesen-

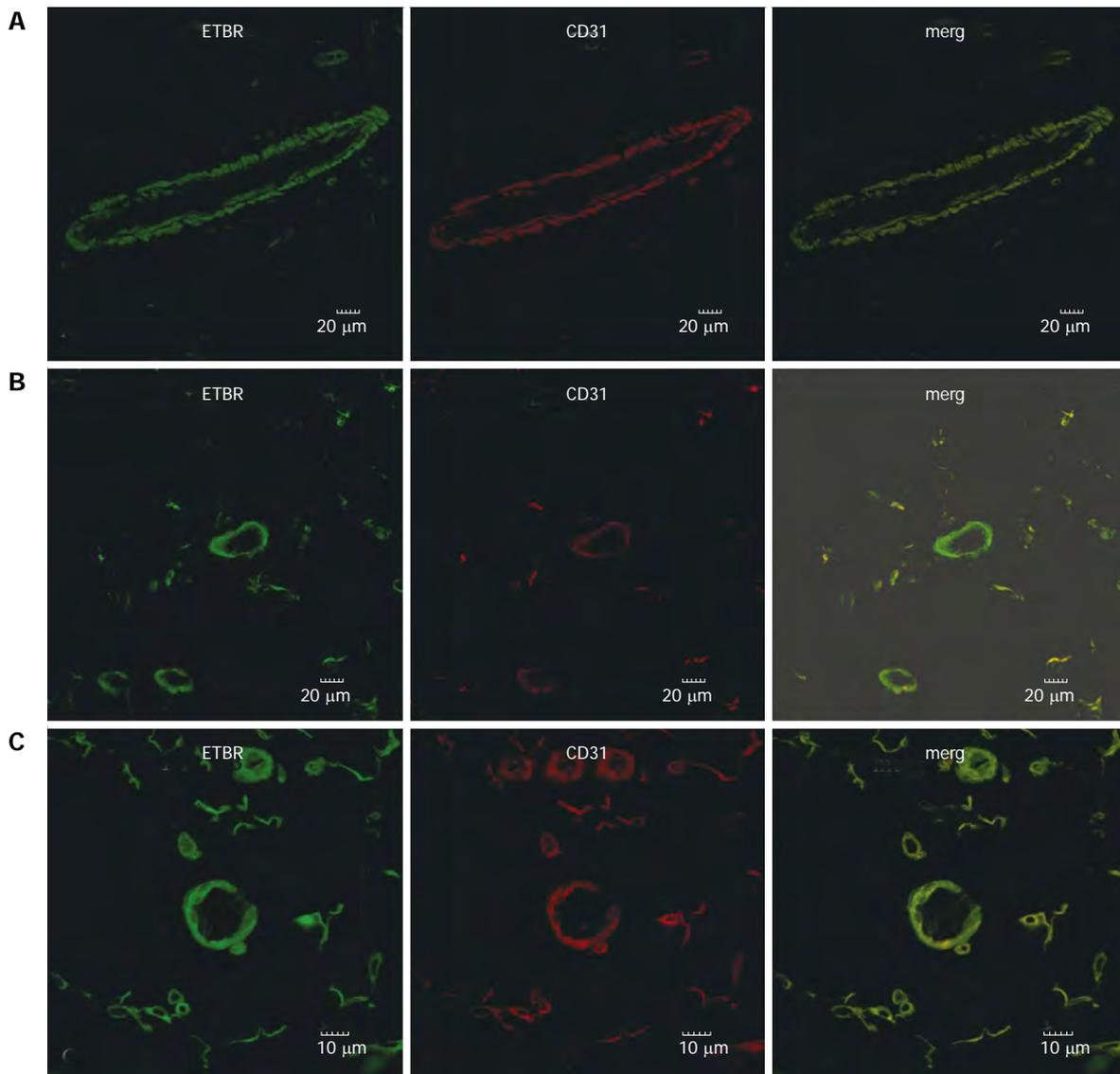


Figure 2 Expression of endothelin B receptor in rat mesentery. Expression of endothelin B receptor (ETBR) in the mesentery of sham-operated rats (A), and common/L on bile duct ligation rats at 2 wk (B) and 4 wk (C). In the mesentery of sham-operated rats, ETBR (green) was mainly expressed in CD31⁺ endothelial cells (red) of the vascular, with weak staining in the microcirculation. With portal pressure rising, ETBR expression was detected in the smooth muscle cells of arterioles and the microcirculation.

tery of PHT rats. Furthermore, the expression of GRK2 increased significantly in CBDL rats, which may imply that desensitization of ETAR and other vasoconstrictor receptors also promotes splanchnic vasodilatation.

The ET-1 signaling system is associated with vascular dysfunction at three levels, changes in: ET-1 concentration, ET-1 receptor expression and sensitivity, and the ET-1 signaling transduction pathway. Thus, the ability to modulate ET-1 signaling at either of these levels may provide ways to improve splanchnic vascular dysfunction. Previous studies have shown that ET-1 was upregulated in the liver tissue and systemic circulation of patients^[9], but the expression level of ET-1 in the splanchnic circulation is not consistent. The observed decrease in ET-1 in the splanchnic circulation 2 and 4 wk after CBDL leads us to speculate that one reason for mesenteric vascular bed dilation in this model is a local

decrease in ET-1.

ET-1 must bind to its receptor to modulate vascular tone, so variations in receptor subtype expression and quantity affect the action of ET-1 on blood vessels. Using an animal model of HPS, which is also applicable to PHT, it was found that the extensive dilation of the pulmonary microcirculation was related to a selective increase in ETBR expression^[16,17]. PHT and HPS can both be induced by CBDL in rats, and dilation of the microcirculation exists in both syndromes, raising the question whether ET-1 signaling through ETBR plays the same role in the hyperdynamic circulation as it does in HPS? Further research will be required to address this important question and establish if this hypothesis is correct. In the present study, we observed that ETAR expression in the mesentery was not different between sham-operated and CBDL rats. Interestingly however, ETBR expression

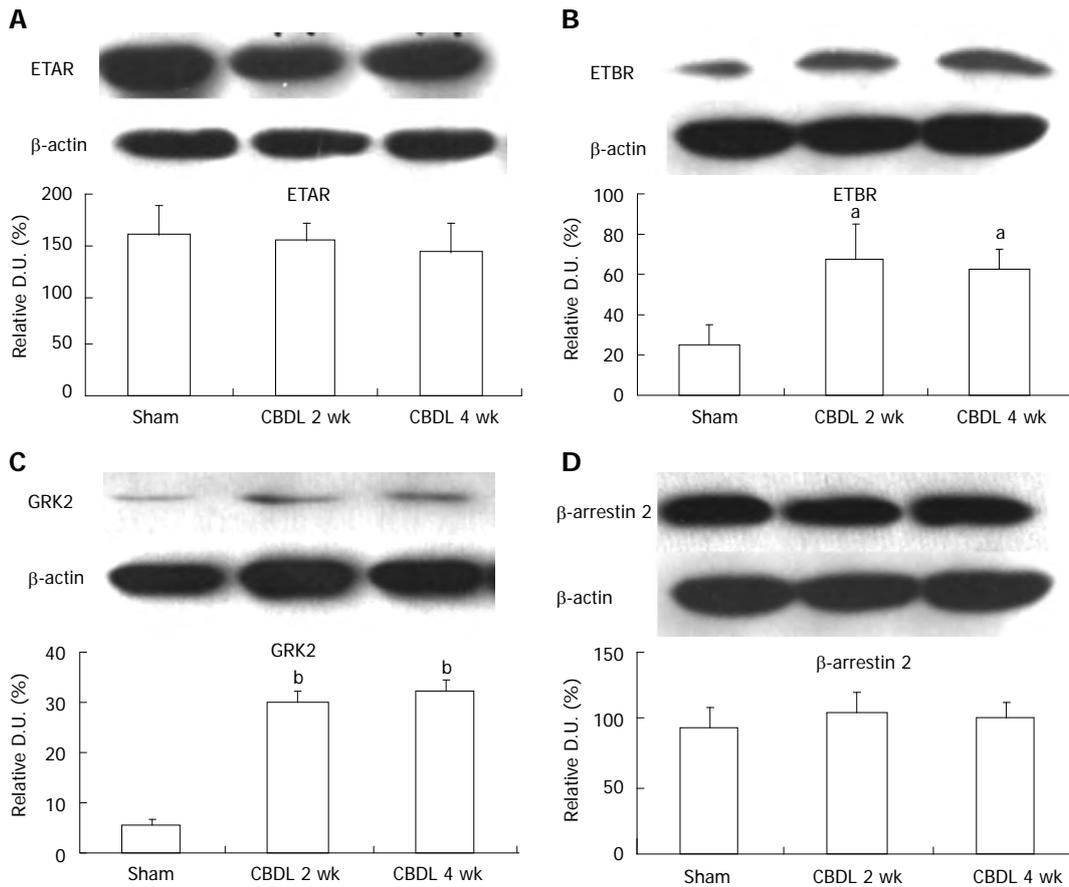


Figure 3 Western blots of endothelin A receptor (A), endothelin B receptor (B), G protein coupled kinase-2 (C) and β -arrestin 2 (D) in rat mesentery. Expression of the investigated proteins in mesentery from sham-operated rats and mesentery from common bile duct ligation (CBDL) rats was compared by Western blot. Representative Western blots are shown for each protein and densitometric quantification of all experiments are provided (data are mean \pm SE; $n = 3-6$ /group). ^a $P < 0.05$, ^b $P < 0.01$ for CBDL rats vs sham-operated rats. ETAR: Endothelin A receptor; ETBR: Endothelin B receptor; GRK2: G protein coupled kinase-2; D.U.: Densitometric units.

was obviously increased by CBDL. Our immunofluorescence data indicates that, under normal physiological conditions, ETBR is primarily expressed in the endothelial cells of the vasculature, whereas the microcirculation also has weak positive staining. With the development of liver fibrosis and liver cirrhosis, ETBR was not only expressed in endothelial cells but was also strongly observed in the microcirculation. Given that the primary function of the microcirculation is to accumulate blood, we interpreted from our data that in the mesentery of CBDL rats, signaling through ETBR mediates vasodilation *via* release of nitrogen oxide from endothelial cells, leading to an increased blood volume. It is possible that ETBR expression in the smooth muscle cells is a compensatory mechanism to facilitate contraction of blood vessels and oppose the overdilation. Analysis of ETBR by Western blot confirmed the increase in expression following CBDL. Though we observed immune-positive staining for both ETAR and ETBR in fat tissue, this would not affect our Western blot data because the fat tissue in mesentery cannot be dissolved by the RIPA lysis buffer and was discarded during homogenization.

ET-1 binds to its Gq protein-coupled receptor to send an extracellular signal into the cell, an event which is limited by the sensitivity of the receptor. Under nor-

mal conditions, the receptor is desensitized to prevent excessive stimulation by vasoactive substances. Available experimental evidence demonstrates that augmentation of receptor sensitivity, by factors like norepinephrine, vasopressin, ET-1 or angiotensin, impairs the transduction of vasoconstrictor signals and promotes dilation of the splanchnic vascular bed^[24]. It is known that GRKs and arrestins are key participants in the canonical pathways leading to phosphorylation-dependent or independent G protein-coupled receptor desensitization and endocytosis^[28]. GRK2 is one member of the GRK family and has been shown to be able to specifically phosphorylate human ETAR and ETBR^[20]. Our findings demonstrate that the increased PP following CBDL resulted in an upregulation of mesenteric GRK2 expression, but not β -arrestin 2. GRK2 and β -arrestin 2 may modulate ETAR and ETBR desensitization through the following mechanisms: (1) phosphorylation of ETAR by GRK2 promotes the receptor binding to β -arrestin 2, which blocks the activation of the G proteins and leads to rapid homologous desensitization; and (2) independent of phosphorylation, GRK2 interacts with G alpha(q) directly, which results in uncoupling of ET-1 receptor and its associated G proteins, thus impairing the ET-1 signal transduction pathway^[29]. Increased GRK2 expression in the mesentery of

PHT rats not only results in desensitization of ETAR, but also the receptors of norepinephrine and angiotensin, which indicates that such vasoactive substances are unable to mediate vasoconstriction, regardless of ligand concentration or the level of receptor expression.

In summary, PHT induced by CBDL in rats was associated with decreased levels of ET-1 in the mesenteric circulation, and increased mesenteric expression of ETBR and GRK2. We conclude that these changes underlie mesenteric vasodilation in an animal model of PHT, and are also applicable to patients with this condition. We interpret our data to indicate that the ET-1 signaling pathway is an important factor in the development of splanchnic vasodilation associated with PHT. These findings have major therapeutic implications not only for individuals with liver disease, but also for other diseases with vascular dysfunction.

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COMMENTS

Background

Portal hypertension (PHT) is a life-threatening condition which frequently develops in patients with liver cirrhosis, and has limited treated options. During PHT, vasodilation results in increased blood flow into the mesenteric circulation, thereby increasing flow into the portal circulation, which can worsen PHT. For many years, endothelin-1 (ET-1) has received considerable interest in the area of liver cirrhosis for its potential contribution to PHT. The aim of the present study was to directly examine the expression of ET-1 and its receptors in the mesentery of rats with PHT, and to clarify how the ET-1 signaling system changed with the development of PHT.

Research frontiers

PHT can give rise to many other severe and often lethal conditions, such as bleeding esophageal varices. Increased resistance to portal blood flow is the primary factor leading to PHT and is aggravated by a hyperdynamic, vasodilated, splanchnic circulation. Though the pathophysiology of PHT is becoming better understood, excepting β -receptor blockers, there is no effective treatment approach for PHT. One reason to explain this phenomenon is that the mechanism of splanchnic vasodilation is unclear. So in this study, the authors choose mesentery tissue of hypertensive rats to research the mechanisms of vasodilation based on ET-1 and its receptors.

Innovations and breakthroughs

The current literature has indicated a possible role for ET-1 signaling in splanchnic vasodilation, though there is a lack of experimental data to support this hypothesis. This is the first *in vivo* study examining the expression of the ET-1 signaling system in the mesentery of PHT rats.

Applications

These findings have major therapeutic implications not only for individuals with liver disease, but also for other diseases with vascular dysfunction.

Terminology

PHT is the main complication of cirrhosis and is defined as a hepatic venous pressure gradient (HVPG) of more than 5 mmHg. Clinically significant PHT is defined as HVPG of 10 mmHg or more; Hyperdynamic circulation: The hyperdynamic circulatory state of PHT is characterized by splanchnic and peripheral vasodilation, increased plasma volume and increased cardiac output.

Peer review

In this paper, the authors show that expression of G protein coupled kinase-2 is downregulated while expression of the endothelin B receptor is increased in the mesentery after common bile duct ligation, a model for PHT. These findings are interesting and might represent some mechanisms underlying vasodilatation in the mesentery.

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Clinicopathological and prognostic significance of galectin-1 and vascular endothelial growth factor expression in gastric cancer

Jie Chen, Su-Jun Zhou, Yun Zhang, Guo-Qiang Zhang, Tian-Zhou Zha, Yi-Zhong Feng, Kai Zhang

Jie Chen, Su-Jun Zhou, Yun Zhang, Guo-Qiang Zhang, Tian-Zhou Zha, Kai Zhang, Department of General Surgery, Yixing People's Hospital, the Affiliated Hospital of Jiangsu University, Yixing 214200, Jiangsu Province, China
Yi-Zhong Feng, Department of Pathology, the Second Affiliated Hospital of Soochow University, Suzhou 215004, Jiangsu Province, China

Author contributions: Chen J carried out data analysis and wrote the manuscript; Zhou SJ designed this study and performed the statistical analyses; Zhang Y, Zhang GQ, Feng YZ and Zha TZ participated in clinicopathological data collection; Chen J and Zhang K scored the immunostained slides, prepared the images and reviewed the manuscript.

Correspondence to: Kai Zhang, MD, Department of General Surgery, Yixing People's Hospital, the Affiliated Hospital of Jiangsu University, No. 75, Tongzhen Guan Road, Yixing 214200, Jiangsu Province, China. yxphpwk@163.com

Telephone: +86-510-87071163 Fax: +86-510-87918767

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Abstract

AIM: To evaluate the expression of galectin-1 and vascular endothelial growth factor (VEGF) in gastric cancer and investigate their relationships with clinicopathologic factors and prognostic significance.

METHODS: Galectin-1 and VEGF were immunohistochemically investigated in tumor samples obtained from 214 gastric cancer patients with all tumor stages. Immunohistochemical analyses for galectin-1 and VEGF expression were performed on formalin-fixed, paraffin-embedded sections of surgical specimens. The relationship between the expression and staining intensity of galectin-1 and VEGF, clinicopathologic variables, and patient survival were analyzed. All patients underwent follow-up until cancer-related death or more than five years after tumor resection. *P* values < 0.05 were considered statistically significant.

RESULTS: Immunohistochemical staining demonstrated that 138 of 214 gastric cancer samples (64.5%) were positive for galectin-1, and 116 out of 214 gastric cancer samples (54.2%) were positive for VEGF. There was a significant association between galectin-1 and VEGF expression; VEGF was detected in 60.1% of galectin-1-positive samples and 43.4% of galectin-1-negative samples (*P* < 0.05). Galectin-1 expression was associated with tumor size, tumor location, stage, lymph node metastases, and VEGF expression (all *P* < 0.05). VEGF expression was related to tumor size, stage, and lymph node metastases (all *P* < 0.05). The 5-year survival rate was 56.6% for galectin-1-positive patients and 69.2% for galectin-1-negative patients, and the prognosis for galectin-1-positive patients was significantly poorer compared with galectin-1-negative patients ($\chi^2 = 13.880$, *P* = 0.000). The 5-year survival rates for VEGF-positive and VEGF-negative patients were 53.4% and 70.5%, respectively ($\chi^2 = 4.619$, *P* = 0.032). The overall survival rate of patients with both galectin-1 and VEGF overexpression in gastric cancer tissue samples was significantly poorer than other groups (both *P* < 0.05).

CONCLUSION: Galectin-1 expression was positively associated with VEGF expression. Both galectin-1 and VEGF can serve as independent prognostic indicators of poor survival for gastric cancer after gastrectomy.

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Key words: Galectin-1; Vascular endothelial growth factor; Gastric cancer; Prognosis

Core tip: Galectin-1 and vascular endothelial growth factor (VEGF) played important roles in angiogenesis and progression of malignant tumor, while their expression in Chinese gastric cancer and relationship between the two parameters and clinicopathological features, as well as prognostic value remained largely unknown. In this present study, we examined 214 gastric cancer samples

for the presence of galectin-1 oncoprotein and VEGF by immunohistochemistry and found that overexpressions of galectin-1 and VEGF were related with tumor progression and poor survival, and our findings supported an association between galectin-1 and VEGF expression. These two molecules may serve as independent predictive markers for patient prognosis in gastric cancer.

Chen J, Zhou SJ, Zhang Y, Zhang GQ, Zha TZ, Feng YZ, Zhang K. Clinicopathological and prognostic significance of galectin-1 and vascular endothelial growth factor expression in gastric cancer. *World J Gastroenterol* 2013; 19(13): 2073-2079 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v19/i13/2073.htm> DOI: <http://dx.doi.org/10.3748/wjg.v19.i13.2073>

INTRODUCTION

Gastric cancer is one of the most common cancers in the world. It is the second leading cause of cancer death after lung cancer^[1,2]. The incidence of gastric cancer is highest in Eastern Asia, including China, Japan and South Korea^[3]. Despite advances in diagnostic techniques, neoadjuvant chemoradiotherapy, and surgery, the 5-year survival rate for gastric cancer remains poor, particularly in more advanced stages^[4]. Recently, therapeutic strategies have been improved by the availability of monoclonal antibodies. Additionally, studies have evaluated new biologic and molecular targets for their potential roles as prognostic markers and targets for therapy in patients with gastric cancer.

Galectin-1, a β -galactoside-binding protein, is a 14 kDa homodimer and the first protein discovered in the galectin family^[5-7]. Accumulating evidence clearly shows that galectin-1 is involved in numerous essential cancer-related processes, including immunosuppression, angiogenesis, and metastasis^[8-15]. Galectin-1 overexpression in tumor stromal cells has been detected in several malignant tumors, such as colon cancer^[16], breast cancer^[17], hepatocellular cancer^[18], prostate cancer^[19], and pancreatic ductal adenocarcinoma^[20]. Furthermore, high galectin-1 expression was shown to correlate with poor survival in several types of cancer^[21-25].

Vascular endothelial growth factor (VEGF) is an angiogenic factor produced by tumor cells that stimulates intratumoral microvessel proliferation. Angiogenesis is a fundamental process in tumor growth and metastasis, and it contributes to the metastatic process by providing large numbers of leaking blood vessels for vascular invasion^[26,27]. VEGF is the most potent and specific promoter of tumor angiogenesis^[28]. It is able to stimulate the growth of epithelial cells of various origins, promote vasculature construction, and enhance blood vessel permeability, especially microvessels^[29]. A few published studies have shown that VEGF overexpression in gastric cancer is associated with poor prognosis^[30-32]. However, no previous studies have clarified the correlation between

galectin-1 and VEGF overexpression in gastric cancer. In this study, we performed immunohistochemical staining and extensively examined galectin-1 and VEGF expression in gastric cancer tissues. The aims of this study were to determine whether the expression levels of galectin-1 and VEGF were correlated with each other and with clinicopathological features and prognosis, including patient survival.

MATERIALS AND METHODS

Patient information

From January 2004 to October 2006, a total of 214 patients with gastric cancer who underwent gastrectomy at the Department of General Surgery of the Affiliated Hospital of Jiangsu University were enrolled in this retrospective study. There were 129 men and 85 women between the ages of 31 and 84 years (mean, 64.5 years). None had received chemotherapy or radiotherapy before surgery. Follow-up was completed on 30 October 2012. Patient clinicopathologic parameters were collected, including age, gender, differentiation, and pathological tumor-node-metastasis classification (according to the International Union Against Cancer).

Immunohistochemistry

Immunohistochemical analyses of galectin-1 and VEGF expression were performed on formalin-fixed, paraffin-embedded sections of surgical specimens. Tissue microarray blocks were serially cut into 4 μ m-thick sections and stained. Paraffin sections were deparaffinized in xylene and rehydrated in a gradient of ethanol solutions. Endogenous peroxidases were blocked with 3% hydrogen peroxide in methanol for 10 min. The slides were immersed in 10 mmol/L citrate buffer (pH 6.0) and heated for 30 min for antigen retrieval. The slides were then cooled at room temperature for 20 min and washed with phosphate-buffered saline (PBS). Non-specific binding was blocked by pre-incubation with 10% fetal calf serum in PBS with 0.01% sodium azide. The slides were then incubated in a humidified chamber for 1 h with antibodies against galectin-1 (titer 1:100, New Castle, United Kingdom) and VEGF (titer 1:50, DakoCytomation, Denmark). After washing three times in PBS, the slides were incubated with the envision-HrP complex (undiluted; Dako) for 60 min and visualized with diaminobenzidine and counterstained with hematoxylin. For substitute negative controls, the primary antibodies were replaced with PBS. Positive controls were provided by the kit supplier. The results were assessed by two independent pathologists who had no knowledge of the patient clinical status.

Evaluation of immunohistochemical staining

A scoring system was used to evaluate the immunoreactivity of gastric cancer. Galectin-1 staining was scored semiquantitatively using the following criteria: 0, no staining and less than 10% of tumor cells or stromal cells with membrane staining; 1+, more than 10% of tumor cells or stromal cells with faint partial membrane staining; 2+,

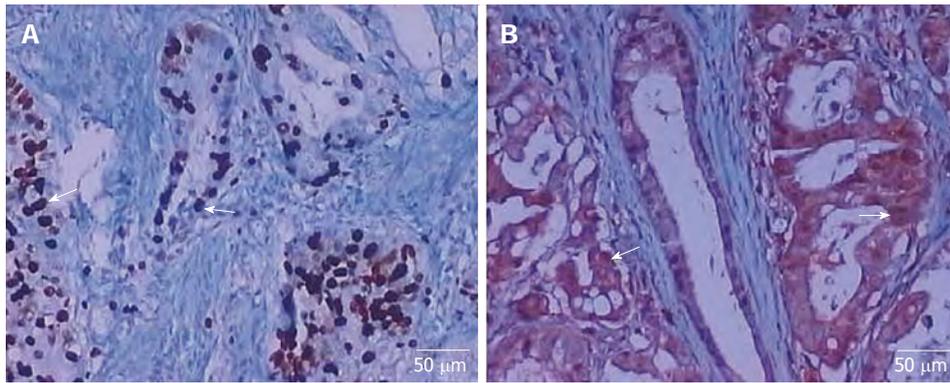


Figure 1 Immunohistochemical staining for galectin-1 and vascular endothelial growth factor (original magnification, $\times 200$). A: Positive galectin-1 expression in gastric cancer tissue; B: Positive vascular endothelial growth factor expression in gastric cancer tissue. The over-expressed markers are shown with arrows.

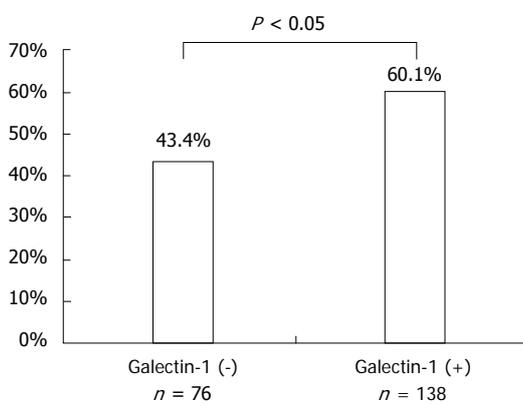


Figure 2 Vascular endothelial growth factor was expressed more frequently in galectin-1-positive gastric cancers than galectin-1-negative.

more than 10% of tumor cells or stromal cells with weak to moderate partial membrane staining; 3+, more than 10% of tumor cells or stromal cells with strong partial membrane staining. Specimens with scores of 0 or 1+ were considered negative, and scores of 2+ or 3+ were considered positive for galectin-1 expression. VEGF staining was considered positive when at least 10% of the tumor cells were stained, as previously described^[33,34].

Follow-up

Patients underwent continuous follow-up up to September 2012. No patients were lost to follow-up. The median follow-up duration was 48.5 mo (range, 0-60 mo) after surgery.

Ethics

This work was performed in accordance with the Declaration of Helsinki of the World Medical Association. This study was ethically approved by the Affiliated Hospital of Jiangsu University (JSUH-EC-189923). All patients provided written informed consent.

Statistical analysis

Statistical analyses were performed with SPSS 16.0 for Windows (SPSS, Chicago, IL, United States). The correlations between galectin-1 and VEGF expression and

clinicopathological features were analyzed using the χ^2 test. The Kaplan-Meier test was employed to evaluate the survival rate, and the survival rate curves were compared using the log-rank test. P values < 0.05 were considered statistically significant.

RESULTS

Galectin-1 and VEGF expression in gastric cancer tissues

Galectin-1 expression in tumor stromal cells was detected in 197 (92.1%) of 214 tumor tissues. Galectin-1 expression was positive in 138 out of 214 gastric cancer samples (64.5%) and negative in the remaining 76 samples (35.5%); 86 samples were 2+ (40.2%), and 52 were 3+ (24.3%). VEGF expression was positive in 116 of 214 gastric cancer samples (54.2%) and negative in the remaining 98 samples (45.8%); 30 samples were 1+ (14.0%), 53 were 2+ (24.8%), and 33 were 3+ (15.4%). Figure 1 shows galectin-1 and VEGF staining in gastric cancer tissues.

Correlation between galectin-1 and VEGF expression and clinicopathological features

There was a significant association between galectin-1 and VEGF expression; VEGF was detected in 60.1% of galectin-1-positive tumors and 43.4% of galectin-1-negative tumors ($P < 0.05$, Table 1, Figure 2). The correlations between galectin-1 and VEGF expression and clinicopathological features are shown in Table 2. Galectin-1 expression was positively associated with tumor size, tumor location, stage, and lymph node metastases (all $P < 0.05$), but it was not correlated with gender, age, or differentiation grade (all $P > 0.05$). VEGF expression was positively correlated with tumor size, stage, and lymph node metastases (all $P < 0.05$), but it was not correlated with the other clinicopathological features assessed (all $P > 0.05$).

Correlation between galectin-1 and VEGF expression and patient survival

All patients underwent follow-up until cancer-related death or more than five years after tumor resection. The median follow-up interval was 48.5 mo. The 5-year sur-

Table 1 Relationship of galectin-1 and vascular endothelial growth factor expression in gastric cancer tissues

| VEGF | Galectin-1 | | P value |
|--------------------|--------------------|-------------------|---------|
| | Positive (n = 138) | Negative (n = 76) | |
| Positive (n = 116) | 83 | 33 | 0.022 |
| Negative (n = 98) | 55 | 43 | |

VEGF: Vascular endothelial growth factor.

vival rate was 56.6% for galectin-1-positive patients and 69.2% for galectin-1-negative patients, and the prognosis for galectin-1-positive patients was significantly poorer than that of galectin-1-negative patients ($\chi^2 = 13.880$, $P = 0.000$). The 5-year survival rates for VEGF-positive and VEGF-negative patients were 53.4% and 70.5%, respectively ($\chi^2 = 4.619$, $P = 0.032$). Additionally, VEGF-positive patients had a shorter survival time than VEGF-negative patients. The cumulative overall survival rates for these two populations were determined (Figure 3A and B).

To evaluate the combined effect of galectin-1 and VEGF expression on the prognosis of gastric cancer, we classified patients into four subgroups according to galectin-1 and VEGF expression: group I, low expression of both markers; group II, high galectin-1 expression and low VEGF expression; group III, low galectin-1 expression and high VEGF expression; and group IV, high expression of both markers. We found that the 5-year survival rate in group IV was 40.9%, which was significantly lower compared with groups I (53.5%), II (49.1%), and III (48.5%) (Figure 3C, $P < 0.05$).

DISCUSSION

Despite the development of surgical techniques and new cytotoxic agents, which have improved the prognosis of gastric cancer, once patients develop resistance to chemotherapeutic regimens, no other treatment options are available. Given the frequent failure of conventional treatment strategies, many cancer-related molecules have been characterized with the goal of developing novel anticancer therapies^[35]. To guide clinical decision making in therapy and prognosis prediction, efforts have been made to identify prognostic biomarkers for patients with gastric cancer.

Galectin-1 is a β -galactoside-binding protein that is abundantly secreted by almost all types of malignant tumor cells. Galectin-1 expression is regulated by hypoxia-inducible factor-1, and it plays vital protumorigenic roles within the tumor microenvironment. Furthermore, galectin-1 suppresses T cell-mediated cytotoxic immune responses and promotes tumor angiogenesis. Recent evidence has demonstrated that galectin-1 plays an important role in tumor progression and metastasis^[36]. The amplification and overexpression of galectin-1 have been demonstrated in several tumors, including colon cancer, breast cancer, and hepatocellular cancer. The frequency

Table 2 Relationship of galectin-1 and vascular endothelial growth factor expression to clinicopathological variables in gastric cancer tissues

| Variable | Galectin-1 | | P value | VEGF | | P value |
|-------------------|------------|-----|---------|------|-----|---------|
| | (+) | (-) | | (+) | (-) | |
| Age (yr) | | | 0.200 | | | 0.784 |
| ≤ 60 | 58 | 39 | | 54 | 48 | |
| > 60 | 80 | 37 | | 62 | 50 | |
| Gender | | | 0.308 | | | 0.583 |
| Male | 87 | 42 | | 62 | 48 | |
| Female | 51 | 34 | | 54 | 50 | |
| Tumor size | | | 0.026 | | | < 0.001 |
| < 3 cm | 43 | 36 | | 48 | 73 | |
| ≥ 3 cm | 95 | 40 | | 68 | 25 | |
| Tumor location | | | 0.004 | | | 0.287 |
| Upper third | 18 | 3 | | 35 | 21 | |
| Middle third | 60 | 50 | | 44 | 38 | |
| Lower third | 60 | 23 | | 37 | 39 | |
| Differentiation | | | 0.112 | | | 0.998 |
| Well | 12 | 14 | | 30 | 25 | |
| Moderate | 53 | 27 | | 26 | 22 | |
| Poor | 73 | 35 | | 60 | 51 | |
| TNM stage | | | < 0.001 | | | < 0.001 |
| T1 | 3 | 37 | | 25 | 65 | |
| T2-T4 | 135 | 39 | | 91 | 33 | |
| Lymph node status | | | < 0.001 | | | 0.002 |
| Positive | 30 | 46 | | 66 | 35 | |
| Negative | 108 | 30 | | 50 | 63 | |

TNM: Tumor-node-metastasis; VEGF: Vascular endothelial growth factor.

of positivity appears to increase with the clinical stage of the disease and is associated with a worse prognosis^[16-18]. However, the prevalence of galectin-1 overexpression in gastric cancer and its relationship with prognosis is not clear. There are only two studies in the literature evaluating the correlation between galectin-1 expression and survival. In these studies, galectin-1 expression in tumor cells was significantly correlated with short survival in astrocytic neoplasms and colon cancer^[16,37]. In the present study, we examined 214 gastric cancer samples for the presence of the galectin-1 oncoprotein by immunohistochemistry. In all, 138 samples (64.5%) showed positive galectin-1 expression, and galectin-1 expression was related to tumor size, differentiation grade, stage, and lymph node metastases, suggesting that this protein may participate in tumor growth and distant metastasis. We also confirmed a significant prognostic value of galectin-1 in gastric cancer using a Kaplan-Meier survival analysis. The outcome of galectin-1-positive patients was significantly poorer than galectin-1-negative patients. Thus, detecting galectin-1 expression in gastric cancer tissues might be helpful for predicting patient prognosis.

Angiogenesis is essential for tumor growth and metastasis^[38]. VEGF is the most potent angiogenic factor identified to date. Tumor angiogenesis and neovascularization require VEGF expression^[39]. VEGF is primarily secreted by tumor cells, and its functions are largely restricted to endothelial cells^[40]. VEGF strongly stimulates the growth of endothelial cells, leading to the formation of new blood vessels and providing essential nutrients

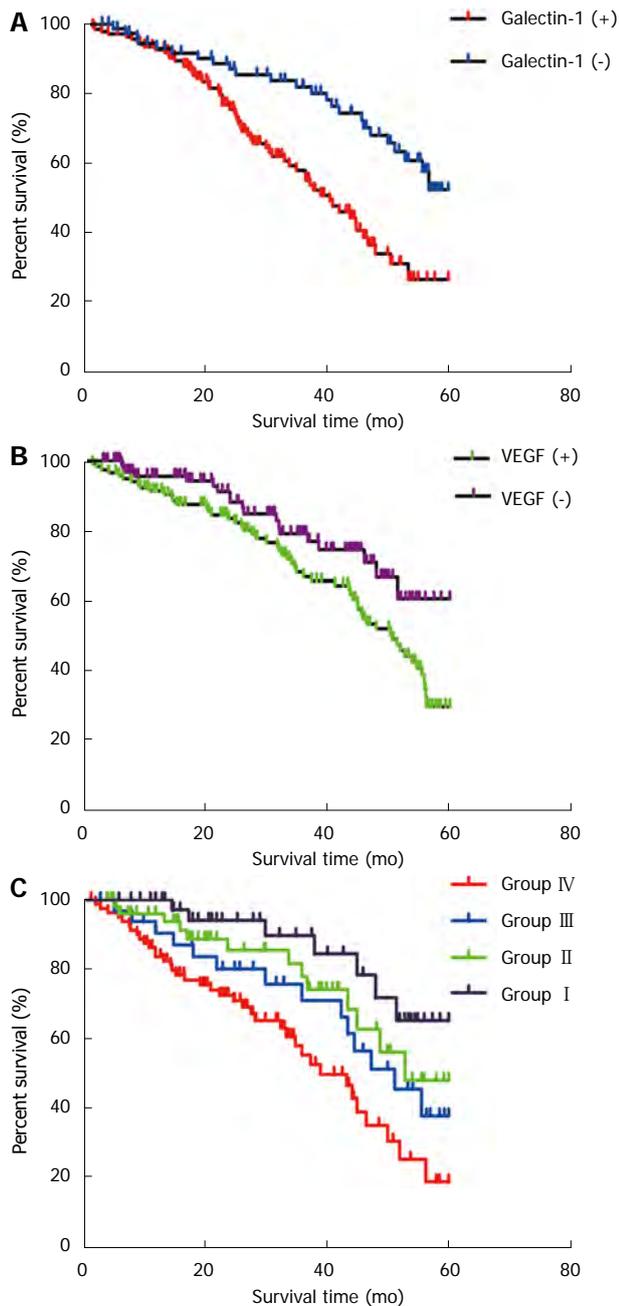


Figure 3 Overall survival rate of patients. A: The overall survival rate of patients relative to galectin-1-positive expression and galectin-1-negative expression in gastric cancer tissue samples. Galectin-1 overexpression was significantly associated with poor patient survival ($P < 0.05$); B: The overall survival rate of patients relative to vascular endothelial growth factor-positive expression and vascular endothelial growth factor-negative expression in gastric cancer tissue samples. Vascular endothelial growth factor overexpression was significantly associated with poor patient survival ($P < 0.05$); C: The overall survival rates of patients relative to groups I, II, III and IV. Both galectin-1 and vascular endothelial growth factor overexpression was significantly associated with poor patient survival ($P < 0.05$).

for tumor growth. Therefore, VEGF-based antiangiogenesis therapy may be of therapeutic benefit against solid tumors and has been tested in several tumor types, including gastric cancers. In our study, VEGF expression was detected in more than half of gastric cancers (54.2%). Both the incidence and proportion of VEGF expres-

sion increased with the progression of gastric cancer, and it was correlated with tumor size, stage, and lymph node metastases. VEGF expression has been identified as a significant marker for tumor recurrence and reduced survival independent of conventional clinicopathological variables in gastric cancer^[41,42]. In this study, using Kaplan-Meier analysis, we also demonstrated a significant association between VEGF expression and poor survival.

VEGF is one of the most potent inducers of angiogenesis, whereas galectin-1 has been implicated in the regulation of VEGF. Koopmans *et al.*^[43] demonstrated that galectin-1 activation led to the translational up-regulation of VEGF and increased angiogenesis through the JAK/STAT pathway in myeloproliferative neoplasia. Fischer *et al.*^[44] demonstrated that galectin-1 inhibited rearranged during transfection and Janus kinase 2 signals and up-regulated vascular endothelial growth factor receptor 3 signaling in trophoblast tumor cells. Hsieh *et al.*^[45] found that galectin-1 was overexpressed in the connective tissue surrounding cancer cells in tumor-associated vascular endothelial cells. Galectin-1 can increase angiogenesis by interacting with neuropilin-1 on the endothelial cell surface. Galectin-1 binding to neuropilin-1, which acts as a co-receptor of VEGF in endothelial cells, enhances VEGF receptor phosphorylation and the subsequent activation of mitogen-activated protein kinases^[16]. However, few studies have evaluated the correlation between VEGF and galectin-1 in gastric cancer.

The present study showed that VEGF expression was increased in galectin-1-positive tumors compared to galectin-1-negative tumors. Meanwhile, galectin-1 expression was also increased in VEGF-positive tumors compared to VEGF-negative tumors. Galectin-1 expression was positively associated with VEGF expression. Galectin-1 and VEGF played concordant roles in tumor angiogenesis, progression, metastasis, and prognosis, which suggests a connection between them. Our results also indicated that galectin-1 and VEGF overexpression was significantly correlated with poor survival in Chinese gastric cancer patients, especially patients with both galectin-1 and VEGF expression. Therefore, detecting galectin-1 and VEGF expression might help to identify gastric cancer patients with a poor prognosis and could therefore be a novel prognostic marker. To date, the galectin-1 regulatory mechanism of VEGF in gastric cancer has not been well explored and requires further study.

COMMENTS

Background

Galectin-1 and vascular endothelial growth factor (VEGF) played important roles in angiogenesis and progression of malignant tumor. The high expression of galectin-1 and VEGF are correlated with disease behavior in some cancers, while their expression in Chinese gastric cancer and relationship between the two parameters and clinicopathological features, as well as prognostic value remained largely unknown.

Research frontiers

Even with the advancement of diagnosis, neoadjuvant chemoradiotherapy and surgery, the 5-year survival for gastric cancer remains poor, especially in more

advanced stages. Recently therapeutic strategies have been improved by the availability of monoclonal antibodies. Researches have been evaluating new biologic and molecular targets for their potential role as prognostic markers and as targets for therapy in patients with gastric cancer.

Innovations and breakthroughs

Given the frequent failure of conventional treatment strategies, many cancer-related molecules have been characterized with the goal of developing novel anticancer therapies. In order to guide clinical decision-making in therapy and prognosis prediction, efforts have been invested in identifying prognostic biomarkers for patients with gastric cancer. Galectin-1 is a β -galactoside binding protein that is abundantly secreted by almost all types of malignant tumor cells. The expression of galectin-1 is regulated by hypoxia-inducible factor-1 (HIF-1) and it plays vital protumorigenic roles within the tumor microenvironment. However, the prevalence of galectin-1 overexpression in gastric cancer as well as its relationship with prognosis is not clear. There are only two studies in the literature evaluating the correlation between galectin-1 expression and survival. In these studies, galectin-1 expression in tumor cells significantly correlated with short survival in astrocytic neoplasms and in colon cancer. In this present study, the authors examined 214 gastric cancer samples for the presence of galectin-1 oncoprotein by immunohistochemistry.

Applications

The study aimed at evaluating the expression of galectin-1 and VEGF in gastric cancer by immunohistochemical methods. The authors found that overexpressions of galectin-1 in tumor stroma cells and VEGF in tumor cells were related with tumor progression and poor survival in gastric cancer, and our findings supported an association between galectin-1 and VEGF expression. These two molecules may serve as independent predicative markers for patient prognosis in gastric cancer.

Terminology

Galectin-1 is a β -galactoside binding protein that is abundantly secreted by almost all types of malignant tumor cells. The expression of galectin-1 is regulated by HIF-1 and it plays vital protumorigenic roles within the tumor microenvironment. Galectin-1 suppresses T cell-mediated cytotoxic immune responses and promotes tumor angiogenesis. VEGF is the most potent angiogenic factor identified so far. Tumor angiogenesis and neovascularization require VEGF expression. VEGF is mainly secreted by tumor cells with its functions largely restricted to endothelial cells, and it strongly stimulate the growth of endothelial cells leading to the formation of new blood vessels, thus providing essential nutrients for tumor growth.

Peer review

This manuscript describes convincingly the expression of galectin-1 and VEGF in gastric cancer patients. The paper is well prepared and its publication in the journal is recommended with minor corrections.

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Synchronous vs sequential laparoscopic cholecystectomy for cholecystocholedocholithiasis

Yan-Bing Ding, Bin Deng, Xin-Nong Liu, Jian Wu, Wei-Ming Xiao, Yuan-Zhi Wang, Jian-Ming Ma, Qiang Li, Ze-Sheng Ju

Yan-Bing Ding, Bin Deng, Jian Wu, Wei-Ming Xiao, Yuan-Zhi Wang, Department of Gastroenterology, Yangzhou No. 1 People's Hospital, The Second Clinical School of Yangzhou University, Yangzhou 225000, Jiangsu Province, China

Xin-Nong Liu, Jian-Ming Ma, Qiang Li, Ze-Sheng Ju, Department of Surgery, Yangzhou No. 1 People's Hospital, The Second Clinical School of Yangzhou University, Yangzhou 225000, Jiangsu Province, China

Author contributions: Ding YB and Deng B contributed equally to this work; Liu XN, Ding YB and Deng B designed the research methods; Wu J, Wang YZ, Ma JM, Li Q and Ju ZS performed the research; Ding YB, Deng B, and Xiao WM analyzed the data; and Liu XN, Ding YB and Deng B wrote the paper.

Correspondence to: Xin-Nong Liu, PhD, Department of Surgery, Yangzhou No. 1 People's Hospital, The Second Clinical School of Yangzhou University, Yangzhou 225000, Jiangsu Province, China. ybding66@163.com

Telephone: +86-514-82981199 Fax: +86-514-82981199

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RESULTS: There were no significant differences between the groups in terms of the numbers of patients, sex distribution, age, American Society of Anesthesiologists score, serum bilirubin, γ -glutamyl transpeptidase, mean diameter of common bile duct stones, and previous medical and surgical history ($P = 0.54$, $P = 0.18$, $P = 0.52$, $P = 0.22$, $P = 0.32$, $P = 0.42$, $P = 0.68$, $P = 0.70$, $P = 0.47$ and $P = 0.57$). There was no significant difference in the surgical operation time between the two groups (112.1 ± 30.8 min vs 104.9 ± 18.2 min). Compared with the sequential operation group, the incidence of pancreatitis was lower (1.4% vs 6.3%), the incidence of hyperamylasemia (1.4% vs 10.0% , $P < 0.05$) was significantly reduced, and the length of the hospital stay was significantly shortened in the synchronous operation group (3 d vs 4.5 d, $P < 0.001$).

CONCLUSION: For treatment of cholecystocholedocholithiasis, synchronous LC combined with EST reduces incidence of complications, decreases length of hospital stay, simplifies the surgical procedure, and reduces operation time.

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Key words: Laparoscopic cholecystectomy; Endoscopic sphincterotomy; Endoscopic retrograde cholangiopancreatography; Cholecystolithiasis; Choledocholithiasis

Ding YB, Deng B, Liu XN, Wu J, Xiao WM, Wang YZ, Ma JM, Li Q, Ju ZS. Synchronous vs sequential laparoscopic cholecystectomy for cholecystocholedocholithiasis. *World J Gastroenterol* 2013; 19(13): 2080-2086 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v19/i13/2080.htm> DOI: <http://dx.doi.org/10.3748/wjg.v19.i13.2080>

Abstract

AIM: To compare synchronous laparoscopic cholecystectomy (LC) combined with endoscopic sphincterotomy (EST) and sequential LC combined with EST for treating cholecystocholedocholithiasis.

METHODS: A total of 150 patients were included and retrospectively studied. Among these, 70 were selected for the synchronous operation, in which the scheme was endoscopic retrograde cholangiopancreatography combined with EST during LC. The other 80 patients were selected for the sequential operation, in which the scheme involved first cutting the papillary muscle under endoscopy and then performing LC. The indexes in the two groups, including the operation time, the success rate, the incidence of complications, and the length of the hospital stay, were observed.

INTRODUCTION

Cholelithiasis, including cholecystolithiasis and common

bile duct stones (CBDSs), is common in clinical practice. The incidence of concurrent cholecystolithiasis and CBDSs is 10%-33%, and varies according to age^[1]. Cholelithiasis can be associated with serious complications, including biliary pancreatitis and suppurative cholangitis. Therefore, it is important to regularize and improve the process of clinical diagnosis and treatment of this disease.

Laparotomy for gallbladder excision, with common bile duct (CBD) exploration or endoscopic sphincterotomy (EST) through duodenal papilla, was once the standard treatment plan for concurrent cholecystolithiasis and CBDSs. In the past 10 years, with the rapid development of laparoscopic techniques, laparoscopic cholecystectomy (LC) has become the main treatment for cholecystolithiasis. However, many studies have shown that LC combined with laparoscopic common bile duct exploration (LCBDE) has a high success rate (up to 83%-89%) for concurrent cholecystolithiasis and CBDSs. It also has many merits, such as a significantly shortened hospital time and synchronous minimally invasive surgery^[2-6]. Moreover, there is no significant difference in the incidence of complications with this technique when compared with EST^[7]. Unfortunately, it is not widely applied because of the complex surgical technique^[3,8].

With the rapid development of endoscopic retrograde cholangiopancreatography (ERCP), a variety of operations can be chosen on the basis of the LC scheme for concurrent cholecystolithiasis and CBDS. Besides LC with LCBDE, the so-called double endoscopy joint operation is also an option, which comprises LC combined with ERCP and EST before, during, or after the operation to remove CBDSs^[9-11]. The most widely used operation scheme is LC combined with preoperative ERCP and EST. This scheme often requires two hospitalizations, longer hospital stays, and correspondingly higher medical costs. Even after strict preoperative screening, a proportion of CBDS cases with preoperative diagnoses is still found to be biliary stone negative during the ERCP process. Therefore, some patients must pay unnecessary ERCP-related medical expenses and undergo potential risks of surgery^[12]. In recent years, there have been reports on the laparoendoscopic rendezvous (LRV) operation to treat concurrent cholecystolithiasis and CBDSs. The LRV operation has the advantages of high stone clearance, a low incidence of complications, and reduced hospital time, but it also has disadvantages that include a complex surgical procedure and a longer single operation time^[13,14].

In our study, we used synchronous LC combined with EST to treat concurrent cholecystolithiasis and CBDSs. This approach combined LRV with conventional surgical procedures to perform endoscopic retrograde bile duct intubation. We compared the efficacy and safety of synchronous LC with LRV *vs* sequential LC with the conventional operation.

MATERIALS AND METHODS

Patients

A total of 167 patients with cholecystolithiasis and CBDSs

were enrolled in this study from June 2009 to October 2012 at the Second Clinical Medical School, Yangzhou University. The preliminary diagnosis was established by the clinical symptoms (abdominal pain and vomiting), signs (right upper-quadrant abdominal pain and jaundice), serum biochemical index (high bilirubin or transaminase level), and abdominal ultrasound (gallstones and suspicious CBDSs, or CBD diameter > 8 mm). All of these cases were further examined by magnetic resonance cholangiopancreatography (MRCP) to diagnose cholecystolithiasis and choledocholithiasis.

The exclusion criteria were: (1) age > 80 years or < 18 years; (2) American Society of Anesthesiologists (ASA) score^[15] ≥ 4; (3) suppurative cholangitis (body temperature > 38.5 °C, with right upper-quadrant abdominal pain and pressure pain, or hyperbilirubinemia); (4) acute pancreatitis (serum amylase 3 times higher than normal); (5) pregnancy; (6) abdominal surgical history; and (7) decompensated cirrhosis that is not suitable for endoscopic and laparoscopic surgery.

A total of 150 patients were retrospectively studied and the treatment procedure is shown in Figure 1. Among these, 70 were selected for the synchronous operation, in which ERCP was combined with EST during LC. The other 80 patients were selected for the sequential operation, in which the papillary muscle was cut under endoscopy, and then LC was performed after 24-72 h. All ERCPs were performed by one of two endoscopic technologists, while LC was performed by one of three expert surgeons. Our study was approved by the Ethics Committee of the Second Clinical Medical School, Yangzhou University, and signed informed consent was obtained from each patient for the operative procedures.

Surgical procedures

The entire procedure was performed with the patient under general anesthesia. Patients in the synchronous group were placed on a C-arm-compatible table. Pneumoperitoneum was routinely established and laparoscopic instruments were put into the peritoneal cavity. The triangle of Calot was first dissected, then the gallbladder artery was ligated close to the gallbladder side, the gallbladder duct was exposed and cut open near the CBD side to make an oblique incision, and the angiographic catheter was inserted (Figure 2A). The contrast agent was injected to confirm the presence of bile duct stones (Figure 2B). The duodenoscope was inserted into the descending part of the duodenum, and a selective CBD intubation was made. Stones were removed by balloon or basket after successful intubation, and lithotripsy or balloon expansion was carried out if it was difficult to remove the stones (Figure 2C). If selective bile duct intubation failed, a yellow zebra guide wire was intubated using an angiographic catheter under laparoscopy (Figure 2D). The yellow zebra was across the duodenal papilla to the descending part of duodenum (Figure 2E), drawn out, and plugged into the duodenum again with the end of guide wire. The duodenoscope was inserted in the descending part of duodenum through the mouth, and the guide wire was pulled

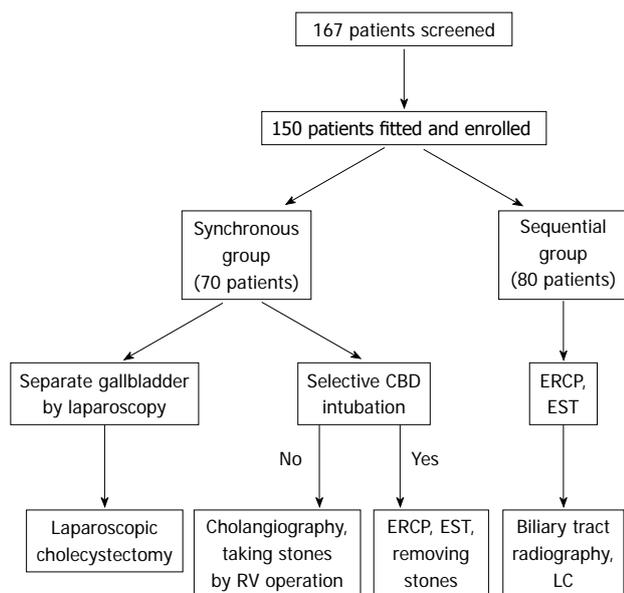


Figure 1 Treatment procedure for this study. CBD: Common bile duct; ERCP: Endoscopic retrograde cholangiopancreatography; EST: Endoscopic sphincterotomy; RV: Rendezvous; LC: Laparoscopic cholecystectomy.

out with the duodenal trap of the duodenoscope. The duodenal papillary muscle was cut with an incision knife, which followed the guide wire retrograde to the duodenal papilla (Figure 2F). Gas inside the gastrointestinal tract was exhausted at the end of the endoscopic operation, the gallbladder duct was ligated by routine laparoscopic procedure, and the gallbladder was removed.

Patients in the sequential operation group were placed in the left supine or prone position. The duodenoscope was inserted, and radiography was performed to confirm the situation of the biliary tract. The duodenal papillary muscle was cut, and stones were removed by balloon or basket. Endoscopic nasobiliary drainage was performed and biliary tract radiography was completed during 24-48 h. Residual stones were removed, and LC was carried out if no residual stone was observed.

Operation time was defined as the time from anesthesia to when the patient awoke after the operation in the synchronous group. In the sequential group, the operation time was the sum of the time for the ERCP operation before LC and the LC operation time. Major complications were defined as any intraoperative or postoperative (42 d) events that altered the clinical course, such as ERCP complications (including pancreatitis, hyperamylasemia, perforation, and bleeding) and LC complications (bile duct leakage, bleeding, pneumonia, and organ failure).

The success rate included the ERCP and LC success rates. ERCP success was defined as smoothly cannulating the CBD and achieving complete CBD stone clearance at the time of final cholangiography. LC success was defined as performing LC smoothly without converting to open surgery. Postoperative hospitalization time was the hospital time for LC combined with ERCP in the synchronous group, while it was the length of the hospital stay after ERCP in the sequential group.

Table 1 Basic characteristics and intraoperative and postoperative parameters of patients who underwent synchronous and sequential operations

| | Synchronous group | Sequential group | P value |
|--------------------------------|-------------------|------------------|---------|
| Total patients | 70 | 80 | |
| M/F ratio | 46/24 | 53/27 | 0.54 |
| Mean age, yr | 59.0 (38-75) | 56.6 (36-74) | 0.18 |
| ASA score (I - II / III) | 62/8 | 70/10 | 0.52 |
| Symptoms | | | |
| Abdominal pain | 59 (84.3) | 72 (90.0) | 0.22 |
| Jaundice | 51 (71.4) | 62 (77.5) | 0.32 |
| Nausea or vomiting | 39 (55.7) | 47 (58.8) | 0.42 |
| Mean serum bilirubin, mg/dL | 5.4 (0.5-24) | 5.9 (0.6-27) | 0.68 |
| Mean γ -GGT, μ /dL | 116.2 (27-342) | 122.8 (35-396) | 0.70 |
| MRCP diagnosis | | | |
| Mean diameter of CBDS, mm | 9.7 (7-21) | 9.2 (6-20) | 0.47 |
| Stone number (single/multi) | 49/21 | 56/24 | 0.57 |
| Mean operative time, min | 112.1 \pm 30.8 | 104.9 \pm 18.2 | 0.08 |
| Success rate | | | |
| Endoscopic sphincterotomy | 70 (100) | 77 (96.3) | 0.15 |
| Laparoscopic cholecystectomy | 69 (98.6) | 80 (100) | 0.74 |
| Major complications rate | | | |
| Acute pancreatitis | 1 (1.4) | 5 (6.3) | 0.14 |
| Hyperamylasemia | 1 (1.4) | 8 (10) | 0.03 |
| Bleeding/perforation/infection | 0 (0) | 0 (0) | |
| Hospital stay, d | 3 (2-6) | 4.5 (3-12) | < 0.001 |

Data are expressed as absolute *n* (%) or median (range). M: Male; F: Female; γ -GGT: γ -glutamyl transpeptidase; ASA: American Society of Anaesthesiologists; MRCP: Magnetic resonance cholangiopancreatography; CBDS: Common bile duct stone.

Follow-up procedure

Patients were scheduled for follow-up 2 and 6 wk after surgery. During that time, no patients were lost to follow-up. The patients were reviewed by color ultrasound and for liver function. MRCP was performed if there was a question of residual bile duct stones, and stones were removed by remedial ERCP if they were confirmed.

Statistical analysis

The SPSS software package (versions 17.0, SPSS, Chicago, IL, United States) was used for all statistical analyses. Categorical variables were compared with the χ^2 test. Continuous variables were compared with the Student's *t* test or the Mann-Whitney *U* test, depending on the distribution. *P* < 0.05 was considered statistically significant.

RESULTS

The baseline characteristics of the patients are shown in Table 1. There were no significant differences between the groups in terms of the numbers of patients, sex distribution, age, ASA score, serum bilirubin, γ -glutamyl transpeptidase, mean diameter of CBDSs, and previous medical and surgical history (*P* > 0.05 each).

The intraoperative and postoperative parameters are shown in Table 1. The mean operation time in the synchronous group was 112.1 \pm 30.8 min. The LRV operation was performed in 15 cases, because it was difficult to complete selective bile duct intubation during the endo-

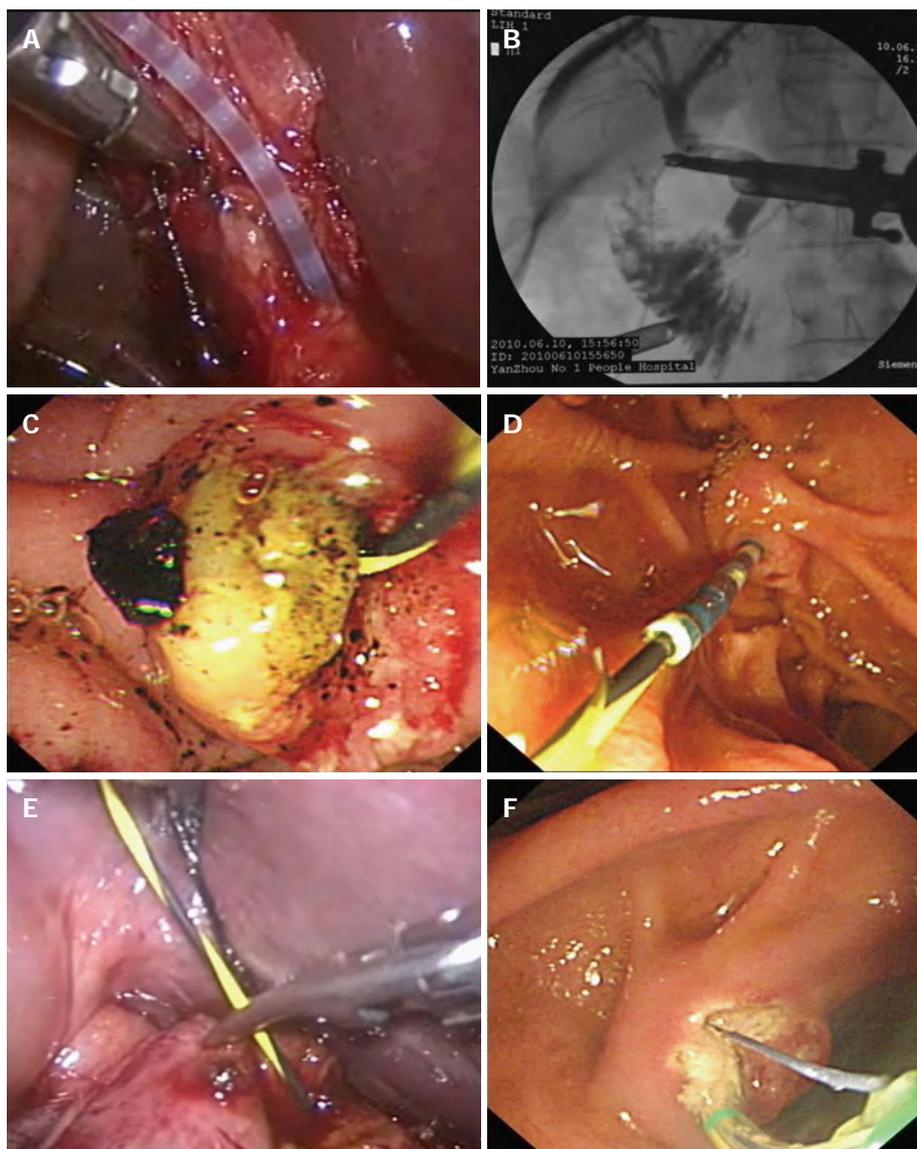


Figure 2 Surgical procedures. A: Angiographic catheter was inserted; B: Cholangiography was performed; C: Stones were removed by balloon or basket; D: Yellow zebra guide wire was inserted into cystic duct; E: Angiographic catheter was inserted following the guide wire; F: Duodenal papillary muscle was cut.

scopic process, and the average operation time of the 15 cases was 132.3 ± 29.0 min. The mean ERCP operation time in the sequential operation group was 38.4 ± 12.1 min, the LC operation time was 66.6 ± 14.4 min, and the overall operation time was 104.9 ± 18.2 min. There was no significant difference in the average operation time between the two groups ($P > 0.05$).

The hyperamylasemia incidence in the synchronous group was 1.4% (1/70), and 10.0% (8/80), in the sequential group, and there was a significant difference in incidence between the two groups ($P < 0.05$). The incidence of acute pancreatitis in the synchronous group was 1.4% (1/70) and 6.3% (5/80) in the sequential group, and there was a trend toward significance between the two groups ($P > 0.05$). Bleeding, perforation, death, and serious complications were not observed in either of the groups. The acute pancreatitis that occurred after the operation was mild, and it did not develop into severe pancreatitis after timely treatment.

The length of the hospital stay in the sequential group was 4.5 d (range, 3-12 d), and five patients with acute pancreatitis had lengthened hospital stays. The length of hospital stay in the synchronous group was 3 d (range, 2-6 d), which was significantly lower than that in the sequential group ($P < 0.001$).

All 150 patients were followed up for a mean 65 wk (range, 8-135 wk). At the 6-wk follow up, color Doppler ultrasound, liver function tests, and MRCP did not identify recurrence of stones and complications related to the operation, except for one patient in the synchronous group. This patient was readmitted 8 wk after the LRV procedure with residual choledocholithiasis and treated successfully with repeat ERCP and CBD clearance.

DISCUSSION

LC combined with EST is the most commonly used minimally invasive treatment for concurrent cholecystolithi-

asis and CBDS^[16,17]. LC combined with postoperative EST is an important remedial treatment measure for stones, which appear in LC but are not removed by instant LCBDE. Its weakness is that EST has a greater need for operative success because, if EST fails to remove stones, patients could require additional surgical procedures. The success rate of ERCP is 85%-90%^[18]. Even if the postoperative ERCP is successful, the hospitalization time is longer than for synchronization^[19,20]. The scheme in most medical units is conventional LC combined with preoperative ERCP, which also has some disadvantages. Even if the preoperative ERCP is successful in removing the stones, the few cases for which LC fails still require laparotomy. If preoperative ERCP is complicated by acute pancreatitis, it is not possible to perform LC. In this study, there were five patients with acute pancreatitis in the sequential group for whom LC had to be delayed, and these patients had extended hospital stays. In addition, intraoperative exploration confirms only 27%-54% of stones, in spite of the clinical history characteristics, medical examination, serum biochemical index, abdominal ultrasound diagnosis, and CBDS preoperative examination, which means that a considerable proportion of patients incur unnecessary ERCP-related medical expenses and potential risks of surgery^[12]. The ERCP serious complication rate was 2.5%-11%, and the mortality rate was 0.5%-3.7%^[18].

In recent years, there have been reports that synchronous ERCP and EST are carried out in LC to treat concurrent cholecystolithiasis with CBDSs^[21]. One meta-analysis of 27 published intraoperative ERCP studies including a total of 795 patients by La Greca *et al.*^[22] showed that the operation success rate was 69.2%-100%, with an average of 92.3%; the average intraoperative endoscopic operation time was 35 min; and the average surgical operation time was 104 min. In these 27 studies, 4.7% of cases required laparotomy, the complication incidence was 5.1%, and the mortality rate was 0.37%. Intraoperative synchronous EST in LC has no obvious differences in terms of complications, such as acute pancreatitis and hyperamylasemia, compared with sequential LC and EST operations, but it significantly improves the operation success rate, shortens the average hospitalization time, and decreases the medical treatment charges^[23]. A randomized study with 120 cases of concurrent cholecystolithiasis with CBDSs observed the risk factors of postoperative ERCP-related pancreatitis, and found that no case was complicated by acute pancreatitis in synchronous surgery, and six patients suffered from iatrogenic acute pancreatitis in sequential surgery^[24]. These data suggest that the synchronous operation has the advantages of high stone clearance, high success rate, and a low complication rate for treating CBDSs when compared to sequential double endoscopy.

Although synchronous surgery has obvious advantages, its implementation faces a few difficulties. First, the synchronous double endoscopy combined operation mostly uses the LRV operation during laparoscopic transcystic intubation into the filar guide and can extend the

operation time. A clinical study with 45 patients showed that the average time for double endoscopy synchronous surgery was 119.09 ± 14.4 min^[13]. Another study showed that the operation time for LC combined with intraoperative ERCP was 192.0 ± 8.9 min, which was 85 min longer than for separate laparoscopic gallbladder resection and CBD exploration^[14]. In the beginning, we used the LRV operation, which is similar to the approach used by ElGeidie's team^[25]. We found that there were certain difficulties in the operation that extended the time required. Now, we prefer LC combined with conventional endoscopic retrograde bile duct intubation, and turn to the LRV operation when there is difficulty in selective intubation. This method can avoid associated risks, including acute pancreatitis and bleeding caused by repeated intubation, contrast agent injection, and pre-cut sphincterotomy. It can also simplify the operation process and reduce the time. In our study, there were difficulties during the selective intubation of 15 patients in the synchronous operation group, so we turned to the LRV operation. There was no difference in the operation time between the synchronous and sequential treatment groups. The incidence of hyperamylasemia and iatrogenic pancreatitis was lower in the synchronous than in the sequential operation group. Besides the operation time, time was required for the positional adjustment of the X-ray machine and endoscopic equipment by the operators. This timing can be addressed after improving the surgical process. Second, the synchronous operation required cooperation between the surgeons and endoscopic physicians. The latter must perform intraoperative ERCP immediately and synchronously with surgery once biliary angiography has confirmed CBDSs. Thus, we can try to reduce the operation time. However, clinical practice often faces certain difficulties. All of the cases in our study were diagnosed with CBDSs by MRCP preoperatively, because the surgeons, endoscopic physicians, and equipment were in the right place from the beginning. This design guaranteed the effective organization of the synchronous double endoscopy operation. The sensitivity and specificity of MRCP diagnosis in CBDS are 95% and 97%, respectively^[26], and all cases diagnosed with CBDSs by MRCP were confirmed in the perioperative period in this study. Third, some researchers think that general anesthesia by endotracheal intubation is an unfavorable factor in duodenoscopy operations^[15], so we used general anesthesia by nasal intubation to reduce this negative influence.

Our study also had several limitations. First, it was a retrospective study that was not performed in a double-blind and randomized fashion. Second, our work was in the preliminary stage, and it did not assess the learning curves for the two types of surgery. Third, the length of follow up was short, and the number of patients was small. Therefore, further studies with larger patient populations are needed to draw more valid conclusions.

In conclusion, we found that both synchronous and sequential laparoscopic operations combined with endoscopic operations were minimally invasive surgical proce-

dures for effective treatment of concurrent cholecystolithiasis and CBDs. Moreover, the synchronous double endoscopy combined operation may selectively apply the LRV scheme. Synchronous surgery has advantages, such as reducing complications and shortening hospital stay, and it can also simplify the operation process and reduce the time required.

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COMMENTS

Background

Cholecystolithiasis, combined with common bile duct stones (CBDs), is common in clinical practice. In the management of cholelithiasis, laparoscopic cholecystectomy (LC) is the treatment of choice, but the ideal management of choledocholithiasis with LC is controversial. Today a number of options exist, including endoscopic sphincterotomy (EST) before LC, laparoscopic common bile duct exploration, and postoperative endoscopic retrograde cholangiopancreatography.

Research frontiers

Several studies have shown the efficacy of the combined laparoendoscopic rendezvous (LRV) technique for treatment of cholecystolithiasis and CBDs. Studies have demonstrated that this method has advantages of easier cannulation, prevention of pancreatic trauma, and reduced hospital time, but it also has disadvantages, including a complex surgical procedure and a longer single operation time.

Innovations and breakthroughs

In this study, the authors used synchronous LC combined with EST to treat concurrent cholecystolithiasis and CBDs, with selective application of the LRV procedure. Study data showed that synchronous surgery had advantages, such as reducing complications and shortening hospital stay, and it also simplified the surgical procedure and reduced the operation time in most cases.

Applications

Elective application of the LRV procedure in a synchronous double endoscopy combined operation is a minimally invasive surgical procedure for the effective treatment of concurrent cholecystolithiasis and CBDs.

Terminology

LRV is a technique in which the sphincterotome is driven across the papilla into the choledochus by a Dormia basket passed into the duodenum through the cystic duct during LC.

Peer review

This was a well-designed retrospective study in which the authors compared the efficacy and safety of synchronous LC with LRV vs sequential LC with the conventional operation. The results are interesting and suggest that synchronous surgery has advantages, such as reducing complications, and shortening operation time and hospital stay.

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Contrast-enhanced ultrasonographic findings of hepatic paragonimiasis

Qiang Lu, Wen-Wu Ling, Lin Ma, Zi-Xing Huang, Chang-Li Lu, Yan Luo

Qiang Lu, Department of Ultrasound, Chinese Evidence-Based Medicine Center, West China Hospital of Sichuan University, Chengdu 610041, Sichuan Province, China

Wen-Wu Ling, Lin Ma, Yan Luo, Department of Ultrasound, West China Hospital of Sichuan University, Sichuan University, Chengdu 610041, Sichuan Province, China

Zi-Xing Huang, Department of Radiology, West China Hospital of Sichuan University, Chengdu 610041, Sichuan Province, China

Chang-Li Lu, Department of Pathology, West China Hospital of Sichuan University, Chengdu 610041, Sichuan Province, China
Author contributions: Lu Q, Ling WW, Ma L, Huang ZX, Lu CL and Luo Y designed and revised the manuscript; Lu Q wrote the manuscript.

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Correspondence to: Dr. Yan Luo, Department of Ultrasound, West China Hospital of Sichuan University, Sichuan University, No. 37, Guoxuexiang, Chengdu 610041, Sichuan Province, China. luoyand@126.com

Telephone: +86-28-85423192 Fax: +86-28-85422192

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Abstract

AIM: To investigate the features of hepatic paragonimiasis on contrast-enhanced ultrasound (CEUS) imaging.

METHODS: Fifteen patients with hepatic paragonimiasis who were admitted to our hospital between March 2008 and August 2012 were enrolled to this study. The conventional ultrasound and CEUS examinations were performed with a Philips IU22 scanner with a 1-5-MHz convex transducer. After conventional ultrasound scanning was completed, the CEUS study was performed. Pulse inversion harmonic imaging was used for CEUS. A bolus injection of 2.4 mL of a sulfur hexafluoride-filled

microbubble contrast agent (SonoVue) was administered. CEUS features were retrospectively reviewed and correlated with pathological findings.

RESULTS: In total, 16 lesions were detected on CEUS. The mean size of the lesions was 4.4 ± 1.6 cm (range, 1.7-6.6 cm). Subcapsular location was found in 12 lesions (75%). All the lesions were hypoechoic. Six lesions (37.5%) were of mixed content, seven (43.8%) were solid with small cystic areas, and the other three (18.8%) were completely solid. Ten lesions (62.5%) were rim enhanced with irregular tract-like nonenhanced internal areas. Transient wedge-shaped hyperenhancement of the surrounding liver parenchyma was seen in seven lesions (43.8%). Areas with hyper- or iso-enhancement in the arterial phase showed contrast wash-out and appeared hypoenhanced in the late phase. The main pathological findings included: (1) coagulative or liquefactive necrosis within the lesion, infiltration of a large number of eosinophils with the formation of chronic eosinophilic abscesses and sporadic distribution of Charcot-Leyden crystals; and (2) hyperplasia of granulomatous and fibrous tissue around the lesion.

CONCLUSION: Subcapsular location, hypoechogenicity, rim enhancement and tract-like nonenhanced areas could be seen as the main CEUS features of hepatic paragonimiasis.

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Key words: Paragonimiasis; Liver; Infection; Contrast-enhanced ultrasonography

Core tip: We retrospectively investigated the contrast-enhanced sonographic features of hepatic paragonimiasis. Hepatic paragonimiasis has its own features on contrast-enhanced ultrasound. Knowledge of these findings is helpful in differentiating hypoechoic lesions in the liver. When a subcapsular hypoechoic lesion with irregular tract-like non-enhancing necrosis is presented in non-

cirrhotic liver, the diagnosis of hepatic paragonimiasis should be suspected.

Lu Q, Ling WW, Ma L, Huang ZX, Lu CL, Luo Y. Contrast-enhanced ultrasonographic findings of hepatic paragonimiasis. *World J Gastroenterol* 2013; 19(13): 2087-2091 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v19/i13/2087.htm> DOI: <http://dx.doi.org/10.3748/wjg.v19.i13.2087>

INTRODUCTION

Paragonimiasis is a parasitic infestation caused by the lung fluke. Although the primary site of paragonimiasis is the lungs, ectopic infestation can occur in locations such as the brain, muscles, retroperitoneum, and liver^[1-6]. The liver is known to be an organ in which ectopic paragonimiasis may occur. Hepatic paragonimiasis often appears as a mass that should be differentiated from other cancerous lesions. Contrast-enhanced ultrasound (CEUS) has been widely used in characterization of focal liver lesions (FLLs)^[7-14]. The enhancement patterns of several FLLs have been described and are well known^[7,15-17]. However, to the best of our knowledge, the CEUS features of hepatic paragonimiasis have not been investigated or reported in the English-language literature. In this study, we retrospectively investigated the CEUS features of hepatic paragonimiasis.

MATERIALS AND METHODS

Patients

We retrospectively reviewed the results of conventional and CEUS examination of 15 patients with hepatic paragonimiasis who were admitted to our hospital between March 2008 and August 2012. There were eight men and seven women with a mean age of 42.5 ± 12.3 years (range, 29-65 years). All patients in this study were residents of China's Sichuan Province, which is an endemic area of paragonimiasis, especially the paragonimiasis skrjabini variety, and a majority of them (10/15) had a history of eating crayfish. The study was approved by the Ethical Committee of the hospital. All the patients underwent surgery and the diagnoses were confirmed histologically.

Ultrasound examination

The conventional ultrasound and CEUS examinations were performed with a Philips IU22 scanner (Philips Medical Solutions; Mountain View, CA, United States) with a 1-5-MHz convex transducer. The CEUS imaging technique used in this study was pulse inversion harmonic imaging. The mechanical index for CEUS was 0.06. After conventional ultrasound scanning was completed, the CEUS study was performed. A bolus injection of 2.4 mL sulfur hexafluoride-filled microbubble contrast agent (SonoVue; Bracco SpA, Milan, Italy) was administered through a 20-gauge needle placed in the antecubital vein.

A flush of 5 mL 0.9% sodium chloride solution was followed after the injection of SonoVue. On completion of the SonoVue injection, the timer was started simultaneously. The target lesion and surrounding liver parenchyma were observed continuously for 6 min. As previously described by Albrecht *et al*^[18], the arterial phase was defined as 7-30 s after contrast agent injection; the portal phase was 31-120 s after injection; and the late phase was 121-360 s after injection. The entire CEUS examination was stored as a dynamic digital video file on the hard disk of the ultrasound system and recorded on a digital video recorder. All of the procedures were performed by Lu Q or Luo Y who had > 5 years of experience of CEUS study of the liver.

Image analysis

The diameters and echogenicity of the tumors on conventional ultrasound were recorded. The enhancing pattern and enhancement level in different phases of CEUS imaging of the lesion were reviewed. The degree of enhancement was divided into nonenhancement, hypoenhancement, isoenhancement, and hyperenhancement according to the enhancement level of the lesion compared with that of the surrounding normal liver parenchyma. Contrast enhancement patterns were classified as homogeneous, heterogeneous, and rim enhancement.

RESULTS

CEUS findings

In total, 16 lesions were detected on CEUS. The mean size of the lesions was 4.4 ± 1.6 cm (range: 1.7-6.6 cm). Subcapsular location was found in 12 lesions (75%). All the lesions were hypoechoic. Six lesions (37.5%) were of mixed content, seven (43.8%) were solid with small cystic areas, and the other three (18.8%) were completely solid. Ten lesions (62.5%) were rim enhanced with irregular tract-like nonenhanced internal areas (Figure 1). Transient wedge-shaped hyperenhancement of the surrounding liver parenchyma was seen in seven lesions (43.8%). Areas with hyperenhancement or isoenhancement in the arterial phase showed contrast wash-out and appeared hypoenhanced in the late phase.

Pathological findings

Microscopy revealed that there was an egg present in one case, but no larvae were present in any of the lesions. There were areas of track-like or sinus structures. The main pathological findings included: (1) coagulative or liquefactive necrosis within the lesion, infiltration of a large number of eosinophils with the formation of chronic eosinophilic abscesses and sporadic distribution of Charcot-Leyden crystals; and (2) hyperplasia of granulomatous and fibrous tissue around the lesion.

DISCUSSION

Hepatic paragonimiasis is an infestation caused by inges-

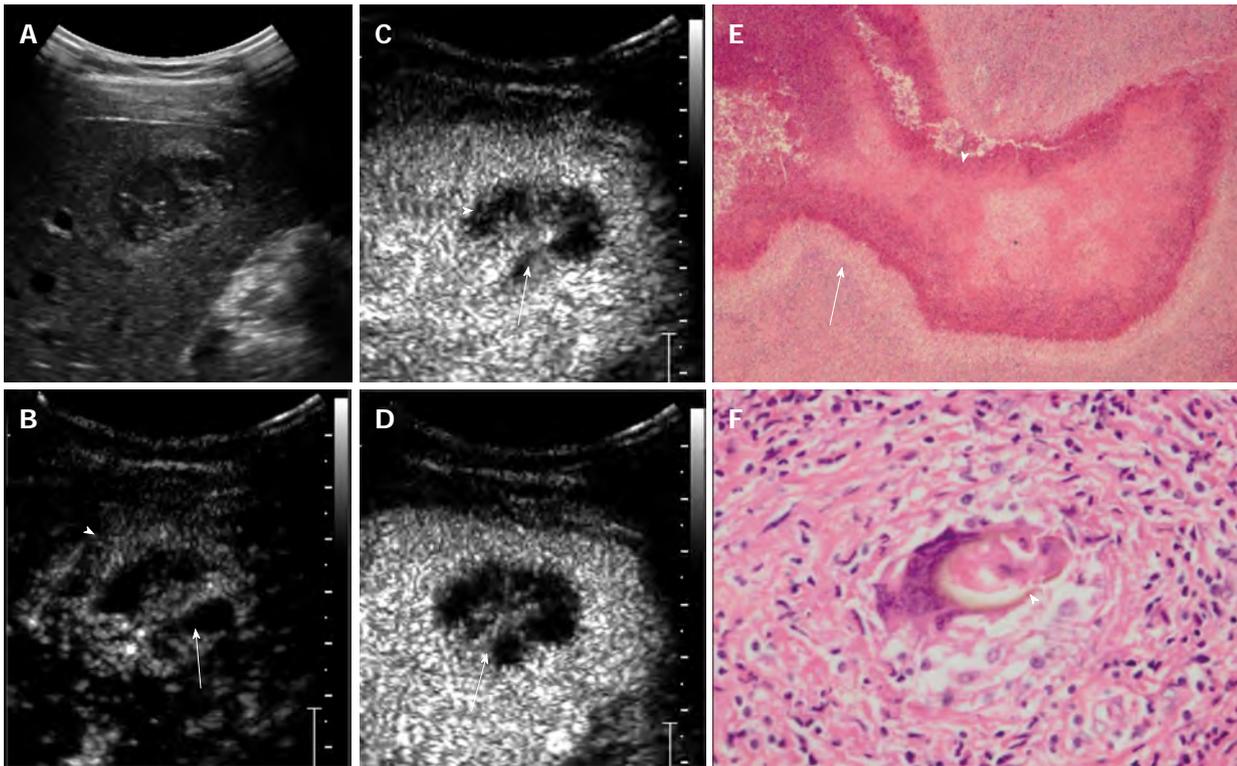


Figure 1 A 29-year-old woman with hepatic paragonimiasis. A: Hypoechoic lesion measuring 3.2 cm × 2.2 cm was seen in the right posterior inferior segment of the liver; B: Contrast-enhanced ultrasound showed rim enhancement (arrow head) and hyperenhanced internal septa (arrow) with irregular unenhanced areas in arterial phase; C and D: In portal phase (C) and late phase (D), contrast agent wash-out was seen at the enhanced septa (arrow), and the unenhanced area remained unenhanced (arrow head); E: Pathological findings showed coagulative necrosis (arrow head) within the lesion, surrounded by infiltration of a large number of barrier-like arrayed epithelioid cells (arrow); F: An egg (arrow head) was engulfed by a macrophage.

tion of raw or incompletely cooked freshwater crabs or crayfish infected with metacercariae. Only two species pathogenic to humans exist in Sichuan Province, namely, *Paragonimus skrjabini* and *Paragonimus westermani*^[19]. During the journey from the intestine to the lung where juvenile worms mature, the juvenile worms often cause damage to the liver capsule and parenchyma^[20,21]. Definitive diagnosis of paragonimiasis is based on the presence of eggs in patients' sputum or feces, or flukes in histological specimens. Polypide and eggs usually cannot be found in most of the lesions. However, with the epidemiological information, diagnosis can be made histopathologically^[22].

The lesion is often incidentally detected by ultrasonography in routine examination. Accurate diagnosis of suspected FLLs is important to determine the most effective therapy. If hepatic infection is correctly diagnosed, the need for surgery can be reduced or even avoided, compared with other abnormalities such as malignant tumors^[12,23].

Like other inflammatory lesions, hepatic paragonimiasis typically shows heterogeneous hyperenhancement in the arterial phase and hypoenhancement in the late phase on CEUS. Pathologically, the imaging feature of these lesions was eosinophilic abscesses, in which the enhanced septa in mixed-content lesions and enhanced area in solid lesions represented hyperplasia of granulomatous and fibrous tissue, whereas the unenhanced area represented necrotic debris, and Charcot-Leyden crystals.

The preponderance of subcapsular involvement and tract-like necrosis is characteristic and it may be attributed to the penetrating behavior of juvenile worms and eosinophilic abscess. The wedge-shaped enhancement in adjacent parenchyma in the arterial phase was similar to that reported by Kim *et al*^[5], and can be explained as inflammatory congestion adjacent to eosinophilic abscess^[19].

When a hypoechoic lesion in the liver is encountered by sonographic imaging, the differential diagnoses should include hepatocellular carcinoma, pyogenic abscesses, and hemangioma. In hepatocellular carcinoma, the hepatic parenchyma is more likely to be cirrhotic^[24]. Necrosis is readily visible by CEUS and is less common in small hepatocellular carcinoma. In pyogenic abscess, fever and pain in the right upper abdomen are more frequent^[12]. On CEUS, nonenhancing abscess and enhancing septa are often seen in pyogenic abscess, and lobulated abscess coalesces into a larger abscess cavity, whereas the eosinophilic abscess of hepatic paragonimiasis is irregular and arranged in tract-like fashion. Hepatic hemangioma may present as a hypoechoic lesion, whereas the CEUS manifestations typically show peripheral nodular enhancement in the arterial phase and gradual filling in the portal phase and hyperenhancement in late phase.

In our review of the literature, besides the imaging findings, blood eosinophilia was often seen in hepatic paragonimiasis patients, which was suggestive of parasitic

infection^[21]. For patients with symptoms of acute infection, praziquantel is the drug of choice to treat paragonimiasis, whereas partial liver resection is more suitable for those who have localized lesions without acute infection symptoms^[25,26].

The main limitation of this study was the small number of patients presented. Although hepatic paragonimiasis is rare, further investigation is mandatory.

In conclusion, hepatic paragonimiasis has its own features at CEUS. Thus, knowledge of these findings is helpful in differentiating hypoechoic lesions found in the liver. When a subcapsular hypoechoic lesion with irregular tract-like nonenhancing necrosis is present in noncirrhotic liver, diagnosis of hepatic paragonimiasis should be suspected.

COMMENTS

Background

Hepatic paragonimiasis is rare, but it often appears as a mass that should be differentiated from other cancerous lesions. Accurate diagnosis of suspected focal liver lesions (FLLs) is important to determine the most effective therapy. For hepatic infection, the need for surgery can be reduced or even avoided if it is correctly diagnosed, as compared with other abnormalities such as malignant tumors. Therefore, it is necessary to investigate the contrast-enhanced ultrasonography (CEUS) features of hepatic paragonimiasis.

Research frontiers

CEUS has been widely used in characterization of FLLs. The enhancement patterns of several FLLs have been described and are well known. However, the CEUS features of hepatic paragonimiasis have not been investigated or reported in the English-language literature.

Innovations and breakthroughs

The CEUS feature of hepatic paragonimiasis has been reported in this study. When a subcapsular hypoechoic lesion with irregular tract-like nonenhancing necrosis is present in noncirrhotic liver, a diagnosis of hepatic paragonimiasis should be suspected.

Applications

CEUS is a convenient and useful method for the detection and discrimination of hepatic paragonimiasis. Hepatic paragonimiasis could be better managed if ultrasound technicians and physicians are familiar with its features on CEUS.

Terminology

CEUS is the application of ultrasound contrast medium to traditional medical sonography. Microbubble contrast agents produce a unique sonogram with increased contrast due to the high echogenicity difference. CEUS can be used to image blood perfusion in organs.

Peer review

The authors described the CEUS findings of hepatic paragonimiasis. They analyzed 16 lesions of hepatic paragonimiasis, and demonstrated several specific findings. The article is well organized and well written.

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Missed diagnosis of early gastric cancer or high-grade intraepithelial neoplasia

Wei Ren, Jin Yu, Zhi-Mei Zhang, Yuan-Kun Song, Yi-Hui Li, Lei Wang

Wei Ren, Jin Yu, Zhi-Mei Zhang, Yuan-Kun Song, Yi-Hui Li, Lei Wang, Department of Gastroenterology, Xin Qiao Hospital, The Third Military Medical University, Chongqing 400037, China

Author contributions: Ren W wrote the paper; Yu J, Zhang ZM analyzed the data; Song YK, Li YH provided the analytic tools; Wang L designed the research.

Correspondence to: Lei Wang, MD, Departments of Gastroenterology, Xin Qiao Hospital, The Third Military Medical University, Chongqing 400037, China. butterfly131@126.com

Telephone: +86-23-68774665 Fax: +86-23-68774665

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Abstract

AIM: To investigate the causes of missed diagnosis of early gastric cancer (EGC) or high-grade intraepithelial neoplasia (HGIN) in Chongqing, China.

METHODS: The present study summarizes 103 cases of EGC/HGIN detected by esophagogastroduodenoscopy (EGD) and pathological analysis from January 2010 to December 2011. Dimethyl silicone oil was administered orally 15 min before the EGD procedures. The stomach was cleaned by repeated washing with saline when the gastroscope entered the stomach cavity. Suspected EGC lesions were subject to conventional biopsy sampling and pathological examinations. The correlation between lesion locations, endoscopic morphology of cancerous sites, training level of the examiners, pathological biopsies, and missed diagnosis was analyzed.

RESULTS: Twenty-three cases were missed among the 103 cases (22.23%) of EGC/HGIN. The rate of missed EGC in the gastroesophageal junction (8/19, 42.1%) was significantly higher than at other sites (15/84, 17.86%) ($\chi^2 = 5.253$, $P = 0.022$). In contrast, the rate of missed EGC in the lower stomach body (2/14, 14.29%) was lower than at other sites (21/89,

23.6%), but there were no significant differences ($\chi^2 = 0.289$, $P = 0.591$). The rate of missed EGC in the gastric antrum (5/33, 15.15%) was lower than at other sites (18/70, 25.71%), but there were no significant differences ($\chi^2 = 1.443$, $P = 0.230$). Endoscopists from less prestigious hospitals were more prone to not diagnosing EGC than those from more prestigious hospitals ($\chi^2 = 4.261$, $P = 0.039$). When the number of biopsies was < 4 , the rate of missed diagnosis was higher (20/23, 89.96%) than for when there were > 4 biopsies (3/23, 13.04%) ($P < 0.001$). In addition, there was no significant difference in the rate of missed diagnosis in patients with 1-3 biopsy specimens ($\chi^2 = 0.141$, $P = 0.932$).

CONCLUSION: Endoscopists should have a clear understanding of the anatomical characteristics of the esophagus/stomach, and endoscopic identification of early lesions increases with the number of biopsies.

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Key words: Missed diagnosis; Early gastric cancer; High-grade intraepithelial neoplasia; Endoscopic diagnosis; Biopsies

Core tip: Early gastric cancer (EGC) detection rate in China is much lower than that in Japan, where $> 80\%$ of EGC is detected. How to avoid missed diagnosis of EGC is most important for digestive endoscopy practice. We found that there were many influencing factors for missed diagnosis of EGC. The most critical issue for endoscopists to avoid missed diagnosis is being cautious about each individual patient.

Ren W, Yu J, Zhang ZM, Song YK, Li YH, Wang L. Missed diagnosis of early gastric cancer or high-grade intraepithelial neoplasia. *World J Gastroenterol* 2013; 19(13): 2092-2096 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v19/i13/2092.htm> DOI: <http://dx.doi.org/10.3748/wjg.v19.i13.2092>

INTRODUCTION

Gastric cancer (GC) is one of the most common malignant carcinomas, which is highly prevalent worldwide. There were 989 600 estimated new cases and 738 000 deaths in 2008, and > 70% of new cases and deaths occurred in developing countries^[1-5]. Although the incidence of GC has been declining in recent years, China still has the most GC patients in the world. Recent statistics show that > 400 000 new cases of GC are confirmed in China annually^[6-8]. The prognosis of advanced GC is poor and its 5-year survival rate is only 20%-40%, but there could be a 5-year survival rate of 90% for early gastric cancer (EGC) after surgical treatment^[9]. Therefore, timely and accurate diagnosis is important for the treatment and prognosis of patients with GC. However, detection rate of EGC in China is generally about 2%-5%. Although at some hospitals in Shanghai, the detection rate of EGC has increased to 20%-28% in recent years, the detection rate is still significantly lower than that in Japan or South Korea^[10,11]. Therefore, it is imperative for endoscopists to make efforts to improve the detection rate of EGC and reduce missed diagnosis in China^[12]. Our study summarizes the missed cases among 103 patients with EGC or high-grade intraepithelial neoplasia (HGIN) admitted to our hospital from 2010 to 2011, and explored the causes of missed diagnosis.

MATERIALS AND METHODS

General information

From January 2010 to December 2010, gastroscopic examinations were performed on 21 500 patients in Xin Qiao Hospital, Chongqing, China and 245 of these were diagnosed with GC. EGC/ HGIN accounted for 17.56% (43/245) of all the cases of GC. From January to December 2011, gastroscopic examinations were performed on 23 000 patients and 230 were found to have GC, and 26.08% (60/230) of them had EGC/HGIN. There were 69 men and 34 women, aged 44-79 years, with an average age of 60.2 years.

Examination methods

In this study, all cases were examined by gastroscopy (Olympus H260 and PENTAX EPK-i-san, Japan). The date of examination and the results, along with the attending doctor and hospital, were recorded. Dimethyl silicone oil was administered orally, 15 min before the esophagogastroduodenoscopy procedures. The stomach was cleaned by repeated washing with saline, when the gastroscope entered the stomach cavity. Suspected EGC lesions were subject to conventional biopsy sampling and pathological examination. The shape and location of the lesions, as well as the extent and site of the biopsies were recorded. Endoscopic diagnosis was performed in accordance with the Paris endoscopic classification of superficial neoplastic lesions^[13]. Pathological diagnosis of EGC/HGIN followed the 2010 version of the World Health Organization classification of tumors of the digestive system^[14].

Missed diagnosis was defined as follows. Patients who were previously diagnosed with other diseases (*e.g.*, gastric polyps or chronic gastritis) at two examinations at < 3 mo apart, and were later confirmed to have EGC/HGIN.

Statistical analysis

Data analysis was conducted using SPSS 20.0 software (Chicago, IL, United States). Comparison between the groups was performed by using χ^2 test or Fisher's exact probability test and $P < 0.05$ was considered statistically significant.

RESULTS

Different lesion locations correlated with different rates of missed diagnosis of EGC/HGIN

There were 23 cases of EGC/HGIN that were not found by endoscopy but were diagnosed later by pathological examination, so the overall rate of missed EGC/HGIN was 22.23% (23/103) (Table 1). In detail, 42.1% (8/19) of cases of EGC/HGIN that occurred in the gastroesophageal junction were missed, and the rate was higher than for other parts of the stomach (15/84, 17.86%; $\chi^2 = 5.253$, $P = 0.022$). The rate of missed EGC in the lower stomach body (2/14, 14.29%) was lower than at other sites (21/89, 23.60%), but there were no significant differences ($\chi^2 = 0.289$, $P = 0.591$). The rate of missed EGC in the gastric antrum (5/33, 15.15%) was also lower than at other sites (18/70, 25.71%), but there were no significant differences ($\chi^2 = 1.443$, $P = 0.230$).

Endoscopists from hospitals of different standing had different rates of missed diagnosis

Among the 23 missed cases of EGC/HGIN, 15 were found to have no abnormalities or were diagnosed with other diseases (*e.g.*, gastric polyps or chronic gastritis) by endoscopy in the less prestigious hospitals (15/23, 65.21%), but were later diagnosed with EGC/HGIN in our hospital (a more prestigious hospital). The other eight cases (8/23, 34.78%) were initially diagnosed with other diseases by endoscopy physicians in our hospital and then with EGC/HGIN after further examinations. The rate of missed diagnosis of EGC/HGIN by endoscopists from less prestigious hospitals was higher than that from more prestigious hospitals ($\chi^2 = 4.261$, $P = 0.039$) (Table 2).

Endoscopic appearance of cancerous lesions affected missed diagnosis of EGC/HGIN

The rate of missed diagnosis of 0-IIc type lesions was 91.3% (21/23), which was higher than that for 0-I (1/23, 4.35%) or 0-IIb (1/23, 4.35%) lesions. However, one 0-IIb lesion in the lesser curvature was missed. At the first gastroscopy examination, no cancerous lesions were found and the patient was treated for gastritis for 1 mo but the symptoms did not improve. The second gastroscopic examination was performed at the request of the

Table 1 Missed diagnosis of early gastric cancer or high-grade intraepithelial neoplasia in different parts of the stomach *n* (%)

| Locations | Total EGC/HGIN | Missed cases |
|---------------------------|----------------|------------------------|
| Gastroesophageal junction | 19 | 8 (42.10) ^a |
| Upper stomach body | 12 | 2 (16.67) |
| Middle stomach body | 11 | 3 (27.27) |
| Lower stomach body | 14 | 2 (14.29) ^b |
| Antrum of stomach | 33 | 5 (15.15) ^b |
| Gastric angle | 14 | 3 (21.42) |
| Total | 103 | 23 (22.33) |

^a*P* < 0.05 *vs* non-missed diagnosis cases; ^bNo statistical differences. EGC: Early gastric cancer; HGIN: High-grade intraepithelial neoplasia.

Table 2 Early gastric cancer or high-grade intraepithelial neoplasia missed by endoscopists at our and other hospitals

| Lesion locations | Other hospitals | Our hospital |
|---------------------------|-----------------|--------------|
| Gastroesophageal junction | 7 | 1 |
| Upper stomach body | 1 | 1 |
| Middle stomach body | 2 | 1 |
| Lower stomach body | 1 | 1 |
| Antrum of stomach | 2 | 3 |
| Gastric angle | 2 | 1 |
| Total | 15 | 8 |

Table 3 Number of biopsies and missed diagnosis *n* (%)

| Biopsies | Missed cases |
|----------|--------------|
| 1 | 7 (30.43) |
| 2 | 7 (30.43) |
| 3 | 6 (26.09) |
| ≥ 4 | 3 (13.64) |
| Total | 23 (100.00) |

patient's family, and flaky red regions were observed in the middle of the gastric body near the lesser curvature. It was further confirmed as intramucosal differentiated-type GC by pathological diagnosis after endoscopic submucosal dissection.

More biopsies resulted in less missed diagnosis

There were 20 patients in whom diagnosis was missed from 1-3 biopsy specimens. The rate of missed diagnosis was 86.96% (20/23). There were three patients in whom diagnosis was missed with four biopsy specimens. The rate of missed diagnosis was 13.04% (3/23). When the number of biopsies was < 4, the rate of missed diagnosis (20/23, 86.96%) was higher than for > 4 biopsies (3/23, 13.04%) (*P* < 0.001). In addition, there was no significant difference in the rate of missed diagnosis in patients with 1, 2 or 3 biopsy specimens ($\chi^2 = 0.141$, *P* = 0.932) (Table 3).

DISCUSSION

The incidence of GC is about 30/100 000 in East Asian countries including China and Japan^[1]. In some regions

of China, the incidence even exceeds 100/100 000^[15,16]. Every year, mass screening in Japan shows the presence of GC in a low proportion of patients receiving gastroscopic examination. It has been reported that in some Japanese hospitals that the diagnosed cases of GC account for only approximately 0.4% of the gastroscopy examinations each year^[17], but the incidence of GC was 1%-1.2% in our endoscopy center, which was significantly higher than that in Japan. In addition, although there are no accurate statistics for EGC detection rate, it is believed that the rate in China is much lower than that in Japan, where > 80% of EGC is detected^[17]. Many factors contribute to the low detection rate of EGC in China, but how to avoid missed diagnosis of EGC is important for digestive endoscopy practice.

We found that lesion location, training level of doctors (doctors from less prestigious hospitals and fewer years of endoscopy experience are considered to have a low level of training), lesion morphology, and the number of biopsies can affect the diagnosis when EGC is not identified. Previous studies have shown that lesion location has a significant effect on EGC missed diagnosis. Hosokawa *et al.*^[18-20] have conducted a survey in Fukui Hospital, where 562 cases of GC were diagnosed from 51 411 (1.05%) gastroscopic examinations, and 188 cases were confirmed as GC within the next 3 years, with an overall missed diagnosis rate of 25.8%. They have also found that doctors with < 10 years experience as an upper gastrointestinal endoscopist missed 32.4% of GC cases, but the missed diagnosis rate was only 19.5% when doctors with > 10 years of experience conducted the examination (*P* < 0.01).

Importantly, EGC in the gastric cardia or body, especially at the lesser curvature or posterior wall, is usually overlooked^[18,19]. Consistent with this, we found that the rate of missed EGC in the gastroesophageal junction near the stomach side was significantly higher than at other sites. Due to the anatomical structure of the cardia, cancerous lesions in these parts are often difficult to observe when the endoscope is withdrawn or reversed. This requires that the endoscopists should carefully investigate the morphological changes in the gastric fundus near the cardia. On one hand, we should not withdraw the gastro-scope too quickly. On the other hand, to avoid the shield of scope itself, it is necessary to observe from both sides of the scope when it is reversed.

Additionally, we found that the proportion of EGC occurring in the gastroesophageal junction was higher than that previously reported. This may have been due to the increased incidence of GC at this site, which needs further large epidemiological investigations. Compared to other sites, the gastric antrum is easy to expose and the rate of missed diagnosis was lower at this site. The rate of missed diagnosis in the gastric antrum was still approximately 15%, therefore, every part of the stomach should be fully and carefully investigated. We also noticed that there was a significant difference in missed diagnosis of EGC between doctors with different training levels in endoscopy, suggesting that the standardization

of endoscopy records and long-term cognitive training for EGC are crucial. The main cause of missed diagnosis of EGC in many primary hospitals is the inadequate knowledge and cognitive ability^[20]. For example, in our study there were several cases of EGC that were misdiagnosed as gastric erosion. Thus, it is urgent for physicians to strengthen their endoscopic training for diagnosis of EGC. Endoscopic appearance also has a significant effect on EGC diagnosis. Compared to the protruding lesions, the depressed lesions were more prone to be missed. This may have been due to the high proportion of depressed lesions in EGC, because we found that superficially depressed lesions accounted for the vast majority of all cases of EGC. However, the rate of missed diagnosis of EGC 0-IIc lesions (IIc, IIc + IIa, IIa + IIc) was still significantly higher than that of 0-II or 0-III lesions. Although the rate of missed diagnosis of 0-IIb EGC was 100% (1/1) in this study, it still calls for more observation on a larger scale. However, we can conclude that 0-II lesions are more easily missed than 0-I and 0-III lesions. Number of biopsies also affected the rate of missed diagnosis. For cases with ≥ 4 biopsies, the rate of missed diagnosis was significantly lower than for those with < 4 biopsies^[21]. In our study, no patients were subjected to other techniques, such as chromoendoscopy, narrow-band imaging (NBI), magnified endoscopy, and NBI + magnified endoscopy, because these new techniques have not been widely adopted in most hospitals in China. If we use these techniques, the results will be better^[22-25]. In addition, whether targeted biopsy sampling can increase the positive rate of EGC/HGIN requires further research.

In summary, there are many influencing factors for the missed diagnosis of EGC. We agreed with Axon that the most critical issue for endoscopists to avoid missed diagnosis is being cautious about each individual patient^[26].

COMMENTS

Background

Gastric cancer (GC) is one of the most common malignant carcinomas. China has the most GC patients in the world. The prognosis of advanced GC is poor but there could be a 90% 5-year survival rate for early gastric cancer (EGC) after surgical treatment. However, detection rate of EGC in China is very low.

Research frontiers

Research of EGC is a hotspot in digestive diseases at present and how to improve the diagnosis rate of EGC is a key problem. This study aimed to analyze the reasons for missed diagnosis of EGC and provide methods to avoid missed diagnosis. With rapid EGC research and development, more new technology will apply.

Innovations and breakthroughs

Few studies have been carried out focusing on the rate of diagnosis of EGC, due to lack of recognition. The present study was a detailed and systematic study in this field. Furthermore, the study also provides a brighter future in the diagnosis and treatment of EGC, with the development of understanding and new technology.

Applications

This study provides reference data for the diagnosis of EGC. It can be applied to gastroenterologists in hospitals of different rank. There are many influencing factors for missed diagnosis of EGC, but the most critical issue for endoscopists

to avoid missed diagnosis is to be cautious about each individual patient.

Peer review

This is an important analysis of the factors involved in the missed diagnosis of EGC. The authors should use other important techniques, for example: chromoendoscopy, narrow-band imaging (NBI), magnified endoscopy, and NBI + magnified endoscopy. These techniques could change the results.

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Chemotherapy and resection for gastric cancer with synchronous liver metastases

Lei Chen, Ming-Quan Song, Hui-Zhong Lin, Lin-Hua Hao, Xiang-Jun Jiang, Zi-Yu Li, Yu-Xin Chen

Lei Chen, Hui-Zhong Lin, Department of Surgery, Qingdao Municipal Hospital, Qingdao 266011, Shandong Province, China

Lei Chen, Shandong University School of Medicine, Jinan 250012, Shandong Province, China

Ming-Quan Song, Xiang-Jun Jiang, Department of Gastroenterology, Qingdao Municipal Hospital, Qingdao 266011, Shandong Province, China

Lin-Hua Hao, First Institute of Oceanography, State Oceanic Administration, Qingdao 266011, Shandong Province, China

Zi-Yu Li, Department of Surgery, Key Laboratory of Carcinogenesis and Translational Research (Ministry of Education), Peking University School of Oncology, Beijing Cancer Hospital and Institute, Beijing 100142, China

Yu-Xin Chen, Department of Surgery, Qilu Hospital Affiliated to Shandong University School of Medicine, Jinan 250012, Shandong Province, China

Author contributions: Chen L, Song MQ and Lin HZ contributed equally to this work; Chen L, Song MQ and Jiang XJ designed the research; Chen L, Lin HZ, Li ZY and Chen YX performed the research; Hao LH analyzed the data; Chen L, Song MQ and Chen YX wrote the paper.

Correspondence to: Yu-Xin Chen, Professor, Department of Surgery, Qilu Hospital Affiliated to Shandong University School of Medicine, 107 Wenhuxi Road, Jinan 250012, Shandong Province, China. yxchen@sdu.edu.cn

Telephone: +86-532-82789562 Fax: +86-532-82789561

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Abstract

AIM: To investigate the effect of surgery and chemotherapy for gastric cancer with multiple synchronous liver metastases (GCLM).

METHODS: A total of 114 patients were entered in this study, and 20 patients with multiple synchronous liver metastases were eligible. After screening with preoperative chemotherapy, 20 patients underwent curative gastrectomy and hepatectomy for GCLM; 14 underwent major hepatectomy, and the remaining six underwent

minor hepatectomy. There were 94 patients without aggressive treatment, and they were in the non-operative group. Two regimens of perioperative chemotherapy were used: S-1 and cisplatin (SP) in 12 patients, and docetaxel, cisplatin and 5-fluorouracil (DCF) in eight patients. These GCLM patients were given preoperative chemotherapy consisting of two courses chemotherapy of SP or DCF regimens. After chemotherapy, gastrectomy and hepatectomy were performed. Evaluation of patient survival was by follow-up contact using telephone and outpatient records. All patients were assessed every 3 mo during the first year and every 6 mo thereafter.

RESULTS: Twenty patients underwent gastrectomy and hepatectomy and completed their perioperative chemotherapy and hepatic arterial infusion before and after surgery. Ninety-four patients had no aggressive treatment of liver metastases because of technical difficulties with resection and severe cardiopulmonary dysfunction. In the surgery group, there was no toxicity greater than grade 3 during the course of chemotherapy. The response rate was 100% according to the Response Evaluation Criteria in Solid Tumors Criteria. For all 114 patients, the overall survival rate was 8.0%, 4.0%, 4.0% and 4.0% at 1, 2, 3 and 4 years, respectively, with a median survival time (MST) of 8.5 mo (range: 0.5-48 mo). For the 20 patients in the surgery group, MST was 22.3 mo (range: 4-48 mo). In the 94 patients without aggressive treatment, MST was 5.5 mo (range: 0.5-21 mo). There was a significant difference between the surgery and unresectable patients ($P = 0.000$). Three patients in surgery group were still alive at the end of the cut-off date.

CONCLUSION: Perioperative weekly DCF and SP achieved a good response, and combined with surgery, they could improve prognosis of GCLM.

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Key words: Gastric cancer; Liver metastases; Surgery; Chemotherapy; Pilot study

Core tip: We investigated the effect of surgery and chemotherapy for gastric cancer with multiple synchronous liver metastases (GCLM). Perioperative weekly docetaxel, cisplatin and 5-fluorouracil and S-1 and cisplatin achieved a good response, and combined with surgery, they could improve prognosis of GCLM.

Chen L, Song MQ, Lin HZ, Hao LH, Jiang XJ, Li ZY, Chen YX. Chemotherapy and resection for gastric cancer with synchronous liver metastases. *World J Gastroenterol* 2013; 19(13): 2097-2103 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v19/i13/2097.htm> DOI: <http://dx.doi.org/10.3748/wjg.v19.i13.2097>

INTRODUCTION

Surgery for gastric cancer with multiple synchronous liver metastases (GCLM) is a major challenge to every surgeon; not only because of coexisting factors, but each GCLM patient has his/her own clinicopathological features. It is difficult to determine the suitable candidates for treatment. At present, the justification for surgical resection is still controversial^[1], and the prognosis is dismal. In contrast, for patients with colorectal carcinoma with liver metastases, a second liver resection is safe and feasible. Hepatic resection has been widely accepted as a potentially curative approach in patients with liver metastases of colorectal carcinoma^[2].

One study demonstrated that patients with GCLM limited to one lobe, who underwent radical gastrectomy with D2 lymphadenectomy, had the most favorable outcomes following hepatic surgical treatment^[3]. A further study found that the number of metastases was no longer considered to be an important predictor of long-term survival^[4]. Some positive effect of liver resection in these patients seemed to imply that hepatic surgical treatment should be recommended for appropriate GCLM candidates^[5-7]. The United Kingdom myoblast autologous grafting in ischemic cardiomyopathy (MAGIC) trial of perioperative chemotherapy in gastric cancer found that perioperative systemic chemotherapy improved 5-year survival from 23% to 36%^[8], compared with surgery alone. What is the optimal dosing appropriate for Chinese patients, and how do we schedule perioperative chemotherapy that could improve tolerability while maintaining efficacy? In our previous pilot study, we found that liver resection combined with a weekly docetaxel-based regimen (docetaxel, cisplatin and 5-fluorouracil, DCF) were well tolerated, with a good response. In the present study, we assessed more GCLM patients who underwent aggressive treatment, in comparison with non-surgical treatment.

MATERIALS AND METHODS

From July 2007 to October 2012, 1821 patients with

gastric cancer were treated in Beijing Cancer Hospital of Beijing University and Qingdao Municipal Hospital. Only patients with adenocarcinoma were enrolled in this study. Among these patients, 114 developed multiple liver metastases. The inclusion and exclusion criteria are described in our previous study^[9]. Patients had adequate physical condition and received two course of preoperative chemotherapy. After effective screening with preoperative chemotherapy, 20 patients underwent curative gastrectomy and hepatectomy for GCLM. Two regimens of perioperative chemotherapy were used. Twelve patients received the S-1 and cisplatin (SP) regimen: 40 mg S-1 orally, twice daily for 3 consecutive weeks, and 60 mg/m² cisplatin intravenously on day 8, followed by a 2-wk rest period, within a 5-wk cycle^[10]. Eight patients received the DCF regimen: 20 mg/m² cisplatin over 1 h; 20 mg/m² docetaxel, over 30 min; and 350 mg/m² 5-fluorouracil over 15 min on day 1. This was administered weekly for 6 wk, followed by a 2-wk break^[9].

According to the Japanese Research Society for Gastric Cancer guidelines, our surgical procedure was total or subtotal gastrectomy, at a minimum of 5 cm clearance. Hepatic resection with D2 lymphadenectomy was performed^[11].

After surgery, two courses of chemotherapy (SP or DCF regimen) were administered. After completion of chemotherapy, patients without other distant disease, except for hepatic metastasis, underwent hepatic arterial infusion (HAI). If liver lesions progressed in the course of postoperative chemotherapy, HAI was commenced immediately. Safety evaluation was standardized by the National Cancer Institute Common Terminology Criteria for Adverse Events version 4.0 (May 28, 2009). Evaluations were classified by the Response Evaluation Criteria in Solid Tumors (RECIST) guidelines^[12]. The study was approved by the medical ethics committees of Qingdao Municipal Hospital and Beijing Cancer Hospital. Written informed consent was obtained according to the principles of the institution.

Evaluation of patient survival was by follow-up contact using telephone and outpatient records. All patients were assessed every 3 mo during the first year and every 6 mo thereafter. Patient follow-up lasted until death or the cut-off date of October 1, 2012. Three patients (2.6%) were lost to follow-up, and survival information was censored at their last visit. Four (3.5%) patients were still alive and were censored at the cut-off date. The median follow-up period for the 114 patients was 10 mo (range: 2-53 mo).

Statistical analysis

Statistical analysis was performed with SPSS version 13.0 (SPSS, Chicago, Illinois, United States). For univariate analysis, binomial and categorical data were evaluated by cross-linked tables and the Fisher's exact test. Results were regarded as being statistically significant when $P < 0.05$. For survival analysis, the Kaplan-Meier method was used.

Table 1 Clinicopathological characteristics of patients with and without hepatectomy

| Clinicopathological characteristics | With hepatectomy | Without hepatectomy |
|--|------------------|---------------------|
| Sex | | |
| Male | 12 | 59 |
| Female | 8 | 35 |
| Primary gastric tumors | | |
| Median diameter of primary gastric tumors (cm) | 4.3 (2.4-8.8) | 4.5 (2.1-9.3) |
| Tumor location | | |
| Upper | 7 | 27 |
| Lower | 13 | 67 |
| Pathological T-stage of the primary ¹ | | |
| pT1 | 2 | 9 |
| pT2 | 4 | 21 |
| pT3 | 12 | 51 |
| pT4 | 2 | 13 |
| N stage of the primary tumor | | |
| N0 | 3 | 18 |
| N1 | 9 | 48 |
| N2 | 5 | 21 |
| N3 | 3 | 7 |
| Differentiation of the primary tumor | | |
| Well | 2 | 9 |
| Moderate | 14 | 64 |
| Poor | 4 | 21 |
| Liver metastases | | |
| Median diameter of liver metastases (cm) | 4.1 (1.7-16) | 4.5 (1.5-18) |
| No. of metastases | | |
| Solitary | 8 | 43 |
| ≥ 2 | 12 | 51 |
| Vascular invasion of metastases | | |
| Present | 3 | 26 |
| Absent | 17 | 68 |
| Site of metastases | | |
| Left lobe | 4 | 18 |
| Right lobe | 7 | 31 |
| Bilobar | 9 | 45 |
| Interruption of hepatic hilum | | |
| Present | 5 | 28 |
| Absent | 15 | 66 |

¹According to tumor-nodes-metastasis-classification.

RESULTS

Patient characteristics

The mean age of the 114 patients was 56.7 years (range: 33-75 years), and the male to female ratio was 3.1:1. Twenty patients underwent gastrectomy and hepatectomy. These 20 patients completed their perioperative chemotherapy and HAI before and after surgery. The other 94 patients were not considered for aggressive treatment of liver metastases. In most cases ($n = 91$), the reason for deciding against aggressive treatment was patient refusal; the remaining three were not eligible for surgery due to severe cardiopulmonary dysfunction. There was no perioperative mortality. There were no obviously different clinicopathological characteristics between patients with and without hepatectomy.

Surgery

There were 12 male and 8 female patients in the surgery

Table 2 Response evaluation after first two courses of preoperative chemotherapy

| No. of cases | Diameter of metastases (mm ³) | | Evaluation of response (according to RECIST) | Adverse events grade |
|--------------|---|-----------|--|----------------------|
| | Pre-chem | Post-chem | | |
| 1 | 128 | 0-10 | CR | 2 |
| 2 | 135 | 56 | PR | 3 |
| 3 | 188 | 65 | PR | 1 |
| 4 | 64 | 22 | PR | 2 |
| 5 | 48 | 24 | PR | 2 |
| 6 | 148 | 38 | PR | 1 |
| 7 | 205 | 83 | PR | 1 |
| 8 | 162 | 65 | PR | 2 |
| 9 | 78 | 30 | PR | 2 |
| 10 | 228 | 94 | PR | 3 |
| 11 | 206 | 108 | PR | 2 |
| 12 | 144 | 56 | PR | 1 |
| 13 | 67 | 41 | PR | 1 |
| 14 | 104 | 67 | PR | 3 |
| 15 | 163 | 103 | PR | 2 |
| 16 | 134 | 92 | PR | 3 |
| 17 | 88 | 61 | PR | 3 |
| 18 | 225 | 134 | PR | 2 |
| 19 | 143 | 96 | PR | 2 |
| 20 | 78 | 43 | PR | 3 |

When the number of liver lesions was > 5, the diameters of the five largest lesions were summed. RECIST: Response Evaluation Criteria in Solid Tumors; CR: Complete response; PR: Partial response; Pre-chem: Pre-chemotherapy; Post-chem: Post-chemotherapy.

group. The median age of this group was 54 years (range: 31-74 years). Seventeen patients had a lymph-node-positive stage of the primary tumor, and only three had no lymph node involvement. There were 13 patients with distal gastric cancer, seven had proximal gastric cancer, and nine had bilobar metastases. The clinicopathological characteristics of the patients who underwent hepatectomy are listed in Table 1.

The patients in the surgery group finished two courses of SP or DCF chemotherapy before the operation. In the two courses of chemotherapy with different regimens, no patients had toxicity greater than grade 3. The most common adverse effects in the two regimens were diarrhea, nausea, leukopenia, neutropenia and thrombocytopenia, at grade 1 or 2 intensity. Most adverse effects could be modified by premedication, such as dexamethasone and antiemetics. Granulocyte colony-stimulating factor support was given to 12 patients. Response to treatment was assessed by monthly magnetic resonance imaging or computed tomography. All patients achieved a partial response according to the RECIST^[12] criteria (Table 2). The response rate was 100% according to the RECIST (Figures 1 and 2). There was no treatment-related mortality.

We performed gastric and liver resection only in cases that were potentially curative. The common complications in the perioperative course were impaired wound healing (surgical therapy in two patients), and pleural effusion in four. Fourteen patients underwent major hepatectomy (hepatic resection of more than three segments:

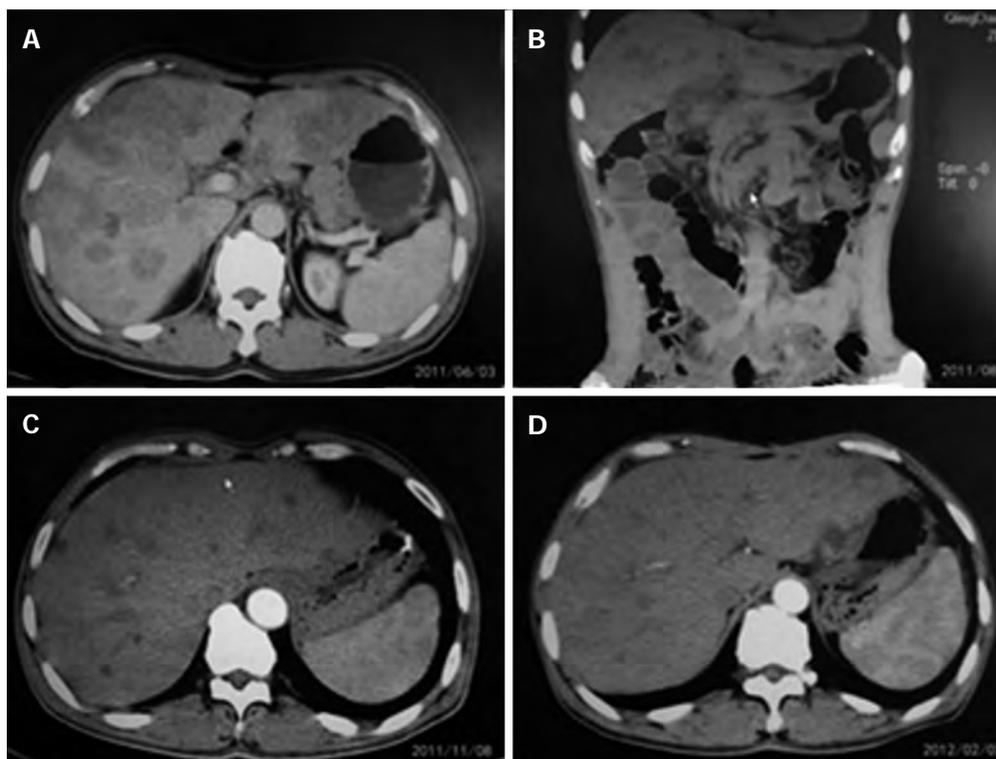


Figure 1 Patients with complete response. A: Abdominal computed tomography (CT) in gastric cancer with multiple synchronous liver metastases (GCLM) patient treated with preoperative chemotherapy (June 3, 2011); B: Abdominal CT in patient with GCLM after neoadjuvant chemotherapy (August 29, 2011); C: Abdominal CT in patient with GCLM after neoadjuvant chemotherapy (November 8, 2011); D: Abdominal CT in patient with GCLM after neoadjuvant chemotherapy (February 2, 2012).

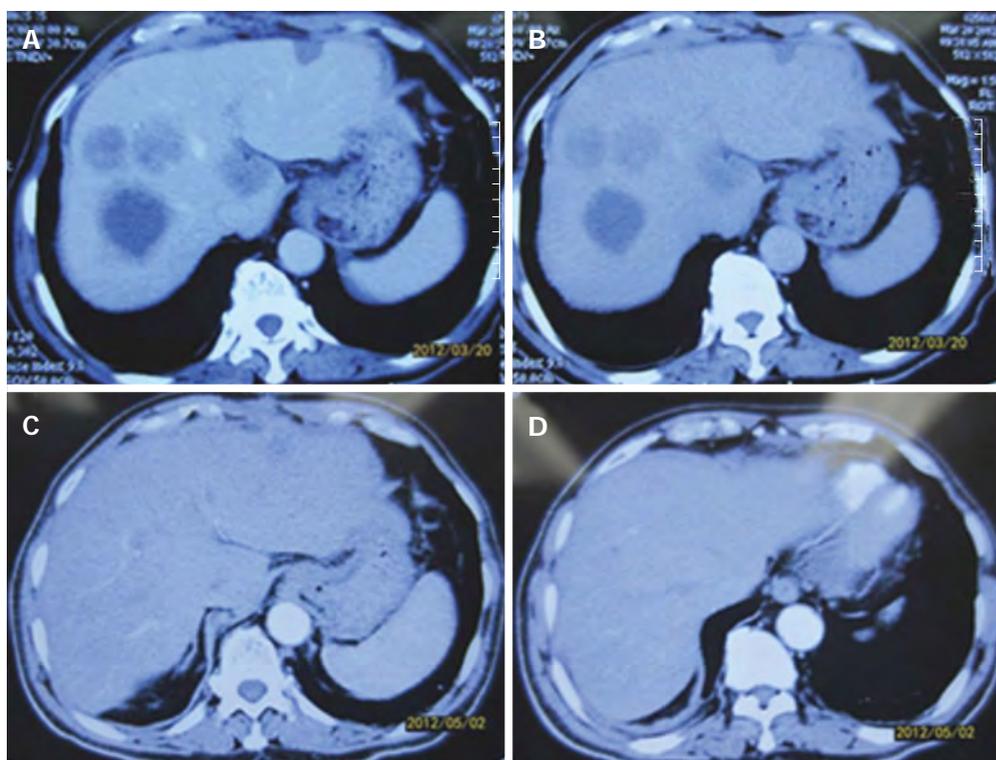


Figure 2 Patients with partial response. A, B: Abdominal computed tomography (CT) in patients with gastric cancer with multiple synchronous liver metastases (GCLM) after preoperative chemotherapy; C, D: Abdominal CT in patients with GCLM after neoadjuvant chemotherapy.

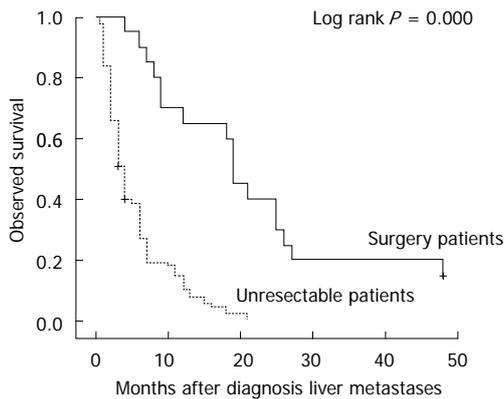


Figure 3 Overall survival of patients with hepatic metastases from gastric cancer.

hemihepatectomy in 12 and trisectionectomy in 2); and the remaining six patients underwent minor hepatectomy (sectionectomy in 2 and limited resection in 4). The types of hepatectomy were classified according to the Brisbane 2000 terminology^[13].

Survival rate in surgery and nonoperative groups

For all 114 patients, the overall survival rate was 8.0%, 4.0%, 4.0% and 4.0% at 1, 2, 3 and 4 years, respectively, with an median survival time (MST) of 8.5 mo (range: 0.5-48 mo). For the 20 patients in the surgery group, MST was 22.3 mo (range: 4-48 mo). In the 94 patients without aggressive treatment, MST was 5.5 mo (range: 0.5-21 mo). A significant difference was observed between the surgery and nonoperative patients ($P = 0.000$, Figure 3). Three patients in the surgery group were still alive at the end of the cut-off date.

DISCUSSION

We reviewed retrospectively 20 macroscopically complete liver resections for patients with GCLM at two institutions. After hepatectomy, their MST was 22.3 mo. These results compare favorably with patients without surgery, whose MST was only 5.5 mo. The survival time in patients with hepatectomy was longer than in those without hepatectomy. However, our MSTs were shorter than the 34 mo reported by Takemura *et al.*^[14]. The discrepancy may have been caused by the different operating procedures. In the Takemura *et al.*^[14] study, 14/64 (21.9%) patients underwent major hepatectomy and the remaining 50 (78.1%) minor hepatectomy. In our study, 70% patients had major hepatectomy and 30% had minor hepatectomy. Both studies indicate that hepatectomy is beneficial for some patients with GCLM despite the remaining controversy surrounding surgical resection.

Liver metastases is reported to develop in 5%-9% of patients with gastric cancer^[15]. One study has shown that only a limited number of GCLM patients are eligible for surgical treatment^[4]. After the promising results of the MAGIC trial, in Europe, current practice for treatment

of GCLM patients has become surgery with perioperative chemotherapy^[10,16]. However, the optimal surgical strategy for GCLM remains a matter of debate. Only some patients with GCLM are ideal candidates for hepatectomy, therefore, many patients are unsuitable for surgical resection, either due to other distant metastases, extensive lymph node metastases, multiple bilateral metastases, or comorbidity.

In recent decades, multimodality approaches using chemotherapy, radiotherapy, or both have been evaluated in an attempt to improve outcomes following gastric cancer surgery. Some benefit has been seen in adjuvant chemotherapy after gastric cancer resection. One recent trial conducted in East Asia, ACTS-GC30, evaluated S-1 chemotherapy and found significant 10% improvement in 3-year overall survival with adjuvant chemotherapy after surgery^[17]. A more compelling study of perioperative chemotherapy was the phase 3 United Kingdom MAGIC trial. This trial demonstrated that perioperative chemotherapy could significantly improve overall survival and progression-free survival in 503 patients with resectable adenocarcinoma. However, this trial also highlighted the challenges involved in delivering postoperative treatment; only 50% of patients were able to receive postoperative chemotherapy, compared with nearly 91% who received preoperative chemotherapy.

In the late stage of gastric cancer, with high rates of toxicity in perioperative chemotherapy, adoption of the perioperative approach could be useful for a large proportion of GCLM patients. Our results also showed that weekly SP and low-dose DCF in perioperative chemotherapy had a positive effect in GCLM. In our study, two patients with initially unresectable multiple liver metastases were converted to resectable after preoperative chemotherapy. Our results also showed that D2 resection provides better locoregional control and significantly better survival compared with unresectable patients. We recommend more personally tailored multimodality treatment approaches (surgery + chemotherapy \pm radiation) in patients with GCLM.

Some researchers have reported that even a generous surgical margin may not be essential for curative hepatic resection of liver metastases, because recurrence is strongly associated with systemic spread rather than local invasion^[6]. This conclusion highlights the essentiality of perioperative chemotherapy. GCLM recurrence after surgery is most likely due to occult metastatic disease in the tumor bed and at distant sites, so locoregional resection alone is not a complete 100% successful procedure. Therefore, multimodality approaches using systemic chemotherapy or radiation, or a combination of both have been used in an attempt to improve outcomes following surgery, especially in patients with multiple metastases.

However, adequate chemotherapy can lead to intolerance and morbidity and mortality. In the present study, we wanted to explore some safe and effective regimens available to Chinese patients with GCLM. We investigated the safety and efficacy of liver resection combined

with perioperative S1 regimen in patients with GCLM. We performed a retrospective analysis based on recent prospectively collected data. S-1 is an orally active combination of tegafur (5-fluorouracil prodrug), gimeracil (an inhibitor of dihydropyrimidine dehydrogenase, which degrades fluorouracil), and oteracil (which inhibits phosphorylation of 5-fluorouracil in the gastrointestinal tract) in a molar ratio of 1:0.4:1. S-1 has been the standard regimen for adjuvant chemotherapy for advanced primary gastric cancer^[18], and its mild side effect profile and ease of administration make it a preferred choice. The DCF regimen has major myelotoxicity^[19-25]. However, weekly DCF in our study was well tolerated, and both the regimens were well tolerated and achieved a good response. All GCLM patients with adequate physical condition obtained a benefit from preoperative chemotherapy, which assisted with their subsequent surgical procedure. Appropriately modified chemotherapy is necessary for the improvement of the GCLM resection rate and complete elimination of micrometastases^[26-31]. In our initial results, weekly DCF yielded an unexpected high response as preoperative chemotherapy for GCLM^[9]. We found that S-1 combined with cisplatin also yielded a high response and had better applicability. These modifications of altering the dose and frequency of the cytotoxic agents are an individualized approach for treatment of GCLM. Our aim is to improve the generally poor prognosis of this aggressive disease and further phase II and III trials are warranted to confirm the feasibility and efficacy of preoperative chemotherapy for GCLM.

COMMENTS

Background

Liver metastasis is a fatal event in gastric cancer patients, and remains a major cause of cancer-related death. Surgery for multiple liver metastases from gastric cancer (GCLM) has favorable outcomes. However, the efficacy and safety of perioperative chemotherapy is still a matter of debate.

Research frontiers

The glycosylated and myristoylated smaller surface antigen trial was conducted to compare gastrectomy with metastasectomy plus systemic therapy versus systemic therapy alone. The results of this trial showed that aggressive surgical resection in combination with systemic chemotherapy may improve the outcomes of the patients with metastatic gastric cancer.

Innovations and breakthroughs

In a previous pilot study, the authors found that liver resection combined with weekly docetaxel-based chemotherapy were well tolerated and had a good response. In the present study, the authors found that perioperative weekly docetaxel, cisplatin and 5-fluorouracil (DCF), and S-1 and cisplatin (SP) in patients with GCLM who underwent aggressive surgical treatment could improve prognosis and overall survival.

Applications

The study results suggest that perioperative weekly DCF and SP could be used to treat GCLM patients who underwent aggressive surgical treatment.

Terminology

Synchronous liver metastases are detected before or during surgery, or occur within 1 year after gastrectomy. Metachronous liver metastases are usually detected within a 2-year period following initial gastrectomy.

Peer review

This is a good study in which the authors evaluated the effect of perioperative weekly DCF and SP in GCLM patients who underwent aggressive surgical treatment. The results are interesting and suggest that perioperative weekly DCF and SP combined with resection could be applied in patients with GCLM.

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Extended antimicrobial prophylaxis after gastric cancer surgery: A systematic review and meta-analysis

Chun-Dong Zhang, Yong-Ji Zeng, Zhen Li, Jing Chen, Hong-Wu Li, Jia-Kui Zhang, Dong-Qiu Dai

Chun-Dong Zhang, Yong-Ji Zeng, Zhen Li, Jing Chen, Hong-Wu Li, Jia-Kui Zhang, Dong-Qiu Dai, Department of Gastrointestinal Surgery, the Fourth Affiliated Hospital of China Medical University, Shenyang 110032, Liaoning Province, China

Author contributions: Zhang CD and Dai DQ conceived the study; Zhang CD and Zeng YJ collected data and performed data analysis; all authors designed the study and wrote the paper, read and approved the final manuscript for submission.

Correspondence to: Dong-Qiu Dai, Professor, Chief Physician, Department of Gastrointestinal Surgery and Cancer Center, the Fourth Affiliated Hospital of China Medical University, Shenyang 110032, Liaoning Province, China. daiq63@163.com

Telephone: +86-24-62043110 Fax: +86-24-62043110

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Abstract

AIM: To investigate the efficacy of extended antimicrobial prophylaxis (EAP) after gastrectomy by systematic review of literature and meta-analysis.

METHODS: Electronic databases of PubMed, Embase, CINAHL, the Cochrane Database of Systematic Reviews, the Cochrane Controlled Trials Register and the China National Knowledge Infrastructure were searched systematically from January 1980 to October 2012. Strict literature retrieval and data extraction were carried out independently by two reviewers and meta-analyses were conducted using RevMan 5.0.2 with statistics tools risk ratios (RRs) and intention-to-treat analyses to evaluate the items of total complications, surgical site infection, incision infection, organ (or space) infection, remote site infection, anastomotic leakage (or dehiscence) and mortality. Fixed model or random model was selected accordingly and forest plot was conducted to display RR. Likewise, Cochrane Risk of Bias Tool was applied to evaluate the quality of ran-

domized controlled trials (RCTs) included in this meta-analysis.

RESULTS: A total of 1095 patients with gastric cancer were enrolled in four RCTs. No statistically significant differences were detected between EAP and intraoperative antimicrobial prophylaxis (IAP) in total complications (RR of 0.86, 95%CI: 0.63-1.16, $P = 0.32$), surgical site infection (RR of 1.97, 95%CI: 0.86-4.48, $P = 0.11$), incision infection (RR of 4.92, 95%CI: 0.58-41.66, $P = 0.14$), organ or space infection (RR of 1.55, 95%CI: 0.61-3.89, $P = 0.36$), anastomotic leakage or dehiscence (RR of 3.85, 95%CI: 0.64-23.17, $P = 0.14$) and mortality (RR of 1.14, 95%CI: 0.10-13.12; $P = 0.92$). Likewise, multiple-dose antimicrobial prophylaxis showed no difference compared with single-dose antimicrobial prophylaxis in surgical site infection (RR of 1.10, 95%CI: 0.62-1.93, $P = 0.75$). Nevertheless, EAP showed a decreased remote site infection rate compared with IAP alone (RR of 0.54, 95%CI: 0.34-0.86, $P = 0.01$), which is the only significant finding. Unfortunately, EAP did not decrease the incidence of surgical site infections after gastrectomy; likewise, multiple-dose antimicrobial prophylaxis failed to decrease the incidence of surgical site infection compared with single-dose antimicrobial prophylaxis.

CONCLUSION: We recommend that EAP should not be used routinely after gastrectomy until more high-quality RCTs are available.

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Key words: Gastric cancer; Gastrectomy; Extended antimicrobial prophylaxis; Intraoperative antimicrobial prophylaxis; Meta-analysis

Core tip: We investigated the efficacy of extended antimicrobial prophylaxis (EAP) after gastrectomy through systematic review of literature and meta-analysis. We recommend that EAP should not be used routinely after

gastrectomy until more high-quality randomized controlled trials are available.

Zhang CD, Zeng YJ, Li Z, Chen J, Li HW, Zhang JK, Dai DQ. Extended antimicrobial prophylaxis after gastric cancer surgery: A systematic review and meta-analysis. *World J Gastroenterol* 2013; 19(13): 2104-2109 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v19/i13/2104.htm> DOI: <http://dx.doi.org/10.3748/wjg.v19.i13.2104>

INTRODUCTION

Although the incidence of gastric cancer is sharply declining, it still remains the second cause of cancer-related death worldwide^[1,2]. Administration of a first-generation cephalosporin as intraoperative antimicrobial prophylaxis (IAP) to prevent surgery-associated infection has been recommended^[3]. Nevertheless, most patients after gastrectomy still receive further extended antimicrobial prophylaxis (EAP) routinely to reduce surgical site infection even until 3-4 postoperative days^[4-6]. Few randomized controlled trials (RCTs) have investigated the efficacy of EAP^[7-10]. Moreover, EAP administration is controversial and there is no worldwide accepted validation as a result of its scarce efficacy.

However, the administration of antimicrobial prophylaxis may result in antibiotics-associated diarrhea (AAD), which can occur as early as few hours after the first dose of antibiotics^[11]. The incidence of AAD varies from 10% to 30%, and AAD has been identified as the leading cause of diarrhea in hospitalized patients, especially in patients with surgery of gastrointestinal tract^[12]. Abuse of antibiotics also aggravates the burden of patient hospital costs.

A total of 21 320 new gastric cancer cases and 10 540 deaths from gastric cancer were projected to occur in the United States in 2012^[2]. Generally, complete surgical resection of gastric cancer with negative margin (R0 resection) and D2 lymphadenectomy is considered as the most effective treatment strategy for gastric cancer in East Asia^[13-15]. Surgical site infections have suggested the essential administration of IAP. However, only few RCTs have investigated the efficacy of EAP^[7-10], and almost no meta-analysis has been conducted to assess the efficacy of EAP. Meta-analysis is considered a more powerful evidence for clinical decision making compared with RCTs. In light of these considerations, we performed this meta-analysis to assess the efficacy of EAP in patients after gastrectomy.

MATERIALS AND METHODS

Literature search

To identify additional studies and published abstracts, electronic databases of PubMed, Embase, CINAHL, the Cochrane Database of Systematic Reviews, the Cochrane Controlled Trials Register and the China National Knowledge Infrastructure were searched systematically

from January 1980 to October 2012. MeSH terms of “stomach neoplasm”, “gastrectomy”, “antibiotic prophylaxis” and “randomized controlled trial” were used. The reference lists of all retrieved articles were reviewed for further identification of potentially relevant trials.

Data collection process

Two reviewers (Zhang CD and Zeng YJ) in our group independently extracted relevant data, including: study and population features, outcomes, titles, abstracts, and even full articles when it was necessary. They compared the results and synthesized the same opinions, and disagreements were solved by discussion with a third reviewer in our group.

Inclusion and exclusion criteria

The inclusion criteria and exclusion criteria were established based on the Cochrane Handbook for Systematic Review of Interventions (Version 5.0.2). The inclusion criteria were: (1) all originally published and unpublished high-quality RCTs; (2) trials concerning antimicrobial prophylaxis after gastrectomy; (3) if studies were from the same author or institution, the most informative and latest ones were selected; and (4) no restriction on publishing language. Exclusion criteria were as follows: (1) studies with little information about the items to be investigated; (2) loss to follow-up exceeding 10%; and (3) non-RCTs.

The following data were acquired: study and year, country, sample size, sex ratio, median age, body-mass index, operation time, blood loss, median follow-up time, participants, interventions, total complications, surgical site infection, incision infection, organ/space infection, anastomotic leakage/dehiscence, and mortality (Tables 1-3).

Quality evaluation

Methodological quality of RCTs was evaluated according to the Cochrane Risk of Bias Tool with regard to randomization, allocation concealment, blind, withdrawal and dropout, and selective reporting bias (Table 4).

Statistical analysis

Data analysis was conducted using Review Manager 5.0.2 (RevMan 5.0.2) with statistics tools risk ratios (RRs). Intention-to-treat analyses were performed. Dichotomous variables were analyzed with RRs. $P < 0.05$ was defined as statistically significant and 95%CI was applied. Fixed model was used if $I^2 < 50\%$ and $P > 0.1$, while random model was selected if $I^2 \geq 50\%$ or $P \leq 0.1$. Likewise, forest plot was conducted to display RR.

RESULTS

Among a total of 52 studies retrieved, 48 studies were found unrelated to our selection criteria after further assessment. Thus, only four RCTs^[7-10] were eligible for the meta-analysis: three RCTs^[7-9] comparing EAP with IAP and one RCT^[10] comparing multiple-dose with single-

Table 1 Primary characteristics of the randomized controlled trails included in the meta-analysis

| Ref. | Country | Sample size | Male | Median age (yr) | Body mass index (kg/m ²) | Operation time (min) | Blood loss (mL) | Median follow-up time (d) |
|--------------------------------------|---------|-------------|------------------|------------------|--------------------------------------|----------------------|------------------|---------------------------|
| Schardey <i>et al</i> ^[7] | Germany | 102 | 60 | 63.7 ± 11.4 | NM | 301.6 ± 87.7 | NM | 42 |
| | | 103 | 59 | 62.6 ± 11.9 | NM | 314.8 ± 107 | NM | |
| | | | <i>P</i> > 0.05 | <i>P</i> > 0.05 | NM | <i>P</i> > 0.05 | NM | |
| Farran <i>et al</i> ^[8] | Spain | 22 | 33 | 57(31-87) | NM | NM | NM | > 22 |
| | | 27 | | | NM | NM | NM | |
| Imamura <i>et al</i> ^[9] | Japan | 179 | 125 | 65 | 22.5 (12.4-32.9) | 200 (64-415) | 210 (1-1700) | 30 |
| | | 176 | 115 | 66 | 22.3 (16.3-33.0) | 209 (58-428) | 200 (1-880) | |
| | | | <i>P</i> = 0.536 | <i>P</i> = 0.429 | <i>P</i> = 0.190 | <i>P</i> = 0.499 | <i>P</i> = 0.903 | |
| Mohri <i>et al</i> ^[10] | Japan | 243 | 174 | 68 (22-91) | 21.6 (13.4-31.6) | 232 (43-70) | 338.0 (10-2811) | 30 |
| | | 243 | 164 | 68 (23-90) | 21.4 (13.6-34.0) | 234 (70-492) | 405.7 (10-2917) | |
| | | | <i>P</i> = 0.375 | <i>P</i> = 0.642 | <i>P</i> = 0.446 | <i>P</i> = 0.798 | <i>P</i> = 0.028 | |

NM: Not mentioned.

Table 2 Secondary characteristics of the randomized controlled trails included in the meta-analysis

| Ref. | Participants | <i>n</i> | Interventions | Complications |
|--------------------------------------|---|----------|--|---|
| Schardey <i>et al</i> ^[7] | 205 patients August 1991-March 1994 Germany, multi-centre, ≥ 18 yr, total gastrectomy | 102 | Polymyxin B 0.1 g, tobramycin 0.08 g, vancomycin 0.125 g and | Infections: Pulmonary, urinary tract; abscess; Insufficiency: Pancreatic, esophagointestinal; miscellaneous; pancreatic fistula |
| | | 103 | amphotericin B 0.5 g four times per day orally from the day before operation until 7 th postoperative day plus perioperative intravenous prophylaxis: cefotaxime 2 × 2 g <i>vs</i> placebo plus perioperative intravenous prophylaxis: cefotaxime 2 × 2 g | |
| Farran <i>et al</i> ^[8] | 49 patients January 2000-March 2005, single centre, ≥ 18 yr, total gastrectomy | 22 | 20 mL oral suspension of erythromycin 0.5 g + gentamicine 0.08 g | Dehiscence; sepsis; abscess; pulmonary infection; pulmonary distress syndrome |
| | | 27 | + nystatin sulfate 0.1 g <i>vs</i> 20 mL placebo solution. Both groups started treatment 12 h before surgery and continued until the 5 th postoperative day | |
| Imamura <i>et al</i> ^[9] | 355 patients June 2005-December 2007, Japan, multi-centre, ≥ 35 yr, distal gastrectomy | 179 | Intraoperative administration plus cefazolin 1 g once after | Anastomotic leakage; remote infections; surgical site infections |
| | | 176 | closure and twice daily for 2 postoperative days <i>vs</i> intraoperative administration: cefazolin 1 g before surgical incision and every 3 h as intraoperative supplements | |
| Mohri <i>et al</i> ^[10] | 486 patients May 2001-December 2004 Japan, single-centre, ≥ 20 yr, elective gastrectomy | 243 | Intraoperative schedule: cefazolin 1 g or ampicillin-sulbactam 1.5 g | Surgical site infection: incision or organ or space; abscess |
| | | 243 | by intravenous infusion > 15 min and an additional dose was administrated if operation > 3 h <i>vs</i> intraoperative schedule plus further treatment at 12-h intervals, a total of 7 doses | |

Table 3 Basic data of the comparisons included in the randomized controlled trails

| Ref. | Total complication | Surgical site infection | Incision infection | Organ/space infection | Remote site infection | Anastomotic leakage/dehiscence | Mortality |
|--------------------------------------|--------------------|-------------------------|--------------------|-----------------------|-----------------------|--------------------------------|-----------|
| Schardey <i>et al</i> ^[7] | 31/102 | NM | NM | NM | 16/102 | NM | 5/102 |
| | 46/103 | NM | NM | NM | 31/103 | NM | 11/103 |
| Farran <i>et al</i> ^[8] | 2/22 | NM | NM | NM | 1/22 | 1/22 | 2/22 |
| | 3/27 | NM | NM | NM | 3/27 | 0/27 | 0/27 |
| Imamura <i>et al</i> ^[9] | 22/179 | 16/179 | 5/179 | 11/179 | 6/179 | 4/179 | NM |
| | 17/176 | 8/176 | 1/176 | 7/176 | 9/176 | 1/176 | NM |
| Mohri <i>et al</i> ^[10] | NM | 23/243 | 14/243 | 12/243 | NM | NM | NM |
| | NM | 21/243 | 11/243 | 10/243 | NM | NM | NM |

NM: Not mentioned.

dose antimicrobial prophylaxis after gastrectomy, including 1095 patients (Tables 1-5).

Primary outcomes: Intraoperative *vs* EAP

Total complications: Three RCTs^[7-9] were included (303 EAP and 306 IAP) and fixed model was applied ($I^2 = 42\%$, $P = 0.18$). No statistically significant difference was detected (RR of 0.86, 95%CI: 0.63-1.16, $P = 0.32$).

Surgical site infection, incision infection and organ/space infection: Only one RCT^[9] comparing EAP and IAP reported surgical site infection, which showed no statistical difference (RR of 1.97, 95%CI: 0.86-4.48, $P = 0.11$). There were also no significant differences in the analysis of incision infection (RR of 4.92, 95%CI: 0.58-41.66, $P = 0.14$) and organ or space infection (RR of 1.55, 95%CI: 0.61-3.89, $P = 0.36$).

Table 4 Quality assessment of the randomized controlled trails included based on the Cochrane Risk of Bias Tool

| Ref. | Randomization | Allocation concealment | Blind | Withdrawal and dropout | Presence of selective reporting bias |
|--------------------------------------|-----------------|------------------------|--------------|------------------------|--------------------------------------|
| Schardey <i>et al</i> ^[7] | Without details | Envelope | Double-blind | Well reported | Unclear |
| Farran <i>et al</i> ^[9] | Well reported | Envelope | Double-blind | Well reported | No |
| Imamura <i>et al</i> ^[9] | Well reported | Envelope | No | Well reported | No |
| Mohri <i>et al</i> ^[10] | Well reported | Without details | No | Well reported | Unclear |

Table 5 Summary of comparisons between extended antimicrobial prophylaxis and intraoperative antimicrobial prophylaxis

| Items | Heterogeneity | | Analysis model | Overall effect | | RR (95%CI) | Ref. |
|--------------------------------|---------------|------|----------------|----------------|------|-------------------|-------|
| | I^2 | P | | Z | P | | |
| Total complications | 42% | 0.18 | Fixed | 0.99 | 0.32 | 0.86 (0.63-1.16) | [7-9] |
| Surgical site infections | NP | NP | Fixed | 1.61 | 0.11 | 1.97 (0.86-4.48) | [9] |
| Incision infections | NP | NP | Fixed | 1.46 | 0.14 | 4.92 (0.58-41.66) | [9] |
| Organ/space infections | NP | NP | Fixed | 0.92 | 0.36 | 1.55 (0.61-3.89) | [9] |
| Remote site infections | 0% | 0.90 | Fixed | 2.58 | 0.01 | 0.54 (0.34-0.86) | [7-9] |
| Anastomotic leakage/dehiscence | 0% | 0.97 | Fixed | 1.47 | 0.14 | 3.85 (0.64-23.17) | [8,9] |
| Mortality | 62% | 0.10 | Random | 0.10 | 0.92 | 1.14 (0.1-13.12) | [8,9] |

NP: Not applicable; RR: Risk ratio.

Remote site infection: Three RCTs (303 EAP and 306 IAP)^[7,9] evaluated remote site infection and fixed model was conducted ($I^2 = 0\%$, $P = 0.90$), however, there was a significantly decreased remote site infection rate in EAP compared with IAP (RR of 0.54, 95%CI: 0.34-0.86, $P = 0.01$).

Anastomotic leakage or dehiscence: Two RCTs (201 EAP and 203 IAP)^[8,9] were evaluated, showing no statistical difference in anastomotic leakage or dehiscence (RR of 3.85, 95%CI: 0.64-23.17, $P = 0.14$).

Mortality: Two RCTs (124 EAP and 130 IAP)^[7,8] were included, which suggested no survival benefit of EAP compared with IAP (RR of 1.14, 95%CI: 0.10-13.12, $P = 0.92$).

Secondary outcomes: Multiple-dose antimicrobial prophylaxis vs single-dose antimicrobial prophylaxis

Only one RCT^[10] compared the efficacy of multiple-dose antimicrobial prophylaxis with single-dose antimicrobial prophylaxis, however, no significant differences were detected in surgical site infection (RR of 1.10, 95%CI: 0.62-1.93, $P = 0.75$), incision infection (RR of 1.27, 95%CI: 0.59-2.75, $P = 0.54$) and organ/space infection (RR of 1.20, 95%CI: 0.53-2.73, $P = 0.66$). The incidence of surgical site infection after gastrectomy was similar by the two antimicrobial prophylaxis regimens.

DISCUSSION

Meta-analysis is considered an ideal statistical tool increasing the statistical power, in other words, meta-analysis is a more powerful evidence for clinical decision making compared with RCTs. In light of these considerations, this meta-analysis was conducted to assess the efficacy of EAP after gastrectomy.

Although it has been widely accepted that patients with gastrectomy will benefit from preoperative antimicrobial prophylaxis and IAP^[16,17], there is still no worldwide accepted validation for EAP. In this meta-analysis, we found that postoperative EAP did not decrease the incidence of total complications in patients with gastrectomy. Additionally, EAP failed to improve surgical site infection rate, including incision infection and organ/space infection; likewise, no significant difference was detected in anastomotic leakage/dehiscence and mortality between EAP and IAP. The same striking finding was that patients did not benefit from multiple-dose antimicrobial prophylaxis compared with single-dose antimicrobial prophylaxis; yet, only one RCT^[10] was included in this meta-analysis. Based on the present evidence, we do not recommend the administration of EAP after gastrectomy; however, our results need to be validated and re-evaluated by more high-level RCTs.

Surgical site infections remain a substantial cause of postoperative mortality^[18]. We therefore conjecture that if EAP can decrease the surgical site infection rate, it may subsequently decrease the postoperative mortality. Unfortunately, EAP failed to decrease the surgical site infection rate. We assessed the mortality of EAP and IAP groups, and found no significant differences between the two groups. In light of these considerations, our findings suggested that EAP fails to decrease mortality in patients after gastrectomy; in other words, no survival benefit can be observed from EAP after gastrectomy based on the present evidence.

Many factors, such as male ratio, median age, obesity, operation time and intraoperative blood loss, may affect the postoperative infection risk^[18-23]. For example, the effect of antibiotics will be diminished as a result of intraoperative blood loss; likewise, longer operation time will increase blood loss; meanwhile, obesity may increase

the difficulty of operation and the operation time. Taking all these factors into consideration, we evaluated the statistical difference systematically; fortunately, we did not detect any significant difference among these items. Therefore, these factors have not affected the outcomes in these RCTs (Table 1).

The only significant difference between EAP and IAP is the remote site infection rate. However, we recommend that EAP should not be applied routinely unless the individuals experience a remote site infection. Therefore, we suggest delivering an “individualized treatment” rather than a routine treatment. The drugs used for EAP in these trials varied from cefazolin 1-1.5 g^[9,10], erythromycin 0.5 g + gentamicine 0.08 g + nystatin sulfate 0.1 g^[8], polymyxin B 0.1 g, tobramycin 0.08 g, vancomycin 0.125 g to amphotericin B 0.5g^[7]. The incidence of surgical site infection in these RCTs ranged from 4.5% to 9.5%, which is in keeping with published rates of 5%-14%^[24,25]. Despite these differences, the infection rates were similar, and no difference was detected (Table 2). However, our results still need to be validated for patients who require surgery on other sites of the body because the micro-flora in these operation sites differs from that in gastrointestinal tract^[26].

There was no country or language restriction in the data search process for this meta-analysis. It is the first meta-analysis concerning the efficacy of EAP after gastrectomy. There are some limitations of these studies, such as various antimicrobial prophylaxis regimens used and disappointing statistical power, thus, more high-level RCTs are needed to validate our results.

Based on the present evidence, EAP fails to decrease the incidence of surgical site infections after gastrectomy; multiple-dose antimicrobial prophylaxis fails to decrease the incidence of surgical site infection compared with single-dose antimicrobial prophylaxis. Therefore, we believe that our findings are significant to all patients with gastrectomy, and suggest that EAP should not be used routinely after gastrectomy until more high-level RCTs are available.

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COMMENTS

Background

Administration of intraoperative antimicrobial prophylaxis (IAP) to prevent surgery-associated infection has been recommended. Although extended antimicrobial prophylaxis (EAP) has been recommended to be discontinued within 24 h of surgery, most patients after gastrectomy still receive further EAP routinely, as a result of insufficient evidence. This meta-analysis was conducted to investigate the effectiveness of EAP aimed at guiding clinical practice.

Research frontiers

Meta-analysis was conducted to evaluate the effectiveness of EAP vs IAP for patients undergoing gastric cancer surgery.

Innovations and breakthroughs

The evidence obtained from this meta-analysis proved that EAP failed to dem-

onstrate the advantages over IAP for patients with gastric cancer surgery with regard to total complications, surgical site infection, incision infection, organ or space infection, anastomotic leakage or dehiscence and mortality. These findings suggested that EAP should not be used routinely after gastrectomy.

Applications

The results of this meta-analysis suggest that EAP should not be administrated routinely after gastric cancer surgery and IAP is a standard treatment strategy for gastric cancer surgery.

Terminology

Surgical site infection: Infection occurs within one month after operation and involves superficial incision, deep incision, organ or space, which may generate some symptoms of infection, such as pain or tenderness, local swelling, redness, heat and so on.

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The period of the investigation extended over 32 years, during which different types and combinations of antibiotics have been used, but does not permit any evaluation of any particular antibiotics which might have been valuable. It is a valuable finding that intra-operative antibiotic prophylaxis is as efficient as an extended post-operative course in preventing post-operative infections.

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Hepatocellular carcinoma in a non-cirrhotic patient with Wilson's disease

Raphael Thattil, Jean-François Dufour

Raphael Thattil, Jean-François Dufour, Department of Hepatology, University Clinic of Visceral Surgery and Medicine, Inselspital University Hospital Bern, CH-3010 Bern, Switzerland
Jean-François Dufour, Division of Hepatology, Department of Clinical Research, University of Bern, CH-3010 Bern, Switzerland

Author contributions: Thattil R acquired data; Dufour JF analysed the case; Thattil R and Dufour JF wrote the paper.

Correspondence to: Dr. Jean-François Dufour, Professor, Department of Hepatology, University Clinic of Visceral Surgery and Medicine, Inselspital University Hospital Bern, CH-3010 Bern, Switzerland. jean-francois.dufour@ikp.unibe.ch

Telephone: +41-31-638026 Fax: +41-31-6329765

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Abstract

We report the exceptional case of hepatocellular carcinoma in a non-cirrhotic patient, whose Wilson's disease was diagnosed at the unusual age of 58 years. The liver histology revealed macrovesicular steatosis with fibrosis, but no cirrhosis. The disease was treated with D-penicillamine for 3 years until acute discomfort in the right upper quadrant led to detection of multifocal hepatocellular carcinoma, which was successfully resected. The histological examination confirmed the malignant nature of the 4 lesions, which were classified according to Edmondson and Steiner as poorly differentiated hepatocellular carcinoma grade 3. The non-tumoral parenchyma showed 80% steatosis with ballooned cells, lobular inflammation, septal fibrosis but no cirrhosis. Hepatocellular carcinoma is rare in Wilson's disease, especially in the absence of cirrhosis. The literature's 28 published cases are reviewed and the contributory role of copper in the hepatocarcinogenic process is discussed.

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Key words: Wilson's disease; Hepatocellular carcinoma; Hepatocarcinogenesis; Copper; Liver; Fibrosis; Cirrhosis

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INTRODUCTION

Wilson's disease is an autosomal recessive disorder of copper metabolism. Wilson's disease has a worldwide prevalence between 1 in 30 000 and 1 in 100 000^[1]. The responsible gene *ATP7B* is located on chromosome 13 and encodes a copper transporter. In Wilson's disease, the copper transporter is mutated and its function is impaired^[1]. Wilson's disease has hepatic, neurological, psychiatric and ophthalmic manifestations. Hepatic manifestations are characterized histologically by steatohepatitis, which evolves into cirrhosis if left untreated. Because most cases of Wilson's disease are diagnosed and treated early, hepatocellular carcinoma is a rare sequela. We report the unusual case of a Wilson's disease patient diagnosed at an advanced age and who developed hepatocellular carcinoma in a non-cirrhotic liver.

CASE REPORT

The patient underwent cholecystectomy due to symptomatic gallstones at 51 years of age. Liver biopsies showed macrovesicular steatosis. The circulating levels of gamma-glutamyltransferase remained chronically elevated and the ALT levels were at the upper limit of the normal. A computed tomography (CT) scan performed 5 years later revealed the presence of a 3 cm subcapsular lesion in liver segment VI as well as several ≤ 1 cm lesions. All lesions displayed a discrete enhancement dur-

Table 1 Synopsis of patients with hepatocellular carcinoma and Wilson's disease

| Ref. | Sex | WD-age (yr) | HCC-age (yr) | Cirrhosis | Medical therapy | Invasive therapy | Status |
|--|--------|-------------|--------------|-----------|---|--|--------|
| Guan <i>et al</i> ^[2] | Female | 23 | 27 | Yes | Penicillamin | Hepatic resection | Alive |
| Iwadate <i>et al</i> ^[3] | Male | 17 | 23 | Yes | Penicillamin, low copper diet | - | Dead |
| Lowette <i>et al</i> ^[4] | - | - | - | Yes | Penicillamin | Transplant | Alive |
| Ikegawa <i>et al</i> ^[5] | Male | 28 | 37 | Yes | Penicillamin, zinc acetate dehydrate | Radiofrequency ablation | Alive |
| Kumagi <i>et al</i> ^[6] | Male | 26 | 66 | Yes | Penicillamin, transplant | Transcatheter arterial chemoembolisation | Dead |
| Kumagi <i>et al</i> ^[6] | Male | 27 | 36 | Yes | Penicillamin, transplant | - | Dead |
| Kumagi <i>et al</i> ^[6] | Male | 27 | 46 | Yes | Penicillamin | - | Alive |
| Lygren <i>et al</i> ^[15] | Male | 15 | 16 | Yes | - | - | Dead |
| Girard <i>et al</i> ^[16] | Male | 22 | 41 | Yes | Penicillamin | - | Dead |
| Kamakura <i>et al</i> ^[17] | Male | 26 | 32 | Yes | Penicillamin | - | Dead |
| Terao <i>et al</i> ^[18] | Male | 29 | 40 | Yes | Penicillamin, dimercaprol potassium sulfate | - | Dead |
| Wilkinson <i>et al</i> ^[19] | Male | 31 | 41 | Yes | Penicillamin | - | Dead |
| Buffet <i>et al</i> ^[20] | Male | 45 | 57 | Yes | Penicillamin | - | Dead |
| Imhof <i>et al</i> ^[21] | Male | 18 | 40 | - | Penicillamin | Hepatic resection | Alive |
| Madden <i>et al</i> ^[22] | Male | 61 | 61 | Yes | Penicillamin | - | Dead |
| Polio <i>et al</i> ^[23] | Male | 32 | 33 | Yes | Penicillamin, low copper diet | Chemotherapy | Dead |
| Cheng <i>et al</i> ^[24] | Female | 39 | 72 | Yes | Penicillamin, dimercaprol potassium sulfate | - | Dead |
| Agret <i>et al</i> ^[25] | Male | 73 | 73 | Yes | - | - | Dead |
| Walshe <i>et al</i> ^[26] | Male | 8 | 46 | Yes | Penicillamin | Transplant | Alive |
| Walshe <i>et al</i> ^[26] | Male | 11 | 42 | Yes | Penicillamin | - | Dead |
| Kumagi <i>et al</i> ^[27] | Male | 66 | 66 | Yes | - | Transcatheter arterial chemoembolisation | Dead |
| Ozçay <i>et al</i> ^[28] | Male | - | 13 | Yes | Penicillamin | Transplant | Alive |
| Aydinli <i>et al</i> ^[29] | Male | 22 | 22 | Yes | - | Radiofrequency ablation, transplant | Alive |
| Xu <i>et al</i> ^[30] | Male | 29 | 29 | Yes | - | Transcatheter arterial chemoembolisation, transplant | Alive |
| Reyes <i>et al</i> ^[31] | Male | 59 | 59 | Yes | - | - | Dead |
| Emlakçioğlu <i>et al</i> ^[32] | Female | 30 | 50 | - | Penicillamin | Transcatheter arterial chemoembolisation | Alive |
| Ikubo <i>et al</i> ^[33] | Female | 28 | 54 | Yes | Penicillamin, pyridoxal-phosphate | Hepatic resection | Alive |
| Savas <i>et al</i> ^[34] | Male | 6 | 12 | Yes | Penicillamin, low copper diet | Transplant | Alive |

WD: Wilson's disease; HCC: Hepatocellular carcinoma diagnosis.

ing the arterial phase without washout during the portal phase. Radiological controls over the next 2 years revealed no evolution of these lesions. The biopsy of the largest lesion showed fibrotic remodelling corresponding to Metavir F3 or a modified Ishak score of 4 without evidence of cirrhosis, a 25% macrovascular steatosis, a moderate chronic hepatic inflammation and an area with small cell dysplasia. A broad clinical examination was negative for neurological and ophthalmic (Kayser-Fleischer-rings) signs of Wilson's disease. However ceruloplasmin levels below the limit of detection (0.1 g/L) and urinary copper excretion was elevated. A genetic test confirmed a frameshift-mutation in exon 14 and 2 missense-mutations in exons 18 and 21 of the *ATP7B* gene. The patient was treated with D-penicillamine and pyridoxal-phosphate. This treatment was well tolerated. An magnetic resonance imaging with hepatocellular specific contrast confirmed the known lesions, which were stable in size and interpreted as regeneration nodules.

The patient worked as a mechanic, never smoked and consumed less than 10 g alcohol per day. His mother had metastatic breast carcinoma and died of a cerebral haemorrhage. His father suffered from an undefined psychiatric condition. His 2 brothers and 1 sister are in good health. He has no children.

At the age of 61 years, the patient presented with

acute pain in the right upper quadrant, which was preceded for several weeks by discomfort. Contrast-enhanced CT revealed 4 hepatic lesions showing enhancement during the arterial phase and washout during the portal phase. Two lesions of 3.7 cm and 2.1 cm were in segment IV, 1 lesion of 3.2 cm was in the segment V and the largest lesion of 9.7 cm was in segments VI and VII. A curative resection was performed. The histological examination confirmed the malignant nature of the 4 lesions, which were classified according to Edmondson and Steiner as poorly differentiated hepatocellular carcinoma grade 3. The non-tumoral parenchyma showed 80% steatosis with ballooned cells, lobular inflammation, septal fibrosis but no cirrhosis. Moreover, there was a mild iron hepatocellular accumulation (Rowe 1), which was absent on the previous biopsy.

DISCUSSION

All the published cases of hepatocellular carcinoma occurring in patients with Wilson's disease are listed in Table 1. As expected for hepatocellular carcinoma, males predominate whereas female constitute a lower than expected percentage (14%) of this group. Females constitute 30% of overall hepatocellular carcinoma cases. The reason may be that cirrhosis initiated by Wilson's disease

is less carcinogenic than that linked to other cirrhotic conditions and that male gender provides additional susceptibility to initiate hepatocarcinogenesis. Because it is uncertain from the information in previous reports whether other risk factors had been considered and excluded^[2-6], it is not possible to fully assess whether common features could link them to the hepatocarcinogenic process in our male patient with longstanding, untreated Wilson's disease. However, our patient was exceptional in that he was non-cirrhotic, whereas all previous cases of hepatocellular carcinoma in Wilson's disease occurred in cirrhotic livers (Table 1).

Long-Evans cinnamon rats, which have a mutated *ATP7B* gene and are therefore an experimental model for Wilson's disease, develop hepatocellular carcinoma spontaneously. However, these animals accumulate iron in addition to copper, and an iron-deficient iron diet can abrogate the development of liver tumors^[7]. This was attributed to the role of iron in promoting reactive oxygen species and DNA strand breaks^[8]. Copper can assume a similar role. Mice receiving copper develop hepatocellular carcinoma, preventable by the concurrent administration of thiamine, which reduces the production of reactive oxygen species in the mitochondria^[9]. In addition, copper stabilizes hypoxia-inducible factor-1 α (HIF-1 α)^[10-12] by restraining the activity of the HIF-1 α -inhibition factor^[10], thereby ensuring the formation of the HIF-1 α transcriptional complex^[11,12] and the expression of target genes important for angiogenesis, such as vascular endothelial growth factor (VEGF)^[10]. Indeed, Martin showed that copper increases VEGF in human hepatoma cells^[12]. Another potential carcinogenic property of copper is its ability to stimulate fibroblast growth factor-2^[13]. Treatment with D-penicillamine promotes hepatocellular iron accumulation^[14]. It is possible that the D-penicillamine treatment of our patient contributed to the oxidative stress through and increase in iron.

When our case is combined with the 28 published cases of hepatocellular carcinoma (Table 1), the mean age at diagnosis of Wilson's disease was 31 ± 18 years: 30 ± 7 years for women and 32 ± 19 years for men. The diagnosis of this genetic disease at such an advanced age suggests that longstanding, untreated Wilson's disease may represent a risk factor for hepatocellular carcinoma. This notion is supported by the observation that the mean age at diagnosis of hepatocellular carcinoma was younger than that observed in patients with other underlying liver diseases (43 ± 18 years).

In conclusion, this case report illustrates that hepatocellular carcinoma does occur in patients with Wilson's disease and that those with longstanding, untreated disease may be particularly vulnerable. Therefore, the importance of determining the fibrosis stage of Wilson's disease patients and of enrolling them in a surveillance program when cirrhotic can only be emphasized.

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Case of rectal angioleiomyoma in a female patient

Goran Z Stanojević, Dragan S Mihailović, Milica D Nestorović, Milan D Radojković, Milan M Jovanović, Miroslav P Stojanović, Branko B Branković

Goran Z Stanojević, Milica D Nestorović, Milan D Radojković, Milan M Jovanović, Miroslav P Stojanović, Branko B Branković, General Surgery Clinic, Niš Clinical Centre, Faculty of Medicine, University of Niš, 18000 Niš, Serbia
Dragan S Mihailović, Institute of Pathology, Niš Clinical Centre, Faculty of Medicine, University of Niš, 18000 Niš, Serbia
Author contributions: Stanojević GZ and Mihailović DS contributed equally to this work; Nestorović MD, Radojković MD, Jovanović MM, Stojanović MP, and Branković BB designed the manuscript and discussed its clinical features; Stanojević GZ wrote the paper.

Correspondence to: Goran Z Stanojević, MD, Professor of Surgery, General Surgery Clinic, Niš Clinical Centre, Faculty of Medicine, University of Niš, Bul. Zorana Djindjića 48, 18000 Niš, Serbia. stgoran@medfak.ni.ac.rs

Telephone: +381-18-4230209 Fax: +381-18-4235186

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Abstract

Angioleiomyoma represents a benign stromal tumor, which usually occurs in the subcutaneous tissue of the extremities, although its occurrence in the gastrointestinal tract is very rare. A case of rectal angioleiomyoma in a 40 year-old female patient is described here. Six months earlier, the patient suffered from periodical prolapse of an oval tumor from the anus, along with difficulties in bowel movement. A transanal extirpation of the tumor was performed. This is the first reported case in the English literature of a patient presenting with prolapsed angioleiomyoma of the rectum. During the immediate postoperative period, as well as 6 mo later, the patient had an unremarkable postoperative recovery.

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Key words: Prolapsed tumor; Angioleiomyoma; Rectum; Leiomyoma

Stanojević GZ, Mihailović DS, Nestorović MD, Radojković

MD, Jovanović MM, Stojanović MP, Branković BB. Case of rectal angioleiomyoma in a female patient. *World J Gastroenterol* 2013; 19(13): 2114-2117 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v19/i13/2114.htm> DOI: <http://dx.doi.org/10.3748/wjg.v19.i13.2114>

INTRODUCTION

Angioleiomyomata are a vascular variant of leiomyomata, which are benign tumors of smooth muscle. Angioleiomyoma of the large bowel is an extremely rare benign tumor. This is the first reported case in the English literature where a patient presenting with perineal discomfort/tenesmus and symptoms with a rectal mass passed via the anus had underlying angioleiomyoma.

CASE REPORT

A 40 year-old woman presented to an examination complaining of pain-related difficulties, feelings of pressure, and a prolapsing rectal tumor during defecation. The patient's difficulties began three years earlier, in the form of a sporadic feeling of discomfort in the anus and an occasional tumor prolapse 3 cm × 2 cm in size, which was spontaneously moving back inside. During the last six months, the tumor has grown up to 7 cm × 5 cm × 6 cm, and had been permanently falling out and hardly withdrawing. Due to bowel movement and personal hygiene problems, the patient came to the proctology unit.

On admission, the woman was hemodynamically stable and the laboratory analyses were within the normal limits, except for a slightly lower level of calcium ions (2.17 mmol/L) and slightly increased chloride (106 mmol/L). She was examined at the Proctology Section of the General Surgery Clinic. The tumor was 7 cm × 5 cm × 6 cm in size, "stuck out" from the anus, had an intact smooth surface, and was painless on palpation, on a 3 cm wide pedicle, and reducible (Figure 1).

After pre-operative preparation, transanal tumor ex-



Figure 1 *In situ* prolapsed angioleiomyoma of the rectum.

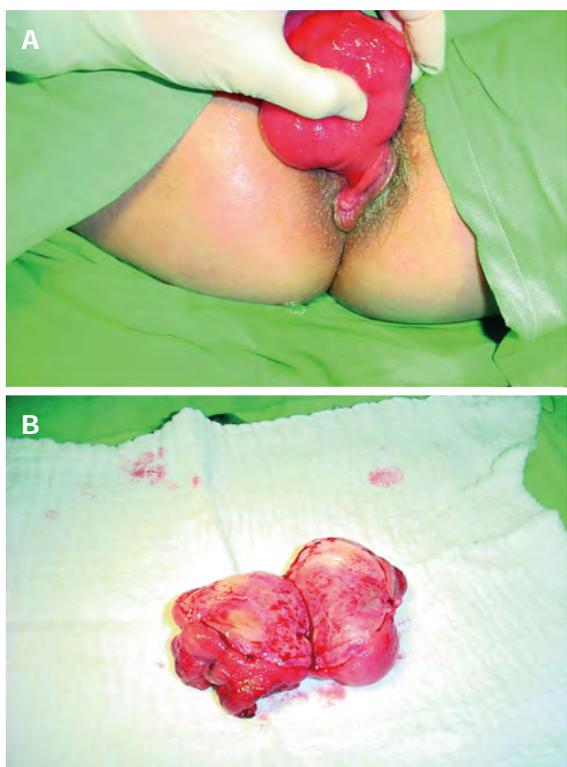


Figure 2 Cross-section of the lobular structure of the tumor showed a visible capsule and a partially bleeding parenchyma. A: Pedunculated tumor; B: Cross-section of the lobular structure tumor with visible capsule and partially bleeding parenchyma.

tirpation surgical intervention was performed. A cross-section of the tumor's lobular structure showed a visible capsule and a partially bleeding parenchyma (Figure 2).

Histopathological examination of the tumor revealed combined capillary and venous angioleiomyoma of the rectum, with thick vascular channels and intervascular smooth muscle bundles (Figure 3).

Results of immunohistochemical analyses revealed that the angioleiomyoma was negative for C-kit and CD34, and positive for smooth muscle actin and desmin (Figure 4).

The postoperative course passed quite regularly, without complications, and the patient was released from

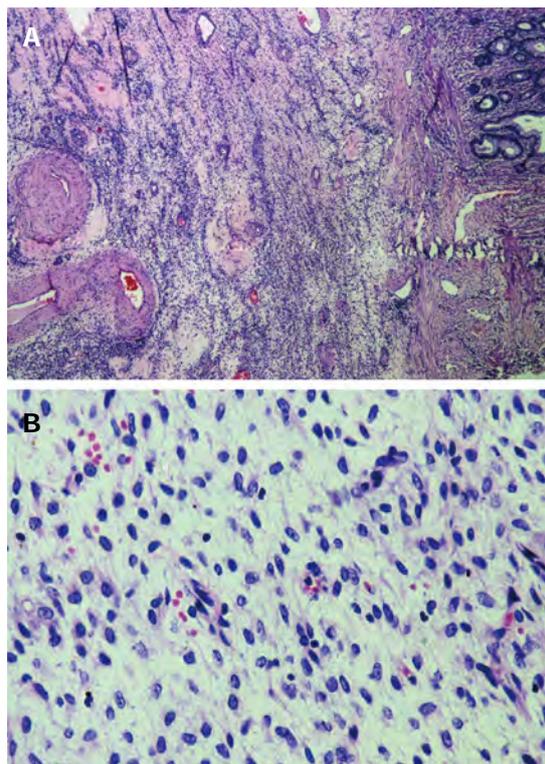


Figure 3 Histopathological examination of a combined capillary and venous angioleiomyoma of the rectum. A: Hematoxylin and eosin staining ($\times 10$); B: Hematoxylin and eosin staining ($\times 40$).

hospital on the second postoperative day. Six months after the operation, during the control examination, the patient was feeling well; digital rectal examination and laboratory findings were in order.

DISCUSSION

Primary leiomyomata are very rare in the gastrointestinal tract; mostly appearing in the stomach and small intestine. They are much less frequent in the esophagus and colon. Leiomyomata in the anorectal region make up 3% of all gastrointestinal benign tumors of smooth muscles (*i.e.*, they appear in 1 out of every 2000-3000 rectal neoplasms)^[1,2]. The first rectum leiomyoma was described by Vander Espt in 1881^[3]. With regard to macroscopic appearance, colon leiomyomata can be sessile (*i.e.*, peduncular, intraluminal, intramural and extraluminal). The clinical picture goes mainly without symptoms, except in the case of large tumors, which present occasional bleeding, a palpable and often prolapsing mass, and occasional pain in the last part of the colon^[1,4].

In 1995, Ezinger divided all leiomyomata into three groups: superficial, vascular (*i.e.*, angioleiomyomata) and deep leiomyomata^[5]. Angioleiomyomata are benign tumors of smooth muscle with expressed abnormal small blood vessels with thickened walls. They usually occur on the skin or in the subcutaneous tissue of the lower extremities, representing one out of five characteristic painful skin lesions, together with angiolipoma, glomus

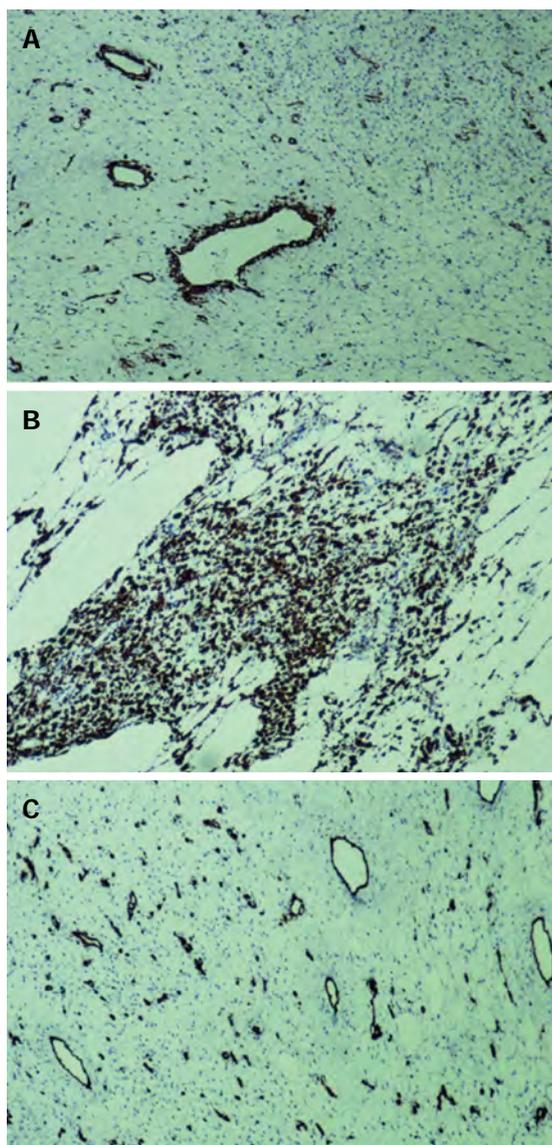


Figure 4 Immunohistochemical analyses. A: Tumor cells positive to actin ($\times 10$); B: Tumor cells positive to desmin ($\times 10$); C: Tumor cells negative for CD34 ($\times 10$).

tumors, spiradenoma, and neurinoma^[6]. Cases of localization have also been described, including the mouth cavity, female reproductive organs, scrotum, and kidneys^[7-11]. They originate from the muscularis mucosa or muscularis propria of the rectum wall. Pathophysiological division includes 4 types of angioleiomyomata: (1) capillary/solid, which are characterized by the existence of stratified smooth muscle fibers that surround a few thin vascular small channels; (2) venous, with numerous vascular small channels with thickened walls; (3) cavernous, with wide vascular small channels, surrounded by a thin layer of smooth muscle cells; and (4) combined capillary and venous angioleiomyomata^[5,12].

The presence of angioleiomyomata in the gastrointestinal tract is exceptionally rare, although in the literature there are several publications which describe cases with complications, such as bleedings, volvulus, perito-

nititis, and perforation^[13-16]. In the English literature, there are no data about the existence of rectum angioleiomyomata, making this the first described case.

Considering the nature of the tumor, the treatment implies that its transanal extirpation was a minimally invasive procedure.

Prolapsed peduncular intraluminal rectal angioleiomyoma represents an exceptionally rare type of tumor, which has now been described in literature for the first time. Its treatment implies transanal extirpation with further patient follow-up.

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Endoscopic drainage for duodenal hematoma following endoscopic retrograde cholangiopancreatography: A case report

Ya-Min Pan, Tian-Tian Wang, Jun Wu, Bing Hu

Ya-Min Pan, Tian-Tian Wang, Jun Wu, Bing Hu, Department of Endoscopy, Eastern Hepatobiliary Hospital, Second Military Medical University, Shanghai 200438, China

Author contributions: All the authors contributed equally to this manuscript.

Correspondence to: Bing Hu, MD, PhD, Department of Endoscopy, Eastern Hepatobiliary Hospital, Second Military Medical University, 225 Changhai Road, Shanghai 200438, China. drhubing@yahoo.cn

Telephone: +86-21-81875221 Fax: +86-21-35030072

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Abstract

Intramural duodenal hematoma (IDH) is a rare complication following endoscopic retrograde cholangiopancreatography (ERCP). Blunt damage caused by the endoscope or an accessory has been suggested as the main reason for IDH. Surgical treatment of isolated duodenal hematoma after blunt trauma is traditionally reserved for rare cases of perforation or persistent symptoms despite conservative management. Typical clinical symptoms of IDH include abdominal pain and vomiting. Diagnosis of IDH can be confirmed by imaging techniques, such as magnetic resonance imaging or computed tomography and upper gastrointestinal endoscopy. Duodenal hematoma is mainly treated by drainage, which includes open surgery drainage and percutaneous transhepatic cholangial drainage, both causing great trauma. Here we present a case of massive IDH following ERCP, which was successfully managed by minimally invasive management: intranasal hematoma aspiration combined with needle knife opening under a duodenoscope.

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INTRODUCTION

Intramural duodenal hematoma (IDH) following endoscopic retrograde cholangiopancreatography (ERCP) is very rare. It is usually caused by blunt damage induced by the endoscope or accessory equipment. The typical clinical symptoms of duodenal hematoma include abdominal pain and vomiting, which are directly induced by duodenal obstruction. The hematoma may also lead to obstruction of the papilla opening, and pancreatitis and cholestasis may follow. The clinical presentations and imaging techniques, *e.g.*, ultrasound, upper gastrointestinal endoscopy, magnetic resonance imaging (MRI) or computed tomography (CT), can confirm the diagnosis. A search of PubMed revealed that there has been no previous report on transnasal endoscopic drainage for treatment of duodenal hematoma. Duodenal hematoma is mainly treated by open surgery drainage and percutaneous transhepatic cholangial drainage (PTCD)^[1,2]. However, both cause great trauma. In this paper we report our successful experience on minimally invasive management of a massive IDH following ERCP using intranasal hematoma aspiration combined with needle knife opening under duodenoscopy, which can be done under direct vision, with less trauma and risk.

CASE REPORT

A 48-year-old male patient presented with epigastric distention and abdominal pain after removing a stone in the common bile duct by ERCP. Before ERCP, the patient had abdominal discomfort, and examination found a common bile duct stone, with no history of hypertension or coagulation disorders. Laboratory findings before ERCP were as follows: total/direct bilirubin (Tbil/Dbil): 10.2/4.0 mmol/L; white blood cells (WBC) $4.0 \times 10^9/L$, 59.4%; γ -glutamyltransferase (γ -GT): 196 U/L; coagulation function: fibrinogen (Fbg): 1.6 g/L; prothrombin time (PT): 12.6 s. The patient began to vomit after 1 wk. A CT scan showed a 59 mm \times 53 mm intact cyst near the head of pancreas in the duodenum (Figure 1A), suspicious of duodenal hematoma. Hepatic function was as follows: γ -GT: 337 U/L; Tbil/Dbil: 19.2/8.0 mmol/L; WBC: $5.6 \times 10^9/L$, 73.4%; Fbg: 2.7 g/L; PT: 13.9 s; and blood amylase was normal.

Liver function examination showed a γ -GT of 196 U/L. Emergency duodenoscope inspection revealed a huge cystic bulging object in the intestinal wall of the duodenum, obstructing the duodenum (Figure 1B). A duodenoscope could not pass the obstruction, and the opening of the duodenal papilla was covered by the hematoma. The diagnosis was confirmed as a duodenum hematoma after ERCP. We punctured the hematoma with a needle knife, then inserted a guidewire and catheter (Figure 2) and extracted 100 mL dark red bloody fluid. The fluid became light red after washing with 1:10 000 epinephrine:saline and metronidazole. When the tension of hematoma became smaller, it was found that the hematoma was located at the lateral descending duodenum, 3 cm from the opening of the main papilla. There was no blood at the opening of papilla, and normal bile flowed out from the opening. X-ray showed the bile duct was full of gas. Then we placed a 8.5F per nasal drainage pipe into the hematoma for continuous drainage (Figure 2).

The epigastric distention and abdominal pain were greatly relieved after hematoma drainage, and the patient could ingest semi-fluid without vomiting. Dark red hydatid fluid (about 20 mL) was continuously extracted from the hematoma each day for 2-3 d, and the drainage volume was 5 mL at 5 d after the operation; the patient developed a low fever (38.2 °C) on the 2nd day after the operation. Duodenoscopy examination was performed again after drainage for 5 d, and it was found that the hematoma was smaller, but the local mucosa still had hyperemia and edema (Figure 3A); the duodenoscope could be easily passed without bleeding and edema at opening of the papilla. The drainage tube was removed and the puncture site was closed with hemostatic clips (Figure 3B). Then we explored the extra-hepatic bile ducts again with a stone basket and found no stones. We placed an endoscopic nasobiliary drainage (ENBD) tube in the common bile duct to avoid puncture site infection caused by bile, and it was pulled out 4 d after ERCP. Then the patient was discharged from hospital without

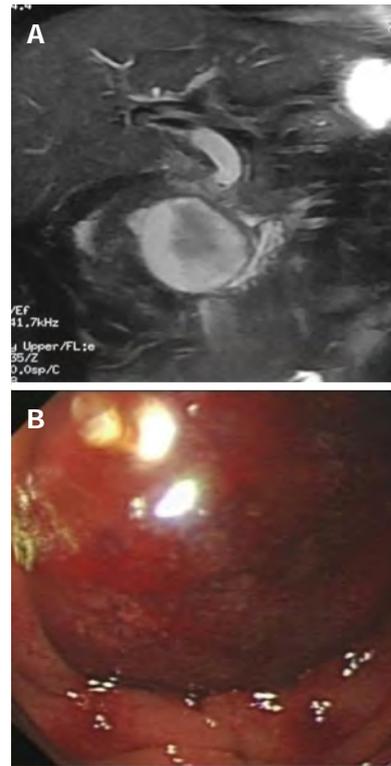


Figure 1 Computed tomography scan and liver function examination of the patient. A: Computed tomography scan showed a 59 mm \times 53 mm intact cyst near the head of the pancreas in the duodenum; B: Duodenoscopy inspection showing a large cystic bulging object in the intestinal wall of the duodenum, obstructing the duodenum.

any symptoms. The hematoma had completely disappeared at 1 mo follow-up.

DISCUSSION

A duodenal hematoma is usually caused by trauma, anticoagulant therapy, rupture of a duodenal aneurysm or biopsy. A duodenal hematoma following ERCP is very rare^[1-4]. Some studies reported that the guidewire was inserted into the liver capsule and caused bleeding beneath the liver capsule due to rupture of small vessels in the liver parenchyma. When it is difficult to insert a duodenoscope into the duodenum, the long route of the endoscope and abdominal pressure may cause hematoma in the peritoneal cavity^[5,6].

The position of the duodenum is generally stationary. When it is compressed, the pylorus will close, and a close circuit is formed between the pylorus and Treiz ligament. The air pressure in the duodenum will be acutely increased, causing the rupture of vessels in the intestinal wall. In some cases, direct damage to the intestinal wall during the operation will cause the hematoma, and if not treated in time, it will result in intestine necrosis and perforation.

The diagnosis of IDH is likely if there are symptoms such as abdominal pain, vomiting and duodenal obstruction after ERCP^[6]. Laboratory evaluations are non-specific and usually show only a mild decrease in hemoglobin

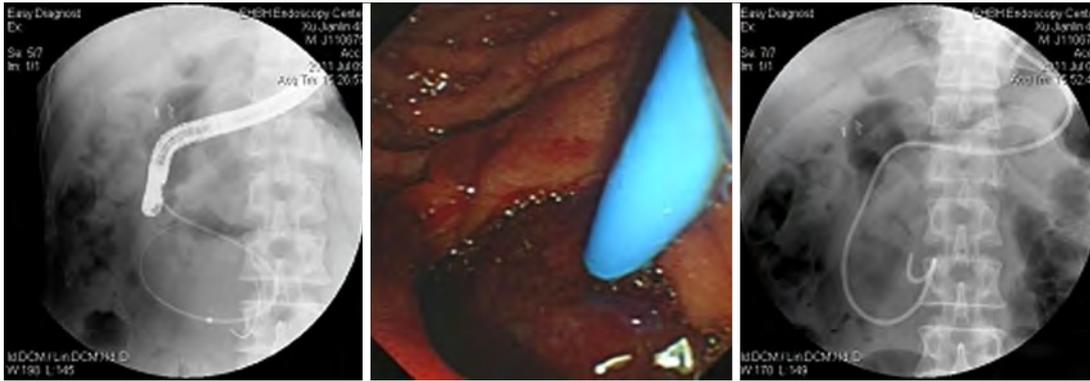


Figure 2 Drainage of the hematoma by the nasal method.

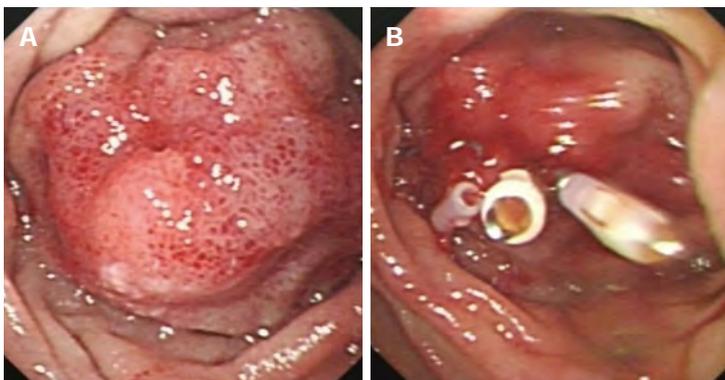


Figure 3 Duodenoscopy examination. A: Hematoma was greatly decreased in size; B: The puncture area was closed with hemostatic clips.

concentration. Imaging techniques, including gastrointestinal endoscopy, CT and MRI, can be used to confirm the diagnosis. Gastrointestinal endoscopy can demonstrate not only duodenal obstruction, but also perforation. CT and MRI can determine the exact extent of the hematoma and may also indicate perforation^[7,8]. Since perforation needs a surgical operation, imaging techniques have to be performed immediately.

Once IDH is confirmed, conservative management with fasting, total parenteral nutrition and nasogastric suction should be given promptly. Traditionally, a duodenal hematoma is mainly treated by open surgery drainage and PTC^D^[9], both causing great trauma. The method used in the present case is minimally invasive and has less risk. We would like to emphasize the following key points in our method. First, the opening should be made at the center of the hematoma with the needle knife under a duodenoscope, and the head of the drainage tube should be soft and flexible to avoid damage to the hematoma wall. Transnasal drainage can be performed by vacuum aspiration, which allows easy observation of the drainage volume. Secondly, after opening the hematoma, the catheter is introduced and the contents are extracted, and then a transnasal guidewire is placed for drainage. Thirdly, the opening on the hematoma should be clamped and an ENBD tube should be placed to drain the bile duct to avoid infection by bile and intestinal juice.

A duodenal hematoma is a rare complication of ERCP, and blunt damage by the endoscope and an accessory should be avoided during ERCP. A CT scan shows specific signs of ERCP-associated duodenal hematoma, such as a high density mass in the enteric cavity and a narrowed enteric cavity. A duodenal hematoma can be easily diagnosed by endoscopy. When the hematoma is very big or causes obstruction of the intestine, a drainage tube can be used to aspirate the contents of the hematoma. Transnasal drainage combined with needle knife puncture under endoscopy has less injury risk and is easy to perform, making it superior to percutaneous drainage. Since ERCP has become a common approach for the treatment of biliary and pancreatic diseases, the incidence of IDH is likely to increase. Our method is worth popularizing in clinical practice.

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Small intestinal tubular adenoma in a pediatric patient with Turner syndrome

Wen-Juan Tang, Ying Huang, Lian Chen, Shan Zheng, Kui-Ran Dong

Wen-Juan Tang, Ying Huang, Department of Gastroenterology of Fudan University Children's Hospital, Shanghai 201102, China

Lian Chen, Department of Pathology of Fudan University Children's Hospital, Shanghai 201102, China

Shan Zheng, Kui-Ran Dong, Department of Surgery of Fudan University Children's Hospital, Shanghai 201102, China

Author contributions: Tang WJ and Huang Y contributed equally to this work; Tang WJ and Huang Y designed the research and wrote the paper; Chen L contributed reagents and supplies; Zheng S and Dong KR performed the surgery.

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Correspondence to: Ying Huang, MD, PhD, Department of Gastroenterology of Fudan University Children's Hospital, 399 Wanyuan Road, Shanghai 201102, China. yhuang815@163.com
Telephone: +86-21-64931990 Fax: +86-21-64931727

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Abstract

Turner syndrome (TS) is a female chromosomal disorder caused by the lack of an X chromosome. The loss of this chromosome may result in the deficiency of tumor-suppressive or DNA repair genes, leading to tumorigenesis. Recombinant human growth hormone (GH) has been popularly used for treatment in TS patients for growth promotion. Although treatment with GH has been correlated with precancerous and cancerous lesions in TS children, its associations with gastric or colonic tumors, especially ileal tubular adenomas, have not been reported frequently. We here report a case of a 16-year-old patient with TS and tubular adenoma of the small intestine. Whether the ileal adenoma was caused by TS itself or GH therapy was discussed.

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Key words: Tubular adenoma; Turner syndrome; Growth hormone; Pediatric patient; Ileocolonoscopy

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INTRODUCTION

Turner syndrome (TS) is a female chromosomal disorder in which all or part of the X chromosome is missing. Clinically, it is not uncommon for patients with TS to develop tumors from various tissues. In adults with TS, gastric or colonic tumors have been reported. However, these tumors are rarely reported in children, and the occurrence of ileal tubular adenoma in pediatric TS patients has not been described. In this case report, ileal tubular adenoma was detected in a 16-year-old girl with TS after receiving growth hormone (GH) therapy for three years. Whether the ileal adenoma was caused by TS itself or GH therapy was discussed.

CASE REPORT

A 16-year-old girl first admitted to the hospital in July 2009 because of short stature. On admission, she was 127.5 cm in height and 25 kg in weight. Her bone age was 9 years. Ultrasonography revealed a small uterus and right ovary and possible absence of left ovary. A chromosomal analysis showed genetic mosaicism of the X chromosome, namely 46Xi (45X, 46XX). Diagnosis of TS was made and daily nocturnal GH injections were initiated. In 2 years, the patient gained 10 cm in height.

In the patient's health history, she experienced per rectal bleeding at the age of 9 mo that had subsided spontaneously and no definitive diagnosis was made. The patient also had recurrent abdominal cramping associated with loose bowel movements, although the pain usually subsided spontaneously. In August 2009, an ultrasono-

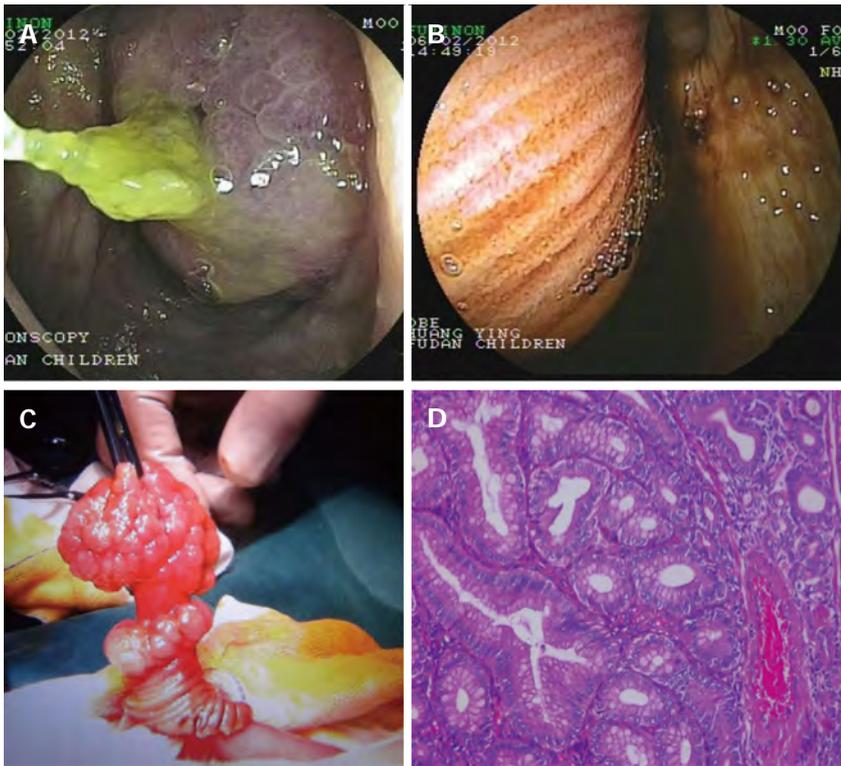


Figure 1 The image of the polyp. A: A large polyp was seen in the terminal ileum when ileocolonoscopy was performed. The tumor appeared hyperemic; B: Double balloon enteroscopy was performed through the anus. The enteroscope was advanced to the ileocecal region and a mass covered with small intestinal mucosa was seen protruding through the ileocecal valve. The appearance was indicative of intussusception; C: A polyp of about 2 cm × 4 cm in diameter and 55 cm from the ileocecal region was seen during operation, with a wide base and multi-nodular appearing surface. The pedicle also appeared nodular; D: Histopathological section of the polyp showing pleomorphic glandular hyperplasia with formation of nodules. The polarity of cell was well presented (hematoxylin and eosin stain, original magnification, × 200).

gram was performed when the abdominal pain became more severe, and an abdominal mass on the right side was noted and suggested intussusception. The pain and mass disappeared spontaneously and no further treatment was undertaken.

The patient again developed abdominal pain on January 27, 2012. The pain was para-umbilical, colicky and associated with bloody stool. There was no tenesmus, and the abdominal colic dissipated after bowel movements. The blood in the stool appeared bright red in color. The patient vomited twice and the vomit contained no blood. She also had a fever of 38 °C and was admitted to the hospital for investigation of gastrointestinal bleeding in February 2012.

On admission, her general condition was normal. There was developmental delay in height and no secondary sexual characteristics were noted. Intellectually, her development was normal. Other features of TS were not evident.

On admission, her blood test revealed a hemoglobin level of 79 g/L, positive stool occult blood, white blood cell 10-15/high power, red blood cell 3-5/high power, and no macrophages. Stool culture was negative. Coagulation function was normal. The patient had several dark-colored, bloody bowel movements after admission and abdominal pain that was relieved after bowel movement. She also vomited several times after admission, and the

vomit consisted of ingested food. No coffee-ground material was noted. A radionuclide scan for Meckel's diverticulum was negative.

Since she has a history of administration of GH, both insulin-like growth factor-1 (IGF-1) and IGF binding protein levels were checked and both were within normal limits (119 ng/mL and 3.05 µg/mL, respectively). Gastroscopy revealed gastritis and a positive rapid urease test for *Helicobacter pylori*. In ileocolonoscopy, the ileocecal valve was petulant and a large polyp was seen in the terminal ileum (Figure 1A). The polyp shifted in position and was soon noted to have retracted to a more proximal position. It was not seen again until the colonoscope had advanced to more than 10 cm proximally.

Enhanced computed tomography and ultrasound of the abdomen were both suggestive of intussusception of the small intestine with a focal segmental increase in the thickness of the bowel wall. A double-balloon enteroscope was advanced through the anus to the ileocecal region. There a mass covered with small intestinal mucosa was seen protruding through the ileocecal valve (Figure 1B). The appearance was indicative of intussusception of the ileum into the colon and an operation was performed to reduce the invagination. Further examination of the small intestine found a mass of about 2 cm × 4 cm in diameter 55 cm from the ileocecal region. The intestinal segment was decompressed and opened. A small

Table 1 Reported cases of gastroenteric tumors in association with Turner syndrome in children

| Ref. | Study location | Age (yr) | Tumor location |
|---|----------------|----------|------------------------------------|
| Siqueira <i>et al</i> ^[6] | Japanese | 14 | Adenocarcinoma of colon |
| Krishnamurthy <i>et al</i> ^[7] | India | 13 | Adenocarcinoma of rectum |
| Eriguchi <i>et al</i> ^[8] | United States | 13 | Carcinoid of appendix |
| Present case | China | 16 | Tubular adenoma of small intestine |

bowel polyp with a wide base and multi-nodular appearing surface was observed (Figure 1C). The pedicle also appeared nodular. The whole segment of small intestine was resected with the polyp *in situ*.

The pathological findings of the polyp are as follows: macroscopically, brownish polyp 3.3 cm × 2.8 cm in size with nodular protuberances on the surface. The cut surface was grayish in color and of medium firmness; microscopically, pleomorphic glandular hyperplasia with formation of nodules, the polarity of the cell was well presented (Figure 1D); immunohistochemically, β -catenin positive, adenomatous polyposis coli protein negative, 10% Ki-67 positive cells, cyclin D1 positive, carcinoembryonic antigen weak positive, neuron specific enolase positive, synaptophysin positive, and CD56 positive. The final diagnosis was small intestinal tubular adenoma.

DISCUSSION

TS is a female chromosomal disorder and is caused by the lack of an X chromosome. It causes delays in both general growth and sexual development. TS is characterized by physical abnormalities such as short stature, swelling of the hands and feet, a broad chest, low hairline, low-set ears, and a webbed neck. Girls with TS typically experience gonadal dysfunction, which results in primary amenorrhea and sterility.

Many tumor-suppressive and DNA repair genes are located on the X chromosome; the lack of one or many genes may result in chromosomal instability and the deficiency of critical DNA repair mechanisms. Therefore, TS may be associated with oncogenesis in some patients^[1]. Clinically, TS has been associated with some tumor types^[2-4] with the most common tumors arising in the sex glands. However, other reports have found that patients with TS have a higher incidence of tumors of the central nervous system (including ophthalmic tumors) and of the bladder and urethra. Some reports indicated that adults with TS have a higher incidence of gastric or colonic tumors, although the mechanism is not clear^[5] and the association was not common in children^[6-8]. Importantly, ileal tubular adenoma in patients with pediatric TS has not been described. The reported and present pediatric cases in the literature are summarized in Table 1.

Recombinant human GH has been popularly used in TS patients for growth promotion. GH exerts its action through binding and activation of its receptor (hGHR), which is ubiquitously expressed in the human body. In

TS patients, GH is therapeutically used for growth enhancement, especially for height. GH also influences many metabolic processes, and its association with tumorigenesis has been the subject of intensive research. In patients with acromegaly, the incidence of precancerous (adenomatous polyps) and cancerous lesions in the colon is increased^[9]. Carroll *et al*^[10] reported that GH deficiency in mice reduced the incidence of colonic tumors. Likewise, hGHR expression in patients with colonic cancer is upregulated, and its expression level is positively correlated with the stage and differentiation of the tumor^[11]. A cohort study by Swerdlow *et al*^[12] that followed 1848 patients receiving GH treatment determined that the incidence and mortality of both rectal and colonic tumors were increased compared with the non-treated patients. These studies suggest that GH may be related to the pathogenesis of gastrointestinal tumors. Moreover, IGF-1 is an important mediator of the action of GH and has been implicated in the pathogenesis, development, invasion and metastasis of many human tumors. In China, studies have demonstrated that IGF-1 mRNA expression level was higher in gastric cancer tissues in contrast to non-tumorigenic tissues^[9]. Other studies have also demonstrated that IGF-1 receptor expression in colonic cancer is elevated and that this over-expression results in tumor cell growth, independent of other growth factors such as platelet-derived growth factor, basic fibroblast growth factor, and epidermal growth factor^[13].

Conversely, insulin-like growth factor binding proteins (IGFBPs), particularly IGFBP-3, can inhibit IGF-stimulated growth of tumor cells, facilitating apoptosis^[14]. Although the role of the GH-IGF-1 axis in gastrointestinal tumors has not been well defined, evidences from both basic and clinical studies^[15] indicate that it may play an important role in the pathogenesis of these tumors because GH enhances tumorigenesis through the action of IGF-1; IGF-1 induces tumorigenesis in the absence of GH; and IGFBP-3 inhibits tumorigenesis.

In conclusion, a small intestinal tumor was detected in a 16-year-old TS patient who had received GH therapy for three years. The patient had a history of per rectal bleeding, recurrent abdominal pain, and intussusception, which indicated the presence of an intestinal polyp. Although neither IGF-1 nor IGFBP-3 levels were elevated, their effects on tumorigenesis could not be ruled out. As the concentration of free IGF is proportional to the ratio of IGF-1/IGFBP-3, and IGF-1 impacts both growth and tumorigenesis, these factors should be closely monitored in patients receiving GH treatment. Future studies should address the relationship between GH therapy, the IGF-axis, and incidence of intestinal carcinomas.

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Obstructive jaundice and melena caused by hemocholecyst: A case report

Ying Fan, Shuo-Dong Wu, Jing Kong

Ying Fan, Shuo-Dong Wu, Jing Kong, the Second Department of General Surgery, Shengjing Hospital of China Medical University, Shenyang 110004, Liaoning Province, China
Author contributions: Fan Y and Wu SD contributed equally to this work; Fan Y and Wu SD designed the research; Kong J discussed the clinical features of the patient and analyzed data; Fan Y, Wu SD and Kong J wrote the paper.
Correspondence to: Dr. Shuo-Dong Wu, the Second Department of General Surgery, Shengjing Hospital of China Medical University, No. 36 Sanhao Street, Heping District, Shenyang 110004, Liaoning Province, China. surgeonfanying@yahoo.com.cn
Telephone: +86-24-96615 Fax: +86-24-96615
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Abstract

A hemocholecyst (HC) is a clot-filled gallbladder caused by bleeding into its lumen. Obstructive jaundice caused by the compression of HC to the hilar biliary tract is likely to be misdiagnosed as cholangiocarcinoma and is extremely rare. We herein report a case of obstructive jaundice and melena caused by HC. A 57-year-old male patient presented with right upper quadrant pain associated with icteric sclera and melena was suspiciously diagnosed as having malignant cholangiocarcinoma by abdominal ultrasonography, computed tomography and magnetic resonance imaging. Laparotomy found a hematoma in the gallbladder. The hematoma spread to the left hepatic lobe forming an exogenous mass which compressed the hilar biliary tract. Radical cholecystectomy and bile duct exploration with T-tube drainage were performed. Histopathological examination revealed massive necrosis of the gallbladder mucosa with inflammatory cells infiltration as well as intraluminal hematoma formation. One month after operation, a T-tube cholangiography revealed a normal biliary tree. We suggest that HC should be considered in patients with obstructive jaundice and melena after common causes are ruled out.

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Key words: Hemocholecyst; Biliary tract; Obstruction; Jaundice; Melena

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INTRODUCTION

A hemocholecyst (HC) is a clot-filled gallbladder caused by bleeding into its lumen. As a cause of hemobilia, HC is rarely reported with a variety of etiology including trauma^[1], iatrogenic manipulation^[2,3], gallbladder tumor^[4,5], cholecystitis^[6] and ruptured cystic artery aneurysm^[7]. Hematological disorders such as hemophilia are also implicated in some cases^[8,9]. Here, we report a rare case of obstructive jaundice and melena caused by HC. Though abdominal ultrasonography (US), computed tomography (CT) and magnetic resonance imaging (MRI) have proved to be highly accurate methods for evaluating gallbladder disorders, a definitive diagnosis cannot be established preoperatively. In addition, our patient had not experienced abdominal trauma, and had no history of obvious bleeding disorders.

CASE REPORT

A 57-year-old man presented at our emergency department with the right upper quadrant pain with icteric sclera for seven days, and melena for two days. The pain was dull in character, sudden in onset, and did not radiate to the back. It was not aggravated by food intake or related to movement. The patient had no history of abdominal trauma or peptic ulcer with bleeding. On admission, the patient was febrile with right upper

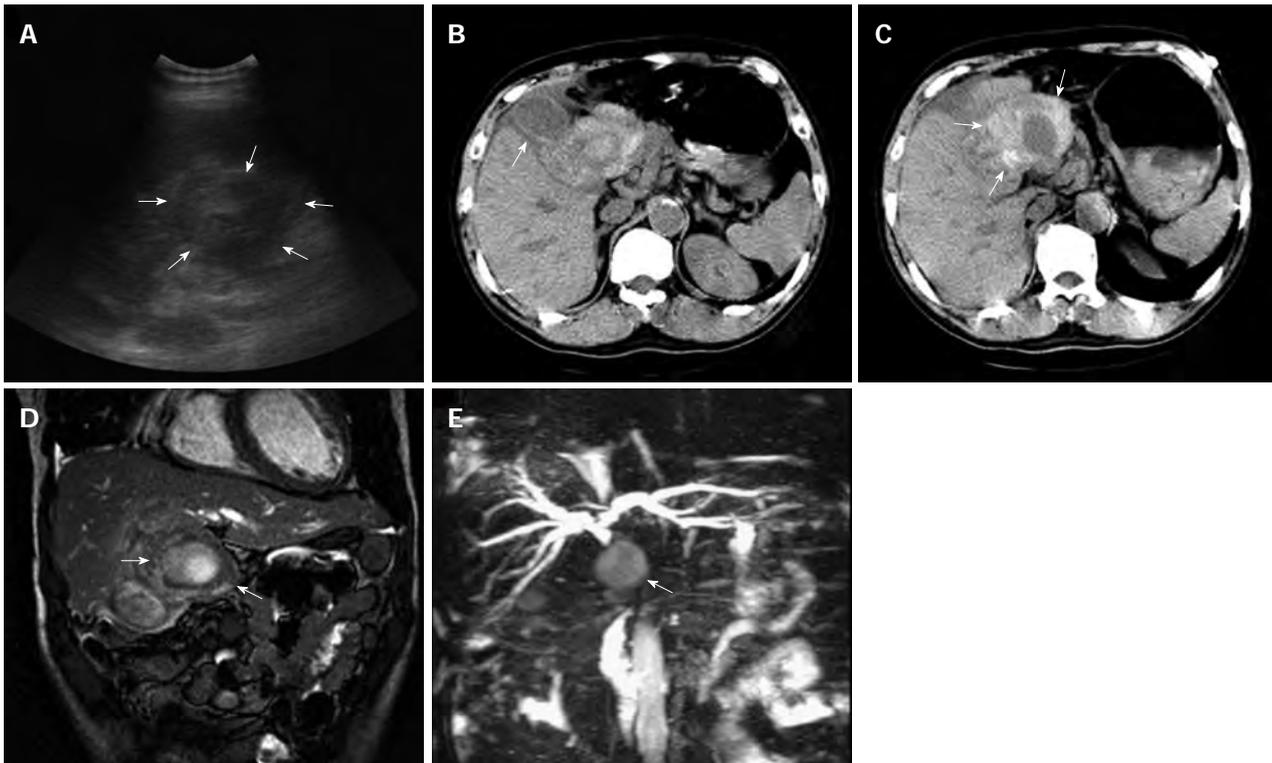


Figure 1 Preoperative imaging findings. A: Ultrasonography revealed a mass with heterogenous echo at the gallbladder region (arrows); B: Computed tomography (CT) showed thickening of the fundus and body of gallbladder wall (arrow); C: CT showed a mass with high-density shadow located at the gallbladder neck without clear demarcation from the left hepatic lobe (arrows); D: Magnetic resonance imaging (MRI) showed a mass located at the gallbladder neck and left hepatic lobe (arrows); E: MRI showed slight dilation of intrahepatic ducts and an obscure image of hilar biliary tract (arrow).

quadrant tenderness, rebound tenderness, slight muscular rigidity and a positive Murphy's sign. Laboratory examinations revealed abnormal hepatic enzyme levels: aspartate aminotransferase 145 U/L (normal, 5-34 U/L), alanine aminotransferase 95 U/L (normal, 0-40 U/L), increased total bilirubin 135.5 $\mu\text{mol/L}$ (normal, 3.4-20.5 $\mu\text{mol/L}$) and increased alkaline phosphatase 240 U/L (normal, 40-150 U/L). The patient had leukocytosis (14 200/ μL), with decreased hemogram (hemoglobin, 8.9 g/dL; hematocrit, 32.7%), but normal platelet count ($242 \times 10^3/\mu\text{L}$). However, prothrombin time and partial thromboplastin time were both normal. Serum amylase and lipase levels were also within normal limits. Tumor markers including alpha foetoprotein, carcinoembryonic antigen and carbohydrate antigen were also negative.

Ultrasound of the abdomen revealed a mass with heterogeneous echo at the gallbladder region (Figure 1A). CT showed thickening of the gallbladder fundus and body wall (Figure 1B) and a mass with high-density shadow located at the gallbladder neck without clear demarcation from the left hepatic lobe (Figure 1C). MRI showed a mass located at the gallbladder neck and left hepatic lobe (Figure 1D) with slight dilation of intrahepatic ducts and an obscure image of hilar biliary tract (Figure 1E). Gastrointestinal tract endoscopic study found no common causes of melena such as malignancy, ischemic colitis, *etc.* Initial diagnosis of malignant cholangiocarcinoma was made. He was treated with antibiotics, hemostatics and fluid supplement before transferred to the operat-

ing room for exploratory laparotomy. On exploration, a hematoma was found in the neck-side lumen of the gallbladder. The hematoma spread to the left hepatic lobe forming an exogenous mass which compressed the hilar biliary tract (Figure 2). Cholecystectomy and bile duct exploration with T tube drainage were performed. Histopathology revealed massive necrosis of the gallbladder mucosa with inflammatory cell infiltration as well as intraluminal hematoma formation. The patient was discharged on the 7th postoperative day in a stable condition. One month after operation, a T-tube cholangiography revealed a normal biliary tree (Figure 3).

DISCUSSION

Hemorrhagic cholecystitis is an extremely rare cause of hemobilia. Up to now, only three cases of hemobilia which was clearly attributable to acalculous cholecystitis, have been reported by Shah *et al.*^[10] and Ellington *et al.*^[11]. Our patient is the 4th reported case. We think that acalculous cholecystitis may cause hemorrhage through mucosal necrosis and ulceration with erosion into one or more vessels in this patient.

Hemobilia classically presents as biliary colic pain, jaundice and gastrointestinal tract bleeding. However, the clinical presentation varies. Depending on the amount and rate of bleeding, blood may clot in various locations. If the blood does not clot in the biliary tract, hematemesis or melena may occur. If blood clots within the bile

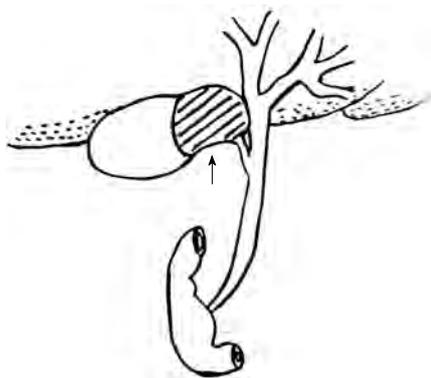


Figure 2 Schema of the hematoma found at the neck of the gallbladder during operation. The arrow indicates hematoma.

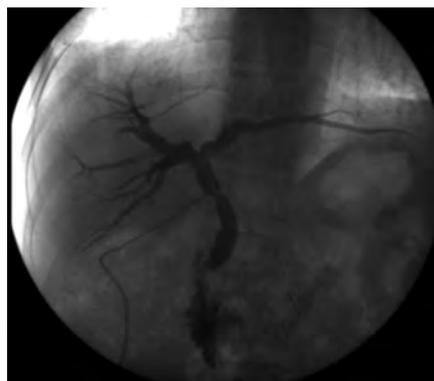


Figure 3 T-tube cholangiography revealed a normal biliary tree after operation.

duct, it may cause obstructive jaundice or pancreatitis. It was unusual in this patient that jaundice was not caused by blood clots within the bile duct, but resulted from the compression of the HC to the hilar biliary tract.

Our patient did not present with melena as a primary symptom. He had symptoms, signs, and laboratory studies that suggested choledocholithiasis and cholangitis. He developed melena only on the 5th day. The preoperative diagnosis of gallbladder hematoma is very difficult, or even impossible, to make in the absence of a history of trauma or bleeding diatheses. Preoperative US, CT and MRI are main techniques for diagnosing gallbladder diseases. Although the US and CT findings of gallbladder hematoma might not be exactly the same as those of gallbladder carcinoma or invasive hilar cholangiocarcinoma, a definitive diagnosis often cannot be made confidently. MRI is useful for differential diagnosis. It was reported that hematomas are similar in signal intensity to skeletal muscle on T1-weighted imaging, with conversion to marked hypointensity on T2-weighted imaging^[8]. The hematoma in our patient was not confined to the gallbladder but spread to the left hepatic lobe forming an exogenous mass which compressed the hilar biliary tract, thus making the diagnosis even more difficult. All preoperative imaging studies gave an impression of a highly suspected cholangiocarcinoma with invasion to the gallbladder neck and left hepatic lobe. In our case, cholangiocarcinoma could not be ruled out. Therefore, the correct diagnosis could only be made through dynamic image observation, aggregate analysis and resolute operation exploration.

In conclusion, we suggest that HC should be considered in patients with obstructive jaundice and melena after common causes are ruled out.

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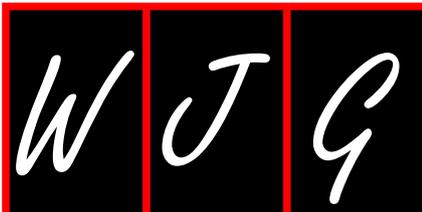
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Alcohol consumption and fatty liver disease

Ren-Nan Feng, Guo-Dong Sun, Yan Zhao, Fu-Chuan Guo, Chang-Hao Sun

Ren-Nan Feng, Yan Zhao, Fu-Chuan Guo, Chang-Hao Sun, Department of Nutrition and Food Hygiene, School of Public Health, Harbin Medical University, Harbin 150081, Heilongjiang Province, China

Guo-Dong Sun, Dean's Office, Harbin Medical University, Harbin 150081, Heilongjiang Province, China

Author contributions: Feng RN, Sun GD, Zhao Y and Guo FC contributed equally to this work; Feng RN and Sun CH designed the study and wrote the manuscript; Feng RN provided financial support for this work; all the authors were involved in revising the manuscript.

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Correspondence to: Chang-Hao Sun, PhD, Department of Nutrition and Food Hygiene, School of Public Health, Harbin Medical University, 157 Baojian Street, Nangang District, Harbin 150081, Heilongjiang Province, China. changhao2002sun@gmail.com

Telephone: +86-451-87502881 Fax: +86-451-87502885

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Abstract

Hamaguchi *et al* recently reported some interesting observations on alcohol consumption and risk of fatty liver disease from a large population. However, we feel that it might be necessary to discuss some concerns in this study. As the alcohol consumption categorization was defined by the same criteria in both men and women, which might affect their results. As another factor is soft drinks consumption. It has been proved that soft drinks, especially fructose, contributes to the development of obesity, diabetes, metabolic syndrome, and nonalcoholic fatty liver disease. However, this confounding factor was not adjusted or discussed in this article. The third is the genetic background, for some genetic factors are related with the development of fatty liver disease, which was also not considered yet.

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Key words: Alcohol; Fatty liver disease; Obesity; Diabetes; Metabolic syndrome

Core tip: Modest alcohol consumption was significantly inversely associated with fatty liver disease in recent studies. However, some studies did not consider some important potential confounding factors when they conclude their findings. Herein, we raised and discussed these important factors in this letter.

Feng RN, Sun GD, Zhao Y, Guo FC, Sun CH. Alcohol consumption and fatty liver disease. *World J Gastroenterol* 2013; 19(13): 2129-2130 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v19/i13/2129.htm> DOI: <http://dx.doi.org/10.3748/wjg.v19.i13.2129>

TO THE EDITOR

We read with great interest the article by Hamaguchi *et al*^[1] published in January 2012 at *World J Gastroenterol*. This cross-sectional study reported some interesting observations on alcohol consumption and risk of fatty liver disease from a large population. However, we feel that it might be necessary to discuss some concerns in this study.

The authors clearly indicated that alcohol consumption was significantly inversely associated with fatty liver disease, especially in men. However, they did not find this association in their previous cohort study^[2]. The reason for this contradiction might be that some important confounding factors were not considered. As the alcohol consumption categorization was defined by the same criteria in both men and women, only 84 female subjects (1.1%) were defined as excess alcohol consumers and 207 (2.7%) were defined as moderate alcohol consumers, the numbers being much lower than those in men (13.5% and 14.7%, respectively). Although the authors analyzed the data in both men and women, the initial categorization was not separated, which might affect their results.

Another factor is soft drinks consumption. It has been proved that soft drinks, especially fructose, contributes to the development of obesity, diabetes, metabolic syndrome, and nonalcoholic fatty liver disease^[3]. However, this confounding factor was not adjusted or discussed in this article. The last point is about the genetic background. Although the mechanisms of this inverse association between alcohol consumption and fatty liver disease are still unclear, some genetic factors are related with the development of fatty liver disease^[4-6], such as peroxisome proliferator-activated receptor gamma and hemochromatosis gene polymorphisms^[7,8]. Therefore, as some genetic factors might interact with alcohol consumption in fatty liver disease, it could be an interesting topic for further investigations.

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World Journal of Gastroenterology
Room 903, Building D, Ocean International Center, No. 62 Dongsihuan Zhonglu, Chaoyang District, Beijing 100025, China
Telephone: +86-10-59080039
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E-mail: wjg@wjgnet.com
http://www.wjgnet.com

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Angiogenic inhibitors for older patients with advanced colorectal cancer: Does the age hold the stage?

Giuseppe Aprile, Caterina Fontanella, Eufemia Stefania Lutrino, Laura Ferrari, Mariaelena Casagrande, Giovanni Gerardo Cardellino, Gerardo Rosati, Gianpiero Fasola

Giuseppe Aprile, Caterina Fontanella, Eufemia Stefania Lutrino, Laura Ferrari, Mariaelena Casagrande, Giovanni Gerardo Cardellino, Gianpiero Fasola, Department of Oncology, University and General Hospital, 33100 Udine, Italy
Gerardo Rosati, Medical Oncology Unit, San Carlo Hospital, 85100 Potenza, Italy

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Correspondence to: Giuseppe Aprile, MD, Department of Oncology, University and General Hospital, Piazzale S Maria della Misericordia 1, 33100 Udine,

Italy. aprile.giuseppe@aoud.sanita.fvg.it

Telephone: +39-432-559308 Fax: +39-432-559305

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Abstract

Although major progress has been achieved in the treatment of advanced colorectal cancer (CRC) with the employment of antiangiogenic agents, several questions remain on the use of these drugs in older patients. Since cardiovascular, renal and other comorbidities are common in the elderly, an accurate assessment of the patients' conditions should be performed before a treatment decision is made. Since most CRC patients enrolled in clinical trials testing antiangiogenic drugs were aged < 65 years, the efficacy and tolerability of these agents in elderly patients has not been adequately explored. Data suggest that patients with advanced CRC derive similar benefit from bevacizumab treatment regardless of age, but the advantage of other antiangiogenic drugs in the same class of patients appears more blurred. Literature data suggest that specific antiangiogenic-related toxicities such as hyperten-

sion or arterial thromboembolic events may be higher in the elderly than in the younger patients. In addition, it should be emphasized that the patients included in the clinical studies discussed herein were selected and therefore may not be representative of the usual elderly population. Advanced age alone should not discourage the use of bevacizumab. However, a careful patients' selection and watchful monitoring of toxicities are required to optimize the use of antiangiogenics in this population.

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Key words: Advanced colorectal cancer; Bevacizumab; Elderly; Antiangiogenesis; Chemotherapy

Core tip: Although promising, limited evidence supports the use of antiangiogenic drugs to treat elderly colorectal cancer patients, that also may have increased toxicities compared to younger subjects. However, advanced age *per-se* should not discourage the use of these drugs. Since older patients constitute a heterogeneous population in terms of overall health status and comorbid conditions, a careful patients' selection and a watchful monitoring of potential treatment-related side effects are recommended to optimize the use of angiogenesis inhibitors in this population.

Aprile G, Fontanella C, Lutrino ES, Ferrari L, Casagrande M, Cardellino GG, Rosati G, Fasola G. Angiogenic inhibitors for older patients with advanced colorectal cancer: Does the age hold the stage? *World J Gastroenterol* 2013; 19(14): 2131-2140 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v19/i14/2131.htm> DOI: <http://dx.doi.org/10.3748/wjg.v19.i14.2131>

INTRODUCTION

Whilst most of cancer diagnosis and deaths occur in

older subjects^[1,2], three major factors are shaping the scenery in which the advanced colorectal cancer (CRC) is managed in all developed countries. Firstly, people are steadily aging and cancer incidence and prevalence are rising among senior citizens^[3,4]. Secondly, the incorporation of new drugs within more complex treatment strategies has raised the median survival of CRC patients to unprecedented figures of 30 mo^[5]. Lastly, more often than before, aggressive surgery and other regional approaches are performed with curative intent in older oligometastatic patients. As a result, the soaring demand for care of senior with CRC is likely to further increase. Although many elderly cancer patients have concurrent chronic disorders or morbidities requiring medical treatment and present with diminished organ functions, impairment of daily vital activities or minor cognitive deficits, the majority of them are treated with systemic chemotherapy and/or biologics^[6,7]. Bevacizumab, a humanized vascular endothelial growth factor (VEGF) inhibitor, has proven efficacy when added to systemic chemotherapy regardless CRC patients' age in first or subsequent lines of therapy^[8]. Specific data regarding its use in the older population are limited. Nevertheless, one out of three patients receive bevacizumab beyond 65 years of age^[9]. Chronological age is still a major barrier that limits the proposal of standard treatment options to the elderly and the harm-to-benefit risk is particularly challenging when treating with noncurative intent^[10]. However, patients' chronologic age does not always reflect their overall health status and older patients are highly heterogeneous because of dissimilar types and grades of concurrent morbidities. All these reasons may increase the difficulty in choosing the most appropriate treatment. Besides, advanced age is a common exclusion criteria to be recruited in clinical trials so that elderly patients have been underrepresented in CRC studies and the few included, usually representing less than 15% of the whole trial population, are highly selected. Despite recent studies have demonstrated the usefulness of a comprehensive geriatric assessment, its adoption in the clinical practice is still limited. Herein, we present the latest data regarding the use of antiangiogenic drugs in older CRC patients, specifically focusing at safety issues and efficacy results of landmark clinical studies.

THE IMPORTANCE OF ANGIOGENESIS IN COLORECTAL CANCERS

Angiogenesis is a cornerstone of tumor mass expansion. In response to hypoxia, the activation of hypoxia-inducible factor (HIF) triggers the expression of VEGF, one of the most important proangiogenic molecules^[11], and its numerous isoforms^[12]. In order to grow, CRCs need to continually acquire new blood supplies throughout the neoangiogenetic process, the formation of new capillaries rising from the splitting of existing ones. In the same way as in other solid tumors, angiogenesis plays an important role in CRC progression and metastatization, and its therapeutic inhibition has become a key component

of anticancer treatment. Bevacizumab, the first Food and Drug Administration-labeled antiangiogenic antibody, was approved for clinical use after showing efficacy in combination with chemotherapy in CRC patients. Still, many issues are unresolved, such as the lack of validated predictive biomarkers^[13], the reasons for initial or acquired resistance to VEGF-inhibitors, and the uncertainty surrounding the opportunity for further antiangiogenic treatment beyond tumor progression. The study of non-endothelial cells involved in the neoangiogenesis through the production of growth factors or the modulation of cell-matrix interactions is of interest^[14]. For example, pericyte recruitment, a key phenomenon in the neovascular formation that is regulated by platelet-derived growth factor (PDGF), transforming growth factor beta (TGF- β) and angiopoietin/Tie2, may be blocked by a number of novel antiangiogenic multitarget tyrosine kinase inhibitors (TKI), including sunitinib, sorafenib, and regorafenib.

ANTIANGIOGENIC DRUGS IN OLDER CRC PATIENTS: FRIENDS OR FOES?

Elderly patients who received 5-FU either alone^[15] or in combination with irinotecan^[16] or oxaliplatin^[17] had similar survival benefits when compared to younger patients, although they may suffer higher rates of specific toxicities^[18]. Despite these reassuring data, clinicians tend to be conservative when considering systemic therapy in the elderly, either not proceeding or upfront reducing chemotherapy doses^[19]. The use of antiangiogenic drugs in patients with advanced CRC is supported by strong scientific evidence and common bevacizumab-related side-effects have been extensively described. Although its treatment effect does not seem to be influenced by patients' age, specific outcome data on the use of bevacizumab in elderly patients derive from retrospective subpopulation analyses of large randomized controlled trials^[20], small phase 2 studies^[21-25], non-randomized community-based registries^[26-29], or cohort studies^[30,31], and have been summarized elsewhere^[32].

In all, available data suggest that medically fit older CRC patients exposed to bevacizumab achieved the same benefits compared to younger patients^[33,34], with a similar toxicity profile, except for a significant increase in arterial thrombosis^[20].

More recently, the randomized phase III AVEX study has prospectively evaluated the additive effect of bevacizumab in the older CRC population. In this trial, 280 elderly patients (median age 76 years, range 70-87 years) received either capecitabine alone (1000 mg/sqm *bid* days 1-14, q21) or combined with bevacizumab (7.5 mg/kg). Over 90% of the enrolled patients had ECOG PS \leq 1. Clinically significant cardiovascular disease was among exclusion criteria. The simultaneous use of bevacizumab produced significant increase of median PFS (9.1 mo *vs* 5.1 mo, HR = 0.53). Interestingly, the RR was twice as high in the combination arm (19.3% *vs* 10.0%) while the safety profile was similar to that previously reported

when testing the combination of capecitabine and bevacizumab. Although the trial was underpowered to detect differences in survival, median OS was longer in the experimental arm (20.7 mo *vs* 16.8 mo, HR = 0.79)^[35]. Nevertheless, senior patients are usually underrepresented in well-designed randomized clinical trials and those enrolled, with good PS and few comorbidities, may not represent the average elderly population. This is the main reason why although skilled in dealing with hypertension, proteinuria, vascular thromboembolic events, bleeding, congestive heart failure, gastrointestinal perforation, and wound-healing complications, most oncologists fear that frequency and intensity of those side-effects might be greater in older CRC patients and the benefit-to-risk ratio less favorable in the general practice.

ANTIANGIOGENIC-INDUCED HYPERTENSION IN OLDER CRC PATIENTS

Epidemiological data collected over the last 30 years have demonstrated that the increasing prevalence of hypertension with age is linked to the combination of increased arterial stiffness, neurohormonal and autonomic dysregulation, and the progressive decline of renal function^[36-38]. In the elderly, hypertension *per-se* is a significant risk factor for cardiovascular morbidity and mortality. The increase of blood pressure, the most frequent side-effect of systemic inhibition of VEGF signaling, may occur at any time during therapy and it is often associated with asymptomatic proteinuria that spontaneously resolves as soon as treatment ends^[39-41]. According to a retrospective review that found an incidence of increased blood pressure of 29% in patients aged > 75 years *vs* 11% in those aged 65 to 75 years^[42], advanced age has to be considered a risk factors for the development of bevacizumab-induced hypertension.

Since hypertension-related disorders, such as stroke or myocardial infarction, have been reported with a higher incidence in older patients, a careful home-based daily blood pressure monitoring is suggested during the whole treatment period^[43,44]. Older patients developing elevated systolic blood pressure may be at particular risk for complication, since this event is even more associated with cardiovascular morbidity and mortality than diastolic hypertension^[45].

Management of antiangiogenic-induced hypertension in older patients usually requires standard treatment and should be promptly adopted^[46]. An 1.5%-3.4% 60-d mortality rate has been reported for CRC patients older than 65 years who developed or worsened preexisting hypertension during exposure to bevacizumab in the BRITe trial^[26]. How to manage this side-effect has been largely discussed. The upfront use of angiotensin-converting enzyme inhibitors is supported by their ability to counteract bevacizumab-induced plasminogen activator inhibitor-1 and this intervention is widely

adopted in the general population^[47]. However, the optimal treatment strategy in the elderly population is unconfirmed and the use of diuretics may be preferred. The JNC-7 hypertension guidelines suggested the use of thiazidic diuretics either alone or in combination as initial therapy for older patients^[48]. Importantly, the Hypertension in the Very Elderly Trial study showed that the use of indapamide, either alone or combined with perindopril, significantly reduced the incidence of stroke and heart failure even in patients aged ≥ 80 years^[49,50]. Although the long term benefits from antihypertensive drug treatment may be relevant for elderly subjects, fit octogenarians with bevacizumab-induced hypertension and a reduced life-expectancy should achieve benefits from intervention as soon as possible. Actually, immediate treatment compared with delayed treatment reduced the occurrence of stroke by 28% and cardiovascular complications by 15% in the Systolic Hypertension in Europe extension trial^[51].

Interestingly, retrospective studies have consistently reported a better survival outcome for patients who had developed bevacizumab-induced hypertension^[52,53]. Inhibition of VEGF signaling may induce a rapid increase in blood pressure, suggesting that hypertension could be a useful pharmacodynamic surrogate marker of VEGF activity^[54]. However, a retrospective analysis of seven randomized phase III trials with bevacizumab in different types of metastatic cancers, showed that the correlation between the vascular side-effect and the clinical outcome was shaggy, since the development of bevacizumab-induced hypertension inconsistently predicted longer PFS and OS^[55].

OTHER CARDIOVASCULAR SIDE-EFFECTS: VENOUS THROMBOEMBOLIC EVENTS, ARTERIAL THROMBOEMBOLIC EVENTS, BLEEDING, AND HEARTH FAILURE

Older cancer patients are at increased risk for vascular thrombosis^[56-59]. More specifically, placebo-controlled trials confirmed that the risk for venous thromboembolic events (VTE) is higher when the patient is aged ≥ 65 , diagnosed with gastrointestinal malignancies, or receiving antiangiogenic drugs^[60]. The average risk for VTE among ambulatory patients undergoing chemotherapy exceeds 12% over one year after treatment initiation, being the use of bevacizumab a potential risk factors^[61,62]. Nevertheless, a pivotal randomized trial enrolling over 800 CRC patients showed similar VTE incidences (19.4% *vs* 16.2%) regardless of bevacizumab exposure^[63]. In addition, a large pooled analysis showed similar incidence of all-grade VTE among CRC cancer patients exposed to bevacizumab (10.9%) compared to the control group (9.8%), with a similar median time to VTE of 2.2 mo *vs* 1.7 mo^[64]. Moreover, a real-practice observational study enrolling 637

advanced CRC patients reported a VTE incidence rate of only 4% in those aged over 65 years^[9]. Taking into account these data, it remains to be clarified if thromboprophylaxis should be considered for all cancer patients^[65,66], or limited to older patients with limited mobility^[67].

Some concerns surround the use of antiangiogenic drugs and the risk of arterial thromboembolic events (ATE) in elderly patients, many of whom may have preexisting cardiovascular risk factors or known cardiovascular disease. Although the event-related death rate remained low, the overall incidence of ATE is close to 4% for advanced CRC patients receiving bevacizumab, and less than 2% in those receiving chemotherapy alone. Significant risk factors for ATE are the history of previous VTE (HR = 2.17) and the older age (HR = 3.65)^[43]. In the BRITe (Bevacizumab regimens: investigation of treatment effects and safety) study, the rate of ATE was identical in patients aged < 65 years old (1.4%) compared with those aged between 65-74 years (1.4%), but it was significantly higher in patients aged > 75 years (4.8%). The analysis of the MAX AGTGC showed that bevacizumab was associated with a modestly higher risk of ATE, but the safety profile was similar regardless of age, previous history of ATE or other vascular risk factors^[68]. Whether the use of low-dose aspirin may be beneficial in reducing the rate of cardiovascular events in cancer patients as well as in the general population^[69] is plausible but unproven.

Atrial fibrillation and coronary artery disease are prevalent with increasing age. Patients on antithrombotic treatment for those conditions should be carefully monitored since bleeding is another potentially severe bevacizumab-induced adverse event^[70]. Whether patients on anticoagulant or antiplatelet therapy could be safely treated with bevacizumab is unclear^[71]. Patients receiving full-dose anticoagulants have a limited risk of severe bleeding (< 1%) regardless concomitant antiangiogenic exposure^[64] and advanced CRC patients treated with bevacizumab while on low-dose acetylsalicylic acid experienced similar rates of bleeding compared to the others (11% *vs* 14%, $P = 0.13$)^[72]. Nonetheless, because of the retrospective nature of the data, a note of caution should be used in patients who are candidates for bevacizumab and are receiving full-dose anticoagulation or antiplatelet therapy.

A large population-based study evaluated the risk of cardiovascular events (ATE, cardiac death, cardiomyopathy or congestive heart failure) among 6803 older CRC patients receiving bevacizumab and chemotherapy^[73]. Median age of included patients 73 years and a fifth were 80 years or older. The cohort study confirmed that the cardiovascular risk of bevacizumab use is modest, reporting no clear association between bevacizumab use and cardiovascular events and a lower than expected increased risk for ATE (HR = 1.82). Accordingly, a large Surveillance, Epidemiology and End-Results Medicare analysis suggested that older CRC patients treated with bevacizumab do not experience an increased risk of cardiovascular adverse events compared with patients not treated

with bevacizumab^[74]. Nevertheless, in the presence of ECGraphic signs of asymptomatic ischemia or in the case of angina or myocardial infarction, antiangiogenic treatment should be immediately discontinued^[75].

ANTIANGIOGENIC-INDUCED PROTEINURIA AND THE AGING RENAL FUNCTION

Animal models showed that VEGF is critical in the regulation of renal vascular network and that perturbations of VEGF expression may damage cellular architecture and function, leading to hypertension and proteinuria^[76]. Clinical data confirmed that bevacizumab may induce thrombotic microangiopathy by reducing glomerular VEGF, and the presence of podocytopathy in patients treated with antiangiogenic drugs suggested that to quantify urinary podocyte excretion may be a highly sensitive indicator of glomerular damage^[77]. A retrospective chart review showed that only 1.6% of patients developed severe proteinuria during bevacizumab administration; baseline chronic kidney disease and the development of hypertension significantly correlated with its occurrence ($P < 0.01$)^[78]. Indeed, a number of factors may increase the chance for antiangiogenic-induced renal toxicity among elderly patients, including age-related renal structural changes and limited nephron reserve, baseline comorbid conditions such as hypertension, diabetes, or cardiovascular diseases, and the use of polypharmacy or potentially nephrotoxic agents^[79]. Since renal failure is initially asymptomatic, a decreased glomerular filtration rate (GFR) or/and an increased albumin-to-creatinine ratio (albuminuria > 30 mg/g of creatinine) may suggest initial kidney damage and forecast later kidney failure^[80]. Therefore, an accurate assessment of renal function is essential during antiangiogenic therapy, especially for elderly people at risk of developing renal dysfunctions. In the clinical practice, elderly CRC patients should be accurately screened for proteinuria before starting bevacizumab or other antiangiogenic drugs by dipstick urine analysis, and a 24-h urine collection is suggested when a 2+ or greater urine dipstick reading is detected. The frequency of the test during the course of therapy should be customized.

THE ISSUE OF THE INTACT PRIMARY TUMOR IN THE ELDERLY

Metastatic CRC patients with intact primary tumor seldom require palliative treatment while on systemic upfront chemotherapy^[81,82]. Although bevacizumab has been associated with a 2% incidence of bowel perforation and a possible increased risk may exist in those with intact primary tumor, upfront noncurative intestinal resection of asymptomatic metastatic CRC patients may be avoided^[83,84]. Among 1953 bevacizumab-treated patients included in the BRITe study, 37 (1.9%) developed gastrointestinal perforation^[85]. Twenty-six of these cases (70%)

occurred within the first 6 mo since treatment start, with a median time to event of 3.5 mo. The presence of an intact primary tumor (HR = 2.0) or having received radiotherapy (HR = 2.1) were significant risk factors for perforation. Interestingly, the study failed to show higher rates of perforation in patients with history of peptic ulcer disease, diverticulosis, or in those who chronically used aspirin (≥ 325 mg/d) or other anti-inflammatory drugs. Moreover, the event was less frequently reported among those aged > 65 (1.1%) compared to those younger than 66 (2.6%) with an HR of 0.49. Similarly, in the MAX AGTGC trial, no gastrointestinal perforations were reported in CRC patients aged over 75 years exposed to bevacizumab, but 4 cases noted in the younger cohort^[34]. Alongside, the rate of intestinal perforation was 3.6% among 223 patients with unresected primary tumor compared to 1.2% among 1373 patients who had been previously resected in the First BEAT study, although an age breakdown was not available^[86].

IS MAINTENANCE WITH BEVACIZUMAB USEFUL IN THE ELDERLY?

Showing a greater benefit if bevacizumab was given until disease progression, results of No. 19966 trial suggested a possible role of antiangiogenic drugs in the maintenance phase^[87]. In addition, a number of randomized studies have been conducted to formally assess the role of bevacizumab as maintenance agent^[88]. In the MACRO (Maintenance in Colorectal) trial, 480 CRC patients were randomly assigned to receive six cycles of bevacizumab, capecitabine, and oxaliplatin every 3 wk followed by bevacizumab either alone or combined with the same chemotherapy regimen until progression^[89]. A slightly longer median PFS was reported in the combination arm (10.4 mo *vs* 9.7 mo), although burdened by a higher rate of severe sensory neuropathy (26% *vs* 8%) and HFS (13% *vs* 7%). Up today, the role of bevacizumab as maintenance therapy is still controversial^[90] and additional randomized maintenance studies, such as the AIO KRK0207, the CAIRO-3, the FFCD Prodigé 9, and the SAKK 41/06 trial, will soon clarify the point. Waiting for more substantial data, small non-randomized studies have investigated the role of bevacizumab as maintenance therapy. In the BOXE study, 44 elderly CRC patients with median age of 74 years (range 70-84 years) received XELOX and bevacizumab at the dose of 7.5 mg/kg every 3 wk for up to 8 cycles followed by maintenance with single-agent bevacizumab at the same dose^[23]. The trial suggested that the combination is feasible and safe in the elderly population and a maintenance with bevacizumab may be offered to responding patients with the intent to prolong PFS.

NOVEL ANTIANGIOGENIC ANTIBODIES: DO THEY FOSTER HOPE FOR OLDER PATIENTS?

Among the more promising novel drugs, aflibercept and

ramucirumab deserve to be presented. Aflibercept, a humanized protein composed of the extracellular domains of VEGFR-1/2 fused onto the constant region of human IgG, was specifically designed to bind VEGF-A, VEGF-B, and PlGF. VELOUR is a phase III placebo-controlled trial that tested the combination of FOLFIRI and aflibercept for advanced CRC patients that had failed an oxaliplatin-based first-line therapy^[91]. The primary endpoint of the trial was OS; secondary endpoints included PFS, overall response rate, and safety. Median age of patients treated with aflibercept was 61 years (range 21-82 years). Patients exposed to aflibercept had longer median OS (13.5 mo *vs* 12 mo, HR = 0.81) and PFS (6.9 mo *vs* 4.6 mo, HR = 0.75) compared to those who were not. However, the toxicity profile was not negligible. While the increases in hypertension and proteinuria were expected as class side-effects, patients receiving aflibercept reported unforeseen significantly higher rates of severe diarrhea (19.3% *vs* 7.8%), fatigue (16.9% *vs* 10.6%), stomatitis (13.7% *vs* 5.0%), and neutropenia (36.7% *vs* 29.5%). This should be considered when offering the treatment to older subjects because they may have increased toxicity when treated with second-line FOLFIRI. Ramucirumab, a VEGFR-2 inhibitor that has shown efficacy in second line gastric cancer, is being studied combined with FOLFOX in the RAISE trial^[92]. In this phase 3 study, over 1000 CRC patients that have previously failed first-line FOLFIRI and bevacizumab are randomized to FOLFOX or FOLFOX plus ramucirumab (8 mg/kg) every 2 wk. Results are eagerly awaited.

IS THERE A ROLE FOR ORAL TKI IN CRC?

In the last few years a number of small molecule inhibiting the VEGF pathway have been tested for advanced CRC patients with disappointing results. A phase III randomized trial compared FOLFIRI plus sunitinib (37.5 mg every 4 out of 6 wk) to FOLFIRI alone in 768 patients with advanced disease^[93]. At the second planned interim analysis the trial was stopped because the data monitoring committee found that the futility boundary had been crossed and more toxicity events were reported in the experimental arm, including neutropenia and severe diarrhea. Final results confirmed no differences in median PFS (7.8 mo *vs* 8.4 mo, HR = 1.05) and a more severe toxic profile.

Vatalanib (PTK787/ZK222584) is an antiangiogenic TKI that blocks VEGFR-1, 2, and 3 by acting as a competitive inhibitor at the adenosine triphosphate-binding site of the receptor kinase. Two randomized, placebo-controlled, large phase 3 trials studied the role of vatalanib in CRC patients treated upfront or in second-line setting^[94,95]. The CONFIRM-1 study showed that the addition of vatalanib to FOLFOX-4 had no impact on median PFS (7.7 mo *vs* 7.6 mo, HR = 0.88) or OS (21.4 mo *vs* 20.5 mo, HR = 1.08) compared with FOLFOX-4 alone as first-line treatment. Similarly, the CONFIRM-2 trial compared FOLFOX plus vatalanib or placebo in

855 advanced CRC patients after the failure of a first-line treatment. Again, marginal differences in terms of PFS (5.6 mo *vs* 4.2 mo, HR = 0.83) were registered with identical survival results (OS 13.1 mo *vs* 11.9 mo). In both trials, more gastrointestinal toxicities, dizziness, anorexia, pulmonary embolism and hypertension were reported in the vatalanib group. Taken together, the results of these trials suggested the uselessness of vatalanib for CRC patients, although a PFS advantage (HR = 0.65) was noted in those patients with higher lactate dehydrogenase baseline values.

The combination of oxaliplatin-based chemotherapy and cediranib, a potent inhibitor of the VEGF family receptor tyrosine kinases with multitarget TKI properties, has been extensively tested. The HORIZON III trial compared FOLFOX plus cediranib (20 mg, daily) or bevacizumab in over 1400 advanced CRC patients in first-line setting^[96]. The study did not meet its primary endpoint and the group exposed to cediranib experienced more toxicity. In addition, the randomized HORIZON II trial showed a marginal improvement in median PFS (8.6 mo *vs* 8.3 mo) when cediranib was added to FOLFOX or XELOX (*vs* placebo), without overall survival differences^[97]. A third randomized trial compared the outcome of advanced CRC patients receiving FOLFOX combined to bevacizumab or cediranib at two different daily doses (20 or 30 mg)^[98]. The trial revealed reduced median PFS for the low-dose cediranib group compared to the standard arm (5.8 mo *vs* 7.2 mo). Similar outcome results and increased toxicity rates were noted when comparing the high-dose cediranib arm to the standard arm.

After all these unsatisfactory data, regorafenib renewed the interest in oral VEGF inhibitors for CRC patients. Regorafenib is an oral multi-kinase inhibitor which targets angiogenic, stromal and oncogenic receptor TK^[99]. In the randomized double-blind, placebo-controlled CORRECT study 760 advanced CRC patients received regorafenib or placebo plus best supportive care after progression to all approved standard therapies^[100]. Overall survival, the primary endpoint, was significantly increased from 5 to 6.4 mo. The most common regorafenib-related AE included fatigue (47.4%), HFSSR (46.6%), diarrhea (33.8%), anorexia (30.4%), voice alteration (29.4%), hypertension (27.8%), mucositis (27.2%), and rash/desquamation (26.0%).

Currently, there are no available data on the specific use of these new drugs in the elderly, and trials designed specifically for older patients are strongly desirable.

CONCLUSION

There is strong evidence for efficacy of bevacizumab and other antiangiogenic drugs in the treatment of advanced CRC. Older age *per-se* should not represent a stringent limit for the employ of these agents. However, the widespread clinical use of antiangiogenetics to treat elderly CRC patients should be cautious and always deserves a personalized benefit-to-risk evaluation along with a care-

ful monitoring of cardiovascular and renal potential side effects.

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Sorafenib and entecavir: The dioscuro of treatment for advanced hepatocellular carcinoma?

Salvatore D'Angelo, Mario Secondulfo, Raffaele De Cristofano, Paolo Sorrentino

Salvatore D'Angelo, Mario Secondulfo, Raffaele De Cristofano, Paolo Sorrentino, Liver Unit, Clinical and Experimental Hepatology, S.G. Moscati Hospital, Contrada Amoretta, 83100 Avellino, Italy

Author contributions: All Authors contributed to patients' follow-up, analyzed data and drafted the paper.

Correspondence to: Salvatore D'Angelo, MD, Liver Unit, Clinical and Experimental Hepatology, S.G. Moscati Hospital, Contrada Amoretta, 83100 Avellino, Italy. sadangelo@aosgmoscati.av.it

Telephone: +39-82-5203859 Fax: +39-82-5203859

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Abstract

Hepatitis B virus (HBV) is responsible for 50%-80% of cases of hepatocellular carcinoma (HCC) worldwide. Entecavir (ET) is a potent inhibitor of chronic HBV-DNA polymerase, inhibiting both the priming and elongation steps of viral DNA replication. Sorafenib (SO) has proven efficacy in prolonging survival in patients with advanced HCC. In this frontier report we discuss a possible way to optimize treatment outcomes in patients with HBV and HCC by treatment with ET and SO, on the basis of our practice and published evidence from the literature.

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Key words: Entecavir; Hepatocellular carcinoma; Hepatitis B virus; Liver function; Sorafenib

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INTRODUCTION

Over 350 million people globally are chronically infected with hepatitis B virus (HBV) and around 25% of these will develop hepatocellular carcinoma (HCC)^[1,2]. HCC is the fifth most common malignancy with approximately 750 000 new cases occurring worldwide each year^[3,4]. Overall 70%-90% of patients with HCC have liver cirrhosis caused mainly by HBV and hepatitis C virus^[5,6]. HBV, an oncogenic virus, can cause HCC in the absence of cirrhosis and the risk of HBV-induced HCC varies depending on the presence or absence of concomitant cirrhosis. Chronic carriers of HBV have up to a 30-fold increased risk of HCC^[7]. In areas of high HBV endemicity, persons with cirrhosis have an approximately 16-fold higher risk of HCC than the inactive carriers, and a 3-fold higher risk for HCC than those with chronic hepatitis but without cirrhosis^[8]. While epidemiological studies provide strong evidence for a causal role of chronic HBV infection in the development of HCC, the pathogenesis of HBV infection and carcinogenesis of HBV-associated HCC are still not fully understood. It is thought that HBV exerts its oncogenic potential through both indirect and direct mechanisms that may act in synergy^[9-11].

In this frontier report we discuss a possible way to optimize treatment outcomes in patients with HBV and HCC by treatment with entecavir (ET) and sorafenib (SO), on the basis of our practice and published evidence from the literature.

POTENTIAL ROLE OF ET AND SO

The most effective way to prevent HBV-related HCC is by vaccination but in patients already infected with HBV, antiviral therapy is the best strategy^[9]. ET, a cyclopentyl guanosine analog, is a potent inhibitor of chronic HBV-DNA polymerase, inhibiting both the priming and elongation steps of viral DNA replication. In clinical trials, ET was superior to lamivudine for all primary end points

evaluated in both nucleoside-naïve and lamivudine-resistant patients as well as being effective in both hepatitis B “e” antigen-positive and -negative nucleoside-naïve patients. Antiviral therapy can reduce, but not eliminate the risk of HCC especially in patients with pre-existing cirrhosis and it is therefore important to maintain virological remission. The use of ET allows long-term HBV-DNA suppression with a low risk of resistance.

SO, a tyrosine kinase inhibitor, has been demonstrated in two large scale randomized, double-blind, placebo-controlled, multicentre, phase III trials (the SHARP trial and the Asia-Pacific trial) to prolong median overall survival and delay the median time to progression in patients with advanced HCC^[12,13]. The SHARP study was the first to show an overall survival benefit for SO in patients with advanced HCC, in which the overall survival was 10.7 mo^[14]. Subanalyses of the SHARP population based on a range of parameters including aetiology (hepatitis B virus present/absent); tumour burden (macroscopic vascular invasion and/or extrahepatic spread present/absent); presence or absence of either lung or lymph node metastasis at baseline, confirmed the efficacy and safety of SO in these subpopulations indicating that SO is effective for patients from the AP region with advanced HCC, irrespective of baseline status^[15,16].

Individually ET and SO have been demonstrated to have important roles in the management of patients with HBV and HCC but how best should we use these agents - in combination or as a sequential strategy. The problem is that although there are a number of published guidelines on the treatment of patients with HBV there are no precise indications on the use of antiviral agents in patients with HBV-related HCC, however it is recognized that the goal of antiviral therapy for HBV is to preserve liver function and prevent the development of cirrhosis and HCC. Early intervention is therefore necessary to prevent liver cell damage and decrease viral genome integration. We believe that it is vital to prevent the deterioration of liver function as modulation of liver function may affect survival directly and indirectly but also it may have an impact on the patient's ability to tolerate subsequent treatments.

In a study by Jin *et al*^[17], first-line ET monotherapy was effective in HBV patients (with and without HCC), improved hepatic function and importantly was associated with increased survival after eradication of HCC - confirming previous results that it improved liver function in patients with decompensated cirrhosis^[18,19]. Considering that liver function is a key factor in deciding treatment options for a given patient and concomitant liver dysfunction often hampers both curative and palliative therapies, the fact that ET can improve hepatic function is decisive in the clinical scenario^[20]. Furthermore, in a study by Chang *et al*^[21] the majority of nucleoside-naïve patients with HBV who were treated with long-term ET achieved substantial histological improvement together with regression of fibrosis or cirrhosis. SO has also shown promising antifibrotic activity with efficacy at

Table 1 Baseline characteristics and main treatment outcomes of our cohort (n = 15) n (%)

| Baseline characteristics | Value |
|---|--------------|
| Characteristic | |
| Male | 1 (6.7) |
| Age, yr (range) | 67 (62-76) |
| BCLC stage | |
| B - intermediate | 10 (66.7) |
| C - advanced | 5 (33.3) |
| Child-Pugh score | |
| 5 | 6 (40) |
| 6 | 9 (60) |
| Treatment outcomes | |
| Overall survival, mo (range) | 26.5 (10-36) |
| Liver decompensation | 4 (26.7) |
| Hepatocellular carcinoma progression | 3 (20.0) |
| Interruption of sorafenib therapy due to adverse events | 0 (0) |

All subjects achieved viral clearance following entecavir treatment before the initiation of sorafenib 800 mg/d.

relatively low doses at the early stage of liver fibrosis^[22].

OUR EXPERIENCE

In our unit, we treated a total of 15 patients (1 male; aged 62-76, median 67 years) with advanced HCC and a history of HBV cirrhosis from October 2008 to December 2011. Diagnosis of advanced HCC was made according to the Barcelona Criteria using contrast enhanced ultrasound, elevated values of alpha-fetoprotein and/or liver biopsy. Ten patients had intermediate BCLC stage B and 5 had advanced BCLC stage C and all had Child Pugh A (9 with an A6, 6 with A5). The baseline characteristics of patients are summarized in Table 1.

All patients achieved a complete clearance of HBV-DNA following the administration of ET (0.5 mg/d) before the initiation of SO. The dosage of SO was gradually increased over a 6-wk period to reach the recommended dosage of 800 mg/d.

The median survival in these patients with HCC and HBV was 26.5 mo (range 10-36 mo). No patient stopped therapy due to AEs (cardiac, gastrointestinal, haematological, neurological or dermatological, or endocrinological). All patients had blood pressure within the accepted recommend range, assumed regular cardiac medication as necessary and were negative for HBV-DNA. Four patients had liver decompensation and three had progression of HCC.

It must be emphasized that our experience is reported here in a very synthetic form, since this paper should be intended as a short commentary addressing how treatment with SO and ET might optimize treatment outcomes in patients with HBV and HCC. In addition, the data reported here present several limitations, which should be taken into account to put the above-mentioned findings in a proper framework. First, the sample observed in our experience is too limited to draw any conclusion. Second, the pure observational nature of our findings does not

allow to retrieve any definite cause-effect relationship.

These limitations taken into account, these results are somehow encouraging: this may be, at least in part, due to the viral clearance achieved by patients. We cannot rule out, however, that the longer survival observed in our patients can be attributed to the high proportion of subject with BCLC-B stage HCC.

CONCLUSION

On the basis of our experience and current literature, therefore, we propose that in patients with HBV monotherapy with ET should be given initially to reduce viral load and preserve liver function thereby allowing follow-up treatment with SO to treat HCC. We believe that this treatment approach may represent a potential improvement in the current management of advanced HCC in patients with concomitant HBV infection. However, further, well-designed studies are needed to investigate the efficacy and safety of this therapy in a large sample of patients. If such study will provide positive results, we feel that SO and ET will be considered the “Dioscuri”, the warrior twins of the Greek mythology, of the treatment of advanced HCC.

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Diagnosis of bowel diseases: The role of imaging and ultrasonography

Davide Roccarina, Matteo Garcovich, Maria Elena Ainora, Gianluigi Caracciolo, Francesca Ponziani, Antonio Gasbarrini, Maria Assunta Zocco

Davide Roccarina, Matteo Garcovich, Maria Elena Ainora, Gianluigi Caracciolo, Francesca Ponziani, Antonio Gasbarrini, Maria Assunta Zocco, Department of Internal Medicine, Catholic University of Rome, 00168 Rome, Italy

Author contributions: Roccarina D wrote the review; Garcovich M, Ainora ME, Caracciolo G, Ponziani F, Gasbarrini A and Zocco MA contributed equally to the overall guidelines and inspiration; Garcovich M also revised the English manuscript.

Correspondence to: Dr. Davide Roccarina, Department of Internal Medicine, Catholic University of Rome, Largo A. Gemelli, 8, 00168 Rome, Italy. davideroccarina@gmail.com

Telephone: +39-6-30156018 Fax: +39-6-30157249

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Abstract

Examinations with a visualisation of the anatomy and pathology of the gastrointestinal (GI) tract are often necessary for the diagnosis of GI diseases. Traditional radiology played a crucial role for many years. Endoscopy, despite some limitations, remains the main technique in the differential diagnosis and treatment of GI diseases. In the last decades, the introduction of, and advances in, non-invasive cross-sectional imaging modalities, including ultrasound (US), computed tomography (CT), positron-emission tomography (PET), and magnetic resonance imaging, as well as improvements in the resolution of imaging data, the acquisition of 3D images, and the introduction of contrast-enhancement, have modified the approach to the examination of the GI tract. Moreover, additional co-registration techniques, such as PET-CT and PET-MRI, allow multimodal data acquisition with better sensitivity and specificity in the study of tissue pathology. US has had a growing role in the development and application of the techniques for diagnosis and management of GI diseases because it is inexpensive, non-invasive, and more comfortable for the patient, and it has sufficient diagnostic accuracy to

provide the clinician with image data of high temporal and spatial resolution. Moreover, Doppler and contrast-enhanced ultrasound (CEUS) add important information about blood flow. This article provides a general review of the current literature regarding imaging modalities used for the evaluation of bowel diseases, highlighting the role of US and recent developments in CEUS.

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Key words: Gastrointestinal tract; Bowel; Imaging; Ultrasound; Colour-Doppler; Contrast-enhancement; Time-intensity curve

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INTRODUCTION

Endoscopy remains the main technique for the diagnosis of gastrointestinal (GI) tract diseases because it allows a direct visualisation of the mucosa and the possibility of taking samples for histological analysis. Moreover, in recent years, improvements in endoscopic techniques have also made it possible to use endoscopy for interventions in some diseases of the GI tract. However, endoscopy has some limitations due to its invasiveness and the difficulty of examining the small bowel, and it does not allow the visualisation of extra-intestinal structures that may be involved.

For many years, traditional radiological techniques played a crucial role in the diagnosis of small bowel diseases. In the last decades, the introduction of, and improvements in, non-invasive cross-sectional imaging tech-

niques including ultrasound (US), computed tomography (CT), positron-emission tomography (PET) and magnetic resonance imaging (MRI), have changed the diagnostic approach to the GI tract^[1]. The high resolution of imaging data, ability to acquire 3D images, enhancement of tissues and additional co-registration techniques (PET-CT, PET-MRI) have improved the diagnostic classification of tissue pathology and performance in terms of sensitivity, specificity and accuracy, depending on the specific method and equipment used, the section of the GI tract investigated, the patient's constitution and preparation, and the type of pathology being studied^[2].

In the last two decades, among the cross-sectional imaging techniques, US has had a growing role in the development and application of techniques for the diagnosis of GI diseases because it is cheap, non-invasive, and more comfortable for the patient, and it has sufficient diagnostic accuracy to provide the clinician with high temporal and spatial resolution image data. Moreover, Doppler and contrast-enhanced ultrasound (CEUS) contribute important information about blood flow.

This article provides a general review of the current literature regarding imaging modalities used for the evaluation of bowel diseases, highlighting the role of US and recent developments in CEUS.

CONVENTIONAL RADIOLOGICAL EXAMINATIONS

Plain-film radiography remains the first-line of investigation in the acute setting. Non-contrast radiography is useful in the initial assessment of various GI diseases, including bowel perforation, obstruction, volvulus, and toxic megacolon^[3].

When detailed luminal evaluation is required, fluoroscopic barium or water-soluble single- and double-contrast studies are the modalities of choice. These techniques are able to visualise transit time, peristalsis, luminal emptying and pathological changes such as stenosis, dilatation, luminal filling defects and external compression. Moreover, double-contrast examinations allow detailed visualisation of the mucosa and the detection of inflammatory and neoplastic changes in the intestine^[4].

Barium swallow studies remain the main investigational tool for dysphagia, allowing direct evaluation and inspection of the oesophageal mucosa and gastro-oesophageal junction, an objective measurement of oesophageal contractibility, assessment of reflux and identification of the presence of strictures, pouches, and hiatal hernia^[5]. With respect to the small intestine, fluoroscopic imaging techniques such as small bowel barium follow-through and conventional enteroclysis are able to detect subtle mucosal abnormalities such as fistulous tracts, adhesions and, more rarely, intraluminal lesions. Functional information about transit time and peristalsis can also be ascertained.

Water-soluble, single-contrast oral studies are gener-

ally performed in the immediate post-operative period to assess anastomotic integrity, due to the potential for free intra-abdominal barium to induce peritonitis^[6].

However, fluoroscopic imaging has several disadvantages: first, it only allows indirect detection of alterations of the small bowel, with no information on deeper wall layers and extramural disease extension; and second, its sensitivity for detecting marginal changes is low compared to direct inspection of the mucosa.

CROSS-SECTIONAL IMAGING

Computed tomography

The development of multi-detector computed tomography (MD-CT) scanners with rapid acquisition of thin slices and multi-planar reconstructions allows a detailed investigation of intestinal loops^[7]. In particular, non-contrast-enhanced CT scanning is replacing plain-film radiography in the evaluation of acute abdominal disease such as intestinal perforation or obstruction^[8]. Intravenous contrast enhancement together with distension of the intestinal lumen by water or positive contrast agents is very useful in the detection of inflammatory and neoplastic intestinal pathologies (fistula, abscess, and phlegmon) as well as in the evaluation of extra-intestinal involvement (mesenteric lymph nodes)^[9].

MD-CT colonography, also known as virtual endoscopy, is a new technique to study the large intestine that is able to detect colonic polyps greater than 6 mm with a similar accuracy to conventional colonography^[10-12]. Similar to CT, it is also important in the detection of extra-colonic pathology^[13,14].

For these reasons, this technique may replace traditional double-contrast examinations as a non-invasive screening test or in acute colonic inflammatory processes when other approaches are contraindicated due to the high perforation risk^[2].

MRI

MRI is generally considered the gold standard examination for TNM staging of rectal cancers because it allows an exact visualisation of the rectal wall and perirectal fat infiltration^[15].

Moreover, MRI is the preferred technique in inflammatory bowel diseases (IBD) because it is able to examine the entire small intestine without radiation hazards^[9,16]. It can detect luminal (stenosis and fissures), mural (wall thickening and wall enhancement after gadolinium administration) and exoenteric (mesenteric inflammation, fibrofatty proliferation, lymph adenopathy, hypervascularity, abscesses and fistulas) pathologies^[16-20]. In particular, MRI is more sensitive than other techniques in the evaluation of anorectal fistulas^[20].

Finally, the administration of intravenous contrast agent and the consequent detection of hypervascular areas are useful in distinguishing between active and inactive disease^[17,21,22].

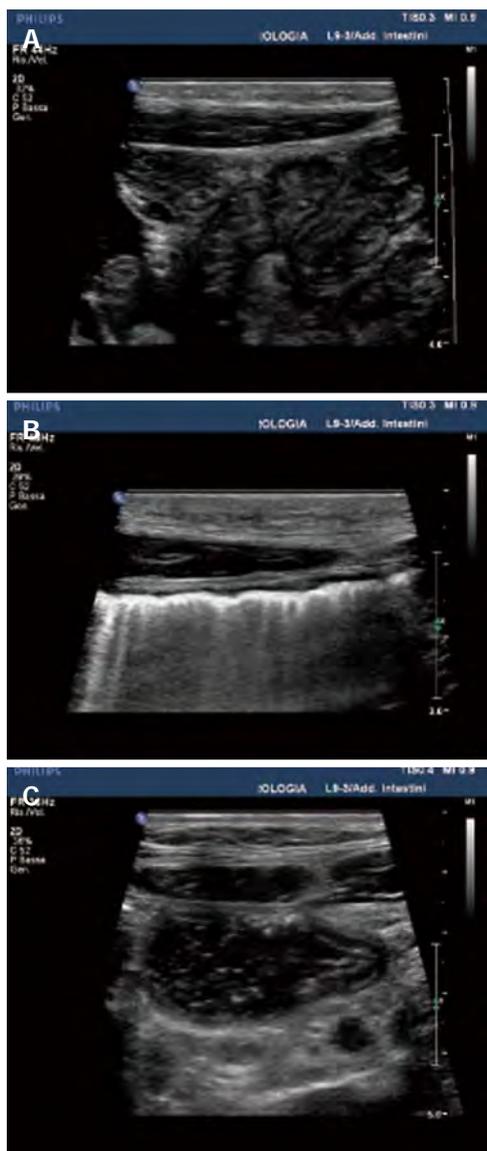


Figure 1 Sonographic appearance of normal bowel. A: Mucus pattern: collapsed bowel containing only a highly reflective core of mucus with target appearance on a transverse section; B: Gas pattern: only the proximal side of the bowel wall is visible due to beam attenuation by gas; C: Fluid pattern: the bowel is filled with fluid and faeces with a tubular appearance on a longitudinal section.

US

Among the cross-sectional imaging techniques, US is less invasive, more comfortable for the patient and has a significant diagnostic accuracy^[23].

The normal bowel wall appears as a multi-layered area with hyperechoic bowel contents at the centre. Five distinct layers can be observed on sonography: an inner hyperechoic layer, the interface between the mucosa and the bowel contents; a second hyperechoic layer, the deep mucosa; a third hyperechoic layer, the submucosa; a fourth hypoechoic layer, the muscle proper; and a last outer hyperechoic layer, the serosa and the serosal fat^[24].

The average wall thickness of the normal gut is 2-4 mm and the US appearance depends not only on the structure of the individual segment but also, more im-

portantly, on its contents and degree of distension. The bowel may be collapsed, containing only a small amount of mucus (mucus pattern), or it may contain fluid or gas (respectively, fluid and gas patterns). The mucus pattern appears as a target with a highly reflective core of mucus. The fluid pattern gives a tubular appearance on a longitudinal section and a rounded pattern on a cross-section. In the gas pattern, only the proximal side of the bowel wall is visible due to beam attenuation by gas (Figure 1).

The jejunum has valvulae conniventes, which produce a ladder pattern, and the ileum has a smooth, featureless wall. The site of the studied bowel must also be inferred from the location of the bowel loop.

The large bowel wall thickness is < 4 mm; it has similar characteristics to the small bowel, but it can be distinguished by its location in paracolic regions and by the presence of haustra.

Similar to the other cross-sectional imaging techniques, US is able to evaluate intestinal findings, such as the bowel wall (in particular, its thickness, layers and perfusion), peristalsis, compressibility, rigidity and extra-intestinal structures, such as perienteric fatty tissues, mesenteric lymph nodes and adjacent organs^[25-29].

US and bowel diseases

The most frequent pathological aspects found by sonography in intestinal diseases are wall thickening, mucosal abnormalities, the absence of peristalsis, mesenteric thickening, lymph node enlargement, vascular alterations, and extra-intestinal complications^[30].

Morphological changes of the bowel wall

Bowel-wall thickening can be found in inflammatory, infectious, ischemic (but only in later stages) and neoplastic diseases. Usually, in inflammation and infections, the wall thickening is regular with preserved stratification, whereas in tumours, the thickness is irregular with loss of normal stratification^[31] (Figure 2).

IBD: Crohn’s disease and ulcerative colitis: The classic sonographic feature of Crohn’s disease (CD) is the “target” sign on transverse images, which means a strong echogenic centre surrounded by a relatively sonolucent rim of more than 5 mm. In a longitudinal section, the sonographic feature is the “sandwich” sign. In CD, transmural inflammation or fibrosis can lead to complete circumferential loss of the typical gut wall layers, which results in a thick hypoechoic rim more than 5 mm. Strictures appear as marked thickenings of the gut wall with a fixed hyperechoic narrowed lumen, dilatation and hyperperistalsis of the proximal gut^[32] (Figure 3).

In expert hands, the distribution of frank lesions of inflammatory bowel disease can be determined with a sensitivity of 73%-87%^[33]. In ulcerative colitis, the sensitivity reaches 89%, and the specificity reaches 100%^[34].

Differentiation between CD and ulcerative colitis based on sonographic findings is based on the location of the disease, the presence of skip lesions and the

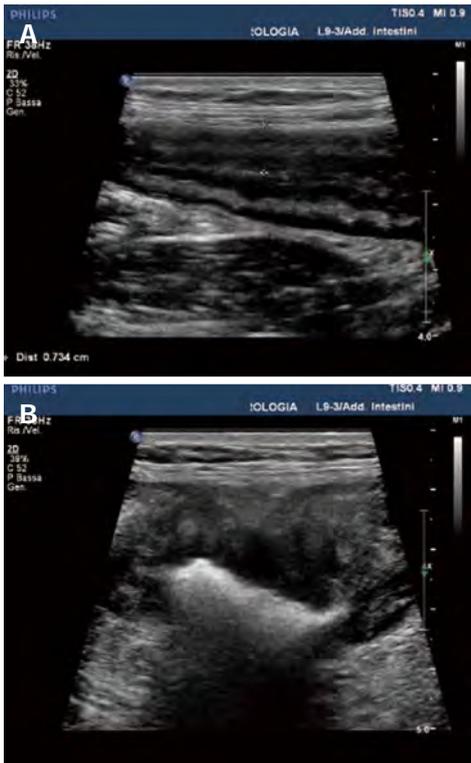


Figure 2 Wall thickening. A: Inflammatory thickening: regular, with preserved wall stratification; B: Neoplastic thickening: irregular with “pseudokidney appearance”.

presence of pericolic abscesses. Bowel-wall thickening is usually less marked in ulcerative colitis with preserved stratification. However, definitive differential diagnosis is difficult on transabdominal sonography^[35-37].

Acute terminal ileitis: Acute terminal ileitis is frequently caused by *Yersinia* species but also by *Campylobacter* and *Salmonella*. Tuberculous enteritis and Behcet’s disease may also affect the ileo-caecal region.

The reported sonographic features include hypoechoic mural thickening of the terminal ileum and caecum between 6 and 10 mm, with hypoechoic swollen ileal folds in the edematous mucosa, and these findings should be related to clinical and laboratory data^[38,39].

Appendicitis: The typical finding of acute appendicitis on a transverse cross-section is the target sign with a hyperechoic centre, an inner hyperechoic ring and an external, thicker hypoechoic ring. In sagittal images, the inflamed appendix is seen as a blind-end, non-compressible tubular structure. Focal or circumferential loss of the inner layer of echoes usually indicates gangrenous inflammation and ulceration of the submucosa. Several studies achieved sensitivities of 80%-93% and specificities of 94%-100% in the sonographic workup of acute appendicitis^[40,41].

Graded compression sonography has gained widespread acceptance as a useful technique for the examination of patients with atypical signs of appendicitis^[42].



Figure 3 Stenosis in patients with Crohn’s disease. A: B-mode aspect: narrow lumen with dilatation of the upstream segments; B: The presence of vascular signals on power Doppler indicates the inflammatory nature of stenosis.

The diagnosis can be established with confidence if the appendix is non-compressible, shows no peristalsis, and measures more than 6 mm in diameter on axial images, and if compression leads to a localised pain response^[43].

A statistically significant association has been found between perforation and two sonographic findings: loculated pericaecal fluid and loss of the echogenic submucosa^[44].

Small bowel tumours: The gut is the most common extranodal site of lymphoma after the stomach^[45]. Eighty percent of gastrointestinal lymphomas have B-cell origins. In patients with underlying coeliac disease, however, a T-lymphocyte origin predominates. In most patients, the US appearance is characterised by transmural hypoechoic wall thickening up to 4 cm in diameter with loss of normal stratification and a central hypoechoic region. This pattern is known as the “pseudokidney” sign^[46,47].

Isolated mucosal involvement is rare and leads to hyperechoic thickening of the mucosa. Sonographic patterns favouring the diagnosis of a non-Hodgkin’s lymphoma over adenocarcinoma are transmural, circumferential, hypoechoic wall thickening with preserved peristalsis, lack of intestinal obstruction, involvement of a long stretch of the gut and the presence of multiple prominent lymph nodes^[48].

Carcinoid is the most frequent small bowel tumour and occurs in 80% of cases in the distal ileum. Usually,



Figure 4 Diverticular disease. A: Reflective outpouchings adjacent to the colonic wall; B: Acoustic shadowing outside the lumen indicating the presence of a coprolith.

small bowel carcinoids appear as hypoechoic, homogenous, predominantly intraluminal masses with smooth intraluminal contours. The tumour is attached to the wall with a broad base, leading to interruption of the submucosa and thickening of the muscularis propria^[49].

Pseudomembranous colitis: The sonographic findings of pseudomembranous colitis (PC) have been described in a number of reports. Striking thickening of the colonic wall with a wide inner circle of heterogeneous medium echogenicity surrounded by a narrow hypoechoic muscularis propria is found in all patients, reflecting the submucosal oedema. The lumen of the colon is almost completely effaced by the mural oedema, and 64%-77% of patients have ascites^[50,51].

Diverticulitis: The sensitivity of US in the diagnosis of acute colonic diverticulitis ranges from 84% to 100% in different studies and is similar to the sensitivity of CT. US features of diverticulitis are the presence of colonic outpouchings associated with bowel-wall thickening and severe local pain induced by graded compression.

Diverticula are round or oval echogenic foci observed in or next to the gut wall, mostly with internal acoustic shadowing^[52-56] (Figure 4).

Colonic carcinoma: There are two possible sonographic appearances of colonic carcinoma. The first is a localised hypoechoic mass up to 10 cm or more with an irregular

shape, lobulated contours and a cluster of high-amplitude echoes (the intramural gas) located eccentrically. The second appearance is a segmental and irregular thickening that could be eccentric or circumferential but is less evident than the first type. The central echo clusters are small because the diseased lumen is usually narrow. This type frequently leads to colonic obstruction. Rectal carcinoma is observed only when the bladder is well-filled^[57-60].

Shirahama *et al*^[61] described four sonographic findings associated with colonic carcinoma in 90% of patients: localised colonic wall thickening with heterogeneous low echogenicity, irregular contour, lack of movement on real-time scanning, and the absence of the layered appearance of the colonic wall. However, negative findings during sonographic examination do not rule out the diagnosis of colonic carcinoma because small masses and overlying bowel gas can lead to false-negative results. Because of these limitations, abdominal sonography cannot be an effective screening technique in colon cancer^[57,62].

Intussusception: Intussusception has a characteristic appearance, and it is usually not mistaken for other bowel abnormalities. Transverse sections reveal a swirled pattern of alternating hyperechogenicity and hypoechogenicity, representing alternating layers of mucosa, muscularis, and serosa: the “doughnut” or “bull’s eye” sign^[63,64]. On longitudinal sections, alternating loops of bowel and a loop-within-loop have a sandwich-like appearance (pseudokidney sign). The outer hypoechoic ring is formed by the intussusciens and the everted returning limb of the intussusceptum with their mucosal surfaces face-to-face. The centre of the intussusception varies with the scan level. At the apex, the centre is hypoechoic because of the entering limb of the intussusceptum. At the base, the entering bowel wall forms a hypoechoic centre that is surrounded by the hyperechoic mesentery^[65,66].

Perfusion of the bowel wall: The role of colour-power Doppler and CEUS

Colour and power Doppler techniques may provide additional information about the macrovascularisation of the bowel wall. In particular, colour and power-Doppler may be helpful in differentiating among ischaemia, inflammation and cancer neovascularisation. The differential diagnosis is possible because ischaemia is characterised by few or no signals, inflammation is characterized by several signals with low resistivity index (RI) (< 60) and symmetric thickening, and cancer neovascularization is characterised by several signals with a high RI (> 60) and asymmetric thickening^[67].

CEUS has recently gained increasing attention because it clearly improves the visualisation of perfusion in various tissues. The development of second-generation, contrast-enhancing agents used in low-mechanical-index harmonic US has enabled real-time assessment of the microvascular circulation and quantification of bowel vascularity^[68-70].

US contrast agents consist of micro-bubbles (1-7

micrometres), often made of a phospholipid shell with a gaseous content that are given intravenously and excreted through the lungs. Obviously, the individual capillaries cannot be discerned, but the micro-bubble content gives rise to a signal “wash” with an intensity that is proportional to the micro-bubble concentration and thus to the blood volume in the portion of the tissue^[71]. This technique has led to important new applications for US. The essential tool is the transit or wash-in, wash-out curve, often referred to as a time-intensity curve (TIC), in which the time course of the transit of micro-bubbles is measured, hence the term “dynamic contrast-enhanced US” (DCE-US). Two categories of information are available from these TICs: results, that depend on timing events such as the arrival time and the time to peak enhancement, and results that depend on the amount of enhancement detected such as the peak enhancement and the area under the TIC.

Such micro-bubble studies have been used to assess inflammatory diseases, giving important information about the severity of the inflammation and its response to therapy^[72-83].

IBD: CD and ulcerative colitis: IBD is associated with hypervascularity of the bowel wall during active disease.

In patients with CD, CEUS is useful for assessing the pattern of neovascularisation within the intestinal layers, allowing better discrimination between active and inactive disease, between inflammatory and fibrotic strictures, and between inflammatory pseudo-tumours and abscesses^[84-89].

In particular, Serra *et al*^[84] prospectively evaluated the vascularisation of the thickened terminal ileum in CD patients using CEUS and compared the clinical activity as measured by the CD activity index (CAI) with the CEUS findings. They used two parameters to assess the vascularisation of the bowel wall: a semi-quantitative method, the pattern of enhancement; and a quantitative method, the E/W ratio, which is the ratio between the major thickness of the enhanced layer (E) and the thickness of the entire wall section (W). The results showed a significant correlation between CAI and the pattern of enhancement. In particular, the frequency of active patients (CAI > 150) was significantly related to the enhancement of the entire wall section and the submucosal enhancement. A positive correlation was observed between the E/W ratio and the CAI values^[84].

Migaleddu *et al*^[90] demonstrated that DCE-US might help in characterising bowel-wall thickening by differentiating fibrosis, oedema and inflammatory neovascularisation and may help to grade disease activity by assessing the presence, initial site, direction and distribution of enhancement.

De Franco *et al*^[91] assessed microvascular activation in the thickened terminal ileal wall in patients with CD using CE-US and evaluated its correlation with a composite index of CD activity (CICDA), the CAI and the simplified endoscopic score for CD (SES-CD). In this study,

unlike the two previously discussed studies, the authors evaluated the mural microvascularity with a quantitative method, analysing software-plotted time-enhancement intensity curves to determine the maximum peak intensity (MPI) and wash-in slope coefficient (β). The MPI and β coefficient were significantly increased in patients with CICDA, CAI and SES-CD scores indicative of active disease^[91].

The introduction of new drugs such as immunomodulators or biological therapies such as monoclonal anti-TNF alpha antibodies in the treatment of CD has led to a need for non-invasive methods to assess the efficacy of pharmacologic treatment. A recent study demonstrated that CEUS could be suitable for evaluating changes in bowel wall vascularisation during anti-inflammatory therapy^[92]. In this study, all of the kinetic parameters (slope, time to peak, and area under the curve) developed from TICs showed significant changes after treatment and were correlated with the CAI score.

Acute appendicitis, acute terminal ileitis, diverticulitis, colitis: In these inflammatory pathologies, especially in the early stages, it is possible to find increased vascularisation with both colour-Doppler and CEUS techniques. The presence of visible hyperaemia or increased flow in the hypoechoic muscular layer of the bowel wall may be a marker of appendicitis, whereas increased flow in the mucosal layer most likely represents enteritis. Increased flow in the fat surrounding the appendix is indicative of transmural extension of the inflammation with mesenteric response. The absence of blood flow indicates gangrenous change or paracolic abscess formation^[93].

Ischaemic disease: In chronic ischaemia of the small bowel, stenotic or occlusive lesions in the coeliac and/or mesenteric arteries are found, and patients typically have postprandial epigastric pain and weight loss. In acute ischaemia, during the first hour, little or no signal from colour-Doppler or echo-enhancing contrast US can be observed. If the ischaemia has lasted a few hours, dilated bowel loops and a thickened bowel wall can be observed, but these signs are non-specific, and the examination is often made difficult by increasing amounts of intraluminal air.

However, Doppler scanning is not the method of choice for diagnosing acute ischaemia of the small bowel because it does not permit the evaluation of the compensatory collateral circulation and distal embolisation. Thus, angiography must be performed for a definite diagnosis^[94,95].

Neoplastic disease: Colour-Doppler and CEUS are not the techniques of choice for the diagnosis of tumours or to differentiate between benign and malignant neoplasia, but, because the tumours are often highly vascularised, these techniques may be helpful to differentiate between tumours and other benign lesions such as abscesses, cysts, and haematomas.

A finding of arterial enhancement with rapid wash-out on CEUS or arterial signs with an RI > 60 on Doppler are highly indicative of a malignant lesion. DCE US with time-intensity curves has recently been used to evaluate tumour responses to anti-vascular therapy^[83].

Extra-intestinal structures: perienteric fatty tissue, mesenteric lymph nodes and adjacent organs

Several intestinal pathologies may involve other structures around the diseased segment such as perienteric fatty tissue, mesenteric lymph nodes, and adjacent or distant organs. The discovery of these findings by US may be helpful for the correct diagnosis.

IBD: Peri-intestinal inflammation leads to the “creeping fat” sign, which appears as a uniform hyperechoic mass typically observed around the ileum and caecum. Mesenteric lymph adenopathy appears as multiple oval hypoechoic masses, usually in the right lower quadrant.

Some of the possible complications of CD are fistula, abscess formation, mechanical bowel obstruction and perforation. Abscesses appear as poorly defined, mostly hypoechoic focal masses that can contain hyperechoic gas. Fistulas are a hallmark of CD and appear in up to one third of patients with advanced disease as hypoechoic tracts with gas inclusion connecting bowel loops or adjacent structures (bladder, abdominal wall, vagina, or the psoas muscle). Detection of gas bubbles in abnormal locations raises the possibility of fistulous communication^[96,97].

Appendicitis: The surrounding mesentery is often inflamed, which can appear as a hypoechoic diffuse halo sign around the appendix.

The presence of a generalised adynamic ileus associated with the presence of free fluid should raise suspicion of perforating appendicitis, even if the appendix has not been found to be enlarged.

Abscess formation is the major complication of a perforating appendicitis. Abscesses may extend into the pelvis or into the peritoneal spaces of the upper abdomen. They may appear as a complex inflammatory mass or localised complex fluid collection. This appearance is indistinguishable from perforated bowel neoplasm. Mesenteric lymph adenopathy may be visualised as multiple oval hypoechoic masses, usually in the right lower quadrant^[98].

Diverticulitis: The sonographic features of acute colonic diverticulitis include inflammatory changes in the pericolonic fat that appear as ill-defined echogenic masses adjacent to the involved thick-walled colonic segments. The most common complication of acute colonic diverticulitis is perforation with abscess formation: this condition is suggested by the presence of an associated localised complex fluid collection.

It is important to note that although sonography can be used to diagnose uncomplicated diverticulitis with

excellent sensitivity and specificity, CT remains the technique of choice for further evaluation of acute colonic diverticulitis, particularly for the assessment of complications such as abscess formation, fistulas, and perforations^[52,56,99,100].

Neoplastic disease: Malignant neoplasia, especially at advanced stages, can extend beyond the intestinal wall to involve perienteric tissues such as in peritoneal carcinomatosis.

The presence of regional malignant lymph adenopathy is highly suggestive of malignant disease. Malignant lymph nodes are larger than 1 centimetre and can measure up to several centimetres. They are round but may colliquate to form large irregular masses with necrotic areas and internal calcifications^[51].

CONCLUSION

In the last decade many cross-sectional imaging techniques have evolved as superior alternatives to fluoroscopic imaging in the examination of the small and large bowels. In particular, transabdominal US may be regarded as the first imaging procedure in the diagnostic work-up and follow-up of bowel diseases. US has gained acceptance, especially in IBD, because it can provide important information including the extent and activity of the disease and the presence of complications. New sonographic techniques combined with the application of intravenous contrast agents increase the accuracy of Doppler US in evaluating bowel wall vascularisation in a real-time manner. The quantitative assessment of bowel wall vascularity by CEUS could provide a useful and simple method to assess the effectiveness of medical treatment.

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Clinicopathological features and outcomes of patients with gastric cancer: A single-center experience

Fatih Selcukbiricik, Evin Buyukunal, Deniz Tural, Mustafa Ozguroglu, Fuat Demirelli, Suheyla Serdengecti

Fatih Selcukbiricik, Evin Buyukunal, Deniz Tural, Mustafa Ozguroglu, Fuat Demirelli, Suheyla Serdengecti, Medical Oncology, Internal Medicine, Cerrahpasa Faculty of Medicine, Istanbul University, Cerrahpasa, 34300 Istanbul, Turkey
Author contributions: Selcukbiricik F, Buyukunal E, Tural D and Ozguroglu M performed the majority of the study and wrote the manuscript; Demirelli F and Serdengecti S conceived the study and finalized the revision; all authors read and approved the final manuscript.

Correspondence to: Fatih Selcukbiricik, MD, Medical Oncology, Internal Medicine, Cerrahpasa Faculty of Medicine, Istanbul University, Cerrahpasa, 34300 Istanbul, Turkey. fsbiricik@yahoo.com
Telephone: +90-212-4143000 Fax: +90-212-4143017
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Abstract

AIM: To evaluate the location, histopathology, stages, and treatment of gastric cancer and to conduct survival analysis on prognostic factors.

METHODS: Patients diagnosed with of stomach cancer in our clinic between 2000 and 2011, with follow-up or a treatment decision, were evaluated retrospectively. They were followed up by no treatment, adjuvant therapy, or metastatic therapy. We excluded from the study any patients whose laboratory records lacked the operating parameters. The type of surgery in patients diagnosed with gastric cancer was total gastrectomy, subtotal gastrectomy or palliative surgery. Patients with indications for adjuvant treatment were treated with adjuvant and/or radio-chemotherapy. Prognostic evaluation was made based on the parameters of the patient, tumor and treatment.

RESULTS: In this study, outpatient clinic records of patients with gastric cancer diagnosis were analyzed retrospectively. A total of 796 patients were evaluated (552

male, 244 female). The median age was 58 years (22-90 years). The median follow-up period was 12 mo (1-276 mo), and median survival time was 12 mo (11.5-12.4 mo). Increased T stage and N stage resulted in a decrease in survival. Other prognostic factors related to the disease were positive surgical margins, lymphovascular invasion, perineural invasion, cardio-esophageal settlement, and the levels of tumor markers in metastatic disease. No prognostic significance of the patient's age, sex or tumor histopathology was detected.

CONCLUSION: The prognostic factors identified in all groups and the proposed treatments according to stage should be applied, and innovations in the new targeted therapies should be followed.

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Key words: Gastric carcinoma; Chemotherapy; Prognostic factors; Treatment; Survival; New agents

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INTRODUCTION

Despite the innovations in treatment, gastric cancer still remains a mortal disease^[1]. Patient, tumor and treatment factors determine the prognosis. In recent years, when there has been an overall reduction in gastric cancer, a moderate increase in proximal stomach and esophagogastric junction region adenocarcinoma has been observed^[2]. While the basic treatment of gastric cancer is complete resection and, following this treatment, if necessary, adjuvant chemoradiotherapy, the standard treatment in meta-

static patients is chemotherapy and palliative treatment. Currently, studies on neoadjuvant therapy are ongoing.

Adjuvant therapy approach

In the intergroup trial (INT 0116), which was a randomized phase III trial, the effectiveness of adjuvant chemoradiotherapy was compared with the monitoring group. In that study, 556 patients were randomized to the adjuvant therapy group, in which the five-year survival rate was 50%, or the surgery group, in which it was 41% (HR = 1.35). That study established the standard adjuvant therapy in gastric cancers. After Macdonald's research^[3], with close to ten years' follow-up demonstrating that survival was 41% after surgery and 50% after adjuvant chemoradiotherapy, this treatment approach has become the standard treatment. However, many studies have been conducted regarding systemic adjuvant treatment^[4].

Neoadjuvant treatment approach

One of the most well-known randomized trials on neoadjuvant treatment for gastric cancer has been reported by Jackson *et al*^[5] and Cunningham *et al*^[6]. The MAGIC study comparing neoadjuvant treatment to surgery alone is the most important work demonstrating a survival advantage for the neoadjuvant treatment approach.

Advanced gastric cancer

Among the forms of treatment of advanced gastric cancer, the best supportive therapies are single-agent chemotherapy, combination chemotherapy and targeted therapies. The five-year survival for stomach cancer is approximately 78%-95% in stage I A, 58%-85% in stage I B, 34%-54% in stage II, 20%-37% in stage IIIA, 8%-11% in stage IIIb, and 5%-7% in stage IV. Wagner *et al*^[7] demonstrated that combination chemotherapy is more beneficial than single-agent chemotherapy (HR = 0.82, 95%CI: 0.74-0.90) Survival with combination treatments *vs* single-agent chemotherapy is 6.7 mo *vs* 8.3 mo. Combination chemotherapies do not provide a significant increase in toxicity but do confer a slight difference in treatment-related mortality (1.1% *vs* 1.5%).

Cisplatin-fluorouracil

Cisplatin-fluorouracil (CF) is the most commonly used regimen for advanced gastric cancer. In 6 basic studies that investigated CF for gastric cancer, the response rate (RR), progression-free survival (PFS) and overall survival (OS) were similar between the CF groups and control groups. In these studies PFS was in the range of 3.7 to 4.1 mo, the median survival was 7.2 to 8.6 mo, and the 2-year survival was 7% to 10%. Addition of docetaxel to CF resulted in a survival advantage^[8]. Kang *et al*^[9] showed similar results for cisplatin ± capecitabine compared with CF. The REAL-2 study compared oxaliplatin combination regimens with regimens containing cisplatin and determined that the latter conferred the best median survival. In phase III of the REAL-2 study, which analyzed the cisplatin ± 5-fluorouracil (5-FU) combination in advanced gastric cancer, the best median survival was 9.9

mo, and two-year survival was 15% [epirubicin-cisplatin-5-FU (ECF) 9.9 mo, cisplatin oxaliplatin 5-FU 9.3 mo, epirubicin oxaliplatin capecitabine cisplatin 9.9 mo and epirubicin-oxaliplatin-capecitabine 11.2 mo]^[10].

Docetaxel-cisplatin-fluorouracil

The TAX 325 study established the standard of phase III trials in advanced gastric cancer. Randomized patients were divided into two arms^[8]. The recurrence rate of the docetaxel-cisplatin-fluorouracil (DCF) arm was reduced approximately 32% compared to the CF arm, and time to progression was 5.6 mo in the CF arm *vs* 3.7 mo in the DCF arm ($P = 0.0004$).

Trastuzumab

HER2 overexpression or amplification is detected in 20% of all gastric cancers. In the ToGA trial in epidermal growth receptor-positive gastric cancer patients, in the first-line treatment, chemotherapy alone was compared with the use of trastuzumab + chemotherapy. Time to progression was 5.5 mo in the patients who received chemotherapy alone 6.7 mo in the chemotherapy + trastuzumab group ($P = 0.0002$). The median survival rate of the patients receiving chemotherapy alone was 11.1 mo *vs* 13.8 mo among patients receiving trastuzumab and chemotherapy together^[11].

MATERIALS AND METHODS

Patients and follow-up

The records of patients with gastric cancer followed by the Department of Medical Oncology were analyzed retrospectively. Patients were recruited to the study if they were treated between 2000 and 2011 by the outpatient clinic. They were followed up by no treatment, adjuvant therapy, or metastatic therapy. We excluded from the study any patients whose laboratory records lacked the operating parameters. According to these criteria, the study sample consisted of the remaining 796 patients (552 male, 244 female, mean age at diagnosis: 58 years).

Patient age, sex, symptoms at diagnosis, localization of the tumor, operative details, histopathological features, AJCC 2010 TNM stage, treatment decisions, sites of metastasis, tumor marker levels at baseline, the presence of adjuvant radiotherapy, PFS, disease-free survival (DFS), and OS were recorded.

The type of surgery in patients diagnosed with gastric cancer was total gastrectomy, subtotal gastrectomy or palliative surgery. Patients with indications for adjuvant treatment were treated with adjuvant and/or radiochemotherapy. The number of patients who received adjuvant treatment was 352 (44.2%). Initially, 394 (49.4%) patients were admitted with metastases, and these patients received chemotherapy. No treatment was initially suggested for 48 patients (6.4%). Each series of chemotherapy treatments received by the patients was recorded.

Statistical analysis

Statistical analysis were performed with SPSS for Win-

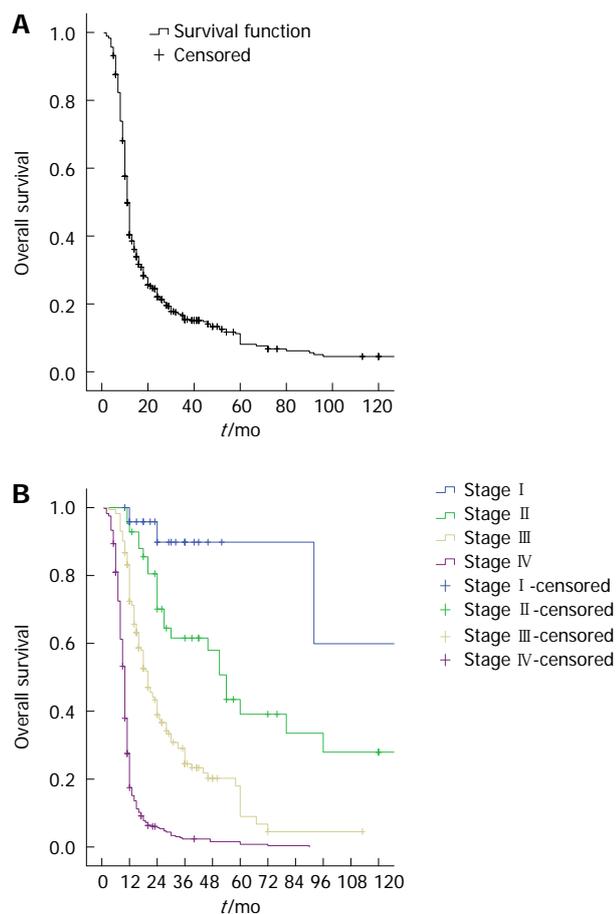


Figure 1 Overall survival in gastric cancer (A) and survival according to gastric cancer stage (B).

dows ver. 15.0 (standard version). Quantitative (numerical) data are reported as the mean ± SD. For two-group comparisons, we used the paired Student's *t*-test or, when necessary, the Mann-Whitney *U* test. For non-numerical data, when suitable for 2 × 2 contingency tables, Yates' corrected χ^2 test and Fisher's exact test were used. Correlations between numerical parameters were analyzed with Spearman's (ρ) correlation test. For the comparison of groups, Student's *t*-test or, when needed, one-way or multi-factor analysis of variance was used.

RESULTS

In this study, outpatient clinic records of patients with gastric cancer diagnosis were analyzed retrospectively. Demographic and clinical characteristics of the 796 gastric cancer cases included in the study were as follows: initial symptoms were dyspeptic symptoms, (39.3%), abdominal pain (24.8%), nausea and vomiting (16.3%), weight loss (7.5%), bleeding (6.4%) and acute abdominal pain (1.6%). The median follow-up period was 12 mo (1-276 mo), the median survival was 12 mo (11.5-12.4 mo), and the 5-year survival rate was 11%. The OS curve is given in Figure 1A, and the survival curve according to stage is given in Figure 1B. The median survival of metastatic patients was 10 mo, compared to 92 mo in stage I patients ($P <$

Table 1 Demographic data of the 796 patients with gastric cancer *n* (%)

| | | |
|-----------------------|----------------------------------|------------|
| Age (yr) | Median | 58 (22-90) |
| Sex | Male | 552 (69) |
| | Female | 244 (31) |
| Median follow-up time | 12 mo (range: 1-276 mo) | |
| Median survival | 12 mo (range: 11.5-12.4 mo) | |
| Tumor location | Pyloric + antrum | 362 (45.4) |
| | Large and small curvature | 252 (31.6) |
| | Cardio-esophageal | 97 (12.2) |
| | Diffuse | 9 (1.1) |
| Stage | Stage I | 29 (3.6) |
| | Stage II | 43 (5.4) |
| | Stage III | 195 (24.5) |
| | Stage IV | 393 (49.3) |
| Type of surgery | Total gastrectomy | 265 (33.2) |
| | Subtotal gastrectomy | 174 (21.8) |
| | Inoperable/palliative | 341 (42.8) |
| Treatment | Adjuvant | 352 (44.2) |
| | Metastatic | 394 (49.4) |
| Histology | Untreated follow-up | 50 (3.9) |
| | Adenocarcinoma (intestinal type) | 493 (61.9) |
| | Signet ring cell (diffuse) | 254 (31.9) |
| | Neuroendocrine | 24 (3) |
| In metastasis | Others | 8 (1.1) |
| | Peritonitis carcinomatosa | 193 (24.2) |
| | Liver | 169 (21.2) |
| | Lymphadenopathy | 73 (9.2) |
| | Liver + peritoneum | 35 (4.4) |
| | Lung | 28 (3.5) |
| | Pleural effusion + acid | 24 (3) |
| Recurrence in | Bone | 23 (2.9) |
| | Others | 17 (2.1) |
| | Peritonitis carcinomatosa | 61 (40.1) |
| | Liver | 36 (23.7) |
| | Lymphadenopathy | 24 (15.8) |
| | Local | 14 (9.2) |
| | Pleural/lung | 12 (7.9) |
| Others | 5 (5) | |

0.0001). The demographic data of the 796 gastric cancer patients are given in Table 1. While the 5-year survival rate with lymphovascular invasion was 18%, this rate was 31% in the patients without lymphovascular invasion (LVI) ($P < 0.0001$). The 5-year survival of patients with perineural invasion (PNI) was 16%, compared to 33.6% without PNI ($P < 0.006$). The 5-year survival rate for patients with negative surgical margins was 28%, which was significantly higher than those with positive margins ($P < 0.0001$). All patients with positive margins died within 5 years.

While the 5-year survival of patients with initially normal crystalline egg albumen (CEA) level was 14.8%, patients with high CEA level all died within 5 years ($P < 0.012$). Five-year survival among patients with initial normal carbohydrate antigen 19-9 (CA 19-9) level was 17.5% in all groups, but for the group with high CA 19-9, 5-year survival was 1.2% ($P < 0.2$). In the evaluation of only stage 4 patients, the tumor marker of high baseline CA 19-9 reached prognostic significance ($P < 0.03$). Gender ($P < 0.2$) and histological subtype had no effect on prognosis ($P < 0.5$). In multivariate analysis, tumor stage had significant effects on overall survival ($P < 0.0001$) and

Table 2 Treatment received by the patients with gastric cancer ($n = 796$) n (%)

| Treatment | | |
|---------------------|------------------------|------------|
| Adjuvant therapy | 5-FU-LV | 222 (27.9) |
| | 5-FU-LV/cisplatin | 43 (5.4) |
| | Untreated follow-up | 58 (7.3) |
| | Others | 17 (2.1) |
| Metastatic series 1 | 5-FU-LV | 32 (4) |
| | DCF | 152 (19.1) |
| | ECF | 77 (9.7) |
| | 5-FU-LV/cisplatin | 121 (15.2) |
| | Palliative treatment | 112 (14.1) |
| | Cisplatin/capecitabine | 20 (2.5) |
| | Others | 31 (3.9) |
| Metastatic series 2 | DCF | 31 (3.9) |
| | 5-FU-LV/cisplatin | 19 (2.4) |
| | ECF | 14 (2.3) |
| | Irinotecan/cisplatin | 17 (2.1) |
| | Supportive | 267 (33.5) |
| | Others | 32 (4) |

5-FU-LV: 5-Fluorouracil plus leucovorin; CF: Cisplatin-fluorouracil; DCF: Docetaxel-cisplatin-fluorouracil; ECF: Epirubicin-cisplatin-5FU.

surgical margin ($P < 0.001$).

The approaches used for gastric cancer treatment are shown in Table 2. A group of patients with gastric cancer without metastasis was followed without medication, and chemotherapy was applied to the others. DFS for approaches to non-metastatic gastric cancer is given in Table 3. The mean survival of the non-treated follow-up group was significantly higher than other groups, primarily because of the survival of the stage I patients ($P = 0.007$). Table 4 shows the effects of chemotherapy or supportive treatment in patients with metastasis. Here, the time to the first progression after initial treatment was defined as PFS1, and the time to the second progression (after the second treatment) was defined as PFS2. PFS1 for patients receiving DCF was 6.56 mo, which was similar to other chemotherapy regimens. The first time to progression in patients receiving supportive therapy was 3.85 mo. After a second round of chemotherapy was started because of progression, DCF significantly prolonged PFS2. Eventually, DCF treatment of metastatic gastric cancer patients significantly prolonged time to progression compared to other approaches. Table 5 compares the results of the 1st and 2nd series of treatments for metastatic cancer. In the first metastatic series, DCF treatment was superior to all other treatments, and the greatest statistical superiority was to ECF and supportive care. DCF was therefore the preferred choice for first-line therapy in our study. A superior PFS was obtained with DCF compared to all other approaches. Supportive treatment was the preferred approach in the second series of our study. This was because of the frequent selection of DCF in the first series and the inability to repeat DCF after progression.

Our study population included 70 patients under age 40 (8.8%), 510 patients between 40 and 65 (64%), and 216 patients over the age of 65 (27.2%). A difference in

Table 3 Disease-free survival with chemotherapy and without chemotherapy in metastasis-free gastric cancer

| Therapeutic approach | n | Average (mo) | Standard deviation | Minimum (mo) | Maximum (mo) |
|----------------------|-----|--------------------|--------------------|--------------|--------------|
| 5-FU-LV | 222 | 21.04 ^b | 19.912 | 2 | 120 |
| Untreated follow-up | 58 | 30.42 | 24.512 | 6 | 120 |
| CF | 43 | 19.00 ^b | 24.452 | 3 | 120 |
| Others | 17 | 21.00 ^b | 24.512 | 3 | 72 |

^b $P < 0.01$ vs untreated follow-up. 5-FU-LV: 5-Fluorouracil plus leucovorin; CF: Cisplatin-fluorouracil.

Table 4 Time to first progression and time to 2nd progression according to treatment (chemotherapy or supportive) care in patients with metastatic gastric cancer ($P < 0.001$)

| Therapeutic approach | n | Average (mo) | Standard deviation | Minimum (mo) | Maximum (mo) |
|---|-----|--------------|--------------------|--------------|--------------|
| First series of chemotherapy and time to progression | | | | | |
| DCF | 152 | 6.56 | 2.869 | 1 | 18 |
| ECF | 77 | 4.56 | 9.021 | 1 | 48 |
| CF | 121 | 4.15 | 5.546 | 1 | 39 |
| Supportive | 112 | 3.85 | 9.951 | 2 | 60 |
| Others | 38 | 5.24 | 11.954 | 1 | 60 |
| Second series of chemotherapy and time to progression | | | | | |
| DCF | 31 | 4.38 | 3.921 | 2 | 15 |
| ECF | 14 | 3.71 | 2.443 | 2 | 10 |
| CF | 19 | 3.76 | 3.914 | 3 | 18 |
| Supportive | 267 | 3.39 | 1.871 | 1 | 12 |
| Others | 17 | 3.75 | 1.528 | 1 | 7 |

5-FU-LV: 5-Fluorouracil plus leucovorin; CF: Cisplatin-fluorouracil; DCF: Docetaxel-cisplatin-fluorouracil; ECF: Epirubicin-cisplatin-5FU.

survival according to age was not observed ($P = 0.8$). In the survival evaluation related to the tumor localization, patients with cardio-esophageal tumors ($P < 0.002$) and patients with linitis plastica ($P < 0.05$) showed the worst survival.

DISCUSSION

This study was designed to determine the prognostic factors of gastric cancer based on tumor location, histological type, stage at diagnosis, and the phases of evaluation of treatment methods.

Talamanti *et al*^[12] explored the relationship between tumor localization and prognosis. Because proximal tumors are more insidious, delay diagnosis, invade more deeply and metastasize to lymph nodes more frequently compared to distal tumors, Talamanti *et al*^[13,14] reported a poorer prognosis for proximal tumors. Furthermore, they demonstrated that the placement of the disease in Caucasian populations significantly affects the prognosis and that tumors with this location show a poor prognosis. In our study, proximal tumors were associated with a worse prognosis than distal tumors, and the frequency of proximal tumors increased significantly after 2005. Proximal tumors required extended gastrectomy, D2 dissection and splenectomy. In this respect, patients with proximal tu-

Table 5 Comparison of treatment approaches in the first and second series of treatments in metastatic gastric cancer patients

| 1 st -series treatment approach | P value | 2 nd -series treatment approach | P value |
|--|---------|--|---------|
| DCF vs 5-FU-LV | 0.043 | DCF vs ECF | 0.050 |
| DCF vs others | 0.010 | DCF vs Supportive | 0.042 |
| DCF vs Supportive | < 0.001 | Supportive vs ECF | 0.500 |
| DCF vs ECF | < 0.001 | DCF vs others | 0.605 |
| DCF vs CF | 0.480 | Irinotecan/Cisp vs ECF | 0.423 |
| ECF vs CF | 0.960 | Supportive vs Irinotecan/Cisp | 0.100 |
| Supportive vs ECF | < 0.01 | DCF vs Irinotecan/Cisp | 0.672 |

5-FU-LV: 5-Fluorouracil plus leucovorin; CF: Cisplatin-fluorouracil; DCF: Docetaxel-cisplatin-fluorouracil; ECF: Epirubicin-cisplatin-5FU.

mors are in serious danger of mortality and morbidity related to surgery as well as delayed diagnosis and increased depth of invasion.

Machara *et al*^[15] and Persiani *et al*^[16] demonstrated the relationship between young age and poor prognosis, but in our study there was no correlation between age and prognosis. In our series this rate was 56% vs 44%. In some studies, the depth of invasion, lymph node metastasis, and distant metastasis were the main prognostic factors^[17]. In our study, the 5-year survival rate of 16% for patients with PNI was significantly lower than those without PNI. Although it is not lymph node metastasis, lymphovascular invasion is a poor prognostic parameter. Patients with LVI had significantly lower 5-year survival than patients without LVI. Ding *et al*^[18] revealed that lymph node metastasis in gastric carcinoma is the most important prognostic factor. In our study, if the node period was increased, survival decreased, and in patients with N2 gastric cancer, 5-year survival decreased to 5%. In the german gastric cancer study, Siewert *et al*^[19] demonstrated, by analyzing the 10-year results of 1654 patients with curative gastrectomy, that lymph node status, invasion depth, the development of postoperative complications, distant metastases and tumor size are associated with prognosis. Maruyama *et al*^[20] showed in 4734 gastric cancer cases that depth of invasion, lymph node metastasis, macroscopic type, localization and histological type are the most important prognostic factors. In our study, while a correlation with the number of lymph nodes removed was not detected, increased node stage affected survival.

The ratio of the number of metastatic lymph nodes to removed lymph nodes is an important prognostic factor. Ding *et al*^[20] demonstrated that the increase of this ratio decreases survival. In our series, as the number of metastatic lymph nodes increased, the 1-year, 3-year, and 5-year survival rates were 97%, 74%, and 63% for N0; 87%, 34.8%, and 18.5% for N1; 73%, 16.4%, and 5% for N2; and 78%, 39% and 0% for N3. In addition, lymph node-negative patients, despite having better prognosis than lymph node-positive patients, experienced recurrence and short survival. After Lauren^[21] demonstrated that gastric carcinoma has two separate histologies, an

intestinal and a diffuse type, the distinct effect of tumor histology on prognosis was investigated. While the intestinal type shows a better prognosis, both histological types can cross the stomach wall and reach the serosal surface and may act metastatic. No difference in survival was observed in any of our patients according to histological type.

When the survival analysis was conducted separately according to the zone of metastasis, we found no differences in survival. However, if carcinoma peritonei was detected, survival averaged less than 8 mo. The role and value of metastasectomy for gastric cancer is not clear. Although there are too few data to draw conclusions about the effect of metastasectomy on survival, Kerkar *et al*^[22] found 1-year, 3-year, and 5-year overall survival rates in 436 patients with liver metastasectomy of 62%, 30% and 26.5%, respectively. Our series included 8 gastric cancer patients with liver metastases who underwent metastasectomy, and the survival data obtained from these patients were consistent with that study. In another study, in the 23-mo follow-up of 43 patients with solitary pulmonary resection, 15/43 (35%) patients were without evidence of disease, and 5-year survival was reported as 33% for gastric cancer^[23]. In our series, there were no cases of metastasectomy for pulmonary metastases of gastric cancer. Dewys *et al*^[24] reported that the gastric cancer symptoms are often nonspecific but can include lumen obstruction, bleeding or acute abdominal pain. Seventy percent of patients initially had symptoms such as abdominal-epigastric pain or discomfort, followed by symptoms such as weight loss, nausea, vomiting, hematemesis and melena. The initial symptoms in our study were consistent with the literature.

In one study, serum CEA was elevated in one-third of gastric cancer patients at diagnosis. Although the CEA level in gastric cancers has low sensitivity as a prognostic marker, high levels are related to the phase of the disease. Higher levels of CA 19-9 and CEA are more sensitive as a combined prognostic factor^[25]. Although in our study population, the initially determined marker values demonstrated no relationship with survival, the prognostic significance of high CA 19-9 at diagnosis in stage IV patients emerged. CA 19-9 was not correlated with the level of CEA-free survival. In gastric cancer, as the stage of the disease progresses, the level of CEA increases. In localized cases, CEA increases by 14%-29%, whereas in patients with metastatic cancer, this figure can reach 85%. Haglund *et al*^[26] and Koga *et al*^[27] reported a 48% sensitivity of CA 19-9 in predicting the prognosis of gastric cancer. Kago and colleagues found high levels of CA 19-9 in 20.9% of stomach cancer patients, including 37% of stage 4 patients and 69.2% of patients with liver metastases.

The median survival of patients with metastatic cancer in this study was 10 mo, and for stage I patients the median survival was 92 mo. We compared our data to the 1-year, 3-year, and 5-year free survival of gastric cancer according to the data Surveillance, Epidemiology and

End Results (SEER) study, covering the years 1975-2008 and a total of 10 601 patients with resected gastric cancer^[28], and found that 1-year survival in stage I, II and III patients of our series was greater, the life span of patients with stage IV; 3-year survival in stage I, II and III patients in our series was greater, whereas stage IV patients showed a worse outcome in our series, and 5-year survival in stage I, II and III patients in our series was better, whereas stage IV patients showed a worse outcome. Comparing all of our study population's survival data with data from the SEER study showed that stage IV patients showed similar survival rates, whereas stage I, II, and III patients seemed to have longer survival times in this series. While local or locoregional recurrence after surgical resection of gastric cancer is a current problem, adjuvant treatment should be administered to patients. Adjuvant therapy, especially in node-positive disease, gives better results. Adjuvant radiotherapy and/or adjuvant chemotherapy has been designed for this purpose in phase III trials.

In a randomized phase III trial, the Intergroup trial (INT 0116), the effectiveness of adjuvant chemoradiotherapy was compared with the observation group and a group treated only with surgery. For resected stage I B-IV (M0), a 5-treatment strategy was planned for gastric and gastroesophageal adenocarcinoma patients, and at the same time, radiotherapy was used. That study reported a statistically significant advantage in median survival. In the current study, 5-year survival for patients receiving adjuvant therapy was 50%, compared to 41% for the surgery group (HR = 1.35)^[5]. In our study, 246 patients were evaluated in terms of the success of adjuvant treatment. A total of 199 patients received adjuvant therapy, but in 99 patients the indication for treatment had not been set. Comparing the types of treatment or follow-up in patients without metastasis at the beginning of the study, the non-treatment group had significantly longer survival than other groups, and significant differences were not found between the other groups. The reason for this most likely is that the patients who received non-adjuvant therapy were already in stage I A, and a longer survival time was expected for these patients. For patients with an indication for adjuvant treatment who underwent a Macdonald regimen, 5-year survival rates were in 90% in stage I, 50% in stage II and 20% in stage III, which are consistent with the literature. The local recurrence rate in the group receiving chemoradiotherapy was 19%. The regional relapse rate was 65% against the 72%. Patients tolerated the regime well. Other adjuvant therapies did not confer a significant increase in survival.

Although some studies have assessed preoperative chemoradiotherapy, the numbers of patients who received neoadjuvant therapy were not large enough for statistical analysis. Compared with general treatment forms in advanced gastric cancer, approaches such as single-agent chemotherapy, combination chemotherapy and targeted therapies can be considered the best adjuvant treatments. Wagner *et al.*^[29], in a meta-analysis, compared

the best adjuvant treatment with chemotherapy regimens and evaluated the median and overall survival rates. Four quality-of-life questionnaires were used to compare chemotherapy with the best supportive care, and chemotherapy was considered better at 12 mo than at 6 mo. In our study, the chemotherapy regimens were superior to supportive care, in accordance with the literature. DCF was used as a metastatic first-line treatment and produced a PFS of 6.5 mo, compared to 4.5 mo using ECF, 4.1 mo using CF, and 3.8 mo using supportive care. In the evaluation of the effectiveness of treatment on survival, using DCF the overall survival was 9.5 mo, 6.5 mo using EC, 5.1 mo using CF and 4.8 mo in patients with only supportive treatment. Any progression under treatment with chemotherapy or supportive care in the second series of treatments was noted, and the PFS2 for DCF was 4.3 mo, for ECF was 3.7 mo and for supportive therapy was 3.3 mo. Considering the effect of combination chemotherapy on PFS, the DCF regimen was superior to all other treatments. Our study was consistent with the results of the TAX 325 study of Van Cutsem *et al.*^[8], which created the standard of advanced gastric cancer care. In their study, DCF was superior to CF in overall survival as well as in time to progression.

Our study evaluated patients treated with different chemotherapy regimens, and DCF showed superior efficacy in all arms in both PFS and overall survival.

The combination of cetuximab with docetaxel and cisplatin does not significantly affect time to progression or overall survival^[30]. Lapatinib, the first dual inhibitor of human epidermal growth factor receptor (HER-1) and HER-2, has been investigated in two phase II studies as a single therapeutic agent, but no survival advantage was observed^[31]. Gefitinib and erlotinib, two tyrosine kinase inhibitors, have been used as a combination treatment for cancer, and in extensive studies, a RR of 9% was obtained^[32]. Bevacizumab, a monoclonal antibody against vascular endothelial growth factor A, was investigated in the AVAGAST study. The combination of bevacizumab + CC conferred no significant survival advantage compared to CC alone. In another study, I C was combined with bevacizumab, and a significant advantage was not observed compared to I C alone^[33]. Sunitinib is an oral inhibitor of VEGFR1, -2, and -3, PDGFR- α and - β and c-kit. Use of second-line sunitinib in phase II trials produced an overall survival of 47.7 wk. In another phase II study using sorafenib in combination with docetaxel and cisplatin, clinical activity was observed^[34]. Everolimus, an oral inhibitor of mTOR, has been effective in gastric cancers in phase I and phase II trials^[35].

Due to the limited number of patients with targeted therapy in this study, HER-2 status and the effectiveness of trastuzumab could not be assessed. Efficacy assessments could not be made also because targeted therapies such as trastuzumab were not used in our series. When HER-2 receptor status is analyzed routinely in stomach cancer patients, targeted therapy may be evaluated more completely.

COMMENTS

Background

In spite of the development of oncology treatments, gastric cancer still has a high mortality. All the prognostic factors should be evaluated before planning the treatment of gastric cancer. It should be kept in mind that there are new treatment modalities for gastric cancer.

Research frontiers

In this study, the authors retrospectively evaluated gastric cancer patients treated in our clinic during the last 10 years. The prognostic factors for these patients were identified and the treatment plan made according to these factors. The treatments of the patients and their survival were evaluated and compared with the literature. Additionally, the importance of targeted therapy is emphasized.

Innovations and breakthroughs

This study has provided new insight into gastric cancer. Properly identifying the prognostic factors and planning the treatment and follow-up according to these factors is suggested. This study has shown that mortality is high in metastatic patients and that clinicians should be more encouraged to use targeted therapy.

Applications

Based on the results, molecular features of metastatic patients, such as human epidermal growth factor receptor (HER-2) receptor status, will be identified and targeted therapy principles will be developed.

Terminology

HER-2 is a member of the epidermal growth factor family. It is involved in tumor proliferation, metastasis and poor prognosis. If a patient is HER-2 positive, then the anti-HER-2 antibody trastuzumab can be useful. Authors need further clinical studies to evaluate other targeted therapy modalities.

Peer review

The authors have identified the prognostic features of gastric cancer patients and compared the standard treatment modalities. They note the importance of molecular studies in gastric cancer patients, and they predict that targeted therapy will be a part of the standard treatment in the future.

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Modulatory effects of *Bifidobacterium longum* BB536 on defecation in elderly patients receiving enteral feeding

Junko Kondo, Jin-Zhong Xiao, Akira Shirahata, Mieko Baba, Akie Abe, Koichi Ogawa, Taeko Shimoda

Junko Kondo, Akira Shirahata, Mieko Baba, Akie Abe, Kitakyushu Hospital Group, Fukuoka 803-8501, Japan
Jin-Zhong Xiao, Food Science and Technology Institute, Morinaga Milk Industry Co. Ltd., Kanagawa 252-8583, Japan
Koichi Ogawa, Clinico Co. Ltd., Tokyo 160-8447, Japan
Taeko Shimoda, Division of Medical Nutrition, Faculty of Healthcare, Tokyo Healthcare University, Tokyo 113-8510, Japan
Author contributions: Kondo J conceived and designed the study, recruited patients, obtained the written consent of the patient or their relatives and drafted the manuscript; Xiao JZ performed the sample analysis and interpretation of data and helped draft the manuscript; Shirahata A, Baba M, Abe A and Ogawa K contributed to patient recruitment and follow-up of the enrolled and managed patients; Shimoda T contributed to the study design, data interpretation, and critical review of the manuscript; all authors approved the final manuscript.

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Correspondence to: Dr. Jin-Zhong Xiao, Food Science and Technology Institute, Morinaga Milk Industry Co., Ltd., 5-1-83 Higashihara, Zama, Kanagawa 252-8583,

Japan. j_xiao@morinagamilk.co.jp

Telephone: +81-46-2523047 Fax: +81-46-2523055

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Abstract

AIM: To investigate the effects of the probiotic *Bifidobacterium longum* BB536 on the health management of elderly patients receiving enteral feeding.

METHODS: Two double-blind, placebo-controlled trials were performed with long-term inpatients receiving enteral tube feeding at Kitakyushu Hospital Group, Fukuoka, Japan. BB536 was administered as BB536-L and BB536-H powders that contained approximately 2.5×10^{10} and 5×10^{10} cfu of BB536, respectively. In the first trial, 83 patients (age range: 67-101 years) were randomized into 2 groups that received placebo (placebo group) or BB536-H (BB536 group) powders. In the second trial, 123 patients (age range: 65-102

years) were randomized into 3 groups, and each group received placebo (placebo group), BB536-L (BB536-L group), or BB536-H (BB536-H group) powders. Each patient received the study medication for 16 wk after 1 wk of pre-observation. Fecal samples were collected from each patient prior to and after the intervention during Trial 2. Clinical observations included body temperature, occurrence of infection, frequency of defecation, and fecal microbiota.

RESULTS: No significant changes were observed in the frequency of defecation for either treatment in Trial 1. However, a significant change was noted in the BB536-L group ($P = 0.0439$) in Trial 2 but not in the placebo or BB536-H groups. Subgroup analyses based on the frequency of defecation for each patient during the pre-observation period for both trials revealed significant increases in bowel movements in patients with a low frequency of defecation and significant decreases in the bowel movements of patients with a high frequency of defecation during the intervention period in the BB536 groups. The combination of Trials 1 and 2 data revealed a modulatory effect of BB536 ingestion on the changes in bowel movements. Significantly increased bowel movements were observed in patients in the low frequency subgroup with significant intergroup differences ($P < 0.01$). Significantly decreased bowel movements were observed in patients in the high subgroup, but no significant intergroup differences were observed compared with the placebo group. BB536 ingestion increased the prevalence of normally formed stools. BB536 intake also significantly ($P < 0.01$) increased the cell numbers of bifidobacteria in fecal microbiota, and significant intergroup differences were observed at week 16. No adverse events were reported in any group.

CONCLUSION: Our results suggest that BB536 ingestion modulated the intestinal environment and may have improved the health care of elderly patients receiving enteral feeding.

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Key words: Probiotics; *Bifidobacterium longum* BB536; Elderly; Defecation

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INTRODUCTION

Enteral feeding is a common method of nutritional support for patients who are unable to achieve their nutritional requirements through an oral diet alone. No accurate data exist on the number of patients who receive enteral feeding. However, the number of new patients requiring enteral feeding in 2007 was approximately 130 000 in Japan^[1], and this number is expected to increase in the future. Elderly people, particularly those who are hospitalized and receiving enteral feeding, exhibit significant problems with defecation, and the consequences of constipation or diarrhea may significantly impact their quality of life^[2,3]. The prevalence of constipation is generally higher in elderly individuals who reside in nursing homes or hospitals compared with elderly individuals in the community^[2]. Diarrhea, which is a potential consequence of enteral feeding, is observed in 2%-95% of patients who receive this therapy^[3,4].

Intestinal microbiota are the largest source of microbial stimulation in the host, and these microbiota affect mucosal and systemic immunity^[5]. The composition of the intestinal microbiota in elderly people is different from that in younger adults, and the number of bifidobacteria decreases with age^[6,7]. Bifidobacteria in the intestinal microbiota may exert beneficial effects in the host, such as the promotion of gut maturation and integrity, antagonism against pathogens, and immune modulation^[8].

Probiotics are currently used in the prevention and treatment of disease, specifically diseases of the intestinal environment. Several studies have investigated the beneficial effects of probiotics in the management of constipation and diarrhea in elderly patients^[9-11]. However, these effects may be strain-dependent, and they are not consistently observed. Therefore, further investigation is required to clarify this relationship.

The probiotic strain *Bifidobacterium longum* (*B. longum*) BB536 was originally isolated from a healthy infant, and it is used in the dairy industry as a probiotic^[12]. Several studies have evaluated the effects of BB536 on the intestinal environment in healthy adults with frequent constipation^[12-14]. Seki *et al.*^[15] reported that the intake of BB536-supplemented milk improved constipation and increased the prevalence of intestinal *Bifidobacterium* in aged indi-

viduals in a preliminary study. Moreover, BB536 intake suppresses antibiotic-induced intestinal disorders^[16].

The present study investigated the efficacy of BB536 in the health care of hospitalized elderly patients receiving enteral nutrition. We performed 2 double-blind, placebo-controlled trials using a 16-wk administration of BB536 to evaluate effects on health, defecation frequency, and the bifidobacterial composition of fecal microbiota in elderly patients receiving enteral nutrition.

MATERIALS AND METHODS

Subjects

Subject recruitment for this study was conducted in long-stay inpatients (age > 65 years) receiving enteral tube feeding at the Kitakyushu Hospital Group (Fukuoka, Japan). The subjects or their relatives provided written informed consent. The following exclusion criteria were used: presence of diabetes, renal dysfunction, severe infectious disease, autoimmune disease, immunodeficiency, pancreatic disease, or hepatic disease prior to the start of the study. The ethics committee of the Kitakyushu Hospital Group approved all study protocols, which followed the Declaration of Helsinki.

Test samples

Three types of study medications were used in the present study: placebo powder, BB536-L powder, and BB536-H powder. BB536-L and BB536-H powders contained lyophilized BB536 at doses of approximately 2.5×10^{10} and 5×10^{10} cfu, respectively, and the placebo powder contained only inactive ingredients (*i.e.*, primarily dextrin). Each dose was supplied in an aluminum sachet (2 g), and all sachets were identical in taste and appearance.

Clinical trials

Two trials were performed in this study, and both trials were performed using a double-blind, placebo-controlled, parallel-group design. Randomization for each group of participants was conducted using a minimization procedure to balance for gender, age, and hospital ward. The trial flows and schedules are presented in Figures 1 and 2, respectively. Routine enteral nutrition was provided to all the subjects during the trial period to maintain nutritional status. Participants, physicians, and other research staff in the study were unaware of treatment assignment. The study powder was suspended in drinking water and administered immediately after enteral feeding. The daily intake of energy and nutrients of each patient group during the trial period are summarized in Table 1. No significant differences in nutrient intake between the groups were observed.

Trial 1: The first trial was performed during the winter from the end of November 2009 to the end of March 2010. This period included one week for pre-observation and 16 wk for the ingestion of study medications. A total of 83 patients were randomized into 2 groups, and each

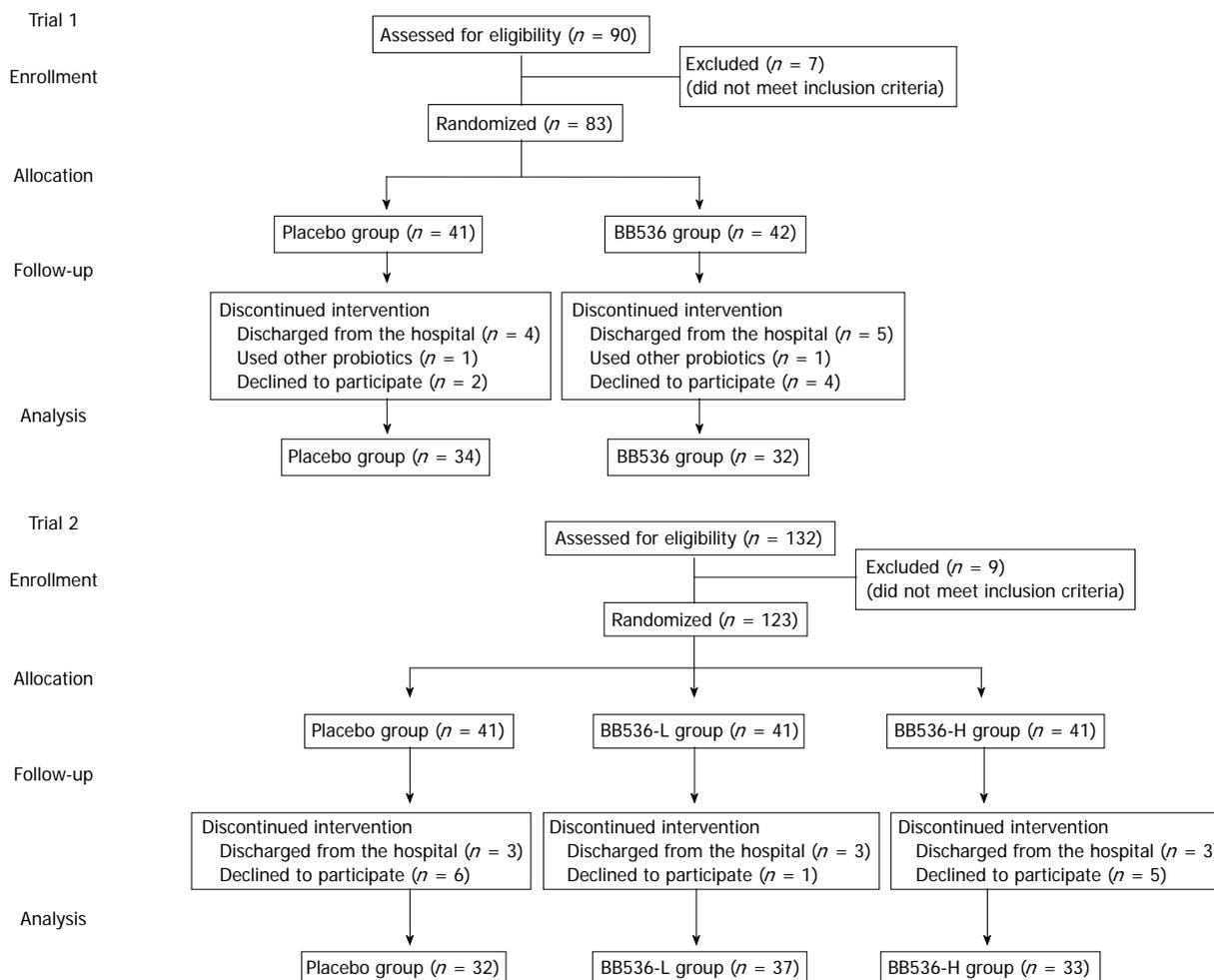


Figure 1 Trial protocol.

Table 1 Subject characteristics and daily intake of energy and nutrients

| Group | n | Gender (M/F) | Age (yr) | Total energy (kcal/d) | Protein (g/d) | Lipid (g/d) | Carbohydrates (g/d) | Dietary fiber (g/d) |
|---------|----|--------------|-------------|-----------------------|---------------|-------------|---------------------|---------------------|
| Trial 1 | | | | | | | | |
| Placebo | 32 | 9/23 | 82.7 ± 9.5 | 884.7 ± 207.2 | 37.2 ± 11.6 | 28.8 ± 9.6 | 118.8 ± 30.6 | 10.8 ± 3.5 |
| BB536-H | 34 | 8/26 | 85.8 ± 7.3 | 917.6 ± 162.6 | 37.5 ± 7.8 | 30.2 ± 10.3 | 124.2 ± 22.5 | 10.3 ± 3.7 |
| Trial 2 | | | | | | | | |
| Placebo | 32 | 9/23 | 83.9 ± 7.5 | 798.1 ± 176.3 | 35.1 ± 11.9 | 24.5 ± 6.1 | 112.3 ± 31.4 | 9.6 ± 3.3 |
| BB536-L | 37 | 9/28 | 84.4 ± 6.8 | 845.6 ± 186.9 | 37.0 ± 10.5 | 26.6 ± 9.9 | 118.0 ± 28.6 | 11.1 ± 4.6 |
| BB536-H | 33 | 10/23 | 84.4 ± 10.1 | 854.8 ± 194.9 | 37.4 ± 10.7 | 26.0 ± 8.0 | 120.1 ± 29.9 | 10.4 ± 3.7 |

M: Male; F: Female.

group was assigned to receive placebo (placebo group) or BB536-H powder (BB536 group) once daily.

Trial 2: The second trial was performed to confirm the results of Trial 1, investigate the dose effect of BB536, and determine any possible influences of treatment on fecal microbiota. This trial was also conducted during the winter from the end of November 2010 to the end of March 2011. The trial period included one week for pre-observation and 16 wk for study medication ingestion. A total of 123 patients were randomized into 3 groups, and each group was assigned to receive the placebo (placebo group), BB536-L (BB536-L group), or BB536-H powder

(BB536-H group) twice daily. Fecal samples were collected from each patient prior to (pre-observation week) and after the intervention (week 16). Fecal samples were collected in plastic tubes, cooled immediately after collection, and stored at -20 °C until analysis.

Clinical observations

Body temperature and the times of defecation were recorded daily. The occurrence of infection and fever and use of other medications, including antibiotics, were also recorded. A trained caregiver monitored stool characteristics during daily care, and stool form and consistency were evaluated using the Bristol Stool Form Scale. The

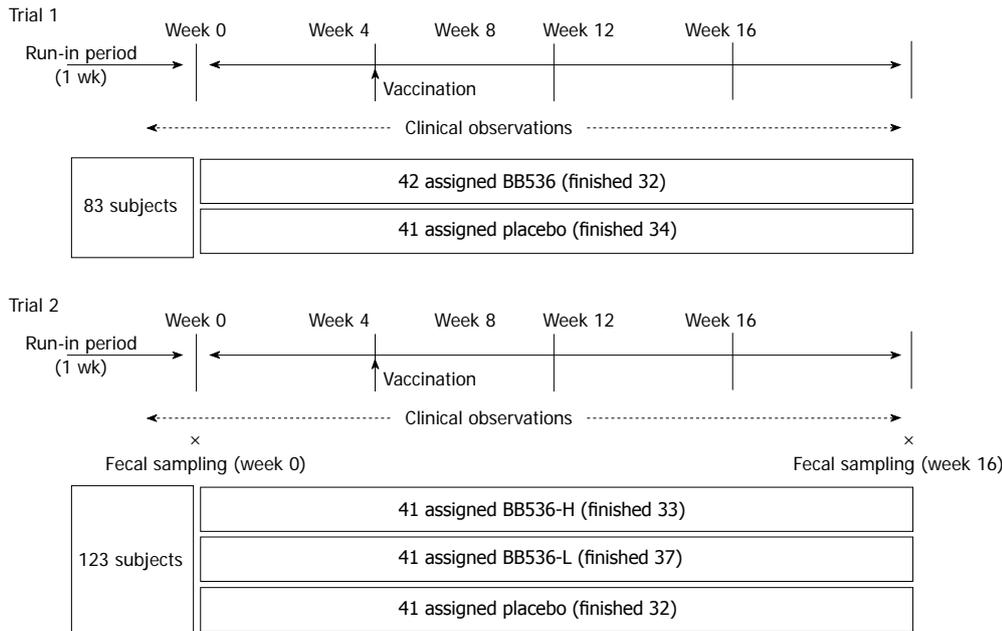


Figure 2 Intervention schedule.

Bristol Stool Form Scale scores range from 1 (separate hard lumps, like nuts and hard to pass) to 7 (watery, no solid pieces, entirely liquid); stools scored at 3 or 4 were considered normal stools^[17].

Analysis of fecal microbiota

DNA was extracted from the fecal samples as described previously^[18]. Briefly, each fecal sample (20 mg) was suspended in 1.0 mL phosphate-buffered saline (PBS) and centrifuged at $14\,000 \times g$. The resulting pellet was washed twice with 1.0 mL PBS and resuspended in 450 μL of an extraction buffer [100 mmol/L Tris-HCl and 40 mmol/L ethylenediaminetetraacetic acid (EDTA) at pH 9.0] with 50 μL of 10% sodium dodecyl sulfate (SDS). Glass beads (300 mg, 0.1 mm diameter) and 500 μL of buffer-saturated phenol were added to the suspension, and the resulting mixture was vigorously vortexed for 30 s with a FastPrepTM FP 100A (Bio 101, Vista, CA, United States) device at a power level of 5.0. The mixture was centrifuged at $14\,000 \times g$ for 5 min, and 400 μL of the supernatant was extracted with phenol-chloroform; 250 μL of the supernatant was precipitated with isopropanol. Purified DNA was dissolved in 200 μL of a Tris-EDTA buffer at pH 8.0.

Real-time polymerase chain reaction (PCR) was performed using an ABI PRISM[®] 7500 Fast Real-Time PCR system (Applied Biosystems, Carlsbad, CA, United States), with SYBR[®] Premix Ex Taq (TaKaRa Shuzo, Japan) and ROX Reference Dye II (TaKaRa Shuzo, Japan) as an internal standard. Primers for the bifidobacterial species and *B. longum* BB536 were used as described previously^[19,20]. The amplification program consisted of 1 cycle at 94 °C for 10 s, followed by 40 cycles at 94 °C for 5 s and 60 °C for 30 s. Fluorescent products were detected at the last step of each cycle. Melting curves were obtained by heat-

ing from 60 °C to 95 °C in 0.2 °C/s increments with continuous fluorescence data collection.

Statistical analysis

Data are expressed as means \pm SD or SE. Daily recorded scores for body temperature and times of defecation were averaged weekly for each individual. Changes in values from baseline (week-1) were calculated based on the weekly scores. Weekly scores or changes were further averaged every 4 wk for analysis. The frequency of each stool type was summed for the total intervention period, and the prevalence of each stool type was calculated. Cell numbers for each bacterial target are expressed as means after logarithmic transformation for each group among individuals with cell numbers that exceeded the detection limit, which was 1×10^6 per gram wet weight of feces. However, statistical analyses were conducted on cell numbers after logarithmic transformation, in which cell numbers below the detection limit were substituted with 1×10^6 . For analysis of sequence differences within a group, two-sequence differences were assessed using the paired Student *t*-test, and multi-sequence differences were analyzed using a repeated measures analysis of variance (ANOVA), followed by Dunnett's test for each time point against the baselines. For analysis of between group differences, two-group differences were evaluated using the Student *t*-test, and multi-group differences were evaluated using a non-repeated measures ANOVA, followed by the Student-Newman-Keuls test for comparisons of each group. Differences in changes from baseline between groups were evaluated using the Student *t*-test at each time point. *P* values less than 0.05 were considered statistically significant. Analyses were performed using SPSS software (Version 15.0J for Windows, Chicago, United States).

Table 2 Bowel movements during the intervention period

| Subgroups of subjects ¹ | Intervention group | Subjects (n) | Bowel movements (times/wk) | | | | | P value ² | |
|------------------------------------|--------------------|--------------|----------------------------|--------------------------|--------------------------|---------------------------|---------------------------|----------------------|--|
| | | | Week-1 | Weeks 1-4 | Weeks 5-8 | Weeks 9-12 | Weeks 13-16 | | |
| Trial 1 | | | | | | | | | |
| Whole | Placebo | 34 | 4.88 ± 2.70 | 5.18 ± 2.57 | 5.20 ± 2.58 | 5.17 ± 2.74 | 4.8 ± 2.2 | 0.326 | |
| | BB536-H | 32 | 5.53 ± 3.76 | 6.62 ± 3.83 | 6.37 ± 3.34 | 6.04 ± 3.25 | 6.0 ± 2.8 | 1.051 | |
| Low | Placebo | 19 | 3.00 ± 0.94 | 4.07 ± 1.37 ^a | 4.25 ± 1.94 ^a | 4.07 ± 1.34 ^a | 3.87 ± 1.13 ^a | 0.002 | |
| | BB536-H | 14 | 2.93 ± 0.92 | 4.41 ± 1.89 ^b | 4.32 ± 1.85 ^b | 4.79 ± 1.92 ^b | 4.82 ± 1.92 ^b | 0.001 | |
| Normal | Placebo | 12 | 6.25 ± 1.36 | 5.46 ± 2.56 | 5.29 ± 2.07 | 5.23 ± 2.40 | 5.21 ± 2.69 | 0.564 | |
| | BB536-H | 14 | 5.79 ± 1.19 | 7.02 ± 2.84 | 7.36 ± 3.16 | 6.27 ± 3.25 | 6.48 ± 2.7 | 0.340 | |
| High | Placebo | 3 | 10.50 ± 1.00 | 11.38 ± 1.77 | 10.38 ± 0.72 | 12.5 ± 3.22 | 8.50 ± 0.58 | 0.786 | |
| | BB536-H | 4 | 13.75 ± 3.77 | 12.94 ± 4.94 | 10.06 ± 3.86 | 9.63 ± 4.75 | 8.75 ± 4.20 ^a | 0.044 | |
| Trial 2 | | | | | | | | | |
| Whole | Placebo | 32 | 5.28 ± 3.34 | 5.02 ± 2.67 | 4.78 ± 2.54 | 4.73 ± 2.80 | 4.60 ± 2.20 | 0.563 | |
| | BB536-L | 37 | 5.51 ± 4.12 | 6.10 ± 3.85 | 5.90 ± 3.43 | 5.11 ± 2.60 | 4.90 ± 3.00 | 0.044 | |
| | BB536-H | 33 | 5.91 ± 4.30 | 6.12 ± 3.89 | 6.20 ± 3.51 | 6.30 ± 3.25 | 5.60 ± 3.80 | 1.075 | |
| Low | Placebo | 20 | 3.05 ± 0.76 | 3.73 ± 0.88 | 3.45 ± 1.05 | 3.41 ± 1.10 | 3.63 ± 1.09 | 0.387 | |
| | BB536-L | 22 | 2.64 ± 1.05 | 3.69 ± 1.22 ^b | 3.74 ± 1.42 ^b | 3.70 ± 1.54 ^b | 3.44 ± 1.32 ^b | 0.001 | |
| | BB536-H | 18 | 3.00 ± 1.03 | 3.96 ± 1.33 ^a | 4.24 ± 1.89 ^b | 4.35 ± 2.09 ^b | 3.83 ± 1.44 ^a | 0.015 | |
| Normal | Placebo | 6 | 6.83 ± 1.33 | 4.79 ± 1.16 | 5.04 ± 1.07 | 4.88 ± 1.61 | 4.79 ± 1.42 | 0.219 | |
| | BB536-L | 7 | 7.00 ± 1.29 | 7.39 ± 3.15 | 7.86 ± 3.58 | 6.46 ± 1.81 | 6.61 ± 2.89 | 0.842 | |
| | BB536-H | 8 | 6.50 ± 1.41 | 6.66 ± 3.70 | 7.47 ± 3.50 | 7.03 ± 3.11 | 5.66 ± 4.18 | 1.217 | |
| High | Placebo | 6 | 11.17 ± 0.98 | 9.54 ± 2.90 | 8.96 ± 2.54 | 9.00 ± 3.57 | 7.92 ± 2.59 ^a | 0.149 | |
| | BB536-L | 8 | 12.13 ± 2.10 | 11.59 ± 3.01 | 10.13 ± 2.12 | 7.81 ± 2.89 ^b | 7.44 ± 4.03 ^b | 0.006 | |
| | BB536-H | 7 | 12.71 ± 3.68 | 11.07 ± 4.08 | 9.79 ± 3.55 ^b | 10.46 ± 3.80 ^a | 10.25 ± 4.12 ^a | 0.040 | |

Values are shown as mean ± SD. ¹Based on the results of bowel movements at week-1. Low, ≤ 4 times; Normal, 5-9 times; High, ≥ 10 times; ²P values are results of repeated measures ANOVA for analyzing the significance of intragroup changes. ^aP < 0.05, ^bP < 0.01 *vs* week-1.

RESULTS

Baseline characteristics of participants and clinical observations

No significant differences in the baseline characteristics of patients were observed between the groups of either trial (Table 1). No significant changes in body temperature during the intervention period were observed between groups in either trial. A few patients experienced body temperatures > 38 °C and received antibiotics, but the incidence of fever was not significantly different between the groups in either trial (data not shown).

Changes in the frequency of defecation

No significant changes in the frequency of defecation were observed following treatments during Trial 1 (Table 2). However, significant changes were observed in the BB536-L group in Trial 2 but not in the placebo or BB536-H groups (Table 2).

The frequency of defecation varied for each patient during the pre-observation period. Therefore, subgroup analyses were performed for patients with infrequent (low) defecation (≤ 4 times a week), normal frequency of defecation (5-9 times a week), and a high frequency of defecation (≥ 10 times a week) at baseline (week-1). We observed significant changes in the frequency of defecation in the low frequency subgroup of the placebo and BB536 groups and the high frequency subgroup of the BB536 group in Trial 1. However, no significant changes were observed in the normal frequency subgroup of either the placebo or BB536 group or the high frequency subgroup of the placebo group during treatment (Table

2). Defecation frequency increased significantly after treatment in the low frequency subgroups of both the placebo and BB536 groups, and the frequency tended to be higher (*P* < 0.1) in the BB536 group compared with the placebo group at weeks 13-16. In contrast, defecation frequency decreased after treatment in the high frequency subgroup of the BB536 group but not in the placebo group, and significant differences were observed at weeks 9-12 and 13-16 in the BB536 group (Table 2).

Significant changes were observed in the frequency of defecation in the low and high frequency subgroups of the BB536 group but not the placebo group in Trial 2 (Table 2). No significant changes in the normal frequency subgroups of any of the three treatment groups were observed (Table 2). Defecation frequency increased significantly after treatment in the low frequency subgroups of both the BB536-L and BB536-H groups, and a trend for a difference was noted in the BB536-H group compared with the placebo group at weeks 9-12 (*P* < 0.1). In contrast, defecation frequency decreased after treatment in the high frequency subgroups of both the BB536-L and BB536-H groups. Significant differences were observed at weeks 9-12 and 13-16 in the BB536-L group and weeks 5-8, 9-12, and 13-16 in the BB536-H group (Table 2).

Combined analyses of Trials 1 and 2 for changes in the frequency of defecation

Figure 3 summarizes the changes in defecation frequency for the three subgroups in the two trials. Defecation frequency increased significantly in the low frequency subgroup of both placebo (*n* = 39) and BB536 (*n* = 54) groups. However, the frequency was significantly higher

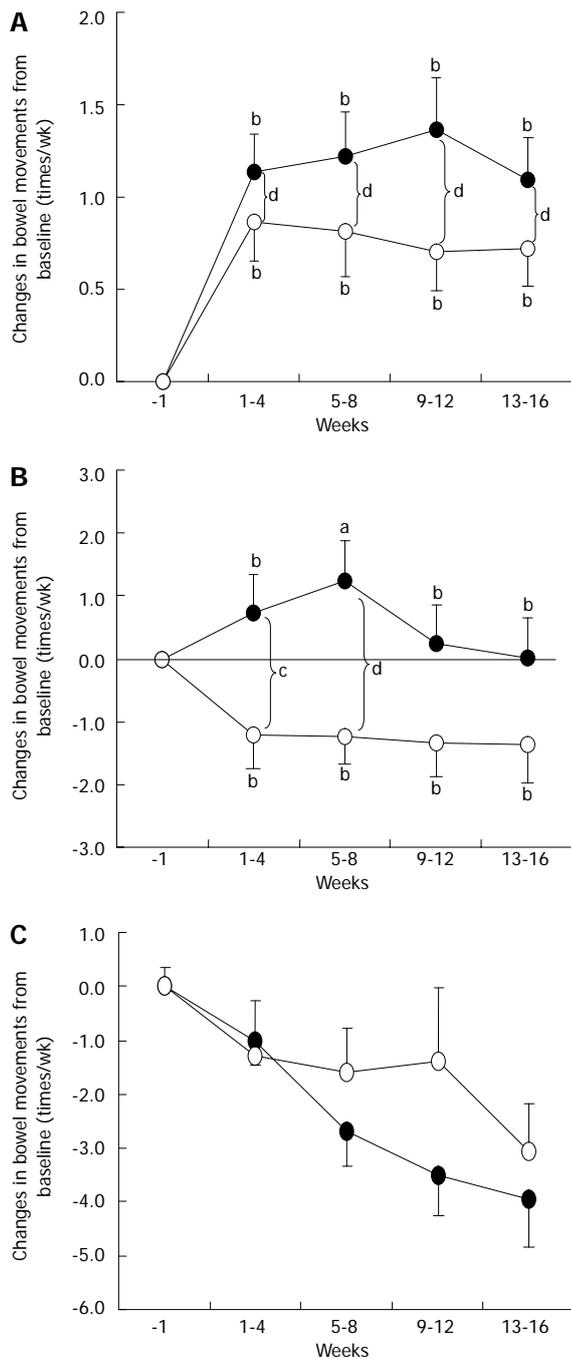


Figure 3 Effects of BB536 intake on changes in defecation frequency. A: Subgroup of patients with low infrequent defecation (≤ 4 times a week); B: Subgroup of patients with normal frequency of defecation (5-9 times a week); C: Subgroup of patients with high frequency of defecation (≥ 10 times a week) at baseline (week-1). Results present the summary of Trials 1 and 2 for the placebo (\circ) and BB536 groups (\bullet) composed of the BB536 group in Trial 1 and BB536-H and BB536-L groups in Trial 2). Times of defecation were averaged weekly for each individual, and changes from baseline (week-1) were calculated. The weekly scores for changes were further averaged every 4 wk. ^a $P < 0.05$, ^b $P < 0.01$ vs week-1 group; ^c $P < 0.05$, ^d $P < 0.01$ between groups.

in the BB536 group compared with the placebo group. Defecation frequency increased significantly at weeks 5-8 in the normal frequency subgroup of the BB536 group ($n = 29$). However, defecation frequency decreased significantly during the intervention period in the placebo group ($n = 18$), and significant intergroup differences

were observed at weeks 1-4 and 5-8. In contrast, defecation frequency decreased during the intervention period in the high frequency subgroup at weeks 5-8, 9-12, and 13-16 for the BB536 group ($n = 19$) but only at weeks 13-16 for the placebo group ($n = 9$). However, no significant intergroup differences were observed due to the small number of patients.

Changes in stool characteristics

Figure 4 presents the incidence of each stool type during the intervention. A significantly higher incidence of stool type 3 (*i.e.*, like a sausage but with cracks on its surface) and type 5 (soft blobs with clear-cut edges that could be passed easily) was observed in the BB536 group than in the placebo group in Trial 1. A significantly higher incidence of stool types 3 and 4 (like a sausage or snake, smooth and soft) was observed in the BB536-L group compared with the placebo group in Trial 2.

Effects on fecal microbiota

Real-time polymerase chain reaction analyses revealed that the cell numbers of total bifidobacteria, *B. longum* subsp. *longum*, and BB536 increased significantly after treatment in all 3 groups, and the cell numbers of these bacterial groups were significantly higher in the BB536 groups than in the placebo group (Table 3). The cell numbers of (*Bifidobacterium breve*) *B. breve* and *B. longum* subsp. *infantis* were significantly higher in the BB536-H group after treatment than before treatment. In addition, the cell numbers of (*Bifidobacterium adolescentis*) *B. adolescentis* were significantly higher in the BB536-H group than the placebo group at week 16. No differences in the cell numbers of the other dominant species of *Bifidobacterium* were observed after treatment.

DISCUSSION

The present results revealed obvious effects of BB536 therapy *vs* placebo in the normalization of defecation frequency in patients who exhibited low and high frequencies of defecation. BB536 administration increased the incidence of close-to-normal stools (types 3-5, Figure 3), which is consistent with the results for defecation frequency. BB536 administration also increased the cell population of bifidobacteria in the microbiota of elderly patients.

The pathogenesis of constipation and diarrhea are multifactorial, and the definition of constipation and diarrhea requires the presence of clinical symptoms and changes in the frequency of defecation^[21]. A careful diagnosis was not possible in the present study because the stools were monitored during daily care. Therefore, we could not classify low or high defecation frequencies as constipation or diarrhea, respectively. However, a defecation frequency ≤ 4 times per week may be considered mild constipation^[22]. The present results suggested a modulatory effect of BB536 in the improving of bowel movements in individuals with a low and high frequency of defecation, which normalized the frequency of def-

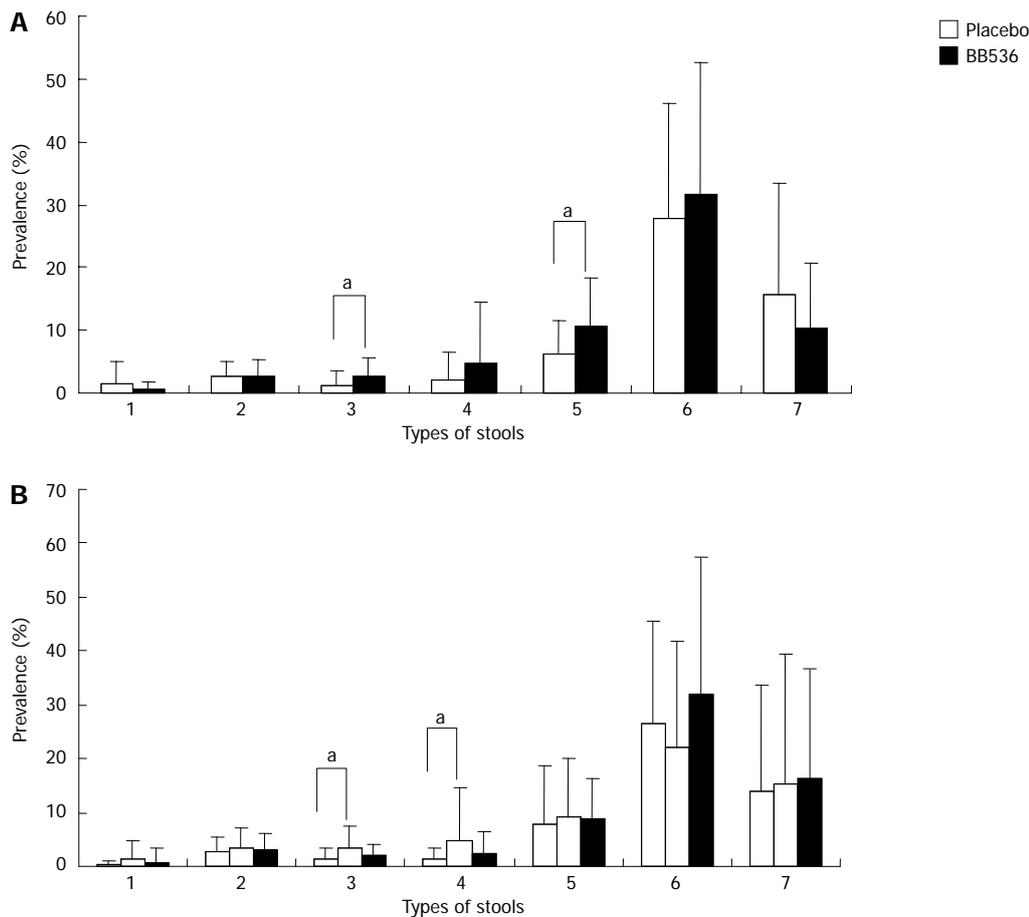


Figure 4 Effects of BB536 intake on stool form during treatment. A: Trail 1; B: Trail 2. Stool types for each bowel movement were recorded using the Bristol Stool Form Scale score, which ranges from 1 to 7. The frequency of each stool type was summed for the total treatment period, and the prevalence of each stool type was calculated. ^a $P < 0.05$ between groups.

ecation.

The effects of BB536 on fecal microbiota were investigated in Trial 2. We focused on the types of *Bifidobacteria* that are the major components of intestinal microbiota in humans and provide beneficial effects to human health^[23]. The number of intestinal bifidobacteria decreases with age^[6,7,24]. The populations of bifidobacteria in feces increased significantly after probiotic ingestion in the present study. These results confirmed previous findings that the ingestion of yogurt containing BB536 increased the population of bifidobacteria in healthy adults with a tendency toward constipation^[13,14]. The administered strain was the primary contributor to this increase in the bifidobacterial microbiota population. However, increases in the cell numbers of *B. breve* and *B. adolescentis* were also observed in the BB536-H group. These results suggest the potential of BB536 administration in the modulation of the intestinal environment, which enhanced the proliferation of endogenous bifidobacterial species.

Trial 2 was performed to confirm the results of Trial 1 (*i.e.*, the beneficial effects on defecation frequency) and investigate the dose effect of BB536. We confirmed the effect of BB536 ingestion on defecation frequency in both trials. However, no significant differences in defecation frequency, stool types, or fecal microbiota were ob-

served between the BB536-L and BB536-H groups, likely because the dose of the probiotic was only doubled. Further studies are required to investigate the dose response of BB536 using a broader dose range.

The present study had several strengths, including randomized treatment allocation, use of placebo controls, assessment of dose effect, evaluation using two successive studies, and evaluation of fecal microbiota during the study. This study also had several limitations, as previously discussed for other probiotic strains^[25]. The results presented herein are applicable only to *B. longum* BB536 and cannot be generalized to other probiotic strains or products. Caution should be exercised in extrapolating these study outcomes to individuals with chronic and/or severe gastrointestinal complications. Another limitation may be the mild effect of the treatment compared with other therapies, such as prokinetics and laxatives, particularly when cost-effectiveness is considered. However, the clinical implications of prokinetic agents are controversial^[26]. In contrast, probiotics are considered to be generally safe. Furthermore, as shown in the present study, *B. longum* BB536 showed a modulatory effect in improving the bowel movements of patients receiving enteral feeding whose bowel movements and frequency were not normal, *i.e.*, patients having either constipation or diar-

Table 3 Cell numbers of the dominant *Bifidobacterium*

| Species of <i>Bifidobacterium</i> | Period | mean (log/g) ± SD (prevalence, %) | | |
|---|---------|-----------------------------------|----------------------------------|----------------------------------|
| | | Placebo | BB536-L | BB536-H |
| All <i>Bifidobacterium</i> | Week-1 | 8.27 ± 1.32 (57.6) | 8.68 ± 1.26 (62.2) | 8.58 ± 0.94 (36.1) |
| | Week 16 | 8.41 ± 1.29 (78.8) ^a | 9.05 ± 0.91 (94.6) ^{bc} | 8.94 ± 0.75 (94.4) ^{bc} |
| <i>B. longum</i> subsp. <i>longum</i> | Week-1 | 6.91 ± 0.41 (15.2) | 7.40 ± 0.88 (27) | 7.29 ± 0.9 (30.6) |
| | Week 16 | 7.56 ± 0.94 (39.4) ^b | 8.13 ± 0.74 (94.6) ^{bd} | 8.26 ± 0.65 (91.7) ^{bd} |
| <i>B. adolescentis</i> | Week-1 | ND (0) | ND (0) | 10.06 (2.8) |
| | Week 16 | 6.64 ± 0.15 (36.4) | 6.57 ± 0.21 (24.3) | 6.97 ± 1.14 (25) |
| <i>B. catenulatum</i> | Week-1 | 11.12 ± 2.24 (9.1) | 12.27 ± 3.01 (8.1) | 12.88 ± 1.2 (5.6) |
| | Week 16 | 9.08 ± 0.36 (6.1) | 8.98 ± 0.28 (8.1) | 8.74 ± 0.05 (5.6) |
| <i>B. breve</i> | Week-1 | 7.91 ± 0.96 (39.4) | 8.04 ± 0.87 (48.6) | 7.84 ± 0.49 (22.2) |
| | Week 16 | 7.77 ± 1.00 (57.6) | 8.34 ± 0.84 (48.6) | 7.82 ± 0.82 (50.0) ^b |
| <i>B. bifidum</i> | Week-1 | ND (0) | 8.63 (2.7) | ND (0) |
| | Week 16 | 9.2 ± 0.66 (6.1) | 7.81 ± 1.30 (5.4) | 7.44 ± 1.24 (8.3) |
| <i>B. longum</i> subsp. <i>infantis</i> | Week-1 | 8.8 ± 0.46 (9.1) | 8.00 ± 1.12 (13.5) | 8.94 ± 0.05 (8.3) |
| | Week 16 | 7.71 ± 1.56 (18.2) | 7.91 ± 1.04 (16.2) | 8.16 ± 0.97 (25) ^a |
| BB536 | Week-1 | 6.42 (3) | 6.71 (2.7) | 6.92 ± 0.26 (5.6) |
| | Week 16 | 6.98 ± 0.77 (24.2) ^a | 7.97 ± 0.70 (89.2) ^{bd} | 8.13 ± 0.63 (91.7) ^{bd} |

^a*P* < 0.05, ^b*P* < 0.01 vs week-1; ^c*P* < 0.05, ^d*P* < 0.01 vs placebo group. *B. longum*: *Bifidobacterium longum*; *B. adolescentis*: *Bifidobacterium adolescentis*; *B. catenulatum*: *Bifidobacterium catenulatum*; *B. breve*: *Bifidobacterium breve*; *B. bifidum*: *Bifidobacterium bifidum*; ND: Not detected (< log 10⁶ cells/g).

rhea. Such effects would contribute to an improved quality of life in the patients and a decreased burden of care for nurses or caregivers. In addition, although an immunoprotective effect was not observed in the present study because no patient experienced influenza infection during the study period, other studies have suggested immunomodulating and anti-infectious effects of BB536^[28,29]. In the present study, we found that probiotic ingestion increased bifidobacteria in the microbiota. In addition, several studies have demonstrated the effects of administration of BB536 in eliminating harmful bacteria^[13,14,30]. Based on these findings, we consider that this probiotic may represent an alternative strategy in the treatment of gastrointestinal disorders and health management in the elderly.

In conclusion, the present findings revealed that the 16-wk long-term ingestion of the probiotic BB536 strain modulated bowel movements and normalized defecation frequency in elderly patients receiving enteral feeding. BB536 administration also significantly increased the population of bifidobacteria in the intestinal microbiota. No adverse effects were associated with the ingestion of BB536. Overall, these results suggest that BB536 ingestion may improve health care in the elderly.

ACKNOWLEDGMENTS

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COMMENTS

Background

Elderly individuals, particularly patients who are hospitalized and receiving enteral nutrition, exhibit significant problems in defecation, which may impact on quality of life due to constipation or diarrhea. The development of novel

therapeutic strategies is necessary to treat these patients more effectively, and probiotics are increasingly used as one alternative in the management of constipation.

Research frontiers

Several studies have investigated the beneficial effects of probiotics in the management of constipation and diarrhea in elderly patients. However, these effects may be strain-dependent, and they are not consistently observed. Therefore, further investigation is required to clarify this relationship. The present study investigated the efficacy of a probiotic *Bifidobacterium* strain in the health management of hospitalized elderly patients receiving enteral nutrition in two double-blind, placebo-controlled trials following a 16-wk administration of BB536.

Innovations and breakthroughs

Authors demonstrated effects of *Bifidobacterium longum* BB536 therapy vs placebo in the normalization of defecation frequency in patients who exhibited low and high frequencies of defecation and increased the cell population of bifidobacteria in fecal microbiota.

Applications

The results of the present clinical trials suggest that the ingestion of the probiotic *Bifidobacterium* BB536 is an alternative strategy for the treatment of gastrointestinal disorders in the elderly.

Peer review

This is a formal good study of double-blind, placebo-controlled trials. The authors should discuss if probiotics should become part of regular EN in the elderly. In conclusion, I think that this had a good study design with interesting results for therapy.

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Correlation of human epidermal growth factor receptor 2 expression with clinicopathological characteristics and prognosis in gastric cancer

Chao He, Xue-Yi Bian, Xing-Zhi Ni, Dan-Ping Shen, Yan-Ying Shen, Hua Liu, Zhi-Yong Shen, Qiang Liu

Chao He, Xing-Zhi Ni, Dan-Ping Shen, Hua Liu, Zhi-Yong Shen, Department of General Surgery, Renji Hospital, Shanghai Jiaotong University School of Medicine, Shanghai 200127, China
Xue-Yi Bian, Department of General Surgery, Suzhou Jiulong Hospital, Shanghai Jiaotong University School of Medicine, Suzhou 215021, Jiangsu Province, China

Yan-Ying Shen, Qiang Liu, Department of Pathology, Renji Hospital, Shanghai Jiaotong University School of Medicine, Shanghai 200127, China

Author contributions: He C, Bian XY and Ni XZ designed the research; He C, Bian XY, Shen DP, Shen YY, Liu H, Shen ZY and Liu Q performed the research; He C, Bian XY, Shen DP and Ni XZ analyzed the data; He C, Bian XY and Ni XZ wrote the paper.

Correspondence to: Xing-Zhi Ni, MD, Professor of Medicine, Department of General Surgery, Renji Hospital, Shanghai Jiaotong University School of Medicine, 1630 Dongfang, Pudong district, Shanghai 200127, China. niyin@yahoo.com

Telephone: +86-21-68383731 Fax: +86-21-58394262

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Abstract

AIM: To investigate human epidermal growth factor receptor 2 (*HER2*) gene amplification and protein expression in Chinese patients with resectable gastric cancer and the association with clinicopathological characteristics and survival.

METHODS: One hundred and ninety-seven gastric cancer patients who underwent curative surgery procedures were enrolled into this study. *HER2* gene amplification and protein expression were examined using fluorescence *in-situ* hybridization (FISH) and immunohistochemistry (IHC) analysis on formalin-fixed paraffin-embedded gastric cancer samples from all patients. For scoring, Hofmann's *HER2* gastric cancer scoring system was adopted. All cases showing IHC3+ or FISH positiv-

ity were defined as *HER2* positive. Patient clinicopathological data and survival information were collected. Finally, χ^2 statistical analysis was performed to analyze the *HER2* positivity rate amongst the subgroups with different clinicopathological characteristics including: gender, age, tumor location, Lauren classification, differentiation, TNM staging, depth of invasion, lymph node metastases and distant metastasis. The probability of survival for different subgroups with different clinicopathological characteristics was calculated using the Kaplan-Meier method and survival curves plotted using log rank inspection.

RESULTS: According to Hofmann's *HER2* gastric cancer scoring criteria, 31 cases (15.74%) were identified as *HER2* gene amplified and 19 cases (9.64%) were scored as strongly positive for *HER2* membrane staining (3+), 25 cases (12.69%) were moderately positive (2+) and 153 cases (77.66%) were *HER2* negative (0/1+). The concordance rate between IHC and FISH analyses was 88.83% (175/197). Thirty-six cases were defined as positive for *HER2* gene amplification and/or protein expression, with 24 of these cases being eligible for Herceptin treatment according to United States recommendations, and 29 of these cases eligible according to EU recommendations. Highly consistent results were detected between IHC3+, IHC0/1 and FISH (73.68% and 95.42%), but low consistency was observed between IHC2+ and FISH (40.00%). The positivity rates in intestinal type and well-differentiated gastric cancer were higher than those in diffuse/mixed type and poorly-differentiated gastric cancer respectively (28.57% vs 13.43%, $P = 0.0103$; 37.25% vs 11.64%, $P < 0.0001$), but were not correlated with gender, age, tumor location or TNM stage, depth of invasion, lymph node metastases and distant metastasis. In poorly-differentiated gastric cancer patients, those without lymph node metastasis showed a higher *HER2* positivity rate than those with lymph node metastasis (26.47% vs 7.14%, $P = 0.0021$). This association was not present in those

patients with well-differentiated gastric cancer (28.57% vs 43.33%, $P = 0.2832$). Within our patient cohort, 26 cases were lost to follow-up. The median survival time for the remaining 171 patients was 18 mo. The median survival times of the HER2 positive and negative groups were 17 and 18.5 mo respectively. Overall survival was not significantly different between HER2-positive and negative groups ($\chi^2 = 0.9157$, $P = 0.3386$), but in patients presenting well-differentiated tumors, the overall survival of the HER2-positive group was significantly worse than that of the HER2-negative group ($P = 0.0123$). In contrast, patients with poorly differentiated and diffuse/mixed subtype gastric cancers showed no significant differences in overall survival associated with HER2. Furthermore, the median survival time of the HER2 positive group did not show any statistically significant differences when compared to the subgroups of gender, age, tumor location, TNM classification, lymph node metastases and distant metastasis.

CONCLUSION: Patients with intestinal type gastric cancer (GC), well-differentiated GC and poorly-differentiated GC without lymph node metastasis, may all represent suitable candidates for targeted therapy using Herceptin.

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Key words: Gastric cancer; Human epidermal growth factor receptor 2; Gene amplification; Protein expression; Clinicopathological characteristics

He C, Bian XY, Ni XZ, Shen DP, Shen YY, Liu H, Shen ZY, Liu Q. Correlation of human epidermal growth factor receptor 2 expression with clinicopathological characteristics and prognosis in gastric cancer. *World J Gastroenterol* 2013; 19(14): 2171-2178 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v19/i14/2171.htm> DOI: <http://dx.doi.org/10.3748/wjg.v19.i14.2171>

INTRODUCTION

Human epidermal growth factor receptor 2 (HER2) is a 185-kDa transmembrane tyrosine kinase receptor^[1] and its gene amplification and protein overexpression play an important role in the proliferation, apoptosis, adhesion, angiogenesis and aggressiveness of many solid tumors^[2], including; breast^[3], colon^[4], bladder^[4], ovarian^[5], uterine cervix^[6], esophageal^[7] and gastric cancer.

Herceptin (trastuzumab) has been approved^[8] in the European Union and the United States for use in combination with 5-fluorouracil (5-FU) or capecitabine plus cisplatin for the first-line treatment of patients with HER2-positive metastatic adenocarcinoma of the stomach or gastroesophageal junction according to the results of the 2010 trastuzumab for gastric cancer (ToGA) trial. However, precise patient inclusion criteria for Herceptin treatment is still not fully defined due to the lack of a standardized HER2 scoring system for gastric cancer^[9,10]. For a clinical,

defining the relationships between HER2 and clinicopathological characteristics can help to select suitable candidates.

Our study aimed to investigate the relationship between *HER2* gene amplification and protein overexpression in resectable gastric cancer patients and determine any correlations with relevant clinicopathological characteristics. Furthermore, we explored the influence of HER2 on disease prognosis in gastric cancer patients. Our study was conducted with a view towards the future introduction of Herceptin targeted therapy for the treatment of gastric cancer patients.

MATERIALS AND METHODS

Patients and tissue specimens

From July 2009 to January 2012, 197 gastric cancer patients who underwent curative surgery at Renji hospital, Shanghai Jiaotong University were enrolled into our study. Formalin-fixed, paraffin-embedded samples of tumors and corresponding normal stomach tissues from 197 gastric cancer patients were evaluated for HER2 protein and gene amplification using immunohistochemistry (IHC) and fluorescence *in-situ* hybridization (FISH) analysis. None of the patients had undergone prior preoperative radiation, chemotherapy or targeted therapy.

The study included 65 women and 132 men, with ages ranging from 22 to 88 years. The median age was 62 years. The tumor sample characteristics of all 197 cases are shown in Table 1. Of all the tumors examined, 31 (15.74%) were located in the cardiac region, 42 (21.32%) in the body, and 122 (61.93%) in the pylorus. The majority (98.98%) of the samples were primary tumors with only 2 recurrent tumors identified. According to Lauren classification, 63 (31.98%) tumors were intestinal-type and 134 (68.02%) were diffuse-type or mixed-type carcinomas. Poorly differentiated tumors (grades I and II) comprised 25.89%, whilst 74.11% of tumors were moderately differentiated (grades III and IV). TNM classification revealed that 13 cases were stage I (6.60%), 46 were stage II (23.35%), 98 were stage III (49.75%) and 40 were stage IV (20.30%). Postoperative follow-up ended in April, 2012.

FISH detection for HER2 gene amplification

FISH was conducted with the HER2 DNA Probe Kit (Invitrogen™ by Life Technologies) according to the manufacturer's instructions. Four- μ m-thick sections were baked overnight at 56 °C, deparaffinized in three 10 min changes of xylene and then rehydrated through two 5-min changes of 100% ethanol. The slides were then reduced for 18 min in SPOT-Light tissue pretreatment solution at > 98 °C, and briefly washed in 3 × PBS at room temperature. The slides were then incubated for 16 min in enzyme reagent solution at 37 °C and washed in 3 × PBS at room temperature, dehydrated through 70%, 85%, and 100% ethanol, and allowed to air dry. After open air drying, the HER2 DNA probe kit (PathVysion HER2 DNA Probe Kit, Abbott Laboratories) which was denatured at

Table 1 Correlation of human epidermal growth factor receptor 2 expression with clinicopathological characteristics *n* (%)

| Clinicopathological characteristics | <i>n</i> | HER2 | | χ^2 | <i>P</i> value |
|-------------------------------------|----------|------------|-------------|----------|----------------|
| | | Positive | Negative | | |
| Sex | | | | 1.2736 | 0.2591 |
| Male | 132 | 27 (20.45) | 105 (79.55) | | |
| Female | 65 | 9 (13.85) | 56 (86.15) | | |
| Age (yr) | | | | 1.3056 | 0.2532 |
| < 60 | 88 | 13 (14.77) | 75 (85.23) | | |
| ≥ 60 | 109 | 23 (21.10) | 86 (78.90) | | |
| Tumor site ¹ | | | | 0.0409 | 0.9798 |
| Cardiac | 31 | 6 (19.35) | 25 (80.65) | | |
| Body | 42 | 8 (19.05) | 34 (80.96) | | |
| Pylorus | 122 | 22 (18.03) | 100 (81.97) | | |
| Lauren classification | | | | 6.5759 | 0.0103 |
| Intestinal | 63 | 18 (28.57) | 45 (71.43) | | |
| Diffuse/mixed | 134 | 18 (13.43) | 116 (86.57) | | |
| Tumor differentiation | | | | 16.6003 | < 0.0001 |
| Well-differentiated | 51 | 19 (37.25) | 32 (62.75) | | |
| Poorly-differentiated | 146 | 17 (11.64) | 129 (88.36) | | |
| TNM classification | | | | 0.6754 | 0.879 |
| I | 13 | 2 (15.38) | 11 (84.62) | | |
| II | 46 | 7 (15.22) | 39 (84.78) | | |
| III | 98 | 20 (20.41) | 78 (79.59) | | |
| IV | 40 | 7 (17.50) | 33 (82.50) | | |

¹Two remnant samples were not included. HER2: Human epidermal growth factor receptor 2.

79 °C for 6 min, was applied onto each slide, a cover slip was added and then sealed with rubber cement. After 16 to 18 h of hybridization at 37 °C, the slides were washed with 73 °C preheated post hybridization buffer for 5 min and dehydrated through 70%, 85% and finally 100% ethanol. After air drying, the slides were counter-stained with 14 μL diaminidino-phenyl-indole, cover slips applied and then slides chilled for 30 min at 4 °C. Finally, the slides were observed through a fluorescence microscope (OLYMPUS BX61).

Immunohistochemical staining

HER2 IHC analysis was performed on 4 μm thick tissue sections. Briefly, after deparaffinization and rehydration steps, the tissue samples were incubated in antigen retrieval solution at 99 °C for 40 min. Endogenous peroxidase activity was quenched by 5 min incubation with hydrogen peroxide. Sections were then incubated with HER2 antibody (Herceptest™, DAKO) for 30 min. Both the primary and secondary antibodies against human HER2 protein were applied for 30 min at room temperature and then the immunocomplexes were visualized with diaminobenzidine for 10 min and placed under a cover slip. Finally, the slides were viewed using light microscopy (LEICA DM2500).

Results scoring

An absolute *HER2* gene copy number lower than 6 or a *HER2*/Chr17 ratio of less than 2 was considered *HER2* negative, whilst cases showing average gene copy numbers of *HER2* ≥ 6 or a gene/CEN17 fluorescence ratio ≥ 2 were considered positive for gene amplification.

Table 2 Immunochemistry-fluorescence *in situ* hybridization concordance *n* (%)

| FISH | IHC | | | | Total |
|----------|-----------|------------|------------|-------------|-------------|
| | 3+ | 2+ | 1+ | 0 | |
| Positive | 14 | 10 | 7 | 0 | 31 (15.74) |
| Negative | 5 | 15 | 21 | 125 | 166 (84.26) |
| Total | 19 (9.64) | 25 (12.69) | 28 (14.21) | 125 (63.45) | 197 |

IHC: Immunochemistry; FISH: Fluorescence *in-situ* hybridization.

Additionally, tight gene clustering of *HER2* signals was also defined as gene amplification. The above criteria are based on Hofmann's criteria in gastric cancer^[9].

In the present study, the IHC score criteria on human gastric cancer also followed Hofmann's criteria^[9]: no staining or < 10% tumor cell positive staining as 0/negative; faintly or barely perceptible staining on > 10% tumor cell membrane as 1+/negative; weak to moderate positive staining on > 10% tumor cells as 2+/(equivocal) positive; cohesive moderate to strong staining on the membrane will be scored as 3+/positive. All cases with IHC3+ or FISH positivity were defined as *HER2* positive.

Statistical analysis

χ^2 statistical analysis was performed to assess the *HER2* positivity rate amongst the subgroups with different clinicopathological characteristics. The probability of survival for different subgroups was calculated using the Kaplan-Meier method and the survival curves plotted using log rank inspection. All statistics were performed using 2-sided analysis, with a significance level of *P* < 0.05, using the "SAS9.13" statistical software package.

RESULTS

HER2 gene amplification and protein expression

The FISH and IHC analysis results for all 197 gastric cancer tissues are shown in Table 2. According to Hofmann's *HER2* FISH scoring criteria, 31 cases (15.74%) were identified as *HER2* gene amplified and the other 166 cases (84.26%) were *HER2* gene amplification negative (Figure 1). Of the 197 samples examined by IHC (following Hofmann's criteria), 19 cases (9.64%) were scored as strongly positive for *HER2* membrane staining (3+), 25 cases (12.69%) were moderately positive (2+), and 153 cases (77.66%) were *HER2* negative (0/1+) (Figure 2).

The concordance rate between IHC and FISH analyses was 88.83% (175/197). Thirty-six cases were defined as *HER2* positive and 24 cases were suitable for Herceptin treatment according to the recommendations of the United States^[11]. However, when applying European Union^[11] recommendations for Herceptin usage, 29 cases were identified as eligible for Herceptin treatment. This difference underscores the requirement for standardized and more precise eligibility criteria for correct identification of patients who are eligible for *HER2* targeted therapy.

Of the 31 FISH-positive cases, 14 cases (45.16%)

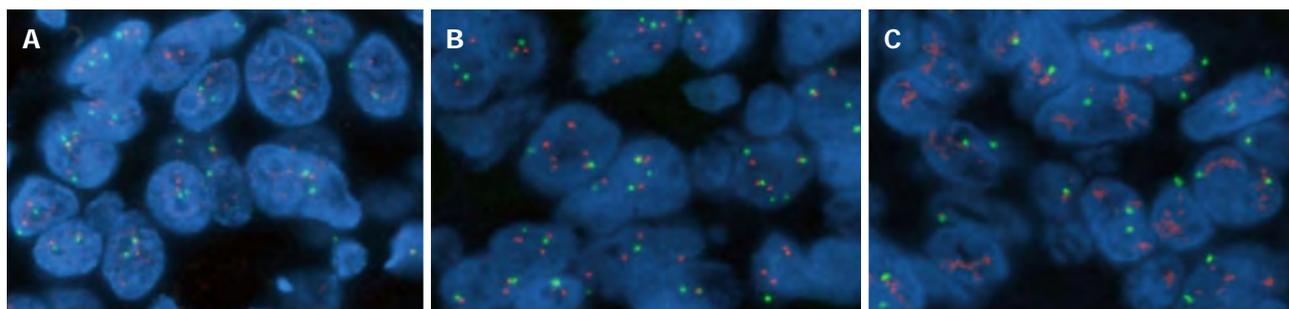


Figure 1 Fluorescent *in-situ* hybridization analysis of human epidermal growth factor receptor 2 gene amplification ($\times 600$). A: Normal human epidermal growth factor receptor 2 (*HER2*) gene expression: Red signals (*HER2* gene), green signals [chromosome enumeration probe 17 (CEP17)], blue signals (nuclei lining dye); B: Positive *HER2* gene amplification: $HER2:CEP17 > 2$; C: Positive *HER2* gene amplification: $HER2:CEP17 > 2$ with clear red cluster signals observed.

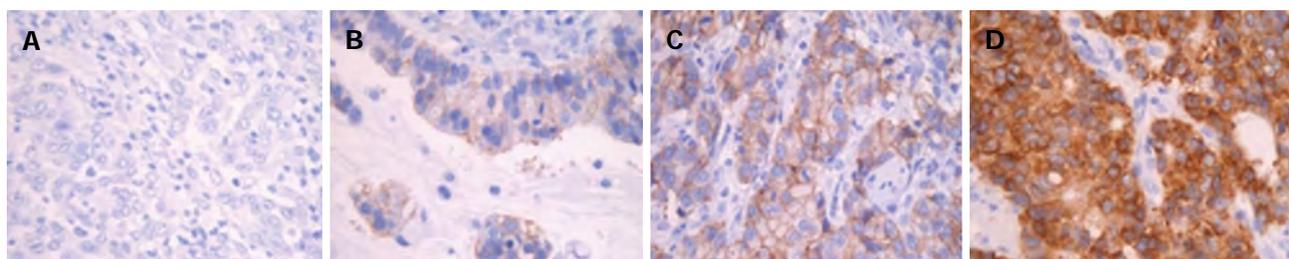


Figure 2 Immunohistochemical analysis of human epidermal growth factor receptor 2 protein expression ($\times 200$). A: Immunohistochemical (IHC) 0: No staining on tumor cell membrane; B: IHC1+: Faintly perceptible staining on $> 10\%$ tumor cell membrane; C: IHC2+: Moderate staining on $> 10\%$ tumor cell membrane; IHC3+: Strong staining on $> 10\%$ tumor cell membrane.

Table 3 Correlation of human epidermal growth factor receptor 2 expression with tumor node metastasis staging *n* (%)

| Clinicopathological characteristics | <i>n</i> | HER2 | | χ^2 | <i>P</i> value |
|-------------------------------------|----------|------------|-------------|----------|----------------|
| | | Positive | Negative | | |
| T | | | | 0.5782 | 0.4470 |
| T1-T2 | 26 | 6 (23.08) | 20 (76.92) | | |
| T3-T4 | 171 | 29 (16.96) | 142 (84.04) | | |
| N | | | | 4.6274 | 0.2012 |
| N0 | 55 | 8 (14.55) | 47 (85.45) | | |
| N1 | 83 | 20 (24.10) | 63 (75.90) | | |
| N2 | 33 | 5 (15.15) | 28 (84.85) | | |
| N3 | 26 | 2 (7.69) | 24 (92.31) | | |
| M | | | | 0.0000 | 1.0000 |
| M0 | 185 | 33 (17.84) | 152 (82.16) | | |
| M1 | 12 | 2 (16.67) | 10 (83.33) | | |

HER2: Human epidermal growth factor receptor 2.

were IHC3+ with a 100% concordance between IHC3+ and FISH, and 10 (32.26%) cases were IHC2+. None of the IHC 0 tumors demonstrated FISH amplification, and only 7 tumors in the IHC1+ group were found to be FISH positive with a ratio of 22.58%. High consistency results was detected between IHC3+, IHC0/1, and FISH scores (73.68% and 95.42%), but low consistency was observed between IHC2+ and FISH (40.00%).

Correlation of HER2 with clinicopathological characteristics

Significantly different HER2 positivity rates were observed when comparing intestinal-type gastric cancers

with diffuse/mixed-type cancers (28.57% *vs* 13.43%, *P* = 0.0103), and well-differentiated cases with poorly-differentiated cases (37.25% *vs* 11.64%, *P* < 0.0001). No relationship was observed between the HER2 positivity rate and sex, age, tumor site and TNM GC classification (*P* > 0.05; Table 1). Furthermore, within the subgroups, no relationship was observed between HER2 positivity and depth of invasion, lymph node metastasis or distant metastasis (Table 3).

Within the poorly-differentiated gastric cancer patient group, those without lymph node metastasis showed a higher HER2 positivity rate than those with lymph node metastasis (26.47% *vs* 7.14%, *P* = 0.0021). This association was not observed in the well-differentiated gastric cancer patient group (28.57% *vs* 43.33%, *P* = 0.2832).

Survival analysis

Of our 197 gastric cancer patients, 26 cases were lost in follow-up. The median survival time for the remaining 171 patients was 18 mo (range: 0-33 mo). During the follow-up time, 60 deaths occurred (35.09%), 57 of which were disease-related. One patient died of perioperative pulmonary infection, and two cases died of heart disease and multiple organ failure, respectively.

The median survival time of the HER2 positive (29 cases) and negative groups (142 cases) was 17 mo and 18.5 mo, respectively. Nevertheless, the HER2 positive gastric cancer patients did not show statistically significant reductions in mean survival times, nor lower 1-year or 2-year survival rates. Furthermore, no statistically significant differences were observed in overall survival

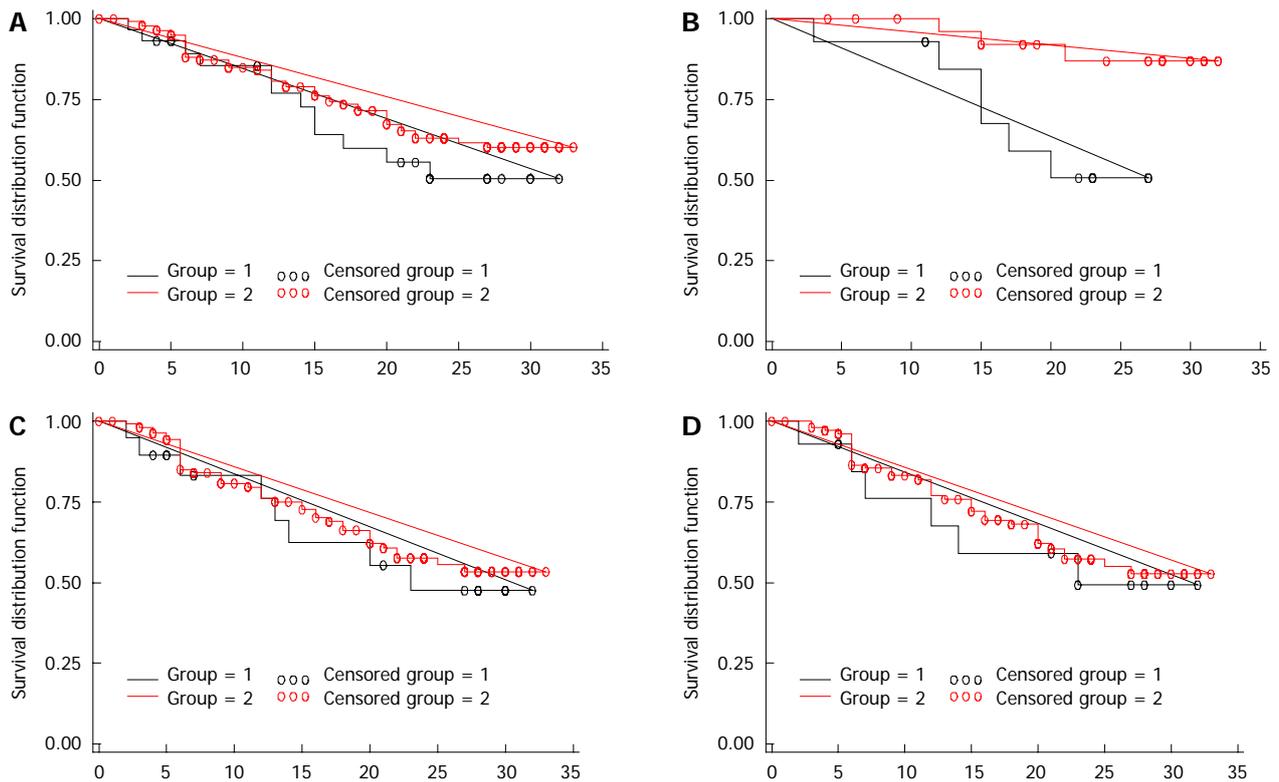


Figure 3 Kaplan-Meier survival analysis. A: Overall survival curves of 171 gastric cancer patients according to human epidermal growth factor receptor 2 (HER2) detection ($P = 0.3386$); B: Survival curve of patients with well differentiated gastric cancer according to HER2 expression ($P = 0.0123$); C: Survival curve of patients with poorly differentiated gastric cancer according to HER2 expression ($P = 0.0988$); D: Survival curve of patients with the diffuse/mixed type gastric cancer according to HER2 expression ($P = 0.6623$).

times between the HER2 positive and negative groups ($\chi^2 = 0.9157$, $P = 0.3386$; Figure 3A).

Within the well differentiated gastric cancer patient group, patients with HER2 tumor positivity had poorer outcomes than those with HER2 negative tumors. The well differentiated HER2 positive patient group exhibited shorter mean survival time (18.5 mo *vs* 27.5 mo) and lower 1-year and 2-year survival rates compared to the HER2 negative group (84.42% *vs* 96.00%; 50.65% *vs* 86.89%; $P = 0.0123$; Figure 3B). The median survival time of the HER2 positive group did not show any statistical associations when compared to the subgroups of sex, age, tumor site, TNM classification, depth of invasion, lymph node metastases and distant metastasis in gastric cancer (Table 4). Within the poorly differentiated and diffuse/mixed type gastric cancer patient groups, no statistically significant differences were observed between the HER2 positive and HER2 negative groups (Figure 3C and D).

DISCUSSION

HER2 gene amplification and protein overexpression in gastric cancer were first reported in 1986^[12,13] and have since been confirmed by numerous studies, highlighting ranges in both HER2 gene amplification rates from 16%-27.1% by FISH analysis and HER2 protein overexpression from 8.2%-53.4% by IHC analysis. The variability within these results is likely due to several fac-

tors including sample size, study design and differences in geographic location^[14]. However, the most important variability factor is likely a consequence of having no standardized HER2 test and scoring criteria^[15]. In the present study, both FISH and IHC scoring criteria followed that of Hofmann^[9] which is considered to be the most appropriate HER2 scoring system in human gastric cancer. Furthermore, to ensure the reliability of our results, we followed the guidelines on HER2 detection in breast cancer, recommended by the American Society of Clinical Oncology/College of American Pathologists (ASCO/CAP)^[16] and used the test kit certified by the United States Food and Drug Administration.

Herceptin (trastuzumab) is a recombinant human monoclonal antibody designed to target and block the function of HER2 by directly binding to the extracellular domain of the receptor^[1,17]. It has been used for the treatment of HER2 overexpressing breast cancer for more than 10 years and was approved by the European Medicines Agency^[18] in 2010 for use in combination with capecitabine or 5-FU and cisplatin for metastatic gastric or GE junction cancers, based on data from the "ToGA" clinical trial. The exact anti-tumor mechanism of Herceptin is not fully understood, however some mechanisms have been postulated^[17,19-23] including interruption of HER2 mediated cell signaling pathways and cell cycle progression; induction of antibody-dependent cell-mediated cytotoxicity and apoptosis; induction of

Table 4 Relationship of different clinicopathological characteristics and prognosis

| Clinicopathological characteristics | HER2 positive | | | HER2 negative | | | χ^2 | P value |
|-------------------------------------|---------------------------|----------------------|----------------------|---------------------------|----------------------|----------------------|----------|---------|
| | Median survival time (mo) | 1-year survival rate | 2-year survival rate | Median survival time (mo) | 1-year survival rate | 2-year survival rate | | |
| Sex | | | | | | | | |
| Male | 20 | 74.34% | 50.18% | 20 | 83.96% | 69.00% | 2.2591 | 0.1328 |
| Female | 10 | 100.00% | 50.00% | 16.5 | 74.50% | 51.79% | 0.0182 | 0.8927 |
| Age (yr) | | | | | | | | |
| ≤ 60 | 23 | 100.00% | 57.14% | 20 | 80.54% | 61.81% | 0.0104 | 0.9186 |
| > 60 | 15 | 67.55% | 49.13% | 18 | 80.62% | 64.35% | 1.6356 | 0.2009 |
| Tumor site | | | | | | | | |
| Cardiac | 19 | 66.67% | 50.00% | 15 | 69.51% | 49.15% | 0.0494 | 0.8242 |
| Body | 16.5 | 62.50% | 62.50% | 14 | 67.80% | 44.07% | 0.1561 | 0.6927 |
| Pylorus | 17 | 85.56% | 46.67% | 20 | 87.00% | 73.25% | 2.3295 | 0.1269 |
| Lauren classification | | | | | | | | |
| Intestinal | 17 | 84.85% | 50.91% | 27 | 89.17% | 76.53% | 2.3604 | 0.1244 |
| Diffues/mixed | 14 | 67.53% | 49.24% | 16.5 | 76.99% | 57.24% | 0.1907 | 0.6623 |
| Tumor differentiation | | | | | | | | |
| Well-differentiated | 18.5 | 84.42% | 50.65% | 27.5 | 96.00% | 86.89% | 6.2701 | 0.0123 |
| Poorly-differentiated | 14 | 67.88% | 49.49 | 17 | 76.56% | 56.71% | 0.0988 | 0.7532 |
| TNM classification | | | | | | | | |
| I and II stages | 18.5 | 68.57% | 57.14% | 21.5 | 93.60% | 79.20% | 2.9813 | 0.0842 |
| III and IV stages | 17 | 82.59% | 45.88% | 16.5 | 73.32% | 54.12% | 0.0263 | 0.8711 |
| T | | | | | | | | |
| T1-T2 | 17 | 66.67% | 66.67% | 28 | 100.00% | 92.31% | 3.4587 | 0.0629 |
| T3-T4 | 15 | 91.30% | 46.99% | 17 | 77.47% | 58.26% | 0.2953 | 0.5869 |
| N | | | | | | | | |
| N0 | 14 | 68.57% | 51.43% | 21 | 90.46% | 74.98% | 2.0667 | 0.1505 |
| N1-N3 | 18.5 | 79.19% | 49.49% | 17 | 75.73% | 57.27% | 0.0531 | 0.8177 |
| M | | | | | | | | |
| M0 | 17 | 78.67% | 54.69% | 20 | 84.41% | 66.01% | 0.7842 | 0.3757 |
| M1 | 11.5 | 50.00% | 0.00% | 5 | 0.00% | 0.00% | 0.5900 | 0.4424 |

HER2: Human epidermal growth factor receptor 2.

anti-angiogenesis effects and increasing receptor turnover by endocytosis. As clinical surgeons, we should be readily and accurately able to identify which patients are suitable for Herceptin treatment. An accurate and reliable HER2 scoring system, together with clinical information, may help us to better determine whether a gastric cancer patient is a potential candidate for targeted therapy using Herceptin.

The relationship between *HER2* gene amplification and protein expression in gastric cancer patients is controversial^[24,25]. Nevertheless, more recent studies have reported a high concordance between gene amplification and protein overexpression using FISH and IHC approaches^[11,26,27]. Indeed, the ToGA trial^[28] (which recruited the largest population of gastric cancer patients to date-3807) reported a HER2 FISH and IHC concordance rate of 87.5%, and further reported that HER2 IHC3+ cases were almost all entirely *HER2* gene amplified (97.5% of cases). However, 22.5% of HER2 FISH positive cases in the ToGA trial were HER2 IHC negative, a finding which differs from the situation observed in breast cancer, where almost all HER2 IHC 0/1+ samples are HER2 FISH negative^[14]. In our study, the overall HER2 positive rate (FISH and IHC combined) was 18.27% while 15.74% of cases showed *HER2* gene amplification by FISH and 9.64% of patients showed HER2 protein overexpression by IHC analysis. The concordance

between the two detection methods was 88.83%. Of the 31 FISH-positive cases, 14 cases (45.16%) were IHC3+, with a 100% concordance between IHC3+ and FISH, and 10 (32.26%) cases were IHC2+. None of the IHC0 tumors showed FISH amplification, and only 7 tumors in the IHC1+ group were found to be FISH positive with a ratio of 22.58%. A high degree of data consistency was observed between IHC3+ and IHC0/1 with FISH (73.68% and 95.42%); however, low scoring consistency was observed between IHC2+ and FISH (40.00%). Thus, our data highlights the need and importance of further clarifying the relationship between *HER2* gene amplification and protein overexpression in gastric cancer.

In our study, no relationship was observed between HER2 positivity and sex, age and TNM classification ($P > 0.05$). However, intestinal-type and well-differentiated gastric cancer cases showed a higher HER2 positive rate than diffuse/mixed-type and poorly-differentiated cancer cases. This finding is in keeping with similar data from the ToGA trial and other published studies^[29,30]. Of interest, the ToGA trial reported a higher HER2 positivity rate in GE junction cancers compared to other gastric cancers (33.2% *vs* 20.9%, $P < 0.001$)^[17]. Our study, as well as that of another group^[31], showed no statistically significant difference between HER2 positivity and the gastric tumor site. Within the poorly-differentiated gastric cancer patient group, those patients without lymph node

metastasis showed a higher HER2 positivity rate when compared to those with lymph node metastasis (26.47% *vs* 7.14%, $P = 0.0021$). No difference in HER2 positivity was observed, however, when comparing lymph node metastasis status in the well-differentiated gastric cancer patient group (28.57% *vs* 43.33%, $P = 0.2832$). The underlying molecular mechanisms behind the varying HER2 positivity rates in the different histological GC subtypes are clearly complex and require further investigation.

The role of HER2 as a prognostic factor in gastric cancer has been controversial due to significant differences in historical study results. More recent studies, however, indicate that HER2 is a poor prognostic factor in gastric cancer patients^[32-35], especially those with liver metastases^[36]. Whilst our study did not show any correlation between HER2 status and overall survival, patients with well-differentiated HER2 positive tumors showed poorer survival times compared to patients with HER2 negative tumors. We speculate that HER2 status has a mild impact on gastric cancer patient survival and may not constitute an independent prognostic factor in gastric cancer patients. Clearly, further research is required to explain the impact of HER2 on development and prognosis of gastric cancer.

In conclusion, an accurate and standardized scoring system for HER2 expression in gastric cancer patients is of clear importance and utility in the optimal selection of patients for Herceptin therapy. Our studies highlight intestinal-type, well-differentiated and poorly-differentiated gastric cancer patients without lymph node metastasis as the three main candidate patient groups for targeted therapy using Herceptin. Finally, we advocate further detailed research on the mechanism(s) through which HER2 expression drives progression of gastric cancer and consideration of additional studies to explore the role of HER2 as an independent prognostic factor.

ACKNOWLEDGMENTS

We thank Dr. Paul R Gavine for valuable suggestions and critical reading of the paper.

COMMENTS

Background

Gastric cancer (GC) is one of the most prevalent cancers worldwide, with poor prognosis. Herceptin (trastuzumab) can improve overall survival without compromising safety in patients with human epidermal growth factor receptor 2 (HER2)-positive metastatic gastric cancer. However, a standardized HER2 scoring system is still required. Studies on the correlation of HER2 and clinicopathological characteristics could help clinicians to optimally select suitable candidates for targeted therapy using Herceptin.

Research frontiers

HER2 inhibition is playing a significant role as a new treatment option for gastric cancer. Numerous countries have approved the use of Herceptin for the treatment of gastric cancer and increasingly, HER2 has become a "hot" research topic. An accurate and reliable HER2 scoring system is necessary to select suitable candidates for Herceptin targeted therapy.

Innovations and breakthroughs

To date, there have been limited studies to determine any correlations of HER2 expression with clinicopathological characteristics and prognosis in Chinese

patients with resectable gastric cancer. Intestinal type gastric cancer patients, well-differentiated gastric cancer patients and poorly-differentiated gastric cancer patients without lymph node metastasis showed a higher HER2 positivity rate and thus could represent ideal candidates for targeted-therapy using Herceptin.

Applications

The study results suggest that an accurate HER2 scoring system plays an important role with clinical significance. Patients with intestinal-type gastric cancer, well-differentiated gastric cancer and poorly-differentiated gastric cancer without lymph node metastasis are ideal candidates for targeted therapy using Herceptin.

Peer review

The paper makes sense to search for the gastric cancer patients in Jiangsu province. The study design is valid and the data is sufficient.

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Involvement of interstitial cells of Cajal in experimental severe acute pancreatitis in rats

Liang-Liang Shi, Ming-Dong Liu, Min Chen, Xiao-Ping Zou

Liang-Liang Shi, Ming-Dong Liu, Min Chen, Xiao-Ping Zou, Department of Gastroenterology, Medical School, the Affiliated Drum Tower Hospital of Nanjing University, Nanjing 210008, Jiangsu Province, China

Author contributions: Liu MD and Zou XP contributed equally to this work; Shi LL, Liu MD and Chen M designed and performed the research; Chen M provided analytical tools and was also involved in editing the manuscript; Shi LL analyzed the data, as well as writing the paper.

Correspondence to: Xiao-Ping Zou, MD, Professor, Department of Gastroenterology, Medical School, the Affiliated Drum Tower Hospital of Nanjing University, Nanjing 210008, Jiangsu Province, China. 13770771661@163.com

Telephone: +86-25-83105206 Fax: +86-25-83105206

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Abstract

AIM: To observe the changes in interstitial cells of Cajal (ICC) in rats with experimental severe acute pancreatitis (SAP).

METHODS: A total of twenty-four SD rats were randomly divided into two groups ($n = 12$), namely the sham (S) group and the SAP group; the SAP rat model was established by retrograde injection of 5% sodium taurocholate (1.0 mL/kg) into the pancreatic duct. Twenty-four hours later intestinal motility was assessed by testing small intestinal propulsion rate, and then the rats were sacrificed. The pancreas and jejunum were resected and underwent routine pathologic examination. Immunohistochemical staining was used to detect c-kit-positive cells in the jejunum. Expression of c-kit mRNA was detected by real-time polymerase chain reaction, and the expression of c-kit protein was evaluated by Western blotting. Ultrastructure of ICC was evaluated by transmission electron microscopy.

RESULTS: There was bleeding, necrosis and a large

amount of inflammatory cell infiltration in pancreatic tissue in the SAP group, while in jejunal tissue we observed a markedly denuded mucosal layer, loss of villous tissue and a slightly dilated muscular layer. The small intestinal propulsion rate was $68.66\% \pm 2.66\%$ in the S group and $41.55\% \pm 3.85\%$ in the SAP group. Compared with the S group, the rate of the SAP group decreased sharply. The density of c-kit-positive cells in the SAP group was significantly lower than in the S group; the respective mean densities were 88.47 ± 10.49 in the S group and 56.11 ± 7.09 in the SAP group. The levels of c-kit protein and mRNA were 0.36 ± 0.04 and 1.29 ± 0.91 in the SAP group, respectively, which were significantly lower than those in the S group (0.53 ± 0.06 , 0.64 ± 0.33 , respectively). In the SAP group, ICC profiles showed the same change tendency, such as vacuolation of mitochondria, irregular vacuoles and loosened desmosome-like junctions.

CONCLUSION: Decreased c-kit-positive cells and ultrastructural changes in ICC resulting from blockade of the c-kit signaling pathway are involved in the intestinal dysmotility associated with SAP.

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Key words: Severe acute pancreatitis; c-kit; Interstitial cells of Cajal; Real-time polymerase chain reaction; Ultrastructure

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INTRODUCTION

In 1893, the Spanish neuro-histologist, Cajal, discovered

interstitial cells of Cajal (ICC) within the gastrointestinal wall. Since then, especially in the most recent two decades, a number of studies have established the roles of ICC in normal functions of the gastrointestinal wall primarily in 4 major groups: ICC in the submuscular plexus; ICC within the circular and longitudinal layers of muscle; ICC in the myenteric plexus (ICC-MY, also called ICC-MP); ICC in the deep muscular plexus. These cells function as pacemaker cells in the gastrointestinal wall to generate slow waves that spread from ICC to smooth muscle cells for triggering calcium entry, as a result of depolarization, and contraction as a basis for peristalsis and segmentation. They maintain normal neurotransmission and regulate mechanical activities in the gastrointestinal tract^[1-5]. More recently, the discovery of c-kit along with its endogenous ligand, stem cell factor (SCF), have dramatically advanced ICC investigations in this field^[6,7]. Presentation of SCF increases expression of c-kit immunoreactive ICC in culture while loss-of-function mutations of the c-kit gene cause deficiency of ICC; these have shown that the SCF/c-kit signal pathway is essential for the maintenance of ICC^[8-11]. Imatinib, a novel and potent inhibitor of c-kit, abolished the spontaneous movements in circular muscles of the mouse small intestine^[12], and this result suggests that the c-kit signaling of ICC plays an essential role in the spontaneous mechanical activity of intestine. Disorders of ICC may result in gastrointestinal motility dysfunctions, which lead to a number of gastrointestinal diseases, including severe acute pancreatitis (SAP). Furthermore, investigators have found that damage of ICC occurred in the muscular layer of the small intestine in experimental acute pancreatitis^[13].

Despite the association of SAP with gastrointestinal motility disturbances on the basis of evidence acquired through both observational clinical^[14] and experimental investigations^[15,16], the detailed mechanisms of the changes in gastrointestinal motility in SAP have not been clearly elucidated. Thus, we hypothesized that ICC might play an important role in the pathogenesis of gastrointestinal dysmotility in SAP. In the present study we tested our hypothesis in a rat model of SAP.

MATERIALS AND METHODS

Animal model establishment

Twenty-four adult male Sprague-Dawley (SD) rats with body weight between 200 g and 250 g were purchased from the animal research center of the affiliated Drum Tower Hospital of Nanjing University Medical School and randomly divided into two groups of equal number ($n = 12$ each): the sham (S) group and the severe acute pancreatitis (SAP) group. To establish the SAP rat model, freshly prepared 5% sodium taurocholate solution was injected at a volume of 1.0 mL/kg from the duodenal papilla into the pancreatic duct. In the S group, the duodenum and pancreas of animals were manually manipulated a few times after laparotomy. All procedures took place under sterile conditions and all animals were housed un-

der pathogen-free conditions in the animal facility with a 12-h light/dark cycle and free access to food and water. The study protocol was approved by the Medical Ethics Committee of the Hospital.

Assessment of small intestinal propulsion rate

Twenty-three hours after the operative procedure for the establishment of the SAP animal model, gastric gavage with 1 mL of methylene blue solution was performed in both groups. One hour later, the rats were euthanized *via* CO₂ asphyxiation and the small intestine in each rat was removed from the abdominal cavity. All the mesentery tissues were stripped and the total length of the small intestine from the pyloric sphincter of the distal stomach to the distal end of the ileum was measured. The movement of the methylene blue solution in the small intestine was observed and recorded. The small intestinal propulsion rate was calculated as the product of the distance of the methylene blue traveled within 30 min immediate after the removal of the small intestine divided by the total length of the small intestine.

Histopathologic examination of the pancreas and the jejunum

Both the pancreas and the jejunum were removed at the time of harvest of the small intestine described above. Four segments of the jejunum 15 cm distal to the ligation of Truiz, approximately 10 mm each in length, were collected for the following study. One segment of the jejunum was opened, cleaned, and inspected macroscopically along with the pancreas that was transversely sectioned, for visible pathologic changes. After gross examination, both organs were fixed with 10% buffered neutral formalin solution for 24 h. The tissue from both organs was sectioned at 4 μ m in thickness. Histology sections were stained with hematoxylin and eosin and evaluated microscopically by experienced pathologists.

Real-time polymerase chain reaction

In the jejunal tissue freshly harvested previously, total RNA was isolated from the jejunum segments with mucosa stripped using TRIzol[®] reagent following the manufacturer's instructions. The reverse transcription (RT) was performed in a 20 μ L reaction mixture containing 1 μ g total RNA by using a PrimeScript RT Reagent Kit (Perfect Real Time, Takara Bio Inc., Otsu, Japan) according to the manufacturer's protocol. The RT reaction product was amplified by using the SYBR Premix Ex Taq (Takara Bio Inc., Otsu, Japan) and ABI PRISM 7500 Real-time PCR system according to the manufacturer's protocol. Primers of c-kit were as follows: 5'-TGGATCAGCAAATGTCA-CAACAAC-3' (forward) and 5'-TAGGCCTCGAACT-CAACAACCA-3' (reverse). The predicted size of the c-kit-PCR product was 132 bp. The primers of β -actin were: 5'-TCGTGCGTGACATTAAAGAG-3' (forward) and 5'-ATTGCCGATAGTGATGACCT-3' (reverse). The predicted size of the β actin-PCR product was 134 bp. Mean fold changes for each sample were calculated

by using the $2^{-\Delta\Delta Ct}$ method as previously described^[17].

Immunohistochemical staining

The segments of jejunum harvested previously were immersed in a fixative containing 4% paraformaldehyde for 6 h at 4 °C. Then the segments were embedded with the optimum cutting temperature compound and sectioned at 10 μm in thickness. The tissue section was mounted on glass slides. For the c-kit staining, tissue sections were incubated with 0.3% Triton X in 10% normal rabbit serum for 60 min and then incubated with the goat anti-c-kit polyclonal antibody (clone sc-1494; Santa Cruz Biotechnology, Inc., Santa Cruz, CA, United States) at 4 °C overnight. Next, we applied a biotin-free polymeric horseradish peroxidase (HRP)-linked antibody conjugate system for 20 min followed by DAB condensed chromogen for 5 min. Tissue sections were counterstained with hematoxylin and eosin (HE). For negative control experiments, the primary antibody was omitted. Images of c-kit-positive cells were taken in 4 randomly chosen fields (× 200 magnification) per tissue section. The positive cell density was assessed with the Image-Pro Plus 6.0 software (Media Cybernetics, Bethesda, MD, United States).

Western blotting

A segment of the jejunum was cut along the mesenteric axis and stripped of the mucosa. The remaining jejunal tissue was immediately snap-frozen in liquid nitrogen and stored at -80 °C. After homogenization in extraction buffer (50 mmol/L Tris-Cl (pH 7.5), 150 mmol/L NaCl, 1% Triton X-100, and 1 mmol/L PMSF), the lysate was collected and centrifuged at 4 °C for 15 min at 10 000 r/min to remove the insoluble material. The protein concentration of the supernatant was measured by spectrophotometry using the BCA protein assay method (Pierce, Rockford, IL, United States). The samples were electrophoresed on a 10% SDS-polyacrylamide gel, and transferred to a PVDF transfer membrane (Millipore, Bedford, MA, United States). The membrane was then incubated with 5% skimmed cow's milk overnight at 4 °C to block nonspecific binding sites and then incubated with the primary c-kit antibody (clone sc-1494; Santa Cruz Biotechnology, Inc., Santa Cruz, CA, United States) applied for 1 h at room temperature. After washing, the secondary matching peroxidase-conjugated antibody was applied to the membrane and incubated for 1 h at room temperature. Specific protein bands were visualized with an X-ray film using the chemiluminescence detection kit (ECL Western blotting detection; Millipore Corp). Optical density of the bands was analyzed with software Quantity One.

Electron microscopy

Immediately after resection, blocks of jejunal tissue were cut and immersed into a fixative containing 5% glutaraldehyde and stored at 20 °C for at least 2 h. Following fixation, tissues were cut into small pieces (1 mm × 2 mm) and further fixed in 5% glutaraldehyde overnight,

and then rinsed for 60 min in 0.1 mol/L phosphate buffer, pH 7.3, and postfixed in 2% OsO₄ in 0.1 mol/L phosphate buffer for 2 h. The tissue specimens were subsequently dehydrated and embedded. Thin sections were cut at 1 μm in thickness and stained with toluidine blue for light microscopy to select suitable areas for ultrathin sectioning. Ultrathin sections were cut at 70-80 nm, mounted onto copper grids, and stained with lead citrate for electron microscopy with a Philips Morgagni 261 EM microscope.

Statistical analysis

The data obtained were expressed as mean ± SD. Comparison between the two groups was performed by using the Student *t*-test, and the differences with *P* < 0.05 were considered as statistically significant. All data were analyzed with SPSS 13.0 software (SPSS Inc., Chicago, IL, United States).

RESULTS

Small intestinal propulsion rate

The small intestinal propulsion rate was significantly lower in the SAP group than in the S group. The respective rate was 68.66% ± 2.66% in the S group and 41.55% ± 3.85% in the SAP group.

Pathological changes

Under gross examination, the pancreas and jejunum in the SAP group appeared edematous at 24 h. The jejunum was full of yellow intestinal juice and ascites, and adhesions of organs were observed in 2 rats of the SAP group. Under light microscope examination, the pancreas from the S group exhibited no signs of pancreatitis (Figure 1A). Histological evaluation of the pancreas in rats with SAP revealed widespread acinar cell necrosis accompanied by edema, visible hemorrhage and inflammatory cell infiltrate (Figure 1B). In the S group, the structure of jejunum was normal (Figure 1C). In the SAP group, the mucosa was markedly denuded and partial loss of villous tissue with crypt layer infarction was also seen. Muscular layers showed slight alteration characterized by dilated thickness (Figure 1D).

Immunohistochemical staining

c-kit-positive cells could be divided into two cell populations. One population was mast cells situated within the mucosa layer (Figure 2A). The second population was ICC with large oval nuclei, sparse cytoplasm and branching processes; these cells were situated in the submuscular plexus (Figure 2B) and the intermuscular septa (Figure 2C). The density of c-kit-positive cells in the SAP group was significantly lower than in the S group; the respective mean densities were 88.47 ± 10.49 in the S group and 56.11 ± 7.09 in the SAP group.

Western blotting

Western blotting analysis using an antibody to c-kit on

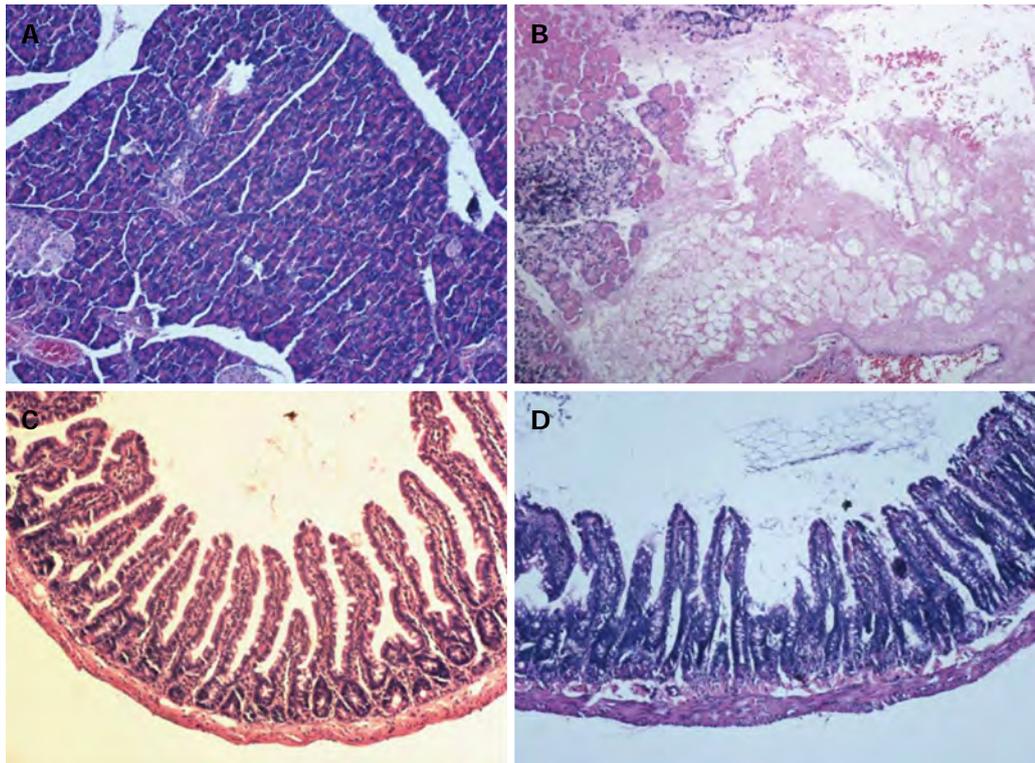


Figure 1 Histological sections from pancreas and jejunum. A: The pancreas of the sham (S) group shows a normal exocrine and endocrine pancreatic architecture; B: The pancreas of severe acute pancreatitis (SAP) rats shows necrosis of the acinar cells accompanied by edema and hemorrhage; C: The structure of jejunum in the S group is normal; D: The mucosa was markedly denuded and the muscular layer was edematous in the SAP group. Magnification $\times 200$.

tissue from the jejunum detected a protein band at approximately 145 kDa that corresponded to the molecular weight of c-kit protein (Figure 3A). The c-kit band density was clearly observed in the S group rats, but significantly reduced in comparison to the SAP group (relative protein expression: the S group, 0.53 ± 0.06 ; the SAP group, 0.36 ± 0.04 , $P < 0.05$; Figure 3B). Consistent with immunohistochemical staining, lower levels of c-kit protein were demonstrated in the SAP group.

c-kit mRNA expression

Decreased expression of c-kit mRNA was demonstrated compared with the S group (Figure 4).

Ultrastructure of ICC

As previously described, ICC in control tissue are present in triangular or fusiform shapes. The nucleus of ICC is very voluminous surrounded by a small perinuclear cytoplasm that expands with long prolongations which are called cytoplasmic processes. The cytoplasm of these cells presents a higher electron density than the cytoplasm of the surrounding muscle cells. ICC contain mitochondria, rough and smooth endoplasmic reticulum, thin and intermediate filaments, caveolae, Golgi apparatus, free ribosomes and cytoplasmic vesicles. They are closely associated with smooth muscle and often network with other ICC. Some of them are intercalated between nerves and smooth muscle cells (Figure 5A-C).

In contrast with control tissues, confluent vacuoles were frequently present in ICC in tissue from the SAP

group (Figure 5D). Mitochondria appeared damaged in some vacuolated processes (Figure 5E). Ultrastructural preservation of other cellular elements and organelles was mostly unaffected. Damage of the desmosome-like junctions between ICC and smooth muscle was also seen (Figure 5F).

DISCUSSION

SAP is a very common clinical disease and its mortality rate ranges from 10% in the case of sterile necrosis to 25% in the case of infected pancreatic necrosis^[18,19]. Many studies have indicated that gastrointestinal dysmotility in rats with SAP could lead to the translocation of bacteria from the gut, thus resulting in pancreatic infections which have been suggested to be a major cause of death in SAP^[20,21]. So it is very important to investigate the possible mechanisms of gastrointestinal dysmotility in SAP in order to reduce the mortality rate of SAP.

We used retrograde injection of 5% sodium taurocholate from the duodenal papilla to establish an SAP rat model. Pancreatic pathological changes, such as pancreatic hemorrhage, necrosis and infiltration of inflammatory cells, could be observed at 24 h after modeling. All these changes were consistent with patients with SAP. This demonstrated that the animal model of SAP was successfully established. In addition, a significantly decreased small intestine propulsion rate in rats with SAP was observed. Our results confirmed that experimental SAP induced intestinal motility disturbances as previously shown^[22].

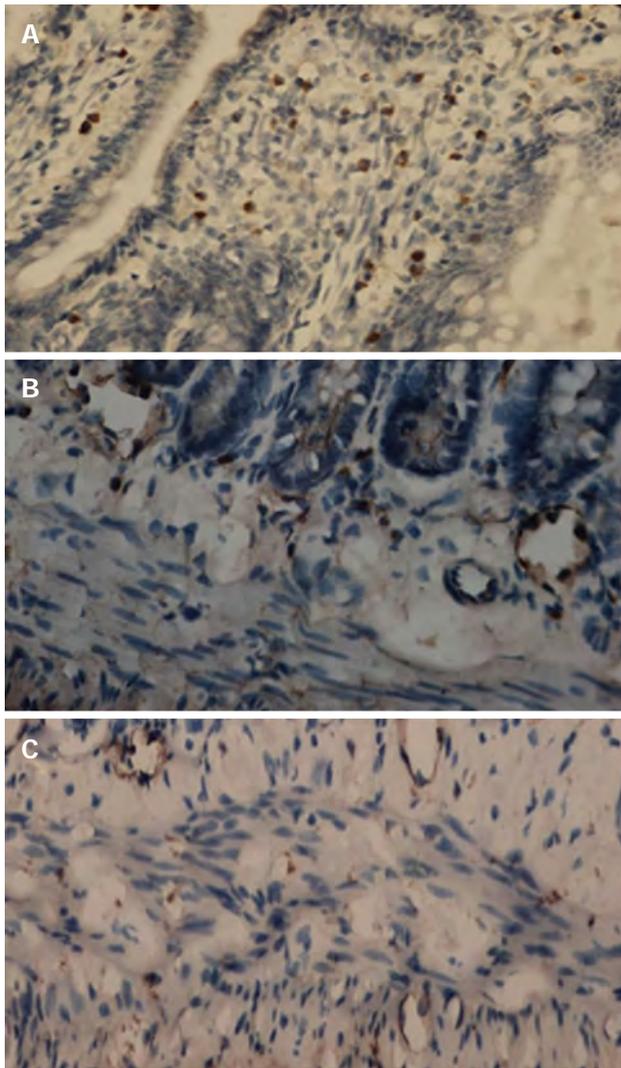


Figure 2 Immunohistochemistry for c-kit. A: Mucosal mast cells in rats retain c-kit positivity (internal control); B: c-kit-positive interstitial cells of Cajal (ICC) in the submuscular plexus in the sham group; C: c-kit-positive ICC in the intermuscular septa in the severe acute pancreatitis group. Magnification $\times 200$.

So far, the pathogenic mechanisms of pancreatitis-induced intestinal motility disturbances are largely unknown. It is well documented that ICC are implicated in the control of gastrointestinal motility. For example, decreased numbers or disrupted networks of ICC are associated with a number of human gastrointestinal motility disorders, including slow transit constipation^[23,24], pseudo-obstruction^[25-27] and diabetic enteropathy^[28]. The potential role of ICC in the pathogenesis of gastrointestinal dysmotility in SAP has attracted attention. ICC can be classified into several subtypes according to their location in the gut wall; ICC at the level of the MY generates slow waves, and studies have confirmed that damage in the network of ICC-MY resulted in change of spontaneous mechanical contractions of the gut in a variety of human disease processes^[29-31]. All these studies were focused on ICC-MY and spontaneous mechanical contractions. In addition to generating slow waves, other subsets of ICC are engaged in mediating enteric neural signals to the

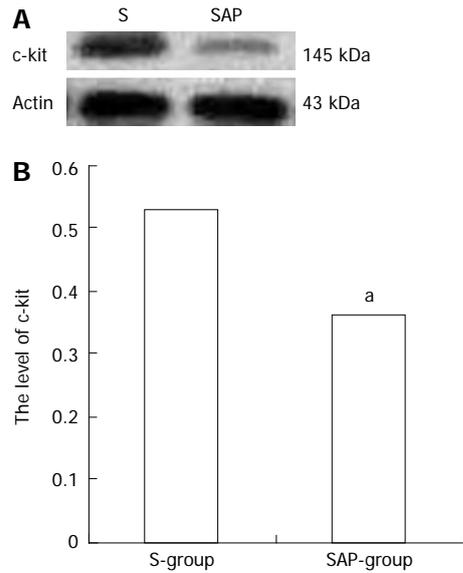


Figure 3 Expression of c-kit protein. A: Bands of Western blotting of c-kit (145 kDa). β -actin is a loading control; B: Statistical analysis of relative density of Western blotting between two groups. Data are represented as mean \pm SD. ^a $P < 0.05$ vs sham (S) group.

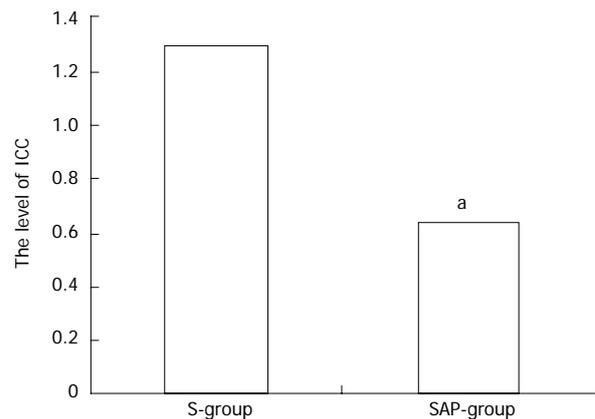


Figure 4 Mean optical density of c-kit mRNA. Each bar represents the mean \pm SD (vertical line). ^a $P < 0.05$ vs sham (S) group. ICC: Interstitial cells of Cajal.

smooth muscles and acting as mechanosensors. In the present study, total ICC were observed by immunohistochemical staining. Around the submuscular plexus and in the intermuscular septa, we have demonstrated a decrease of c-kit-positive cells in these regions in the SAP group. Consistent with immunohistochemical staining, lower levels of c-kit protein were demonstrated in the SAP group.

Investigators have examined the ultrastructure of ICC by transmission electron microscopy in their intestinal obstruction model^[30] and surgical resection model^[31]. These findings all suggested that an actual change in ICC phenotype occurred from the ultrastructural appearance. Moreover, functionally mature ICC redifferentiated toward a smooth muscle cell phenotype when kit receptors were blocked^[32]. Similarly, in our study, morphological changes such as vacuolation of mitochondria, irregular vacuoles and loosened desmosome-like junctions were

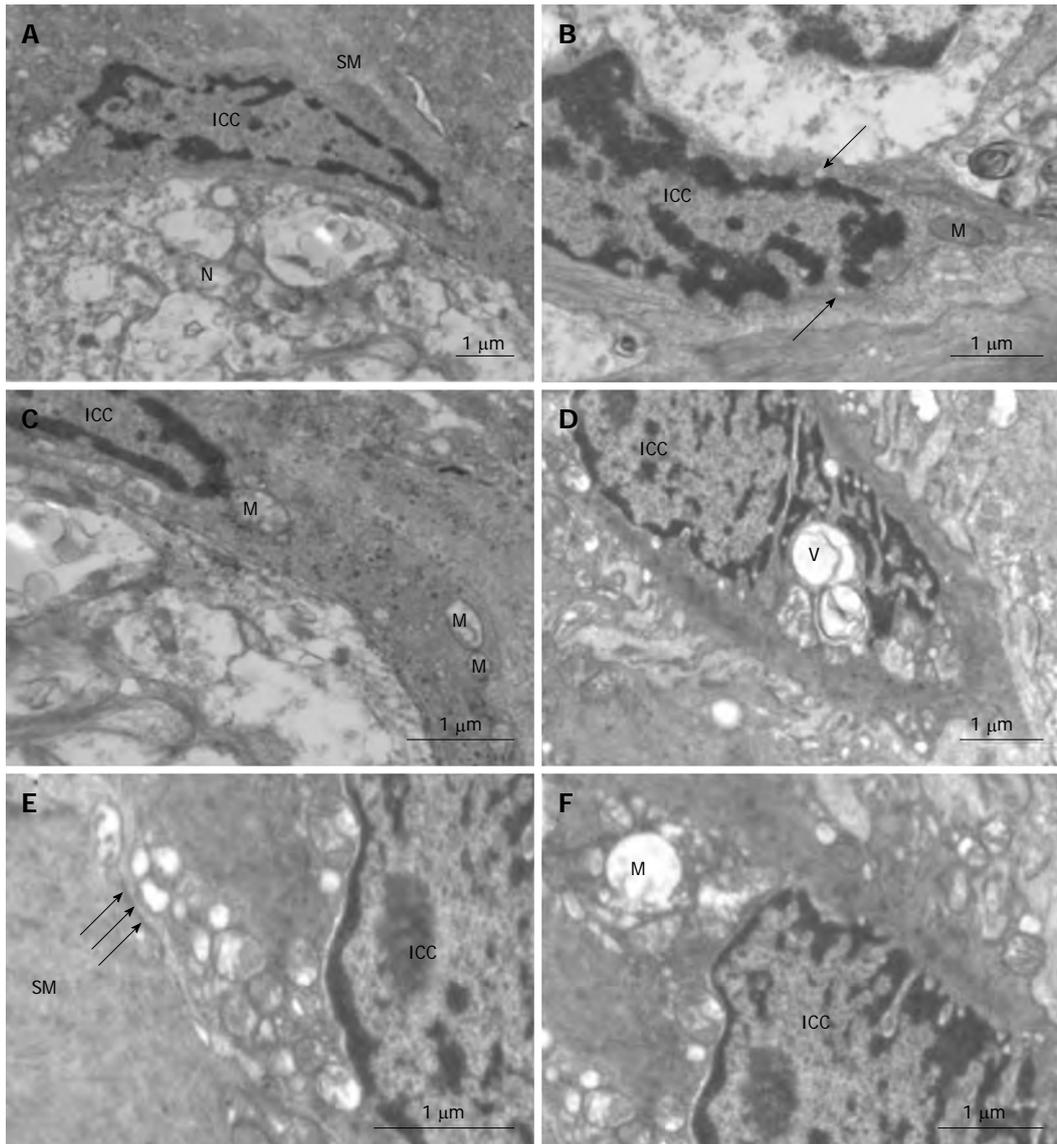


Figure 5 Ultrastructure of interstitial cells of Cajal. A-C: Control. A: Interstitial cells of Cajal (ICC) with fusiform nuclear morphology show an elongated nucleus with scarce perinuclear cytoplasm, and are situated between the smooth muscle (SM) and the enteric nerve (N). The cytoplasmic processes surround the external contours of the enteric nerve; B: Caveolae are lining cytoplasmic membrane (arrows); C: The processes of some ICC have characteristically numerous mitochondria (M); D-F: Severe acute pancreatitis. D: Vacuoles (V) are present in the ICC; E: The density of desmosome-like junction between ICC and smooth muscle is lower (arrows); F: Vacuolated mitochondria are present in the processes of ICC.

present in ICC in the SAP group, while the ultrastructure of ICC is normal in the S group. However, there is not sufficient evidence to support the theory that ICC transdifferentiate towards a smooth muscle cell phenotype. Although we did not investigate the amplitudes and frequencies of slow waves of the jejunum generated by ICC, it could be speculated that loss of ICC and changes of the ultrastructure influenced the function of ICC and eventually resulted in gastrointestinal dysmotility. The precise cellular changes that occur in response to the blockade of the c-kit signaling pathway are an extremely interesting direction for future investigation.

The present study further demonstrated that the expression of c-kit mRNA was significantly down-regulated in the SAP group. These data are consistent with previous reports that c-kit is down-regulated in the sigmoid colon

of patients with slow transit constipation^[33] and in the gallbladders of guinea pigs on a high cholesterol diet^[34]. In the gastrointestinal tract, development and maintenance of the ICC phenotype have been linked to intracellular signaling *via* c-kit. Beckett *et al*^[35] have shown that blocking c-kit signaling during late gestation results in failure of ICC networks and pacemaker function to develop in the murine small intestine. Other investigators have shown that blockade of c-kit signaling caused redifferentiation of functionally mature ICC toward a smooth muscle cell phenotype^[32]. In the present study, we provide additional evidence that the c-kit signaling pathway may be responsible for development and maintenance of the ICC. However, further studies are needed to demonstrate whether and when these changes could be restored to normal.

In conclusion, this study has disclosed that decreased

c-kit-positive cells and degenerative ultrastructural changes of ICC were present in the jejunum of rats with SAP, and that all these changes resulted from blockade of the c-kit signaling pathway. This study may provide new insights into pathological mechanisms of gastrointestinal motility disturbances in SAP. Since loss and proliferation of c-kit-positive cells lead to a variety of human gastrointestinal motility disorders^[36-38] and gastrointestinal stromal tumors^[39,40], thus developing the means to manipulate the ICC phenotype may have profound therapeutic benefits for these patients.

COMMENTS

Background

The incidence of intestinal dysmotility increases the mortality of patients with severe acute pancreatitis (SAP), but until now, the mechanism of this dysmotility is largely unknown. Many studies have reported that interstitial cells of Cajal (ICC), which are known as pacemaker cells, are associated with gastrointestinal dysmotility diseases.

Research frontiers

Loss and proliferation of ICC lead to a variety of human gastrointestinal motility disorders and gastrointestinal stromal tumors. However, the detailed changes of ICC in SAP are not clearly elucidated. In this study, the authors demonstrate that the loss and ultrastructural changes of ICC could be a potential mechanism for intestinal dysmotility in SAP.

Innovations and breakthroughs

Recent reports have highlighted the importance of ICC in gastrointestinal motility disorders and gastrointestinal stromal tumors. In gastrointestinal motility disorders, loss of ICC was present. This is the first study to report that loss of ICC was also present in SAP. Furthermore, the studies would suggest that the loss of ICC may result from blockade of the c-kit signaling pathway.

Applications

This study provided new insights into pathological mechanisms of gastrointestinal motility disturbances in SAP. Developing the means to manipulate the ICC may have profound therapeutic benefits.

Terminology

ICC were firstly described by the Spanish neuro-histologist Cajal. ICC are involved in processes such as generation of slow waves, neurotransmission and regulation of mechanical activities; all these processes are thought to be crucial in intestinal motility. SAP is a special type of acute pancreatitis accounting for 10% to 20% of all acute pancreatitis episodes; it is a dangerous condition with more complications and higher mortality.

Peer review

The authors examined the expression of c-kit and ultrastructural changes of ICC in jejunum in rats with experimental severe acute pancreatitis. The study revealed that decreased c-kit positive cells and degenerative ultrastructural changes of ICC were present; these changes were correlated to blockade of c-kit signaling pathway. The results are interesting and may provide new insights into pathological mechanisms of gastrointestinal dysmotility in SAP.

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Comparative evaluation of intragastric bile acids and hepatobiliary scintigraphy in the diagnosis of duodenogastric reflux

Teng-Fei Chen, Praveen K Yadav, Rui-Jin Wu, Wei-Hua Yu, Chang-Qin Liu, Hui Lin, Zhan-Ju Liu

Teng-Fei Chen, Praveen K Yadav, Rui-Jin Wu, Wei-Hua Yu, Chang-Qin Liu, Hui Lin, Zhan-Ju Liu, Department of Gastroenterology, Shanghai Tenth People's Hospital, Tongji University, Shanghai 200072, China

Author contributions: Chen TF and Yadav PK contributed equally to this work; Chen TF and Yadav PK collected and analysed data and drafted manuscript; Wu RJ and Lin H contributed to revision of manuscript; Yu WH and Liu CQ contributed to the collection of the data; Liu ZJ contributed by developing study concept and design, interpretation of data, and critical revision of manuscript.

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Correspondence to: Dr. Zhan-Ju Liu, Professor, Department of Gastroenterology, Shanghai Tenth People's Hospital, Tongji University, Shanghai 200072, China. zhanjuli@yaho.com
Telephone: +86-21-66301164 Fax: +86-21-66303893

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Abstract

AIM: To assess the diagnostic value of a combination of intragastric bile acids and hepatobiliary scintigraphy in the detection of duodenogastric reflux (DGR).

METHODS: The study contained 99 patients with DGR and 70 healthy volunteers who made up the control group. The diagnosis was based on the combination of several objective arguments: a long history of gastric symptoms (*i.e.*, nausea, epigastric pain, and/or bilious vomiting) poorly responsive to medical treatment, gastroesophageal reflux symptoms unresponsive to proton-pump inhibitors, gastritis on upper gastrointestinal (GI) endoscopy and/or at histology, presence of a bilious gastric lake at > 1 upper GI endoscopy, pathologic 24-h intragastric bile monitoring with the Bilitec device. Gas-

tric juice was aspirated in the GI endoscopy and total bile acid (TBA), total bilirubin (TBIL) and direct bilirubin (DBIL) were tested in the clinical laboratory. Continuous data of gastric juice were compared between each group using the independent-samples Mann-Whitney *U*-test and their relationship was analysed by Spearman's rank correlation test and Fisher's linear discriminant analysis. Histopathology of DGR patients and 23 patients with chronic atrophic gastritis was compared by clinical pathologists. Using the Independent-samples Mann-Whitney *U*-test, DGR index (DGRi) was calculated in 28 patients of DGR group and 19 persons of control group who were subjected to hepatobiliary scintigraphy. Receiver operating characteristic curve was made to determine the sensitivity and specificity of these two methods in the diagnosis of DGR.

RESULTS: The group of patients with DGR showed a statistically higher prevalence of epigastric pain in comparison with control group. There was no significant difference between the histology of gastric mucosa with atrophic gastritis and duodenogastric reflux. The bile acid levels of DGR patients were significantly higher than the control values (Z : TBA: -8.916, DBIL: -3.914, TBIL: -6.197, all $P < 0.001$). Two of three in the DGR group have a significantly associated with each other (r : TBA/DBIL: 0.362, TBA/TBIL: 0.470, DBIL/TBIL: 0.737, all $P < 0.001$). The Fisher's discriminant function is followed: Con: $Y = 0.002TBA + 0.048DBIL + 0.032TBIL - 0.986$; Reflux: $Y = 0.012TBA + 0.076DBIL + 0.089TBIL - 2.614$. Eighty-four point zero five percent of original grouped cases were correctly classified by this method. With respect to the DGR group, DGRi were higher than those in the control group with statistically significant differences ($Z = -5.224$, $P < 0.001$). Twenty eight patients (59.6%) were deemed to be duodenogastric reflux positive by endoscopy, as compared to 37 patients (78.7%) by hepatobiliary scintigraphy.

CONCLUSION: The integrated use of intragastric bile acid examination and scintigraphy can greatly improve the sensitivity and specificity of the diagnosis of DGR.

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Key words: Duodenogastric reflux; Diagnosis; Intragastric bile acids; Hepatobiliary scintigraphy

Core tip: The study results suggest that total bile acid is the most important factor of the bile acids to determine duodenogastric reflux (DGR) by using a variety of statistical methods. Using the receiver operator curve, we found the hepatobiliary scintigraphy is better than the examination of gastric juice in the diagnosis of DGR. From this study, the biggest revelation is that we can research other medical problems particularly using many statistical methods.

Chen TF, Yadav PK, Wu RJ, Yu WH, Liu CQ, Lin H, Liu ZJ. Comparative evaluation of intragastric bile acids and hepatobiliary scintigraphy in the diagnosis of duodenogastric reflux. *World J Gastroenterol* 2013; 19(14): 2187-2196 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v19/i14/2187.htm> DOI: <http://dx.doi.org/10.3748/wjg.v19.i14.2187>

INTRODUCTION

Duodenogastric reflux (DGR) is a natural physiological phenomenon which is commonly defined as the transport of duodenal contents from the duodenum to the stomach^[1]. Chernov *et al*^[2] concluded that DGR was involved in the formation of the internal gastric environment, which played a significant role in gastric digestion and that its regulation was affected by the coordinated motor and evacuated performance of the gastroduodenal junction and duodenum. Duodenal fluid causes an increase in inflammatory cells in the gastric mucosa, decrease in parietal cells, hyperplasia of mucous cells and changes in glandular morphology. Patients with DGR will feel heartburn, nocturnal cough and chest pain, nausea, epigastric pain, gassy or bloating feelings, vomiting and so on. DGR has been implicated in the pathogenesis of a variety of upper gastrointestinal disorders including esophagitis, gastritis, duodenal and gastric ulcers^[3].

With the increasing number of research in this field, reliable, repeatable and simple methods of assessment of DGR are required, especially in the early stage. Earlier used techniques included radiology, endoscopy and intubation methods such as nasogastric aspiration of bile marker or the measurement of bile acids in fasting gastric aspirates. At present, several methods are available to detect duodenogastric reflux in general hospital. For example, intubation of the upper gastrointestinal tract is the essential method to assess the extent and severity of tissue damage of duodenogastric reflux disease in our daily work. This intubation should be gentle because it

may cause disturbances in gastric and duodenal motility. The conventional and most widely accepted method of diagnosing DGR is the measurement of intragastric bile acid in the gastric juice aspirated through nasogastric tube and hepatobiliary scintigraphy. In the last few years, scintigraphic radiological techniques, such as imaging with hepatobiliary scintigraphy, has become available to study dynamic duodenogastric reflux^[4-6], but they also have limitations^[7,8].

The aim of this study was to represent the visualization of endoscopy, to measure intragastric bile acids aspirated at endoscopy and to compare them with DGR index (DGRi) assessed by hepatobiliary scintigraphy to assess the sensitivity and specificity of these two techniques in the diagnosis of DGR.

MATERIALS AND METHODS

Patients and methods

A total of 99 patients (41 male and 58 female) with DGR were undergoing esophagogastroduodenoscopy (EGD) from September 2011 to March 2012 at Shanghai Tenth People's Hospital, Tongji University. The diagnosis of DGR was based on the combination of the following arguments: a long history of gastric symptoms poorly responsive to prokinetics, mucosa-protective medicines, H₂-blockers and/or proton-pump inhibitors (PPI), gastroesophageal reflux symptoms unresponsive to PPI, gastritis on upper GI endoscopy, and/or at histology, presence of a large amount of bile in the gastric cavity at > 1 endoscopic examination, pathologic at 24-h intragastric bile monitoring with the Bilitec device. The gastric juice was often lucidity or light yellow-green and/or associated mucosal change in these patients' endoscopic images. Before investigation, all patients were interviewed by the senior author for the presence of both upper abdominal symptoms (heartburn, regurgitation, nocturnal cough and chest pain) and dyspeptic symptoms (nausea, epigastric pain, gassy or bloating feelings, vomiting). None of the patients had diabetes mellitus, neurological disorders, vascular diseases, collagen diseases, neoplastic diseases or inflammatory bowel disease. Acute cases and patients who had previously undergone gastrectomy or esophagotomy were excluded.

As a control group, 70 consecutive patients (35 male and 35 female) who needed EGD for an annual medical check-up were enrolled. None had undergone earlier esophageal, gastric or biliary surgery; and none had earlier gastrointestinal diseases or was on medication which would influence gastric acidity or motility. After this, all patients underwent upper gastrointestinal endoscopy and found gastric juice was normal and the gastric mucosa was not damaged obviously under the macroscopic observation.

The protocol of this study was approved by the ethics committee of the Shanghai Tenth People's Hospital. Written informed consent was obtained from all participants.

Endoscopic study

Endoscopic examination was performed to find the evidence of DGR in all patients using fiber optic gastro-duodenoscopy (The GIF-H260 and Q260 endoscopes, Olympus Medical Systems Co., Tokyo, Japan). To ensure the most accurate results possibly, every patient was not taken any food or drink for 8-10 h before examination to allow a valid examination of the upper gastric intestinal (GI) tract and to lower the risk of vomiting.

The doctor explained the test to everyone, including the possibility of biopsy and risks such as the need to remove polyps or other surgical procedures and asked to sign a consent form agreeing to the procedure. At the same time, all the participants informed the endoscopy team about any medications he/she was taking and any allergy or bad reactions in previous tests. People who have had cardiac valve replacement or blood vessel graft suggested to continue medications to prevent infection. All dentures and eyeglasses prior to begin an upper endoscopy were removed. Each of the subjects was given a topical anesthetic before the test to numb his/her throat to prevent gagging. The patient was placed on his/her left side and had a plastic mouthpiece placed between his teeth to keep his mouth opening that makes easier to pass the tube. The doctor lubricated the endoscope, passed it through the mouthpiece, and then asked him to swallow it. The doctor guided the endoscope under direct visualization through his esophagus to the first part of small intestine (duodenum). Any saliva was cleared using a small suction tube that was removed quickly and easily after the test.

The doctor inspected portions of the linings of everyone's esophagus, stomach, and the first part of small intestine and then re-inspects them as the instrument is withdrawn. To determine the presence and severity of DGR, biopsies of gastric inflammation was necessary to be performed in the antrum of the stomach. All endoscopic examinations were done by well-trained endoscopists, and three expert endoscopists examined the endoscopy photographs to determine whether the attending endoscopists had diagnosed accurately. The endoscopic diagnosis was established by consensus of two or three expert endoscopists and the attending endoscopist.

Histopathology

Biopsy samples, no less than four sequential sections, were taken from the inflammatory mucosa for each enrolled patient. Mucosal erythema, erosion or ulcerations of the gastric wall were usually considered signs of gastric inflammation. Biopsy specimens were immediately placed in a 10% buffered formalin solution, routinely processed, and embedded in paraffin in the department of Pathology. Two sections were stained with hematoxylin and eosin (HE). At the same time, 23 patients with chronic atrophic gastritis were reviewed for comparison. The estimation of inflammatory was made only when the biopsy specimen consisted of intestinal columnar epithelial cells with goblet cells. All biopsy examinations were

done by well-trained clinical pathologists and the pathological diagnosis was established by consensus of two or three expert pathologists.

Determination of bile acids in gastric juice

For all patients, resting gastric juice was aspirated through a sterile wash tube inserted down the biopsy channel of the gastroscope. The gastric aspirate was stored at -20°C until batch analysis. The concentration of free and total bile acid was made by the steroid dehydrogenase method (Modular P800, Hoffmann-La Roche Ltd, Basel, Switzerland), performed in duplicate with a mean coefficient of variation of 5% for each patient. The mean overall percentage recovery was 89 percent and the variance was less than 10 percent in duplicate analyses. In the present study, three bile acids were analyzed in accordance with the clinical processes: total bile acid (TBA), total bilirubin (TBIL) and direct bilirubin (DBIL).

Duodenogastric reflux imaging

Twenty eight patients of DGR group and 19 persons of control group were subjected to hepatobiliary scintigraphy for the diagnosis of DGR. $^{99\text{m}}\text{Tc}$ -ethyl hepatic iminodiacetic acid (EHIDA) imaging was performed using single-photon emission-computed tomography (SPECT)/CT (PHILIPS Precedence 16 SPECT/CT, Koninklijke Philips Electronics NV, The Netherlands) in accordance with our institution's standard protocol. Stress and rest images were acquired 1 h after injecting 111-185 MBq (3-5mCi) of technetium $^{99\text{m}}\text{Tc}$ ethyl hepatic iminodiacetic acid, [$^{99\text{m}}\text{Tc}(\text{CO})_3(\text{EHIDA})$].

Patients were in fasting, non-smoking for 4-12 h and oral potassium perchlorate 400 mg was taken to close the thyroid function before examination. DGR was studied scintigraphically using a modified and extended version of the conventional hepatobiliary scintigraphy. The study was conducted with the patient in the supine position and the gamma camera detector placed above the patients' abdomen. About 111-185 MBq [$^{99\text{m}}\text{Tc}(\text{CO})_3(\text{EHIDA})$] ($^{99\text{m}}\text{Tc}$ -EHIDA) was injected intravenously. Gallbladder contraction was then stimulated by a fatty meal and/or intravenous cholecystokinin (1-5 units/kg). SPECT was performed by acquiring 32 projections over 180° (from 45°RAO to 45°LPO) on a circular, 400-mm field of view gamma camera. Serial images of the liver and hepatobiliary system were obtained at every 5 min up to one hour, followed by imaging at every 10 min for the next two hours. At the end of the study, 20-40 MBq $^{99\text{m}}\text{Tc}$ -EHIDA was given orally to confirm the location of the stomach if necessary.

In this research, the films of all participants, showing both SPECT and planar projection image, were evaluated retrospectively by two nuclear consultant radiologists working together. Scans were scored as positive for DGR only if the two physicians agreed on the presence of DGR. Retrograde movement of radioactivity from the duodenum into the stomach was considered abnormal and diagnostic of DGR. DGRi was calculated to estimate

Table 1 Comparison of demographic and clinical characteristics of duodenogastric reflux group and control group

| | DGR group (n = 99) | Control group (n = 70) |
|--------------------------|--------------------|------------------------|
| Age (mean ± SD) | 48.6 ± 16.2 | 50.1 ± 13.2 |
| Gender (male/female) | 41/58 | 35/35 |
| Epigastric pain (yes) | 72.7% ¹ | 17.10% |
| Nausea/vomit (yes) | 20.2% ¹ | 7.10% |
| Bitter taste (yes) | 31.3% ¹ | 4.30% |
| Sour regurgitation (yes) | 23.2% ¹ | 8.60% |
| Retrosternal pain (yes) | 18.2% ¹ | 1.40% |
| Anorexia (yes) | 26.30% | 10.00% |

¹Statistically significant differences ($P < 0.05$). DGR: Duodenogastric reflux.

the severity of DGR, following the formula:

$$\text{DGRi}(\%) = \frac{\text{Supreme count rate in the stomach}}{\text{Intrahepatic supreme count rate}} \times 100\%$$

Statistical analysis

All statistical analyses were performed using Statistical Analysis Software IBM SPSS Statistics 20 (Chicago, IL, United States). The significance level was set at 0.05 for all statistical tests. Values are expressed as mean ± SD or stand error of mean. Continuous data of gastric juice and DGRi were using the Independent-samples Mann-Whitney *U*-test between DGR and control group. The relationship among the TBA, DBIL and TBIL of DGR group was analysed by Spearman's rank correlation test and Fisher's linear discriminant analysis. The comparison between intragastric bile acids and hepatobiliary scintigraphy in the diagnosis of DGR was demonstrated by receiver operating characteristic (ROC) curve.

RESULTS

Characteristics of enrolled patients

Characteristics of the enrolled patients are shown in Table 1. The group of patients with DGR was 41 males and 58 females, with a mean ± SD age of 48.62 ± 16.20 years (95%CI: 45.39-51.85). The group of patients without DGR was 35 males and 35 females, with a mean ± SD age of 50.16 ± 13.23 years (95%CI: 47.00-53.31). The group of patients with DGR showed a statistically higher prevalence of epigastric pain in comparison with that without DGR.

Endoscopic study and histopathology

The images of patients which were got in the endoscopic examination were revealed in Figure 1. The gastric juice of DGR patients was lucidity or light yellow-green and/or associated mucosal changes. Pathologically the reflux was associated with infiltration of mononuclear leukocytes, neutrophilic granulocytes, and eosinophilic granulocytes and with foveolar hyperplasia in the gastric mucosa. Our results suggest that postprandial duodenogastric bile reflux is characterized by superficial inflam-

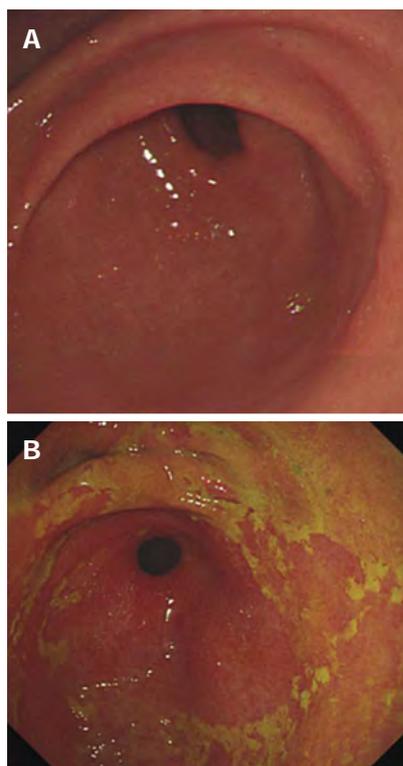


Figure 1 Comparison between the review of endoscopic evaluation control group (A) and duodenogastric reflux group (B). Compared with control group, the gastric mucous paste of duodenogastric reflux patient is usually yellow or green and has bile dyeing like islands.

matory changes in the gastric mucosa. Reviewed with past recording, there is no significant difference between atrophic gastritis and duodenogastric reflux (Figure 2).

Determination of bile acids in gastric juice

Gastric juice was successfully collected from all enrolled patients, and the concentration of bile acids in gastric juice was measured in the clinical laboratory. Analysis of the gastric aspirates was described in the Table 2, Figure 3. The bile acids levels of DGR patients were significantly higher than the control values (Z : TBA: -8.916, DBIL: -3.914, TBIL: -6.197, all $P < 0.001$). Using Nonparametric correlations, two of three in the DGR group have a significantly associated with each other (r : TBA/DBIL: 0.362, TBA/TBIL: 0.470, DBIL/TBIL: 0.737, all $P < 0.001$). Using the Fisher's linear discriminant analysis, we found the canonical correlation is 0.631 ($P < 0.001$). The standardized canonical discriminant function coefficient of TBA, DBIL and TBIL is individually 0.899, 0.084 and 0.152, from which we found TBA is the most important factor in the diagnosis of DGR in the examination of gastric juice. The Fisher's discriminant function is followed: Con: $Y = 0.002\text{TBA} + 0.048\text{DBIL} + 0.032\text{TBIL} - 0.986$; Reflux: $Y = 0.012\text{TBA} + 0.076\text{DBIL} + 0.089\text{TBIL} - 2.614$. Eighty-four point zero five percent of original grouped cases were correctly classified by this method. In other words, the result of endoscopy and gastric juice biochemistry detection were consistent more than 80%

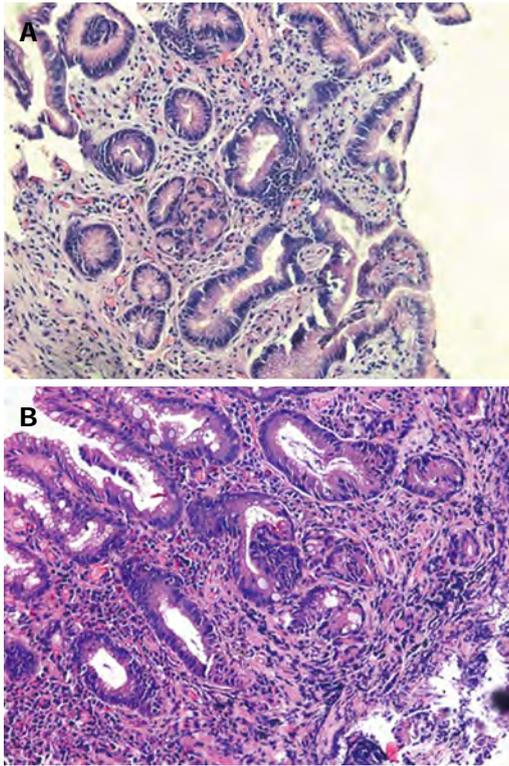


Figure 2 Representative hematoxylin and eosin staining of gastric tissue from chronic atrophic gastritis (A) and duodenogastric reflux (B). Isolated metaplasia of glandular epithelium and mild inflammation of the lamina propria was found in the tissue of duodenogastric reflux patients (original magnification, $\times 200$).

| Table 2 Results of gastric juice analyses between duodenogastric reflux group and control group | | | | | | |
|---|--------|---------------------|-------------|---------------|--------|-------|
| Type | | mean \pm SD | Range | 95%CI | Z | Sig. |
| TBA | Con | 44.51 \pm 56.53 | 0.80-235.80 | 31.05-58.01 | -8.916 | 0.000 |
| | Reflux | 263.64 \pm 171.61 | 0.70-660.50 | 229.41-297.87 | | |
| DBIL | Con | 1.87 \pm 1.85 | 0.00-8.90 | 1.43-2.31 | -3.914 | 0.000 |
| | Reflux | 5.43 \pm 6.12 | 0.00-23.70 | 4.20-6.65 | | |
| TBIL | Con | 1.63 \pm 1.34 | 0.10-7.40 | 1.31-1.94 | -6.197 | 0.000 |
| | Reflux | 5.49 \pm 5.51 | 0.30-21.90 | 4.39-6.59 | | |

Sig.: Asymp. Sig. (2-tailed) or exact Sig.; TBA: Total bile acid; TBIL: Total bilirubin; DBIL: Direct bilirubin.

by this method. The sensitivity and the specificity is separately 83.8% and 84.3%.

Duodenogastric reflux imaging

When hepatobiliary scintigraphy was administered by constant intravenous infusion it resulted in an increased elimination in bile for the first 80-100 min, and the concentration in bile then remained relatively constant for the rest of the test. Normally no increase in radioactivity in the stomach can be recorded, while the local radioactivity of the stomach increased during the investigation in DGR patients (Figure 4). The DGRi of DGR group were higher than those of the control group significantly ($Z = -5.224, P < 0.001$) (Figure 5). Twenty eight patients (59.6%) were deemed to be duodenogastric reflux posi-

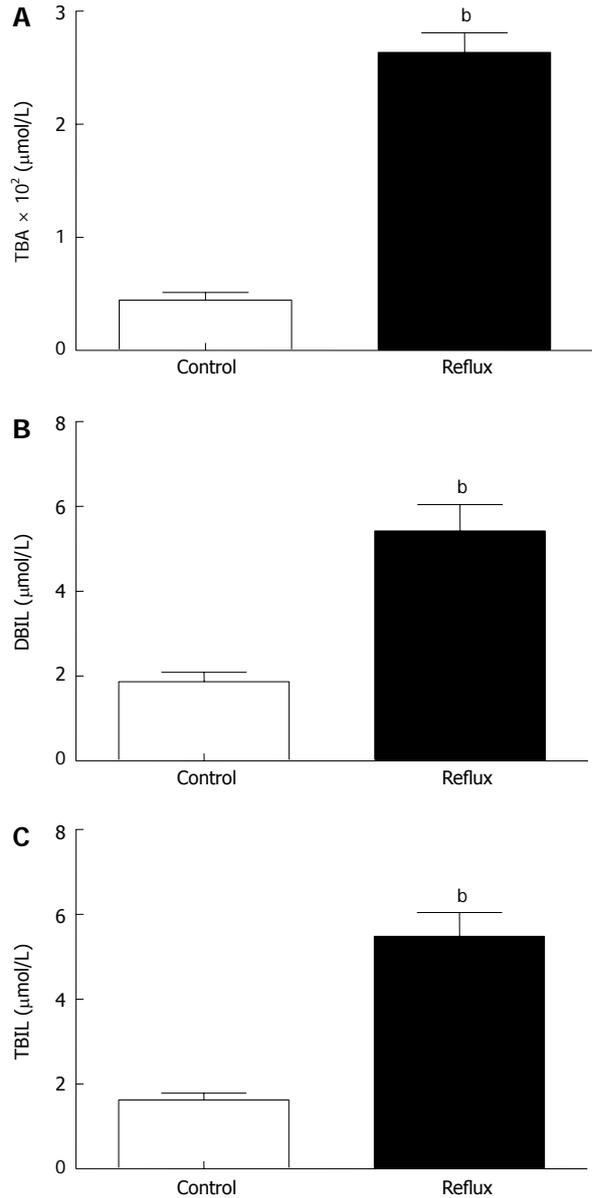


Figure 3 Intra-gastric concentrations of total bile acid (A), direct bilirubin (B) and total bilirubin (C) aspirated in endoscopy examination in duodenogastric reflux group and control group. Patients with duodenogastric reflux had a significantly higher total bile acid (TBA), total bilirubin (TBIL) and direct bilirubin (DBIL) compared to controls ($^bP < 0.001$ vs control group). Data are expressed as mean \pm SE and difference was calculated using the independent-samples Mann-Whitney *U*-test.

tive by endoscopy, as compared to 37 patients (78.7%) by hepatobiliary scintigraphy. In this study, we also found some patients who were not determined with DGR by endoscopy were found the clue of duodenogastric reflux in the hepatobiliary scintigraphy. Furthermore, 11 patients were evaluated twice by the hepatobiliary scintigraphy at intervals ranging from 3-14 d. The result was identical in 8 patients, from which it indicates the good reproducibility of the test.

DISCUSSION

DGR is a natural physiological phenomenon often oc-

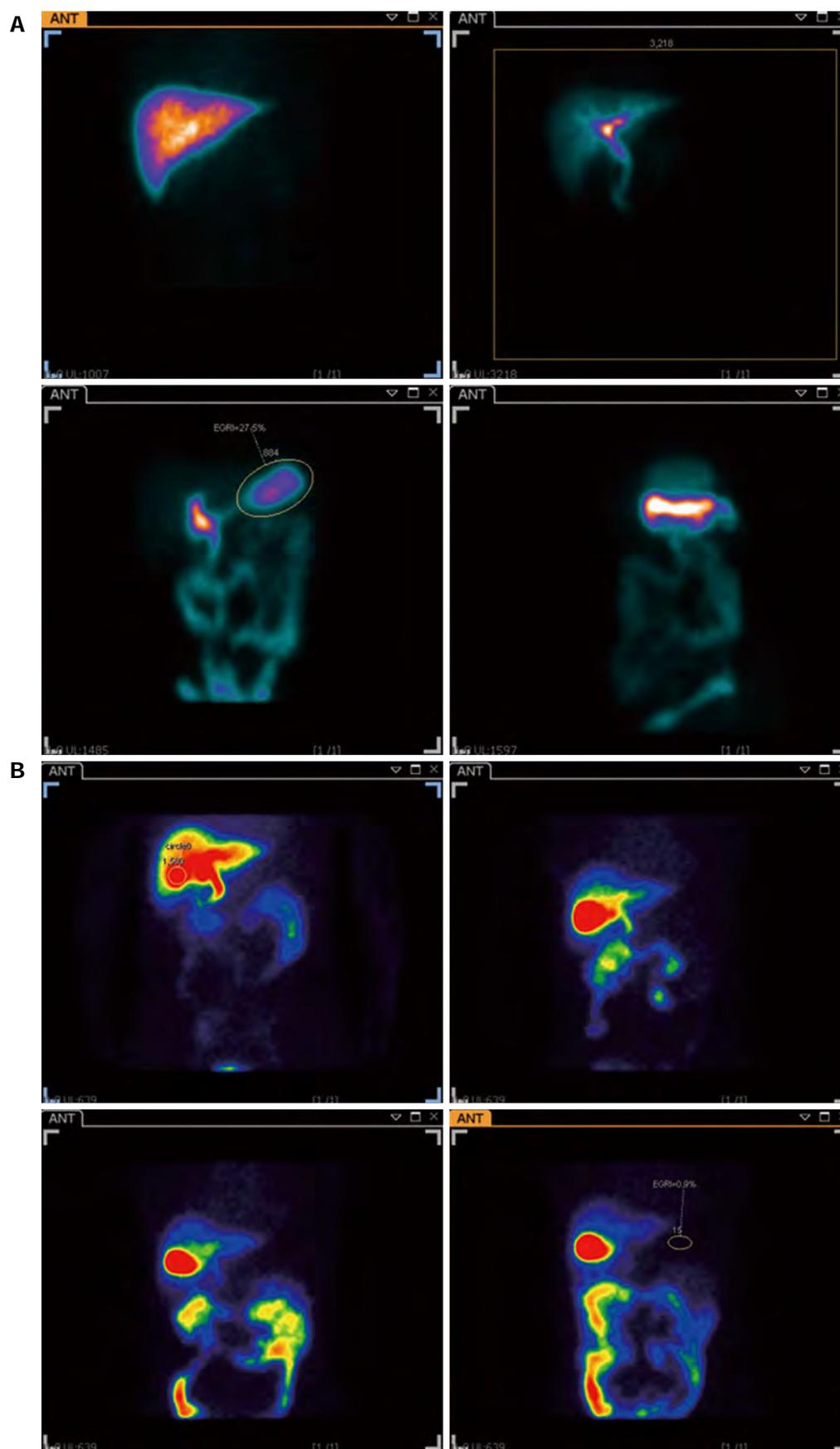


Figure 4 Examination of duodenogastric reflux by ^{99m}Tc -ethyl hepatic iminodiacetic acid test. A: One episode of duodenogastric reflux, of which the duodenogastric reflux index is 27.5%, is shown in the gastric localization (yellow circle) in the third image; B: A normal study in which no reflux is seen in the gastric region (yellow circle) outlined in the last picture.

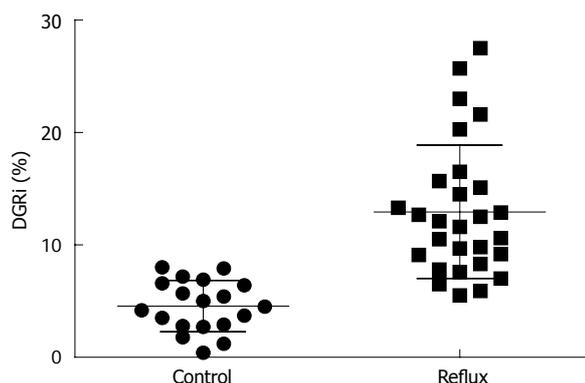


Figure 5 Comparison of duodenogastric reflux group and control group in the scintigraphy. The reflux rates of duodenogastric reflux group in the patients were higher than those in the control group with statistically significant differences ($Z = -5.224$, $P < 0.001$). Data was calculated using the independent-samples Mann-Whitney U -test. DGRI: Duodenogastric reflux index.

curing during the early hours of the morning and postprandial period^[9]. It is commonly understood to mean the passing into the stomach of duodenal fluid containing secretions from the intestinal mucosa, bile and pancreatic fluid^[10]. The prevalence of upper gastrointestinal symptoms and frequency of established diagnosis of upper gastrointestinal disease is greatest for the patients with marked DGR, being approximately twice that of patients without evidence of DGR^[11]. For over a century DGR has been considered the main cause of the primary or secondary alkaline gastritis and plays the basic role in the pathogenesis of gastritis and other GI tract diseases (reflux oesophagitis, gastric ulcer, progressing metaplasia or oesophageal and gastric cancer). In the previous researches, DGR occurred in 30% to 40% of adult patients presenting with acid reflux esophagitis or gastroesophageal reflux disease^[12,13]. It is common even in asymptomatic subjects, especially in gastric and duodenal ulcer patients, gastric surgery, gallstone patients, patients undergoing gallbladder operations and cases of chronic pulmonary disease. DGR is a physiologic event, but also that the pathologic presence of duodenal juice in the foregut lumen may account for the development of Barrett's metaplasia and dysplasia^[14,15], and for that of gastric polyps^[16], as well. Excessive DGR has been associated with the development of antral gastritis, gastric ulcers, alkaline esophagitis, esophageal or gastric adenocarcinoma, and intestinal metaplasia of the gastric mucosa^[17-20]. Gastric mucosal damage induces mast cell degranulation and a release of vasoactive mediators, such as histamine, leading to vascular congestion and lamina propria edema^[21]. Accurate detection of DGR has been a major problem for many years. The exact pathogenic features of bile reflux in unoperated stomach as well as its contributions to gastric mucosal lesions in chronic gastritis are still remaining unrevealed^[22]. The clinical diagnosis of excessive DGR is usually based on endoscopic observation of bile reflux found in the stomach, antral gastritis or ulceration, or the histologic documentation of foveolar hyperplasia, vascular congestion, lamina propria edema or chemical

gastritis^[23-25].

The various techniques employed to detect DGR are endoscopy gastroduodenal intubation and direct sampling, gastric pH monitoring, ambulatory gastric bilirubin monitoring and hepatobiliary scintigraphy. Among them, the use of the intubation technique is considered non-physiologic since it is invasive and thereby may spuriously provoke reflux. Gastric pH monitoring is cumbersome, entails the use of sophisticated instruments and is uncomfortable for the patients. Scintigraphic documentation of DGR is technically easy, simple and physiologic as it is noninvasive^[3]. Bilitec method reliably identified the presence of bilirubin and it has made feasible to quantitatively detect duodenogastroesophageal reflux of bile^[26]. Just *et al*^[27] showed that there was no correlation between an alkaline pH and the presence of bilirubin. Due to methodological discrepancies, research into the significance of duodenogastric reflux in the diagnosis of DGR has yielded varying results. Combined with past researches and practice, we think the diagnosis of DGR is still based on the systematic analysis of endoscopy, gastric fluid samples obtained by intubation and hepatobiliary scintigraphy, a more physiological, non-invasive method.

Endoscopy is one of the principal means of studying upper abdominal complaints for routine clinical purposes and is considered as a minimally invasive procedure, since it does not require an incision into one of the major body cavities and does not require any significant recovery after the procedure (unless sedation or anesthesia has been used). Stein *et al*^[28] reported that upper gastrointestinal endoscopy had lower accuracy and predictive value than scintigraphy or gastric pH monitoring in the assessment of DGR. We can find duodenogastric reflux under direct visualization in our daily clinical work. But this is only a temporary phenomenon for the most part, and not on behalf of the patient's disease status. The chief source of error in this technique is the possible effect of the intubation in either promoting or hindering reflux. Therefore, endoscopic findings only give us an intuitive, subjectivity evidence of the bile reflux and the test is largely a qualitative one.

In addition to the observation of DGR situation, we did pathological examinations during the routine gastroscopy examination. It has been demonstrated in animal experiments that duodenal fluid caused an increase in inflammatory cells in the gastric mucosa, a decrease in parietal cells, a hyperplasia of mucous cells and changes in glandular morphology. The important factor is the antrum which serves to protect the mucosa of the gastric body from the toxic effects of DGR. In our research, we found the atrophic gastritis was very common in patients with severe reflux in endoscopy, and we didn't found the histopathology significantly different between DGR and atrophic gastritis, which was consistent with the previously research^[29].

The assessment of gastric fluid, an important work in the endoscopic progress, is another important impact in the diagnosis of DGR. The surfactant effect of bile acids

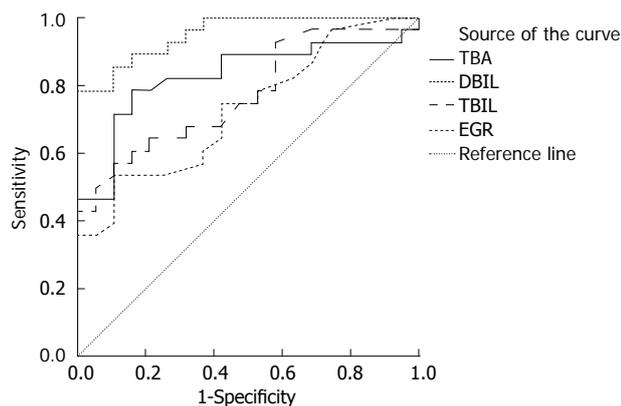


Figure 6 Receiver operator curve for ^{99m}Tc -ethyl hepatic iminodiacetic acid test and gastric juice analyses in the diagnosis of duodenogastric reflux. Area under the curve was of 0.953 for ethyl hepatic iminodiacetic acid scintigraphy ($P < 0.001$, 95%CI: 0.901-1.000), 0.830 for total bile acid (TBA) ($P < 0.001$, 95%CI: 0.709-0.950), 0.722 for direct bilirubin (DBIL) ($P = 0.008$, 95%CI: 0.587-0.872) and 0.773 for total bilirubin (TBIL) ($P = 0.002$, 95%CI: 0.642-0.905).

is closely related to their hydrophobic-hydrophilic balance. Bile acids have a surfactant effect for lipid absorption^[30], and they may have a cytotoxic action if the surfactant effect is too strong^[31,32]. Indeed, Heuman reported that the hydrophilic-hydrophobic balance of bile acids correlates with their toxicity, and increasing hydrophobicity was associated with increasing cytotoxicity towards the gastrointestinal epithelium^[33]. Therefore, the bile acids may also have some roles in the formation of duodenogastric gastritis and in the diagnosis of DGR. In our study, we found there was a good correlation between TBA and DBIL, TBA and TBIL, DBIL and TBIL in DGR group. When we used Fisher's linear discriminant analysis to analyze the three indexes in the determination of DGR, we found TBA was the most important factor in the diagnosis and created two formats to discriminant the diagnosis of DGR. The consistency between the direct vision of endoscopy and gastric juice examination was nearly 84%. By this method, the sensitivity and the specificity was separately 83.8% and 84.3% and this is the first time that we used this method to determine DGR.

Hepatobiliary scintigraphy, using ^{99m}Tc -EHIDA derivatives, is superior to upper gastrointestinal endoscopy in the detection of DGR and also has the advantage of being non-invasive and physiological. A hepatobiliary tracer is injected intravenously and ^{99m}Tc -EHIDA excreted through the liver into the biliary tract and further into the duodenum in cholescintigraphy. When DGR happened, ^{99m}Tc -EHIDA passes into the duodenum and via reflux into the stomach. About 60% (28/47) of the isotope dose was secreted into the bile in 1.5 h. In the past researches, a good correlation was shown between the severity of mucosal changes on histology and the presence of DGR on scintigraphy^[34,35]. The present study not only confirms this sensitive method for the diagnosis of DGR, but also proves its superiority over intragastric bile acids estimation (Figure 6). When we used ROC

curve, we found the hepatobiliary scintigraphy was better than the examination of gastric juice (Figure 6). This means the hepatobiliary scintigraphy has better sensitivity and specificity. From the statistical analysis, we also found TBA was the most important factor of bile acids to determinate the diagnosis of DGR, which was in accordance with the result of the standardized canonical discriminant function coefficient of TBA. But the method gave no information on the nature of the reflux fluid, *i.e.*, the substances neither contained in bile, nor did it measure anything more than bile reflux. It is well accepted that hepatobiliary scintigraphy recorded only a relatively short period. Being noninvasive, physiological and good repeatability, hepatobiliary scintigraphy appears suitable for routine clinical use in the diagnosis of DGR^[36,37].

All in all, the results of endoscopy, discriminant function of intragastric bile acid examination and scintigraphy were correlated with the final diagnosis of DGR. Integrated use of these three methods will help improve the accuracy of diagnosis of DGR.

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COMMENTS

Background

Duodenogastric reflux (DGR) is a natural physiological phenomenon often occurring during the early hours of the morning and postprandial period, which is commonly understood to mean the passing into the stomach of duodenal fluid containing secretions from the intestinal mucosa, bile and pancreatic fluid. Earlier used techniques employed to detect DGR included gastroduodenal intubation and direct sampling, gastric pH monitoring, endoscopy, gastric mucosal biopsy and hepatobiliary scintigraphy, but every method has its limit in the diagnosis of DGR.

Research frontiers

Gastric pH monitoring is cumbersome, entails the use of sophisticated instruments and is uncomfortable for the patients. Bilitex method reliably identified the presence of bilirubin and it has made feasible to quantitatively detect duodenogastroesophageal reflux of bile. Due to methodological discrepancies, research into the significance of duodenogastric reflux in the diagnosis of DGR has yielded varying results.

Innovations and breakthroughs

This is the first time that we used the Fisher's linear discriminant analysis to determine the bile acids in gastric juice and found total bile acid is the most important factor in the diagnosis of DGR. Using the Receiver operator curve, authors found the hepatobiliary scintigraphy is better than the examination of gastric juice.

Applications

By understanding the advantages and disadvantages of intragastric bile acids and scintigraphy, this study demonstrates the hepatobiliary scintigraphy have better sensitivity and specificity than intragastric bile acids in the diagnosis of DGR and the integrated use of these two methods can greatly improve the accuracy and sensitivity of the diagnosis of DGR.

Terminology

Hepatobiliary scintigraphy is a radionuclide diagnostic imaging study that evaluates hepatocellular function and patency of the biliary system by tracing

the production and flow of bile from the liver through the biliary system into the small intestine. Sequential images of the liver, biliary tree and gut are obtained. Computer acquisition and analysis as well as pharmacological interventions are frequently employed.

Peer review

Many reports evaluate duodenogastric reflux with endoscopic examination or gastric juice examination. Hepatobiliary scintigraphy can check objectively dynamic duodenogastric reflux and is no invasive method. This report results hepatobiliary scintigraphy is a useful method for evaluating duodenogastric reflux and help improve the accuracy of diagnosis of duodenogastric reflux with integrated use of endoscopy and intragastric bile examination.

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miRNA-338-3p suppresses cell growth of human colorectal carcinoma by targeting smoothened

Kai Sun, Hai-Jun Deng, Shang-Tong Lei, Jing-Qing Dong, Guo-Xin Li

Kai Sun, Hai-Jun Deng, Shang-Tong Lei, Jing-Qing Dong, Guo-Xin Li, Department of General Surgery, Nanfang Hospital of Southern Medical University, Guangzhou 510515, Guangdong Province, China

Author contributions: Sun K designed and performed the study, analyzed the data, and wrote the paper; Deng HJ, Lei ST, Dong JQ and Li GX helped perform a portion of the study.

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Correspondence to: Dr. Kai Sun, Department of General Surgery, Nanfang Hospital of Southern Medical University, Guangzhou Dadao North Street No. 1838, Guangzhou 510515, Guangdong Province, China. sunkai9602@sina.com

Telephone: +86-20-62787170 Fax: +86-20-61641683

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Abstract

AIM: To investigate the regulative effect of miRNA-338-3p (miR-338-3p) on cell growth in colorectal carcinoma (CRC).

METHODS: The lentiviral vector pLV-THM-miR-338-3p and pLV-THM-miR-338-3p-inhibitor were constructed. The recombinant viral vector encoding the pre-miR-338-3p or miR-338-3p-inhibitor and the two packaging plasmids psPAX2 and pMD2.G were cotransfected into human embryonic kidney 293T cells to package lentivirus. The supernatant containing the lentivirus particles was harvested to determine the viral titer, and this supernatant was then used to transduce CRC-derived cell line, SW-620. Flow cytometry was utilized for sorting the green fluorescent protein (GFP)⁺ cells to establish the SW-620 cell line stably expressing pre-miR-338-3p or miR-338-3p-inhibitor. Moreover, the expression of miR-338-3p was determined by real-time reverse transcriptase polymerase chain reaction, and

Western blotting was used to detect the expression of the smoothened (SMO, the possible target of miR-338-3p) protein in SW-620 cells. Furthermore, the status of CRC cell proliferation and apoptosis were detected by 3-(4,5-dimethyl-2 thiazoyl)-2,5-diphenyl-2H-tetrazolium bromide assay and flow cytometry, respectively.

RESULTS: Restriction enzyme digestion and DNA sequencing demonstrated that the lentiviral vector pLV-THM-miR-338-3p and pLV-THM-miR-338-3p-inhibitor were constructed successfully. GFP was expressed after the SW-620 cells were transduced by the lentivirus. Expression of miR-338-3p in SW-620 cells transduced with the lentivirus pLV-THM-miR-338-3p was significantly increased (relative expression 3.91 ± 0.51 vs 2.36 ± 0.44 , $P < 0.01$). Furthermore, overexpression of miR-338-3p inhibited the expression of SMO protein in SW-620 cells, which showed obviously suppressed proliferation ability [cellular proliferation inhibition rate (CPIR) $61.9\% \pm 5.2\%$ vs $41.6\% \pm 4.8\%$, $P < 0.01$]. Expression of miR-338-3p in SW-620 cells transduced with the lentivirus pLV-THM-miR-338-3p-inhibitor was significantly decreased (relative expression 0.92 ± 0.29 vs 2.36 ± 0.44 , $P < 0.01$). Moreover, the downregulated expression of miR-338-3p caused upregulated expression of the SMO protein in SW-620 cells, which showed significantly enhanced proliferation ability (CPIR $19.2\% \pm 3.8\%$ vs $41.6\% \pm 4.8\%$, $P < 0.01$). However, anti-SMO-siRNA largely, but not completely, reversed the effects induced by blockage of miR-338-3p, suggesting that the regulative effect of miR-338-3p on CRC cell growth was indeed mediated by SMO.

CONCLUSION: miR-338-3p could suppress CRC growth by inhibiting SMO protein expression.

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Key words: Colorectal carcinoma; Hsa-miRNA-338-3p; Smoothened; Lentivirus

Core tip: The previous study has shown that loss of miR-338-3p expression is associated with clinical aggressiveness of colorectal carcinoma (CRC). In this study, the authors demonstrated that forced expression of miR-338-3p in CRC cells suppressed cell proliferation and induced apoptosis, whereas inhibition of miR-338-3p in CRC cells promoted growth. We described miR-338-3p as a direct regulator of smoothed (SMO) expression in CRC, showing a new mechanism responsible for SMO upregulation in CRC. This study provides evidence for antiangiogenic activity of miR-338-3p in the development of CRC and it may develop as a useful biomarker or therapeutic target in CRC.

Sun K, Deng HJ, Lei ST, Dong JQ, Li GX. miRNA-338-3p suppresses cell growth of human colorectal carcinoma by targeting smoothed. *World J Gastroenterol* 2013; 19(14): 2197-2207 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v19/i14/2197.htm> DOI: <http://dx.doi.org/10.3748/wjg.v19.i14.2197>

INTRODUCTION

Colorectal carcinoma (CRC) is one of the leading causes of cancer-related death worldwide with an estimated one million new cases and 500 000 deaths annually. The CRC incidence and mortality in China have increased rapidly in the past few decades^[1]. Screening for CRC allows early-stage diagnosis of the malignancy and potentially reduces mortality. New targeted therapies directed against molecules involved in the pathogenesis of CRC have recently been reported to be safe and effective^[2,3]. With the advent of new chemotherapeutic agents, such as angiogenesis inhibitor and transforming growth factor- α inhibitors, there is growing interest to identify new prognostic biomarkers and therapeutic targets for this disease^[4].

miRNAs are a new class of small noncoding RNAs that regulate the expression of target genes through translational repression or mRNA cleavage/decay^[5,6]. Genome-wide studies have demonstrated that miRNA genes are frequently located at cancer-associated genomic regions or in fragile sites, and in minimal regions of loss of heterozygosity or of amplification, or in common breakpoint regions, indicating the potential roles of miRNAs in tumorigenesis^[7,8]. miRNAs have been demonstrated to play an important role in the multi-step processes of carcinogenesis, either by oncogenic or tumor suppressor function^[9]. Studies of miRNAs have been extended to many types of tumors, including CRC. These studies have revealed that miRNAs may be potential diagnostic or prognostic tools for cancer, and the identification of target mRNAs is a key step for assessing the role of aberrantly expressed miRNAs in human cancer^[10].

miR-338-3p has recently been discovered and is involved in cell growth. Although miR-338-3p is known to be specifically expressed in neuronal tissue, little is known about its abundance and function during carcinogenesis^[11,12]. We have found that miR-338-3p is downregulated in several CRC samples compared with adjacent non-tumorous tissues, suggesting that miR-338-3p might act as tumor suppressor in CRC, however, the targets that it regulates in CRC have not been established. Smoothed (SMO) protein is related to G-protein-coupled receptors, and is the key activator of the Hedgehog (Hh) signaling pathway^[13,14]. Upregulation of SMO in CRC is correlated with higher biological aggressiveness, advanced stage, poor differentiation, larger tumor size, and high proliferative activity^[15]. Furthermore, it is also well known that SMO regulation, both in physiological and pathological conditions, is mostly at a post-transcriptional level^[16]. Moreover, with the application of bioinformatics predictions, we have found that miR-338-3p and SMO mRNA 3'-untranslated region (UTR) have complementary binding sites. Thus, we inferred that the noncoding RNA, miR-338-3p, acts as a local regulator of SMO by binding to the 3'-UTR of its mRNA, thereby modulating CRC development. In order to verify this hypothesis, we investigated the regulative effect of miR-338-3p on cell proliferation and apoptosis in CRC. We aimed to reveal a new regulatory mechanism of miR-338-3p in the development of CRC, and provide a new miRNA and target gene for clinical application.

MATERIALS AND METHODS

Construction of transfer vector pLV-THM-miR-338-3p and pLV-THM-miR-338-3p-inhibitor

The lentiviral vectors used in this study were pLV-THM, psPAX2, and pMD2.G, which were a transfer vector, packaging plasmid, and envelope plasmid, respectively. The sequences of interest were inserted into the transfer vector between the *Mlu*I and *Cla*I restriction sites according to the Addgene protocol. The third generation of self-inactivating, lentivirus plasmid, pLV-THM (HIV-1-based vector; Addgene, Cambridge, MA, United States), which contains a CMV-driven enhanced green fluorescence protein (GFP) reporter and an H₁ promoter upstream of the restriction sites (*Mlu*I and *Cla*I), was used as the transfer plasmid and was linearized by digesting the vector with the restriction enzymes. The sequence of the mature miR-338-3p (5'-UCCAGCAU-CAGUGAUUUUGUUG-3') was obtained from miR-Base (<http://www.mirbase.org/>). The pre-miR-338-3p and miR-338-3p-inhibitor oligonucleotides were chemically synthesized by Sangon Biotech Co. Ltd. (Shanghai, China) and were inserted between the *Mlu*I and *Cla*I sites of the pLV-THM plasmid. After the pre-miR-338-3p and miR-338-3p-inhibitor lentiviral-based vector were transformed into competent *Escherichia coli* DH5 α cells

using the calcium chloride method, antibiotic-resistant colonies were selected on LB-ampicillin agar plates. After colony selection and further propagation, the plasmid was extracted using the alkaline lysis method. The plasmid DNA was then analyzed by restriction enzyme digestion and sequence analysis. The plasmid containing the target gene was digested with the restriction enzymes and amplified by polymerase chain reaction (PCR). The clones with positive PCR results were subjected to DNA sequencing.

Cell lines and culture

Human embryonic kidney 293T (HEK-293T) cells (Invitrogen, Carlsbad, CA, United States) and the human CRC-derived cell line SW-620 (Shanghai Institutes for Biological Science, CAS, China) were cultured in Dulbecco's Modified Eagle's Medium high glucose supplemented with 10% heat-inactivated fetal bovine serum (FBS; Hyclone, Logan, UT, United States) at 37 °C in a humidified incubator with 5% CO₂. The medium was changed every 3 d, and the cells were trypsinized with trypsin/ethylene diamine tetraacetic acid when 80%-90% confluence was reached. Cells at passages 4-8 were used for the experiments.

Lentiviral packaging and virus collection

Twenty-four hours prior to transfection, the HEK-293T cells in logarithmic growth phase were trypsinized, and the cell density was adjusted to 1.0×10^6 cells/mL with complete culture medium. The cells were reseeded into 15-cm cell culture dishes and cultured for 24 h prior to transfection. The cells were 90%-95% confluent on the day of transfection. The recombinant viral vector encoding the miR-338-3p or miR-338-3p-inhibitor and the two packaging plasmids psPAX2 and pMD2.G were extracted with a plasmid extraction kit (Invitrogen) and cotransfected into HEK-293T cells according to the manufacturer's instructions. After 8 h transfection, the cell culture medium was replaced with fresh complete medium. After 24 h transfection, the expression of GFP was determined. After 48 h transfection, the culture medium was collected and centrifuged at $4000 \times g$ at 4 °C for 10 min to remove any cellular debris. The supernatant was filtered through a 0.45- μ m filter into a Plus-20 centrifugal ultrafiltration unit and centrifuged at $4000 \times g$ to obtain a high-titer lentivirus stock. The lentivirus without the transgene was used as the negative control and was produced in the same manner.

Virus transduction and fluorescent cell selection

SW-620 cells were seeded at 1.0×10^5 cells per well in 24-well plates in DMEM containing 10% FBS. After 24-h incubation, the cells were transduced with each lentivirus stock (3.0×10^5 Titer Units). The SW-620 cells were then incubated for an additional 48-72 h prior to identifying the GFP⁺ cells by flow cytometry (Becton Dickinson, San Jose, CA, United States).

Detection of miR-338-3p expression by real-time reverse transcriptase RT-PCR

Total RNA from SW-620 cells was prepared using the TRIzol reagent (Invitrogen) after viral transduction. The precipitate was dissolved in diethylpyrocarbonate-treated water, and a nucleic acid protein analyzer (Beckman Coulter, Fullerton, CA, United States) was used to determine the RNA concentration. The purity and integrity of the RNA were identified as follows: the A_{260nm}/A_{280nm} was ≥ 1.8 , and the band ratio of 28 S RNA to 18 S RNA was ≥ 1.5 in formaldehyde denaturing gel electrophoresis. Accurate quantitation of the mature miR-338-3p was obtained using the TaqMan MicroRNA Assays (Applied Biosystems, Foster City, CA, United States). The reverse transcription reaction was performed using 10 ng total RNA and the looped primers. Real-time PCR was performed using the standard TaqMan MicroRNA Assays protocol on the iCycler iQ Real-Time PCR Detection System (Bio-Rad, Hercules, CA, United States). The PCR reaction (20 μ L) included 1.33 μ L reverse transcription product, 1 \times TaqMan Universal PCR Master Mix, No AmpErase UNG, 0.2 μ mol/L TaqMan probe, 1.5 μ mol/L forward primer, and 0.7 μ mol/L reverse primer. The reactions were incubated in a 96-well plate at 95 °C for 10 min followed by 40 cycles of 95 °C for 15 s and 60 °C for 1 min. The miR-338-3p expression level was measured using the Ct (threshold cycle) method. Ct is the fractional cycle number at which the fluorescence of each sample passes the fixed threshold. The $\Delta\Delta$ CT method for relative quantitation of gene expression was used to determine the miR-338-3p expression levels. The Δ CT was calculated by subtracting the Ct of U6 from the Ct of the miR-338-3p. The $\Delta\Delta$ CT was calculated by subtracting the Δ CT of the reference sample from the Δ CT of each sample. The fold change was calculated using the equation $2^{-\Delta\Delta CT}$. The TaqMan MicroRNA Assays for U6 RNA was used to normalize the relative abundance of miR-338-3p.

miRNA target prediction

The analysis of miR-338-3p-predicted targets was performed using the algorithms TargetScan (<http://targetscan.org/>), PicTar (<http://pictar.mdc-berlin.de/>) and MiRanda (<http://www.microrna.org/microrna/home.do>).

Detection of SMO protein expression by Western blotting

SW-620 cells were rinsed twice with cold PBS and were then lysed in ice-cold lysis buffer containing 150 mmol/L NaCl, 50 mmol/L Tris-HCl (pH 7.6), 0.1% SDS, 1% Nonidet P-40, and protease inhibitor cocktail (Boehringer Mannheim, Lewes, United Kingdom). The samples were cleared by centrifugation at $13\,000 \times g$ for 10 min. The cellular protein (50 μ g) was subjected to SDS-PAGE and electrotransferred to polyvinylidene fluoride membranes (Immobilon, Bedford, MA, United States). After blocking in 20 mmol/L Tris-HCl, (pH 7.6) containing 150

mmol/L NaCl, 0.1% Tween-20, and 5% nonfat dry milk, the membranes were incubated with primary antibodies against SMO or β -actin, which was used as a sample loading control, overnight at 4 °C. The membranes were then incubated with horseradish-peroxidase-conjugated secondary antibody. The blot was developed using the ECL detection kit (Amersham Pharmacia Biotech Inc., Piscataway, NJ, United States) according to the manufacturer's instructions.

Cell proliferation assay

The status of cell proliferation was determined by 3-(4,5-dimethyl-2 thiazoyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT; Amresco, Solon, OH, United States) assay. Exponentially growing SW-620 cells were adjusted to 2.5×10^4 cells/mL with DMEM, plated in 96-well plates (Corning, Corning, NY, United States) at 200 μ L/well and then incubated for 12 h according to routine procedure. After being transduced with each lentivirus stock and incubated for 48 h (5 duplicate wells for each sample), 20 μ L/well MTT (5 g/L) was added to each well. The medium was then removed after 4 h incubation and 100 μ L/well dimethyl sulfoxide was added to dissolve the reduced formazan product. Finally, the plate was read in an enzyme-linked immunosorbent microplate reader (Bio-Rad 2550) at 490 nm. The cellular proliferation inhibition rate (CPIR) was calculated using the following formula: $CPIR = (1 - \text{average } A \text{ value of experimental group} / \text{average } A \text{ value of control group}) \times 100\%$.

Apoptosis assay

The effects of miR-338-3p on CRC cell cycle and apoptosis were examined by flow cytometry. Pretreated SW-620 cells were harvested and washed twice with PBS, fixed with 70% ethanol at -20 °C for 30 min, and stored at 4 °C overnight, then washed with PBS again, treated with 100 mL 100 mg/L RNase at 37 °C for 30 min, and stained with 100 mL 50 mg/L propidium iodide at 4 °C for 30 min in the dark. The multiplication cycle and apoptotic rate were assayed using flow cytometry, and the data were analyzed using CellQuest software. The percentages of cells in the G₀/G₁ phase and S phase, and the apoptotic rate were measured by calculating the ratio of the number of corresponding cells to the number of total cells. For each sample, 10 000 cells were measured.

Statistical analysis

The relative expression analysis of the target gene was performed using REST-XL (Relative Expression Software Tool, available at <http://www.wzw.tum.de/genequantification>). All data in the experiment were presented as the mean \pm SD. Comparisons between the groups were analyzed with one-way ANOVA and Student-Newman-Keuls *Q* test, using SPSS version 13.0 software (SPSS Inc., Chicago, IL, United States). *P* < 0.05 was considered statistically significant.

RESULTS

Lentivirus package and transduction

HEK-293T cells were cotransfected with the transfer plasmid, pLV-THM-transgene, the packaging plasmid, psPAX2, and the envelope plasmid, pMD2.G. The high-titer lentivirus was harvested as the stock virus solution. GFP was expressed 48 h after the SW-620 cells were transduced by the lentivirus, and the cells were observed under a fluorescence microscope (Figure 1A, B). This suggests that the miR-338-3p or miR-338-3p-inhibitor vector was successfully transduced into the SW-620 cells, which provides the basis for further studies regarding the molecular function of miR-338-3p in CRC cells. The GFP⁺ fluorescent cells were then identified and harvested using flow cytometry for the next experiment (Figure 1C-E).

Real-time reverse transcriptase-PCR detecting miR-338-3p expression in CRC cells after lentivirus transduction

To study the expression pattern of miR-338-3p in SW-620 cells after lentivirus transduction, we performed real-time reverse transcriptase (RT)-PCR to detect miR-338-3p expression in the SW-620 cells. Real-time RT-PCR indicated that the miR-338-3p cDNA increased exponentially and then reached a plateau. The miR-338-3p amplification curve was a typical reverse S pattern (Figure 2A) and showed higher amplification efficiency. The miR-338-3p PCR product was 72 bp long, the corresponding T_m was 84.09 ± 0.15 °C, the melting temperature was even, and the shape of the peak was sharp (Figure 2B). As shown in Figure 2C, the expression level of miR-338-3p in the pLV-THM-miR-338-3p group was more than one-third of the expression in the control cells that were transduced with the blank pLV-THM vector, whereas the expression level of miR-338-3p in the pLV-THM-miR-338-3p-inhibitor group decreased significantly compared with the control group (*P* < 0.01). Thus, we established the SW-620-miR-338-3p and SW-620-miR-338-3p-inhibitor cell lines successfully to observe the corresponding biological effect.

SMO is a target of miR-338-3p in CRC

Most miRNAs are thought to control gene expression by base-pairing with the miR-recognizing elements found in their messenger target. We then used all three currently available major prediction programs, including TargetScan, Miranda and PicTar, to analyze the potential interaction between miR-338-3p and SMO. SMO mRNA was predicted by all of the algorithms and revealed potential miR-338-3p target sites in its 3'-UTR (Figure 3A).

To check if miR-338-3p actually affected SMO expression in CRC cells, we analyzed the consequence of the ectopic expression of miR-338-3p. We transfected the pre-miR-338-3p and miR-338-3p-inhibitor into SW-620 cells by lentivirus transduction as described above, and we searched for changes in SMO protein

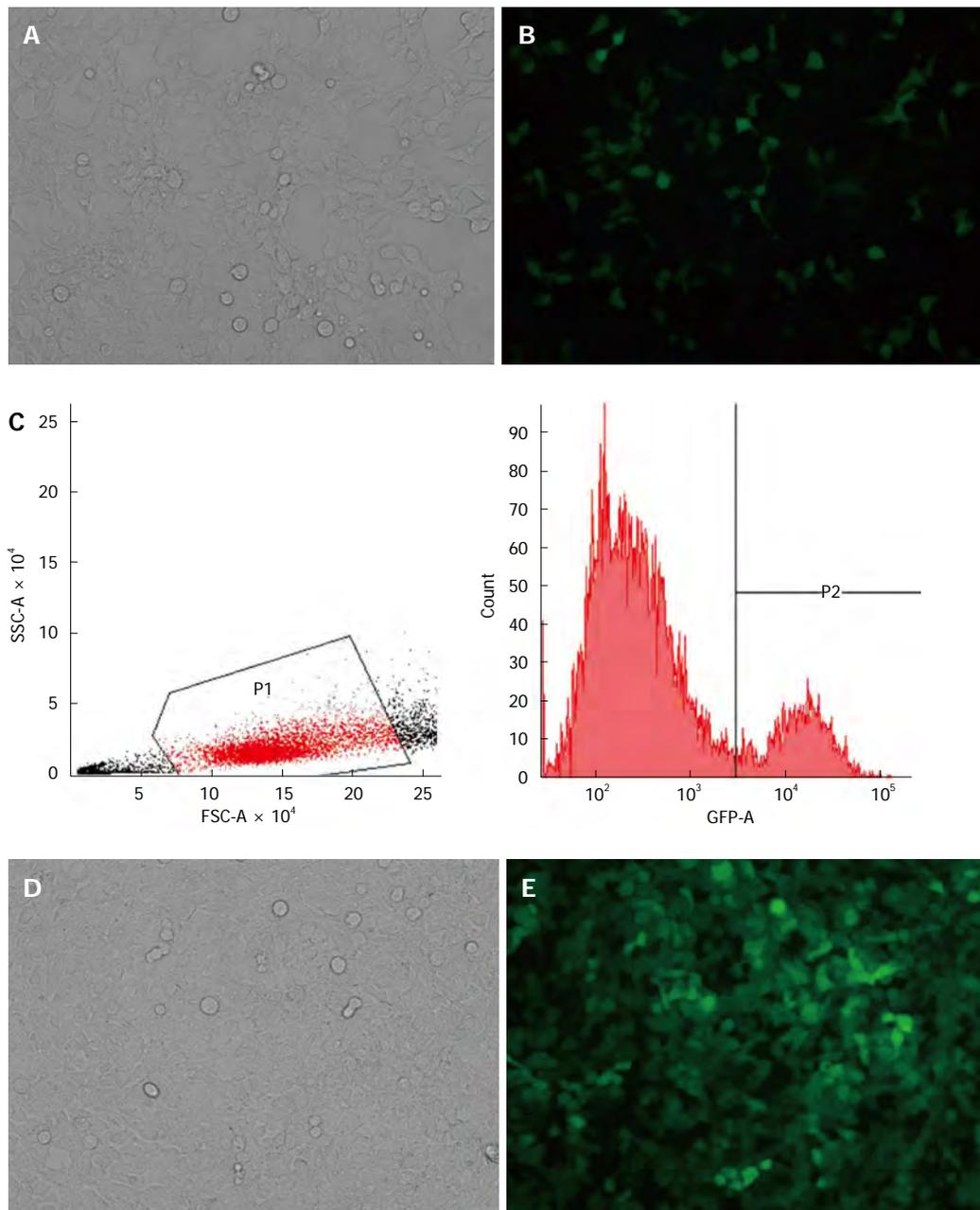


Figure 1 SW-620 cells transduced by lentivirus before and after flow cytometry selection. A, B: SW-620 cells transduced by lentivirus before flow cytometry selection (A: Light microscopy; B: Fluorescent microscopy $\times 40$); C: SW-620 cells with green fluorescent protein⁺ were distinguished by flow cytometry; D, E: SW-620 cells transduced by lentivirus after flow cytometry selection (D: Light microscopy; E: Fluorescent microscopy $\times 40$).

levels by Western blotting analysis. Introduction of pre-miR-338-3p caused a significant increase of miR-338-3p value and decreased SMO protein levels in SW-620 cells. Conversely, miR-338-3p-inhibitor caused a significant decrease of miR-338-3p value and increased SMO protein level (Figure 3B). This result strongly validates a post-transcriptional regulation of SMO protein by miR-338-3p.

miR-338-3p suppresses proliferation and induces apoptosis in CRC cells

SMO has a key role in the cell cycle, particularly in the growth arrest at the G₁/S transition, therefore, we

further tested if the cell growth potential of stably transduced CRC cells expressing miR-338-3p or miR-338-3p-inhibitor was modified as a consequence of the demonstrated SMO alteration. First, to evaluate the effect of miR-338-3p on CRC cell proliferation, growing SW-620 cells were transduced with lentivirus pLV-THM-miR-338-3p or pLV-THM-miR-338-3p-inhibitor for 48 h and the cell proliferation was determined by MTT assay. We observed a significant increase in proliferation after transduction of pLV-THM-miR-338-3p-inhibitor (Figure 4A, $P < 0.01$). In contrast, pre-miR-338-3p significantly inhibited cell proliferation (Figure 4A, $P < 0.01$). These data indicate that cell proliferation can

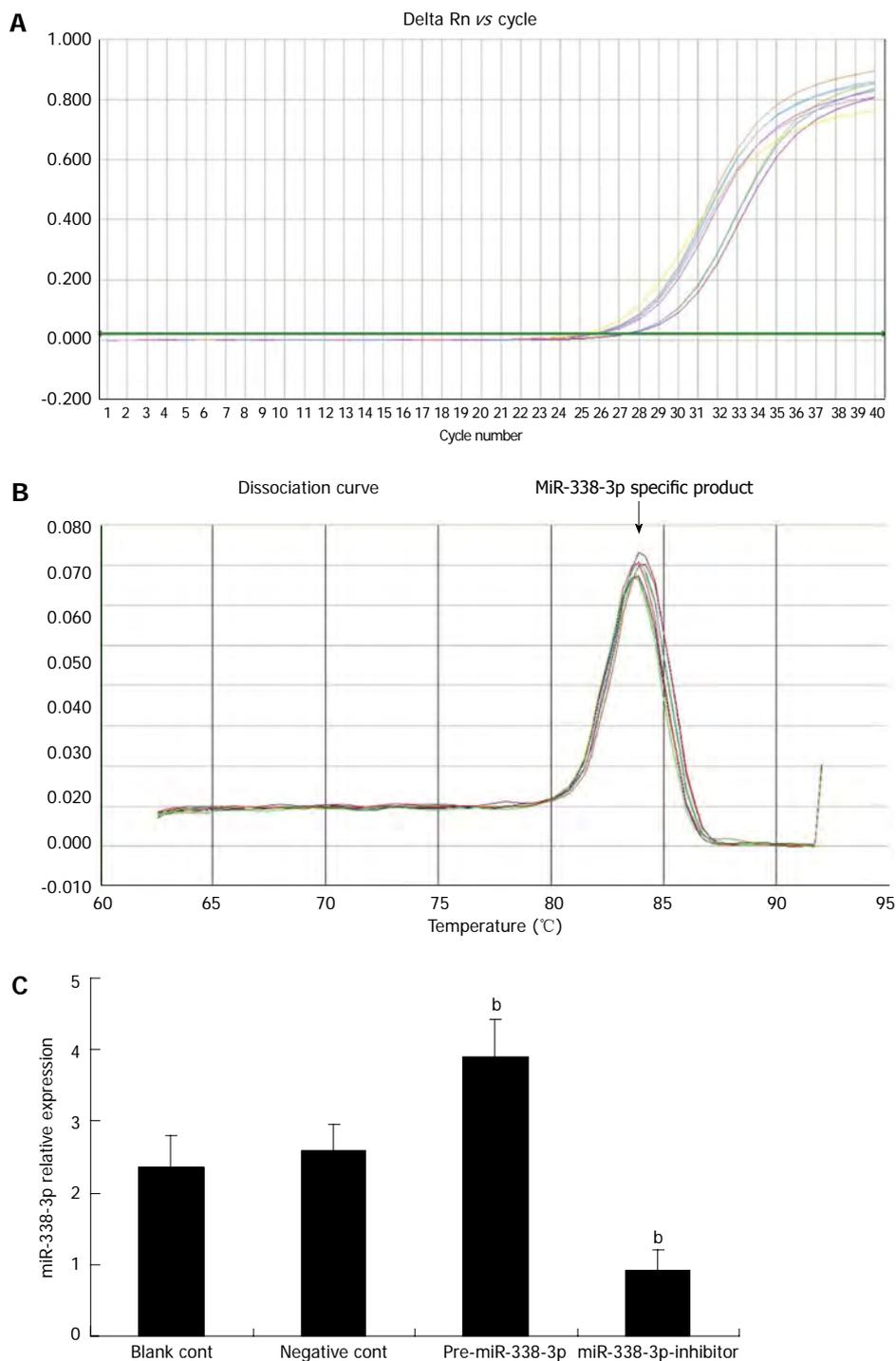


Figure 2 Real-time reverse transcriptase-polymerase chain reaction analysis detecting miRNA-338-3p expression in SW-620 cells. A: miRNA-338-3p (miR-338-3p) cDNA concentrations, Log value as ordinate, Ct value as abscissa; B: T_m of miR-338-3p was 84.09 °C; C: Expression of miR-338-3p detected by real-time reverse transcriptase-polymerase chain reaction. Expression of U6 snRNA was used as an internal control. ^b*P* < 0.01 vs control group.

be significantly suppressed by increased miR-338-3p expression. Second, we performed flow cytometry analysis after exposure to miR-338-3p or miR-338-3p-inhibitor to investigate CRC cell-cycle phase distribution. SW-620 cells overexpressing miR-338-3p had a significant decrease in the S-phase population and a increase in the G₀/G₁ population compared with cells transduced with negative control lentivirus (Figure 4B, *P* < 0.01). On the

contrary, miR-338-3p-inhibitor significantly increased the S-phase and decreased the G₀/G₁ population (Figure 4B, *P* < 0.01). Third, we investigated the effect of miR-338-3p on apoptosis by flow cytometry and found that apoptosis increased dramatically in SW-620 cells after transduction with lentivirus pLV-TM-miR-338-3p, suggesting that miR-338-3p may function as a strong apoptotic inducer in human CRC cells (Figure 4C-F). These

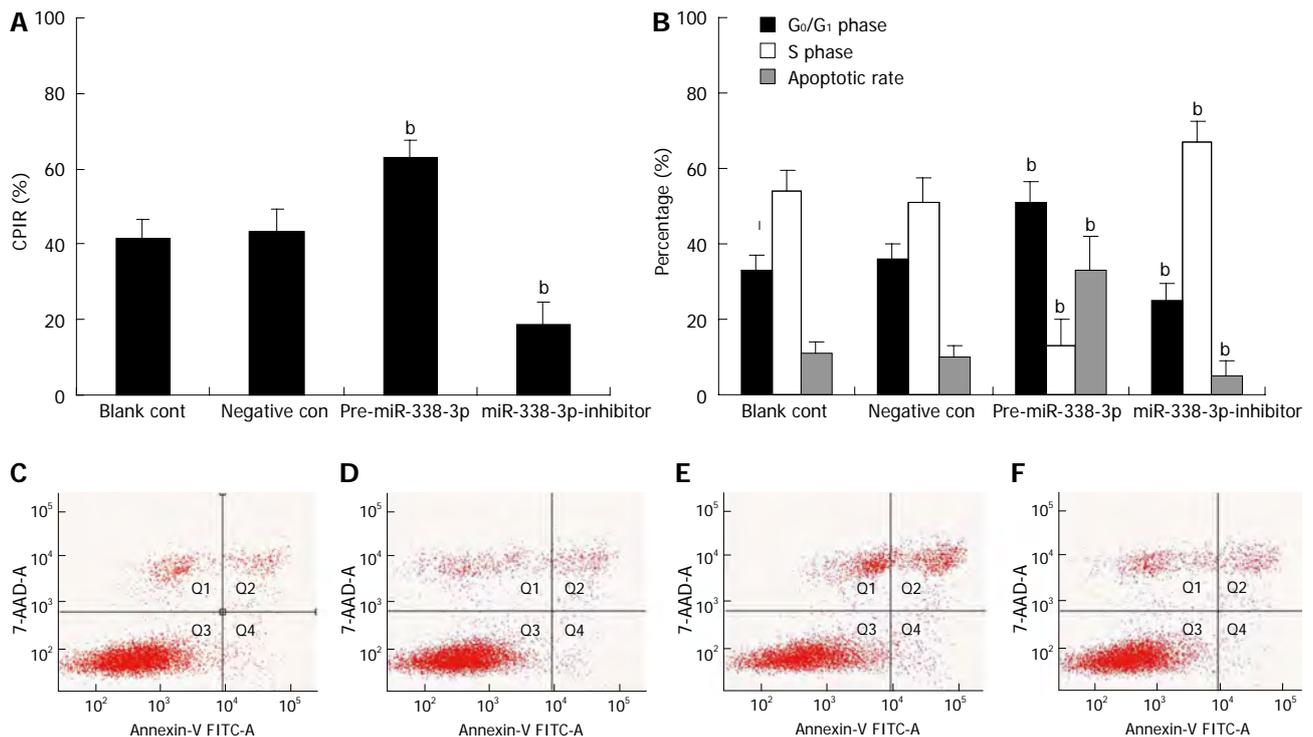


Figure 4 Effects of miRNA-338-3p on cell proliferation and apoptosis in colorectal carcinoma cells. **A:** Cell proliferation was determined by 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide assay. Cellular proliferation inhibition rate (CPIR) in the presence of pre-miRNA-338-3p (miR-338-3p) or miR-338-3p-inhibitor was compared with that of the controls; $n = 6$, mean \pm SD. ^b $P < 0.01$ vs control group; **B:** Effects of pre-miR-338-3p and miR-338-3p-inhibitor on cell-cycle in SW-620 cells. The percentages of cells in G₀/G₁ phase and S phase and apoptotic rate were measured by computing the ratio of the number of corresponding cells to total cells; $n = 3$, mean \pm SD. ^b $P < 0.01$ vs control group; **C-F:** Apoptosis analysis of transduced cells by flow cytometry. **C:** Blank control; **D:** SW-620 cells transduced with lentivirus pLV-THM-control; **E:** SW-620 cells transduced with lentivirus pLV-THM-miR-338-3p; **F:** SW-620 cells transduced with lentivirus pLV-THM-miR-338-3p-inhibitor. The right lower quadrant (FITC⁺/PI) shown as apoptotic cells.

and apoptosis in CRC. We found that the proliferative potential was suppressed after restoration of miR-338-3p expression in CRC cells transduced by lentivirus vector, pLV-THM-miR-338-3p. However, the downregulation of miR-338-3p, due to transducing by lentivirus vector pLV-THM-miR-338-3p-inhibitor into SW-620 cells, induced CRC cell proliferation. Cell cycle status and apoptosis are usually closely associated. Cells failing to progress to mitosis are destined for apoptosis. Besides cell-cycle arrest, the inhibition of cell growth observed in CRC cells with pre-miR-338-3p may also be a result of increased apoptosis. In this study, treatment of lentivirus pLV-THM-miR-338-3p caused G₀/G₁ phase arrest and blocked cells from entering S phase. Interestingly, as seen in other tumor cells, we clearly demonstrated that pre-miR-338-3p induced significant apoptosis in CRC cells, as demonstrated by flow cytometry. These data demonstrate that miR-338-3p is a potential tumor suppressor for CRC. However, the exact mechanisms of miR-338-3p remain unknown.

With the application of bioinformatics prediction programs, such as TargetScan, PicTar and MiRanda, we found that miR-338-3p and the 3'-UTR of SMO mRNA had complementary binding sites. From this, we hypothesized that SMO may be a new target of miR-338-3p in CRC; however, this finding has not yet been reported.

SMO, a seven-membrane-spanning receptor is a fundamental component of the Hh signaling pathway and an important anticancer drug target^[25-27]. Once activated, SMO triggers a series of intracellular events with resultant activation of the zinc finger transcription effectors including Gli, which in turn regulates cell proliferation, differentiation, apoptosis and invasion^[28-30]. It has been reported that 3-Keto-N-(aminoethyl-aminocaproyl-dihydrocinnamoyl) cyclopamine (KAAD-cyclopamine), a synthetic specific antagonist of SMO, markedly inhibits hepatocellular carcinoma cell growth and motility by binding to SMO^[31]. Indeed, in our study, downregulation of SMO occurred in response to lentivirus vector pLV-THM-miR-338-3p transduction into CRC cells, and significant upregulation of SMO occurred in response to lentivirus vector pLV-THM-miR-338-3p-inhibitor transduction. Consistent with Huang *et al.*^[32], our results suggest that SMO is a direct target of miR-338-3p in CRC cells.

We deduced that miR-338-3p inhibited CRC cell proliferation, likely through downregulating SMO. To confirm this, we performed RNA interference to knock down SMO in CRC cells before transduction with miR-338-3p-inhibitor. We showed that anti-SMO-siRNA could significantly, but not completely, inhibit miR-338-3p-inhibitor-induced proliferation of CRC cells.

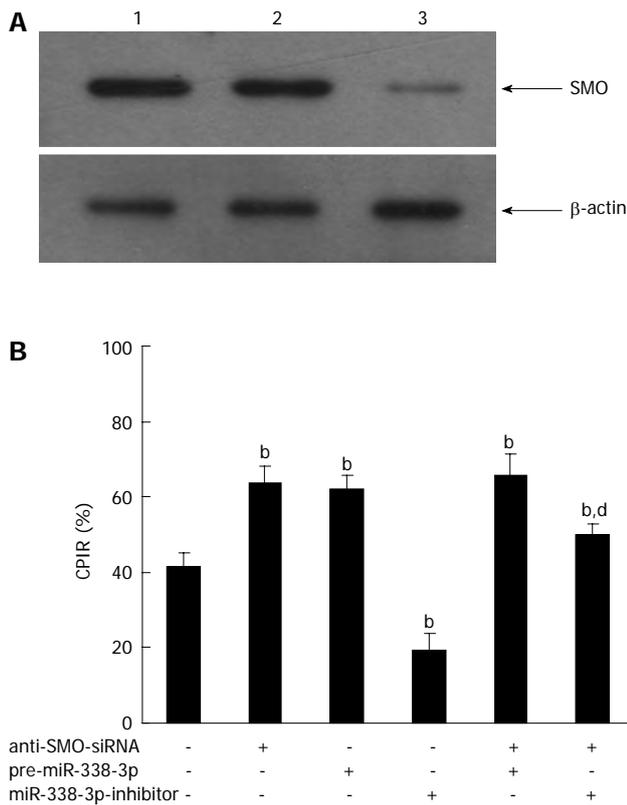


Figure 5 Ectopic expression of miRNA-338-3p affects proliferation of colorectal carcinoma cells by targeting smoothened. SW-620 cells were pretreated with or without anti-smoothened (SMO)-siRNA (50 nmol/L) for 24 h prior to transduction with lentivirus pLV-TM-miRNA-338-3p (miR-338-3p) or pLV-TM-miR-338-3p-inhibitor. **A:** Western blotting analysis showing that SMO protein reduced markedly after transfection with anti-SMO-siRNA. Equal loading was confirmed by using β -actin. Lane 1, blank control; lane 2, SW-620 cells transfected with control siRNA; lane 3, SW-620 cells transfected with anti-SMO-siRNA; **B:** Cell proliferation was determined by 3-(4,5-dimethyl-2 thiazoyl)-2,5-diphenyl-2H-tetrazolium bromide assay. Enhancement of SW-620 cell proliferation by miR-338-3p-inhibitor was largely, but not completely, abrogated by anti-SMO-siRNA [cellular proliferation inhibition rate (CPIR) from 19.2% to 50.9%]; $n = 3$, mean \pm SD. ^b $P < 0.01$ vs negative control group. ^d $P < 0.01$ vs sole miR-338-3p-inhibitor group.

These results confirmed that the inhibitory effect of miR-338-3p on CRC cell proliferation was largely, but not completely, mediated by SMO, suggesting that miR-338-3p could regulate other SMO-independent signaling pathways to promote CRC growth. We think that our results, which identify SMO as a target for miR-338-3p in the context of CRC cell line, fit well within a dynamic view of the miRNA-mediated regulation of gene expression. It is well known and widely predicted that the relationship between miRNAs and target mRNAs is not a “one to one” connection, because the same mRNA can be regulated by more than one miRNA, and that the choice of how many and which miRNAs target one 3'-UTR is strongly determined by the specific cellular environment^[33-35]. An miRNA that regulates targets playing opposite roles in the control of cell proliferation may act as a tumor suppressor in some cancers and as an oncogene in others, depending on which targets are driving tumorigenesis in that specific

cellular milieu^[36].

In summary, we have described miR-338-3p as a direct regulator of SMO expression in CRC, showing a new mechanism responsible for SMO upregulation in CRC. These findings further outline the importance of miR-338-3p in CRC carcinogenesis. However, it should be emphasized that our results were generated from cultured CRC cells and that they might not necessarily and comprehensively reflect the situation *in vivo*^[37]. Further experiments, beyond the scope of this study, are required to elucidate the antitumor mechanisms of miR-338-3p in athymic mice.

COMMENTS

Background

miRNAs regulate gene expression by mainly binding to the 3'-untranslated region (UTR) of the target mRNAs, leading to mRNA degradation or translation inhibition. miRNAs are aberrantly expressed in various cancers, suggesting that they play a vital role as a novel class of oncogenes or tumor suppressor genes, depending on the targets they regulate.

Research frontiers

Colorectal carcinoma (CRC) is one of the most serious malignancies in China. Our previous study has shown that loss of miRNA-338-3p (miR-338-3p) expression is associated with clinical aggressiveness of CRC. In this study, the authors report the regulatory effect of miR-338-3p on proliferation and apoptosis of CRC cells.

Innovations and breakthroughs

Some human miRNAs are consistently deregulated in human cancer, suggesting a role for these genes in tumorigenesis. Authors previous study has also shown that loss of miR-338-3p expression is associated with clinical aggressiveness of CRC. The authors demonstrated that forced expression of miR-338-3p in CRC cells suppressed cell growth, whereas inhibition of miR-338-3p promoted cell growth. Furthermore, smoothened (SMO) was identified as a direct target of miR-338-3p. The antiangiogenic role of miR-338-3p was determined as tumor suppressor.

Applications

This study indicates that miR-338-3p suppresses cell growth by targeting the SMO gene in CRC *in vitro* and miR-338-3p might be a novel potential strategy for CRC treatment.

Terminology

Most miRNAs are thought to control gene expression by base-pairing with the miR-recognizing elements, 3'-UTR, found in their messenger target. Not surprisingly, with the application of bioinformatics predictions, we find that miR-338-3p and SMO mRNA 3'-UTR has complementary binding sites.

Peer review

miR-338-3p could suppress CRC growth ability by inhibiting SMO protein expression. This study provides evidence for antiangiogenic activity of miR-338-3p in the development of CRC, and may be developed as a useful biomarker or therapeutic target in CRC.

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Non-invasive panel tests for gastrointestinal motility monitoring within the MARS-500 Project

Aldo Roda, Mara Mirasoli, Massimo Guardigli, Patrizia Simoni, Davide Festi, Boris Afonin, Galina Vasilyeva

Aldo Roda, Mara Mirasoli, Massimo Guardigli, Department of Chemistry "G. Ciamician", University of Bologna-Alma Mater Studiorum, 40126 Bologna, Italy

Patrizia Simoni, Davide Festi, Department of Medical and Surgical Sciences, University of Bologna-Alma Mater Studiorum, 40138 Bologna, Italy

Boris Afonin, Galina Vasilyeva, State Scientific Center of Russian Federation-Institute of Biomedical Problems of the Russian Academy of Sciences (IBMP), 123007 Moscow, Russia

Author contributions: Roda A designed the research; Mirasoli M, Simoni P and Festi D developed experimental protocols and materials; Afonin B and Vasilyeva G supervised the experiments during the simulation of a manned mission to Mars; Guardigli M analyzed the data; Roda A, Mirasoli M and Guardigli M wrote the paper; Roda A and Afonin B revised the final version of the paper.

Correspondence to: Aldo Roda, Professor, Department of Chemistry "G. Ciamician", University of Bologna-Alma Mater Studiorum, Via Selmi 2, Bologna 40126, Italy. aldo.roda@unibo.it

Telephone: +39-51-343398 Fax: +39-51-343398

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Abstract

AIM: To develop an integrated approach for monitoring gastrointestinal motility and inflammation state suitable for application in long-term spaceflights.

METHODS: Breath tests based on the oral administration of ^{13}C -labeled or hydrogen-producing substrates followed by the detection of their metabolites ($^{13}\text{CO}_2$ or H_2) in breath were used to measure gastrointestinal motility parameters during the 520-d spaceflight ground simulation within the MARS-500 Project. In particular, the gastric emptying rates of solid and liquid contents were evaluated by ^{13}C -octanoic acid and ^{13}C -acetate breath tests, respectively, whereas the oro-cecal transit time was assessed by an inulin H_2 -breath test, which was performed simultaneously with the ^{13}C -

octanoic acid breath test. A ready-to-eat, standardized pre-packaged muffin containing 100 mg of ^{13}C -octanoic acid was used in the ^{13}C -octanoic acid breath test to avoid the extemporaneous preparation of solid meals. In addition, a cassette-type lateral flow immunoassay was employed to detect fecal calprotectin, a biomarker of intestinal inflammation. Because no items could be introduced into the simulator during the experiment, all materials and instrumentation required for test performance during the entire mission simulation had to be provided at the beginning of the experiment.

RESULTS: The experiments planned during the simulation of a manned flight to Mars could be successfully performed by the crewmembers without any external assistance. No evident alterations (*i.e.*, increasing or decreasing trends) in the gastric emptying rates were detected using the ^{13}C -breath tests during the mission simulation, as the gastric emptying half-times were in the range of those reported for healthy subjects. In contrast to the ^{13}C -breath tests, the results of the inulin H_2 -breath test were difficult to interpret because of the high variability of the H_2 concentration in the breath samples, even within the same subject. This variability suggested that the H_2 -breath test was strongly affected by external factors, which may have been related to the diet of the crewmembers or to environmental conditions (*e.g.*, the accumulation of hydrogen in the simulator microenvironment). At least in closed microenvironments such as the MARS-500 simulator, ^{13}C -breath tests should therefore be preferred to H_2 -breath tests. Finally, the fecal calprotectin test showed significant alterations during the mission simulation: all of the crewmembers were negative for the test at the beginning of the simulation but showed various degrees of positivity in at least one of the subsequent tests, thus indicating the onset of an intestinal inflammation.

CONCLUSION: Breath tests, especially those ^{13}C -based, proved suitable for monitoring gastrointestinal motility in the 520-d isolation experiment within

MARS-500 project and can be applied in long-term spaceflights.

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Key words: Breath test; Gastrointestinal inflammation; Gastrointestinal motility; Spaceflight; Stress

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INTRODUCTION

A manned mission to Mars is currently starting to garner a consistent level of support, as exploration roadmaps are under study by various space agencies. Nevertheless, several issues related to the health of humans during such a long space mission still must be solved.

Extended-duration space missions expose the crewmembers to microgravity, radiation and a stressful environment due to mission-related factors (*e.g.*, confinement, isolation, anxiety, physiologic stress, sleep deprivation and modifications of their nutrition regimes, circadian rhythms and microbial environments) that affect their physiological status^[1]. To properly monitor the crewmembers' health status during a real space mission, a suitable panel of biochemical tests and related analytical instrumentation should be developed, implemented in the space module and validated for its clinical utility and applicability in spaceflight. These tests should be easily performed onboard by the crewmembers on non-invasively collectable biological samples (*e.g.*, saliva, breath expatriate, urine, or stool) and employing compact devices in a point-of-care format.

Among the alterations that might occur in long-term spaceflights, changes in the gastrointestinal (GI) motility and related gut inflammatory states are of particular relevance. The main factors affecting GI motility are the physical properties of the solid and liquid contents of the stomach and intestine and the functional, hormonal and enzymatic changes in those organs. Spaceflight-related changes in GI function, such as fluid shifts, combined with reduced fluid intake, would tend to decrease GI motility. Although GI motility has not been systematically studied in spaceflight, a significant increase in the mouth-to-caecum transit time has been demonstrated in ground simulations (10 d of -6° head-down bed resting^[2,3] and water immersion^[4]).

Previous studies have demonstrated that adequate nutritional status is critical to maintaining crew health during extended-duration spaceflight^[5-8], and a common cause of reduced dietary intake, especially during the first d of a mission, is space motion sickness^[9]. The impact of

psychological, physical, and immunological stressors on GI motility, duodenal and biliary secretion, epithelial permeability, and inflammation is currently thoroughly documented, and stress has a major influence on digestive diseases. Gastrointestinal motor dysfunctions, mainly caused by stress conditions, alteration of circadian rhythms and nutritional regimen, may also represent themselves as additional stress factors^[10,11]. Decreased GI motility will, in turn, result in delayed intestinal absorption, alterations in the intestinal microflora and decreased bioavailability of orally administered drugs^[12]. Such possible alterations must be expeditiously and continuously detected to guide the adoption of the actions necessary to avoid negative consequences to the crewmembers' health and, more generally, wellness (and thus to the crew's efficiency).

In this work, we present an integrated approach to the non-invasive monitoring of GI motility and inflammation state that was optimized in the frame of the MARS-500 project. This project was realized by the State Scientific Center of the Russian Federation-Institute of Biomedical Problems of the Russian Academy of Sciences (IBMP), under the auspices of Roscosmos and the Russian Academy of Sciences and in collaboration with the European Space Agency and other space agencies and institutions from all over the world. The project consisted of several isolation experiments, including a final 520-d isolation (the longest spaceflight ground simulation ever conducted) designed to simulate a round-trip manned mission to Mars. The project aimed at obtaining useful information about physical and psychological problems that astronauts might face during a long stay onboard an interplanetary space vehicle and to set up technologies for monitoring their health status with possible application in real space missions.

The integrated approach herein described employed breath tests (BTs) for the evaluation of GI motility. Indeed, ^{13}C - and H_2 -BTs based on the oral administration of ^{13}C -labeled or hydrogen-producing substrates followed by the detection of the metabolites of these substrates ($^{13}\text{CO}_2$ or H_2 , respectively) in the breath represent a convenient, non-invasive and efficient procedure for obtaining information on motor and organ functions of the GI system. Such tests are routinely used for the detection of alterations in GI motility, bacterial overgrowth, and lactose intolerance, among other issues, and for the diagnosis of infection with *Helicobacter pylori*^[13-15]. We evaluated the gastric emptying rates of solid and liquid content by ^{13}C -octanoic acid and ^{13}C -acetate BT, respectively, whereas the orocecal transit time was assessed by an H_2 -BT that used inulin as the hydrogen-producing substrate (the latter BT was performed simultaneously with the ^{13}C -octanoic acid BT for the measurement of the gastric emptying rate of solids). In addition, a cassette-type lateral flow immunoassay was employed for detecting fecal calprotectin, a biomarker of intestinal inflammation.

Because they are non-invasive and easily self-performed, BTs are potentially transferrable to the space environment, provided protocol standardization and the

development of compact on-board instrumentation. Miniaturized instrumentation based on electrochemical gas sensors is available for H₂-BT, whereas compact instrumentation based on non-dispersive infrared spectroscopy (NDIRS) has been developed as an alternative to isotope ratio mass spectrometry (IRMS) for the measurement of ¹³CO₂ in breath^[16]. In perspective, miniaturized dedicated analytical instrumentation suitable for on-board operation by the crewmembers will make this integrated approach applicable in real space missions, thus providing a useful tool for the early detection of dysfunctions of the GI system and the adoption of suitable countermeasures, such as diet adjustments or pharmacological interventions.

MATERIALS AND METHODS

Subjects

The crew was composed of six male subjects, who at the beginning of the experiment had a median age of 31 years (range 27-38 years), median body weight of 81 kg (range 74-100 kg), and median body mass index of 26.3 kg/m² (range 23.6-32.3 kg/m²). During the mission simulation, all of the crewmembers received the same diet, the composition of which was almost identical to that of the diet used in the International Space Station^[17].

Ethics

All of the scientific investigations performed in the frame of the MARS-500 experiments were reviewed and approved by the IBMP Committee on Bioethics, and all of the volunteers signed the written informed consent for participation in the experiment.

Materials employed for diagnostic tests

A standard muffin meal (EXPIROGer[®], manufactured and packaged by Sofar SpA, Milan, Italy) containing 100 mg of ¹³C-octanoic acid was employed in the ¹³C-BT for the measurement of the gastric emptying rate of solid meals. The muffin (weight 100 g) had a 378 kcal (1589 kJ) calorie content and the following composition: 5.5 g of proteins, 57.5 g of carbohydrates, 14.0 g of fats (corresponding to 5.8%, 60.8%, and 33.3% of the total calories, respectively), 1.1 g of dietary fiber and 16.7% moisture. Stable ¹³C-isotope-labeled sodium acetate (99% isotope purity) was purchased from Cambridge Isotope Laboratories (Andover, MA). Inulin (Beneo[™] HP-Gel) with a degree of polymerization of 5-60 was obtained from Orafit (Oreya, Belgium). The enteral nutrition solution Nutrizon standard was manufactured by Otsuka Pharmaceutical (Tokyo, Japan) and had (for 100 mL) a 110 kcal (420 kJ) calorie content, 15% of which were from proteins and 55% from carbohydrates. The semiquantitative rapid immunochromatographic test for the detection of calprotectin in feces (PreventID[®] Cal Detect[®]) was produced by Preventis GmbH, Wiesenstr, Germany. The test allowed an easy visual evaluation of fecal calprotectin, providing three degrees of positivity: low (< 15 µg/g),

medium (15-60 µg/g), and high (> 60 µg/g).

Assay protocols

Breath tests were performed during the Baseline Data Collection period (BDC; before the start of the simulation) and in three separate experimental sessions at approximately d 100, 240 and 475 of the mission simulation. During each experimental session, different ¹³C-BTs performed on the same subject were staggered by at least 3 d to allow the washout of the administered substrates and the recovery of basal ¹³C levels.

The combined ¹³C- and H₂-BT for the measurement of the gastric emptying rate of solids and the orocecal transit time consisted of the simultaneous administration of the EXPIROGer[®] standard meal and inulin, followed by the measurement of the kinetics of the appearance of ¹³CO₂ and H₂ in the breath. In preparation for the test, the crewmembers were requested to refrain from fatty meals or a high intake of dietary fiber the day before the test. Antibiotics, fermented milk products and laxatives were also avoided during the 10-d period preceding the test. After an overnight fast, breath samples were collected to measure the basal levels of ¹³CO₂ and H₂. Subsequently, the subjects received the EXPIROGer[®] standard meal and 5.0 g of inulin dissolved in 200 mL of water. Breath samples for ¹³CO₂ analysis were collected up to 240 min after substrate ingestion in 12-mL glass tubes, which were then transferred outside the simulator for analysis. Samples for the evaluation of breath H₂ content were collected in plastic bags up to 440 min after substrate ingestion, and the concentration of H₂ was measured on-board immediately after each breath sample had been collected. During the test, the subjects were allowed to drink water and, after 4 h, to resume their usual dietary regimens.

The ¹³C-BT for the measurement of the gastric emptying rate of liquids consisted of the administration of sodium ¹³C-acetate followed by the measurement of the kinetics of the appearance of ¹³CO₂ in the breath. After an overnight fast, breath samples were collected to measure the basal level of ¹³CO₂. Subsequently, the subjects orally received 150 mg of sodium ¹³C-acetate dissolved in 500 mL of Nutrizon enteral nutrition solution, and breath samples were collected up to 240 min after substrate ingestion in 12-mL glass tubes, which were then transferred outside the simulator for analysis. After assuming the substrate, the subjects were requested not to ingest any additional food or drink until the end of the test.

The fecal calprotectin test was performed directly by the crewmembers during the BDC and on day 130, 220, and 475 of the mission simulation following the instructions provided by the manufacturer (the test was repeated twice in each experimental session).

Sample analysis

For the measurement of the ¹³CO₂/¹²CO₂ ratio, breath samples were analyzed using a BreathMAT IRMS (Ther-

Table 1 Dynamics of the body mass (kg) of the crewmembers

| | Crewmember | | | | | |
|---------------------------|------------|-------|------|------|------|------|
| | A | B | C | D | E | F |
| BDC | 81.5 | 99.5 | 76.6 | 86.9 | 82.5 | 73.5 |
| Exp. session 1 | +3.5 | -2.0 | +4.3 | -1.0 | +0.1 | +0.2 |
| Exp. session 2 | +3.3 | -8.2 | +4.0 | -4.6 | -2.0 | -4.7 |
| Exp. session 3 | +1.5 | -20.4 | -3.8 | -6.9 | -1.2 | -6.8 |
| End of mission simulation | -1.1 | -22.6 | -5.4 | -9.7 | -4.0 | -7.2 |

BDC: Baseline Data Collection.

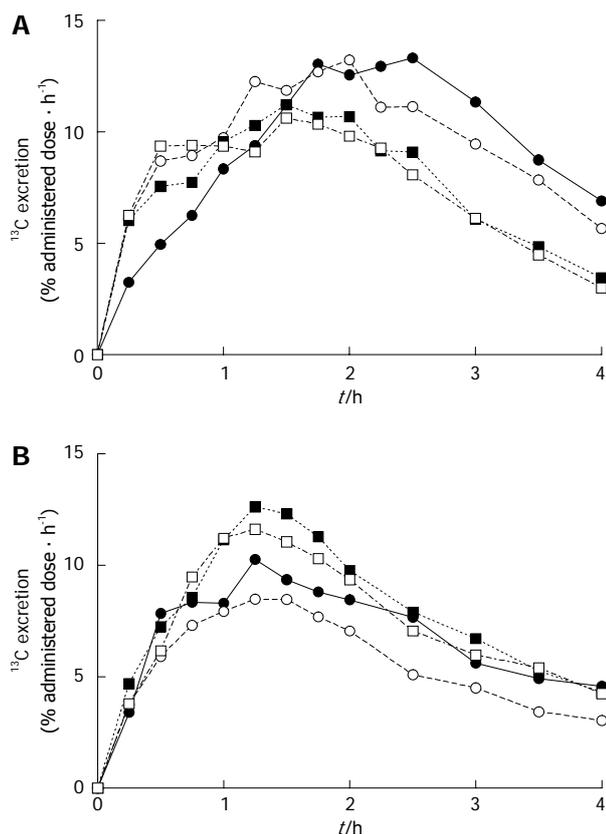


Figure 1 ^{13}C -breath test for the evaluation of gastric emptying rates. Representative $^{13}\text{CO}_2$ excretion kinetic profiles obtained in the ^{13}C -breath test for the evaluation of the gastric emptying rates of (A) solids and (B) liquids performed during the Baseline Data Collection period (\bullet) and during the mission simulation (experimental session 1: \circ ; experimental session 2: \square ; experimental session 3: \triangle).

mo Finnigan MAT GmbH, Bremen, Germany). The measurement of breath H_2 levels was performed on-board by the crewmembers using a portable H_2 analyzer equipped with a miniaturized electrochemical cell (Lactotest 102, Medical Electronic Construction R&D sprl, Brussels, Belgium).

Statistical analysis

The ^{13}C -BT results, given as the $^{13}\text{CO}_2$ content of the exhaled CO_2 expressed in $\delta\text{‰}$ PDB units (zero $\delta\text{‰}$ PDB corresponds to 1.12372% ^{13}C atoms), were processed to evaluate the rate of excretion of $^{13}\text{CO}_2$ produced by the metabolism of the ^{13}C -labeled substrate, which was expressed as a percentage of the administered dose per

hour. To this purpose, the total expiratory CO_2 production of each subject was assumed to be 300 mmol/m^2 of body surface/h^[18], and the body surface was computed as described by Haycock *et al*^[19]. For the evaluation of the relevant gastric emptying parameters, the excretion kinetics were analyzed by a least-square fitting procedure using a suitable equation^[18], and the gastric emptying half-times were calculated from the coefficients of the equation^[20].

The H_2 -BT results, given as the H_2 breath concentrations in ppm, were processed for evaluating the enrichment of H_2 in the breath over the basal value due to the fermentation of inulin by the intestinal microflora and then plotted as a function of time; the orocecal transit time was assessed as the time at which the breath hydrogen content rose 10 ppm above the basal value^[21].

To assess alterations in GI motility, the results of the breath tests performed during the BDC and during the mission simulation were compared by one-way ANOVA for matched data with Dunnett's post-test using GraphPad Prism version 5.03 (GraphPad Software, San Diego, CA). Values of $P < 0.05$ were considered to be statistically significant.

RESULTS

Crew health status

The periodic blood biochemical function tests and clinical examinations during the mission simulation did not show any significant pathology or physiological alteration. Comparison of the body weights of the crewmembers during the BDC and at the end of the mission simulation indicated that one subject (B) displayed a significant reduction in weight (-21%), whereas for the other subjects, the reduction was lower (C, D, E and F) or negligible (A). Although no net increases in body weight were observed, subjects A and C experienced a rise in body mass during the first part of the experiment (Table 1).

^{13}C -BT for gastric emptying rate

Figure 1 shows representative $^{13}\text{CO}_2$ excretion kinetic profiles obtained in the ^{13}C -BT for the evaluation of the gastric emptying rates of solids and liquids performed during BDC and in the different experimental sessions during the mission simulation. The gastric emptying half-times obtained for the six crewmembers by analyzing the $^{13}\text{CO}_2$ excretion kinetic profiles using the procedure described in the Statistical Analysis section are reported in Table 2.

It can be observed that at the beginning of the simulation (BDC), certain subjects (*i.e.*, A and D) had long gastric emptying half-times of solids (*e.g.*, 4.4 and 5 h for A and D, respectively) and that this behavior was maintained in most of the experimental sessions performed during the mission simulation. As a general rule, long gastric emptying half-times of solids were paralleled (albeit to a lesser extent) by relatively long gastric emptying half-times of liquids, although a large variability in the differences between the two times was observed. Nevertheless,

Table 2 Gastric emptying half-times (h) evaluated by ¹³C-breath test

| Experimental session | Crewmember | | | | | | mean ± SD |
|----------------------|------------|-----|-----|-----|-----|-----|-----------|
| | A | B | C | D | E | F | |
| Solids | | | | | | | |
| BDC | 4.4 | 2.8 | 3.3 | 5.0 | 3.2 | 2.8 | 3.5 ± 1.0 |
| Exp. session 1 | 2.7 | 2.7 | 2.2 | 6.2 | 2.9 | 2.9 | 3.2 ± 1.5 |
| Exp. session 2 | 3.7 | 2.2 | 2.9 | 3.5 | 2.8 | 2.5 | 2.8 ± 0.5 |
| Exp. session 3 | 4.9 | 2.2 | 2.7 | 4.8 | 3.4 | 2.6 | 3.3 ± 1.2 |
| Liquids | | | | | | | |
| BDC | 2.6 | 2.3 | 2.4 | 3.0 | 2.8 | 1.9 | 2.5 ± 0.4 |
| Exp. session 1 | 2.6 | 2.0 | 2.2 | 2.8 | 2.6 | 2.5 | 2.5 ± 0.3 |
| Exp. session 2 | 2.6 | 2.1 | 2.7 | 2.6 | 2.6 | 2.5 | 2.5 ± 0.2 |
| Exp. session 3 | 2.9 | 2.0 | 2.6 | 4.0 | 2.8 | 2.6 | 2.8 ± 0.7 |

BDC: Baseline Data Collection.

no evident increasing or decreasing trend in the gastric emptying half-times was detected for any crewmember during the mission simulation; most of the measured gastric emptying half-times were in the range of those reported for healthy subjects^[18,22], although in certain cases, rather high values were obtained.

H₂-BT for orocecal transit time

Figure 2 shows the H₂ excretion kinetic profiles obtained in the H₂-BT for the evaluation of the orocecal transit time. The H₂ breath concentrations showed a large variability, sometimes decreasing below the basal level, which increased the difficulty of identifying the H₂ excretion kinetic profiles and evaluating the orocecal transit time by applying the standard criteria reported in the literature (*i.e.*, by identifying the first time at which the breath hydrogen concentration increased by at least 10 ppm above the baseline value).

Although, in several cases, acceptable H₂ excretion profiles were obtained (for example, crewmember D showed high H₂ breath concentrations at long times after substrate ingestion, which were paralleled by a delayed gastric emptying of solids), the overall results suggested that the inulin H₂-BT was negatively affected by external factors, which may have been related to the simulation environment, such as the closed chamber simulating the space station.

Fecal calprotectin test

Table 3 summarizes the results of the fecal calprotectin test for the evaluation of intestinal inflammation performed during the BDC and during the mission simulation. The results are given as scores according to the semi-quantitative evaluation of calprotectin concentration in fecal samples that was performed with the test. Notably, the crewmembers were negative for the fecal calprotectin test during the BDC, but for all of them positive results were obtained in at least one of the tests performed during the mission simulation. The observed degrees of intestinal inflammation varied from low (in two subjects) to high (in four subjects).

Table 3 Results of the fecal calprotectin test¹

| Experimental session | Crewmember | | | | | |
|----------------------|------------|--------------------|---|---|------------------|------------------|
| | A | B | C | D | E | F |
| BDC | - | - | - | - | - | - |
| Day 130 | - | +++ | + | + | - | +++ |
| Day 220 | - | -/+ ² | - | - | +++ | -/+ ² |
| Day 475 | +++ | -/+++ ² | + | - | -/+ ² | +++ |

¹Legend: (-) negative, (+) low positivity (< 15 µg/g), (++) medium positivity (15-60 µg/g), (+++) high positivity (> 60 µg/g); ²The repeated tests gave different results. BDC: Baseline Data Collection.

DISCUSSION

Continuous and non-invasive monitoring of the health status of the crewmembers during space missions requires the development of cutting-edge technologies; their requirements (simple analytical procedures, possibility of self-administration, use of portable point-of-care instrumentation, long shelf-life of reagents) are similar to those faced in critical medicine (*e.g.*, clinical medicine in emergency situations, remote field locations or third-world countries). Thus, new technological solutions that are suitable for the space environment will benefit medical diagnostics for all of us.

In this work, ¹³C- and H₂-BT were employed for the non-invasive monitoring of GI motility during the MARS-500 project. The accuracy of ¹³C- and H₂-BT for the measurement of motor functions of the GI system has been demonstrated by several studies^[21,23-25]. However, the application of BT in the space environment still requires certain improvements. For example, the ¹³C-octanoic acid BT is typically performed using extemporaneously prepared meals (*e.g.*, ¹³C-octanoic acid is incorporated into egg yolk, which is then pan-cooked and consumed with bread and butter), which makes meal standardization difficult and limits test reproducibility. To overcome this drawback, we employed a ready-to-eat test meal (a muffin containing 100 mg of ¹³C-octanoic acid) with carbohydrate, lipids, proteins and calorie content optimized for the BT performance. The long-term stability of this test meal and its suitability for the measurement of the gastric emptying rate of solids have been evaluated in a multicenter study^[26]. Moreover, the muffin is designed for diagnostics; thus, it is gluten-, lactose- and glucose-free to enable its administration to subjects who are affected by celiac disease, lactose intolerance or diabetes, and the unpleasant taste and odor that are characteristic of short-chain fatty acids are efficiently masked. We also combined the ¹³C-octanoic acid BT for measuring the gastric emptying rate of a solid meal and the inulin H₂-BT for measuring the orocecal transit time into a single test to reduce the number of experimental sessions in the mission simulation and to allow the direct comparison of two different indexes of GI motility, avoiding subject day-to-day variability.

Regarding the instrumentation employed for the an-

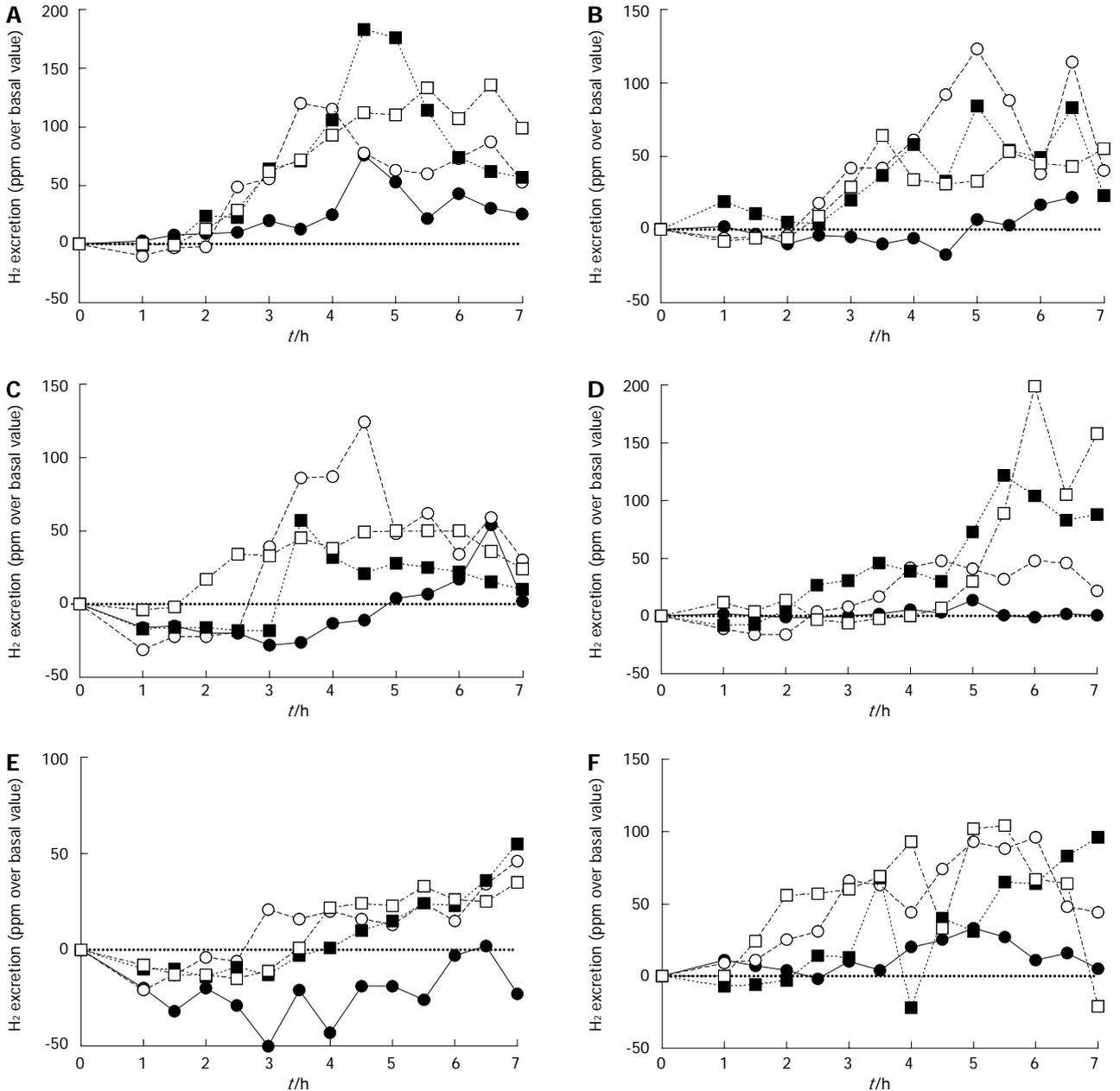


Figure 2 H₂-breath test for the evaluation of orocecal transit time. Hydrogen excretion kinetic profiles obtained in the H₂-breath test (BT) for the evaluation of the orocecal transit time performed during the Baseline Data Collection period (•) and during the mission simulation (experimental session 1: ◦; experimental session 2: ◼; experimental session 3: ◻). This BT was performed simultaneously with the ¹³C-octanoic acid BT for the evaluation of the gastric emptying rate of solids (A-F).

analysis of the breath samples, the measurement of the ¹³CO₂/¹²CO₂ ratio was performed by IRMS in an external laboratory. However, NDIRS, which is more amenable to miniaturization, could also be used [17,20]. Work is in progress to develop a miniaturized hybrid analytical device combining the NDIRS technology for ¹³CO₂ measurement with the fuel cell technology for H₂ measurement employed in the Lactotest 102 H₂ breath analyzer. Such a device will allow the simultaneous onboard measurement of the ¹³CO₂/¹²CO₂ ratio and H₂ concentration in a single breath sample, thus avoiding the need for separate breath sample collection in dual BT.

The results obtained during the MARS-500 experiments did not show significant alterations in the gastric

emptying rates of solids and liquids (researchers are currently increasingly inclined to use only gastric emptying half-times when reporting the results of the ¹³C-octanoic acid BT; therefore, we do not discuss other gastric emptying parameters, such as the lag time). Subjects A and D presented long gastric emptying half-times of solids with high variability, but no unambiguous trends were observed. Moreover, it should be taken into account that Choi *et al.* [27,28] suggested that the truncation of the observation period of ¹³C-octanoic acid BT to four hours could lead to an overestimation of gastric emptying half-times. Therefore, the long half-times measured for subjects A and D could be at least in part ascribed to this factor (indeed, these gastric emptying half-times were

close to or even longer than the observation period).

In contrast, the results of the H₂-BT for the oro-cecal transit time, performed simultaneously with the ¹³C-octanoic acid BT, were difficult to interpret because the high variability of the H₂ concentration in the breath samples did not allow a reliable evaluation of the oro-cecal transit times. Nevertheless, certain results suggested, as expected, a positive correlation with gastric emptying half-times. For example, in subject D, who showed the longest gastric emptying half-times for solids, the highest concentrations of H₂ in the breath were often detected at longer times in comparison with the other subjects. These results suggested that the H₂-BT was strongly affected by external factors, such as the diet of the crewmembers (hydrogen can be produced by the fermentation of other food sugars and related substances, such as dietary fiber) and the environmental conditions (*e.g.*, the possible accumulation of hydrogen in the simulator microenvironment). Indeed, hydrogen concentrations up to 30-40 ppm were recorded inside the simulator, whereas external values remained below 1.0 ppm. Moreover, the portable H₂ analyzer employed in this experiment required manual injection of the breath sample; thus, the reproducibility of the measurement could be improved by implementing automated sample management procedures. Nevertheless, in the absence of further information, it might be concluded that in closed microenvironments, such as the MARS-500 simulator, ¹³C-BTs should be preferred to H₂-based tests. In particular, the lactose ¹³C-ureide BT, which has been established as a reliable test for the assessment of oro-cecal transit time^[29,30], could represent an alternative to the inulin H₂-BT.

In contrast to ¹³C-BTs, the fecal calprotectin test detected significant alterations during the mission simulation: all of the crewmembers were negative for the test during the BDC but showed various degrees of positivity (from low for subjects C and D to high for subjects A, B, E, and F) in at least one of the tests performed during the mission simulation. Calprotectin is a sensitive fecal marker of intestinal inflammation that is used to differentiate between organic intestinal diseases (*e.g.*, chronic inflammatory diseases, infectious diseases, or colon cancer) and functional intestinal diseases (*e.g.*, irritable bowel syndrome)^[31,32]. Application of calprotectin test for screening asymptomatic subjects has also been reported^[33,34]. Fecal calprotectin can be determined with high specificity and sensitivity using the CalDetect[®] lateral flow immunoassay^[35]. Because it has been already demonstrated in animal models and humans that stress influences the inflammatory response^[36,37], the stress conditions experienced by the crewmembers could be responsible for the observed intestinal inflammation, although external factors related to diet and environment, as well as possible alterations in the intestinal microflora, cannot be excluded.

In conclusion, the results obtained in the MARS-500 mission simulation suggested that the stress level experienced by crewmembers during the mission simulation had no significant impact on the GI motility. Because

previous experiments performed in microgravity conditions showed alterations in the GI motility^[38,39], it could be concluded that microgravity should have a major impact on GI motor functions, whereas stress-related factors might contribute to the onset of motility alterations but are not the primary cause. Nevertheless, useful information on the possible application of BTs in future isolation experiments or real space missions has been obtained. Due to their simplicity of performance, ability to be performed repeatedly, safety, and non-invasiveness, ¹³C-BTs represent a promising approach for the monitoring of alterations of motor and/or organ functions of the GI system, thus moving space medicine closer to clinical observation systems used on Earth. In the MARS-500 experiments, ¹³CO₂ analysis in breath samples was performed by IRMS in an external analysis facility, but portable analytical instruments for ¹³CO₂ breath analysis (for example, based on the NDIRS technology) integrated within an informatics framework for data acquisition, analysis, and remote transmission will allow crewmembers to perform such tests autonomously. Regarding H₂-BT, suitable portable H₂ breath analyzers are already available, but the results suggested that the performance of this BT is strongly affected by external factors; thus, it could be concluded that in this type of application, ¹³C-BTs should be preferred to H₂-based tests. In addition, the measurement of fecal calprotectin by a cassette-type lateral flow immunoassay evidenced a significant degree of intestinal inflammation in all the crewmembers. Although no clinical symptoms associated with intestinal inflammation were reported during the mission simulation, the possibility that a combination of isolation, stress and dietary factors (*i.e.*, prolonged nutrition with canned and preserved foods) could favor the onset of this pathological status should be considered in future mission simulations or real space flights.

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COMMENTS

Background

Extended-duration space missions expose the crewmembers to microgravity, radiation, stress and other factors that can affect their physiological status. For instance, changes in gastrointestinal motility may result in the reduced intestinal

absorption of nutrients, alterations in the intestinal microflora and decreased bioavailability of orally administered drugs. Such possible alterations must be detected expeditiously to avoid negative consequences to the crewmembers' health and, more generally, wellness.

Research frontiers

The evaluation of the gastrointestinal motility during a real space mission requires biochemical tests that can be easily performed onboard by the crewmembers. Biological samples should be easily collectable in a microgravity environment (*e.g.*, saliva or breath expatriate) and analyzed using compact devices in a point-of-care format. Tests and related analytical instrumentation are to be implemented in the space module and validated for its clinical utility and applicability in spaceflight.

Innovations and breakthroughs

In this study, ¹³C- and H₂-breath tests for the monitoring of gastrointestinal motility have been designed to be self-performed without any external assistance by the subjects participating in the final 520-d isolation experiment in the frame of the MARS-500 project. The reagents for breath test performance have been optimized for long-term storage (no materials could be introduced into the simulator during the isolation period) and minimum preparation required before use; a portable H₂ analyzer equipped with a miniaturized electrochemical cell has been provided to allow the onboard measurement of breath H₂ levels by the crewmembers. A commercially available cassette-type lateral flow immunoassay was also employed for detecting fecal calprotectin, a biomarker of intestinal inflammation.

Applications

The study suggested that breath tests, especially those based on ¹³C, could be employed for the monitoring of alterations of motor and/or organ functions of the gastrointestinal system in future isolation experiments or real space missions.

Peer review

The authors present an interesting application of non-invasive gastrointestinal (GI) motility and lower intestinal inflammation tests in a closed-chamber space simulation. Although the results overall reveal no significant change in gastric emptying and require additional confirmation, this study represents an interesting demonstration of how GI monitoring may be achieved with very limited resources. This battery of tests could find application not only in outer space but also in bedside testing in a variety of clinical environments, both inpatient and outpatient.

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Evolution of disease phenotype in adult and pediatric onset Crohn's disease in a population-based cohort

Barbara Dorottya Lovasz, Laszlo Lakatos, Agnes Horvath, Istvan Szita, Tunde Pandur, Michael Mandel, Zsuzsanna Vegh, Petra Anna Golovics, Gabor Mester, Mihaly Balogh, Csaba Molnar, Erzsebet Komaromi, Lajos Sandor Kiss, Peter Laszlo Lakatos

Barbara Dorottya Lovasz, Michael Mandel, Zsuzsanna Vegh, Petra Anna Golovics, Lajos Sandor Kiss, Peter Laszlo Lakatos, First Department of Medicine, Semmelweis University, H-1083 Budapest, Hungary

Laszlo Lakatos, Istvan Szita, Tunde Pandur, Department of Medicine, Csolnoky F Province Hospital, H-8200 Veszprem, Hungary
Agnes Horvath, Department of Pediatrics, Csolnoky F Province Hospital, H-8200 Veszprem, Hungary

Gabor Mester, Mihaly Balogh, Department of Medicine, Grof Eszterhazy Hospital, H-8500 Papa, Hungary

Csaba Molnar, Department of Infectious Diseases, Magyar Imre Hospital, H-8400 Ajka, Hungary

Erzsebet Komaromi, Department of Gastroenterology Municipal Hospital, H-8100 Varpalota, Hungary

Author contributions: Lovasz BD and Lakatos L contributed equally to this work; Lovasz BD contributed to supervision, patient selection and validation, database construction and manuscript preparation; Lakatos L contributed to study design, data collection, supervision, patient selection and validation, database construction, and manuscript preparation; Pandur T, Mester G, Balogh M, Szita I, Molnar C, Komaromi E, Mandel M, Vegh Z, Golovics PA and Kiss LS contributed to database construction and manuscript preparation; Lakatos PL contributed to study design, data collection, supervision, patient selection and validation, database construction, statistical analysis, and manuscript preparation; all authors have approved the final draft submitted.

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Correspondence to: Peter Laszlo Lakatos, MD, PhD, First Department of Medicine, Semmelweis University, Korányi S. 2/A, H-1083 Budapest,

Hungary. lakatos.peter_laszlo@med.semmelweis-univ.hu

Telephone: +36-1-2100278 Fax: +36-1-3130250

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in adult and pediatric onset Crohn's disease (CD) populations, diagnosed between 1977 and 2008.

METHODS: Data of 506 incident CD patients were analyzed (age at diagnosis: 28.5 years, interquartile range: 22-38 years). Both in- and outpatient records were collected prospectively with a complete clinical follow-up and comprehensively reviewed in the population-based Veszprem province database, which included incident patients diagnosed between January 1, 1977 and December 31, 2008 in adult and pediatric onset CD populations. Disease phenotype according to the Montreal classification and long-term disease course was analysed according to the age at onset in time-dependent univariate and multivariate analysis.

RESULTS: Among this population-based cohort, seventy-four (12.8%) pediatric-onset CD patients were identified (diagnosed \leq 17 years of age). There was no significant difference in the distribution of disease behavior between pediatric (B1: 62%, B2: 15%, B3: 23%) and adult-onset CD patients (B1: 56%, B2: 21%, B3: 23%) at diagnosis, or during follow-up. Overall, the probability of developing complicated disease behaviour was 49.7% and 61.3% in the pediatric and 55.1% and 62.4% in the adult onset patients after 5- and 10-years of follow-up. Similarly, time to change in disease behaviour from non stricturing, non penetrating (B1) to complicated, stricturing or penetrating (B2/B3) disease was not significantly different between pediatric and adult onset CD in a Kaplan-Meier analysis. Calendar year of diagnosis ($P = 0.04$), ileal location ($P < 0.001$), perianal disease ($P < 0.001$), smoking ($P = 0.038$) and need for steroids ($P < 0.001$) were associated with presence of, or progression to, complicated disease behavior at diagnosis and during follow-up. A change in disease location was observed in 8.9% of patients and it was associated with smoking status ($P = 0.01$), but not with age at diagnosis.

Abstract

AIM: To investigate the evolution of disease phenotype

CONCLUSION: Long-term evolution of disease behavior was not different in pediatric- and adult-onset CD patients in this population-based cohort but was associated to location, perianal disease and smoking status.

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Key words: Crohn's disease; Age at diagnosis; Disease behavior; Disease course

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INTRODUCTION

Inflammatory bowel disease (IBD) is multifactorial: both genetic and environmental risk factors (*e.g.*, smoking, or appendectomy) contribute to its pathogenesis^[1]. During the past two decades, the incidence pattern of IBD has changed significantly^[2], showing both common and distinct characteristics. The phenotypic classification of Crohn's disease (CD) plays an important role in patient management, and may help predict the clinical course in CD patients^[3]. In 2005, the Montreal revision of the Vienna classification system was introduced^[4]. Although the broad categories for CD classification remained the same, changes were made within each category. Upper gastrointestinal (GI) disease is now classified independently of, or alongside, disease at more distal locations. Finally, perianal disease, which occurs independently of small bowel fistulae, is no longer classified as penetrating disease. Instead, a perianal modifier has been introduced, which may coexist with any disease behavior.

Using the Vienna classification system, it has been shown in clinical cohorts that there can be a significant change in disease behavior over time, whereas disease location remains relatively stable^[3,5]. In a landmark paper by Cosnes *et al*^[6], up to 70% of CD patients developed either penetrating or stricturing disease. Louis *et al*^[5] reported similar results in a Belgian study, in which 45.9% of patients had a change in disease behavior ($P < 0.0001$) during 10 years of follow-up, especially from non-stricturing, non-penetrating disease to either stricturing (27.1%; $P < 0.0001$) or penetrating (29.4%; $P < 0.0001$) forms. Age at diagnosis (before or after age 40) had no influence on either disease location or behavior. In contrast, disease location remained relatively stable during follow-up, with only 15.9% of patients exhibiting a change in disease location during the first 10 years. In addition, the probability of change in disease behavior in patients with initially non-stricturing, non-penetrating disease was 30.8% over nine years in a more recent Hun-

garian study^[7], in which data were obtained from referral centers.

More recently, authors from New Zealand^[3] showed in a population-based cohort study, that although > 70% percent of CD patients had inflammatory disease at diagnosis, 23% and 40% of patients with initial inflammatory disease progressed to complicated disease phenotypes after five and ten years of follow-up, respectively. This was not associated with age at onset. In contrast, disease location remained stable in 91% of patients with CD. Of note however, the median follow-up of CD patients was only 6.5 years. Similarly, in the IBSEN cohort, 36%, 49% and 53% of CD patients diagnosed between 1990 and 1994 initially had or developed either stricturing or penetrating complications^[8]. In addition, recent data suggest a change in the natural history of CD as shown by decreasing surgical rates^[9].

According to the available literature, pediatric onset CD runs a more aggressive course, including more extensive disease location, more upper GI involvement, growth failure, more active disease, and need for more aggressive medical therapy, in predominantly referral-center studies^[10-12]. However, data so far have been partly contradictory, and pediatric disease behavior seems to parallel that of adults^[13]. A Scottish study simultaneously compared disease behavior and location in pediatric and adult onset IBD patients^[14]. In childhood-onset patients, there was a clear difference in disease location at onset and after five years; with less ileum- and colon-only location among pediatric-onset patients, but more ileocolonic and upper gastrointestinal involvement ($P < 0.001$ for each). In addition, disease behavior after five years did not differ between the two groups. In contrast, disease phenotype was associated with location. However, the evolution of disease phenotype was not studied.

Because only limited data are available on the evolution of disease phenotype in patients with a pediatric- and adult-onset CD in from a single population-based cohort over a long-term follow-up, the aim of this study was to analyze the evolution of disease behavior and location in a population-based Veszprem province database according to the age-group at diagnosis, which included incident adult- and pediatric-onset CD populations diagnosed between January 1, 1977 and December 31, 2008.

MATERIALS AND METHODS

Patients

A well-characterized Hungarian cohort of 1420 incident cases of inflammatory bowel disease diagnosed between January 1, 1977 and December 31, 2008 were included. In total, 506 CD patients [CD, male: female: 251:255, age at diagnosis: 28.5 years, interquartile range (IQR): 22-38 years] were diagnosed during the inclusion period. Patients were followed until December 31, 2009 or death. All patients had at least one year of follow-up data avail-

Table 1 Clinical characteristics of patients with Crohn's disease

| | CD (n = 506) |
|---------------------------------------|---------------|
| Male/female | 251/255 |
| Age at presentation (yr) ¹ | 28.5 (22-38) |
| Follow-up (yr) ¹ | 13.5 (6-19.5) |
| Familial IBD | 12.90% |
| Location at diagnosis | |
| L1 | 32.80% |
| L2 | 35.90% |
| L3 | 30.60% |
| L4 only | 0.70% |
| L4 | 4.80% |
| Behavior at diagnosis | |
| B1 | 56.90% |
| B2 | 19.80% |
| B3 | 23.30% |
| Frequent relapse | 13.10% |
| Perianal disease | 25.50% |
| Arthritis | 26.70% |
| PSC | 1.80% |
| Ocular | 4.70% |
| Cutaneous | 9.30% |
| Steroid use | 68.60% |
| Azathioprine use | 45.80% |
| Biological use | 10.70% |
| Resection/re-operation | 41.3%/28.2% |

¹Median (interquartile range). L1: Ileal; L2: Colon; L3: Ileocolon; L4: Upper gastrointestinal; B1: Inflammatory; B2: Stenosing; B3: Penetrating; PSC: Primary sclerosing cholangitis.

able. Patients with indeterminate colitis at diagnosis were excluded from the analysis. Patient clinical data is summarized in Table 1. The ratio of urban-to-rural residence was also relatively stable (55% urban).

Methods

Data collected from 7 general hospitals and gastroenterology outpatient units (Internal Medicine Departments, Surgery Departments, Paediatric Departments and Outpatient Units) from Veszprem County (Veszprem, Papa, Tapolca, Ajka, Varpalota, Zirc). A more detailed description of the data collection and case assessment methods used, as well as the geographical and socioeconomic background of the province and the Veszprem Province IBD Group was published in previous epidemiological studies by this group^[15].

The majority of patients (94% of CD and 71% of ulcerative colitis patients) were monitored at the Csolnoky F Province Hospital in Veszprem. This hospital also serves as a secondary referral center for IBD patients in the province. Data collection was prospective since 1985; prior to that, only in Veszprem were data collected prospectively. In other sites throughout the province, data for this period (1977-1985) were collected retrospectively in 1985. Both in- and outpatients permanently residing in the area were included in the study. Diagnoses (based on hospitalization records, outpatient visits, endoscopic, radiological, and histological evidence) generated in each hospital and outpatient unit

were reviewed thoroughly, using the Lennard-Jones^[16] or the Porto criteria^[17], as appropriate. At the Veszprem pediatric IBD clinic, all probable cases of IBD are evaluated in a single unit by a pediatric gastroenterologist with experience in the diagnosis and treatment of IBD together with adult gastroenterologists. In addition, all endoscopies for pediatric patients are performed and all follow-up is conducted by two expert adult gastroenterologists, and pediatric cases were followed together by pediatric and adult gastroenterologists. According to the Montreal classification, an age at diagnosis < 17 years was defined as pediatric onset.

Age, age at onset, the presence of familial IBD, presence of extraintestinal manifestations (EIM) including: arthritis, conjunctivitis, uveitis, episcleritis, erythema nodosum, pyoderma gangrenosum, primary sclerosing cholangitis (PSC), and the frequency of flare-ups (frequent flare-up: > 1/year^[18]) were registered. Disease phenotype (age at onset, duration, location, and behavior) was determined according to the Montreal classification^[4] (based on: age at onset, location, and behavior, with perianal and upper GI disease as additional modifiers). Non-inflammatory behavior was defined as either stricturing or penetrating disease. Perianal disease and behavior change (from B1 to B2 or B3) or location during follow-up was also registered. Every significant flare or new symptom was meticulously investigated by gastroenterology specialists. Morphological investigations included proctosigmoidoscopy, colonoscopy, computed tomography (CT) scan, small-bowel ultrasound and small bowel X-ray. Patients in clinical remission had regular follow-up visits including laboratory and imaging studies (annual abdominal ultrasound). Endoscopy and CT-scans were only occasionally performed in patients in clinical remission. Of note, upper GI symptoms were carefully evaluated. Only indisputable manifestations were classified as upper GI involvement (*e.g.*, stenosis, ulcers), but not small erosions, or even simple gastric or duodenal ulcers, the later occurring shortly after the start of high dose systemic steroid therapy. Upper GI endoscopy was performed regularly at the time on the diagnosis of CD only in the last ten years, earlier only in case of gastroesophageal symptoms.

Medical therapy was thoroughly registered (*e.g.*, steroid, immunosuppressive, or biological use, azathioprine intolerance as defined by the European Crohn's and Colitis Organization, Consensus Report 28), need for surgery or reoperation (resections in CD), development of colorectal and small bowel adenocarcinoma, other malignancies, and smoking habits, were investigated by reviewing medical records during follow-up and by the completion of a questionnaire. Only patients with a confirmed diagnosis for more than one year were enrolled.

In addition, due to Hungarian health authority regulations, a follow-up visit is obligatory for IBD patients at a specialized gastroenterology center every six months. Otherwise, under the conditions of the Hungarian na-

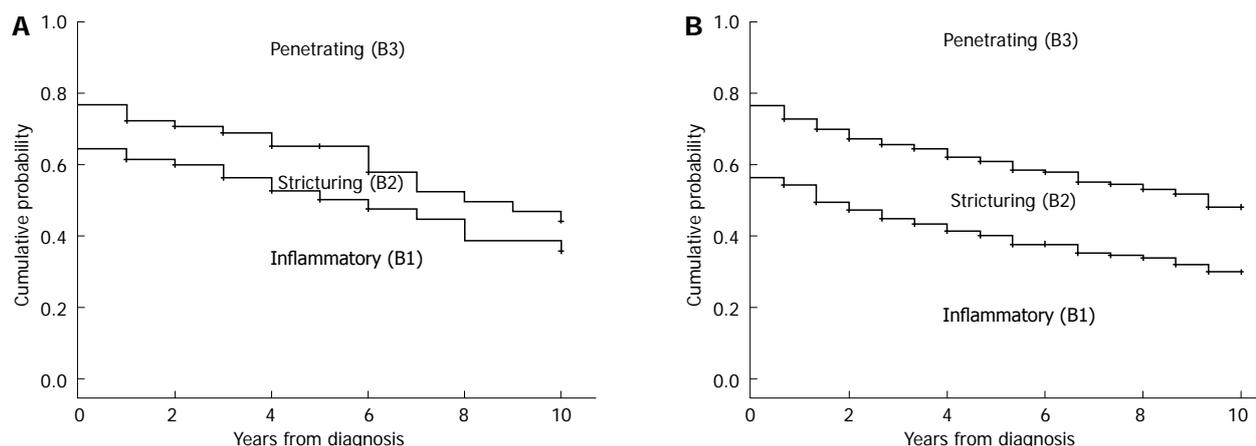


Figure 1 The evolution of disease behavior in patients with Crohn's disease according to the age at diagnosis. A: Pediatric onset; B: Adult onset.

tional health insurance system, patients forfeit their right to ongoing subsidized therapy. Consequently, the relationship between IBD patients and specialists is a close one.

The study was approved by the Semmelweis University Regional and Institutional Committee of Science and Research Ethics and the Csolnoky F Province Hospital Institutional Committee of Science and Research Ethics.

Statistical analysis

Variables were tested for normality by Shapiro Wilk's *W*-test. The distribution of disease behavior at different time points and between subgroups of CD patients was compared by χ^2 -test with Yates correction. Odds ratios (OR) were calculated. Kaplan-Meier survival curves were plotted for analysis with LogRank and Breslow tests to determine probability of disease behavior change in patients with inflammatory (B1) behavior at diagnosis. Additionally, Cox-regression analysis using the enter method was used to assess the association between categorical clinical variables and time to disease behavior or location change. Variables with a *P* value < 0.2 in univariate analysis were included in the multivariate testing results for continuous variables are expressed as median (IQR) unless otherwise stated. Peter Laszlo Lakatos performed all statistical analysis. For statistical analysis, SPSS® 15.0 (SPSS Inc., Chicago, IL) was used. A *P* value of < 0.05 was considered significant.

RESULTS

Evolution of disease phenotype in CD patients according to age at onset

Five hundred six residents of the Veszprem province were diagnosed with CD during the 32-year period from 1977 to 2008. The clinical characteristics of these patients are shown in Table 1. Sixty-five (12.8%) CD patients were diagnosed < 17 years of age. Follow-up information was collected up to December 31 2009, equaling 5758 patient-years of follow-up.

There was no significant difference in the distribution of disease behavior between pediatric (B1: 62%, B2: 15%, and B3: 23%) and adult onset CD patients (B1: 56%, B2: 21%, and B3: 23%) at diagnosis (*P* = NS). In addition, the distribution of disease behavior after 1, 3, 5, 7, 10 and 15 years and the probability of developing penetrating or complicated (stenosing/penetrating) disease behavior during follow-up did not significantly differ in patients with pediatric and adult onset disease by χ^2 and Kaplan-Meier analysis (Figure 1, *P*LogRank = NS, *P*Breslow = NS) Because the length of follow-up differed between the groups, statistical analysis was not performed using final disease behavior data.

Similarly, the probability and time to change in disease behavior from B1 to B2/B3 disease was not significantly different between pediatric- and adult-onset CD in a Kaplan-Meier analysis (Figure 2). The probability of complicated disease behavior for patients who initially exhibited inflammatory disease behavior was 7.6%, 27.5%, and 42.0% in the pediatric and 12.1%, 26.4%, and 37.5% in the adult-onset patients after 1, 5, and 10 years of follow-up (*P*LogRank = NS, *P*Breslow = NS).

In contrast, the distribution of disease location at diagnosis was different between pediatric- and adult-onset CD patients (L3 pediatric-onset: 41.3%, *vs* adult-onset: 28.8% *P* = 0.05, Figure 3). A change in disease location was observed 8.9% of the CD patients. The probability of change in disease location was 5.2%, 8.9%, and 10.8% after 5, 10, and 15 years of follow-up, respectively. However, this did not differ according to age at onset (*P*LogRank = NS).

Predictors of progression of disease behavior and location

The calendar year of diagnosis and location were associated with presence of or progress to complicated disease behavior at diagnosis and during follow-up. There was a significant difference in the distribution of disease behavior in patients diagnosed from 1977 to 1998 (*n* = 273, B1: 50%, B2: 22%, and B3: 28%) and from 1999 to 2008 (B1: 65%, B2: 17% and B3: 18%) at diagnosis (*P*

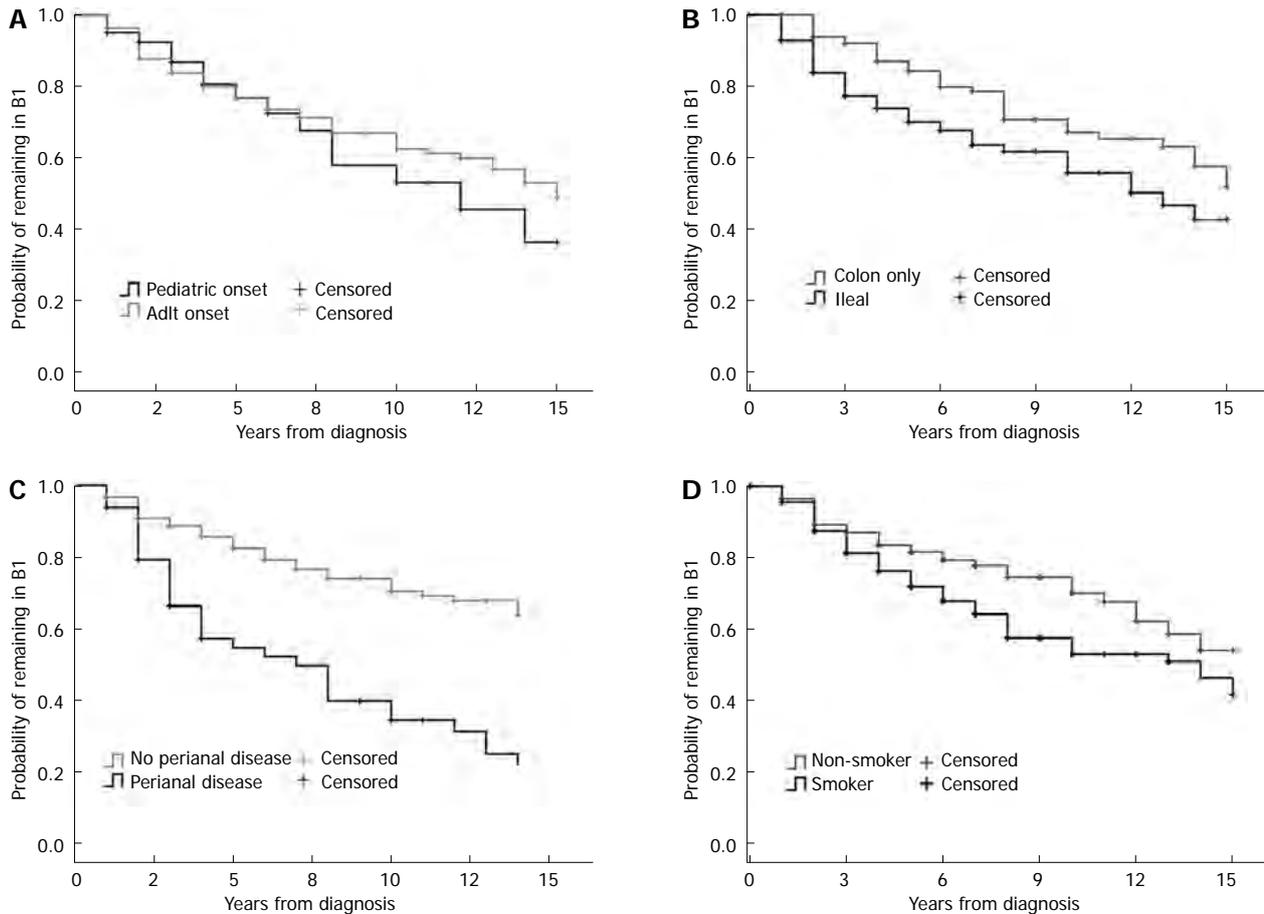


Figure 2 The probability of remaining in inflammatory (B1) disease behavior in patients with Crohn's disease according to the age at diagnosis (A), location (B), presence of perianal disease (C) and smoking status (D). A: $P_{\text{LogRank}} = 0.40$, $P_{\text{Breslow}} = 0.62$; B: $P_{\text{LogRank}} = 0.013$, $P_{\text{Breslow}} = 0.002$; C: $P_{\text{LogRank}} < 0.001$, $P_{\text{Breslow}} < 0.001$; D: $P_{\text{LogRank}} = 0.038$, $P_{\text{Breslow}} = 0.051$.

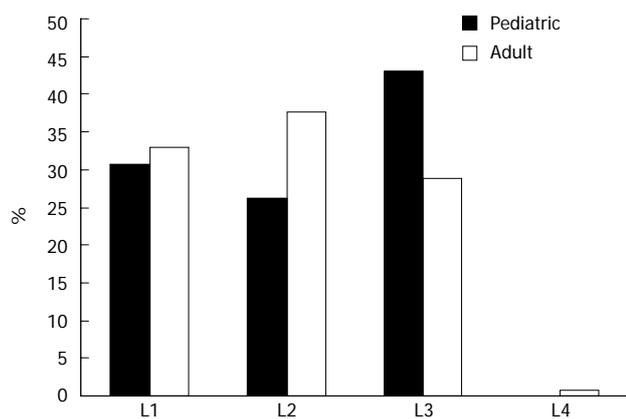


Figure 3 Distribution of disease location in Crohn's disease patients at diagnosis according to the age at onset.

= 0.003) and after one, three, and five years of follow-up ($P_{1\text{-year}} = 0.007$, $P_{3\text{-years}} = 0.002$, $P_{5\text{-years}} < 0.001$ by χ^2 analysis, and in the probability of developing penetrating or complicated (stenosing/penetrating) disease behavior during follow-up in a Kaplan-Meier analysis [$P_{\text{LogRank}} < 0.001$, $P_{\text{Breslow}} < 0.001$ (Figure 4)]. The probabilities of penetrating or complicated (stenosing/penetrating)

disease behavior after three and seven years of follow-up were 37.4% and 44.8%, and 58.4% and 66.2%, in the 1977-1998 cohort, while this was 26.5% and 34.4%, and 43.6% and 50.6%, in the 1999-2008 cohort.

Trends were similar when pediatric-onset and adult-onset patients were analyzed separately. The disease behavior pattern at diagnosis did not differ significantly between the two groups diagnosed in 1977-1998 (pediatric-onset $n = 33$, B1: 51%, B2: 21%, and B3: 28%, adult-onset $n = 240$, B1: 50%, B2: 22%, and B3: 28%) and in 1999-2008 (pediatric-onset $n = 32$, B1: 72%, B2: 9%, and B3: 19%, adult-onset $n = 201$, B1: 64%, B2: 18%, and B3: 18%). The evolution of disease behavior was also similar to the full cohort (data not shown).

In addition, disease location and presence of perianal disease was associated with disease behavior at diagnosis (colon only B1: 73%, B2: 12%, and B3: 15%, vs ileal involvement B1: 48%, B2: 24%, and B3: 28%, $P < 0.001$; perianal disease absent B1: 66%, B2: 21%, and B3: 13%, perianal disease present B1: 30%, B2: 15%, and B3: 55%, $P < 0.001$). Probability of change in disease behavior from B1 to B2/B3 disease was significantly higher in patients with ileal involvement ($P_{\text{LogRank}} = 0.013$, $P_{\text{Breslow}} = 0.002$, $\text{HR}_{\text{L1 or L3}} = 2.27$, 95%CI: 1.32-3.92)

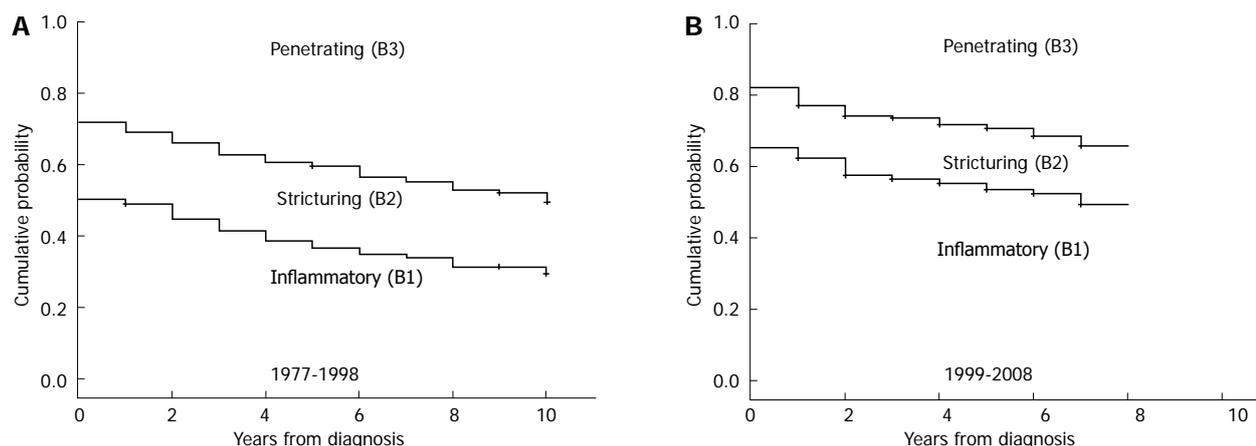


Figure 4 The evolution of disease behavior in patients with Crohn's disease according to the year of diagnosis. A: 1977-1998; B: 1999-2008. *P*LogRank < 0.001, *P*Breslow < 0.001 for complicated behavior between groups A and B.

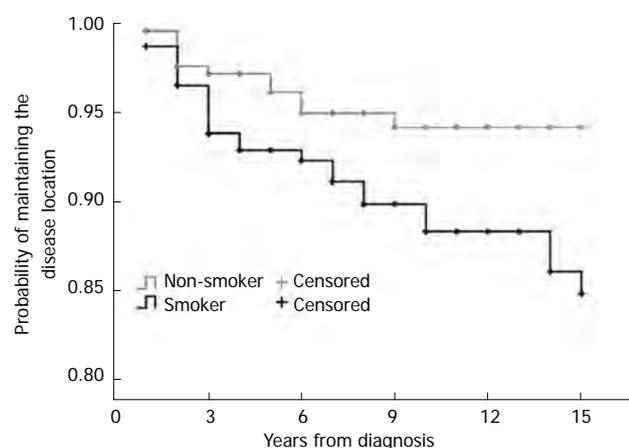


Figure 5 The probability of maintaining the disease location in patients with Crohn's disease according to the smoking status. *P*LogRank = 0.011, *P*Breslow = 0.002.

and perianal disease (*P*LogRank < 0.001, *P*Breslow < 0.001, HR_{perianal} = 2.98, 95%CI: 2.21-4.03) (Figure 2B-D). Similarly, need for steroids, either at diagnosis or during follow-up, was associated with an increased risk of disease progression (*P*LogRank < 0.001, *P*Breslow < 0.001, HR = 3.66, 95%CI: 1.67-8.04), but not early azathioprine exposure. The same trend was observed for smoking (*P*LogRank = 0.038, *P*Breslow = 0.051, HR_{smoking} = 1.482, 95%CI: 0.96-2.37).

In contrast, calendar year of diagnosis was associated with the progression to non-inflammatory disease behavior (*P*LogRank = 0.04, *P*Breslow = 0.04, HR_{after 1998} = 0.73, 95%CI: 0.55-0.97) in patients with initially inflammatory disease. The probability of progression to complicated disease behavior after five and seven years was 15.1% and 21.8% in patients diagnosed after 1998 while this was 27.4% and 33.3% in patients diagnosed between 1977 and 1998. In a multivariate Cox regression analysis, after excluding steroid exposure at any time point from the variables, the effect of location (*P* < 0.001; for L1 *P* < 0.001, HR = 2.19, 95%CI: 1.50-3.09; for L3

P = 0.01, HR = 1.59, 95%CI: 1.12-2.28), perianal disease (*P* < 0.001, HR = 3.11, 95%CI: 2.23-4.34) and smoking (*P* = 0.031, HR = 1.42, 95%CI: 1.04-1.96) remained significant.

Interestingly, the probability of disease location change differed according to smoking status (*P*LogRank = 0.011, *P*Breslow = 0.03, HR_{smoking} = 2.35, 95%CI: 1.19-4.63, Figure 5), but not according to gender, initial disease location, behavior, presence of perianal disease or calendar year of diagnosis.

DISCUSSION

This current, population-based, prospective study reveals that evolution of disease behavior and location do not differ significantly between CD patients with adult or pediatric onset during long-term follow-up, as the change of disease phenotype is not different significantly in pediatric- and adult-onset CD, in contrast to previous, large, multicenter studies. There were no significant differences in disease behavior between pediatric- and adult-onset patients at the time of diagnosis or during follow-up. In contrast, findings presented here confirm the results of previous studies, namely, that disease location was significantly different according to the age at diagnosis. Pediatric patients presented more frequently with extensive disease. A change in disease location was relatively rare and it was associated with smoking status.

The same, progressive characteristics of CD were described by Cosnes *et al*^[6] in a study of patients treated at a French referral center. More than 80% of patients developed complications with time. After 5 and 20 years' disease duration, the risk for stricturing disease complications were 12% and 18% respectively, whereas 40% and 70% of patients developed penetrating complications, respectively. An association was reported, however, with disease location; the probability of complicated disease was as high as 94% after 20 years in patients with ileal disease. Results were comparable in a Belgian study^[5]. In this study, 45.9% of patients had a change in disease be-

havior after 10 years of follow-up, especially from non-stricturing, non-penetrating disease to either stricturing (27.1%) or penetrating (29.4%) disease. The frequency of complicated disease was somewhat lower in the present population-based study. The probability of developing penetrating complications was 35.4% and 58.2% after 5 and 20 years' disease duration, while 55.9% and 73.7% of patients diagnosed between 1977-2008 developed either penetrating or stricturing complications. Finally, 53% of patients developed stricturing or penetrating disease during a 10-year follow-up in the population-based prospective IBSEN cohort in CD patients diagnosed between 1990 and 1994^[8]. Of note, however, in the study by Cosnes *et al*^[6], classification of patients according to disease behavior was a poor predictor of disease activity during the next five years. A similar proportion of patients required immunosuppressive drugs and surgery.

According to previous data, the natural history was reported to be more severe in pediatric CD. Extensive, complicated disease phenotypes were reported to be frequent in a population-based study by Vernier-Massouille *et al*^[19]. In this study, the prevalence of B2 and B3 phenotypes increased from 25% to 44%, and from 4% to 15%, while the frequency of B1 disease decreased from 71% to 41%, respectively, from diagnosis until approximately 10 years of follow-up. In addition, according to a recent French study by Pigneur *et al*^[10] patients with early childhood-onset CD often have more severe disease, increased frequency of active periods, and increased need for immunosuppressants. In contrast, in the present study, disease behavior at diagnosis and the rate of progression to complicated disease did not differ between pediatric- and adult-onset CD patients. Similarly, in a population-based cohort in New Zealand^[3], age at diagnosis was not predictive of the rate of progression from inflammatory to complicated disease behavior. Until now, this was the only study that investigated the importance of age at onset according to the Montreal classification including pediatric onset patients. However, significant data were collected retrospectively and the median follow-up was 6.5 years which is half of the median follow-up of patients in the present study. In addition, > 70% of CD patients had inflammatory disease at diagnosis, with 23% and 40% of patients with initial inflammatory disease progressing to complicated disease phenotypes after five and ten years of follow-up.

Previous studies suggested that the disease location was different between pediatric and adult onset patients with more ileocolonic and upper GI disease in pediatric patients^[3,6,19], in concordance with the present study. In a French population-based pediatric CD study^[19] the most frequent location at diagnosis was ileocolonic disease (63%). Disease extension was observed in a surprisingly large proportion of pediatric patients (31%) during follow-up. In addition, in a population-based New-Zealand CD cohort, authors have reported an association be-

tween initial disease location and probability of disease extension. Patients with colon-only location progressed more rapidly to ileocolonic disease than those with ileal disease ($P = 0.02$). Of note, the rate of disease location change at 10 years in this study (9%) was in the range reported in the present study (8.9% during a median 13 years), although somewhat higher rates were reported in the study by Louis *et al*^[5] (15.9% during 10-years). In the latter study, 20.3% of patients with an initial L1 location changed to another location, while the proportion of patients changing from L2 was 16.7%. In the present study, the probability of disease behavior change was 8.8% and 10.9% after 10 and 15 years of disease duration. The change in disease location was not different between patients with pediatric or adult onset, nor between patients with L1 and L2 disease. In contrast, a novel finding of the present study was that change in disease location was associated with smoking status (HR = 2.35, $P = 0.01$). The probability of a change in disease location was 5.8% and 5.8% in non-smokers, and 11.7% and 15.1% in smokers after 10 and 15 years' disease duration.

Additional predictors of disease behavior change identified in the present study included presence of ileal involvement, perianal disease, smoking and calendar year of diagnosis, with perianal involvement being the most important predictor. The role of initial ileal involvement, extensive disease, and perianal disease as a possible predictor of non-inflammatory behavior was first suggested in a landmark study by Cosnes *et al*^[6]. Additionally age < 40 years at diagnosis was associated with the development of penetrating complications (HR = 1.3). Similar findings were presented from the New Zealand cohort^[3], where patients with ileal (L1) disease progressed most quickly to non-inflammatory disease behavior, followed by patients with upper GI (L4) or ileocolonic (L3) disease ($P < 0.0001$). The probability of progression to penetrating disease was similar to that of progression to stenosing disease after 10 years. Overall, the proportion of penetrating disease was highest in those with ileocolonic (27%) or ileal disease (21%) compared to patients with colon-only disease (7%, $P = 0.006$). Patients with perianal disease were at risk of a change in disease behavior (HR = 1.62, 95%CI: 1.28-2.05). In a subsequent population-based study from the IBSEN group^[8], non-inflammatory disease behavior during follow-up was associated with initial L1 (86%) *vs* L2 (30%, $P < 0.001$) or L3 location (60%, $P < 0.005$). Finally, in a previous publication by our group^[7], ileal disease location (HR = 2.13, $P = 0.001$), presence of perianal disease (HR = 3.26, $P < 0.001$), prior steroid use (HR = 7.46, $P = 0.006$), early AZA (HR = 0.46, $P = 0.005$) and smoking (HR = 1.79, $P = 0.032$) were independent predictors of disease behavior change in a referral CD cohort. Data regarding the effect of smoking are equivocal, however. A recent review^[20] and previous studies have demonstrated that smoking was associated with complicated disease, penetrating intestinal complications^[21], and greater likelihood

of progression to complicated disease, as defined by development of strictures or fistulae, a higher relapse rate, and need for steroids and immunosuppressants^[22]. In a recent study by Aldhous *et al.*^[23], the deleterious effect of smoking was only partially confirmed. Current smoking was associated with less colonic disease, however smoking habits at diagnosis were not associated with time to development of stricturing, penetrating disease, nor with perianal penetrating disease or time to first surgery. Of note, a possible neutralizing effect of immunosuppressant therapy was reported in some studies^[24,25].

Conclusions were slightly different if authors assessed the factors associated with the development of disabling disease. In the paper by Loly *et al.*^[26] stricturing behavior at diagnosis (HR = 2.11, $P = 0.0004$) and weight loss (> 5 kg) at diagnosis (HR = 1.67, $P = 0.0089$) were independently associated with time to the development of severe disease in multivariate analysis. The definition of severe, non-reversible damage was, however, much more rigorous. It was defined by the presence of at least one of the following criteria: the development of complex perianal disease, any colonic resection, either two or more small-bowel resections or a single small-bowel resection measuring more than 50 cm, or the construction of a definite stoma. In a similar study by Beaugerie *et al.*^[27], with a different definition of disabling disease, initial requirement for steroid use (OR = 3.1, 95%CI: 2.2-4.4), an age below 40 years (OR = 2.1, 95%CI: 1.3-3.6), and the presence of perianal disease (OR = 1.8, 95%CI: 1.2-2.8) were associated with the development of disabling disease^[27]. The positive predictive value of disabling disease in patients with two and three predictive factors of disabling disease was 0.91 and 0.93, respectively. Concordantly, in the present study, need for steroids was identified as a risk factor for progression of disease behavior (HR = 3.66, $P < 0.001$).

Finally, the calendar year of diagnosis was associated with disease behavior at diagnosis and the progression to non-inflammatory disease behavior ($P_{\text{LogRank}} = 0.04$, $P_{\text{Breslow}} = 0.04$, $HR_{\text{after 1998}} = 0.73$, 95%CI: 0.55-0.97) in patients with initially inflammatory disease in the present study, suggesting a change in the natural history of the disease in the last decade. Trends were similar in the pediatric- and adult-onset patients. However, although azathioprine was started more frequently and earlier in the last decade^[28], the change in disease behavior progression was not directly associated with the increased and earlier use of azathioprine, pointing to the fact that probably the change in the patient management is far more complex. Of note, distribution of disease location was not different in patients with a diagnosis before or after 1998. In contrast, presence of perianal disease was less prevalent in the later group (17.9% *vs* 31.5%, $P < 0.001$, OR = 0.48), suggesting an increased awareness and probably earlier diagnosis.

Authors are aware of possible limitations of the present study. The treatment and monitoring paradigm for CD patients has changed significantly over the last three

decades. The majority of patients received maintenance therapy with sulfasalazine or a 5-aminosalicylic acid derivative (mesalazine or olsalazine), if tolerated, especially until the mid-1990s. Azathioprine or 6-mercaptopurine were used as maintenance therapy for steroid dependent, steroid-refractory, or fistulizing patients in selected cases, mainly after resective surgery until the late-1980s, but on a more widespread basis and earlier in the disease course only from the mid-to-late 1990s. Short-term oral corticosteroid treatment was used for clinical exacerbations, usually at initial doses of 40-60 mg of prednisone per day, which was tapered and discontinued over 2 to 3 mo. Infliximab (and later adalimumab) has been used for both induction and maintenance therapy in selected cases since the late 1990s. Similarly, small-bowel follow through was replaced by CT or MR-enterography from the 1990s. The strengths of the study include long-term prospective follow-up, the fact that leading IBD specialists were involved during the entire follow-up, and also that the evaluation and monitoring of pediatric-onset patients was managed jointly by pediatric and adult gastroenterologists using similar principles.

In conclusion, the long-term evolution of disease behavior in pediatric- and adult-onset CD patients did not differ in this population-based incident cohort. In contrast location, smoking, and need for steroids were associated with presence of, or progression to, complicated disease behavior at diagnosis and during follow-up, in concordance with previous referral and population-based studies. In addition, there was a change in the evolution of the disease behavior according to the calendar year of diagnosis. Progression to complicated disease phenotype was less likely in patients diagnosed after 1998, however this was at least partly associated with a milder disease phenotype at diagnosis including a decreased prevalence of perianal disease in the later group. A novel finding of the present study was that the change in disease location was associated with smoking status.

COMMENTS

Background

According to the available literature, pediatric onset Crohn's disease (CD) runs a more aggressive course, including more extensive disease location, more upper gastrointestinal involvement, growth failure, more active disease, and need for more aggressive medical therapy, in predominantly referral-center studies.

Research frontiers

Limited data are available on the long-term disease course in pediatric and adult patient cohorts with inflammatory bowel diseases from the same geographic area in population-based cohorts.

Innovations and breakthroughs

Some new data indicate that pediatric disease may parallel that of adults, however data so far are conflictive. The present study reports that the long-term evolution of disease behavior was not different in pediatric- and adult-onset CD patients in this prospective population-based incident cohort from Eastern Europe. Interestingly, change in disease location was associated with smoking status.

Applications

Understanding the evolution of the disease course in CD may lead to more optimized patient management and follow-up.

Terminology

Disease phenotype is categorized according to the Montreal classification and includes age at onset (A1: < 17 years, A2: 17-40 years and A3: > 40 years) location (L1: Ileal, L2: Colon, L3: Ileocolon, L4: Upper gastrointestinal) and behavior (B1: Inflammatory, B2: Stenosing, B3: Penetrating). While disease location is thought to be more stable, a change in the disease behavior is a rather frequent event.

Peer review

This is a prospective, well-designed study, with a remarkable number of patients with CD reporting that the risk for developing complicated disease phenotype is not different between pediatric onset and adult onset CD patients.

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Comparative analysis of endoscopic precut conventional and needle knife sphincterotomy

Andrzej Jamry

Andrzej Jamry, 2nd Surgical Department, District Hospital Radomska, 27-200 Starachowice, Poland

Author contributions: Jamry A solely contributed to this paper.
Correspondence to: Andrzej Jamry, MD, 2nd Surgical Department, District Hospital Radomska, Langiewicza 30, 27-200 Starachowice, Poland. jamry@tlen.pl
Telephone: +48-60-2795259 Fax: +48-41-2736158
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Abstract

AIM: To compare the efficacy, complications and post-procedural hyperamylasemia in endoscopic pre-cut conventional and needle knife sphincterotomy.

METHODS: We performed a retrospective analysis of two pre-cut sphincterotomy (PS) techniques, pre-cut conventional sphincterotomy (PCS), and pre-cut needle knife (PNK). The study included 143 patients; the classic technique was used in 59 patients (41.3%), and the needle knife technique was used in 84 patients (58.7%). We analyzed the efficacy of bile duct access, the need for a two-step procedure, the rates of complications and hyperamylasemia 4 h after the procedure, "endoscopic bleeding" and the need for bleeding control. Furthermore, to assess whether the anatomy of the Vater's papilla, indications for the procedure or the need for additional procedures could inform the choice of the PS method, we evaluated the additive hyperamylasemia risk 4 h after the procedure with respect to the above mentioned variables.

RESULTS: The bile duct access efficacy with PNK and PCS was 100% and 96.6%, respectively, and the difference between the two groups was not significant ($P = 0.06$). However, the needle knife technique required two-step access significantly more often, in 48.8% vs

8.5% of cases ($P < 0.0001$). The only complication noted was post-ercp pancreatitis (PEP), which was observed in 4/84 (4.8%) and 2/59 (3.4%) patients submitted to PNK and PSC, respectively; the difference between the two procedures was not significant ($P = 0.98$). An analysis of other consequences of the techniques yielded the following results in the PNK and PCS groups: hyperamylasemia 4 h after the procedure > 80 U/L, 41/84 vs 23/59 ($P = 0.32$); hyperamylasemia 4 h after the procedure > 240 U/L, 19/84 vs 11/59 ($P = 0.71$); pancreatic pain, 13/84 vs 7/59 ($P = 0.71$); endoscopic bleeding, 10/84 vs 8/59 ($P = 0.97$); and the need for bleeding control, 10/84 vs 7/59 ($P = 0.79$). In the next part of the study, we analyzed the influence of the method chosen on the risk of hyperamylasemia with respect to an indication for endoscopic retrograde cholangiopancreatography, papillary anatomy and concomitant procedures performed. We determined that the hyperamylasemia risk was increased by more than threefold [odds ratio (OR) = 3.38; $P = 0.027$] after PCS in patients with a flat Vater's papilla and more than fivefold (OR = 5.3; $P = 0.049$) after the PNK procedure in patients who required endoscopic homeostasis.

CONCLUSION: PCS and PNK do not differ in terms of efficacy or complication rates, but PNK is more often associated with the necessity for a two-step procedure.

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Key words: Sphincterotomy; Endoscopic; Endoscopic retrograde cholangiopancreatography; Complications; Hyperamylasemia

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INTRODUCTION

Pre-cut sphincterotomy (PS) may increase the efficacy of the ineffective conventional endoscopic cannulation of biliary ducts by 64%-91%, and some studies have reported increases in efficacy of up to 95%-99%^[1,2]. However, the efficacy and technical details of PS remain controversial, and the reported complication rates range from 3.78% to 19.2%, with an odds ratio (OR) of 0-2.71^[3-7]. Therefore, PS accounts for 0% to 44% of all sphincterotomies at various centers^[3,8-10]. The procedure may be performed by one of two methods, using either a non-needle knife or a needle knife^[3,5,6,11]. The first procedure is performed using a shallowly anchored conventional structure cannulotome [pre-cut conventional sphincterotomy (PCS)]^[11-13]; the second procedure (PNK), relies on a needle knife incision of the intramural part of Vater's papilla^[11,14]. Both methods are performed with various modifications that can independently influence complications^[3]. For example, a variation of PNK proceeds without the distal broadening of the incision to avoid Wirsung's duct orifice damage^[15]. In comparison with the conventional incision, this modification produces different anatomical results with an unknown impact on the complication rate and pancreatic juice efflux. The available literature presents only two studies comparing the PCS and PNK methods. However, in these trials, PCS was performed as a trans pancreatic sphincterotomy, and PNK began at the orifice^[13,16]. Different approaches to PS indication, different indications for the switch from conventional to PS procedures, technical PS variations, the rules of two-step procedure implementation and different definitions of complications at various centers explain the limited value of data reported by different authors^[13]. The lack of interpretable data by different authors prompted retrospective comparison of PCS and PNK (modified, without the distal broadening of the cut). The analysis included the efficacy of access to the common bile duct (CBD), the necessity to initiate a two-step procedure, the frequency of typical complications [post-ercp pancreatitis (PEP), bleeding, and perforation], the frequency of "endoscopic" bleeding, and the need for haemostasis, the influence on impaired pancreatic juice efflux and the need for further hospitalization. Additionally, we assessed the degree to which the effect of the PS method affects on the impaired efflux of pancreatic juice was dependent on papillary anatomy, procedurale indications or concomitant procedures. The aim of the study was to answer two questions: (1) is there a difference between the efficacy and safety of the analyzed pre-cut methods; and (2) should Vater's papilla anatomy, procedural indications and concomitant procedures influence the choice of pre-cut method?

MATERIALS AND METHODS

Inclusion criteria

We included patients with ineffective common bile duct deep cannulation using a conventional cannulotome (O-

lympus KD 301Q-0729) and guide-wire (MET 35-380 Cook) after a 10 min procedure. Sphincterotomy using a conventional cannulotome was performed if sufficient anchoring in the papilla orifice was possible; the needle knife procedure was used in the remaining patients. Exclusion criteria: Patients with invasive procedures on Vater's papilla in the past, and acute pancreatitis before the endoscopic retrograde cholangiopancreatography (ERCP) procedure were excluded.

Study material

The study included patients submitted to ERCP from one center over 21 mo (February 2010 to November 2011). Papillotomy was performed on 402 patients, during that time frame, of whom 165 qualified for the pre-cut procedure. However, 22 were excluded from analysis because of a previous endoscopic attempt on the papilla or symptoms of acute pancreatitis before ERCP. Finally, 143 (35.6%) patients were admitted to the study. A conventional cannulotome was used in 59 (41.3%) of the patients in this group, the needle knife technique was used in 84 patients (58.7%).

Technique

The conventional pre-cut procedure was performed with a cannulotome (Olympus KD 301Q-0320). A 2 to 3 mm long incision was made after anchoring the cannulotome, and the end of the device was continuously repositioned toward the CBD orifice for deep cannulation. The patient was referred to a two-step procedure, if, a maximum of five trials of cannulation were ineffective. The next ERCP was performed 4-7 d later, after the tissue edema had regressed. The needle knife technique was performed with a KD-441Q Olympus cannulotome at the midway point between the papilla's orifice and the transverse fold. Catheterization was performed when the CBD orifice was exposed, and the sphincterotomy was proximally broadened with a conventional cannulotome with the distal fragment left intact. The procedure was postponed 4-7 d in cases with five ineffective trials of cannulation. For each PS technique, we used prosthesis if a randomly contrasted Wirsung's duct exhibited impaired retrograde contrast efflux. We performed endoscopic hemostasis with an HES solution (hypertonic 5.6% NaCl solution with adrenaline 1:20 000) for cases of bleeding for more than 2 min, which made further cannulation trials impossible. Serum amylase levels were measured in every patient to assess pancreatic juice efflux impairment 4 h after the procedure. Pancreatic pain requiring analgesics was assessed 24 h after the procedure, and pancreatic pain was an indication for subsequent serum amylase level assessment. The analysis: The PCS and PNK techniques were assessed according to the efficacy of CBD access, the necessity of a two-step procedure, pancreatic juice efflux impairment (amylase level > 80 UI after 4 h), hospitalization indications (amylase level > 240 IU after 4 h)^[3,17-19], and PEP, which was defined as an amylase level three times the upper limit with concomitant pancreatic

Table 1 Patient characteristics-risk factor

| | Needle-knife PNK (n = 84) | Conventional PCS (n = 59) | P value |
|--------------------------------|------------------------------|------------------------------|---------|
| Female | 43 | 38 | 0.16 |
| Age < 50 yr | 16 | 7 | 0.36 |
| Bilirubin level (norm) | 8 | 10 | 0.30 |
| Concomitant systemic diseases | 20 | 6 | 0.06 |
| Neoplasms | 26 | 17 | 0.96 |
| Retention cause | | | |
| Cholelithiasis | 24 | 23 | 0.26 |
| Papillar stenosis | 14 | 10 | 0.85 |
| Distal stenosis | 18 | 13 | 0.70 |
| Middle stenosis | 3 | 5 | 0.37 |
| Hilum stenosis | 5 | 4 | 0.88 |
| Anatomy of the papilla | | | |
| Flat | 28 | 23 | 0.60 |
| Prominent | 32 | 23 | 0.94 |
| In diverticulum | 12 | 10 | 0.84 |
| Tumor | 18 | 5 | 0.06 |
| Biliary duct diameter | | | |
| (mean) | 14.49 | 14.07 | 0.42 |
| (< 9 mm) | 18 | 13 | 0.90 |
| Accessory procedures | | | |
| Prosthesis implantation | | | |
| CBD | 65 | 51 | 0.83 |
| Wirsung | 11 | 2 | 0.08 |
| Prosthesis in CBD diam. (mean) | 6.01 | 5.89 | 0.59 |
| Pathological sampling | 12 | 3 | 0.13 |

CBD: Common bile duct; PCS: Pre-cut conventional sphincterotomy; PNK: Pre-cut needle knife.

pain requiring analgesics 24 h after the procedure^[19,22]. We also analyzed bleeding, which was defined as the presence of clinical symptoms of blood extravasation into the alimentary tract^[20], the frequency of “endoscopic” bleeding (without clinical symptoms), and the necessity of hemostasis (no spontaneous regression 2 min after the incision). We compared perforations, which were defined as contrast extravasation out of the duodenal lumen during ERCP or gas in the retroperitoneal space on imaging^[8,9,22,23]. The PCS and PNK methods were submitted to logistic regression analysis to assess their influence on pancreatic juice efflux and the effect of Vater’s papilla anatomy (flat, prominent, inside diverticulum, or tumor), ERCP indications (choledocholithiasis, distal stenosis, or CBD diameter) and concomitant procedures (prosthesis, hemostasis, or pathological specimen sampling)^[19,23].

Statistical analysis

Frequency tables as well as χ^2 and Mann-Whitney *U* tests were used for the statistical analyses where appropriate. Unifactor and multifactor models of logistic regression were used to assess the probability that the analyzed parameters influenced the presence of hyperamylasemia. The measure of hyperamylasemia risk was expressed as an odds ratio (OR) with 95% confidence intervals. *P* values less than 0.05 were considered to be statistically significant, and the statistical analyses were performed using MedCalc ver. 12.3.

Table 2 Comparison of the efficacy and complication rates of pre-cut conventional sphincterotomy and pre-cut needle knife procedures

| Procedure | PNK (n = 84) | PCS (n = 59) | P value |
|------------------------------|--------------|--------------|----------|
| Two-step access | 41 | 5 | < 0.0001 |
| Efficacy | 84 | 57 | 0.58 |
| Amylase level (after 4 h) | | | |
| > 80 U/L | 41 | 23 | 0.32 |
| > 240 U/L | 19 | 11 | 0.71 |
| Pancreatic pain (after 24 h) | 13 | 7 | 0.71 |
| PEP | 4 | 2 | 0.98 |
| “Endoscopic” bleeding | 10 | 8 | 0.97 |
| Endoscopic homeostasis | 10 | 7 | 0.79 |
| Perforation | 0 | 0 | - |

PEP: Post endoscopic pancreatitis; PCS: Pre-cut conventional sphincterotomy; PNK: Pre-cut needle knife.

RESULTS

The study included 143 patients; the conventional pre-cut technique was used in 59 patients (41.3%), and the needle knife was used in 84 patients (58.7%). The clinical characteristics and risk factors were compared between the PCS and PNK groups, and the parameters are presented in Table 1. There were no significant differences between the groups with respect to the following parameters: sex, age < 50 years, percentage of patients with normal bilirubin levels, concomitant systemic and tumor diseases, cause of icterus (choledocholithiasis, or distal CBD stenosis), Vater’s papilla anatomy (flat, prominent, inside a diverticulum, or tumor) or the diameter of the common biliary duct. The frequency of prosthesis implantation in the CBD and pancreatic duct, the diameter of the prosthesis introduced to the biliary duct and the pathological specimen sampling did not differ between the groups. In the second part of the study, we assessed both techniques according to the necessity of introducing a two-step procedure, the efficacy of the endoscopic approach to the bile ducts and the consequences of the pre-cut procedures (Table 2). A two-step procedure was performed significantly more frequently in the PNK group, than in the PCS group (48.8% vs 8.5%, *P* < 0.0001). The two-step procedure allowed for CBD catheterization in all 45 patients in the PNK group, and 2 out of 5 patients in the PCS group. Biliary tree access was achieved in all patients treated with PKN and in 96.6% of patients treated with the PCS technique. Differences in the efficacies of the two methods were not statistically significant; however, in the PNK group, successful access was more frequently associated with a two-step procedure. There were no significant differences in the number of patients with elevated amylase levels exceeding 80 IU and 240 IU 4 h after the procedure. Pancreatic pain was observed 24 h after the procedure in 15.5% and 11.9% of patients in the PNK and PCS groups, respectively, and the differences were not statistically significant. Moreover, there were no notable differences in the rates of PEP. PEP was observed in 4.8% of the patients in the PNK group, and 3.4% in the PCS group. All PEP cases exhibited a mild or mod-

Table 3 Logistic regression-Hyperamylasemia (> 80 U/L) 4 h after the procedure and its association with indication, Vater’s papilla anatomy and additional procedures

| Parameter | Conventional (PCS) | | Needle knife (PNK) | |
|--------------------------|--------------------|------|--------------------|------------------|
| | P | OR | P | OR |
| Indications | | | | |
| Lithiasis | 1.4 | 0.73 | 0.19 | 0.52 |
| Distal stenosis | 0.98 | 1.01 | 0.98 | 0.96 |
| Middle stenosis | 0.38 | 0.52 | 0.55 | 0.67 |
| CBD diam. < 9 mm | 0.96 | 0.97 | 0.9 | 1.06 |
| Bilirubin level - N | 0.43 | 1.72 | 0.42 | 1.8 |
| Vater’s papilla anatomy | | | | |
| Flat | 0.027 ¹ | 3.38 | 0.22 | 0.56 |
| Prominent | 0.034 | 0.28 | 0.86 | 1.0 |
| In diverticulum | 0.52 | 0.62 | 0.93 | 1.05 |
| Tumor | 0.96 | 1.04 | 0.9 | 1.06 |
| Additional procedures | | | | |
| CBD prosth. diam. < 6 Fr | 0.13 | 5.3 | 0.56 | 0.7 |
| Endoscopic haemostasis | 0.82 | 1.2 | 0.049 | 5.4 ² |
| Specimen sampling | 0.84 | 0.78 | 0.92 | 1.06 |

¹Statistically significant only in unifacto logistic regression; ²Statistically significant only in multifactor logistic regression. CBD: Common bile duct; OR: Odds ratio; PCS: Pre-cut conventional sphincterotomy; PNK: Pre-cut needle knife.

erate course and were not treated surgically. All bleeding events observed in both groups were qualified as “endoscopic”. There were no significant differences between the two groups. The pre-cut procedure was not complicated by perforation or bleeding associated with clinical symptoms in any patient in either group. Additionally, the risk of hyperamylasemia 4 h after the procedure was evaluated for any association with procedural indications, papillary anatomy, common bile duct prosthesis, prosthesis diameter, bleeding hemostasis, or the collection of pathological specimens. Logistic regression analysis (Table 3) revealed a three fold increase in the risk of hyperamylasemia after the PCS technique in patients with a flat papilla (OR = 3.38), whereas the hyperamylasemia risk in PNK patients was 5 times higher (OR = 5.38) after endoscopic hemostasis.

DISCUSSION

The pre-cut procedure is used following an unsuccessful conventional cannulation attempt of the biliary tree. The procedure is performed using a variety of techniques that can roughly be divided into two major groups: PNK and PCS^[15]. Some authors believe that the conventional pre-cut procedure is the method of choice, and in case of its failure, needle cannulotome is recommended^[12]. PCS is widely believed to offer better direction and depth for the incision, which should decrease the risk of perforation and bleeding. However, each consecutive unsuccessful cannulation increases the risk of post-endoscopic pancreatitis (OR = 1.39), and can reach OR = 9.4 after more than 15 ineffective cannulations^[4,7]. PNK may be modified in the manner in which the incision is made, halfway between the orifice and the transverse fold with no distal

Table 4 Frequency of pre-cut sphincterotomy with a two-step approach, and efficacy of common bile duct cannulation (pre-cut conventional sphincterotomy and pre-cut needle knife procedures)

| Ref. | PS freq. | PS technique | Two-step | Efficacy |
|--|----------|--------------|----------|----------|
| Slot <i>et al</i> ^[1] | 16.5% | PNK | 12% | 99% |
| Kasmin <i>et al</i> ^[14] | 18.0% | PNK | 32% | 93% |
| Huigbregtse <i>et al</i> ^[21] | 19.2% | PNK | 47% | 91% |
| Dowsett <i>et al</i> ^[25] | 12.8% | PNK | 54% | 96.2% |
| Shakoor <i>et al</i> ^[26] | 3.8% | PNK | 13% | 85% |
| Leung <i>et al</i> ^[27] | 3.9% | PNK | 15% | 95% |
| Own material | 20.9 % | PNK | 48% | 100% |
| | 14.7% | PCS | 8.5% | 94.4% |
| Binmoeller <i>et al</i> ^[11] | 38% | PCS | 9% | 100% |
| Goff <i>et al</i> ^[12] | 44.0% | PCS | 14% | 97% |

PS: Pre-cut sphincterotomy; PCS: Pre-cut conventional sphincterotomy; PNK: Pre-cut needle knife.

elongation. This technique avoids manipulations in the vicinity of Wirsung’s duct orifice, which is believed to decrease the risk of PEP. Nevertheless, inferior maneuverability in the direction and depth of the cut may increase the perforation rate^[14,15]. We found only two publications directly comparing the efficacy and safety of needle knife and non-needle knife PS methods^[16,13]. Therefore, we aimed to compare PCS and PNK (modified, without the distal broadening cut) in the present work. The limitations of the present study are the small number of patients and the retrospective format of the study. At least 8422 patients need to be analyzed to assess the lack of difference in PEP frequency between the two groups; however, this would be difficult to accomplish in a single study. The second limitation of the present analysis is the retrospective format of the study, which restrains the exclusion of all of the parameters that indirectly influence the results. This retrospective design explains the difference in the frequency of Vater’s papilla tumor in the analyzed groups 18 (PNK) vs 5 (PCS) patients, which may suggest that the needle-knife technique was used more often in patients with Vater’s papilla tumor. The difference was not statistically significant (P = 0.06); however, the possibility that the difference may become significant in larger sample sizes cannot be excluded. These conditions suggest the necessity of performing larger, prospective, randomized and multi-center studies.

There is a significant discrepancy in the frequencies of PS among various centers. The percentage of patients submitted to PNK in our study was comparable to other reports; however, conventional pre-cut papillotomy is rare. Nevertheless, both PS techniques were employed relatively often, (35.6% of all patients with sphincterotomy) (Table 4). This relatively high frequency was most likely the result of an early switch from the conventional method to the needle knife technique to lower the risk of PEP after multiple ineffective cannulation attempts^[24]. In the present study, the the efficacy results demonstrated no significant differences between the PCS and PNK methods, nevertheless, needle knife incision more often

Table 5 Complication rates after pre-cut sphincterotomy

| Ref. | Pts. No. | PS type | Start of cut | All complications | PEP | Bleeding | Perforation |
|--|----------|---------|--------------|-------------------|------|----------|-------------|
| Slot <i>et al</i> ^[1] | - | PNK | Orifice | 12% | 0.5% | 5.5% | 3% |
| Kasmin <i>et al</i> ^[14] | 72/398 | PNK | Centre | 11% | 3.8% | 3.8% | 3.8% |
| Huibregtse <i>et al</i> ^[221] | 190/987 | PNK | Orifice | 2.6% | 1.0% | 1.5% | 0% |
| Dowsett <i>et al</i> ^[25] | 96/748 | PNK | Orifice | 5.20% | 1.0% | 4% | 0% |
| Shakoor <i>et al</i> ^[26] | 53/1367 | PNK | Orifice | 11% | 5.5% | 3.7% | 1.8% |
| Leung <i>et al</i> ^[27] | 20/510 | PNK | Centre | 20% | 0% | 20% | 0% |
| Donnellan <i>et al</i> ^[28] | 352/2603 | PNK | Centre | 4.8% | 1.0% | 4.2% | 0.3% |
| Our data | 84/402 | PNK | Centre | 4.80% | 5.4% | 0% | 0% |
| | 59/402 | PCS | - | 3.4% | 3.4% | 0% | 0% |
| Binmoeller <i>et al</i> ^[11] | 123/327 | PCS | - | 5.3% | 2.7% | 2.4% | 0% |
| Goff <i>et al</i> ^[12] | 32/110 | PCS | - | 12% | 12% | 0% | 0% |

PS: Pre-cut sphincterotomy; PEP: Pos-ercp pancreatitis; PCS: Pre-cut conventional sphincterotomy; PNK : Pre-cut needle knife.

requires a two-step implementation procedure. The overall efficacy of cannulation did not depend on the technique used and was not influenced by the higher frequency of the two-step procedure in PNK, which is similar to data from other centers (Table 4). The aim of the second part of the study was to compare the groups with respect to typical complications (PEP, bleeding, and perforation) and additional parameters, including increased amylase levels 4 h after the procedure, “endoscopic” bleeding confirmed in ERCP and the necessity for endoscopic hemostasis. We did not observe significant differences in any of the above mentioned variables. Similarly, investigators in Helsinki and a multicenter trial performed in China also reported no differences in the complication rates in a direct comparison of needle knife and non-needle knife PS^[16,13]. It should be noted that this study compared two different technical modifications of PCS and PNK. The pre-cut method with conventional sphincterotomy was performed after anchoring the cannulotomy in Wirsung’s duct; meanwhile, the PNK method the cut initiated in the orifice. These technical modifications explain why a direct comparison with the present trial is impossible. The data presented above also demonstrate a unique distribution of complications compared with other available reports (Table 5). We noted one complication, PEP, that fulfilled Cotton’s consensus criteria^[20]. A credible reason for the absence of clinically significant bleeding may stem from frequent endoscopic haemostasis in extravasation observed during the procedure. In contrast, there are various definitions of bleeding after ERCP, which may result in discrepancies in the presented data and make reliable comparisons impossible.

The most probable reason for the lack of perforation in all analyzed patients might be the frequent use of a two-step procedure. This method avoids of further cannulation trials in regions of edematous tissues with altered anatomy. It appears that repeating the procedure after edema regression, 4-7 d after the first procedure, may be safer than repeated cannulation trials, and the visible bile streak may facilitate proper localization of the CBD orifice. This idea is only partially supported by data from different centers. Dowsett *et al*^[25] and Huibregtse *et al*^[21] did not report perforation using a two-step procedure

after PN in almost half their patients; Shakoor *et al*^[26], Donnellan *et al*^[27] and Bruins Slot *et al*^[11], who described the use of a two-step procedure relatively rarely, reported perforation rates in 1.8% and 3% of their patients, respectively. However, Kazimin *et al*^[14] and Leung *et al*^[28] reports, did not report a similar relationship, which may be the result of their relatively low rates of PNK and the technical modifications in their methodology (Tables 4 and 5). One example of such a modification is Doswett’s suggestion^[25] to elevate the upper part of the papilla with the needle knife during PNK cutting, which should lower the risk of duodenal wall penetration. The lack of a standard procedure precludes reliable comparisons of results. Nevertheless, our data seem to validate the statement that the PCS and PNK methods do not differ in terms of complication rates, and that the PNK technique is more often associated with a two-step procedure, justifying the strategy to attempt PCS first and switch to PNK in case of PCS failure. It should be noted that the switch to PNK from PCS was performed relatively early in the presented material, because many ineffective cannulation trials may increase the risk of PEP^[24]. In contrast, the PCS procedure is not feasible in all patients including; for example, in cases of duodenum lumen stenosis in presence of a pancreatic head tumor or obstructed papillary orifice due to the deposit. Other situations that may indicate the use of different types of the pre-cut procedure may depend on papilla anatomy, procedural indications or concomitant actions. In the third part of our study, we attempted to determine which factors should impact the choice of the type of pre-cut procedure. For this reason, we assessed the procedural differences with respect to impaired post-procedural pancreatic juice efflux. The study revealed an additional risk (OR = 3.38) of impaired pancreatic juice efflux 4 h after the procedure in PCS patients with a flat papilla (Table 3). This finding suggests that specific anatomy should prompt special precautions in multiple cannulations, and that specific anatomy indicates an early switch to the needle knife technique, if feasible. However, a flat papilla is a contraindication for the PNK method due to the unsatisfactory depth control during the incision and the higher risk of duodenal wall penetration. We have performed the PNK procedure in 23 patients with flat

papillae without any perforations. It appears that in these patients, the omission of the PCS step and the direct conversion to PNK is reasonable. The second indication of a high risk (OR = 5.4) of hyperamylasemia 4 h after the procedure was hemostasis in patients after analysed PNK pre-cut modification. This result suggests that the necessity for hemostasis requires the distal upper part of the papilla to be incised to ensure proper pancreatic juice efflux in an environment of hemostatic edema. However, the relatively small sample of patients and retrospective character of the present study require further prospective research.

COMMENTS

Background

The pre-cut procedure allows access to the bile ducts in cases of conventional technique failure. However, there are variations in the detailed technique for this procedure, which are generally divided into the non-needle knife procedure using a cannulotome with a conventional structure, and the needle knife using a needle-shaped device. Both techniques have been widely modified, and there are no firm rules defining the indications for the type of technique of choice.

Research frontiers

Author assessed the efficacy and safety profiles of both techniques and estimated their influence on pancreatic juice efflux (on the basis of amylase levels 4 h after the procedure) according to papillary anatomy, indications and the type of concomitant procedures.

Innovations and breakthroughs

In the first part of the study, they compared two modifications of pre-cut sphincterotomy. In contrast to Halttunen's and Wang's studies, the conventional incision was performed without Wirsung's duct cannulation; In addition, another difference was the needle knife incision was initiated from the middle of the intramural portion without a distal incision. The second part of the study concerned the influence of the procedural technique used on pancreatic juice efflux impairment depending on papillary anatomy, indications for the procedure and concomitant procedures. The risk of hyperamylasemia is three times higher after the conventional precut technique in patients with flat papilla. In the group of patients treated with the precut needle knife technique, the risk of hyperamylasemia was five times higher after endoscopic hemostasis.

Applications

The primary part of the study revealed that both analyzed methods may be used interchangeably, as they exhibit no differences in complication rates. Nevertheless, the needle knife technique often requires two endoscopic retrograde cholangiopancreatography (ERCP) procedures and should be the method of choice in cases of conventional pre-cut incision failure. The reason for the increased risk of hyperamylasemia is hemostasis after the needle knife procedure. This finding suggests that leaving the distal papilla intact may impair pancreatic juice efflux. This could be addressed by an incision in the distal part of the papilla, which requires further study.

Terminology

Pre-cut conventional sphincterotomy describes the incision performed with a cannulotome in cases of unfeasible deep cannulation of bile ducts. Pre-cut needle knife procedure is performed with a cannulotome a protruding distal portion responsible for intramural incision of the papilla. The incision may be initiated from the orifice of the papilla (orifice cut) or in the middle segment of the intramural part (middle cut). In the second modification, the distal part may be dissected or intact (as in their data). Two-step procedure-describes the situation after the pre-cut procedure and failure to access bile ducts. The second ERCP is performed 4-7 d after the first one and avoids cannulation in the region of oedematous tissues with altered anatomy.

Peer review

In this retrospective paper, the authors compared safety and efficacy of two different precut technique for biliary access. The authors conclude that the two techniques are basically similar concerning biliary cannulation success and complication rate, except for the need of a second intervention which was more needed in the needle knife group. This is an interesting paper.

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Epithelial markers of colorectal carcinogenesis in ulcerative colitis and primary sclerosing cholangitis

Pavel Wohl, Tomas Hucl, Pavel Drastich, David Kamenar, Julius Spicak, Eva Honsova, Eva Sticova, Alena Lodererova, Jan Matous, Martin Hill, Petr Wohl, Milos Kucera

Pavel Wohl, Tomas Hucl, Pavel Drastich, David Kamenar, Julius Spicak, Department of Hepatogastroenterology, Institute for Clinical and Experimental Medicine, Videnska 1958, 14021 Prague 4, Czech Republic

Eva Honsova, Eva Sticova, Alena Lodererova, Department of Pathology, Institute for Clinical and Experimental Medicine (IKEM), Videnska 1958, 14021 Prague 4, Czech Republic

Jan Matous, 2nd Department of Internal Medicine, Third Faculty of Medicine, Charles University, 11640 Prague 1, Czech Republic
Martin Hill, Department of Biostatistics, Institute of Endocrinology, Charles University, 11640 Prague 1, Czech Republic

Petr Wohl, Milos Kucera, Institute for Clinical and Experimental Medicine (IKEM), Videnska 1958, 14021 Prague 4, Czech Republic
Author contributions: Wohl P, Honsova E, Sticova E and Lodererova A performed the majority of experiments and wrote the manuscript as main author; Spicak J, Kucera M, Drastich P, Hucl T and Kamenar D co-ordinated and provided the collection of all the human material; Wohl P and Matous J contributed the study and wrote the manuscript; Hill M performed biostatistics.

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Correspondence to: Pavel Wohl, MD, Department of Hepatogastroenterology, Institute for Clinical and Experimental Medicine, Videnska 1958, 14021 Prague 4, Czech Republic. pawo@ikem.cz

Telephone: +420-26136-2139 Fax: +420-26136-2697

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Abstract

AIM: To evaluate the expression of epithelial markers of colorectal carcinogenesis in patients with long-term ulcerative colitis (UC) and primary sclerosing cholangitis (PSC) before and after transplantation.

METHODS: Eight patients with UC and PSC prior to liver transplantation (PSC-UC), 22 patients with UC after liver transplantation for PSC (OLT), 9 patients with active ulcerative colitis without PSC (UCA), 7 patients with

UC in remission (UCR) and 10 controls (N) underwent colonoscopy with multiple biopsies. Specimens were analysed histologically and semi-quantitatively immunohistochemically for p53, Bcl-2 and cyclooxygenase-2 (COX-2) markers. Statistical analysis was performed by Kruskal-Wallis and Fisher's exact tests.

RESULTS: PSC-UC had a statistically significantly higher expression of p53 in the nondysplastic mucosa as compared to OLT, UCA, UCR and N ($P < 0.05$). We also found a statistically significant positive correlation between the incidence of PSC and the expression of p53 ($P < 0.001$). UCA had a higher p53 expression as compared to UCR. OLT had a significantly lower expression of p53 as compared with PSC-UC ($P < 0.001$). Bcl-2 had a significant higher bcl-2 expression as compared with controls. No difference in COX-2 expression between PSC-UC, UCR and UCA was found. UCA had higher COX-2 expression as compared to UCR. We also found a statistically significant positive correlation between the expression of COX-2 and p53. Patients after liver transplantation for PSC had a statistically significantly lower expression of the p53 compared with PSC-UC ($P < 0.001$). PSC-UC had the same inflammatory endoscopic activity as OLT and UCR when evaluated with the Mayo score.

CONCLUSION: Our study shows that the nondysplastic mucosa of UC patients with PSC is characterised by a higher expression of the tumour suppressor gene p53, suggesting a higher susceptibility of cancer. This p53 overexpression correlates with the presence of PSC whilst it is not present in patients with UC after liver transplantation for PSC.

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Key words: Immunohistochemistry; Ulcerative colitis; Primary sclerosing cholangitis; Colorectal carcinoma; Liver transplantation; p53; bcl2; Cyclooxygenase-2

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INTRODUCTION

Patients with ulcerative colitis (UC) have an increased risk of colorectal cancer (CRC). Primary sclerosing cholangitis (PSC) is a chronic inflammatory disease often associated with inflammatory bowel disease (IBD)^[1]. PSC patients have a greater risk of potential malignant impact^[1]. IBD may be diagnosed at any time during the course of PSC but, in most cases, IBD is recognised first^[2]. Although both diseases run distinct courses with no direct relationship between their severities there are some features that distinguish patients with PSC and UC. Some authors have even suggested that PSC with UC represents a distinct disease phenotype^[3,4].

The increased risk of CRC is associated with long-term UC, the activity of the disease, the extent of the disease, the presence of PSC and the family incidence of CRC^[5,6]. In a population-based Swedish study, the cumulative incidence of CRC in UC patients with PSC was 33% at 20 years^[7]. Moreover, dysplasia and cancer in patients with a combined diagnosis of PSC-UC have recently been found even in patients with a shorter duration of the disease^[8]. Liver transplantation for PSC in UC patients has also been shown as a risk factor for CRC^[9-12]. However, some reports have not yet confirmed this data^[2,13].

Colitis-associated carcinoma (CAC) has several distinguishing clinical features when compared with sporadic colorectal carcinoma (SCC). CAC progresses to invasive adenocarcinoma from flat and nonpolypoid dysplasia more frequently than SCC. CAC may also be multifocal, likely due to the broad field-effect of mucosal inflammation contributing to the development of neoplasia^[14]. Standard colonoscopy is thus insufficient in detecting flat dysplasia and regenerative changes in colonic mucosa. Consequently, multiple biopsies or advanced endoscopic techniques such as chromoendoscopy, narrow band imaging or autofluorescence have been used^[14,15]. Recently, it has been indicated that early detection of premalignant changes in the nondysplastic mucosa of UC by immunohistochemistry and polymerase chain reaction methods might be possible^[8]. Epithelial histopathological markers of colorectal carcinogenesis, which have thus far been utilised especially in advanced dysplastic changes, may also now have clinical impact in nondysplastic mucosa^[8]. To the best of our knowledge, PSC-UC patients at present have not been studied in the context of histopathological markers in nondysplastic mucosa.

The tumour suppressor gene p53 is a 53 kDa nuclear

protein involved in the control of the cell cycle, apoptosis and the maintenance of genomic stability^[16-18]. p53 plays an active role in both DNA repair and the induction of apoptosis^[19]. It is mutated in a variety of cancers including colorectal carcinoma^[20-22]. Abnormal p53 expression, detected by immunohistochemistry, is often used as a marker of p53 mutation and thus found in dysplastic or cancerous tissue. Surprisingly, high p53 expression has been found in chronic UC patients with severe disease without cancer^[14,23]. Interestingly, alterations of p53 were reported to occur early in the carcinogenesis of CAC compared to SCC, where they seem to be a late event. Bcl-2 is an important antiapoptotic gene. Some of the effects of p53 may be at least partially mediated by the downregulation effect on bcl-2. Bcl-2 has been shown to be overexpressed in SCC; however, its role in CAC is uncertain.

Cyclooxygenase-2 (COX-2) is an important inflammatory mediator which might play a role in the pathophysiologic processes of inflammatory bowel disease and the development of neoplasia as well^[24]. COX-2 is induced upon cellular activation by hormones, proinflammatory cytokines, growth factors and tumour promoters^[25]. COX-2 overexpression occurs early in UC-associated neoplasia and the COX-2 increase cannot be explained only by inflammatory activity alone^[26].

Liver disease (PSC) might influence colonic mucosa by an unknown mechanism. One of the possible explanations of this mechanism is bile acid.

The aim of this study was to evaluate the expression of epithelial markers of colorectal carcinogenesis (p53, bcl-2, COX-2) in UC patients with or without the presence of PSC and after liver transplantation for PSC and correlate this expression with clinical and histopathological parameters.

MATERIALS AND METHODS

Patients

Eight patients with UC and PSC without liver transplantation (PSC-UC), 22 patients with UC after liver transplantation for PSC (OLT), 9 patients with active ulcerative pancolitis (UCA), 7 patients with UC in remission (UCR) and 10 controls (N) were included into the study (Table 1). UC activity was evaluated by the endoscopic Mayo score (0-remission, 1-mild, 2-moderate, 3-severe). The diagnosis of PSC was confirmed by ERCP or MR-CP and liver biopsy. All subjects gave their informed consent with the study protocol which had been reviewed and approved by the local ethics committee. The study was performed in accordance with the Helsinki Declaration and Title 45, Code of Federal Regulations, Part 46, Protection of Human Subjects.

Histopathology evaluation

All patients underwent a colonoscopy with a standard white light endoscope. All UC patients, regardless of PSC diagnosis, that were included into the study suffered pan-

Table 1 Demographic features of participating patients

| | <i>n</i> | Age (yr) | Sex (M/F) | Duration of UC (yr) | PSC | Duration after OLT (yr) | Histology | Endoscopy score (Mayo) |
|--------|----------|---------------|-----------|---------------------|---------------------|-------------------------|------------|------------------------|
| N | 10 | 52.2 ± 14.09 | 4/6 | 0 | No | 0 | 0 | 0 |
| UCR | 7 | 41.57 ± 13.35 | 3/4 | 9.57 ± 1.59 | No | 0 | 0.42 ± 0.4 | 0.71 ± 0.45 |
| UCA | 9 | 45.88 ± 17.62 | 5/4 | 9.56 ± 2.41 | No | 0 | 2.7 ± 0.4 | 2.5 ± 0.49 |
| PSC-UC | 8 | 37.12 ± 6.8 | 5/3 | 8.75 ± 1.56 | Yes (<i>n</i> = 8) | 0 | 2.1 ± 0.59 | 1.12 ± 0.33 |
| OLT | 22 | 43.33 ± 12.11 | 11/11 | 12.4 ± 5.24 | No | 5.19 ± 2.61 | 1.4 ± 0.49 | 1.09 ± 0.29 |

N: Controls; M: Male; F: Female; UC: Ulcerative colitis; N: UCR: UC in remission; PSC: Primary sclerosing cholangitis; OLT: UC after liver transplantation for PSC; UCA: UC active disease.

Table 2 Monoclonal and polyclonal antibodies used in this study

| Specificity | Origin | Company | Antigen retrieval | Dilution of antibodies |
|----------------------|--------|---------------------------------|----------------------|------------------------|
| bcl-2 Oncoprotein | Mouse | DakoCytomation, Denmark | Buffer EDTA, pH 8 | 20 × |
| p53 | Mouse | DakoCytomation, Denmark | Tris/EDTA, pH 9 | 40 × |
| COX-2 | Mouse | Cayman, Michigan, United States | Citrate buffer, pH 6 | 40 × |

COX-2: Cyclooxygenase-2.

colitis. Biopsies were taken from the entire colon in 10cm intervals (approximately 40 samples). Neither dysplasia nor cancer was detected. Semiquantitative evaluation of p53, bcl-2 and COX-2 immunoreactivity was performed independently by two the hispathologists (Eva H, Eva S) in a blinded fashion. There was a general agreement between these observers. For the few discrepancies, a second evaluation was undertaken to find an agreement. Biopsies were analysed histologically and semi-quantitatively immunohistochemically for p53, bcl-2 and COX-2 with a scoring scale comparable to other studies^[19,21,26,27]. The expression of antigens was analysed on 4 µm thick sections by a two-step indirect immunoperoxidase method. Slides were deparaffinised in xylene and rehydrated in graded ethanol. After deparaffinisation and rehydration, the slides were cooked in a microwave oven (buffers used for antigen retrieval are listed in Table 2). Endogenous peroxidase was blocked by 0.3% H₂O₂ in 70% methanol for 30 min. Next, the specimens were incubated with a primary antibody for 30 min. The antibody was detected by incubation with a secondary antibody (Histofine Simple Stain MAX PO, Nichirei, Japan) for 30 min and incubation with Dako Liquid DAB+ Substrate-Chromogen System (DakoCytomation, Denmark). Afterwards, the specimens were counterstained with Haematoxylin and mounted in Entellan (Merck, Germany). Monoclonal antibodies (Ab) used in this study are listed in Table 2. p53 was evaluated in the intranuclear region, whereas bcl-2 and COX-2 were examined by immunohistochemistry in colonic cytoplasmic region of the epithelial cells.

The immunohistochemistry scoring scale was based on the evaluation of the percentage of staining of positive cells, 0, no staining, 1+, mild 1%-32% of epithelial cell population, 2+, moderate from 33% to 66% of cell

population and 3+, the highest staining from 67% to 100%. A positive result was considered as staining of more than 33% of the epithelial cells. Staining intensity was evaluated as weak, moderate and strong. Histological and endoscopic disease activity (Mayo score, also known as the Mayo Clinic Score and the Disease Activity Index) were evaluated (0-no inflammation, 1, mild, 2, medium and 3, severe inflammation)^[28,29].

Statistical analysis

The data were evaluated using a robust Kruskal-Wallis test followed by Dunn’s multiple comparison with Bonferroni correction. The relationships between positivity in epithelial markers was evaluated by Fisher’s exact test (*P* value < 0.05 was considered significant). The relationships between the continuous variables were evaluated using Spearman’s correlation.

RESULTS

p53

PSC-UC had a significantly higher expression of p53 in the nondysplastic mucosa as compared to OLT, UCA, UCR and N (*P* < 0.05) (Figure 1A). We also found a statistically significant positive correlation between the presence of PSC and the expression of p53 (*P* < 0.001) (Table 3). UCA had a higher p53 expression as compared to UCR (*P* < 0.05). Correlation between p53 expression and duration of UC did not reach significance (*r* = -0.014, *n* = 55).

bcl-2

UCA had a significantly higher bcl-2 expression as compared to controls (Figure 1B).

COX-2

The expression of COX-2 did not differ in PSC-UC as compared to OLT, UCA and UCR. UCA had a higher COX-2 expression as compared to UCR (*P* < 0.05) (Figure 1C). We also found a statistically significant positive correlation between the expression of COX-2 and p53 (*P* < 0.05) (Table 3).

Disease activity

PSC-UC, UCA and OLT did not significantly differ in histological disease activity. However, their histological activity was significantly higher when compared with

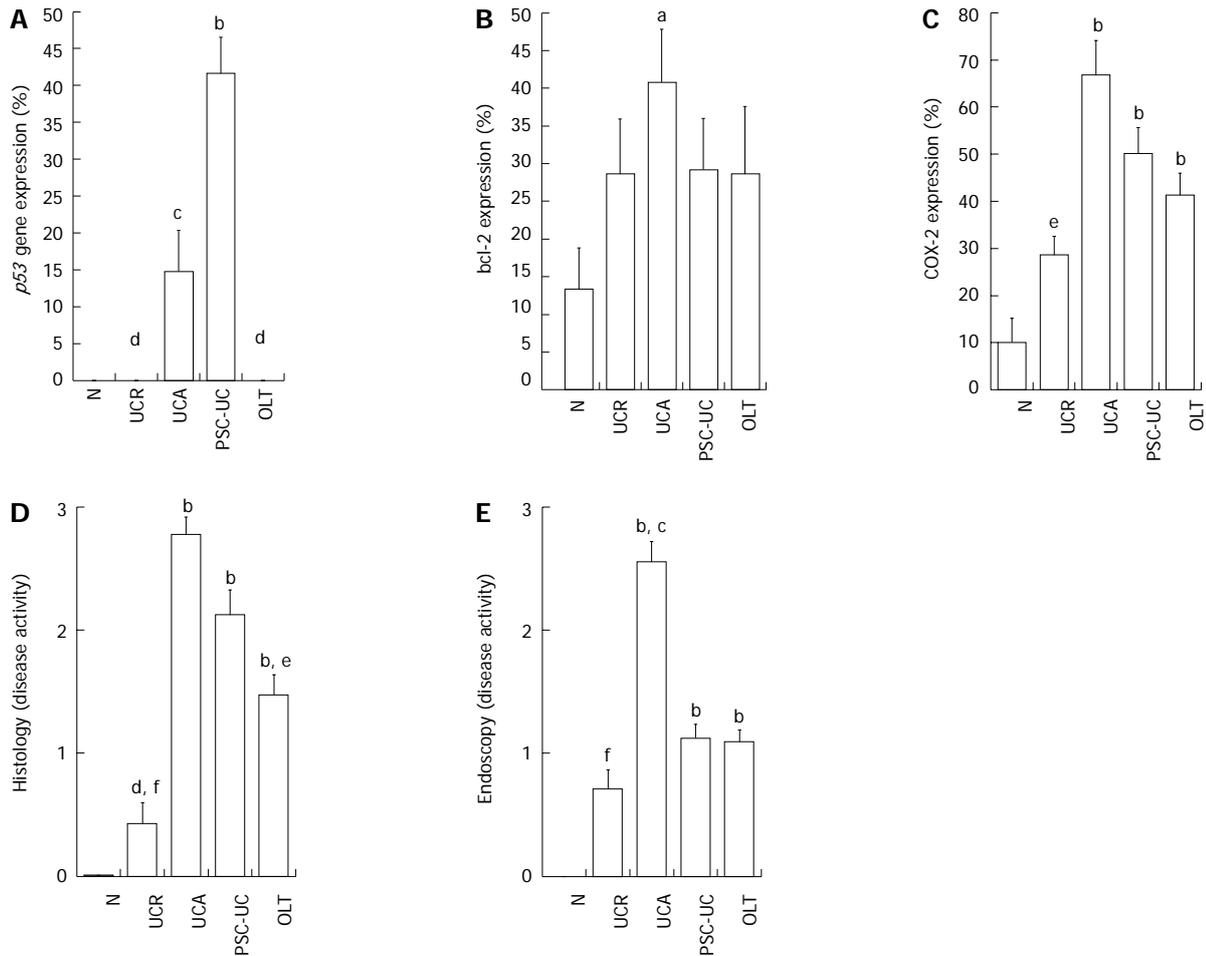


Figure 1 Comparison of findings in percent (%) in all tested group. A: Comparison of intranuclear *p53* gene expression in percent (%) in all tested group; B: Comparison of *bcl-2* expression in percent (%) in all tested group; C: Comparison of COX-2 expression by immunohistochemistry in percent (%) in all tested group; D: Comparison of inflammatory disease activity by histology (0 = nonactive, 1 = mild, 2 = moderate, 3 = severe) in all tested group; E: Comparison of endoscopic findings by Mayo score in all tested group in all tested group. The bars with error bars represent group means with SEM. Differences between groups were evaluated using Dunn's multiple comparisons with Bonferroni correction. ^a $P < 0.05$, ^b $P < 0.01$ vs N group; ^c $P < 0.05$, ^d $P < 0.01$ vs PSC-UC group; ^e $P < 0.05$, ^f $P < 0.01$ vs UCA group. UC: Ulcerative colitis; UCR: UC in remission; PSC: Primary sclerosing cholangitis; OLT: UC after liver transplantation for PSC; UCA: UC active disease; COX-2: Cyclooxygenase-2.

UCR and N ($P < 0.001$) (Figure 1D). PSC-UC had the same inflammatory endoscopic activity as OLT and UCR when evaluated with the Mayo score but this activity was lower when compared with UCA ($P < 0.05$) (Figure 1E).

Liver transplantation

Patients after liver transplantation for PSC had a statistically significantly lower expression of the *p53* gene compared with PSC-UC. These two groups of patients did not differ in the other tested parameters (*bcl-2*, COX-2, histology and endoscopy) (Table 4).

DISCUSSION

The presence of PSC in UC patients is generally considered as a risk factor for colorectal cancer. However, comprehension of the specific mechanisms involved in CAC pathogenesis in PSC patients remains limited. The role of colonic mucosal markers such as *p53*, *bcl-2* and COX-2 based on immunohistochemistry evaluation in PSC-UC

patients has not yet been reported.

Our study shows that PSC-UC is characterised by a higher expression of the tumour suppressor gene *p53* in nondysplastic mucosa as compared with OLT, UCA, UCR and controls which suggests a higher neoplastic potential of PSC-UC. Moreover, we found a statistically significant positive correlation between the incidence of PSC and *p53* expression. The observed expression of *p53* is driven mainly by inflammation while it did not correlate either with histological or endoscopic activity. Surprisingly, we found a lower *p53* expression in OLT when compared to PSC-UC. To our knowledge, this finding has not been previously described in the literature and suggests the hypothesis that liver disease (PSC) is associated by an unknown mechanism with increased expression of *p53* in the intestinal mucosa. In addition, *p53* expression correlates with higher COX-2 expression suggesting that inflammation may contribute to the amount of *p53* gene expression. On the other hand, the expression of COX-2 did not differ between PSC-UC

Table 3 Relationship between p53, cyclooxygenase-2 and primary sclerosing cholangitis n (%)

| | PSC- | PSC+ | Row total | COX-2- | COX-2+ | Row total |
|--------------|------------|-----------|------------|------------|------------|------------|
| p53- | 43 (78.18) | 0 (0) | 43 (78.18) | 34 (61.82) | 5 (9.09) | 39 (70.91) |
| p53+ | 4 (7.27) | 8 (14.55) | 12 (21.82) | 9 (16.36) | 7 (12.73) | 16 (29.09) |
| Column total | 47 (85.45) | 8 (14.55) | 55 (100) | 43 (78.18) | 12 (21.82) | 55 (100) |

Statistical significance (Fisher's exact test) $P < 0.001$. PSC: Primary sclerosing cholangitis; COX-2: Cyclooxygenase-2.

Table 4 Comparison of primary sclerosing cholangitis-ulcerative colitis and ulcerative colitis after liver transplantation for primary sclerosing cholangitis

| | p53 | COX-2 | bcl-2 | Histology score | Endoscopy score (Mayo) |
|---------|----------------|-------|-------|-----------------|------------------------|
| PSC-UC | ↑ ^a | ↔ | ↔ | ↔ | ↔ |
| OLT | ↓ | ↔ | ↔ | ↔ | ↔ |
| P value | $P < 0.001$ | NS | NS | NS | NS |

^a $P < 0.05$ vs PSC-UC group. COX-2: Cyclooxygenase-2; UC: Ulcerative colitis; PSC: Primary sclerosing cholangitis; OLT: UC after liver transplantation for PSC.

and OLT, UCA and UCR. This finding might advance the hypothesis that the COX-2 mediated inflammatory pathway could play a similar role in PSC-UC and UC patients irrespective of the presence of PSC. We have also confirmed the previously described higher expression of p53 and COX-2 in the active disease^[23,30,31].

The importance of the p53 tumour suppressor gene in PSC associated carcinogenesis has been demonstrated for hepatobiliary malignancies including cholangiocarcinoma and CAC without PSC^[17,32,33]. Increased p53 gene expression in the colonic mucosa in UC patients has been reported; however, patients with PSC have not been evaluated in these studies^[14,15,17,29,34-38]. Our data clearly show a positive correlation between the presence of PSC and the level of expression of p53 in the intestinal nondysplastic mucosa of UC patients. These results thus support the hypothesis that PSC plays a role in UC associated colorectal carcinogenesis. We suggest that this happens, at least in part, through the overexpression of p53.

Alterations in the p53 gene predispose to colonocytes dysplasia^[25]. Mutations of the p53 gene seem to occur at an early stage in CAC carcinogenesis compared to it being a late event in CRC^[19,27]. Previously, we confirmed this by showing p53 overexpression in nondysplastic mucosa in a disease with high risk of cancer development. There is an ongoing debate in the literature whether p53 alterations can occur in nondysplastic epithelium. Patients with longstanding UC without dysplasia showing p53 overexpression may develop neoplasia 5 times more likely than those without^[17]. Other studies reported p53 mutations in nondysplastic epithelium in patients with or without colorectal cancer^[35,39]. In contrast, p53 was found only in dysplastic mucosa by others^[25,36-37]. Accordingly, p53 expression clearly preceded dysplasia. It also appeared earlier in the course of the disease than previously reported^[38]. However, we did not confirm this data in our study. One explanation is the small number of the tested group. In addition, the early expression of p53 in nondysplastic mucosa might make it a high risk marker of premalignant

epithelium. The hypothesis of a p53 driven carcinogenesis in PSC-UC is further supported by the fact that we found a difference between p53 expression in PSC-UC patients and in patients after liver transplantation. Surprisingly, we found no p53 expression in the OLT group. Liver transplantation may contribute to the reduction of p53 gene expression by eliminating the causative liver disease (PSC?). Liver transplantation could thus be viewed as having a temporary protective effect in the CAC pathogenesis. However, this finding needs to be verified in further studies. The mechanism of this phenomenon remains unknown. It would be interesting to see whether p53 expression diminishes in the same patients following transplantation or whether it comes back as in the case of the recurrence of PSC. In a 6 year follow up of our tested group, we observed no PSC recurrence. Another factor that may contribute to the different expression is the use of immunosuppressive therapy in patients after transplantation.

The mechanisms involved in the pathogenesis of CAC may be different in patients with PSC as compared to UC alone^[10,40]. The effects of hepatobiliary factors may be one explanation. Bile acids play an important role in PSC-UC^[1,2,17,40]. Secondary bile acids have been shown to result in hyperproliferation and thus play a role in PSC-UC and CAC pathogenesis^[11,2,17]. Reduction of the incidence of CAC was achieved in PSC-UC with the use of ursodeoxycholic acid^[41,42]. Unfortunately, the effects of ursodeoxycholic acid cannot be judged from our study as all our patients with PSC, prior or after transplantation, received it.

The inflammatory theory is still considered to be important in the process of colorectal carcinogenesis in UC^[14,22]. The mechanisms of COX-2 driven carcinogenesis are still not fully understood, though studies suggest that an increased expression of COX-2 as a consequence of inflammation reduces apoptosis and increases angiogenesis^[26,31]. In our study, we confirmed higher COX-2 expression in UCA compared to UCR. PSC-UC did not

differ in the expression of COX-2 when compared with OLT, UCA and UCR. However, PSC-UC was identical in histological inflammatory activity to UCA, but had a higher activity in comparison to UCR despite similar COX-2 expression. The COX-2 expression thus did not fully correlate with histological inflammatory activity alone. Interestingly, in the study of Agoff *et al.*^[26], COX-2 overexpression occurred early in UC-associated neoplasia; however, the cancer risk increase could not be explained solely by inflammatory activity alone. In their study, overall neoplastic change explained the majority of the variation in COX-2 expression, whereas inflammatory activity explained only 11%^[26].

Bcl-2 is considered as an important antiapoptotic gene which is in reciprocal relation with p53^[43]. Ilyas *et al.*^[34] have shown that bcl-2 plays an important role in UC associated carcinogenesis. We found a higher bcl-2 expression in UCA as compared to controls. Inflammation could be one possible explanation. In contrast to p53, no association with the presence of PSC was observed. We also did not find negative regulation of bcl-2 and p53 as previously described in breast cancer or adenomas. Thus, the impact of bcl-2 on colorectal cancer pathogenesis of PSC-UC based on our findings is still unclear.

We could also suggest, as other authors have, that PSC-UC might be a subgroup of UC^[3,4,44]. PSC-UC is characterised by the same histological inflammatory activity as UCA but differs from UCR and N. PSC-UC had a higher p53 expression as compared to UCA, UCR, OLT and N; however, no difference between these groups was observed in COX-2 expression. PSC-UC thus shows signs of both UCA and UCR characteristics. Because of the known mild clinical course of PSC-UC as compared to UC alone, it may be underdiagnosed with unfavourable clinical consequences. Accordingly, regular colonoscopy has been recommended for all PSC patients. For that reason, p53 overexpression might be a useful predictor of potential carcinogenesis of colorectal mucosae in PSC-UC patients. In addition, according to our study, routine clinical and endoscopic indexes of colitis without PSC (*e.g.*, Mayo, UCDAI) cannot be used in PSC-UC. The presence of PSC in patients with UC should be taken into account especially in clinical and experimental studies.

Our study has several limitations. We investigated only a small group of subjects and used the immunohistochemical method for detection of mucosal markers. It should be noted, however, that immunohistochemical investigations and mutation analysis rely on samples of mucosa obtained by colonoscopic biopsy and thus are subject to the same sampling error^[27]. In addition, we may have missed some non-sense mutations resulting in a truncated protein^[23]. We also did not detect dysplasia in any of our patients. However, it might have been interesting to compare the expression of these markers in nondysplastic and dysplastic mucosa. Moreover, it would have been better to analyse the same patients with PSC and UC before and after OLT. This was not possible since the PSC-UC patients had not yet undergone OLT,

but these patients will be included into a subsequent study.

In conclusion, PSC-UC was characterised by a higher expression of the tumour suppressor gene *p53* in nondysplastic mucosa explaining, at least in part, the higher neoplastic potential of PSC-UC. Furthermore, this overexpression was not present in UC patients who underwent liver transplantation for PSC. The expression of p53 thus correlated with the presence of PSC, suggesting a carcinogenic effect of the liver disease on colonic mucosa. The presence of p53 expression in nondysplastic mucosa may support its use as a marker of increased susceptibility to cancer that may enable detection of premalignant epithelium. It may be the use of epithelial markers of carcinogenesis which may in the future be used to better predict the risk preneoplastic lesions and CAC in UC patients with PSC and after liver transplantation. Our results need to be verified in larger future studies.

COMMENTS

Background

The presence of primary sclerosing cholangitis (PSC) in ulcerative colitis (UC) patients is generally considered a risk factor for colorectal cancer. However, comprehension about the specific mechanisms involved in colitis associated carcinoma (CAC) pathogenesis in PSC patients remains limited. The aim of this study was to evaluate the expression of epithelial markers of colorectal carcinogenesis in patients with long-term UC and PSC before and after transplantation.

Research frontiers

CAC has several distinguishing clinical features when compared with sporadic colorectal carcinoma (SCC). CAC progresses to invasive adenocarcinoma from flat and nonpolypoid dysplasia more frequently than SCC. CAC may also be multifocal likely due to the broad field-effect of mucosal inflammation contributing to the development of neoplasia. Standard colonoscopy is thus insufficient in detecting flat dysplasia and regenerative changes in colonic mucosa. Recently, it has been indicated that early detection of premalignant changes in the nondysplastic mucosa of UC by immunohistochemistry and polymerase chain reaction methods might be possible. Epithelial histopathological markers of colorectal carcinogenesis, which have thus far been utilised especially in advanced dysplastic changes, may also now have clinical impact in nondysplastic mucosa. The role of colonic mucosal markers such as p53, bcl-2 and cyclooxygenase-2 (COX-2) based on immunohistochemistry evaluation in UC and PSC prior to liver transplantation (PSC-UC) patients has not yet been reported.

Innovations and breakthroughs

Data clearly show a positive correlation between the presence of PSC and the level of expression of p53 in the intestinal nondysplastic mucosa of UC patients. These results thus support the hypothesis that PSC plays a role in UC associated colorectal carcinogenesis. The authors suggest that this happens, at least in part, through the overexpression of p53.

Applications

Because of the known mild clinical course of PSC-UC as compared to UC alone, it may be underdiagnosed with unfavourable clinical consequences. Accordingly, regular colonoscopy has been recommended for all PSC patients. For that reason, p53 overexpression might be a useful predictor of potential carcinogenesis of colorectal mucosae in PSC-UC patients. In addition, according to their study, routine clinical and endoscopic indexes of colitis without PSC (*e.g.*, Mayo, UCDAI) cannot be used in PSC-UC. The presence of PSC in patients with UC should be taken into account especially in clinical and experimental studies. The presence of p53 expression in nondysplastic mucosa may support its use as a marker of increased susceptibility to cancer that may enable detection of premalignant epithelium. It may be the use of epithelial markers of carcinogenesis which may in the future be used to better predict the risk preneoplastic lesions and CAC in UC patients with PSC and after liver transplantation.

Peer review

The study shows that the nondysplastic mucosa of UC patients with PSC is characterised by a higher expression of the tumour suppressor gene p53, suggesting a higher susceptibility of cancer. This p53 overexpression correlates with the presence of PSC, whilst it is not present in patients with UC after liver transplantation for PSC.

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Therapeutic efficacy of transarterial chemo-embolization with a fine-powder formulation of cisplatin for hepatocellular carcinoma

Kazuhiro Kasai, Akira Ushio, Yukiho Kasai, Kei Sawara, Yasuhiro Miyamoto, Kanta Oikawa, Yasuhiro Takikawa, Kazuyuki Suzuki

Kazuhiro Kasai, Akira Ushio, Yukiho Kasai, Kei Sawara, Yasuhiro Miyamoto, Kanta Oikawa, Yasuhiro Takikawa, Kazuyuki Suzuki, Division of Gastroenterology and Hepatology, Department of Internal Medicine, Iwate Medical University, Iwate 020-8505, Japan

Author contributions: Kasai K and Ushio A contributed equally to this work; Kasai K, Ushio A, Sawara K, Miyamoto Y, Kasai Y, Oikawa K, Takikawa Y and Suzuki K designed research; Kasai K and Ushio A performed research; Kasai K and Ushio A analyzed data; Kasai K wrote the paper.

Correspondence to: Kazuhiro Kasai, MD, Division of Gastroenterology and Hepatology, Department of Internal Medicine, Iwate Medical University, Uchimaru 19-1, Morioka, Iwate 020-8505, Japan. kaz-k@yc4.so-net.ne.jp

Telephone: +81-19-6515111 Fax: +81-19-6524666

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Abstract

AIM: To evaluate the efficacy of transarterial chemoembolization (TACE) using a suspension of a fine-powder formulation of cisplatin (DDPH) in lipiodol (LPD) in the treatment of hepatocellular carcinoma (HCC).

METHODS: The subjects were 262 HCC patients treated with TACE using a DDPH-LPD suspension. The DDPH-LPD suspension was prepared by mixing 50 mg of DDPH into 10 mL of LPD. TACE was repeated when treated lesions relapsed and/or new hepatic lesions were detected. These patients received additional TACE using the same agent. TACE was repeated until complete regression of the tumor was obtained. The primary efficacy endpoint of the current study was the objective early response rate. Secondary efficacy endpoints were progression-free survival (PFS) and overall survival.

RESULTS: The objective early response rate was 43.6%. Cumulative PFS rates were 56.7% at 6 mo, 23.1% at 12 mo, 13.4% at 18 mo, and 10.5% at 24 mo. The median PFS was 6.6 mo. Cumulative survival rates were 90.6% at 6 mo, 81.9% at 12 mo, 70.5% at 24 mo, and 58.8% at 36 mo. Median survival time was 46.6 mo. All adverse reactions were controllable by temporary suspension of treatment. No serious complications or treatment-related deaths were observed.

CONCLUSION: TACE using a suspension of DDPH in LPD may be a useful treatment for HCC.

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Key words: Cisplatin; DDPH; Hepatocellular carcinoma; Portal vein tumor thrombosis; Transarterial chemoembolization

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INTRODUCTION

Hepatocellular carcinoma (HCC) is a common primary liver cancer with a rising incidence worldwide^[1]. In Japan, more than 30 000 people die of HCC each year^[2]. Curative therapies such as resection, liver transplantation, and local ablative treatments may offer a chance of improved life expectancy, but these treatment modalities are applicable to only a small proportion of HCC patients. As a result, in patients with advanced HCC who are not

eligible for these curative therapies, transarterial chemoembolization (TACE) has been the mainstay treatment option with proven survival benefits^[3,4]. Many studies of TACE have been reported; a method using lipiodol (LPD), an oily contrast medium used as a drug delivery system, is now widely used, and anticancer drugs such as doxorubicin (ADM), epirubicin, and other anthracyclines are often used^[5,6]. However, the tumors have a high frequency of recurrence after TACE^[5,7]. Moreover, HCC is not necessarily sensitive to these drugs^[8,9]. Therefore, the therapeutic results of TACE for HCC should improve as anticancer drugs become more effective.

Cisplatin (CDDP), a platinum compound, is an effective anticancer agent used in the treatment of various malignancies^[10]. Researchers have reported that TACE using a suspension of CDDP powder in LPD may be more effective against unresectable HCC than TACE using an ADM-LPD emulsion^[11,12]. However, only a few institutions have used this for TACE because it is difficult to refine the CDDP powder. Since 2004, a fine-powder formulation of CDDP (DDPH, IA-call; Nippon Kayaku, Tokyo, Japan) has been available as a therapeutic agent for intra-arterial infusion in Japan. As a result, TACE using DDPH has become widespread in Japanese institutions. We have already used TACE with DDPH for HCC patients and reported favorable results^[13]. The aim of this study was to elucidate the efficacy of this therapy by analyzing the clinical results of 262 HCC patients treated in this manner.

MATERIALS AND METHODS

Study design and patient eligibility

This clinical investigation was approved by the ethics committee of our institution, and informed consent was obtained from all patients. The study was designed as a single-institution, open clinical study. The primary efficacy endpoint of the current study was the objective response rate. Secondary efficacy endpoints were progression-free survival (PFS) and overall survival (OS).

Eligibility criteria were as follows: (1) Eastern Cooperative Oncology Group performance status of 0-2; (2) age over 20 years; (3) diagnosis of HCC based on imaging or histological findings; (4) no indication for surgical resection or local ablation therapy such as radiofrequency ablation (RFA); (5) bidimensionally measurable hepatic lesions; (6) adequate hepatic function (serum total bilirubin < 3.0 mg/dL), and adequate renal function (serum creatinine < the upper normal limit); (7) no extrahepatic metastasis; (8) no tumor thrombus in the main trunk of the portal vein; or (9) no HCC treatment for 4 wk before study entry.

Enrolled were patients with HCC suitable for curative treatments such as surgical resection and local ablation therapy but who were of high risk for these therapies. A total of 262 consecutive patients who were to undergo TACE using DDPH between January 2006 and May 2011 were enrolled. All of the enrolled patients met the inclusion criteria.

Diagnosis

The diagnostic criteria for HCC *via* imaging were based on hyperattenuation in the arterial phase and hypoattenuation in the portal phase on dynamic computed tomography (CT) or magnetic resonance imaging (MRI), and tumor stain on angiography. When HCC could not be diagnosed by imaging alone, fine-needle biopsy using abdominal ultrasonography (US) was performed to obtain histological proof. Further assessment of HCC was conducted by measuring levels of α -fetoprotein (AFP) and des- γ -carboxy prothrombin (DCP).

Liver function was evaluated according to the Child-Pugh classification^[14]. Tumor stage was assessed based on the tumor node metastasis (TNM) staging system of the Liver Cancer Study Group of Japan^[15]. Portal vein tumor thrombosis (PVT) grade was classified as follows: Vp0, no invasion of the portal vein; Vp1, invasion of the third or more distal branch of the left or right portal vein; Vp2, invasion of the second branch of the portal vein; Vp3, invasion of the first branch of the portal vein; and Vp4, invasion of the trunk of the portal vein.

Preparation of the agents for TACE

DDPH was mixed with LPD (iodized oil, Lipiodol Ultra-Fluide; Andre Guerret, Aulnay-sous-Bois, France). The DDPH-LPD suspension was prepared by mixing 50 mg of DDPH into 10 mL of LPD. The dosage of DDPH-LPD suspension was adjusted depending on the tumor size, number of tumors, degree of liver impairment, and renal function, but the maximum dose of DDPH-LPD suspension was not allowed to exceed 10 mL.

Treatment procedures

In all TACE procedures, hepatic angiography was performed by the femoral approach using a 4-Fr catheter and a 1.8-Fr to 2.4-Fr microcatheter. After confirming the hepatic arteries supplying the target tumor, a catheter was selectively inserted into the hepatic artery supplying the target tumor, and the DDPH-LPD suspension was injected. In patients with several tumors in the liver, superselective catheterization was performed for each lesion. If superselective catheterization was not possible, the DDPH-LPD suspension was injected into the right and left main hepatic arteries distal to the origin of the cystic artery. After the injection, arterioembolization was performed using porous gelatin particles (Gelpart; Nippon Kayaku, Tokyo, Japan) mixed with contrast medium.

All patients were followed up with US, CT, and/or MRI after 1 mo and then every 3 mo thereafter. Treatment was repeated by TACE alone when treated lesions relapsed and/or new hepatic lesions were detected. These patients received additional TACE using the same agent during the follow-up period unless the tumors progressed. TACE was repeated until complete regression of the tumor was obtained.

Evaluation of therapeutic efficacy

Tumor response was assessed by US, CT, and/or MRI at 1 mo from the start of treatment and every 3 mo thereaf-

Table 1 Baseline characteristics of the 262 patients

| Characteristics | | |
|---------------------------------------|-------------------|----------------------|
| Enrolled patients | | 262 |
| Age (yr) | Median (range) | 70 (32-92) |
| Sex | Male/female | 176/86 |
| Etiology | HBV/HCV/NBNC | 30/170/62 |
| Child Pugh classification | A/B/C | 147/93/22 |
| Number of tumors | < 10/≥ 10 | 114/148 |
| Maximum tumor size (mm) | Median (range) | 32.5 (8.0-300.0) |
| Stage ¹ | I / II / III / IV | 17/45/136/64 |
| PVTT grade | Vp0/Vp1-2/Vp3 | 202/27/33 |
| Total bilirubin (mg/dL) | mean ± SD | 1.0 ± 0.7 |
| Albumin (g/dL) | mean ± SD | 3.4 ± 0.6 |
| Prothrombin time (%) | mean ± SD | 91.1 ± 8.4 |
| Platelet count (× 10 ⁴ /L) | mean ± SD | 8.9 ± 5.0 |
| AFP (ng/mL) | median (range) | 31.6 (1.0-1 000 000) |
| AFP-L3 (ng/mL) | median (range) | 4.0 (0-91.8) |
| DCP (mAU/mL) | median (range) | 60 (0-928 900) |
| Previous treatment | Yes/no | 107/155 |

Data are expressed as median values with ranges, mean ± SD, or number of patients. ¹According to the modified RECIST (mRECIST) criteria. HBV: Hepatitis B virus; HCV: Hepatitis C virus; NBNC: Negative for hepatitis B surface antigen and HCV antibody; PVTT: Portal vein tumor thrombosis; AFP: α -fetoprotein; AFP-L3: Lens culinaris agglutinin-reactive fraction of α -fetoprotein; DCP: Des- γ -carboxy prothrombin.

ter. The response was classified according to the modified RECIST (mRECIST) criteria^[16], which take into account only the viable (arterially enhancing) component of the target tumors, and grade tumor response as follows: complete response (CR)-disappearance of any intratumoral arterial enhancement in all target lesions; partial response (PR)-at least a 30% decrease in the sum of diameters of viable target lesions, taking as reference the baseline sum of the diameters of target lesions; progressive disease (PD)-increase (at least 20%) in the sum of the diameters of viable target lesions, taking as reference the smallest sum of the diameters of viable (enhancing) target lesions recorded since treatment started, or appearance of new lesions; stable disease (SD)-all other cases.

Toxicity was evaluated using the National Cancer Institute-Common Terminology Criteria for Adverse Events, version 3.0 (CTCAE v3.0).

Statistical analysis

Baseline data are expressed as means ± SD or as medians and range. Statistical analysis was performed in September 2011. The cumulative survival rate and PFS were calculated from the date of therapy initiation and assessed by the Kaplan-Meier life-table method, and differences were evaluated using the log-rank test. Univariate analysis of predictors for survival of patients was assessed using the Kaplan-Meier life-table method, and differences were evaluated using the log-rank test. Multivariate analysis of predictors for survival was assessed by the Cox proportional hazards model. Significance was accepted at $P < 0.05$. All analyses were performed using SPSS version 11 software (SPSS, Chicago, IL, United States).

RESULTS

Patient characteristics

The characteristics of the 262 patients are listed in Table 1. There were 176 male and 86 female patients, ranging in age from 32 to 92 years (median age, 70 years). There were 147 (56.1%), 93 (35.5%), and 22 (8.4%) patients with Child-Pugh Stages A, B, and C, respectively. The median diameter of the largest tumor was 32.5 mm (range, 8-300 mm). Serum AFP levels were > 10 ng/mL in 182 patients, and 146 patients were DCP-positive (> 40 mAU/mL).

Clinical efficacy

The median duration of follow-up was 17.0 mo (range, 2.0-64.0 mo). A total of 682 TACE procedures were performed in 262 patients. The median number of TACE procedures was 2 cycles (range, 1-13 cycles). Early response status in the 262 patients was assessed after the first course of therapy. As a result, 34 patients (13.0%) had CR, 80 patients (30.6%) had PR, 69 patients (26.3%) had SD, and 79 patients (30.1%) had PD [response rate (CR + PR/all cases) = 43.6%]. The disease control rate (CR + PR + SD/all cases) was 69.9%.

PFS

The median PFS was 6.6 mo. The PFS rates at 6, 12, 18, and 24 mo were 56.7%, 23.1%, 13.4%, and 10.5%, respectively (Figure 1).

Survival

The median survival time (MST) was 46.6 mo. The cumulative survival rates at 6, 12, 24, and 36 mo were 90.6%, 81.9%, 70.5%, and 58.8%, respectively (Figure 1).

Cumulative survival rates of patients with no PVTT were 96.3% at 6 mo, 90.4% at 12 mo, 79.7% at 24 mo. On the other hand, cumulative survival rates of patients with PVTT were 68.6% at 6 mo, 49.0% at 12 mo, 33.7% at 24 mo. The survival rate was significantly higher in patients with no PVTT than in patients with PVTT ($P < 0.001$, Figure 2A).

Moreover, cumulative survival rates were determined by PVTT grade in 60 patients with PVTT. Cumulative survival rates of patients with Vp1-2 were 87.6% at 6 mo, 67.0% at 12 mo, 52.8% at 24 mo. On the other hand, cumulative survival rates of patients with Vp3 were 51.0% at 6 mo, 32.5% at 12 mo, 16.2% at 24 mo. The survival rate was significantly higher in patients with Vp1-2 than in patients with Vp3 ($P = 0.009$, Figure 2B).

Prognostic factors affecting patient survival were analyzed by examining 17 potential parameters (Table 2). Univariate analysis revealed 12 significant prognostic factors related to survival: stage ($P < 0.001$), Child Pugh classification ($P = 0.005$), JIS score ($P < 0.001$), total bilirubin ($P = 0.031$), albumin ($P = 0.012$), number of tumors ($P < 0.001$), maximum tumor size ($P < 0.001$), PVTT grade ($P < 0.001$), tumor distribution ($P < 0.001$),

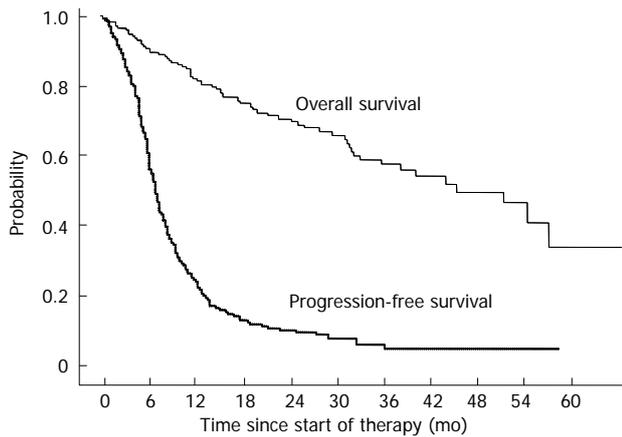


Figure 1 Overall survival and progression-free survival curves of 262 patients treated with transarterial chemoembolization using DDPH.

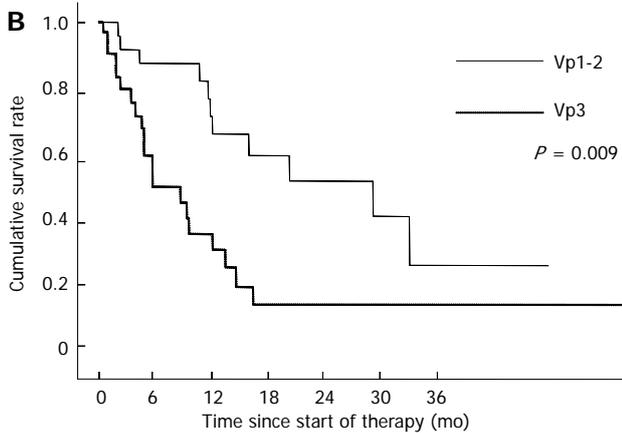
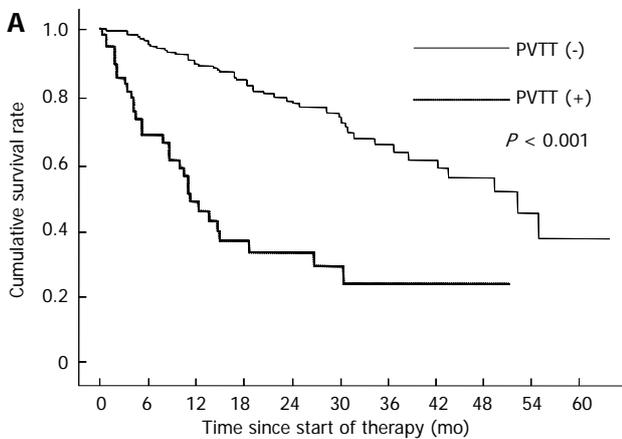


Figure 2 Cumulative survival rates. A: Comparison of cumulative survival rates between patients with no portal vein tumor thrombosis and those with portal vein tumor thrombosis; B: Comparison of the cumulative survival rates between patients with Vp1-2 and patients with Vp3. PVTT: Portal vein tumor thrombosis.

AFP ($P < 0.001$), DCP ($P < 0.001$), and therapeutic effect ($P < 0.001$).

Multivariate analysis showed 3 significant prognostic factors related to survival: PVTT grade ($P = 0.010$), AFP ($P = 0.002$), and therapeutic effect ($P < 0.001$).

Adverse effects

Table 3 summarizes the adverse effects. No treatment-

Table 2 Univariate and multivariate analysis of predictors of survival

| Variable | Hazard ratio | 95%CI | P value |
|--|--------------|-------------|-----------|
| Univariate analysis of predictors of survival | | | |
| Age (≤ 65 vs > 65 yr) | 1.085 | 0.772-1.525 | 0.638 |
| Gender (M vs F) | 0.861 | 0.607-1.222 | 0.403 |
| Previous treatment (no vs yes) | 1.258 | 0.911-1.738 | 0.164 |
| HCV antibody (negative vs positive) | 0.951 | 0.673-1.344 | 0.776 |
| Stage (I, II, III vs IV) | 2.705 | 1.919-3.814 | < 0.001 |
| Child Pugh classification (A vs B or C) | 1.584 | 1.149-2.184 | 0.005 |
| JIS score (0-2 vs 3-5) | 2.285 | 1.638-3.189 | < 0.001 |
| Total bilirubin (≤ 1.5 mg/dL vs > 1.5 mg/dL) | 1.578 | 1.044-2.385 | 0.031 |
| Albumin (> 3.5 mg/dL vs ≤ 3.5 mg/dL) | 1.527 | 1.100-2.119 | 0.012 |
| Number of tumors (< 10 vs ≥ 10) | 1.920 | 1.378-2.675 | < 0.001 |
| Maximum tumor size (≤ 50 mm vs > 50 mm) | 2.052 | 1.404-2.998 | < 0.001 |
| PVTT grade (Vp0, 1, 2 vs Vp3) | 4.142 | 2.754-6.230 | < 0.001 |
| Tumor distribution (Unilateral vs Bilateral) | 2.237 | 1.464-3.420 | < 0.001 |
| AFP (≤ 100 ng/mL vs > 100 ng/mL) | 2.131 | 1.539-2.949 | < 0.001 |
| AFP-L3 ($\leq 50\%$ vs $> 50\%$) | 1.664 | 0.957-2.894 | 0.071 |
| DCP (≤ 100 mAU/mL vs > 100 mAU/mL) | 2.201 | 1.588-3.051 | < 0.001 |
| Therapeutic effect (CR + PR vs SD + PD) | 3.419 | 2.382-4.909 | < 0.001 |
| Multivariate analysis of predictors of survival | | | |
| PVTT grade (Vp0, 1, 2 vs Vp3) | 2.310 | 1.217-4.384 | 0.010 |
| AFP (≤ 100 ng/mL vs > 100 ng/mL) | 1.856 | 1.265-2.724 | 0.002 |
| Therapeutic effect (CR + PR vs SD + PD) | 3.392 | 2.240-5.135 | < 0.001 |

The JIS score is obtained by simply adding both scores for the tumor, lymph node, metastasis (TNM) stage and Child-Turcotte-Pugh stage. HCV: Hepatitis C virus; JIS: Japan integrated staging; PVTT: Portal vein tumor thrombosis; AFP: α -fetoprotein; DCP: Des- γ -carboxy prothrombin; CR: Complete response; PR: Partial response; SD: Stable disease; PD: Progressive disease.

related deaths occurred, and no grade 4 treatment-related adverse effects were noted. Fever and nausea were seen transiently in 50% or more patients, but both were mild. Leucopenia and thrombocytopenia occurred in 15 (5.7%) and 18 (6.9%) patients, respectively; these were also mild and transient. Although grade 2 or higher liver abscess and hepatic/renal failure were observed in 4 (1.4%) and 1 (0.4%) patients, respectively, these adverse reactions were controllable by medical treatment. In addition, hepatic arterial damage (HAD) after TACE was observed in one patient. Although one patient was observed to have slight wall irregularity of the hepatic artery, HAD associated with TACE did not interfere with catheterization at the next TACE session.

DISCUSSION

TACE plays a crucial role in the treatment of HCC without surgical resection or RFA. The survival benefit of TACE has also been confirmed by randomized, controlled trials and a meta-analysis^[3,4]. The most commonly

Table 3 Adverse effects among the 262 patients *n* (%)

| Adverse effect | Grade 1 | Grade 2 | Grade 3 | Grade 4 |
|-----------------------|------------|-----------|---------|---------|
| Nausea/vomiting | 160 (61.1) | 48 (18.3) | - (-) | - (-) |
| General fatigue | 28 (10.7) | 17 (6.5) | 1 (0.4) | - (-) |
| Fever | 168 (64.1) | 27 (10.3) | - (-) | - (-) |
| Abdominal pain | 121 (46.1) | 54 (20.6) | - (-) | - (-) |
| Leucopenia | 13 (4.9) | 2 (0.7) | - (-) | - (-) |
| Thrombocytopenia | 16 (6.1) | 2 (0.7) | - (-) | - (-) |
| AST/ALT | 154 (58.8) | 46 (17.6) | - (-) | - (-) |
| Liver abscess | - (-) | 2 (0.7) | 2 (0.7) | - (-) |
| Hepatic/Renal failure | - (-) | - (-) | 1 (0.4) | - (-) |

Data are expressed as number of patients with percentages in parentheses. AST: Aspartate aminotransferase; ALT: Alanine aminotransferase.

used agent used in TACE for HCC treatment is ADM-LPD emulsion, followed by embolization with a gelatin sponge^[12,13,17,18]. The MST ranged from 18 to 34 mo with the use of TACE with ADM-LPD emulsion, but there is no clear evidence identifying the best chemotherapeutic agent for TACE.

CDDP is an effective anticancer agent used in the treatment of various malignancies^[10]. CDDP has been reported to exert its actions by binding to the DNA in cancer cells, inhibiting DNA synthesis and subsequent cellular division. The antitumor activity of CDDP is closely associated with the serum concentration of the drug^[19].

The key point of intra-arterial infusion chemotherapy is the selective retention of anticancer drugs at a high concentration in the HCC for a long time. LPD shows very selective deposition within HCC, in which it remains for several months after intra-arterial injection, whereas it disappears more rapidly from the nontumorous parenchyma^[20]. Consequently, augmented antitumor efficacy and milder side effects were expected with the use of this substance for TACE. In fact, Morimoto *et al.*^[21] investigated the pharmacological advantages of TACE using DDPH for hypervascular hepatic tumors in animal experiments. They reported that the tumor concentration of the platinum agent in the DDPH-LPD-TACE group was about 14 times higher than that in the DDPH-hepatic arterial infusion (HAI) group. In addition, they reported that the plasma concentrations of the platinum agent were lower in the DDPH-LPD-TACE group than in the DDPH-HAI group.

Ono *et al.*^[12] reported that TACE using a suspension of CDDP powder in LPD was more effective against unresectable HCC than that using ADM-LPD emulsion. However, because CDDP was only available as a solution, it was difficult to prepare a high-dose CDDP suspension using LPD.

A fine-powder formulation of CDDP, namely DDPH, for intra-arterial infusion has been available for HCC treatment since 2004 in Japan. Dispensing of CDDP powder improved with the development of DDPH, and DDPH has now come to replace CDDP powder. Since DDPH-LPD suspension for TACE in HCC patients was expected to yield better therapeutic outcomes, TACE using DDPH-LPD suspension became widespread in

Japanese institutions. We have already used TACE with DDPH-LPD suspension for HCC patients and reported favorable results^[13]. This article focused on the efficacy of this therapy by analyzing the clinical results of 262 HCC patients treated in this manner.

The MST in the current study was 46.6 mo. The cumulative survival rates at 6, 12, 24, and 36 mo were 90.6%, 81.9%, 70.5% and 58.8%, respectively. The outcome in the present study was superior to previous trials of TACE using ADM, epirubicin, and other anthracyclines^[5,6,13]. This could be explained as being due to the fact that TACE with ADM cannot be repeated as required because of the high frequency of adverse effects of ADM, such as leucopenia, severe vascular changes, and hepatic artery occlusion^[12,13,22]. In the current study, leucopenia and HAD were observed in only 15 (5.7%) and 1 (0.4%) patients, respectively. Considering that TACE is often repeated in most patients, longer patency of the hepatic artery is preferable for properly deploying the lipiodol mixture and embolic agents into the tumor. In addition, we concluded that anthracyclines such as ADM may be relatively less effective against HCC; this is because of the high expression level of P-glycoprotein, which transports antitumor agents such as anthracyclines or vinca alkaloids from cells with a high active efflux mechanism in HCC tumors^[23].

Moreover, survival in the present study was superior to previous trials of TACE using drug-eluting beads^[24-29]. In these outcomes of previous trials of TACE using drug-eluting beads, the response rates were superior to the current study. Nevertheless, the cumulative survival rates of the patients in the current study were higher than those of the patients in the previous trials. Drug-eluting beads are known to give more distal vessel occlusion for a long-term period^[30]. Therefore, it is possible that TACE with drug-eluting beads could have a greater embolizant effect than TACE with DDPH-LPD suspension, and this would lead to increased tumor growth factor release in response to hypoxia, with a consequent probability of recurrence and reduced overall survival.

The presence of PVTT has traditionally been considered a contraindication for transarterial therapy^[31]. However, a recent study has revealed that TACE for patients with PVTT had survival benefits over conservative treatment^[32]. Compared with this recent study, cumulative survival rates of patients with PVTT in the present study were better. On the other hand, cumulative survival rates of patients with Vp3 in subgroup analysis of the present study were 51.0% at 6 mo, 32.5% at 12 mo, and 16.2% at 24 mo. In our previous study of hepatic arterial infusion chemotherapy (HAIC) with 5-fluorouracil (5-FU) and pegylated IFN- α 2b (PEG-IFN α -2b) for HCC patients with Vp3/4, cumulative survival rates were 83.8% at 6 mo, 77.8% at 12 mo, 55.6% at 24 mo^[33]. Although it is impossible to compare the results of TACE using a suspension of DDPH in LPD and HAIC using 5-FU and PEG-IFN α -2b for HCC patients with Vp3, we think that a randomized controlled study comparing these therapies in patients with Vp3/4 will be needed in the future.

The prognosis of HCC patients depends on many factors, such as tumor stage and liver function. In the current study, the prognostic factors in patients treated with TACE with DDPH-LPD suspension were investigated. Among the variables examined, PVTT grade (Vp0-2), AFP (≤ 100 ng/mL), and therapeutic effect (CR+PR) were identified as being significantly associated with longer survival times on multivariate analysis. These results were similar to the result of a nationwide prospective cohort study by Takayasu *et al.*^[34], which was performed in 8510 patients with unresectable HCC who underwent TACE using an emulsion of lipiodol and anti-cancer agents followed by gelatin sponge particles.

Considering these facts, we conclude that TACE using DDPH-LPD suspension could be a useful treatment strategy for HCC patients. To confirm these results, randomized controlled trials comparing TACE using DDPH-LPD suspension with TACE using ADM-LPD emulsion or TACE using drug-eluting beads for patients with HCC are mandatory. Moreover, we think that a randomized controlled study comparing these therapies and HAIC for HCC patients with PVTT will be needed in the future.

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COMMENTS

Background

In recent years, transcatheter arterial chemoembolization (TACE) using an emulsion of doxorubicin (ADM) with lipiodol (LPD) (ADM-LPD emulsion) followed by embolization with a gelatin sponge has been commonly employed for hepatocellular carcinoma (HCC) treatment. However, HCC is not necessarily sensitive to these drugs.

Research frontiers

Cisplatin, a platinum compound, is an effective anticancer agent used in the treatment of various malignancies. Recently, a fine-powder formulation of cisplatin (DDPH, IA-call; Nipponkayaku, Tokyo, Japan) has also been available since 2004 as a therapeutic agent for intra-arterial infusion in Japan. Researchers have recently reported that TACE using a suspension of cisplatin powder in LPD may be more effective against unresectable HCC as compared with that using ADM-LPD emulsion. Therefore, TACE using DDPH has become widespread in Japanese institutions.

Innovations and breakthroughs

In this article, the authors evaluated the effectiveness of TACE using DDPH-LPD for 262 HCC patients. The objective early response rate was 43.6%. Cumulative survival rates were 90.6% at 6 mo, 81.9% at 12 mo, 70.5% at 24 mo, and 58.8% at 36 mo. Median survival time was 46.6 mo. All adverse reactions were controllable by temporary suspension of treatment. No serious complications or treatment-related deaths were observed. The outcome in the present study was superior to previous trials of TACE using ADM-LPD. Moreover, survival in the present study was superior to previous trials of TACE using drug-eluting beads.

Applications

Although randomized, controlled trials comparing TACE using DDPH-LPD suspension with TACE using ADM-LPD emulsion or TACE using drug-eluting beads for patients with HCC are mandatory, the authors conclude that TACE using DDPH-LPD suspension could be a useful treatment strategy for HCC patients.

Terminology

TACE is a minimally invasive medical procedure to restrict a tumor's blood

supply. TACE is an interventional radiology procedure. The procedure involves gaining percutaneous access to the hepatic artery. When a blood vessel supplying tumor has been selected, alternating aliquots of the chemotherapy dose and of embolic particles, or particles containing the chemotherapy agent, are injected through the catheter. CDDP is a chemotherapy drug. It was the first member of a class of platinum-containing anti-cancer drugs that now also includes carboplatin and oxaliplatin. These platinum complexes react *in vivo*, binding to and causing crosslinking of DNA, which ultimately triggers apoptosis.

Peer review

This paper is well written. The clinical results are appropriately described. The authors present clinical evaluation of TACE of DDPH in lipiodol in the treatment of HCC. The data indicate that the treatment in HCC patients resulted in significantly better early response rate, overall survival, progression free survival and cumulative survival rates.

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Effects of Lizhong Tang on cultured mouse small intestine interstitial cells of Cajal

Min Woo Hwang, Jung Nam Kim, Ho Jun Song, Bora Lim, Young Kyu Kwon, Byung Joo Kim

Min Woo Hwang, Department of Sasang Constitutional Medicine, College of Korean Medicine, Kyung-hee University, Seoul 130-701, South Korea

Jung Nam Kim, Ho Jun Song, Bora Lim, Young Kyu Kwon, Byung Joo Kim, School of Korean Medicine, Pusan National University, Yongsan 626-870, South Korea

Author contributions: Kwon YK and Kim BJ designed the research; Hwang MW, Kim JN and Song HJ performed the experiments; Lim B, Kwon YK and Kim BJ analyzed the data; and Hwang MW and Kim BJ wrote the paper.

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Correspondence to: Byung Joo Kim, PhD, School of Korean Medicine, Pusan National University, Beomeori, Mulgeum-eup, Yongsan 626-870, South Korea. vision@pusan.ac.kr

Telephone: +82-51-5108469 Fax: +82-51-5108420

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Abstract

AIM: To investigate the effects of Lizhong Tang, an herbal product used in traditional Chinese medicine, on mouse small intestine interstitial cells of Cajal (ICCs).

METHODS: Enzymatic digestions were used to dissociate ICCs from mouse small intestine tissues. The ICCs were morphologically distinct from other cell types in culture and were identified using phase contrast microscopy after verification with anti c-kit antibody. A whole-cell patch-clamp configuration was used to record potentials (current clamp) from cultured ICCs. All of the experiments were performed at 30-32 °C.

RESULTS: ICCs generated pacemaker potentials, and Lizhong Tang produced membrane depolarization in current-clamp mode. The application of flufenamic acid (a nonselective cation channel blocker) abolished the generation of pacemaker potentials by Lizhong Tang. Pretreatment with thapsigargin (a Ca²⁺-ATPase inhibi-

tor in the endoplasmic reticulum) also abolished the generation of pacemaker potentials by Lizhong Tang. However, pacemaker potentials were completely abolished in the presence of an external Ca²⁺-free solution, and under this condition, Lizhong Tang induced membrane depolarizations. Furthermore, When GDP-β-S (1 mmol/L) was in the pipette solution, Lizhong Tang still induced membrane depolarizations. In addition, membrane depolarizations were not inhibited by chelerythrine or calphostin C, which are protein kinase C inhibitors, but were inhibited by U-73122, an active phospholipase C inhibitors.

CONCLUSION: These results suggest that Lizhong Tang might affect gastrointestinal motility by modulating pacemaker activity in interstitial cells of Cajal.

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Key words: Interstitial cells of Cajal; Lizhong Tang; Motility; Gastrointestinal tract; Whole-cell patch clamp configuration

Core tip: The gastroprokinetic effects of Lizhong Tang are mediated by the induction of pacemaker potentials in interstitial cells of Cajal (ICCs). Taken together, our data suggest that the gastroprokinetic effects of Lizhong Tang are mediated by the induction of pacemaker potentials in ICCs. Considering the effects of this drug on ICCs, further research is required to identify the compounds responsible for the effects of Lizhong Tang and to determine their mechanisms of action.

Hwang MW, Kim JN, Song HJ, Lim B, Kwon YK, Kim BJ. Effects of Lizhong Tang on cultured mouse small intestine interstitial cells of Cajal. *World J Gastroenterol* 2013; 19(14): 2249-2255 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v19/i14/2249.htm> DOI: <http://dx.doi.org/10.3748/wjg.v19.i14.2249>

INTRODUCTION

Lizhong Tang, was first reported 1800 years ago in “Shanghan Lun”, and it remains a classical herbal product in traditional Chinese medicine. Lizhong Tang is composed of Radix Ginseng (*Panax ginseng* C.A. Meyer), Rhizoma Zingiberis (*Zingiber officinale* Roscoe), Rhizoma Atractylodis Macrocephalae (*Atractylodes macrocephala* Koidz.) and Radix Glycyrrhizae (*Glycyrrhiza uralensis* Fisch)^[1], and it is widely used in traditional medicine to treat spleen deficiency patterns in many diseases with common symptoms, such as vomiting, diarrhea, stomach pain, poor appetite, cold limbs, and stomach bleeding which, are caused by cold and weak organs^[1,2]. However, little is known of the molecular basis of the effects of Lizhong Tang on gastrointestinal (GI) motility.

Interstitial cells of Cajal (ICCs) are pacemaker cells in GI muscles that generate rhythmic oscillations in membrane potentials known as slow waves^[3-5]. Slow waves propagate within ICC networks and are conducted into smooth muscle cells *via* gap junctions. Furthermore, they initiate phasic contractions by activating Ca²⁺ entry through L-type Ca²⁺ channels. Pacemaker activity in the murine small intestine is mainly due to periodic activations of nonselective cation channels^[6,7] or Cl⁻ channels^[8,9]. ICCs also mediate or transduce inputs from the enteric nervous system. However, the effects of Lizhong Tang in mouse small intestine ICCs have not been investigated, and therefore, we undertook this study to investigate the characteristics of Lizhong Tang in mouse small intestine ICCs.

MATERIALS AND METHODS

Preparation of cells and cell cultures

Balb/c mice (3-7 d old) of either sex were anesthetized with ether and sacrificed by cervical dislocation. The small intestines, from 1 cm below the pyloric ring to the cecum, were removed and opened along the mesenteric border. The luminal contents were removed by washing with Krebs-Ringer bicarbonate solution. The tissues were then pinned to the base of a Sylgard dish, and the mucosae were removed by sharp dissection. Small tissue strips of intestinal muscle (consisting of both circular and longitudinal muscles) were equilibrated in Ca²⁺-free Hanks solution (containing the following in mmol/L: KCl 5.36, NaCl 125, NaOH 0.34, NaHCO₃ 0.44, glucose 10, sucrose 2.9, and HEPES 11) for 30 min, and then, the cells were dispersed using an enzyme solution containing collagenase (Worthington Biochemical Co., Lakewood, NJ, United States) 1.3 mg/mL, bovine serum albumin (Sigma Chemical Co., St. Louis, MO, United States) 2 mg/mL, trypsin inhibitor (Sigma) 2 mg/mL and ATP 0.27 mg/mL. The cells were plated onto sterile glass coverslips coated with murine collagen (2.5 µg/mL, Falcon/BD, Franklin Lakes, NJ, United States) in a 35-mm culture dish and then cultured at 37 °C in a 95% O₂, 50 mL/L CO₂ incubator in smooth muscle growth medium (Clo-

netics Corp., San Diego, CA, United States) supplemented with 2% antibiotics/antimycotics (Gibco, Grand Island, NY, United States) and murine stem cell factor (SCF 5 ng/mL, Sigma). ICCs were identified immunologically using an anti-c-kit antibody (phycoerythrin-conjugated rat anti-mouse c-kit monoclonal antibody; eBioscience, San Diego, CA, United States) at a dilution of 1:50 for 20 min^[10]. The ICCs were morphologically distinct from other cell types in culture and were identified using phase contrast microscopy after verification with anti c-kit antibody.

Patch-clamp experiments

A whole-cell patch-clamp setup was used to record the membrane potentials (current clamp) of cultured ICCs. An axopatch ID (Axon Instruments, Foster, CA, United States) was used to amplify membrane currents and potentials. The command pulse was applied using an IBM-compatible personal computer running pClamp software (version 6.1; Axon Instruments). The data obtained were filtered at 5 kHz displayed on an oscilloscope and a computer monitor, and printed using a pen recorder (Gould 2200, Gould, Valley View, OH, United States). The results were analyzed using pClamp and Origin (version 6.0) software. All of the experiments were performed at 30-32 °C.

Solutions and drugs

The physiological salt solution used to bathe cells (Na⁺-Tyrode) contained the following (in mmol/L): KCl 5, NaCl 135, CaCl₂ 2, glucose 10, MgCl₂ 1.2 and HEPES 10, adjusted to pH 7.4 with NaOH. The pipette solution contained the following (in mmol/L): KCl 140, MgCl₂ 5, K₂ATP 2.7, NaGTP 0.1, creatine phosphate disodium 2.5, HEPES 5 and EGTA 0.1, adjusted to pH 7.2 with KOH. Lizhong Tang was purchased from I-WORLD Pharmaceuticals (South Korea). Lizhong Tang is composed of Radix Ginseng (*Panax ginseng* C.A. Meyer), Rhizoma Zingiberis (*Zingiber officinale* Roscoe), Rhizoma Atractylodis Macrocephalae (*Atractylodes macrocephala* Koidz.) and Radix Glycyrrhizae (*Glycyrrhiza uralensis* Fisch.). The adult dosage is 10-15 g (crude material) per day. More information about Lizhong Tang can be obtained at the I-WORLD Pharmaceuticals Homepage (<http://i-pharm.koreasme.com>). The pills were dissolved with distilled water at a concentration of 0.5 g of crude drug/ml and stored in the refrigerator. All of the drugs were obtained from Sigma (Sigma Chemical Co., United States). The drugs were dissolved in distilled water, and added to the bathing solution to the desired concentrations immediately prior to use. The addition of these chemicals to the bathing solution did not alter its pH. Thapsigargin, U-73122, and U-73343 were dissolved in dimethyl sulfoxide (DMSO) to produce 50 and 100 mmol/L stock solutions and added at 1000 times dilutions to the bathing solution on the day of the experiment. The final concentration of DMSO in the bathing solution was always < 0.1%, and we confirmed that this

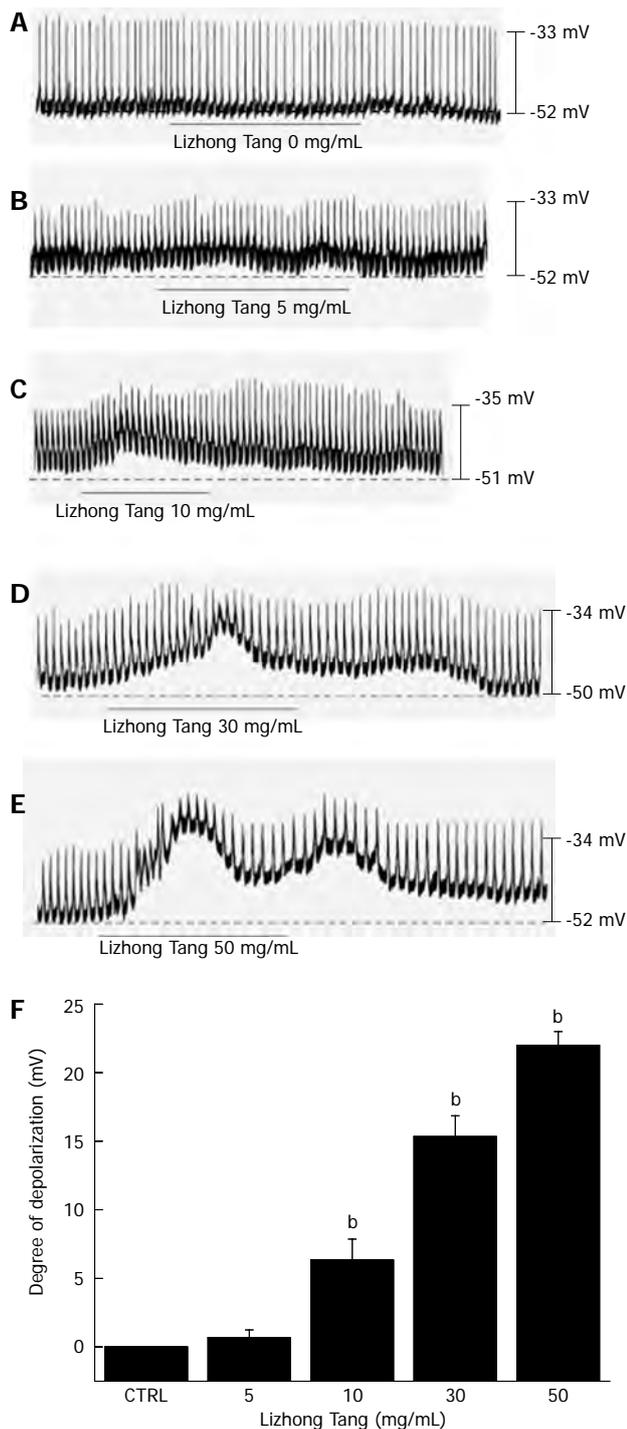


Figure 1 Effects of Lizhong Tang on pacemaker potentials in cultured interstitial cells of Cajal from murine small intestines. A-E: Pacemaker potentials in interstitial cells of Cajal exposed to Lizhong Tang (0-50 mg/mL) in current-clamp mode ($I = 0$); F: The responses to Lizhong Tang are summarized. Bars represent mean \pm SE. ^b $P < 0.01$ vs control group. CTRL: Control.

concentration did not affect the results.

Statistical analysis

All of the data are expressed as the mean \pm SE. Student's *t*-test for unpaired data was used to compare the control and experimental groups. Statistical significance was accepted for *P* values < 0.05 .

RESULTS

Effect of Lizhong Tang on the pacemaker potentials of cultured ICCs

The patch-clamp technique was tested on ICCs, which had formed network-like structures in culture after 2-4 d. Spontaneous rhythms were routinely recorded from cultured ICCs under current- and voltage-clamp conditions; ICCs within networks displayed more robust electrical rhythms. Tissue-like spontaneous slow waves have been previously recorded from these cells^[11]. To understand the relationship between Lizhong Tang and the modulation of pacemaker activity in ICCs, we examined the effects of Lizhong Tang on pacemaker potentials. Recordings from cultured ICCs under current-clamp mode ($I = 0$) showed spontaneous pacemaker potentials. The mean resting membrane potential was -52 ± 1.3 mV, and the mean amplitude was 23 ± 2 mV. In the presence of Lizhong Tang (0-50 mg/mL), the membrane potentials were depolarized to 0.6 ± 0.5 mV at 5 mg/mL, 6.3 ± 1.5 mV at 10 mg/mL, 15.2 ± 1.3 mV at 30 mg/mL, and 22.1 ± 1.2 mV at 50 mg/mL (Figure 1A-E). Summarized values and a bar graph of the effects of Lizhong Tang on pacemaker potentials are provided in Figure 1F ($n = 4$).

Effects of non-selective cation channel blocker or Cl channel blocker on Lizhong Tang-induced pacemaker potentials in cultured ICCs

To determine the characteristics of the membrane depolarizations induced by Lizhong Tang, flufenamic acid (a non-selective cation channel blocker)^[12,13] and niflumic acid (a Cl channel blocker)^[12,14] were used. In the presence of flufenamic acid (5 μ mol/L), pacemaker potentials were abolished and the subsequent application of Lizhong Tang (30 mg/mL) did not produce membrane depolarization (Figure 2A). In the presence of flufenamic acid, the membrane depolarizations produced by Lizhong Tang were 0.6 ± 0.4 mV, which was significantly different from the control values obtained in the absence of flufenamic acid ($n = 4$, Figure 2C). Pacemaker potentials were also abolished in the presence of niflumic acid (5 μ mol/L), but Lizhong Tang still produced membrane depolarization (Figure 2B). In the presence of niflumic acid, the mean membrane depolarization produced by Lizhong Tang was 15.3 ± 0.4 mV, which was not significantly different from the control condition ($n = 4$, Figure 2C).

Effects of external Ca^{2+} -free solution and Ca^{2+} -ATPase inhibitor in the endoplasmic reticulum on Lizhong Tang-induced pacemaker potentials in cultured ICCs

External Ca^{2+} influx is necessary for GI smooth muscle contractions and is essential for the generation of pacemaker potentials by ICCs. The generation of pacemaker currents is known to be dependent on intracellular Ca^{2+} oscillations^[15]. To investigate the roles of external and of internal Ca^{2+} , Lizhong Tang was tested under external Ca^{2+} -free conditions and in the presence of thapsigargin, an inhibitor of Ca^{2+} -ATPase in the endoplasmic reticu-

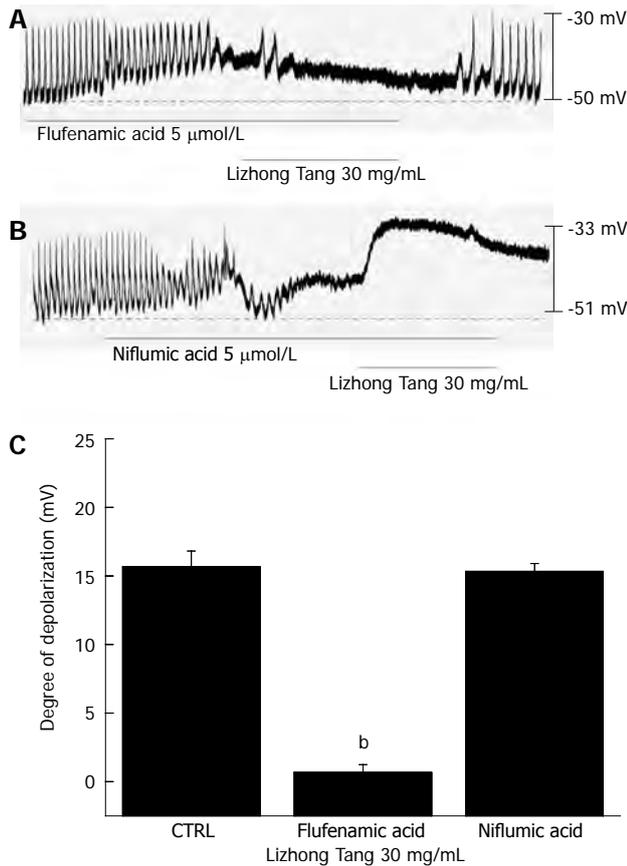


Figure 2 Effects of flufenamic acid (a nonselective cation channel blocker) or niflumic acid (a Cl⁻ channel blocker) on Lizhong Tang-induced pacemaker potentials in cultured interstitial cells of Cajal from murine small intestines. **A:** The application of flufenamic acid (5 μmol/L) abolished the generation of pacemaker potentials, and in the presence of flufenamic acid, Lizhong Tang (30 mg/mL) did not cause membrane depolarization; **B:** In contrast, although niflumic acid (5 μmol/L) abolished the generation of pacemaker potentials, it did not block Lizhong Tang-induced (30 mg/mL) membrane depolarization; **C:** The responses to Lizhong Tang in the presence of flufenamic acid or niflumic acid are summarized. Bars represent mean ± SE. ^b*P* < 0.01 vs control group. CTRL: Control.

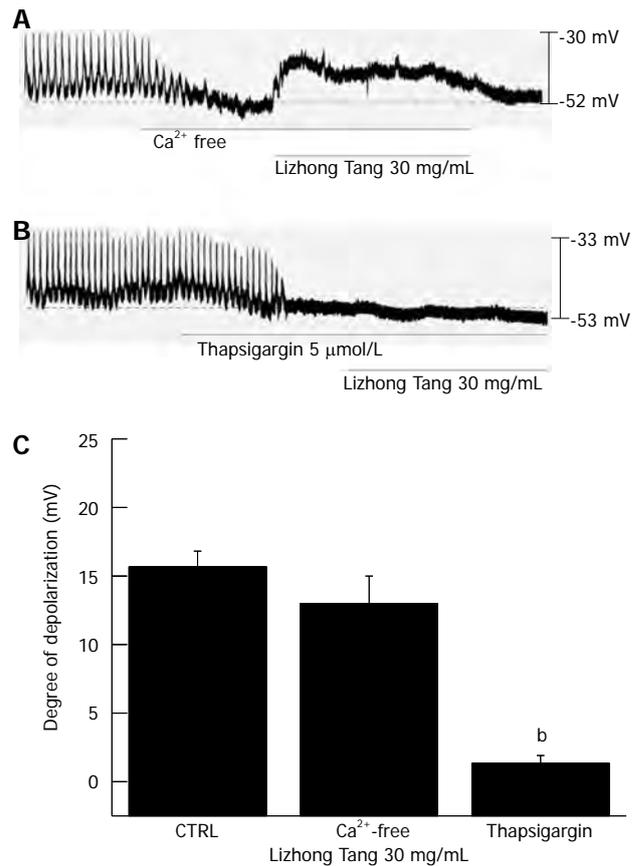


Figure 3 Effects of an external Ca²⁺-free solution, thapsigargin (an inhibitor of Ca²⁺-ATPase in the endoplasmic reticulum), or U-73122 (an active phospholipase C inhibitor) on Lizhong Tang-induced pacemaker potentials in cultured interstitial cells of Cajal. **A:** External Ca²⁺-free solution abolished the generation of pacemaker potentials, but failed to block Lizhong Tang-induced (30 mg/mL) membrane depolarization; **B:** Thapsigargin (5 μmol/L) abolished the generation of pacemaker potentials, and blocked Lizhong Tang-induced (30 mg/mL) membrane depolarization; **C:** The responses to Lizhong Tang in external Ca²⁺-free solution in the presence of thapsigargin are summarized. Bars represent mean ± SE. ^b*P* < 0.01 vs control group. CTRL: Control.

lum^[6,12]. Pacemaker potentials were completely abolished in the presence of an external Ca²⁺-free solution, and under this condition, Lizhong Tang induced membrane depolarizations (*n* = 4, Figure 3A). However, under external Ca²⁺-free conditions, membrane depolarizations by Lizhong Tang (30 mg/mL) were not significantly different from the depolarizations induced by Lizhong Tang (30 mg/mL) under normal Ca²⁺ conditions (*n* = 4, Figure 3C). In addition, Lizhong Tang-induced membrane depolarizations were inhibited by thapsigargin pretreatment (Figure 3B). Furthermore, the membrane depolarizations induced by Lizhong Tang were significantly affected by the presence of thapsigargin (*n* = 4, Figure 3C).

The involvement of G protein on Lizhong Tang-induced pacemaker potentials in cultured ICCs

The effects of GDP-β-S (a non-hydrolysable guanosine 5'-diphosphate analogue that permanently inactivates G-protein binding proteins^[16]) were examined to determine whether G-proteins are involved in the effects of

Lizhong Tang on ICCs. When GDP-β-S (1 mmol/L) was in the pipette solution, Lizhong Tang (30 mg/mL) still induced membrane depolarizations (Figure 4A). However, the membrane depolarizations induced by Lizhong Tang were not significantly affected by the presence of GDP-β-S (1 mmol/L) in the pipette solution (*n* = 4, Figure 4C).

Effects of phospholipase C inhibitor on Lizhong Tang-induced pacemaker potentials in cultured ICCs

Because membrane depolarizations induced by Lizhong Tang are related to intracellular Ca²⁺ mobilization, we examined whether the effects of Lizhong Tang on pacemaker potentials required phospholipase C (PLC) activation. To test this possibility, Lizhong Tang-induced membrane depolarizations were measured in the absence or presence of U-73122 (an active PLC inhibitor^[17]). Pacemaker membrane depolarizations currents were completely abolished by U-73122 (5 μmol/L), and under these conditions, Lizhong Tang-induced (30 mg/mL)

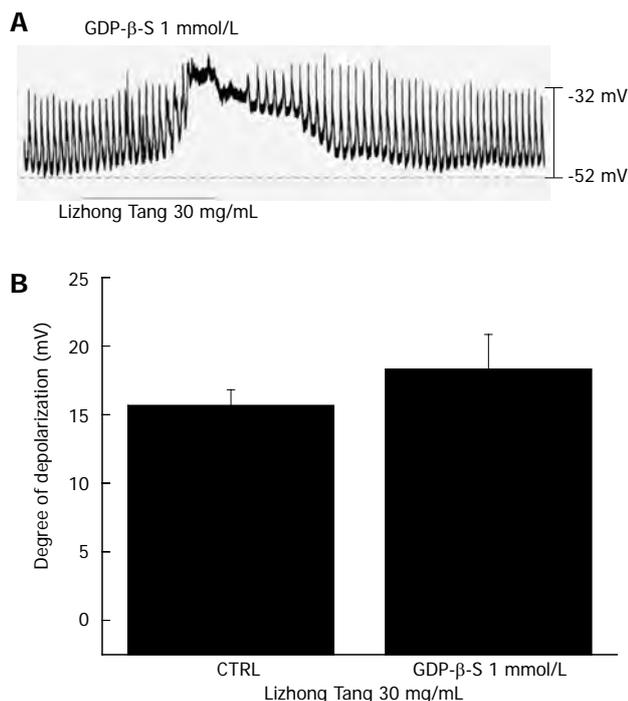


Figure 4 Effects of GDP-β-S in the pipette on Lizhong Tang-induced pacemaker potentials in cultured murine small intestine interstitial cells of Cajal. **A:** Pacemaker potentials in interstitial cells of Cajal exposed to Lizhong Tang (30 mg/mL) in the presence of GDP-β-S (1 mmol/L) in the pipette. Under these conditions, Lizhong Tang (30 mg/mL) caused membrane depolarization; **B:** The responses to Lizhong Tang in the presence of GDP-β-S in the pipette are summarized. Bars represent mean ± SE. CTRL: Control.

membrane depolarizations were suppressed ($n = 4$, Figure 5A). In the presence of U-73122, the mean membrane depolarization produced by Lizhong Tang was 0.5 ± 0.3 mV, and this was significantly different than the depolarization observed in the absence of U-73122 ($n = 4$, Figure 5C). Treatment with U-73343 (5 μmol/L; an inactive analog of U-73122) had no influence on Lizhong Tang-induced pacemaker potentials, and Lizhong Tang-induced (30 mg/mL) membrane depolarizations were not suppressed by U-73343 (Figure 5C).

Effects of protein kinase C inhibitor on Lizhong Tang-induced pacemaker potentials in cultured ICCs

We tested the effects of chelerythrine and of calphostin C (both inhibitors of protein kinase C (PKC)^[12,18]) to investigate whether Lizhong Tang-induced pacemaker potential responses are mediated by the activation of PKC. Neither chelerythrine (1 μmol/L) nor calphostin C (1 μmol/L) had any effect on membrane depolarizations induced by Lizhong Tang (30 mg/mL; Figure 6) and the value was also not significantly different when compared with the membrane depolarizations induced by Lizhong Tang in the absence of chelerythrine or calphostin C ($n = 5$, Figure 6C).

DISCUSSION

The GI tract exhibits spontaneous mechanical con-

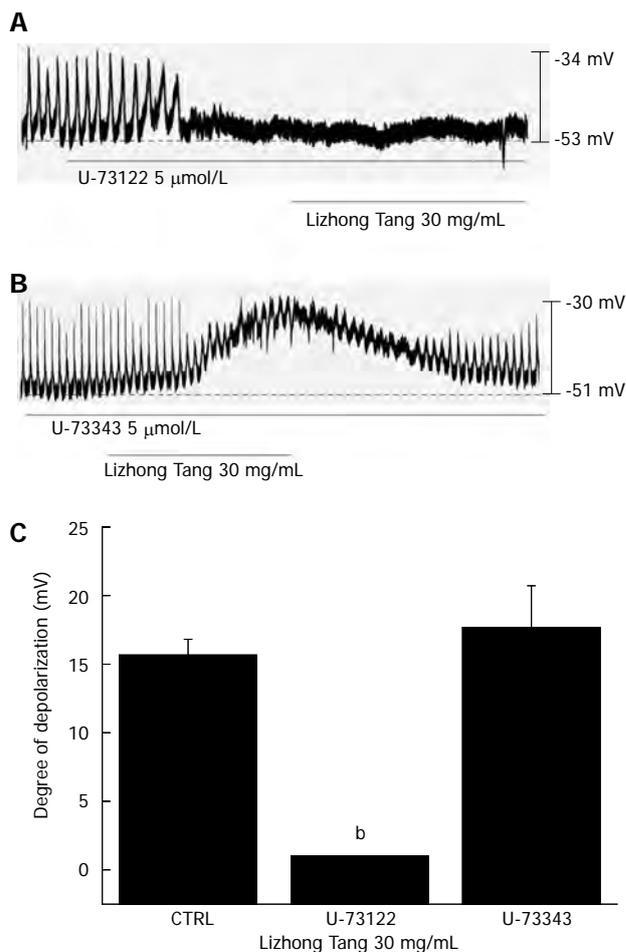


Figure 5 Effects of phospholipase C inhibitors on Lizhong Tang-induced potentials in cultured interstitial cells of Cajal. **A:** U-73122 (5 μmol/L; a phospholipase C inhibitor) abolished the generation of pacemaker potentials, and blocked Lizhong Tang-induced (30 mg/mL) membrane depolarization; **B:** The application of U-73343 (5 μmol/L) did not influence the generation of pacemaker currents or block Lizhong Tang-induced (30 mg/mL) membrane depolarization; **C:** The responses to Lizhong Tang in the presence of phospholipase C inhibitors are summarized. Bars represent mean ± SE. ^b $P < 0.01$ vs control group. CTRL: Control.

tractions that are mediated by the periodic generation of electrical pacemaker potentials, which are the basic determinant of GI smooth muscle activity^[3]. Recent studies have shown that the ICCs act as the pacemakers and conductors of electrical slow waves in GI smooth muscles^[3-5]. Moreover, evidence indicates that endogenous agents, such as, neurotransmitters, hormones, and paracrine substances modulate GI tract motility by influencing ICCs^[5-7,19,20]. Therefore, one of the best ways to investigate the role of GI motility is to use ICCs. Many types of ICCs with different immunohistochemical and electrical properties, including myenteric ICCs (ICC-MY), intramuscular ICCs (ICC-IM), deep muscular plexus ICCs (ICC-DMP), and submucosal ICCs (ICC-SM), are distributed throughout the GI tract^[16]. In animal models lacking ICC-MY, slow waves in the small intestine are strongly attenuated, which shows that these cells are indeed essential for pacemaker activity in the GI tract^[21]. Furthermore, ICCs are involved in physiological GI mo-

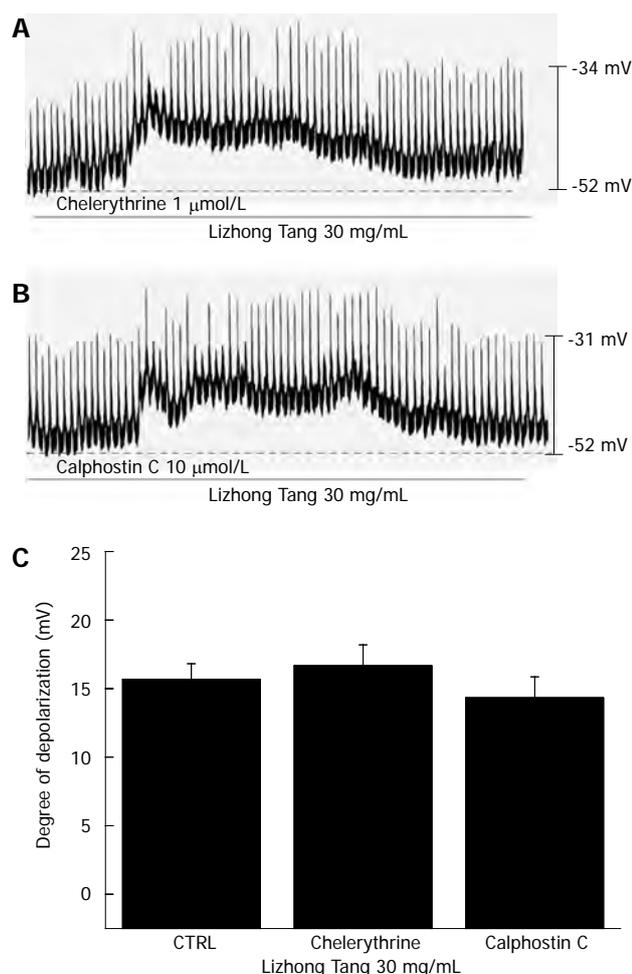


Figure 6 Effects of chelerythrine or calphostin C (inhibitors of protein kinase C) on Lizhong Tang-induced pacemaker potentials in cultured interstitial cells of Cajal. A, B: Pacemaker potentials in interstitial cells of Cajal exposed to Lizhong Tang (30 mg/mL) in the presence of chelerythrine (1 $\mu\text{mol/L}$) or calphostin C (10 $\mu\text{mol/L}$). Lizhong Tang caused membrane depolarization in the presence of both inhibitors; C: The responses to Lizhong Tang in the presence of chelerythrine or calphostin C are summarized. Bars represent mean \pm SE. CTRL: Control.

tility and are therefore clinically important in many bowel disorders, including inflammatory bowel disease, chronic idiopathic intestinal pseudo-obstruction, intestinal obstruction with hypertrophy, achalasia, Hirschsprung's disease, juvenile pyloric stenosis, juvenile intestinal obstruction, and anorectal malformation^[16].

Lizhong Tang warms the liver and spleen and strengthens the spleen and stomach. It has been widely used as treatment from deficiency, diarrhea with watery stool, nausea and vomiting. In addition, it also has ameliorative effects on loss of appetite, abdominal pain, acute or chronic gastritis gastric or duodenal ulcers, irritable bowel syndrome, chronic colitis, chronic bronchitis, oral herpes, and functional uterine bleeding^[1,22,23]. However, the effects of Lizhong Tang on GI tract motility and ICCs have not been investigated.

In this study, Lizhong Tang produced membrane depolarization in current-clamp mode, and the application of flufenamic acid (a non-selective cation channel

blocker), but not niflumic acid (a Cl^- channel blocker), abolished the generation of the pacemaker potentials induced by Lizhong Tang, suggesting that the Lizhong Tang-induced membrane depolarizations may be mediated by non-selective cationic channels. In addition, pretreatment with a Ca^{2+} -free solution or with thapsigargin (a Ca^{2+} -ATPase inhibitor in the endoplasmic reticulum), abolished the generation of pacemaker potentials. Under Ca^{2+} -free conditions, Lizhong Tang also showed membrane depolarization; however, in the presence of thapsigargin, Lizhong Tang did not show membrane depolarization, suggesting that intracellular calcium release is necessary. Furthermore, pacemaker membrane depolarizations were inhibited by U-73122 (PLC inhibitor), but not by GDP- β -S, which permanently binds G-binding proteins. In addition, the PKC inhibitors chelerythrine and calphostin C did not block Lizhong Tang-induced pacemaker potentials, suggesting that PLC is involved in the induction of the pacemaker potentials, but that PKC is not. In summary, Lizhong Tang affects GI motility by modulating pacemaker activity in ICCs, and this activation is associated with non-selective cationic channels via phospholipase C activation, and Ca^{2+} release from internal storage in an external Ca^{2+} -, G-protein-, and PKC-independent manner.

Taken together, our data suggest that the gastroprokinetic effects of Lizhong Tang are mediated by the induction of pacemaker potentials in ICCs. Considering the effects of this drug on ICCs, further research is required to identify the compounds responsible for the effects of Lizhong Tang and to determine their mechanisms of action.

COMMENTS

Background

Interstitial cells of Cajal (ICCs) are the pacemaker cells that generate slow waves in the gastrointestinal (GI) tract. Lizhong Tang is a classic herbal product in traditional Chinese medicine. However, the effects of Lizhong Tang in mouse small intestine ICCs have not been investigated.

Research frontiers

The gastroprokinetic effects of Lizhong Tang are mediated by the induction of pacemaker potentials in ICCs.

Innovations and breakthroughs

Lizhong Tang affects GI motility by modulating pacemaker activity in ICCs, and this activation is associated with non-selective cationic channels via phospholipase C activation, and Ca^{2+} release from internal storage in an external Ca^{2+} -, G-protein-, and protein kinase C-independent manner.

Applications

Lizhong Tang may be a new target for pharmacological treatment of GI motility disorders.

Peer review

In their manuscript, authors studies the effect of Lizhong Tang, an herbal product used in traditional Chinese medicine, on the pacemaking activity of mouse small ICCs. It was well written.

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E- Editor Zhang DN



Disease progression in chronic hepatitis C patients with normal alanine aminotransferase levels

Dong Hyun Sinn, Geum-Youn Gwak, Jae-uk Shin, Moon Seok Choi, Joon Hyeok Lee, Kwang Cheol Koh, Seung Woon Paik, Byung Chul Yoo

Dong Hyun Sinn, Department of Internal Medicine, Sanggye Paik Hospital, Inje University School of Medicine, Seoul 139-707, South Korea

Geum-Youn Gwak, Jae-uk Shin, Moon Seok Choi, Joon Hyeok Lee, Kwang Cheol Koh, Seung Woon Paik, Byung Chul Yoo, Department of Medicine, Samsung Medical Center, Sungkyunkwan University School of Medicine, Seoul 135-710, South Korea

Author contributions: Sinn DH and Gwak GY designed the research, analyzed the data, and wrote the paper; Shin J, Choi MS, Lee JH, Koh KC, Paik SW and Yoo BC provided data and critically revised the paper; all authors approved the final version of the paper.

Correspondence to: Geum-Youn Gwak, MD, Department of Medicine, Samsung Medical Center, Sungkyunkwan University School of Medicine, 50 Ilwon-dong, Gangnam-Gu, Seoul 135-710, South Korea. gy.gwak@samsung.com

Telephone: +82-2-34103409 Fax: +82-2-34103849

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Abstract

AIM: To investigate whether the disease progression of chronic hepatitis C patients with normal alanine aminotransferase (ALT) levels differs by ALT levels.

METHODS: A total of 232 chronic hepatitis C patients with normal ALT (< 40 IU/L) were analyzed. The patients were divided into "high-normal" and "low-normal" ALT groups after determining the best predictive cutoff level associated with disease progression for each gender. The incidence of disease progression, as defined by the occurrence of an increase of ≥ 2 points in the Child-Pugh score, spontaneous bacterial peritonitis, bleeding gastric or esophageal varices, hepatic encephalopathy, the development of hepatocellular carcinoma, or death related to liver disease, were compared between the two groups.

RESULTS: Baseline serum ALT levels were associated

with disease progression for both genders. The best predictive cutoff baseline serum ALT level for disease progression was 26 IU/L in males and 23 IU/L in females. The mean annual disease progression rate was 1.2% and 3.9% for male patients with baseline ALT levels ≤ 25 IU/L (low-normal) and > 26 IU/L (high-normal), respectively ($P = 0.043$), and it was 1.4% and 4.8% for female patients with baseline ALT levels ≤ 22 IU/L (low-normal) and > 23 IU/L (high-normal), respectively ($P = 0.023$). ALT levels fluctuated during the follow-up period. During the follow-up, more patients with "high-normal" ALT levels at baseline experienced ALT elevation (> 41 IU/L) than did patients with "low-normal" ALT levels at baseline (47.7% vs 27.9%, $P = 0.002$). The 5 year cumulative incidence of disease progression was significantly lower in patients with persistently "low-normal" ALT levels than "high-normal" ALT levels or those who exhibited an ALT elevation > 41 U/L during the follow-up period (0%, 8.3% and 34.3%, $P < 0.001$).

CONCLUSION: A "high normal" ALT level in chronic hepatitis C patients was associated with disease progression, suggesting that the currently accepted normal threshold of serum ALT should be lowered.

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Key words: Alanine aminotransferase; Upper limits of normal; Disease progression; Hepatitis C virus; Hepatocellular carcinoma

Core tip: Recent studies have indicated that the upper limit of normal for the serum alanine aminotransferase (ALT) level should be lowered. However, outcome studies based on the development of adverse events during long-term follow-up are limited. In this present study, among patients infected with chronic hepatitis C virus who had normal ALT levels, the risk of disease progression differed between patients with "high-normal" and "low-normal" ALT levels, even within the currently ac-

cepted normal levels. This finding suggests that lowering the normal threshold of ALT levels may be necessary to better identify patients who are at increased risk for disease progression.

Sinn DH, Gwak GY, Shin J, Choi MS, Lee JH, Koh KC, Paik SW, Yoo BC. Disease progression in chronic hepatitis C patients with normal alanine aminotransferase levels. *World J Gastroenterol* 2013; 19(14): 2256-2261 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v19/i14/2256.htm> DOI: <http://dx.doi.org/10.3748/wjg.v19.i14.2256>

INTRODUCTION

Serum alanine aminotransferase (ALT) is an easily available, low-cost screening tool for detecting hepatocellular disease^[1,2]. Currently, the upper limit of normal (ULN) of ALT has been set at a mean value \pm 2SD in a group of healthy individuals^[1], usually at approximately 40 IU/L in many hospitals, including our hospital, although this value varies slightly between laboratories. However, several recent studies have demonstrated that the ULN of ALT should be lower than the currently accepted thresholds^[3-7]. In these studies, the ULN of ALT was assessed in the standard manner (set at a mean value \pm 2SD for healthy individuals); however, by defining a “new” healthy reference population, largely by excluding metabolically abnormal individuals^[6]. If the ULN of ALT, often interchangeably used with the healthy level, is defined in this manner, it will vary according to the chosen reference class.

Another way of defining the ULN of ALT, or healthy levels, involves outcome studies, which are based on the development of adverse events during long-term follow-up^[8-11]. The ULN of ALT can be set at a level that places individuals at increased risk of adverse consequences. In fact, a “high-normal” ALT level, even within the currently accepted normal range, has been associated with increased liver disease-related mortality^[12,13], suggesting that the “healthy” ALT level should be lower than the currently accepted thresholds. In the present study, we aimed to assess whether a “high-normal” ALT level is associated with an increased risk of disease progression among patients with chronic hepatitis C.

MATERIALS AND METHODS

Patients

Previously, we reported the incidence and risk factors of disease progression in 1137 patients with chronic hepatitis C virus (HCV) infections^[14]. The present study is a subgroup analysis of our previous study. In our previous study, we enrolled 1137 chronic hepatitis C patients who had no history or evidence of advanced liver disease. The detailed inclusion and exclusion criteria are described in our previous report^[14]. Briefly, patients exhibited evidence of chronic HCV infection but had no history or evidence

of cirrhotic complications, including a Child-Pugh score of $>$ 5 points, esophageal or gastric variceal bleeding, spontaneous bacterial peritonitis, hepatic encephalopathy, and HCC. For the present study, out of a total of 1137 patients, we selected 232 patients who did not receive antiviral therapy for chronic HCV infection and exhibited normal ALT levels ($<$ 40 IU/L) at enrollment.

Follow-up and endpoint assessment

Follow-up data collection and endpoint assessment followed the protocol of our previous study^[14]. Briefly, all patients were followed-up at least every 3-6 mo, or more frequently as required, for at least 1 year. Follow-up tests included conventional biochemical tests and abdominal ultrasonography screening. Endoscopic examination was performed when patients exhibited any symptoms or signs suggesting gastrointestinal bleeding, such as hematemesis, melena, hematochezia or sudden drops in blood hemoglobin levels. If patients did not exhibit any indications of gastrointestinal bleeding, endoscopic examination was not performed routinely or regularly. Patients who dropped out during the follow-up or who died without reaching the endpoint were classified as either withdrawals or censored cases, respectively. All ALT levels during the follow-up were obtained from each patient, and the changes in ALT levels during the follow-up were also assessed. Blood samples of the patients were collected after $>$ 8 h of fasting and analyzed within 24 h. Plasma concentrations of ALT were measured using an autoanalyzer (Hitachi Modular D2400, Roche, Tokyo, Japan).

The primary endpoint was the time to disease progression, as defined by the first occurrence of any of the following: an increase of at least 2 points in the Child-Pugh score, spontaneous bacterial peritonitis, bleeding gastric or esophageal varices, hepatic encephalopathy, the development of HCC or death related to liver disease^[14]. HCC was diagnosed by histological evaluation or was diagnosed clinically according to the 1st edition of guidelines for the diagnosis of HCC of the Korean Association for the Study of the Liver^[15]. As one of the potential risk factors for disease progression, alcohol consumption was assessed as all-or-none from available medical records. The Institutional Review Board at Samsung Medical Center reviewed and approved this study protocol.

Statistical analysis

The cumulative incidence rate of disease progression was calculated and plotted by using the Kaplan-Meier method. Differences in the incidence rate between the groups were analyzed using a log-rank test. A receiver operating curve (ROC) analysis was performed for ALT levels to estimate the best predictive cut-off values. Multivariate analysis was performed using the Cox proportional hazard model for variables with *P*-values of $<$ 0.05 for univariate analysis to identify factors associated with disease progression. *P*-values less than 0.05 were considered significant.

Table 1 Baseline characteristics of the 232 patients

| Characteristics | (n = 232) |
|--|------------------|
| Age (yr, mean \pm SD) | 57.2 \pm 10.7 |
| Gender, male/female | 89 (38):143 (62) |
| Weight (kg, mean \pm SD) | 61.6 \pm 9.3 |
| Alcohol consumption | 32 (14) |
| Diabetes | 29 (13) |
| Estimated duration of infection (mo, median, range) | 18 (0–398) |
| Alanine aminotransferase (IU/L, median, quartile) | 25 (19–32) |
| Aspartate aminotransferase (IU/L, median, quartile) | 30 (23–48) |
| Platelet ($10^3/\text{mm}^3$, median, quartile) | 179 (13–224) |
| Aspartate aminotransferase: platelet ratio index (median, range) | 0.4 (0.1–7.5) |
| > 1 | 50 (22) |
| Genotype ¹ | |
| 1b | 12 (57) |
| 1 others | 1 (5) |
| 2 | 8 (38) |

¹Percent value refers to percentage within studied patients. Data are expressed as absolute numbers (percentage) or mean \pm SD.

RESULTS

Patient characteristics and incidence of disease progression

Table 1 presents the clinical features of the patients at study entry. Disease progression was noted in 33/232 patients (14.2%) during the median follow-up of 54.1 mo (range: 12–151 mo). The mean annual incidence rate of disease progression was 3.1%. The cause of disease progression (first occurrence) was HCC in 27 patients (11.6%), bleeding varices in 2 patients (0.9%), \geq a 2 point increase in the Child-Pugh score in 2 patients (0.9%), and hepatic encephalopathy in 2 patients (0.9%).

Disease progression according to the baseline serum ALT levels

Because previous studies have suggested differing normal ALT thresholds in males and females^[16–18], we analyzed data separately by gender. The ALT level (tested as a numeric variable) was significantly associated with the disease progression in both males [hazard ratio (HR) = 1.09; 95%CI: 1.01–1.21, $P = 0.048$] and females (HR = 1.07; 95%CI: 1.01–1.13, $P = 0.040$). When ALT levels were stratified, the incidence rates of disease progression were 5%, 11%, and 21% in male patients with ALT levels of < 20 IU/L ($n = 20$), 20–39 IU/L ($n = 27$), and 30–39 IU/L ($n = 42$), respectively ($P = 0.191$) (Figure 1A). In female patients, the incidence rates were 2%, 18%, and 20% with ALT levels of < 20 IU/L ($n = 42$), 20–39 IU/L ($n = 56$), and 30–39 IU/L ($n = 45$), respectively ($P = 0.034$) (Figure 1B).

We performed ROC curve analysis to determine the best ALT cutoff value associated with disease progression. The best cutoff value was 26 IU/L in males (area = 0.722, $P = 0.011$, sensitivity = 0.85, specificity = 0.53) and 23 IU/L in females (area = 0.634, $P = 0.055$, sensitivity = 0.80, specificity = 0.47). The cumulative incidence

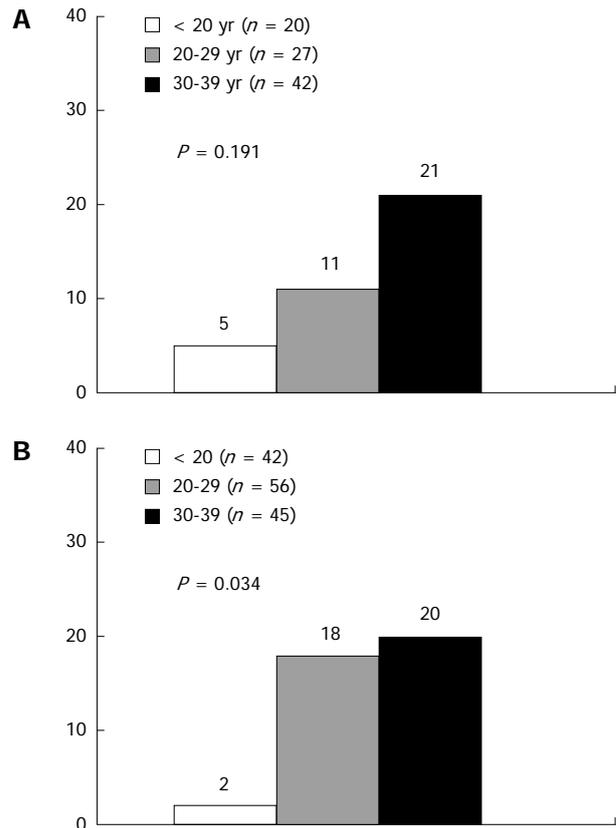


Figure 1 Incidence of disease progression according to serum alanine aminotransferase levels. There was an increase in the incidence of disease progression along with an increase in serum alanine aminotransferase (ALT) values for males (A) and for females (B). White, gray and black bars represent patients with ALT levels < 20 IU/L, 20–29 IU/L, and 30–39 IU/L, respectively.

of disease progression was significantly higher in patients with “high-normal” ALT levels than in those with “low-normal” ALT levels in both males and females (Figure 2).

Risk of disease progression according to baseline ALT levels

The potential risk factors assessed for disease progression included the following variables: age, gender, diabetes mellitus, alcohol intake, body weight, estimated duration of infection, platelet levels, ALT levels, aspartate aminotransferase (AST) levels, and α -fetoprotein levels. Because HCV RNA quantitation and genotype data were available for only a few patients, these variables were not included in our analysis.

Univariate Cox proportional-hazard regression analyses revealed that age, platelet levels, AST levels, and ALT levels were significantly associated with disease progression in males (Table 2). In females, age and diabetes as well as platelet, AST, and ALT levels were associated with disease progression (Table 2). Multivariate Cox proportional-hazard regression analyses were performed for the above-mentioned variables. After adjusting for potential confounders, the baseline ALT levels remained a significant factor associated with disease progression in both genders (Table 2).

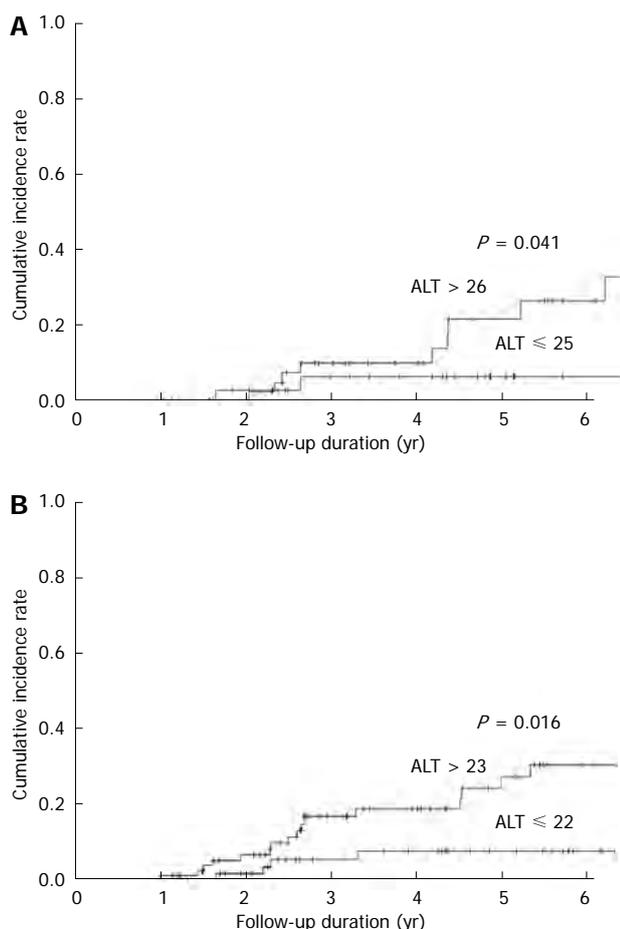


Figure 2 Cumulative incidence of disease progression according to alanine aminotransferase levels. The incidence rate differed between patients with “high-normal” and “low-normal” alanine aminotransferase (ALT) levels. A: Males; B: Females.

Change of ALT level during follow-up and risk of disease progression

ALT levels fluctuated during the follow-up period. During the follow-up, ALT remained “low-normal” in 41 patients (17.7%), “high-normal” in 101 patients (43.5%), and elevated over > 41 IU/L in 90 patients (38.8%). More patients with “high-normal” ALT levels at baseline experienced an ALT elevation (> 41 IU/L) during follow-up than did patients with “low-normal” ALT levels at baseline (47.7% *vs* 27.9%, $P = 0.002$). The 5-year cumulative incidence of disease progression was significantly lower in patients whose ALT levels remained “low-normal” than in those patients whose ALT levels were “high-normal” or who exhibited ALT elevation > 41 U/L during follow-up (0%, 8.3% and 34.3%, $P < 0.001$, Figure 3).

DISCUSSION

The present study demonstrated a significant difference in the disease progression rate in chronic hepatitis C patients with “high-normal” and “low-normal” ALT levels for both genders. Because the long-term prognosis significantly differs between patients with “high-normal” ALT

Table 2 Disease progression hazard ratio for each factor according to gender

| | Univariate | | Multivariate | |
|---------------------------------------|----------------------|-----------|----------------------|-----------|
| | Hazard ratio (95%CI) | P value | Hazard ratio (95%CI) | P value |
| Male | | | | |
| ALT (IU/L) > 26 <i>vs</i> ≤ 25 | 4.67 (1.03-21.1) | 0.045 | 5.35 (1.05-27.3) | 0.043 |
| Platelet ($10^3/\text{mm}^3$) | 0.98 (0.97-0.99) | 0.002 | 0.98 (0.96-0.99) | 0.012 |
| Age (yr) | 1.07 (1.01-1.13) | 0.030 | 1.06 (0.98-1.14) | 0.12 |
| AST (IU/L) | 1.02 (1.01-1.03) | < 0.001 | 1.00 (0.99-1.02) | 0.77 |
| Female | | | | |
| ALT (IU/L) > 23 <i>vs</i> ≤ 22 | 3.51 (1.17-10.5) | 0.025 | 4.40 (1.12-15.8) | 0.023 |
| Platelet ($10^3/\text{mm}^3$) | 0.97 (0.96-0.98) | < 0.001 | 0.97 (0.96-0.98) | < 0.001 |
| Age (yr) | 1.06 (1.01-1.10) | 0.017 | 1.04 (0.98-1.09) | 0.18 |
| AST (IU/L) | 1.02 (1.01-1.03) | < 0.001 | 1.00 (0.99-1.01) | 0.97 |
| Diabetes (yes <i>vs</i> no) | 3.23 (1.24-8.41) | 0.016 | 2.57 (0.83-7.92) | 0.10 |

AST: Aspartate aminotransferase; ALT: Alanine aminotransferase.

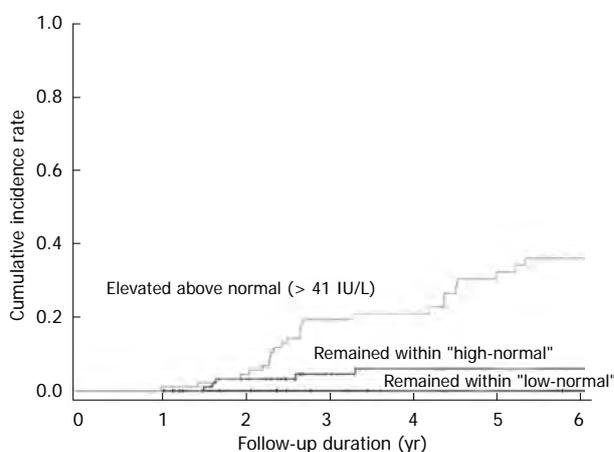


Figure 3 Cumulative incidence of disease progression according to changes in alanine aminotransferase levels. The incidence rate differed among patients who exhibited persistently “low-normal” alanine aminotransferase (ALT) levels or “high-normal” ALT levels and patients who exhibited ALT elevation (> 41 IU/L) during follow-up.

values and patients with “low-normal” ALT values, these findings strongly suggest that the currently used normal ALT range warrants further stratification^[19-22]. This finding is consistent with findings by Lee *et al*^[13], who investigated the incidence of HCC in a prospective cohort of chronic HCV-infected patients. The authors reported that elevated serum ALT levels were an independent risk factor for the development of HCC and that the risk begins to rise in patients with ALT levels of 15 IU/L, far below the currently used ULN for ALT levels^[13]. Thus, patients with “high-normal” ALT (26 to 40 IU/L in male and 23 to 40 IU/L in female in this study) should not be considered “normal” or “healthy”, and lowering the ‘healthy’ ULN of ALT is advisable.

In this study, we enrolled patients who exhibited normal ALT levels at baseline. However, during the follow-up, many patients experienced ALT elevation. Overall, 90 of 232 patients (38.8%) exhibited ALT elevation > 41 IU/L during the follow-up. Patients with “high-normal” ALT levels exhibited a higher incidence of ALT flare

(> 41 IU/L) than did patients with “low-normal” ALT levels. During follow-up, only 17.7% of patients with initially “low-normal” ALT levels persistently expressed “low-normal” ALT levels. The risk of disease progression differed significantly among patients who exhibited ALT elevation (> 41 IU/L), patients who persistently exhibited “high-normal” ALT, and patients who persistently exhibited “low-normal” ALT levels (34.3%, 8.3% and 0%, respectively). This finding emphasizes the importance of serial ALT follow-ups^[20,23,24], even for patients with normal ALT levels, because ALT levels can change and disease can progress in these patients.

It is noteworthy that best cutoff value of ALT for disease progression differed according to gender in this study. Several factors influence serum ALT values, including age, race, gender and body mass index^[16-18,25]. Currently, many laboratories do not assign different ULN of ALT by gender; however, several studies support the consideration of gender when setting the ULN of ALT^[3,5]. In this study, we also observed different cutoff values according to gender (26 IU/L in males and 23 IU/L in females) when the ULN of ALT was calculated in terms of predicting adverse consequences. Gender-specific ULN values of ALT appear more reasonable and should be applied in clinical practice.

There are limitations to this study that require careful interpretation of our results. The mean annual incidence rate of disease progression in this study was 3.09%. Previous studies on the natural history of HCV have used various outcome measures, making it difficult to compare to this study. Nevertheless, the annual progression rate to cirrhosis in chronic HCV infection has been reported to be 0.1% to 1%^[26-28]. As the endpoint of this study was advanced cirrhotic complications, the reported incidence rate in this study was very high. This study is a retrospective study that was performed at a tertiary referral center. Hence, selection bias may account for the high incidence rate of disease progression. Furthermore, a considerable proportion of the patients may have had significant fibrosis at baseline. Although baseline liver biopsies were not performed in most patients, an aspartate aminotransferase: platelet ratio index > 1, a noninvasive marker that can predict fibrosis in chronic hepatitis C patients^[29], was noted in 22% of the patients at baseline.

In summary, the present study demonstrated that patients with “high-normal” ALT levels, even within the currently accepted normal range, exhibit a significantly higher risk of ALT elevation and disease progression. The optimal ALT cutoff value to predict adverse outcomes differed by gender. Thus, gender-specific and lower ALT cutoffs seem more appropriate than the currently used ALT cutoff (40 IU/mL, regardless of gender).

COMMENTS

Background

Serum alanine aminotransferase (ALT) is an easily available, low-cost screening tool for detecting hepatocellular disease. Currently, an upper limit of normal (ULN) of ALT has been set at the mean value \pm 2SD in a group of healthy

individuals. However, the ULN of ALT should also be set at a level that identifies individuals at risk for developing adverse consequences during follow-up.

Research frontiers

Several previous studies, in which the ULN of ALT was defined as the mean value \pm 2SD, have demonstrated that the ULN of ALT is lower than the currently accepted thresholds (40 IU/L).

Innovations and breakthroughs

The present study, a long-term outcome study, demonstrated that there was a significant difference in the rate of disease progression between chronic hepatitis C patients with “high-normal” and “low-normal” ALT levels for both genders.

Applications

Because the long-term prognosis significantly differs between patients with “high-normal” ALT values and patients with “low-normal” ALT values, these findings strongly suggest that the currently used normal ALT range warrants further stratification.

Terminology

“High-normal” and “low-normal” refers to ALT levels that are associated with disease progression for each gender within the currently accepted normal ALT level range (< 40 IU/L).

Peer review

The hepatic enzymes is indicator of liver damage, its changes must have personal specificity, individual variations is fact, regardless the gender, however, findings in this study is important.

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Hepatitis B virus induces expression of cholesterol metabolism-related genes *via* TLR2 in HepG2 cells

Ying-Ju Li, Ping Zhu, Yu Liang, Wei-Guo Yin, Jian-Hua Xiao

Ying-Ju Li, Yu Liang, Wei-Guo Yin, Jian-Hua Xiao, Institute of Pathogenic Biology, University of South China, Hengyang 421001, Hunan Province, China

Ying-Ju Li, Department of Infectious Diseases, the First Affiliated Hospital of University of South China, Hengyang 421001, Hunan Province, China

Ping Zhu, Guangdong Cardiovascular Institute, Guangdong General Hospital, Guangdong Academy of Medical Sciences, Guangzhou 510080, Guangdong Province, China

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Correspondence to: Jian-Hua Xiao, Professor, Institute of Pathogenic Biology, University of South China, Hengyang 421001, Hunan Province, China. jhxiao223@163.com

Telephone: +86-734-8282232 Fax: +86-734-8282232

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Abstract

AIM: To investigate whether hepatitis B virus (HBV) exacerbates hepatic cholesterol accumulation, and explore the underlying mechanisms.

METHODS: HepG2 cells were infected with adenovirus (Ad) containing 1.3-fold overlength HBV genome. Real-time polymerase chain reaction and Western blotting were used to measure mRNA and protein expression of target genes. Cholesterol accumulation was measured by fluorescence microscopy. Cell toxicity due to Ad-HBV treatment was determined by the mitochondrial tetrazolium assay. The protein levels of toll-like receptors (TLRs) were determined by Western blotting.

RESULTS: Ad-HBV increased hepatic cholesterol accumulation and enhanced the mRNA and protein levels of

low-density lipoprotein receptor (LDLR) and 3-hydroxy-3-methylglutaryl-coenzyme A reductase (HMGCoAr) mRNA and protein expression in HepG2 cells. In addition, these inductive effects were partly offset by suppressing TLR2 expression levels by small interfering RNA in HepG2 cells.

CONCLUSION: Ad-HBV increases LDLR and HMGCoAr expression, resulting in exacerbated cholesterol accumulation in HepG2 cells, which was mediated *via* the TLR2 pathway.

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Key words: Hepatitis B virus; Toll-like receptors; Low-density lipoprotein receptor; 3-hydroxy-3-methylglutaryl-coenzyme A reductase

Core tip: This study investigated whether hepatitis B virus (HBV) exacerbates hepatic cholesterol accumulation and explored the underlying mechanisms. The authors found that adenovirus HBV increased low-density lipoprotein receptor and 3-hydroxy-3-methylglutaryl-coenzyme A reductase expression, resulting in exacerbated cholesterol accumulation in HepG2 cells, which was mediated *via* the toll-like receptor 2 pathway. These results may also have implications in the treatment of atherosclerosis.

Li YJ, Zhu P, Liang Y, Yin WG, Xiao JH. Hepatitis B virus induces expression of cholesterol metabolism-related genes *via* TLR2 in HepG2 cells. *World J Gastroenterol* 2013; 19(14): 2262-2269 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v19/i14/2262.htm> DOI: <http://dx.doi.org/10.3748/wjg.v19.i14.2262>

INTRODUCTION

Hepatitis B virus (HBV) infection is a major public

health problem worldwide^[1]. A possible role for infections in atherosclerosis has been deeply scrutinized since the demonstration that herpes virus induced atherosclerosis in chickens in 1978^[2]. It has been shown that the incidence of hepatic steatosis in HBeAg-negative chronic hepatitis B patients is about 32%^[3]. A published study from a health-screening test cohort showed that there was a strong association between hepatitis virus carriers and carotid atherosclerosis^[4,5].

It is still controversial as to whether HBV-induced inflammation correlates with disease in organs other than the liver. To date, there are few data available to prove the association between HBV infection and atherosclerosis. Kiechl *et al*^[6] found no significant association between chronic hepatitis and the development of new carotid atheromatous plaques, although they did not specify the type of hepatitis virus. However, another study in Japan demonstrated an increased prevalence of carotid atherosclerosis in HBV carriers^[7].

Research has revealed that HBV-induced inflammation correlates with disease in organs other than the liver^[8]. A previous report indicated that inflammation plays an important role in atherosclerosis^[9]. In addition, this adverse impact of virus infection is partly, if not all, mediated by toll-like receptors (TLRs)^[10-13]. For instance, Zhang *et al*^[14] found that TLR2/4 signaling involved in the adaptive immune response plays a role in chronic HBV infection.

However, the role of HBV infection in hepatic cholesterol accumulation is still unclear. Therefore, the aims of the present investigation were to test: (1) whether HBV affects the expression of genes related to cholesterol metabolism such as low-density lipoprotein receptor (LDLR) and 3-hydroxy-3-methylglutaryl-coenzyme A reductase (HMGCoAr) in hepatocytes; and (2) whether TLRs are involved in lipid metabolism disorders caused by HBV.

MATERIALS AND METHODS

Cell culture

HepG2 and AD293 cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum and 1% (v/v) penicillin-streptomycin at 37 °C in a humid atmosphere of 5% CO₂. HepG2 cells were switched to serum-free medium 24 h before treatment.

Amplification and quantification of adenoviral vector-hepatitis B virus

In this study, adenoviral vectors were designed, which initiated HBV replication from a 1.3-fold overlength HBV genome. A control adenoviral vector (Ad) was also included. AD293 cells were infected with 1×10^5 to 1×10^7 copies of Ad-HBV for 72 h. Cells were harvested and counted under the microscope. An equal number of cells were maintained in phosphate buffered saline

Table 1 Primers for real-time polymerase chain reaction

| Gene | Primers for real-time polymerase chain reaction |
|---------|--|
| LDLR | Sense: 5'-TCAACACACAACAGCAGATGGCAC-3' Antisense: 5'-AAGGCTAACCTGGCTGTCTAGCAA-3' |
| HMGCoAr | Sense: 5'-TATGTGCTGCTTGGCTGCATGTC-3' Antisense: 5'-ATACCAAGGACACACAAGCTGGGA-3' |
| TLR2 | Sense: 5'-ACCTGTCCAACAACAGGATCACCT-3' Antisense: 5'-TGTTC AAGACTGCCAGGGAAGAA-3' |
| TLR4 | Sense: 5'-GCCGAAAGGTGATTGTTGTGGTGT-3' Antisense: 5'-ACTGCCAGGTCTGAGCAATCTCAT-3' |
| GAPDH | Sense: 5'-AGGAGTAAGAAACCCTGGAC-3' Antisense: 5'-CTGGGATGGAATTGTGAG-3' |

LDLR: Low-density lipoprotein receptor; TLR2: Toll-like receptor 2; TLR4: Toll-like receptor 4. HMGCoAr: 3-hydroxy-3-methylglutaryl-coenzyme A reductase; GAPDH: Glyceraldehyde-3-phosphate dehydrogenase.

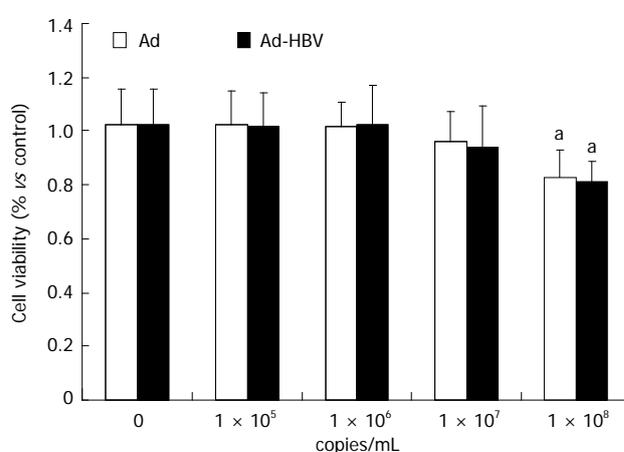


Figure 1 Cell toxicity by adenovirus hepatitis B virus. The data are presented as means \pm SE. ^a $P < 0.05$ vs control group, $n = 6$. Ad: Adenovirus; HBV: Hepatitis B virus.

(PBS) and underwent three freeze-thaw cycles. Lysates were cleared by centrifugation at $14\,000 \times g$, divided into equal volumes and used for real-time polymerase chain reaction (PCR) and infecting HepG2 cells. The HBV DNA was quantified using the BIO-RAD iCycler real-time PCR system and the qPCR Master Mix (Da An Gene Co., Ltd, Guangzhou, China). The copy of viral genome equivalents was determined using a calibration curve containing known amounts of HBV DNA. Ad was amplified in AD293 cells. To quantify Ad, the infected efficiency of Ad in AD293 cells was measured and normalized to the infected efficiency of Ad-HBV.

Cytotoxicity assay

Cell toxicity due to Ad-HBV treatment was determined by the mitochondrial tetrazolium assay (MTT). HepG2 cells were grown in media with Ad-HBV at various concentrations for 4 d before addition of the MTT agent. Optical density was read at 570 nm using the BiotekElx-800 plate reader. Cells treated with vehicle served as controls, and the cell viability of the Ad-HBV treated group was normalized to that of the control group.

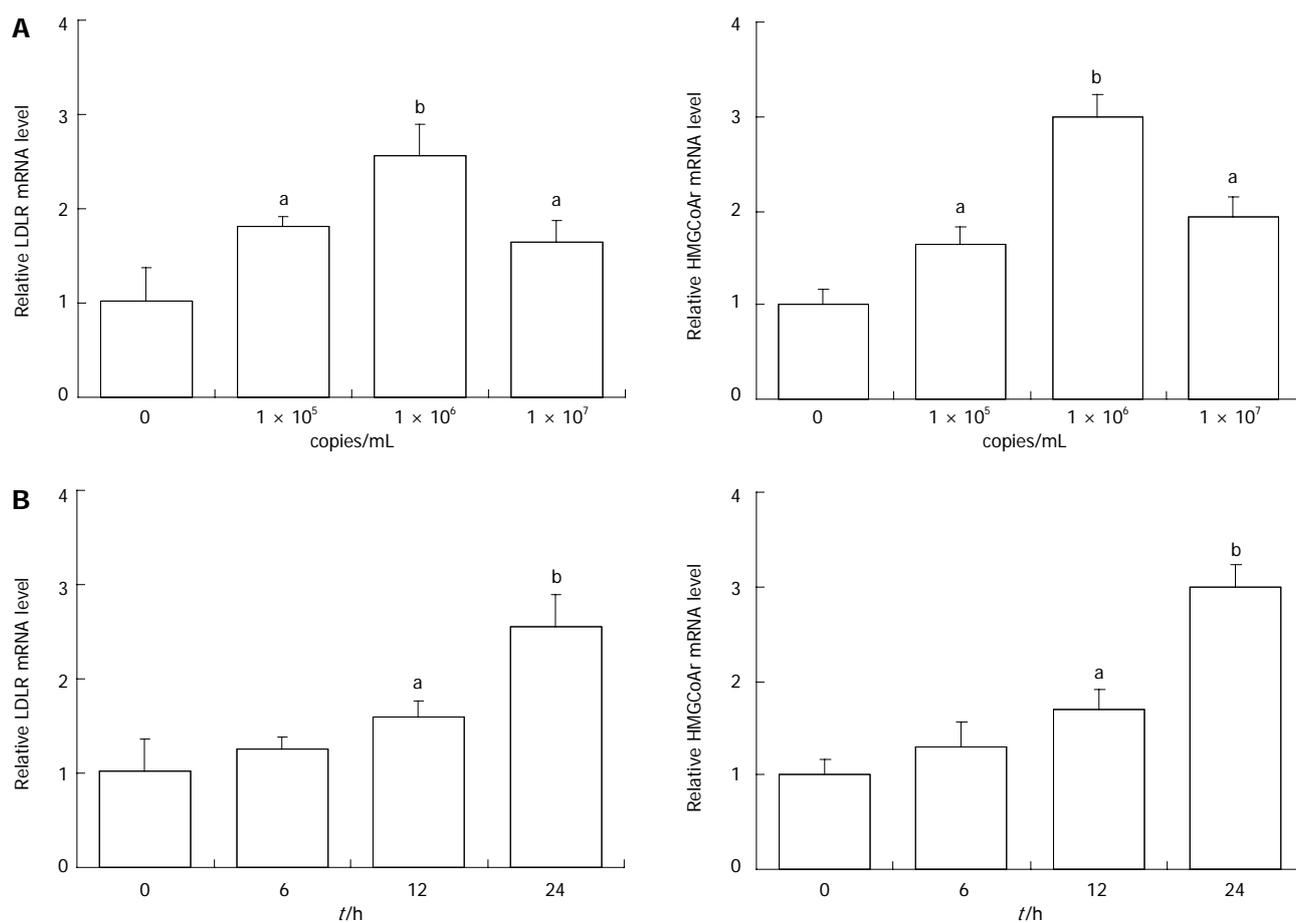


Figure 2 Effects of adenovirus hepatitis B virus treatment on the mRNA levels of genes related to cholesterol metabolism in HepG2 cells. A: HepG2 cells were treated with different concentration of adenovirus-hepatitis B virus (Ad-HBV) (1×10^5 , 1×10^6 , 1×10^7 copies) for 24 h; B: HepG2 cells were incubated for 0-24 h with 1×10^6 copies Ad-HBV. ^a $P < 0.05$, ^b $P < 0.01$ vs control group. LDLR: Low-density lipoprotein receptor.

Quantitative RT-PCR

Total RNA was isolated from cultured HepG2 cells treated with different concentration of Ad-HBV (1×10^5 , 1×10^6 , 1×10^7 copies) for 24 h as well as HepG2 cells incubated for 0-24 h with 1×10^6 copies Ad-HBV using the guanidinium-phenol-choloroform method^[15]. Total RNA (500 ng) was used as a template for RT-PCR. The RT reaction was set up using a kit from TaKaRa (PrimeScript™ RT reagent Kit, Dalian, China). Following synthesis, cDNA was split for the separate amplification of target genes using specific primers as shown in Table 1. All primers were designed using the following website (www.idtdna.com/Primerquest/). Real-time PCR was performed in a BIOD using SYBR Premix Ex Taq™ (TaKaRa, Dalian, China) according to the manufacturer's protocol. After PCR, a dissociation curve (melting curve) was constructed at the temperature ranging from 60 °C to 95 °C. Ct values were averaged and normalized to GAPDH. Relative expression was determined by the $\Delta\Delta C_t$ comparative threshold method.

TLRs-siRNA

Sequences of TLR2/TLR4 siRNA were purchased from Santa Cruz (United States). The transfection of siRNA

was performed using a Lipofectamine kit (Invitrogen, United States) according to the manufacturer's instructions. The medium was changed 6 h after transfection, and the cells were incubated with Ad-HBV or Ad for a further 24 h. The cells were then harvested and the protein levels of TLRs were determined by Western blotting. Cholesterol uptake measurements were then carried out.

Western blotting

Cells were washed with PBS, scraped into lysis buffer (Tris-EDTA + Complete protease inhibitor; Roche, United States) and mechanically homogenized. Total protein samples (40 μ g per well) were electrophoresed on 8% SDS-polyacrylamide gel and transferred to polyvinylidene difluoride (PVDF) membranes at 100V for 100 min. Membranes were incubated overnight with anti-LDLR (1:2000, Millipore), anti-HMGCoAr (1:1000, Millipore), anti-TLR2 (1:1000, Millipore), anti-TLR4 (1:1000, Santa Cruz), or anti-GAPDH (1:10 000, Sigma). Anti-mouse secondary antibody conjugated with horseradish peroxidase (Promega, United States) and Super-Signal West Pico Chemiluminescent Substrate (Pierce, United States) were used for detection.

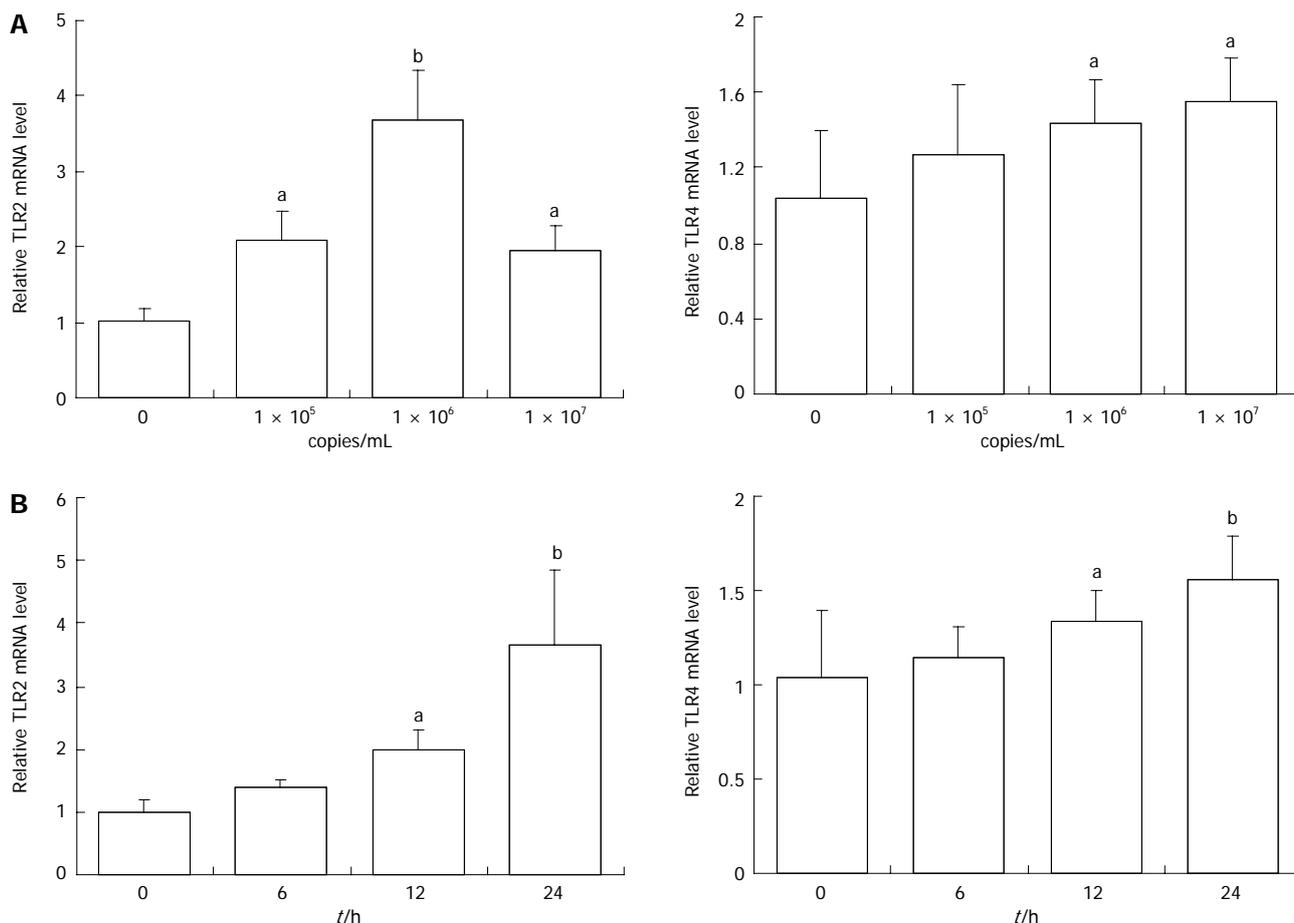


Figure 3 Effects of adenovirus hepatitis B virus treatment on toll-like receptors mRNA levels in HepG2 cells. A: Adenovirus-hepatitis B virus (Ad-HBV) at 10^5 and 10^6 copies profoundly augmented the mRNA levels of toll-like receptor 2 (TLR2). While the concentration of Ad-HBV up to 10^7 copies, the mRNA expression were suppressed instead. B: The mRNA of TLR4 were increased in HepG2 cells treated with Ad-HBV, which was in a dose-dependent manner. Results were representative of three similar experiments. ^a $P < 0.05$, ^b $P < 0.01$ vs control group.

Modified LDL uptake measurements

HepG2 cells were incubated with human 1^2 -dioctadecyl-1-3,3,3',3'-tetramethyling-docarbocyanine perchlorate (DiI)-labeled acetylated low density lipoprotein (DiI-acl-LDL) (10 μ g/mL) for 4 h, DAPI staining was used to detect the nucleus. The two color images were visualized under a fluorescence microscope. ZEN 2008 and Image software were used to analyze the quantity of DiI-acl-LDL.

Statistical analysis

Results were shown as mean \pm SE, and all experiments were run in triplicate. The statistical significance of differences between groups was determined using the Student *t* test. ^a $P < 0.05$, ^b $P < 0.01$, vs the control group.

RESULTS

Cell toxicity of Ad-HBV

The toxicity of Ad-HBV against HepG2 cells used for propagation of viral infections was measured. Both Ad and Ad-HBV below 10^7 copies/mL did not exhibit either toxic or proliferative effects on HepG2 cells, while

1×10^8 copies/mL of the virus reduced HepG2 cell survival rates (Figure 1).

Ad-HBV changes the mRNA levels of genes related to cholesterol metabolism in HepG2 cells

Whether HBV leads to cholesterol metabolism disorders in the liver is still controversial. Therefore, we determined the effects of Ad-HBV on the mRNA levels of genes related to cholesterol metabolism in HepG2 cells. As shown in Figure 2A, Ad-HBV at 1×10^5 and 1×10^6 copies/mL significantly augmented the mRNA levels of LDLR and HMGCoAr. When the concentration of Ad-HBV reached 10^7 copies/mL, the mRNA expression was suppressed. Ad-HBV at 1×10^6 copies/mL significantly induced mRNA expression of LDLR (2.56 ± 0.33 vs 1.03 ± 0.25 , $P < 0.01$, $n = 3$) and HMGCoAr (2.98 ± 0.25 vs 1.01 ± 0.18 , $P < 0.01$, $n = 3$). Ad-HBV upregulated the mRNA levels of LDLR and HMGCoAr in a time-dependent manner. HepG2 cells were maintained with 1×10^6 copies/mL of Ad-HBV for different time periods and values were expressed as fold changes relative to the controls. After 24 h of incubation the mRNA expression of LDLR (2.56 ± 0.33 vs 1.03 ± 0.34 , $P < 0.01$, $n =$

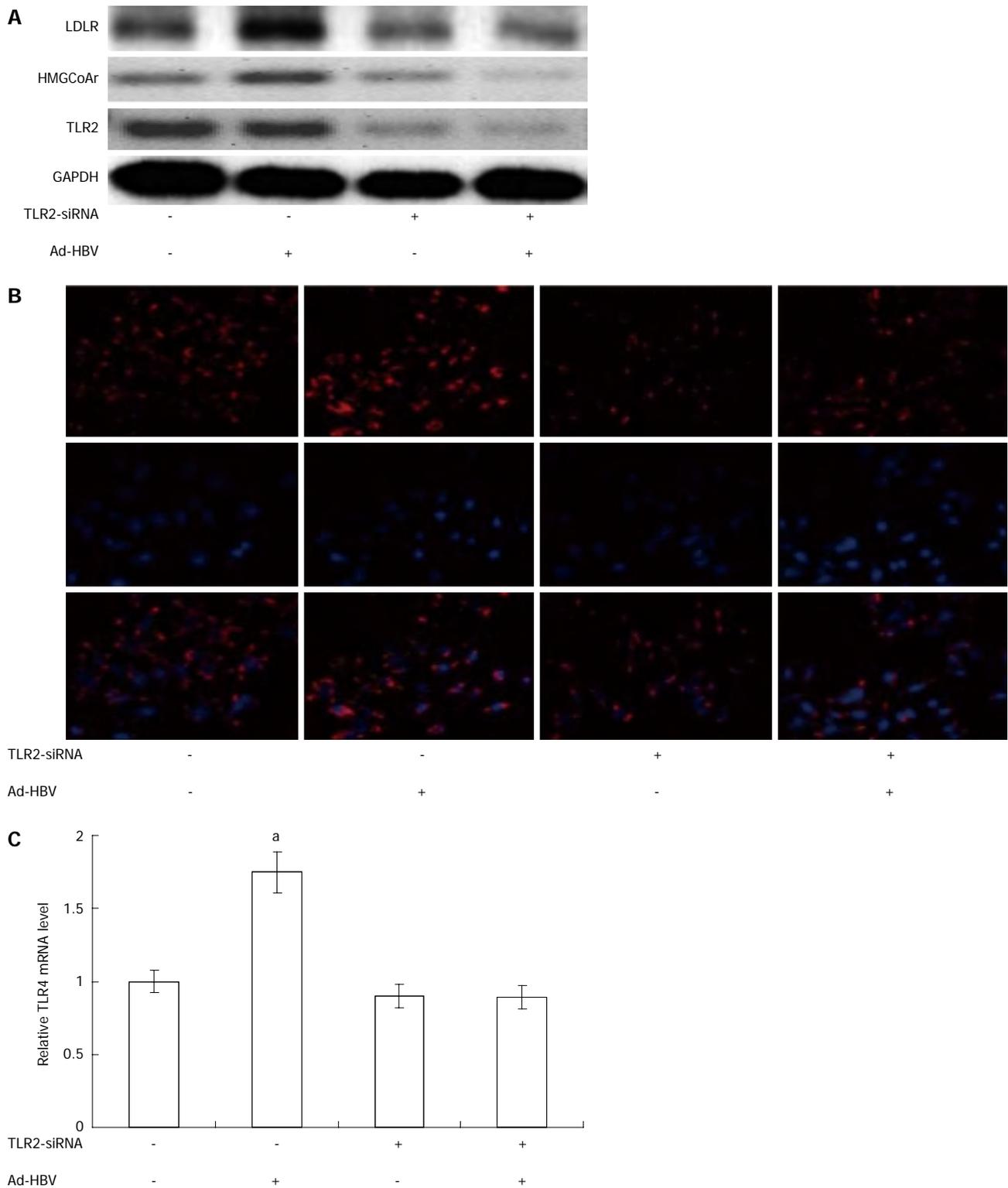


Figure 4 Effects of toll-like receptor 2-SiRNA on the expression of proteins related to cholesterol metabolism and intake of cholesterol by HepG2 cells treated with adenovirus hepatitis B virus. **A:** The protein expression of low-density lipoprotein receptor (LDLR) and 3-hydroxy-3-methylglutaryl-coenzyme A reductase (HMGCoAr) were assayed; **B:** HepG2 Cells were exposed for 4 h to Dil-acLDL (10 $\mu\text{g}/\text{mL}$), after thorough washing with phosphate buffered solution, fluorescence of Dil-acLDL was detected in cytoplasm of cells by fluorescence microscopy; **C:** Relative quantity of Dil-acLDL in HepG2 cells were analyzed by software. Results were representative of three similar experiments. ^a $P < 0.05$, ^b $P < 0.01$ vs control group. TLR2: Toll-like receptor 2; LDLR: Low-density lipoprotein receptor; LDL: Low density lipoprotein; Ad-HBV: Adenovirus hepatitis B virus.

3) and HMGCoAr (2.99 ± 0.25 vs 1.01 ± 0.18 , $P < 0.01$, $n = 3$) reached a peak (Figure 2B).

Ad-HBV changes the mRNA levels of TLRs in HepG2 cells

Researchers recently showed that TLRs were involved in viral infection and its downstream effects. To investigate whether Ad-HBV upregulated the mRNA levels of genes related to cholesterol metabolism in HepG2 cells, TLRs mRNA expression was determined. Ad-HBV at 1×10^5 copies/mL and 1×10^6 copies/mL significantly induced the mRNA levels of TLR2. Values were expressed as fold changes relative to the controls. When the concentration of Ad-HBV was increased to 1×10^7 copies/mL, mRNA expression was suppressed (Figure 3A). These changes were consistent with the changes in cholesterol metabolism-related genes. The mRNA levels of TLR4 were increased in HepG2 cells treated with Ad-HBV in a dose-dependent manner (Figure 3B).

TLR2 mediated the effects of Ad-HBV on the expression of proteins related to cholesterol metabolism and intake of cholesterol in HepG2 cells

To clarify the mechanism of Ad-HBV-upregulated mRNA levels of proteins related to cholesterol metabolism in HepG2 cells, we used siRNA-mediated down-regulation of TLR2/TLR4 to confirm our hypothesis that TLR2/TLR4 may participate in this process. To evaluate the involvement of TLR2/TLR4 in the effects of Ad-HBV, we attenuated the expression of TLR2 using human TLR2-SiRNA, which suppressed TLR2 protein level by up to 80% (Figure 4). Transient transfection of HepG2 cells with TLR2-siRNA substantially abolished Ad-HBV-mediated upregulation of LDLR and HMGCoAr and the uptake of Dil-acl-LDL (Figure 4). TLR4-SiRNA did not change the expression of proteins related to cholesterol metabolism during Ad-HBV infection (data not shown).

DISCUSSION

There is increasing evidence to indicate that hepatic lipid accumulation is related to hepatic fibrosis and inflammation, resulting in cell apoptosis and cancer^[16,17]. In particular, it is assumed that lipid accumulation is a prerequisite for subsequent events leading to liver injury in nonalcoholic fatty liver disease^[18]. In addition, it was recently shown that hepatic steatosis may be a factor in HCV-induced liver pathogenesis and may impair the response to interferon-based therapy^[19,20]. Due to the importance of lipid accumulation, the mechanism by which nonalcoholic fatty liver disease and HCV infection cause hepatic steatosis has been studied intensively^[4]. However, the molecular mechanisms by which HBV infection causes hepatic steatosis have been poorly investigated.

According to current knowledge, liver LDLR is the most important receptor of binding and internalization of plasma-derived LDL-cholesterol and regulates plasma

LDL concentration. Changes in receptor activity alter the rates of LDL uptake by the liver with a corresponding increase or decrease in plasma LDL levels^[21,22]. Our results showed that the mRNA and protein levels of LDLR were increased following infection of HepG2 cells with Ad-HBV.

3-hydroxy-3-methylglutaryl coenzyme A reductase (HMGCoAr) is an enzyme which catalyzes the conversion of HMGCoA to mevalonate, the rate limiting step in cholesterol biosynthesis. Normally, in mammalian cells this enzyme is suppressed by cholesterol and its derivatives from the LDLR mediated-internalization of LDL as well as oxidized species of cholesterol. Competitive inhibition of the reductase upregulates the expression of LDLR in the liver, which in turn increases the catabolism of plasma LDL and lowers the plasma concentration of cholesterol, an important determinant of atherosclerosis^[23-25].

HBV, like many other microorganisms that contribute to the pathogenesis of atherosclerosis, can colonize the vascular tissues^[26], induce vasculitis^[27], and stimulate inflammatory and immune responses that may lead to vascular damage and precipitate atherosclerosis. It was reported in one Japanese study that HBV infection can be atherogenic in otherwise healthy subjects with preserved liver function^[7]. We reasoned that HBV would be a rational candidate pathogen among the stimuli that contribute to atherosclerosis. The mechanism behind this association is still unclear, and additional studies are required.

TLRs have been reported to play an important role in liver damage after infection with HBV, and the mechanisms may involve virus-induced immune modulation^[14,28,29]. TLRs are also involved in other immune diseases mediated by HBV. TLR4 had been reported to not only inhibit HBV replication, but also to induce immune injury in cells^[30]. Consistent with previous studies, we found that Ad-HBV increased the expression of TLR2/4. Most importantly, we observed that the upregulation of LDLR and HMG-CoAr *via* Ad-HBV can be partially blocked by silencing TLR2, but not TLR4. Taken together, these results suggest that Ad-HBV infection-induced cholesterol accumulation in hepatocytes is mediated by TLR2.

In conclusion, our data indicate that HBV is able to induce the gene expression of TLR2, thereby causing hepatic lipid accumulation by increasing genes related to cholesterol absorption and metabolism. Because increased lipid deposition is involved in the progression of severe liver injury such as hepatitis and hepatocellular carcinoma, our results provide important information in understanding the development and progression of HBV-induced pathogenesis.

COMMENTS

Background

Cholesterol accumulation plays an important role in the progression of atherosclerosis. This study was undertaken to investigate whether hepatitis B

virus (HBV) exacerbates hepatic cholesterol accumulation and the underlying mechanisms were examined.

Research frontiers

Previous studies have mainly focused on the potential effect of HBV in the progression of atherosclerosis. The authors hypothesized that HBV increases low-density lipoprotein receptor and 3-hydroxy-3-methylglutaryl-coenzyme A reductase expression resulting in exacerbated cholesterol accumulation in HepG2 cells, which was mediated *via* the toll-like receptor 2 (TLR2) pathway.

Innovations and breakthroughs

To further clarify the potential effect of HBV in the progression of atherosclerosis, the authors examined the effects of adenovirus hepatitis B virus (Ad-HBV) in the progression of atherosclerosis which is partly mediated *via* the TLR2 pathway.

Applications

The results show that Ad-HBV up-regulates the expression of genes related to cholesterol metabolism *via* the TLR2 pathway. Further studies are required to evaluate the mechanism by which HBV regulates the TLR2 pathway.

Terminology

There are some associations between hepatitis virus and carotid atherosclerosis. Hepatitis virus causes liver and even systemic inflammatory reactions, and inflammation is one the pathophysiological changes in atherosclerosis.

Peer review

This manuscript presented that Ad-HBV up-regulated the expression of genes related to cholesterol metabolism in HepG2 cells. Furthermore, the atherosclerosis effect of Ad-HBV is *via* TLR2 pathway. The experiment seems to be correct. The study appears well conducted and the results discussed with honesty, and caution.

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Habitual rapid food intake and ineffective esophageal motility

Kong-Ling Li, Ji-Hong Chen, Qian Zhang, Jan D Huizinga, Shawn Vadakepeedika, Yu-Rong Zhao, Wen-Zhen Yu, He-Sheng Luo

Kong-Ling Li, Ji-Hong Chen, Qian Zhang, Jan D Huizinga, Shawn Vadakepeedika, Yu-Rong Zhao, Wen-Zhen Yu, He-Sheng Luo, Department of Gastroenterology and Hepatology, Renmin Hospital of Wuhan University, Wuhan 430060, Hubei Province, China

Jan D Huizinga, On sabbatical at Wuhan University from McMaster University, L8N3Z5 Hamilton, Ontario, Canada

Author contributions: Li KL analyzed the data and wrote the manuscript; Chen JH performed the esophageal manometry, analyzed the data and wrote the manuscript; Zhang Q, Zhao YR and Yu WZ assisted with the manometry; Huizinga JD and Vadakepeedika S contributed to manuscript writing; Luo HS designed the study.

Correspondence to: Ji-Hong Chen, MD, PhD, Professor of Medicine, Director of GI Motility Lab, Department of Gastroenterology and Hepatology, Renmin Hospital of Wuhan University, No. 238 Jiefang Road, Wuchang, Wuhan 430060, Hubei Province, China. chenjihong2@medmail.com.cn

Telephone: +86-27-88041911 Fax: +86-27-88042292

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Abstract

AIM: To study non-cardiac chest pain (NCCP) in relation to ineffective esophageal motility (IEM) and rapid food intake.

METHODS: NCCP patients with a self-reported habit of fast eating underwent esophageal manometry for the diagnosis of IEM. Telephone interviews identified eating habits of additional IEM patients. Comparison of manometric features was done among IEM patients with and without the habit of rapid food intake and healthy controls. A case study investigated the effect of 6-mo gum chewing on restoration of esophageal motility in an IEM patient. The Valsalva maneuver was performed in IEM patients and healthy controls to assess the compliance of the esophagus in response to abdominal pressure

increase.

RESULTS: Although most patients diagnosed with NCCP do not exhibit IEM, remarkably, all 12 NCCP patients who were self-reporting fast eaters with a main complaint of chest pain (75.0%) had contraction amplitudes in the mid and distal esophagus that were significantly lower compared with healthy controls [(23.45 mmHg (95%CI: 14.06-32.85) vs 58.80 mmHg (95%CI: 42.56-75.04), $P < 0.01$ and 28.29 mmHg (95%CI: 21.77-34.81) vs 50.75 mmHg (95%CI: 38.44-63.05), $P < 0.01$, respectively)]. In 7 normal-eating IEM patients with a main complaint of sensation of obstruction (42.9%), the mid amplitude was smaller than in the controls [30.09 mmHg (95%CI: 19.48-40.70) vs 58.80 mmHg (95%CI: 42.56-75.04), $P < 0.05$]. There was no statistically significant difference in manometric features between the fast-eating and normal-eating groups. One NCCP patient who self-reported fast eating and was subsequently diagnosed with IEM did not improve with proton-pump inhibition but restored swallow-induced contractions upon 6-mo gum-chewing. The Valsalva maneuver caused a markedly reduced pressure rise in the mid and proximal esophagus in the IEM patients.

CONCLUSION: Habitual rapid food intake may lead to IEM. A prospective study is needed to validate this hypothesis. Gum-chewing might strengthen weakened esophageal muscles.

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Key words: Esophageal manometry; Ineffective esophageal motility; Non-cardiac chest pain; Rapid food intake; Valsalva maneuver

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INTRODUCTION

Non-cardiac chest pain (NCCP) and ineffective esophageal motility (IEM) are often associated with gastroesophageal reflux disease (GERD). Although esophageal dysmotility is considered an uncommon cause of non-GERD-related NCCP^[1,2], in our practice it is not infrequent. In recent years, we noted that some patients with a primary complaint of chest pain or discomfort had a life long habit of rapid food intake. Their esophageal manometry exhibited low esophageal contraction amplitudes during wet swallows. This initiated the current investigation into a possible relationship between habitual rapid food intake, symptoms and motility dysfunction.

In a recent study, a possible association was investigated between self-reported eating behavior and metabolic risk factors (overweight, hypertension, hyperglycemia, hypertriglycerolemia, low levels of high-density lipoprotein (HDL) cholesterol, hyperuricemia and fatty liver)^[3]. The conclusion was that rapid eating increases metabolic risk factors although the mechanism was not investigated. In the present study, we analyzed the clinical and manometric characteristics of IEM patients with and without a habit of rapid eating. Our main objective was to investigate a possible correlation between rapid food intake and IEM. Our hypothesis was that rapid eating is associated with less swallow-induced contractions, contributing to IEM through disuse of the esophageal musculature; hence we predicted that patients with IEM and rapid eating should have more severe ineffective esophageal motility compared to IEM patients without the habit of rapid eating. We also report a case-study of an IEM patient whose symptoms were improved by 6 mo of gum-chewing exercise.

MATERIALS AND METHODS

Subjects

Data were collected from patients in our department with various symptoms including chest pain or discomfort, dysphagia, heartburn that lasted from 1 mo to 30 years who underwent esophageal manometry. Some patients volunteered information about their fast eating habits as a cause of their symptoms. We collected information about the manometry tests of all patients whose eating habits were recorded at the first visit, and in addition, we obtained information about eating habits by telephone interview of 9 additional patients.

Two groups of volunteers participated in the study: Group V1 as healthy controls in the manometric analysis; Group V2 recruited to record healthy Chinese people's daily meal duration. Group V1 were without any digestive or systemic symptoms and the volunteers underwent the same manometric procedures as the patients. Group V2

were sent out to canteens, fast-food restaurants, Chinese restaurants and local families to time the duration of the meal intake.

Written informed consent was provided by all the participants. This study was approved by the Ethics Committee of Renmin Hospital of Wuhan University.

Study protocol

The patients' current symptoms, medical history and basic information including age, gender, body mass index (BMI) was obtained and standard esophageal manometry was performed. During the manometry testing, the patients were instructed to perform the Valsalva maneuver. Volunteers in Group V1 underwent the same manometric procedure after their basic information was obtained. Each of them performed the Valsalva maneuver. Group V2 was assigned to the above-mentioned dining locations to record the meal duration.

Esophageal manometry

Following an overnight fast and 48-h discontinuation of any medication that may interfere with esophageal motility, conventional stationary esophageal manometry was performed using a 3.5 mm diameter, eight-lumen, sleeve sensor catheter assembly (Mui Scientific, Mississauga, Ontario, Canada) with eight side-holes arranged in radial form and located 2-7 cm apart. Manometric data were recorded and analyzed by means of the Polygram 98 and the Polygram Net Esophageal Manometry Testing Application Software (Medtronic A/S, Tonsbakken, Skovlunde, Denmark). The catheter was inserted transnasally into the stomach and intragastric pressure (GP) was obtained in a supine position. The lower esophageal sphincter (LES) resting pressure was defined as the mid-respiratory LES pressure compared with GP. Patients or healthy volunteers were then instructed to perform ten wet swallows (10 mL water each, separated by an interval of 30 s) to measure and calculate the contraction amplitude, duration and velocity in the proximal, mid and distal esophagus. When calculating the velocity, we did not incorporate data indicative of simultaneous (*i.e.*, velocity > 8 cm/s) contractions. The existence of double-peaked or multi (≥ 3)-peaked waves was also noted.

The manometric criteria for the diagnosis of IEM were no fewer than 30% of the wet swallows featuring one or more of the following characteristics: (1) contraction amplitude < 30 mmHg at either or both of the distal points 5 and 10 cm above the LES; (2) simultaneous contraction (distal velocity between 5 and 10 cm above the LES > 8 cm/s) with amplitude < 30 mmHg; and (3) absent or non-transmitted peristalsis^[4,5].

The valsalva maneuver

After wet swallows, 12 patients and all the volunteers in Group V1 were instructed to perform the Valsalva maneuvers in the supine position, exhaling forcibly with the mouth closed and the nose pinched shut^[6,7]. Data on the pressure changes in the esophageal body and the LES

Table 1 Esophageal manometry results, expressed as mean (95%CI) in ineffective esophageal motility patients and healthy

| | IEM patients with the habit of fast eating (<i>n</i> = 12) | IEM patients without the habit of fast eating (<i>n</i> = 7) | Healthy controls (<i>n</i> = 10) |
|------------------------------|---|---|-----------------------------------|
| LES pressure (mmHg) | 12.71 (6.80-18.62) | 11.08 (-0.59-22.76) | 14.94 (10.38-19.49) |
| Distal esophagus | | | |
| Amplitude (mmHg) | 28.29 (21.77-34.81) ^b | 33.78 (19.56-48.00) | 50.75 (38.44-63.05) |
| Duration (s) | 3.04 (2.44-3.65) | 2.74 (1.32-4.15) | 3.09 (2.30-3.88) |
| Velocity (cm/s) ¹ | 1.42 (1.14-1.70) | 3.33 (0.79-5.86) | 1.57 (0.89-2.24) |
| Mid esophagus | | | |
| Amplitude (mmHg) | 23.45 (14.06-32.85) ^b | 30.09 (19.48-40.70) ^a | 58.80 (42.56-75.04) |
| Duration (s) | 3.12 (2.32-3.91) | 3.18 (1.81-4.55) | 2.45 (2.13-2.79) |
| Velocity (cm/s) ² | 2.18 (1.23-3.12) | 3.76 | 2.35 (1.38-3.33) |
| Proximal esophagus | | | |
| Amplitude (mmHg) | 36.75 (22.93-50.57) | 41.47 (8.79-74.15) | 49.96 (36.28-63.64) |
| Duration (s) | 2.42 (1.84-3.00) | 3.13 (1.48-4.77) | 2.25 (1.80-2.71) |
| Velocity (cm/s) ³ | 3.66 (1.71-5.60) | 2.56 | 2.42 (1.75-3.09) |

¹Velocity in the distal esophagus of 3 fast-eating and 3 normal-eating IEM patients could not be calculated due to simultaneous contractions; ^{2,3}Velocity in the mid (proximal) esophagus of 6 fast-eating and 5 normal-eating IEM patients and 2 healthy controls could not be calculated due to simultaneous contractions. Contraction data were in response to wet swallows. Data indicative of simultaneous (*i.e.*, velocity > 8 cm/s) or other non-propulsive contraction were excluded when calculating the mean velocity. ^a*P* < 0.05, ^b*P* < 0.01 *vs* control. IEM: Ineffective esophageal motility; LES: Lower esophageal sphincter.

were collected.

Rapid food intake measurement

We used the length of time it took for a patient to finish an average meal as the indicator of the speed of eating. Meal lengths of the healthy population were recorded when they had regular Chinese meals with or without water. None of them took alcohol or had chat time included.

Statistical analysis

Except for age which was presented as median and range, the other data were expressed as means and 95%CI. Kolmogorov-Smirnov analysis was applied to determine data distribution. Student's *t* test was employed for the comparison of data. Statistical significance was acknowledged if *P* < 0.05.

RESULTS

Meal lengths in IEM patients with or without the habit of rapid food intake

Ten NCCP patients mentioned their eating habits specifically during initial evaluation, six of whom reported a habit of rapid eating and were all diagnosed with IEM according to the manometric criteria. We managed to obtain information from 9 other IEM patients by telephone calls. Among these 19 patients, 12 (63.2%) (7 males and 5 females, median age 44.5 years, range 18-57 years, mean BMI 22.52; 95%CI: 20.45-24.59) volunteered the fact that they had been eating much faster than normal speed for a long time from 5 to 31 years. The other seven (36.8%) patients (2 males and 5 females, median age 52 years, range 45-74 years, mean BMI 22.48 (95%CI: 20.56-24.39) reported no habit of rapid eating.

Ten [4 males and 6 females, median age 22 years, range 20-33 years, mean BMI 20.69 (95%CI: 19.39-21.87)] healthy volunteers were recruited into Group V1. Group

V2 consisted of 91 (50 males and 41 females) healthy volunteers.

For the self-reporting fast-eaters, meals all lasted no more than 8 min (3 min in one patient; 4 min in one; 5 min in five; 6 min in three; 7 min in one and 8 min in one). Their average meal duration was significantly shorter than that of the healthy volunteers (5.42 min, 95%CI: 4.58-6.25 *vs* 16.58 min, 95%CI: 14.21-18.94, *P* < 0.01). The meal lengths in the IEM patients with normal eating habits ranged from 10 to 30 min and their mean meal length was not statistically different from healthy volunteers (18.86 min, 95%CI: 12.31-25.41 *vs* 16.58 min, 95%CI: 14.21-18.94, *P* > 0.05). We found that the meal lengths of all IEM patients with normal eating habits were longer than those of the self-reporting rapidly eating patients.

Some fast-eating patients reported that while eating fast they spent shorter time chewing. They also swallowed more rapidly and frequently though they did not quantify it.

Clinical characteristics

The predominant clinical manifestation in the fast-eating group was chest pain or discomfort (9/12, 75.0%), followed by sensation of obstruction (5/12, 41.7%), heartburn (2/12, 16.7%), acid reflux (1/12, 8.3%), dysphagia (1/12, 8.3%), chest tightness (1/12, 8.3%), food regurgitation (1/12, 8.3%), abdominal discomfort (1/12, 8.3%), nausea (1/12, 8.3%) and eructation (1/12, 8.3%). In the normal-eating group, sensation of obstruction was the most common (3/7, 42.9%), followed by heartburn (2/7, 28.6%), acid reflux (2/7, 28.6%) and chest pain or discomfort (1/7, 14.3%).

Manometric features

Table 1 shows the IEM patients' manometric features. The contraction amplitudes in the distal and mid esophagus of the fast-eating IEM patients were significantly

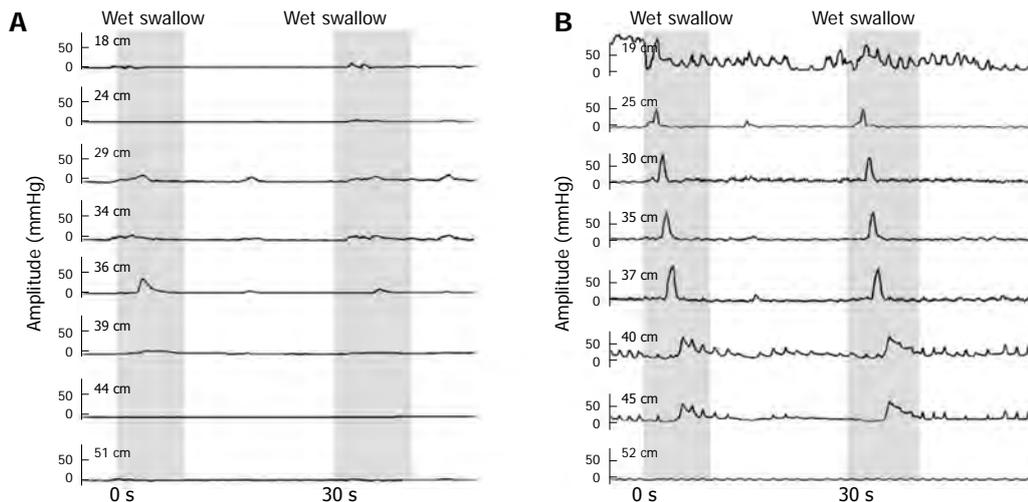


Figure 1 Manometric tracings of a fast-eating ineffective esophageal motility patient (A) and a healthy control (B). The numbers (in cm) on the left indicate the distances from the nose to each side-hole on the catheter.

lower ($P < 0.01$) than in the control group. The amplitude in the mid esophagus of the normal-eating IEM patients was also significantly lower ($P < 0.05$) than in the controls. There was no statistically significant difference in manometric features between the IEM patients with and without the habit of rapid food intake.

Simultaneous contractions were observed in 11 fast-eating and 6 normal-eating IEM patients (91.7% and 85.7% respectively *vs* 30% in healthy controls) and non-pulsive (but not simultaneous) contractions in 1 fast-eating patient (8.3% *vs* 0% in controls). Seven fast-eating and 6 normal-eating patients (58.3% and 85.7% *vs* 20% in controls) exhibited double-peaked waves and 3 fast-eating and 3 normal-eating patients (25.0% and 42.9% *vs* 20% in controls) had multi-peaked waves during certain wet swallows. A typical manometric tracing from one of the fast-eating IEM patients is shown in Figure 1.

Short swallowing interval caused prevention of peristalsis

According to our protocol, wet swallows should be separated by an interval of 30 s. However, in some patients and healthy controls, the interval between certain swallows happened to be shorter than 10 s or even near zero. We observed that in pairs of short-interval swallows, only one peristalsis appeared in response to the first or the second swallow while the response to the other swallows was only contraction in the proximal esophagus, and the contraction in the distal part was prevented, as shown in Figure 2.

Response of the esophageal musculature to the Valsalva maneuver

Pressure alterations in the LES and distal, mid and proximal esophagus during the Valsalva maneuver between IEM patients and healthy controls were compared (Table 2), and the manometry tracings are illustrated in Figure 3. IEM patients showed a much lower increase in esophageal

pressure due to the Valsalva maneuver compared with controls. Mean changes in LES pressure of IEM patients were not statistically different from that of healthy volunteers.

Esophageal motility improved by gum-chewing exercise: a case report

A 57-year-old male with a history of rapid food intake for more than 30 years, with each meal lasting less than 5 min, presented to our outpatient department with 2 years of moderate retrosternal chest pain, sensation of obstruction and occasional dysphagia. The initial esophageal manometry revealed that his swallow-induced esophageal contraction amplitude was extremely low (distal amplitude 10.42 mmHg on average). He was advised to slow down his speed of eating and to take proton-pump inhibitor (PPI) for 4 mo, but resulting in no benefit. The drug was discontinued. Then a gum-chewing exercise (about 10 times a day, 15 min each time, for 6 mo) was recommended. The patient returned to the hospital 6 mo later, reporting that his symptoms had been relieved. The contraction amplitude of his repeat manometry was improved (distal amplitude 58.03 mmHg on average). His manometric tracings before and after the gum-chewing exercise are shown in Figure 4. A repeat manometry after another 6 mo revealed continued normalized esophageal motility (distal amplitude 60.07 mmHg on average), though he had reduced the frequency of gum-chewing exercise since the previous manometry. During the manometry this time, the patient was also asked to perform 10 pairs of wet swallows at the interval of 2 s, 8 of which failed to initiate any peristalsis and only 2 of which were observed with peristaltic contraction at the end of the second pair of wet swallows.

DISCUSSION

Of the 19 IEM patients whose eating habits were investi-

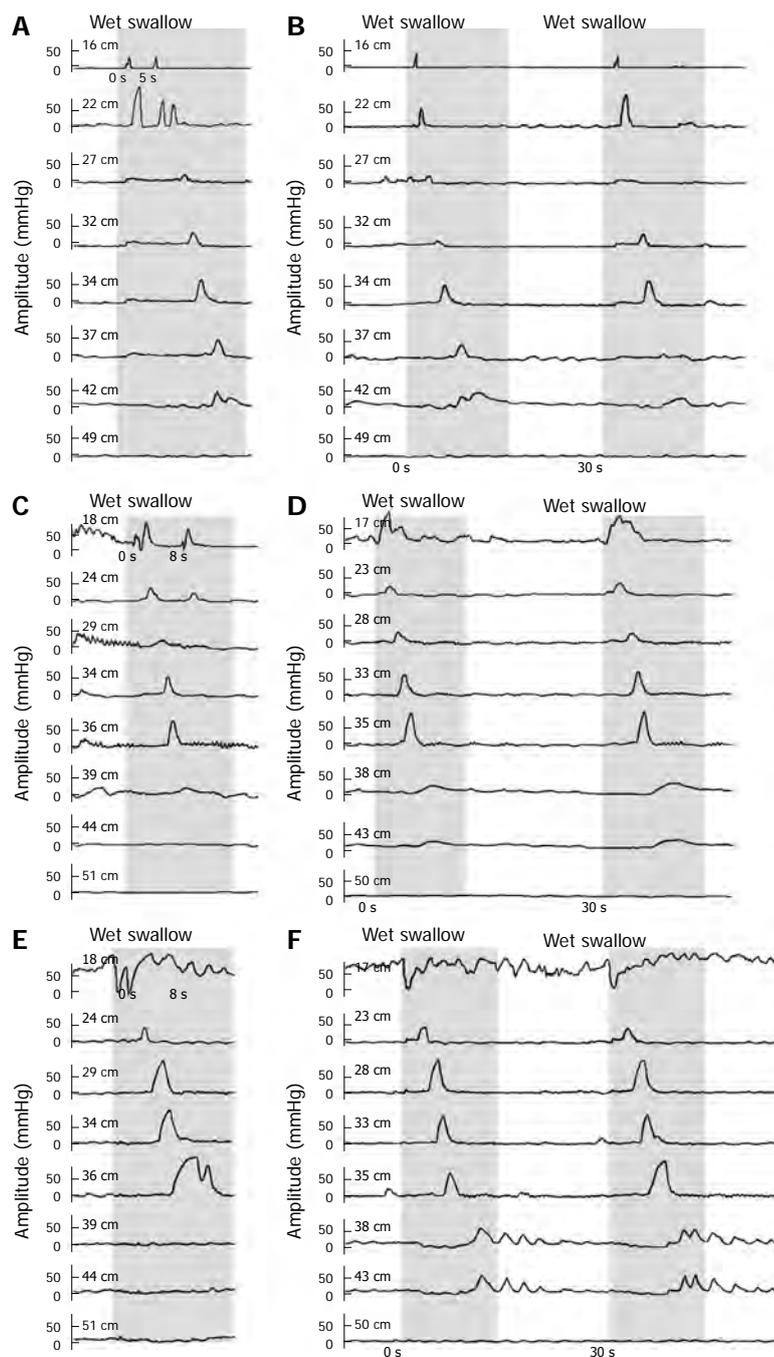


Figure 2 Manometric tracings of swallowing at an interval < 10 s (A, C, E) in comparison with swallowing at the interval of 30 s (B, D, F). The numbers (in cm) on the left indicate the distances from the nose to each side-hole on the catheter. In an ineffective esophageal motility patient who was habitually rapidly eating (A, B), only one peristaltic contraction appeared in response to the second of a pair of wet swallows at the interval of 5 s (A). Similar finding was observed in one healthy control (E, F) whose two wet swallows were almost continuous (E). In another control (C, D), peristalsis was only seen in response to the first of a pair of wet swallows at the interval of 8 s (C).

gated, 12 were fast eaters. The main presenting symptom of the fast eaters was chest pain or discomfort; the main symptom of the normal-eating patients was sense of obstruction. Although the average values of all swallow-induced contraction amplitudes were lower in the fast-eating group, there was no statistically significant difference compared with the normal-eating IEM patients. There are two possible explanations for this result. One is that factors other than fast eating were the dominant cause of weakened esophageal muscle in both groups. The other

is that the weakened esophageal muscle could be due to fast eating (disuse of musculature) in fast-eating patients while other causes may contribute to the similar weakening in normal-eating patients. The other causes likely include acid reflux since 57% of the patients in this group reported heartburn or acid reflux, whereas only 25% of the fast eating group reported this symptom. The present study cannot distinguish between these two possibilities although it is very striking that all fast eaters showed dramatic weakening of the esophageal muscle. When

Table 2 Effects of Valsalva maneuver on esophageal pressure, expressed as mean (95%CI) in ineffective esophageal motility patients and controls

| | Increase in LES pressure (mmHg) | Increase in distal pressure (mmHg) | Increase in mid pressure (mmHg) | Increase in proximal pressure (mmHg) |
|-------------------------------|---------------------------------|------------------------------------|----------------------------------|--------------------------------------|
| IEM patients (<i>n</i> = 12) | 11.56 (0.57-22.54) | 21.73 (15.46-27.99) | 21.18 (12.28-30.08) ^a | 19.07 (11.41-26.74) ^a |
| Control (<i>n</i> = 10) | 7.81 (-0.86-16.48) | 39.43 (15.37-63.49) | 43.44 (22.85-64.03) | 34.18 (23.41-44.95) |

^a*P* < 0.05 *vs* control. IEM: Ineffective esophageal motility; LES: Lower esophageal sphincter.

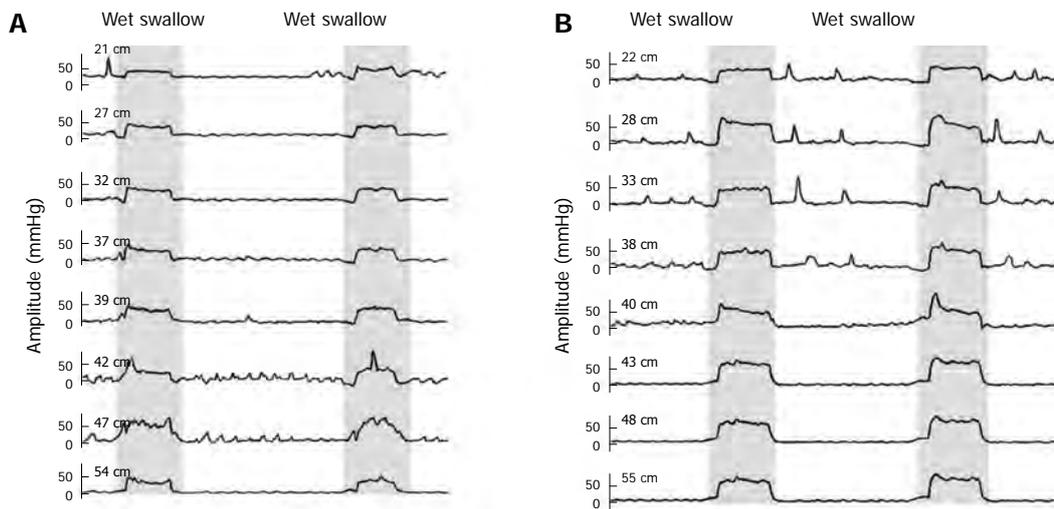


Figure 3 Effects of the Valsalva maneuver on the esophageal manometric tracings in a patient with ineffective esophageal motility (A) and a healthy control (B). The numbers (in cm) on the left indicate the distances from the nose to each side-hole on the catheter.

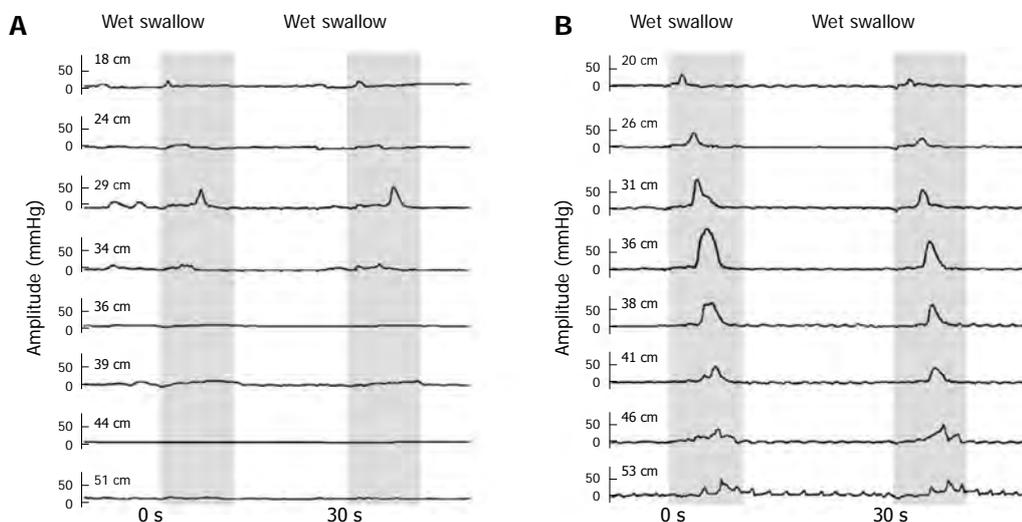


Figure 4 Manometric tracings of a fast-eating non-cardiac chest pain patient diagnosed with ineffective esophageal motility before (A) and after (B) 6-month gum-chewing exercise. The numbers (in cm) on the left indicate the distances from the nose to each side-hole on the catheter.

NCCP patients are evaluated for esophageal dysmotility, only a few are subsequently diagnosed with IEM. The fact that all NCCP patients who self-reported fast eating were diagnosed with IEM suggests but does not prove a causal relationship. The case study suggests, but does not yet prove, that gum-chewing strengthens the esophageal muscle and it is consistent with the hypothesis that the weakening of the musculature was due to non-use of the

musculature because of reduced swallow-induced contractions, although the weakened musculature may have been caused by other factors. In summary, although it is possible that fast eating is associated with weakening of the musculature, the present study does not provide direct evidence for it.

Habitual fast eating associated with rapid swallowing may limit the number of swallow-induced contractions

since only the first or the last bolus are associated with a propulsive contraction. We have observed this phenomenon in this study, which is consistent with previous reports^[8-11]. The contractions may become weaker with time. Another feature of rapid eating is insufficient mastication. Reduced duration of chewing prevents the optimization of the size, the softness and the lubrication of food boluses ready for swallowing^[12]. Vagus nerve activity, which plays a vital role in the regulation of salivation^[13] and esophageal peristalsis^[14] and is enhanced by mastication^[13], may also be less activated by inadequate chewing. To provide further evidence for or against the hypothesis that fast eating contributes to IEM, a prospective study is needed where the meal composition is standardized and the actual timing of swallows is measured.

The case report suggests that gum-chewing may strengthen the esophageal musculature. It would be important to find out if this is true independent of the cause of IEM. In this patient, PPI treatment did not relieve symptoms, and regular daily gum-chewing restored muscle contractile activity. Chewing gum on a regular basis is a stimulus that induces mastication-associated vagal activation^[15] and swallow-associated propulsive contractions.

In the distal and mid esophagus, the contraction amplitudes in the fast-eating IEM patients were significantly reduced. However, their proximal manometric features were not statistically different from controls. This was probably due to the special musculature of the human esophagus, whose upper one-third is composed of striated muscle whereas the lower one-third is made up of smooth muscle and in between both types exist. Peristalsis in the striated muscle portion is induced by the sequential activation of neurons in the ambiguous nucleus which is solely a central mechanism; while in the smooth muscle portion, the peripheral intramural and central mechanisms cooperate to control peristalsis^[14]. Considering the different manometric presentation of the distal and proximal esophagus in IEM, it is probable that a disorder in the peripheral neural control of esophageal smooth muscle contributes to the development of IEM in these patients.

Consensus on a causal relationship between NCCP and IEM has not hitherto been reached. Heartburn, dysphagia and regurgitation, reported by our patients, are possible risk factors for NCCP, in addition to psychological factors such as anxiety and depression^[16] which often haunt our patients and aggravate their symptoms. Hence, NCCP in IEM is a result of many complex interactions and evidence is insufficient to assert that NCCP is caused by IEM. NCCP is often associated with GERD and IEM is the most common form of dysmotility in GERD and is correlated with more GERD episodes and prolonged acid clearance in a posture-dependent manner^[17]. Although 24 h pH monitoring was not carried out, most of our patients did not suffer from GERD and the LES pressure was normal in our patients. Nevertheless, a contribution of gastroesophageal reflux to the symptoms of our IEM patients cannot be excluded.

The habit of rapid eating is a common phenomenon in China and may originate from periods in China when food supply was limited and collective dining was the main form of meal, so to ensure that sufficient food could be secured, many people developed the habit of rapid eating that eventually persisted for years. In addition, certain occupations in China, such as waiters/waitresses in restaurants and sales assistants in shops may not get sufficient free time to eat meals relaxed and hence quick eating may become a habit. We now investigate eating habits routinely in association with IEM and recommend changes in life style and exercise to alleviate their symptoms by strengthening their esophageal musculature.

The Valsalva maneuver increases the intrathoracic^[18] and intra-abdominal pressure and leads to the activation of the diaphragm muscle^[19]. Both the LES musculature and the crural diaphragm can contribute to the increase in LES pressure in response to an increased intra-abdominal pressure although evidence suggests that no active contraction of the smooth muscle is involved^[20,21]. Most of our patients did not show decreased LES, but those who did might benefit from the Valsalva maneuver since it does increase the pressure of the esophageal junction. Previous studies in humans and animals showed that adjusted respiration could increase the pressure around the LES^[22-25]. The effect of the Valsalva maneuver on esophageal muscle contraction is rarely mentioned. Our IEM patients showed a dramatic reduction in the proximal and mid esophageal response to the Valsalva, suggesting a weakened adaptive response of the esophageal musculature, at least the skeletal muscle.

In summary, inquiry into eating behavior is an important part of examination of patients with NCCP. Eating fast increases metabolic risk and should be discouraged. Eating fast may lead to ineffective esophageal motility, but more studies are needed to prove a direct causal relationship.

COMMENTS

Background

Esophageal dysmotility is considered an uncommon cause of non-cardiac chest pain (NCCP), but in our practice it is not infrequent. Previous studies have reported the correlation between eating behaviors and development of diseases, but the role of rapid eating in ineffective esophageal motility (IEM) and related symptoms has not been investigated.

Research frontiers

Both IEM and NCCP are often associated with gastroesophageal reflux disease, but the pathophysiological mechanisms underlying IEM and NCCP are still poorly understood.

Innovations and breakthroughs

This study raises the possibility that rapid eating leads to IEM and attaches importance to inquiry into eating behavior as part of the examination of patients with NCCP.

Applications

Clinicians can take into account rapid eating as a potential cause of IEM, and the test in esophageal function. Further studies are needed to prove a direct causal relationship between rapid food intake and IEM.

Terminology

IEM: IEM is defined manometrically as esophageal body contractions with $\geq 30\%$ of wet swallows at an amplitude < 30 mmHg in the distal esophagus.

Peer review

The concept is interesting, as the next step the authors should approach it prospectively, applying an objective definition of eating patterns rather than self-reporting.

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Esophageal lichen planus: A case report and review of the literature

Jennifer A Nielsen, Robert M Law, Keith H Fiman, Cory A Roberts

Jennifer A Nielsen, Division of Research, ProPath, Dallas, TX 75247, United States

Robert M Law, Division of Dermatopathology, ProPath, Dallas, TX 75247, United States

Keith H Fiman, Gastroenterology Consultants Southwest, LLP, Sugar Land, TX 77478, United States

Cory A Roberts, Division of Gastrointestinal Pathology, ProPath, Dallas, TX 75247, United States

Author contributions: Nielsen JA, Law RM, Fiman KH and Roberts CA contributed to the conception, design and acquisition of data; Law RM analyzed and interpreted the dermatopathology; Fiman KH analyzed and interpreted the endoscopy; Roberts CA analyzed and interpreted the gastrointestinal pathology; Nielsen JA and Roberts CA drafted the article and revised it critically for important intellectual content; Nielsen JA, Law RM, Fiman KH and Roberts CA approved the final version to be published.

Correspondence to: Cory A Roberts, MD, Division of Gastrointestinal Pathology, ProPath, 1355 River Bend Dr., Dallas, TX 75247, United States. cory.roberts@propath.com

Telephone: +1-214-2371641 Fax: +1-214-2371743

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Abstract

Esophageal involvement by lichen planus (ELP), previously thought to be quite rare, is a disease much more common in women and frequently the initial manifestation of mucocutaneous lichen planus (LP). Considering that the symptoms of ELP do not present in a predictable manner, ELP is perhaps more under-recognized than rare. To date, four cases of squamous cell carcinoma in association with ELP have been reported, suggesting that timely and accurate diagnosis of ELP is of importance for appropriate follow-up. In this case report, a 69-year-old female presented with dysphagia and odynophagia. She reported a history of oral LP but had no active oral or skin lesions. Endoscopic examination revealed severe strictures and web-like areas in the esophagus. Histologic examination demonstrated

extensive denudation of the squamous epithelium, scattered intraepithelial lymphocytes, rare eosinophils and dyskeratotic cells. Direct immunofluorescence showed rare cytoid bodies and was used to exclude other primary immunobullous disorders. By using clinical, endoscopic, and histologic data, a broad list of differential diagnoses can be narrowed, and the accurate diagnosis of ELP can be made, which is essential for proper treatment and subsequent follow-up.

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Key words: Esophageal lichen planus; Esophagus; Immunofluorescence; Immunobullous disorders; Diagnostic accuracy

Core tip: Lichen planus is an idiopathic disorder that generally affects middle-aged patients with clinical manifestations in the skin, mucous membranes, genitalia, hair, and nails. It is fairly common as a skin disease, affecting 0.5% to 2% of the population, the mouth being the most common site of involvement. We present one such case, diagnosed using clinical, endoscopic, and histologic data, and distinguished from primary immunobullous disorders by immunofluorescence.

Nielsen JA, Law RM, Fiman KH, Roberts CA. Esophageal lichen planus: A case report and review of the literature. *World J Gastroenterol* 2013; 19(14): 2278-2281 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v19/i14/2278.htm> DOI: <http://dx.doi.org/10.3748/wjg.v19.i14.2278>

INTRODUCTION

Lichen planus (LP) is an idiopathic disorder that generally affects middle-aged patients with clinical manifestations in the skin, mucous membranes, genitalia, hair, and nails^[1]. Proposed etiologies include reaction to medica-

tion, Hepatitis C or other viral infections, bacteria such as *Helicobacter pylori*, or autoimmune processes; however, the exact etiology and pathogenesis are still unknown^[1,2]. It is fairly common as a skin disease, affecting 0.5% to 2% of the population^[3], the mouth being the most common site of involvement^[1]. Conversely, esophageal involvement by LP (ELP) has previously been considered quite rare, with fewer than 50 cases reported in the literature before 2008 and a predilection for women^[4]. In 2010 the Mayo Clinic published a series of 27 cases within a 10-year period, suggesting that it is perhaps more under-recognized than rare and often the initial manifestation of mucocutaneous LP^[1]. Subsequently, there is often a significant delay between onset of symptoms, dysphagia being the most common, and diagnosis^[3]. Considering that four cases of squamous cell carcinoma (SCC) in association with ELP have been confirmed to date^[5-7], the seriousness of this diagnostic delay should move physicians to take greater precautions to rule out ELP. We present one such case, diagnosed using clinical, endoscopic, and histologic data, and distinguished from primary immunobullous disorders by immunofluorescence.

CASE REPORT

A 69-year-old female presented with dysphagia and odynophagia that had been ongoing for years. She reported a history of oral LP, but had no active oral or skin lesions, and a previously normal upper gastrointestinal series X-ray. The patient initially declined endoscopy and took proton-pump inhibitors without benefit. Later endoscopic examination revealed severe strictures and rings throughout the length of the esophagus with web-like areas; however, the gastroesophageal junction was spared and appeared essentially normal. The mucosa showed severe, diffuse sloughing with passage of the endoscope (Figure 1). Esophageal biopsy was obtained for routine histology and submitted in 10% buffered formalin. Esophageal dilation was not performed.

Histologically, the esophageal tissue demonstrated extensive denudation of the surface epithelium. The mucosa was detached from the subepithelial tissue in several areas without preservation of the basal layer (Figure 2A). Where attached, the squamous (esophageal) epithelium was somewhat atrophic with diffuse spongiotic change, scattered intraepithelial lymphocytes, rare eosinophils, and dyskeratotic cells (Civatte bodies) (Figure 2B, black circle). The subepithelial tissue showed edema and a diffuse lichenoid infiltrate including lymphocytes, eosinophils, and occasional mast cells (Figure 2C). There was no evidence of *Candida* by virtue of a negative alcian blue/periodic acid-Schiff stain. The absence of significant intraepithelial acute inflammation and/or viral cytopathic effect in conjunction with the lichenoid infiltrate and Civatte bodies excluded a viral infection. However, while a definitive diagnosis of LP could not be made on routine histology alone, it was suggested. The patient was promptly re-biopsied a month later from the middle and upper esophagus. The biopsies were submitted in Zeus



Figure 1 Endoscopy showing webs.

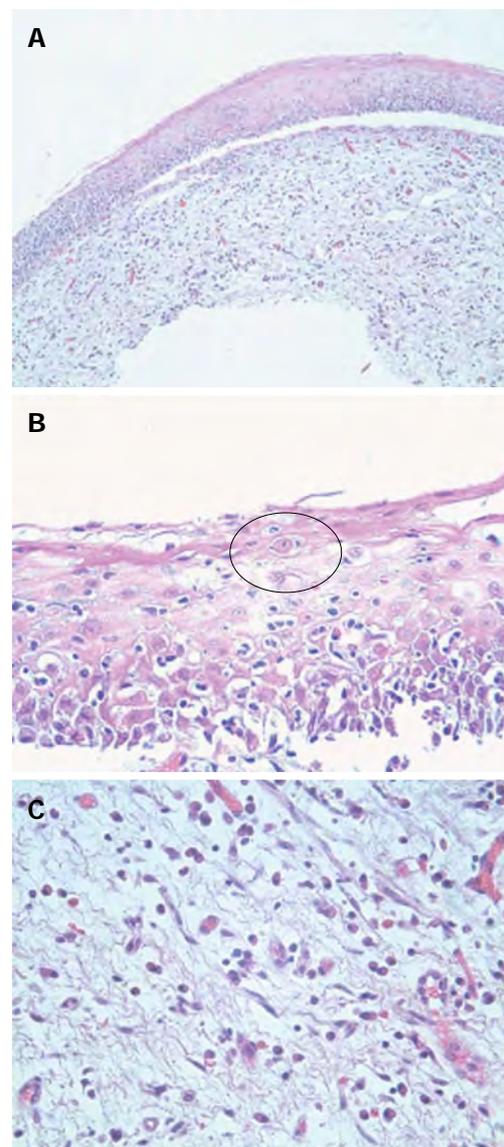


Figure 2 Histologically, the esophageal tissue demonstrated extensive denudation of the surface epithelium. A: Subepithelial separation, HE stain, $\times 100$; B: Civatte bodies (black circle), HE stain, $\times 400$; C: Subepithelial edema and inflammation, HE stain, $\times 400$.

transport media for immunofluorescence.

Direct immunofluorescence revealed fibrillar deposi-

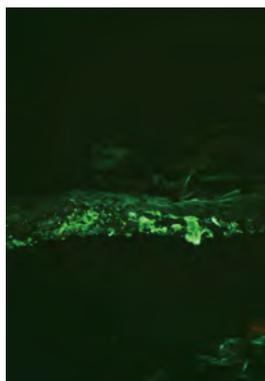


Figure 3 Immunofluorescence, fibrinogen.

tion of fibrinogen along the basement membrane zone (Figure 3), characteristic of but not specific for LP. IgG, IgA, IgM, and C3 showed rare cytooid bodies in the same areas without any evidence of a primary immunobullous disorder such as pemphigus or pemphigoid. The basic histomorphology in conjunction with the clinical history of oral lichen planus and the negative immunofluorescence excluded immunobullous disorders, such as esophageal pemphigus vulgaris.

DISCUSSION

First described by Al-Shihabi *et al*^[8] and Lefers^[9] simultaneously but separately, over 80 cases of ELP have been reported in both English and foreign-language literature to date, only 8 of which are male^[3]. Multiple retrospective studies have shown that ELP is under-recognized^[1,3,10], since the esophageal symptoms can present before, concurrently, or develop after the diagnosis of extra-ELP^[1,3]. In his review of 79 patients that developed ELP, Fox noted that 14 patients developed ELP as the first and only manifestation of LP. Oral LP has been long known to predispose 2%-3% of cases to the development of oral SCC^[10]; however, with documentation of 4 cases of ELP progressing to esophageal SCC, early diagnosis and accurate therapy for ELP patients has become a more serious issue^[2]. One of these esophageal SCC cases was reported in a series of 8 patients, the mean delay between symptom onset and diagnosis of which was 27 mo^[6]. Additionally, Katzka *et al*^[11] found in his review of 27 patients with ELP that this delay in diagnosis not only resulted in increased length of time with symptoms (range: 0.33-30 years, mean: 4.72 years) but also increased the number of failed treatments before diagnosis (range: 0-15, mean: 2.5), including prior dilatations, medications such as proton-pump inhibitors, and fundoplication.

Because the symptoms of ELP are not distinctive, many clinicians recommend physicians maintain a low threshold for performing endoscopies to rule out ELP in patients experiencing dysphagia with a history of mucocutaneous LP^[3,10]. Esophageal sloughing and refractory strictures in a middle-aged or older female even in the absence of extra-ELP should raise ELP as a diagnostic

consideration, as less than half of those with mucosal LP will exhibit concomitant skin lesions^[2,10]. Additionally, easy peeling of the esophageal mucosa with minimal contact and formation of “tissue paper-like membranes” is a frequently observed characteristic^[11]. Suspecting a more common culprit such as gastroesophageal reflux disease (GERD), endoscopists oftentimes focus on the lower esophagus and could potentially miss proximal lesions caused by ELP^[3]. In general, GERD can be distinguished from ELP by the sparing of the gastroesophageal junction in ELP^[3]. In a study using magnification chromoendoscopy on 24 consenting patients with cutaneous and/or oral LP, the University Medical Center Utrecht (Netherlands) found that 5 (21%) had ELP, 5 (21%) had GERD, and 7 (29%) had both, with no differences in symptoms amongst the groups^[10]. Early diagnosis may be improved by new diagnostic modalities such as chromoendoscopy or magnification endoscopy^[2].

The final diagnosis can be reached by combining the historic, endoscopic, and histologic data; whereas the routine light microscopy, while unusual, is not pathognomonic for ELP. The most indicative characteristics of ELP are a lymphohistiocytic interface inflammatory infiltrate and dyskeratotic cells (Civatte bodies)^[10]. Other common disorders that affect both esophagus and skin are bullous disorders, such as pemphigus vulgaris, paraneoplastic pemphigus, epidermolysis bullosa aquistia, mucous membrane pemphigoid, bullous pemphigoid, and Hailey-Hailey disease^[3]. The lack of specific immunofluorescent staining in conjunction with the subepithelial as opposed to suprabasal separation and history of oral LP clearly excluded the bullous disorders in this case^[3,11]. While pityriasis lichenoides chronica (PLC) shows similar histology in cutaneous biopsies, there are no published reports of PLC occurring on mucosal surfaces such as the esophagus^[12]. Even more importantly, the patient had no cutaneous lesions or history to support this diagnosis. A viral cause was excluded due to the lack of erosion/ulceration, viral cytopathic effect, and acute inflammation. Further, Civatte bodies are not typically seen in viral infections. More remote possibilities, such as graft-versus-host disease (GVHD) and toxin-associated damage, are characterized by apoptosis, which is absent in this case. Furthermore, Civatte bodies, while characteristic of ELP, are not seen in GVHD or toxic-injury, such as caused by the drug mycophenolate which essentially mimics GVHD. Finally, historical data showing a lack of transplant history or mycophenolate use serves to remove GVHD and/or mycophenolate from a diagnostic consideration here^[12]. By taking into consideration the various diagnostic methods and suggestions, other possible diagnoses can be ruled out, and the diagnostic delay of ELP can be decreased, which is essential for appropriate treatment and clinical follow-up, potentially preventing more serious sequelae, including SCC^[2].

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Sarcina ventriculi of the stomach: A case report

Shiva K Ratuapli, Dora M Lam-Himlin, Russell I Heigh

Shiva K Ratuapli, Russell I Heigh, Division of Gastroenterology and Hepatology, Mayo Clinic Arizona, Scottsdale, AZ 85259, United States

Dora M Lam-Himlin, Department of Pathology, Mayo Clinic Arizona, Scottsdale, AZ 85259, United States

Author contributions: Ratuapli SK performed literature search and drafted the manuscript; Heigh RI saw the patient, conceived the idea and critically revised the manuscript; Lam-Himlin DM reviewed stomach biopsies and critically revised the manuscript.

Correspondence to: Russell I Heigh, MD, Division of Gastroenterology and Hepatology, Mayo Clinic Arizona, 13400 East Shea Blvd, Scottsdale, AZ 85259,

United States. heigh.russell@mayo.edu

Telephone: +1-480-3016737 Fax: +1-480-3016990

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Key words: *Sarcina ventriculi*; Gram negative; Emphysematous gastritis; Gastric perforation; Bacterial overgrowth

Core tip: *Sarcina ventriculi* is a rare bacterium, seen in gastric biopsies of patients with gastroparesis. Only eight cases have been reported so far, where in it has been implicated in the development of gastric ulcers, emphysematous gastritis and gastric perforation. In our case, gastric erythema improved with antibiotic treatment. Given its association with life threatening illness in two reported cases, it may be prudent to treat with antibiotics and anti-ulcer therapy, until further understanding is achieved.

Abstract

Sarcina ventriculi is a Gram positive organism, which has been reported to be found rarely, in the gastric specimens of patients with gastroparesis. Only eight cases of *Sarcina*, isolated from gastric specimens have been reported so far. *Sarcina* has been implicated in the development of gastric ulcers, emphysematous gastritis and gastric perforation. We report a case of 73-year-old male, with history of prior Billroth II surgery and truncal vagotomy, who presented for further evaluation of iron deficiency anemia. An upper endoscopy revealed diffuse gastric erythema, along with retained food. Biopsies revealed marked inflammation with ulcer bed formation and presence of *Sarcina* organisms. The patient was treated with ciprofloxacin and metronidazole for 1 wk, and a repeat endoscopy showed improvement of erythema, along with clearance of *Sarcina* organisms. Review of reported cases including ours suggests that *Sarcina* is more frequently an innocent bystander rather than a pathogenic organism. However, given its association with life threatening illness in two reported cases, it may be prudent to treat with antibiotics and anti-ulcer therapy, until further understanding is achieved.

Ratuapli SK, Lam-Himlin DM, Heigh RI. *Sarcina ventriculi* of the stomach: A case report. *World J Gastroenterol* 2013; 19(14): 2282-2285 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v19/i14/2282.htm> DOI: <http://dx.doi.org/10.3748/wjg.v19.i14.2282>

INTRODUCTION

Sarcina ventriculi is a Gram positive anaerobic bacterium, with carbohydrate fermentative metabolism as its sole energy source^[1], and is able survive in very low pH environment^[2]. Even though it is similar in appearance to *Micrococcus* species, certain morphological features (*i.e.*, larger size, non-cluster forming pattern) help differentiate it from the latter organism^[3].

Various reports in veterinary literature have implicated *Sarcina* in the development of gastric dilatation^[4] and death of livestock, cats and horses^[5,6]. *Sarcina* has also been reported to be found in feces of healthy humans consuming a predominantly vegetarian diet^[7]. Recently, several reports have shown an association between *Sarcina* in the stomach and chronic nausea, dyspepsia, abdominal pain, gastric ulcers^[3], and rarely emphysematous gastritis^[8] and

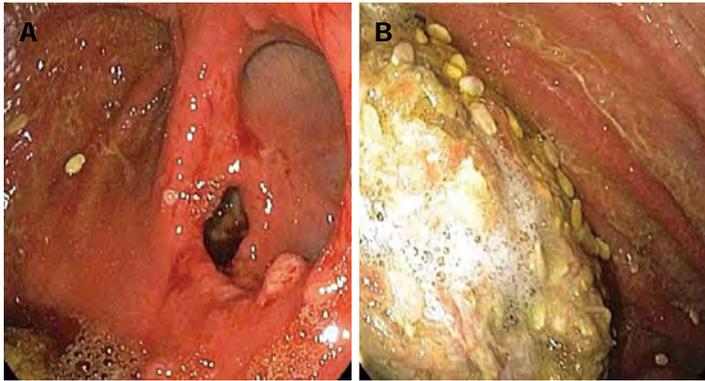


Figure 1 Esophagogastroduodenoscopy. A: Polyps at the anastomosis; B: Gastric erythema and food bezoar.

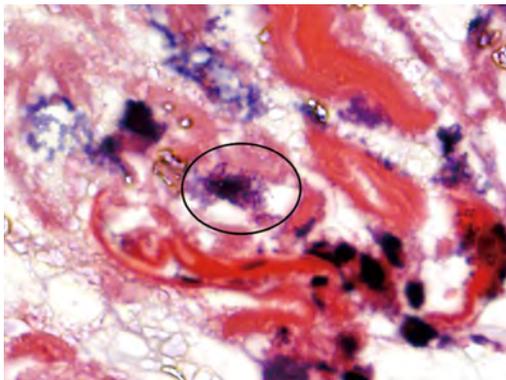


Figure 2 Characteristic 8-10 micron tetrads of *Sarcina* organisms (circled) were identified on endoscopic biopsy. The background shows abundant bacterial overgrowth and debris from retained food. Separate fragments of ulcer bed were present (not pictured) (hematoxylin and eosin; original magnification $\times 1000$ oil lens).



Figure 3 Repeat esophagogastroduodenoscopy showing improvement of gastric erythema.

gastric perforation^[9]. However *Sarcina* has also been found in gastric specimens without any other pathologic changes^[5], suggesting that it may be a bystander rather than a pathogenic organism. To date, only eight cases of *Sarcina ventriculi* isolated from gastric biopsy specimens have been reported. We now report a case of *Sarcina ventriculi* of the stomach, associated with iron deficiency anemia and gastroparesis.

CASE REPORT

A 73-year-old male presented to the clinic for further evaluation of iron deficiency anemia. The patient had a history of medically refractory gastric ulcers in his 20 s, for which he underwent antrectomy and gastrojejunostomy (Billroth II) along with truncal vagotomy in 1985. He continued to be anemic since the surgery, with intermittent intake of oral iron replacement.

On initial evaluation for an incidentally detected anemia prior to unrelated urologic surgery, he did not have any gastrointestinal symptoms. The patient specifically denied nausea, vomiting, abdominal pain or weight loss. His complete blood count revealed decreased hemoglobin of 8.5 g/dL, decreased mean corpuscular volume of 63.2 fL, normal white cell count of 9.8×10^9 L (normal 4.2×10^9 - 10.2×10^9 L) and elevated platelet count 415×10^9 L (normal 151×10^9 - 355×10^9 L). Iron studies showed

markedly reduced iron level of 12 mg/dL (normal 50-150 mg/dL), with an elevated total iron binding capacity of 490 mg/dL (normal 250-400 mg/dL) and reduced iron % saturation of 2% (normal 14%-50%).

Three years prior, an esophagogastroduodenoscopy (EGD) had revealed an anastomotic ulcer and polyps at the anastomotic site, the biopsies of which showed acute inflammation, but were otherwise unremarkable. A colonoscopy at that time was unremarkable except for diverticulosis. An EGD done during the current evaluation demonstrated diffuse gastric erythema, along with two 4mm polyps at the anastomosis (Figure 1). There was also a large amount of retained food in the stomach.

Tissue biopsies of the erythematous stomach revealed marked inflammation with ulcer bed formation, along with abundant bacterial overgrowth including the presence of *Sarcina* organisms (Figure 2). The *Sarcina* organisms were identified on routine hematoxylin and eosin (HE) stain, and no additional special stains or immunolabeling was performed. Based on prior studies^[3], the tetrad morphology and size are characteristic enough to establish a diagnosis without further ancillary testing. Biopsies were negative for *Helicobacter pylori*, both by routine HE staining and by immunohistochemical staining. Aspirates from the small bowel also came back positive for small intestinal bacterial overgrowth, with $> 100\,000$ cfu/mL of mixed Gram-positive and Gram-negative flora.

The patient was treated with metronidazole 250 mg three times a day and ciprofloxacin 250 mg twice daily

Table 1 Clinical, endoscopic and histological features of the eight reported cases of *Sarcina ventriculi* in the literature

| Case No. | Age | Sex | Symptoms/clinical findings | Endoscopic findings | Histologic findings | Treatment | Follow-up |
|----------|-----|--------|--|--|---|--|---|
| 1 | 14 | Male | Abdominal pain CT showed pneumoperitoneum. Intraoperatively there was necrotic stomach and gastric perforation and peritonitis | Not performed | Diffuse acute hemorrhagic gastritis and <i>Sarcina</i> organisms | Gentamicin and metronidazole | Symptoms improved after 5 d and patient discharged |
| 2 | 50 | Male | Chronic nausea, vomiting | Esophagitis, duodenal lesion | Chronic superficial gastritis and ulcer with <i>Sarcina</i> organisms | Not available | Not available |
| 3 | 3 | Female | Vomiting, hematemesis X-ray showed dilated stomach with intramural air | Gastric inflammation, blackening of mucosa, cobblestone appearance | Polymorphic inflammatory infiltrate with <i>Sarcina</i> organisms and gas bubbles | Imipene, fluconazole and omeprazole | Repeat endoscopy 6 mo later showed complete normalization |
| 4 | 58 | Female | Nausea and vomiting | Gastritis, food bezoar, inflammatory mass in duodenum | Active chronic gastritis with <i>Sarcina</i> organisms | Partial gastrectomy for obstruction | Treated for adenocarcinoma of pylorus |
| 5 | 44 | Female | Dyspepsia and substernal burning | Gastric ulcer and retained food | Non malignant gastric ulcer with <i>Sarcina</i> organisms | Omeprazole, ranitidine, metoclopramide | Symptoms improved |
| 6 | 36 | Male | Nausea, vomiting, epigastric pain in the setting of narcotic use | Retained food | <i>Sarcina</i> organisms without other histologic abnormalities | Received jejunostomy for malnutrition | Repeat biopsy negative for <i>Sarcina</i> organisms |
| 7 | 12 | Female | Dysphagia in the setting of esophageal atresia status post gastric pull through | Retained food, anastomotic stricture | Reflux esophagitis, <i>Sarcina</i> organisms | Information unavailable | Information unavailable |
| 8 | 46 | Female | Epigastric pain in the setting of pancreatic adenocarcinoma status post pancreatico-duodenectomy | Retained food and bile | Active chronic duodenitis with <i>Sarcina</i> organisms | No treatment | Continues spasms after 1 mo |

CT: Computed tomography.

for 1 wk, along with daily sucralfate. He also received intravenous (IV) iron 300 mg × 2 doses followed by oral iron and achieved normal iron stores and hemoglobin levels. Subsequent follow up with a repeat EGD 3 mo later showed improvement of gastric erythema, and absence of food bezoar (Figure 3). Aspirates from the small bowel continued to suggest small intestinal bacterial overgrowth, with > 100 000 cfu/mL. However, repeat biopsies from the stomach were negative for *Sarcina* organisms, and showed features of chronic gastritis. Clinically, the patient's perception of overall health improved with the above treatment, and he continued to be free of gastrointestinal symptoms.

DISCUSSION

While the pathogenic role of *Sarcina* in the veterinary literature is well established, its role in human disease is not entirely clear. Since the initial description in 1842, the pathogenic role in humans has been questioned, as it has been found in the blood^[10] and feces^[7] of healthy humans.

Over the last three years, 8 cases^[3,8,9] of *Sarcina* associated with endoscopic biopsies have been reported. While all these patients presented with various gastrointestinal symptoms (nausea, vomiting, epigastric pain, dyspepsia) only two patients had associated life threatening complications of emphysematous gastritis^[8] and gastric perfora-

tion^[9] (Table 1). Our patient did not have any gastrointestinal symptoms, and *Sarcina* was found incidentally, when gastric biopsies were performed for erythematous mucosa.

Another interesting feature is the presence of delayed gastric emptying in five of the eight reported cases. All of these patients had retained food in stomach during endoscopic examination. Similarly, our patient had a Bill-roth II with truncal vagotomy, which predisposed him to have delayed gastric emptying, as was evident by the gastric bezoar seen during endoscopic examination. Hence impaired emptying of stomach could potentially lead to the growth of *Sarcina* in the stomach.

The need for antibiotic treatment, when *Sarcina* is found in endoscopic biopsies of clinically stable patients is unknown. Of the reported cases, two patients with associated life threatening disease (*i.e.*, emphysematous gastritis and gastric perforation) received intravenous antibiotics and recovered. One patient with non-life threatening disease was treated with combination of proton pump inhibitors and prokinetics, with good relief of symptoms. Some authors suggest that an underlying mucosal defect, such as erosion or ulceration, may predispose patients to more serious sequelae, from this otherwise ubiquitous organism^[3]. We elected to treat our patient with antibiotics, as there was significant gastric erythema and ulceration, as well as small intestinal bacterial overgrowth.

In summary, *Sarcina ventriculi* is a rare bacterium, seen

predominantly in patients with delayed gastric emptying. Review of the published cases along with our case suggests that it is more frequently an innocent bystander rather than a pathogenic organism. Given its association with life threatening illness in two reported cases, it may be prudent to treat with antibiotics and anti-ulcer therapy, until further understanding is achieved.

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E- Editor Zhang DN



Aggressive juvenile polyposis in children with chromosome 10q23 deletion

Seth Septer, Lei Zhang, Caitlin E Lawson, Jose Cocjin, Thomas Attard, Holly H Ardinger

Seth Septer, Jose Cocjin, Thomas Attard, Department of Gastroenterology and Hepatology, Children's Mercy Hospital and Clinics, Kansas City, MO 64108, United States

Lei Zhang, Division of Cytogenetics, Department of Pathology, Children's Mercy Hospital and Clinics, Kansas City, MO 64108, United States

Caitlin E Lawson, Holly H Ardinger, Division of Clinical Genetics, Department of Pediatrics, Children's Mercy Hospitals and Clinics and University of Missouri, Kansas City School of Medicine, Kansas City, MO 64108, United States

Author contributions: Septer S, Cocjin J, Attard T and Ardinger HH designed the research; Septer S, Zhang L, Lawson CE, Attard T and Ardinger HH wrote the paper.

Correspondence to: Dr. Seth Septer, Department of Gastroenterology and Hepatology, Children's Mercy Hospital and Clinics, 2401 Gillham Road, Kansas City, MO 64108,

United States. ssepter@cmh.edu

Telephone: +1-816-2343016 Fax: +1-816-2341553

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Abstract

Juvenile polyps are relatively common findings in children, while juvenile polyposis syndrome (JPS) is a rare hereditary syndrome entailing an increased risk of colorectal cancer. Mutations in *BMPR1A* or *SMAD4* are found in roughly half of patients diagnosed with JPS. Mutations in *PTEN* gene are also found in patients with juvenile polyps and in Bannayan-Riley-Ruvalcaba syndrome and Cowden syndrome. Several previous reports have described microdeletions in chromosome 10q23 encompassing both *PTEN* and *BMPR1A* causing aggressive polyposis and malignancy in childhood. These reports have also described extra-intestinal findings in most cases including cardiac anomalies, developmental delay and macrocephaly. In this report we describe a boy with a 5.75 Mb deletion of chromosome 10q23 and a 1.03 Mb deletion within chromosome band 1p31.3

who displayed aggressive juvenile polyposis and multiple extra-intestinal anomalies including macrocephaly, developmental delay, short stature, hypothyroidism, atrial septal defect, ventricular septal defect and hypospadias. He required colectomy at six years of age, and early colectomy was a common outcome in other children with similar deletions. Due to the aggressive polyposis and reports of dysplasia and even malignancy at a young age, we propose aggressive gastrointestinal surveillance in children with 10q23 microdeletions encompassing the *BMPR1A* and *PTEN* genes to include both the upper and lower gastrointestinal tracts, and also include a flowchart for an effective genetic testing strategy in children with juvenile polyposis.

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Key words: Polyposis; Genetics; Cancer; Endoscopy pediatric

Core tip: Children with aggressive juvenile polyposis related to microdeletions of chromosome 10q23 and involving *PTEN* and *BMPR1A* are rare, however this deletion conveys significant gastrointestinal and extraintestinal risks. Children with this gene mutation are at significant risk for extensive polyposis, early colectomy and gastrointestinal malignancy. This work describes the clinical manifestations associated with these deletions. We also suggest genetic testing strategies for those with juvenile polyps and also propose gastrointestinal surveillance for patients with chromosome 10q23 deletions encompassing *PTEN* and *BMPR1A*.

Septer S, Zhang L, Lawson CE, Cocjin J, Attard T, Ardinger HH. Aggressive juvenile polyposis in children with chromosome 10q23 deletion. *World J Gastroenterol* 2013; 19(14): 2286-2292 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v19/i14/2286.htm> DOI: <http://dx.doi.org/10.3748/wjg.v19.i14.2286>

INTRODUCTION

Classification of polyps in children and subsequent attempts to diagnose hereditary polyposis syndromes begin with histologic sub-typing. The juvenile polyp was first described by Diamond^[1] and is characterized histologically by an edematous lamina propria with inflammatory cells and cystically dilated glands which are lined by cuboidal to columnar epithelium^[2]. While sporadic juvenile polyps are fairly common in the first decade of life and may be found in up to 2% of the pediatric population^[3,4], juvenile polyposis syndrome (JPS) is a rare hereditary polyposis syndrome occurring in 1:100 000^[5] and entails an increased risk of colorectal cancer and to a lesser degree gastric cancer. Juvenile polyposis syndrome is defined (Jass Criteria) by the presence of five or more juvenile polyps in the colorectum, any number of juvenile polyps proximal to the colorectum or any number of juvenile polyps with a positive family history of juvenile polyposis^[3,4,6]. JPS typically presents in adolescence or adulthood. Treatment consists of surveillance endoscopy with polypectomy. Endoscopy is typically performed on a regular basis after polyps are found. Prophylactic surgery is indicated if polyp burden is unmanageable endoscopically, when juvenile polyps display dysplasia or in the case of severe gastrointestinal bleeding. A severe form of JPS called juvenile polyposis of infancy (JPI) has also been described and is characterized by its early manifestations of generalized polyposis, diarrhea, gastrointestinal bleeding and protein losing enteropathy in the first two years of life resulting in death in infancy in some patients^[7].

Three genes have been associated with juvenile polyps. In 45%-60% of patients with typical JPS a mutation can be found in either the *SMAD4* or *BMPR1A* genes^[8-12]. *SMAD4* is a tumor suppressor gene located on chromosome 18q21 and is associated with hereditary hemorrhagic telangiectasia in some individuals in addition to JPS. *BMPR1A* is located on chromosome 10q22-23^[12] and encodes for a receptor important in the BMP/growth factor signaling pathways. Additionally, a third gene, *PTEN*, also located on chromosome 10q22-23, has been associated with juvenile polyps, in association with Cowden syndrome, a familial cancer syndrome, and the lesser known Bannayan-Riley-Ruvalcaba syndrome^[13] associated with macrocephaly, developmental delay and some minor dysmorphia. These conditions, now grouped together as the “*PTEN*-hamartoma syndrome” (PHTS)^[14], may present with juvenile or hamartomatous polyps. In addition to an increased risk for breast, thyroid, colorectal and endometrial cancers in adulthood, skin lesions such as lipomas, trichelomomas and papillomatous lesions, and penile macules are common findings.

Of considerable interest are a small group of patients who have been reported to have a chromosome 10q23 deletion involving both the *BMPR1A* and *PTEN* genes and also developed juvenile polyposis. Less than twenty patients have been described with these mutations^[8,15-26]. Many of these patients were originally tested by chromo-

some analysis or more recently by chromosome microarray due to congenital anomalies, macrocephaly and/or developmental delay. Many of them also developed aggressive juvenile polyposis and in some cases required colectomy. The most common physical finding in these children was macrocephaly and the majority also had developmental delay. Other findings seen in multiple patients were atrial septal defect and/or ventricular septal defect, hemangioma, club foot, hypotonia and speckling of the penis.

Herein, we describe a patient who presented with a microdeletion of chromosome 10q23 which resulted in the deletion of both *BMPR1A* and *PTEN* genes and an additional microdeletion involving chromosome 1p31.3 of uncertain significance. His polyposis history is compared to that of others with similar 10q23 deletions and contrasted with those with mutations in either *BMPR1A*, *PTEN* or *SMAD4* alone. We have developed an algorithm for genetic testing for patients presenting with juvenile polyposis since those with microdeletions are subject to significant health risks, including malignancy. We will also focus on the optimal long term gastrointestinal surveillance for these patients.

CASE REPORT

The patient was delivered at 37 wk gestation by Caesarean delivery due to macrocephaly and weighed 9 lbs, 12 oz. Head circumference at birth was 38.7 cm (90th percentile), and at 11 mo of age he was significantly macrocephalic (+4 SD). At a few days of age, a heart murmur was noted and echocardiogram revealed a large ventricular septal defect (VSD), atrial septal defects (ASD) and several smaller VSDs. Repair was performed at 10 d of age. His postoperative course was complicated by ectopic atrial tachycardia which required short term amiodarone therapy. Tracheostomy was performed at 11 mo of age due to multiple episodes of respiratory distress, multiple pneumonias and a diagnosis of tracheobronchomalacia. Other phenotypic characteristics in this child were hypospadias requiring repair, sagittal craniosynostosis requiring surgical correction, exotropia, midface hypoplasia with large cheeks, a prominent Cupid's bow of the upper lip, and deep palmar creases. Additionally, his medical history included adenoidal hypertrophy necessitating adenoidectomy and fundoplication for medically refractory gastroesophageal reflux exacerbating respiratory compromise. Developmental delay was also present with delayed speech and gross motor delays. Endocrine issues included short stature (< 3rd percentile), obesity (body mass index, BMI > 97 kg/m²), growth hormone deficiency and primary hypothyroidism. The patient was treated with growth hormone and *L*-thyroxine and his height eventually reached the 10th percentile.

Due to the multiple anomalies found in this patient, genetic consultation had been obtained at 4 years of age. He was reported to have had a normal 46, XY karyotype on previous testing. Microarray comparative genomic

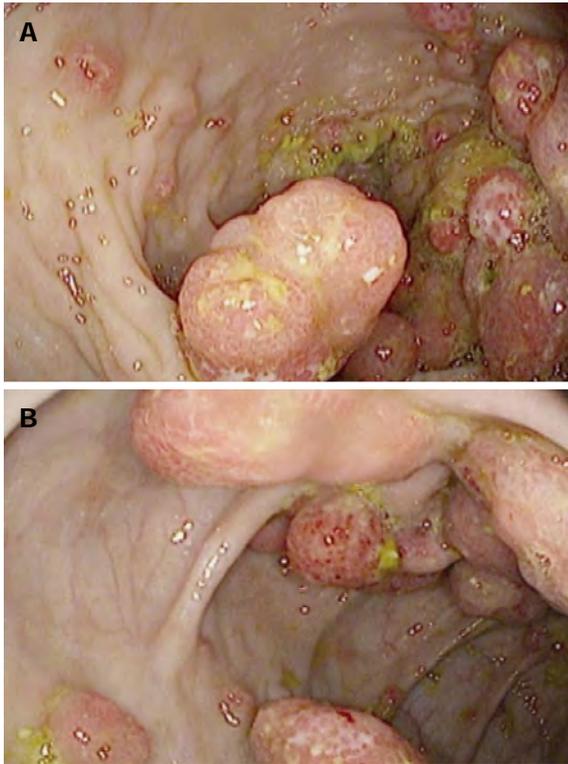


Figure 1 Endoscopic view. A: Polyps noted during colonoscopy, prior to colectomy; B: Endoscopic view of colonic polyps in the patient described.

hybridization (aCGH) analysis was performed (Agilent 244k platform) and two genomic deletions were found in this patient. One is a 1.03 Mb deletion within chromosome band 1p31.3 involving seven annotated genes and transcripts: *CACHD1*, *RAVER2*, *JAK1*, *AK3L1*, *DNAJC6*, *LEPR*, *LEPROT* [chr1:64870449-65897852 (hg18)]. The other one is a 5.75 Mb deletion of chromosome 10q23.1q23.31 involving 26 annotated genes and transcripts including *BMPR1A* and *PTEN* [chr10:84311235-90064565 (hg18)]. Parental analyses of these two deletions showed normal results indicating these deletions are *de novo* in origin.

At 4 years of age the patient was seen in our Pediatric Gastroenterology Clinic for consultation due to the concern regarding polyposis with the 10q23 deletion and also for his symptoms of abdominal distension of uncertain etiology. At age 5 years he underwent esophagogastroduodenoscopy (EGD) and colonoscopy with significant findings of five small (4-5 mm) duodenal polyps and approximately 30 polyps in the colon, from rectum to cecum. Histopathology revealed juvenile polyps in all cases, without any adenomatous transformation. Growth hormone was stopped at this point due to a concern for increased polyp growth.

Four months later blood was noted in the stool and repeat endoscopy was performed. The polyp burden had increased to approximately 50 small polyps (4-6 mm) in the duodenum and 75-100 polyps in the colon. The majority of these colonic polyps were less than 6 mm, however there were five to six larger polyps 1-2 cm in

size. Histology of all polyps was consistent with juvenile polyps.

A third endoscopy was performed six months later and again 50 polyps were noted in the duodenum, with several of them increased in size to 8 mm. Colonoscopy revealed 50-100 polyps from sigmoid to cecum (Figure 1). Approximately half of these polyps were now > 1.5 cm with several larger than 3 cm in diameter. Subsequently, as a result of the polyp burden which precluded endoscopic removal, the child was referred for laparoscopic subtotal colectomy with ileorectal anastomosis. The resected colon contained greater than 50 polyps, ranging in size from 0.6-3.1 cm in diameter. The polyps were juvenile in all cases and there was no dysplasia found. Post-operatively the patient struggled with frequent stooling and skin breakdown but is improved with use of fiber and loperamide.

DISCUSSION

The 10q23 deletion encompassing both *PTEN* and *BMPR1A* is rare, but conveys significant multisystem health problems and is known to have a variable phenotype with many individuals harboring juvenile polyps. Some individuals with this deletion fit the description of JPI with aggressive and early onset gastrointestinal polyposis. Our patient did not meet the criteria for JPI, as there was not diarrhea, bloody stools or hypoalbuminemia in the first two years of life. However, he did have extensive juvenile polyposis at a young age which led to colectomy. This aggressive gastrointestinal phenotype is not expected in generalized JPS, which is typically diagnosed in adolescence or adulthood. Additional clinical features in our patient included cardiac defects, macrocephaly, developmental delay, tracheobronchomalacia necessitating tracheostomy, medically refractory gastroesophageal reflux requiring fundoplication, thyroid and growth hormone deficiency and hypospadias. Whether these additional features represent the variability of the 10q23 deletion syndrome or whether they are associated with the additional 1p31.3 deletion is unknown at this time.

This is the first report of co-existing deletions of 10q23 and 1p31.3. The 1.03 Mb deletion of 1p31.3 has not been reported before. There are no known benign copy number variants in the region (<http://projects.tcag.ca/variation/>). Petti *et al*²⁷¹ reported a 15-year-old boy who carried a heterozygous 3.2 Mb deletion, which covers and extends beyond the deletion in our patient and had obesity, behavioral problems, mild intellectual impairment and facial dysmorphism. Vauthier *et al*²⁸¹ reported a three-year-old boy with an 80 kb homozygous deletion of 1p31.3 which included part of *DNAJC6* and *LEPR* genes. This patient showed early onset obesity, mild dysmorphic features, intellectual disability, and epilepsy. Eight additional family members were heterozygous for the 80 kb 1p31.3 deletion. Seven of the eight were either overweight or obese and none had intellectual impairment. Our current patient has a heterozygous deletion

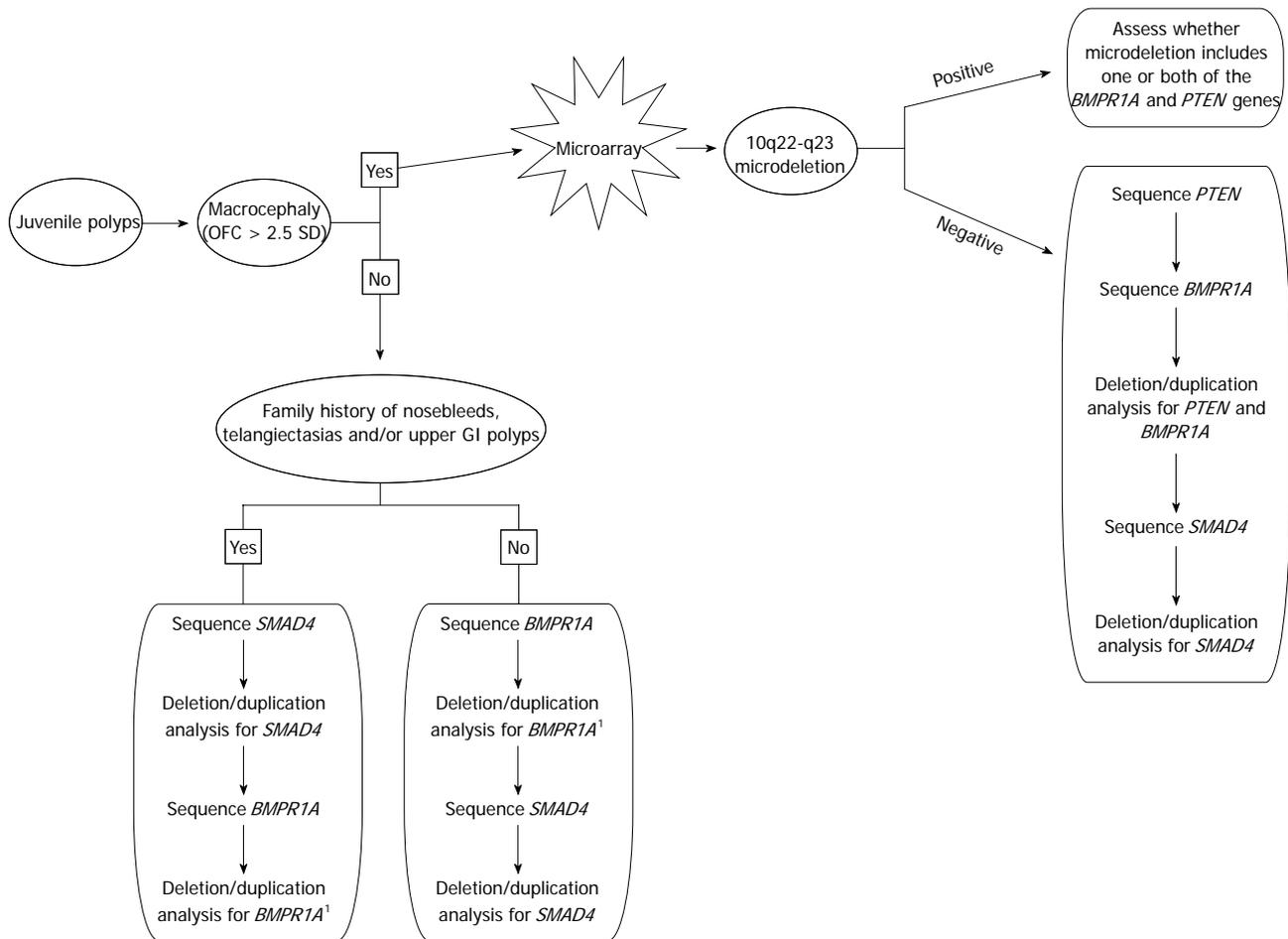


Figure 2 Algorithm for genetic testing and diagnosis of individuals with juvenile polyyps. ¹If a deletion in *BMPR1A* is found, ask the testing laboratory if a microarray is indicated based on the location of the deletion. OFC: Occipital-frontal circumference. GI: Gastrointestinal.

of the *DANJC6* and *LEPR* genes. Since age 2 years, his weight has tracked above the 75th percentile and height at or below the 10th percentile with a BMI at the 99th percentile for age which may reflect the effect of the deleted *LEPR* gene as high BMI is not typically associated with the 10q23 deletion phenotype. Heterozygous loss of the *DNAJC6* gene in the current patient is of unknown significance. A literature review did not reveal any significant clinical associations of other genes deleted in the region within 1p31.3. Therefore, it is unclear how the 1p31.3 deletion may have impacted the phenotype of 10q23 deletion in our patient other than contributing to his elevated BMI.

This patient's most significant medical issues, including polyyps and subsequent colectomy, pertain to his chromosome 10q23 deletion and its disruption of the function of *BMPR1A* and *PTEN*. *PTEN* is an important tumor suppressor gene. Mutations (including sequence changes or deletions) of the *PTEN* gene are associated with PHTS as previously described. Both hamartomatous and juvenile polyyps are seen in PHTS. Sequence changes or partial deletions of the *BMPR1A* gene result in loss-of-function of that gene and typically result in juvenile polyyps syndrome. Interestingly, among patients reported to have a deletion of chromosome 10q23 which

includes *BMPR1A* but does not include *PTEN*^[17,29], none have been reported to have polyyps thus far^[30]. A combined and synergistic effect of the deletion of both *BMPR1A* and *PTEN* in 10q23 microdeletion may be involved in this aggressive polyyps. The functions of the *PTEN* protein include phosphatase activity down-regulating the PI3K/Akt pathway, which helps regulate cell growth, proliferation, and apoptosis^[31]. The *BMPR1A* gene encodes a receptor for the BMP pathway binding proteins and this pathway inhibits cell proliferation, especially of the gastrointestinal tract^[32,33]. Therefore, the deletion of both of these genes may lead to increased proliferation of gastrointestinal cells predisposing to polyyps and potential gastrointestinal malignancies.

Gastrointestinal management of patients with 10q23 microdeletions is determined on an individual basis due to the variability in onset of polyyps and severity of progression. In many patients with this deletion, including the patient described in this report, there is an accelerated rate of polyp development that occurs at a very early age and is more aggressive than that seen in PHTS or in *BMPR1A*-associated JPS. In fact, nearly half of the reported patients have been referred for colectomy^[16-18,20,23,25,26] in childhood, with several requiring surgery before 2 years of age. When contemplating colectomy, the number of

polyps, size of polyps, associated symptoms and level of concern for malignancy are all considered. Although there is an increased risk of colorectal cancer in adults with PHTS^[34] and JPS^[35], children are rarely diagnosed with gastrointestinal cancer and do not routinely undergo colectomy. However, in those with 10q23 microdeletions there are reports of early colorectal dysplasia and malignancy. Dysplastic polyps or colonic epithelial dysplasia were noted in the colon in three children^[22,23,25] and the duodenum in one^[20]. An additional patient developed rectal cancer at age 24 years^[23] and is now deceased. These observations suggest children with 10q23 deletions require frequent endoscopic surveillance of not only the lower but also the upper gastrointestinal tracts. We propose yearly EGD and colonoscopy after diagnosis of these mutations. Some patients, such as the one described in this paper, may require more frequent endoscopy if polyps are rapidly increasing in size or number and all polyps cannot be removed during one endoscopy. Small bowel surveillance with capsule endoscopy should also be considered. As in our patient, this risk for early gastrointestinal malignancy should prompt consideration for colectomy when the polyp burden becomes too great to manage through serial polypectomy or when dysplasia develops. Additionally, post-colectomy endoscopic surveillance is also warranted by the presence of upper intestinal polyps in a majority of reported cases, the high recurrence rates of polyps in the remnant rectum and the pouch and the fact that even after colectomy there is continued risk for duodenal or rectal cancer.

Extra-intestinal workup should also be considered due to the frequent non-gastrointestinal manifestations. In the patients' reported, common findings include cardiac (ASD, VSD), developmental delay, hypotonia, lipoma and hemangioma. Extraintestinal malignancies reported in 10q23 microdeletions include thyroid cancer^[8] and mucinous cystadenoma of the ovaries^[26]. These observations suggest neurodevelopmental assessment, close monitoring of growth parameters, careful dermatologic exam, thyroid exam and/or ultrasound and echocardiography should all be considered in these patients both at time of diagnosis and throughout life.

Genetic testing plays a critical role in establishing the correct diagnosis for patients who have features of JPS, PHTS, or both since this will have an impact on the surveillance and management of the patient. We propose the following algorithm to achieve a genetic diagnosis in the most timely and cost-effective manner (Figure 2). Macrocephaly of greater than 2.5 SD is a common feature seen in PHTS and is not commonly associated with JPS so it is a reasonable starting point in making a diagnosis. Additionally, in patients with JPS, a mutation in *SMAD4* is more likely when there is family history of polyps when compared to *BMPR1A*. A *SMAD4* mutation is also more likely when there is a positive family history of nosebleeds and/or telangiectasias, as *SMAD4* mutations are also associated with hereditary hemorrhagic telangiectasia syndrome, along with features of JPS. Immunohisto-

chemistry for SMAD4 may also be done in some centers, and if positive, guide genetic testing^[36]. *BMPR1A* is located more proximal to the centromere on chromosome 10 than *PTEN* and there are several genes located between these two. Therefore, if a deletion is detected in either *BMPR1A* or *PTEN*, it is important to assess the precise location of this deletion in the event it could represent a larger 10q23 microdeletion. For this reason, we recommend a microarray analysis (if one has not already been completed) if a deletion is detected in either *BMPR1A* or *PTEN*. Both PHTS and JPS are inherited in an autosomal dominant pattern and both can either be inherited from a parent or occur as a *de novo* event. Once a genetic diagnosis is established in a presenting patient, parental studies may be critical to assess if either parent is at risk for medical complications that are associated with these conditions.

Our report highlights the phenotypic diversity of deletions including chromosome 10q23 and involving *PTEN* and *BMPR1A*. These patients are at risk for cardiac, endocrine, gastrointestinal and neurodevelopmental abnormalities. They have a heightened risk of accelerated polyposis, in some cases conforming to the traditional definition of JPI and, in addition harboring an increased risk of gastrointestinal malignancy that appears greater than if there is a mutation or deletion in either gene alone. Multidisciplinary assessment of these patients is an early prerequisite for care.

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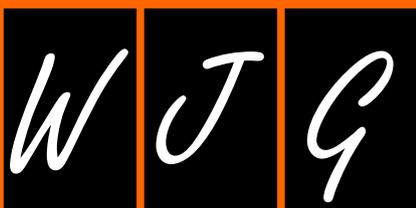
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APPENDIX I-VI Instructions to authors

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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
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PUBLISHER
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Colorectal anastomotic leakage: Aspects of prevention, detection and treatment

Freek Daams, Misha Luyer, Johan F Lange

Freek Daams, Misha Luyer, Department of Surgery, Catharina Ziekenhuis, 5623 EJ Eindhoven, The Netherlands

Johan F Lange, Department of Surgery, Erasmus Medical Center, 3015 CE Rotterdam, The Netherlands

Author contributions: Daams F and Luyer M performed the review of literature and wrote the paper; Lange JF supervised the data collection and revised the paper

Correspondence to: Freek Daams, MD, Department of Surgery, Catharina Ziekenhuis, Michelangelolaan 2, 5623 EJ Eindhoven, The Netherlands. freekdaams@gmail.com

Telephone: +31-6-55688542 Fax: +31-40-2443370

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motic leakage. New anastomotic techniques and risk scores should improve incidence numbers and early detection, whereas future research could focus on preservation of the anastomosis in case of leakage.

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Abstract

All colorectal surgeons are faced from time to time with anastomotic leakage after colorectal surgery. This complication has been studied extensively without a significant reduction of incidence over the last 30 years. New techniques of prevention, by innovative anastomotic techniques should improve results in the future, but standardization and "teachability" should be guaranteed. Risk scoring enables intra-operative decision-making whether to restore continuity or deviate. Early detection can lead to reduction in delay of diagnosis as long as a standard system is used. For treatment options, no firm evidence is available, but future studies could focus on repair and saving of the anastomosis on the one hand or anastomotic breakdown and definitive colostomy on the other hand.

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Key words: Colorectal surgery; Complications; Postoperative care; Anastomotic leakage; Prevention

Core tip: This editorial covers the past achievements and future challenges in the field of colorectal anasto-

INTRODUCTION

Anastomotic leakage after colorectal resection (CAL) is a dreaded complication and is reported to have a significant mortality (6%-22%)^[1]. Morbidity is dramatically increased opposed to patients without CAL and leads to reoperations, radiological interventions and permanent stoma in 56%^[2]. CAL is the leading cause of postoperative death after colorectal surgery, increases the risk of a permanent stoma significantly. Although available data on the effect of CAL on long-term oncologic outcome is not univocal, most papers on this topic report worse oncologic outcome in terms of increased local recurrence and negative association with survival^[3]. Despite great numbers of studies investigating risk factors, surgical techniques and prevention of CAL, over the last three decades incidence has not reduced. In a recent publication by the Dutch Surgical Colorectal Audit incidence of CAL after restorative colon and rectum resections in 9192 registered patients in The Netherlands over 2010 was 8.7% (Table 1). Additionally, with patients expected to become older and to have more co morbidities, every patient but also every colorectal surgeon will increasingly be exposed to CAL and forthcoming difficulties in diagnosis and treatment. Incidence should be reduced and outcome must improve. Understanding current developments and its omissions will lead to design of relevant future research.

RISK FACTORS

Extensive literature is available on the topic of risk factors for anastomotic leakage. Among other factors are male gender, smoking, obesity, alcohol abuse, preoperative steroid and non-steroidal anti-inflammatory drugs use, longer duration of operation, preoperative transfusion, contamination of the operative field and timing during duty hour^[4-7]. Increasingly, aspects of case volume for rectal surgery are discussed in respect to postoperative complications. Asteria *et al*^[8] described case volume per centre < 20 is correlated to CAL. In line with this finding, Biondo *et al*^[9] described in their study over 1046 emergency colorectal resection that CAL occurred less frequent in patients who were treated by specialized colorectal surgeons. Recently, risk factor studies have also been undertaken for laparoscopic colorectal surgery, identifying body mass index, tumour distance from the anal verge, tumour depth, and pelvic outlet as independent predictors for increased operative time and morbidity after laparoscopic total mesorectal excision^[10]. Furthermore, American Society of Anesthesiologists III/IV patients and operative time longer itself are risk factors for CAL after laparoscopic colorectal surgery^[11]. It is debatable whether leakage rates might have been lower if preoperative radio-chemotherapy is not applied as widely as is done nowadays, since neo-adjuvant therapy is one of the strongest risk factors amongst the above mentioned. This great abundance of literature does not provide colorectal surgeons with clear guidance in the decision of when to renounce from restorative surgery. To provide an objective assessment of the risk of anastomotic leakage, Dekker *et al*^[12] developed and tested the Colon Leakage Score (CLS). In this score multiple risk factors were taken up and points were attributed to the patients per risk factor. As a predictor, CLS had an excellent area under the curve of the receiver-operating characteristics curve (AUC 0.95, 95%CI: 0.89-1.00), and an odds ratio of 1.74 (95%CI: 1.32-2.28). To our knowledge this tool is unique in its ability to detect high-risk patients preoperatively and objectively assesses the necessity for diverting ileostomy or non-restorative surgery.

SURGICAL TECHNIQUE

A recent review from our group addresses all the important steps that surgeons need to take into mind when creating a colorectal anastomosis^[13]. Although some prerequisites should be present as adequate blood flow, without any tension in the absence of peritonitis, no clear value can be given for these aspects. When the little evidence that is available for the hand-sewn anastomosis is evaluated, it can be concluded that an inverting single layer continuous suture technique with slowly absorbable monofilament material seems preferable. Strong evidence lacks for other important aspects as distance from the suture to the edge of the anastomosis, distance between the sutures, layers included in the suture, suture tension and the optimal configuration. The highest level of evidence

Table 1 Number of colon and rectum resections in The Netherlands in 2011 and percentage anastomotic leakage *n* (%)

| | Colon | | Rectum | |
|---------------------|--------------|-------------|-------------|-------------|
| | < 75 yr | ≥ 75 yr | < 75 yr | ≥ 75 yr |
| Resections | 10249 (59.0) | 7246 (41.0) | 5076 (72.0) | 1933 (28.0) |
| Anastomotic leakage | 666 (7.4) | 449 (7.3) | 310 (11.4) | 55 (8.1) |

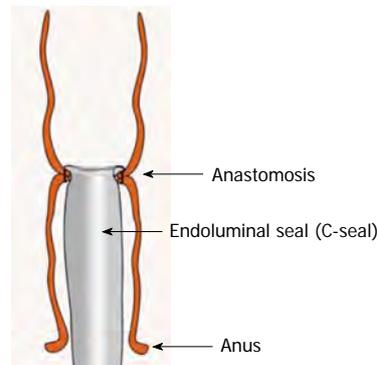


Figure 1 C-seal. Endoluminal biodegradable anastomotic cover. Printing with permission from Bakker *et al*^[17].

exists for the equality regarding to CAL of stapling *vs* hand sewn anastomosis, without evidence for one technique being superior to the other^[14]. Following the above mentioned statements, currently stapling techniques might be of preference since the technique is uniform and easy to learn, making it ideal for comparing results between hospitals and surgeons and for teaching young surgeons.

There is a need for development of new techniques since all previous research has not lead to radically decreased leakage rates. Many experimental techniques have been investigated and some have shown at least equal result in comparison to hand-sewn techniques. Not many techniques tested in animal experiments have been translated to the human setting. Reasons for this could be that no standard models and robust translatable outcome measures exist for colorectal experiments. In humans, the so-called compression anastomosis is shown to have similar leakage rates compared to hand-sewn anastomosis^[14,15]. Extra-luminal sealing using fibrin glue or acrylates have been reported mostly in animal studies, few reports on their use in human colorectal anastomosis have not shown beneficial effects on CAL^[16]. Endo-luminal sealing by means of a biodegradable barrier has shown to be successfully applied in humans and a multicentre randomised clinical trial is currently being undertaken (Figure 1)^[17].

Future studies should in our opinion focus on techniques that are easy to learn and have high reproducibility. To enhance reproducibility, animal studies should use the same animal models that are currently available or under construction.

EARLY DETECTION

Anastomotic leakage typically becomes clinically appar-

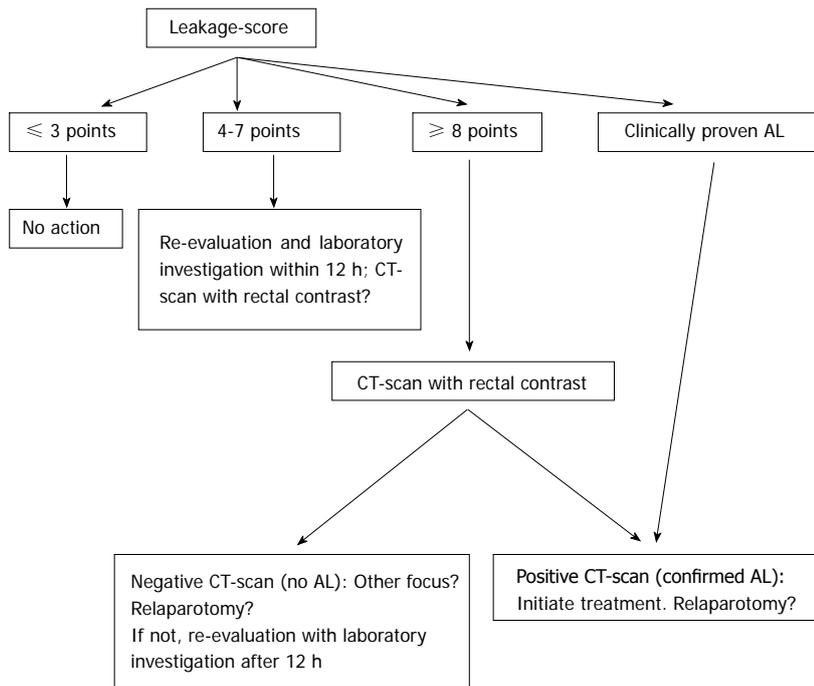


Figure 2 The Dutch Leakage Score. According to the points attributed to the patients on the basis of clinical symptoms, treating doctors can follow this diagnostic flowchart. Reprinted from den Dulk *et al*^[19]. CT: Computed tomography; AL: Anastomotic leak.

ent between the 5th and the 8th postoperative day, but many exceptions exist, with one study even reporting a mean of the 12th postoperative day for the diagnosis of CAL^[18]. Clinical signs of systemic inflammatory response syndrome, fever, ileus and pain are frequent but have low positive predictive value for CAL, when observed separately. In a study by den Dulk *et al*^[19] these clinical features were combined into a clinical scoring system (Dutch Leakage Score), with which patients were scored daily in a systematical and uniform way. Points are attributed to certain clinical symptoms (*i.e.*, fever, heart rate), nutritional status (signs of ileus, gastric retention, type of intake) and laboratory findings [*i.e.*, C-reactive protein (CRP) level, leucocytes, kidney function]. After applying the score system retrospectively on a historical cohort, the score was used prospectively. It was shown that patients with a higher score were prone to CAL requiring intensive clinical observation or radiological evaluation (Figure 2). This scoring system reduced delay in diagnosis of anastomotic leak from 4 to 1.5 d, decreasing false negative diagnostic imaging representing a major factor of delay in diagnosis^[20]. Although it is not known if the application of the score leads to an increase of negative imaging, the score could be especially beneficial in daily clinical practise where young doctors and nursing staff could identify high risk patients very easily and in a standardised manner. Furthermore, it could improve comparability of studies when applied more universally.

This interval between surgery and clinical onset suggests a preclinical phase in which non-clinical methods could be used to predict CAL. Consequently, routine postoperative measurement of serum level CRP is studied for infectious complications after colorectal surgery in general and CAL in particular. In a meta-analysis by Warschkow *et al*^[21] including six studies, a cut-off of 135

mg/L on postoperative day 4 resulted in a negative predictive value of 89% for infectious complications. CRP and other biochemical parameters detect systemic reactions, while other techniques are recently applied to detect local, juxta-anastomotic changes in metabolism and ischemia. Microdialysis of the peritoneal cavity is such a technique using an indwelling two-lumen catheter that detects changes in oxygenation locally at the site of anastomosis. Few studies have shown the ability to distinct patients with CAL after rectum resection from patients with an uncomplicated course, although these have insufficient samples to provide predictive values^[22,23]. Future studies should focus on preclinical detection of CAL, since patients that are reoperated in an early phase could be protected from septic sequelae of clinical CAL.

TREATMENT

When facing and treating patients with CAL, surgeons have to take into account many different aspects, *i.e.*, age, health status and current clinical condition of the patient, extent of dehiscence, time between operation and reoperation, indication of primary resection, presence of diverting stoma and localisation of the anastomosis. These variables lead to individualisation of treatment strategies and incomparable outcome. However, few studies, showing that surgeons believe that the anastomosis can be repaired rather than dismantled, have paved the way for a trial in which next to mortality and morbidity, preservation of the anastomosis could be one of the endpoints^[24,25]. Difficulties in designing such a trial are the aforementioned large variety of clinical course, the unpredictability of CAL and the relatively low incidence of CAL per centre.

CONCLUSION

Colorectal anastomotic leakage is a serious complication that has great clinical impact on patients, putting surgeons in dilemmas of prevention, diagnosis and treatment. Many aspects of colorectal anastomotic leakage like etiology remain unclear. Current practise however should comprise intra-operative risk assessment and subsequent adaptation of operative technique when necessary. Current optimal suture technique appears to be using slowly absorbable monofilament sutures applied in a continuous, inverting, single layer manner or stapling. Postoperatively, early detection plays a key role and a leakage score system and routine laboratory tests (CRP at postoperative day 3-4) contribute strongly to it. When reoperating, sparing the anastomosis should be kept in mind as a valid treatment option, although more research is needed on which clinical state allows this option.

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Dipeptidyl peptidase-4: A key player in chronic liver disease

Minoru Itou, Takumi Kawaguchi, Eitaro Taniguchi, Michio Sata

Minoru Itou, Eitaro Taniguchi, Division of Gastroenterology, Department of Medicine, Kurume University School of Medicine, Kurume 830-0011, Japan

Takumi Kawaguchi, Michio Sata, Division of Gastroenterology, Department of Medicine and Department of Digestive Disease Information and Research, Kurume University School of Medicine, Kurume 830-0011, Japan

Author contributions: Itou M and Kawaguchi T collected the materials and wrote the manuscript; Taniguchi E discussed the topic; Sata M supervised the manuscript.

Correspondence to: Takumi Kawaguchi, MD, PhD, Division of Gastroenterology, Department of Medicine and Department of Digestive Disease Information and Research, Kurume University School of Medicine, 67 Asahi-machi, Kurume 830-0011, Japan. takumi@med.kurume-u.ac.jp

Telephone: +81-942-317902 Fax: +81-942-317820

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Abstract

Dipeptidyl peptidase-4 (DPP-4) is a membrane-associated peptidase, also known as CD26. DPP-4 has widespread organ distribution throughout the body and exerts pleiotropic effects *via* its peptidase activity. A representative target peptide is glucagon-like peptide-1, and inactivation of glucagon-like peptide-1 results in the development of glucose intolerance/diabetes mellitus and hepatic steatosis. In addition to its peptidase activity, DPP-4 is known to be associated with immune stimulation, binding to and degradation of extracellular matrix, resistance to anti-cancer agents, and lipid accumulation. The liver expresses DPP-4 to a high degree, and recent accumulating data suggest that DPP-4 is involved in the development of various chronic liver diseases such as hepatitis C virus infection, non-alcoholic fatty liver disease, and hepatocellular carcinoma. Furthermore, DPP-4 occurs in hepatic stem cells and plays a crucial role in hepatic regeneration. In this review, we described the tissue distribution and various biological effects of DPP-4. Then, we discussed the impact of DPP-4 in chronic liver disease and the possible thera-

peutic effects of a DPP-4 inhibitor.

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Key words: Incretin; Viral hepatitis; Insulin resistance; Steatohepatitis; Cancer; Sitagliptin; Vildagliptin; Alogliptin; Teneligliptin; Linagliptin

Core tip: Dipeptidyl peptidase-4 (DPP-4) is a membrane-associated peptidase, also known as CD26. DPP-4 has widespread organ distribution throughout the body and exerts pleiotropic effects *via* its peptidase activity. In this review, we described the tissue distribution and various biological effects of DPP-4. Then, we discussed the impact of DPP-4 in chronic liver disease and the possible therapeutic effects of a DPP-4 inhibitor.

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DISTRIBUTION OF DIPEPTIDYL PEPTIDASE-4

Dipeptidyl peptidase-4 (DPP-4, enzyme code number 3.4.14.5) is a 110 kDa membrane-associated peptidase, which was originally identified in 1966 as a dipeptide naphthylamidase that hydrolyzed glycyl-prolyl-beta-naphthylamide^[1]. DPP-4, also known as CD26^[2-4], is expressed on the apical surfaces of epithelial and acinar cells and in endothelial cells, fibroblasts, and lymphocytes^[5-8]. DPP-4 also exists as a soluble circulating form in plasma^[9].

DPP-4 occurs in all organs including the small intestine, biliary tract, exocrine pancreas, spleen, and brain in both rodents and humans^[10-12]. This widespread organ distribution indicates that DPP-4 has pleiotropic biological activities. The liver is one of the organs that highly ex-

Table 1 Target peptide of dipeptidyl peptidase-4

| Category | Peptide | Ref. |
|---------------------------------------|------------------------------|-------------------|
| Glucose metabolism | GLP-1 | [94-97] |
| | GIP | [97-99] |
| | Glucagon | [97,100,101] |
| | PACAP-38 | [102-104] |
| Gut motility | GLP-2 | [97,105,106] |
| | VIP | [107-109] |
| | NPY | [109,110] |
| | GRP | [103] |
| Appetite regulation | Peptide histidine methionine | [94,102] |
| | Peptide YY | [107,111] |
| Chemokine | CCL5/RANTES | [112-114] |
| | CCL11/eotaxin | [115,116] |
| | CCL22/MDC | [112] |
| | CXCL9/MiG | [117-119] |
| | CXCL10/IP10 | [114,119,120] |
| | CXCL11/I-TAC | [121,122] |
| Growth | CXCL12/SDF-1 | [93,113] |
| | IGF-1 | [123,124] |
| Reproduction | GHRH | [94,125] |
| | Prolactin | [126-128] |
| | hCG α | [129] |
| Vasodilation | LH α | [123] |
| | CGRP | [107,110] |
| Pain regulation | Bradykinin | [107,108] |
| | Enkephalin | [130] |
| | Endomorphins | [109,130-132] |
| Homeostasis | Substance P | [109,110,133,134] |
| | Thyotropin α | [123,135] |
| Inhibition of endothelial cell growth | Vasostatin- I | [136] |

GLP: Glucagon-like peptide; GIP: Glucose-dependent insulinotropic peptide; PACAP-38: Pituitary adenylate cyclase-activating polypeptide-38; VIP: Vasoactive intestinal peptide; NPY: Neuro-peptide Y; GRP: Gastrin-releasing peptide; CCL: Chemokine (C-C motif) ligand; RANTES: Regulated upon activation; MDC: Macrophage-derived chemokine; CXCL: Chemokine (C-X-C motif) ligand; MiG: Monokine induced by gamma interferon; IP-10: Protein 10 from interferon (γ)-induced cell line; I-TAC: Interferon-inducible T-cell α chemoattractant; SDF-1: Stromal-derived factor-1; IGF-1: Insulin-like growth factor-1; GHRH: Growth hormone releasing hormone; hCG α : Human chorionic gonadotropin α subunit; LH α : Leutinizing hormone α chain; CGRP: Calcitonin-related peptide.

presses DPP-4^[8]. In the healthy human liver, intense staining for DPP-4 is seen in hepatic acinar zones 2 and 3, but not in zone 1. Similar lobular heterogeneity is also seen in the expression of cytochrome p450, gamma-glutamyl-transpeptidase (GGT), and glutamine synthetase^[13-15]. This heterogeneous lobular distribution suggests that DPP-4 may be involved in the regulation of hepatic metabolism.

BIOLOGICAL ACTIVITIES OF DPP-4

Peptidase

DPP-4 is an enzyme that cleaves N-terminal dipeptides of proline or alanine-containing peptides including incretin, appetite-suppressing hormones (neuropeptide), and chemokines as listed in Table 1. Representative targets are glucagon-like peptide (GLP)-1, GLP-2, peptide YY, chemokine ligand 12/stromal-derived factor-1 (CXCL12/SDF-1), and substance P. Thus, DPP-4 exerts pleiotropic effects on glucose metabolism, gut motility, appetite regu-

lation, inflammation, immune system function, and pain regulation though its peptidase activity (Figure 1).

Immune stimulation

DPP-4 expression is downregulated in the resting state of T-cells; however, expression is upregulated by antigenic or mitogenic stimulation via an interleukin-12-dependent mechanism^[16-18]. DPP-4 activates intracellular molecules including p56^{lck}, phospholipase C- γ , and mitogen-activated protein kinase (MAPK)^[11]. This activation enhances T-cell maturation and migration, cytokine secretion, antibody production, immunoglobulin isotype switching of B cells, and activation of cytotoxic T cells^[19,20]. In addition, soluble CD26 binds to mannose 6-phosphate receptor and is taken up by CD14 positive monocytes, increasing their antigen presenting activity and T-cell proliferation^[11,21,22].

Binding to extracellular matrix

DPP-4 has the ability to bind to the Binding to extracellular matrix (ECM), preferentially to the collagens I and III, and fibronectin^[23,24], and is involved in hepatocyte-extracellular matrix interactions^[23,25]. The putative collagen binding site of DPP-4 is located at the C-terminal portion of the molecule, separate from the peptidase catalytic site^[26].

In a mouse xenograft model, treatment with anti-DPP-4 monoclonal antibody inhibits the growth of renal cell carcinoma via disruption of binding to the extracellular matrix^[27]. On the other hand, over expression of DPP-4 induces apoptosis of prostate cancer cells by inhibition of cell migration and invasion through down-regulation of MAPK-extracellular signal-regulated kinase-1/2 activation^[28]. Thus, the role of DPP-4 may differ in different types of cancer.

Degradation of ECM

DPP-4 binds to adenosine deaminase and activates plasminogen-2, leading to an increase in plasmin levels^[11]. The increased plasmin degrades type IV collagen, fibronectin, laminin, and proteoglycan, and activates matrix metalloproteinases. These changes result in the degradation of the ECM^[29,30].

Resistance to anti-cancer agents

DPP-4 is thought to be associated with sensitivity to anti-cancer agents in hematologic malignancies. DPP-4 has been linked to high topoisomerase II α levels, resistance to anti-cancer agents, and the malignant potential of T-cell lymphoma^[11,21,22]. Treatment with anti-DPP-4 monoclonal antibody causes dephosphorylation of both MAPK and integrin β 1 in T-cell lymphoma, leading increased sensitivity to anti-cancer agents and greater survival^[31]. Similar beneficial effects of anti-DPP-4 monoclonal antibody have been reported in renal cell carcinoma^[27] and malignant mesothelioma tumors^[32].

Lipid accumulation

DPP-4 affects lipid metabolism by the inactivation of

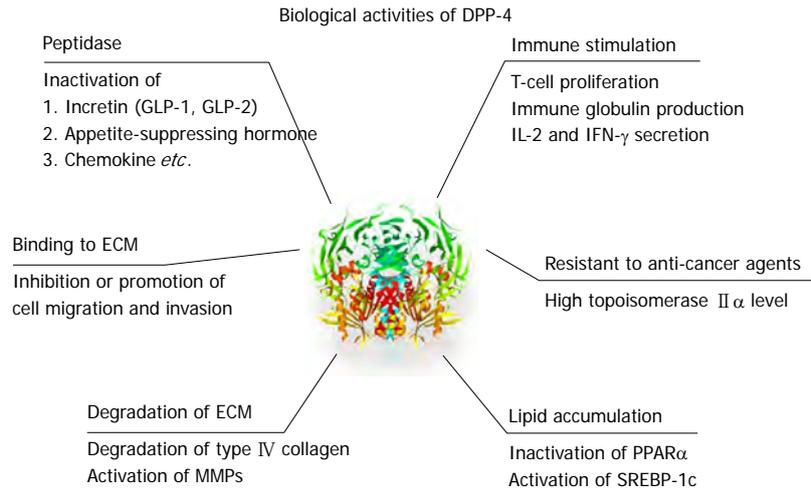


Figure 1 Pleiotropic effects of dipeptidyl peptidase-4. Dipeptidyl peptidase-4 (DPP-4) exerts various effects on metabolism and chemokine through peptidase activity. In addition, DPP-4 is involved in immune stimulation, binding to and degradation of extracellular matrix, and resistant to anti-cancer agents. DPP-4 also directly affects lipid accumulation. GLP: Glucagon-like peptide; ECM: Extracellular matrix; MMPs: Metalloproteinases; IL: Interleukin; IFN: Interferon; PPAR: Peroxisome proliferator-activated receptor; SREBP: Sterol regulatory element binding protein.

peptides such as GLP-1, neuropeptide Y, and peptide YY. In addition, DPP-4 is known to directly affect lipid metabolism. Knock-out of the gene encoding DPP-4 directly causes activation of the peroxisome proliferator-activated receptor- α pathway and inactivation of the sterol regulatory element binding protein-1 pathway^[33], thereby increasing lipid oxidation, reducing lipogenesis, and resulting in the prevention of high-fat diet-induced hepatic steatosis.

CHANGES IN DPP-4 IN PATIENTS WITH LIVER DISEASE

Serum level of DPP-4 is elevated in patients with liver cirrhosis^[34,35] and up-regulation of hepatic DPP-4 expression is thought to be responsible for this elevation^[36]. Here, we describe the effects of DPP-4 according to each pathophysiology.

Hepatitis C virus infection

Patients with hepatitis C virus (HCV) infection show increased serum DPP-4 expression in hepatocytes^[37,38]. Lymphocyte subset analysis has also shown that CD8+ T-cells, which express DPP-4, are present in the portal and periportal areas in patients with HCV infection^[39]. Since HCV infects CD8+ T-cells^[39-41], HCV-infected T-cells may be responsible for the increased serum DPP-4 activity in patients with HCV infection.

In addition, glucose intolerance with insulin resistance is a feature of HCV infection and is associated with disease progression as well as prognosis^[42-52]. Besides hepatic inflammation and steatosis, HCV itself is involved in the development of insulin resistance through the impairment of insulin receptor substrate-1/2^[52-54]. Moreover, HCV infection is known to be associated with increased DPP-4 expression in the ileum, liver, and serum^[38]. Transfection with cDNA encoding part of the HCV non-structural genome region 4B/5A induces expression of DPP-4 in hepatocyte cell lines^[55]. Furthermore, eradication of HCV by interferon therapy results in a decrease in serum DPP-4 levels^[56-61] and administration of sitagliptin

significantly improves HCV-related glucose intolerance^[62]. Since there is no significant association between serum DPP-4 activity and severity of liver disease in patients with HCV infection^[38], HCV infection may directly up-regulate DPP-4 activity, leading to impairment in glucose metabolism.

Non-alcoholic fatty liver disease

Non-alcoholic fatty liver disease (NAFLD) is a hepatic manifestation of metabolic syndrome and the most common cause of chronic liver disease^[63-66]. Although various factors are responsible for the development of NAFLD, a high glucose load is known to induce DPP-4 expression in HepG2 cells^[67] and hepatic DPP-4 mRNA expression level in the livers is significantly higher in patients with NAFLD, compared to healthy subjects^[67]. In fact, serum DPP-4 activity and hepatic expression of DPP-4 are correlated with hepatic steatosis and NAFLD grading^[68]. Moreover, DPP-4 deficient rats show lower levels of hepatic proinflammatory and profibrotic cytokines and reduced hepatic steatosis compared to wild type rats. These favorable changes in lipid metabolism are independent of glucose metabolism^[69]. Similar to these results from animal experiments, in patients with NAFLD, DPP-4 activity in serum and liver specimens correlate with markers of liver damage such as serum GGT and alanine aminotransferase levels, but do not correlate with fasting blood glucose levels and glycosylated hemoglobin (HbA1c) values^[68,70]. Thus, hepatic DPP-4 expression in NAFLD may be directly associated with hepatic lipogenesis and liver injury.

Recently, DPP-4 inhibitor has been reported to improve hepatic steatosis in mice and humans^[71]. We also experienced a case of refractory NAFLD that was successfully treated with sitagliptin, a DPP-4 inhibitor^[72]. Moreover, it is reported that sitagliptin ameliorates liver enzymes and hepatocyte ballooning in patients with non-alcoholic steatohepatitis^[73]. Taken together, these findings may indicate that DPP-4 inhibitors ameliorate hepatic injury and glucose impairment in patients with NAFLD.

Hepatocellular carcinoma

Increased DPP-4 expression is seen in various malignant

tumors, such as breast cancer^[74,75], brain glioma^[76], malignant mesothelioma^[77], and squamous cell laryngeal carcinoma^[78]. In hepatocellular carcinoma (HCC), increased DPP-4 expression is also seen in liver specimens and serum from both rats^[79] and humans^[80].

Inhibition of DPP-4 in human hepatoma cells is reported to suppress tyrosine kinase, leading to anti-apoptotic effects^[81]. However, Yamamoto *et al*^[82] recently reported a case in which dramatic regression of HCC was seen after four weeks' treatment with DPP-4 inhibitor in a patient with HCV-related chronic hepatitis. Although it is unclear whether DPP-4 inhibitor is directly involved in the regression of HCC, marked invasion of CD8+ T-cells was seen around the HCC tissue^[82], suggesting that the DPP-4 inhibitor may have improved immune response, which has been impaired by chronic HCV infection^[38]. Although exogenous insulin or sulfonylurea treatment increases the risk of HCC^[83], treatment with DPP-4 inhibitor does not show any tumor promoting effects in mice^[84]. Thus, a DPP-4 inhibitor may safely exert beneficial effects on HCV-related HCC through modulation of immunity.

Stem cell and hepatic regeneration

Increased hepatic DPP-4 expression has been reported to occur in the cirrhotic liver^[85,86]. Although the impact of increased DPP-4 expression remains unclear, Lee *et al*^[87] recently reported that human liver stem cells express DPP-4, but not CD34 and CD45, which are hematopoietic stem cell and endothelial progenitor cell markers. Thus, DPP-4 is a specific marker of adult hepatic stem and progenitor cells, indicating that DPP-4 may be involved in the regeneration in chronically inflamed liver.

CXCL12/SDF-1 causes hematopoietic stem cell (HSC) homing and is an important chemokine for hepatic regeneration^[88-91]. CXCL12/SDF-1 is a target peptide of DPP-4 and the inhibition of the cell-surface DPP-4 activity of HSC/hematopoietic progenitor cell populations increases their CXCL12/SDF-1 directed chemotaxis, homing, and engraftment^[92]. Therefore, inhibition of DPP-4 may be an effective therapy for increasing the efficacy and success of HSC/hematopoietic progenitor cell transplantation^[92]. DPP-4 inhibition also increases number of progenitor cells, and stabilization of endogenous CXCL12/SDF-1 by DPP-4 inhibition is achievable and may be a promising strategy to intensify sequestration of regenerative stem cells^[93].

CONCLUSION

In this review, we described the tissue distribution and biological effects of DPP-4. Then, we discussed the impact of DPP-4 in chronic liver disease and the possible effects of a DPP-4 inhibitor. DPP-4 plays crucial roles in the development of various chronic liver diseases, and DPP-4 inhibition seems to have beneficial effects in chronic liver diseases. However, DPP-4 inhibitors also modulate the immune system, and further studies will be focused on

the effects of long-term administration of a DPP-4 inhibitor on infection and carcinogenesis.

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Role of interleukin-6 in Barrett's esophagus pathogenesis

Katerina Dvorak, Bohuslav Dvorak

Katerina Dvorak, Department of Cellular and Molecular Medicine, The University of Arizona, Tucson, AZ 85724, United States

Bohuslav Dvorak, Department of Pediatrics and Steele Children's Research Center, The University of Arizona, Tucson, AZ 85724, United States

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Correspondence to: Katerina Dvorak, PhD, Associate Professor, Department of Cellular and Molecular Medicine, The University of Arizona, Tucson, AZ 85724, United States. kdvorak@email.arizona.edu

Telephone: +1-520-9710255 Fax: +1-520-6265009

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Abstract

Barrett's esophagus (BE) is a metaplastic lesion of the distal esophagus arising as a consequence of chronic gastroesophageal reflux disease. Multiple studies show that BE is associated with increased risk of esophageal adenocarcinoma (EAC). Epidemiological studies and animal models demonstrate that chronic inflammation triggered by repeated exposure to refluxate predisposes to the development of BE and EAC. The chronic inflammation is associated with cytokine alterations. Interleukin 6 (IL-6) is a cytokine that stimulates cell proliferation and apoptosis resistance is frequently increased in different cancers. Importantly, IL-6 and transcriptional factor signal transducer and activator of transcription 3 (STAT3) that is activated by IL-6 are also increased in BE and EAC. This review critically appraises the role of IL-6/STAT3 pathway in progression of BE to EAC from the published evidence currently available.

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Key words: Barrett's esophagus; Interleukin 6; Bile acids; Inflammation; Apoptosis

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INTRODUCTION

Barrett's esophagus (BE) is a condition where normal squamous epithelium is replaced by metaplastic intestinal-like columnar epithelium containing goblet cells. This premalignant lesion is associated with a nearly 40-fold increased risk for the development of esophageal adenocarcinoma (EAC), a cancer with poor prognosis, and a median survival of less than one year^[1]. EAC is most common in the Caucasian population in the western countries. EAC incidence increased almost six fold between 1975 and 2001^[2] and EAC mortality also increased more than sevenfold^[2]. Currently, EAC has the fastest growing incidence rate of all cancers in the United States. Approximately 17000 patients will be diagnosed with esophageal cancer in 2012 and about 14600 patients will die of this cancer in the United States^[3].

There is overwhelming evidence that BE arises as a consequence of chronic gastroesophageal reflux disease (GERD). GERD is a very common medical condition in the United States affecting 40% of the adult population at least monthly. One third of these patients have erosive esophagitis and 6%-14% of patients undergoing endoscopy for symptomatic GERD have BE^[1]. This represents about 2 million people in the United States alone^[4]. The rate of transformation to cancer is about 0.1%-0.2% per year^[5].

Histopathologic steps in the progression of BE include: (1) metaplasia of the normal esophageal squamous epithelium to a specialized intestinal glandular epithelium; (2) low-grade dysplasia; (3) high-grade dysplasia; and (4) esophageal adenocarcinoma with invasive and metastatic potential. However, little is known, about the signaling pathways promoting the development of metaplasia and dysplasia.

CHRONIC INFLAMMATION AND CYTOKINE DYSREGULATION IN BE

Epidemiological studies and animal models demonstrate that chronic inflammation predisposes to the development of various forms of cancer including gastrointestinal malignancies^[5]. In the esophagus, chronic inflammation is triggered by repeated exposure to components of refluxate such as gastric acid and bile acids. Indeed, chronic reflux is the strongest risk factor for the development of BE and EAC^[6]. A major regulatory pathway linking inflammation and cancer is activation of nuclear factor κ B (NF- κ B) signaling. The same pathway initiates transcription of cytokines. In agreement with the inflammatory hypothesis of BE/EAC development, NF- κ B is constitutively activated in BE or EAC but is not detected in esophagitis or the adjacent normal esophageal mucosa^[7].

Esophageal mucosa damaged by refluxate is commonly infiltrated by inflammatory cells of different lineages. First, the damaged site is infiltrated by neutrophils and monocytes (acute inflammation) followed by lymphocytes and plasma cells primarily at the site of metaplasia (chronic inflammation)^[8]. Cytokines that are produced by the inflammatory cells and by Barrett's epithelium play a crucial role in BE carcinogenesis^[9]. Furthermore, noxious compounds, such as reactive oxygen and nitrogen species, released during chronic inflammation may damage DNA and induce mutations that subsequently promote cancer development.

Interestingly, Barrett's esophagus is characterized by a unique cytokine environment compared to erosive esophagitis. While BE is associated with Th2 cytokines, erosive esophagitis is distinguished primarily by a Th1 cytokines profile^[10]. This difference in the cytokine profile does not seem to be simply a result of the development of intestinal metaplasia since the cytokine profile is completely different in the duodenum or the gastric antrum^[10]. We analyzed multiple cytokines in human tissues using cytokine arrays^[11]. Interleukin-6 (IL-6) levels were consistently increased in BE compared to control tissues. The expression of other cytokines, such as IL-8, was variable and inconsistent.

IL-6 AND CANCER

This review is focused on the IL-6/signal transducer and activator of transcription 3 (STAT3) pathway. IL-6 is a potent, pleiotropic Th2 cytokine that regulates immune defense response. Its release is triggered by tissue damage or infection. IL-6 acts as both a pro-inflammatory and anti-inflammatory cytokine. IL-6 plays a central role in the transition from the acute to the chronic phase of the inflammatory process^[12]. Importantly, the IL-6 pathway is one of the most important mechanisms linking inflammation to cancer^[13].

IL-6 overexpression is implicated in the pathogenesis of different tumors, including cancers of the ovary, prostate, breast, kidney and lung^[14]. IL-6 is also associated with

the development of colon cancer, predominantly colitis-associated colon cancer. Recent *in vivo* evidence shows that IL-6 controls tumor formation and growth in a mouse colitis-associated colon cancer^[15]. These studies indicate that the ablation of IL-6 reduces tumor burden, while the elevation of IL-6 levels accelerates tumor formation. The effects of IL-6 are mediated by STAT3. As expected, STAT3 deficiency reduced tumor incidence and growth, while STAT3 hyperactivation had an opposite effect in this model^[15]. These studies clearly indicate that IL-6/STAT3 signaling is crucial in the carcinogenesis that is linked to inflammation, such as colitis-associated colon cancer.

Only a few studies investigating the role of IL-6 in esophageal carcinogenesis were reported^[11,16,17]. We have shown that IL-6 is secreted from BE and EAC tissues and that phosphorylated STAT3 is expressed in BE and EAC^[11,16]. These studies were confirmed by Zhang *et al.*^[17]. Non-transformed and transformed human Barrett's epithelial cell lines were used in this study. Phospho-STAT3 was expressed only by transformed Barrett's cells, which also exhibited higher levels of IL-6 mRNA and of IL-6 and Mcl-1 proteins than non-transformed Barrett's cells.

In a recent study, serum IL-6 was significantly increased in esophageal cancer (86%) as compared to carcinoembryonic antigen (30%) and squamous cell cancer antigen (24%)^[18]. This was noted for both squamous cell carcinoma of the esophagus (87.1%, 23% and 33%, respectively) and EAC (7%, 39% and 13%, respectively). Interestingly, concentrations of IL-6 depended on distant metastases and patient's survival^[18]. Importantly, both colitis-associated colon cancer and esophageal adenocarcinoma are associated with chronic inflammation. Therefore, elevated IL-6/STAT3 signaling is one of the key pathways involved in esophageal tumorigenesis.

AUTOCRINE PRODUCTION OF IL-6 BY CANCER CELLS

One strategy used by cancer cells to upregulate growth and survival pathways is through autocrine production of growth and survival factors. IL-6 is produced by different cells, including immune cells and epithelial cells^[19]. Expression of IL-6 by cancer cells suggests that IL-6 acts as an autocrine growth factor to promote tumorigenesis^[20].

But why is IL-6 a crucial factor in tumorigenesis if STAT3 can be activated by other cytokines? Grivnennikov *et al.*^[21] suggested that tumors choose IL-6 to constitutively activate STAT3, because immune cells together with malignant cells are capable of producing massive amounts of "start-up" IL-6 (but not other family members) required for tumor progression. Indeed, both IL-6 and the IL-6 receptor are expressed in intestinal epithelial cells and these proteins are also increased in colorectal cancers^[22]. Importantly, our studies indicate that premalignant BE tissue expresses membrane-bound IL-6 receptor as well as soluble IL-6 receptor (sIL-6R) and secretes increased amounts of IL-6 as BE progresses to esophageal adenocarcinoma (unpublished data)^[11,16].

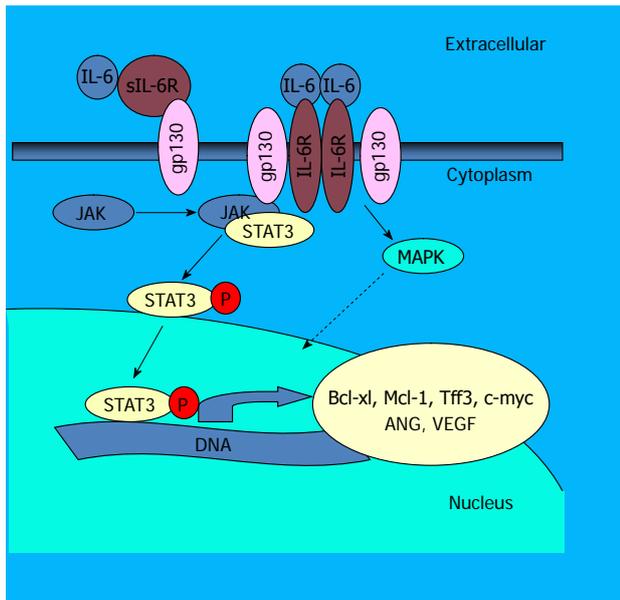


Figure 1 Interleukin 6 signaling scheme of the interleukin 6/signal transducer and activator of transcription 3 signaling pathway. IL-6: Interleukin 6; STAT3: Signal transducer and activator of transcription 3; sIL-6R: Soluble IL-6 receptor; IL-6R: IL-6 receptor; JAK: Janus kinase; MAPK: Mitogen-activated protein kinase; VEGF: Vascular endothelial growth factor; ANG: Angiopoietin.

STAT3 AND CANCER

IL-6 activity is mediated through activation of at least three different pathways. First, IL-6 binds to either cognate IL-6 receptor (IL-6Ra) or sIL-6R. Followed by binding to the receptors: (1) IL-6 induces association of signal transducer gp130 and ErbB, which leads to the activation of the MAP kinase pathway and activation of transcription factor NF-IL-6; (2) IL-6 promotes activation of Phosphatidylinositol 3-kinases, a prominent kinase associated with NF- κ B activation and apoptosis resistance^[23]; and (3) IL-6 signaling is primarily mediated by the Janus kinase (JAK)/STAT pathway (Figure 1). In this pathway the complex of IL-6 and its receptor interacts with the membrane bound gp130^[24]. This event leads to the phosphorylation of JAKs and subsequent phosphorylation of the transcription factor STAT3. Activated STAT3 then forms dimers and translocates from the cytoplasm to the nucleus. In the nucleus, STAT3 activates the transcription of specific genes by binding to consensus DNA elements.

There are six essential alterations to normal cell physiology, which together define a cancer cell. These include: evasion of apoptosis, self-sufficiency in growth signals, insensitivity to growth-inhibitory signals, limitless replicative potential, tissue invasion and metastasis and sustained angiogenesis^[25]. STAT3 participates in the regulation of these processes^[26]. Particularly, STAT3 increases the expression of genes that are required for angiogenesis, uncontrolled proliferation and survival^[27]. These include genes such as anti-apoptotic genes (*Bcl-xL*, *Mcl1* and *survivin*), or genes involved in proliferation (*c-MYC*, *cyclin D1*) or angiogenesis (vascular endothelial growth factor). All

these proteins are associated with tumorigenesis and they are expressed in BE or EAC^[11,28-31].

In addition, STAT3 contributes to constitutive NF- κ B activation in tumor cells. Recent studies show that STAT3 prolongs NF- κ B nuclear retention through acetyltransferase p300-mediated RelA acetylation, thereby interfering with NF- κ B nuclear export and thus inducing permanent NF- κ B activation. Another important effect of STAT3 is that STAT3 negatively regulates the expression of tumor suppressor gene p53^[27]. Importantly, p53 activity can be restored in cells by inhibiting STAT3 signaling^[27].

STAT3 REGULATION

The activation of STAT3 is regulated by suppressors of cytokine signaling (SOCS) and protein inhibitors of activated STATs (PIASs). These proteins are often deregulated in different cancers. SOCS-3 negatively regulates activated receptor complexes by inactivating JAKs or by blocking recruitment sites for STAT3^[32]. It also target signaling complexes for ubiquitination and degradation. PIAS3 blocks the DNA-binding activity of STAT3 and inhibits STAT3-mediated gene activation^[33]. Silencing of SOCS3 expression due to aberrant methylation of the gene in various cell lines and cancers was reported by He *et al*^[34]. Inactivation of SOCS-3 is frequently observed also in dysplastic Barrett's esophagus and EAC due to promoter hypermethylation^[35]. In normal squamous epithelium and normal gastric mucosa, SOCS-3 methylation was not observed. The expression of PIAS3, another inhibitor of activated STAT3 protein, was also decreased in various cancers including prostate, colon, gastric or brain cancer^[36]. However, such studies have not been performed in BE or EAC.

INCREASE IN IL-6 ASSOCIATED WITH CANCER IN MALE

The reasons for the higher prevalence of BE in males are not clear. Similarly to esophageal adenocarcinoma, hepatocellular carcinoma (HCC) is more prevalent in the male population. Recently, Naugler *et al*^[37] identified a possible mechanism for this gender disparity in HCC. They found in a mouse model of HCC that administration of diethylnitrosamine induced an increase in serum IL-6 in males compared to females. In wild type animals the incidence of HCC was 100% in males and only 13% in females. In contrast IL-6^{-/-} males and females exhibited a similar very low incidence of HCC and longer survival^[37]. The absence of IL-6 resulted in almost complete inhibition of diethylnitrosamine-induced hepatocarcinogenesis. Their study indicated that estrogen mediated suppression of IL-6 is crucial in preventing hepatocellular carcinoma. Perhaps, a similar mechanism is involved in esophageal tumorigenesis, and that is why males are affected by this disease more often than women.

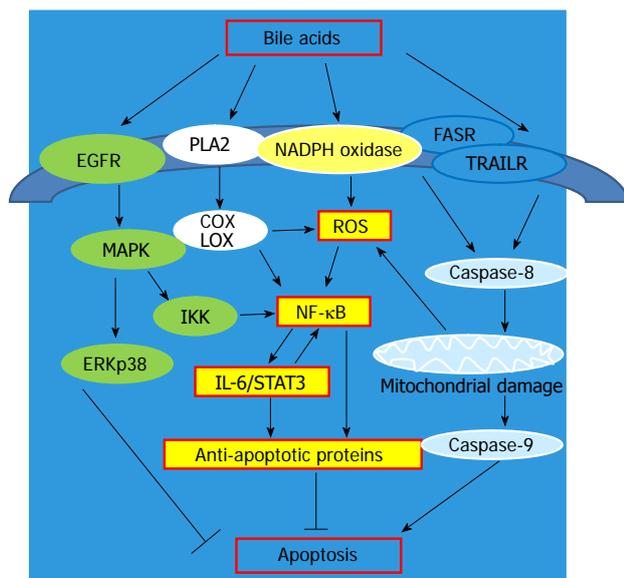


Figure 2 Apoptosis and the signaling pathways activated by bile acids. EGFR: Epidermal growth factor receptor; MAPK: Mitogen-activated protein kinase; IL-6: Interleukin 6; STAT3: Signal transducer and activator of transcription 3; COX: Cyclooxygenase; LOX: Lipoxygenase; IKK: IκB kinase; ROS: Reactive oxygen species; NF-κB: Nuclear factor kappa B; PLA2: Phospholipase A2; NADPH: Nicotinamide adenine dinucleotide phosphate; ERK: Extracellular-signal-regulated kinase; FASR: FAS receptor; TRAILR: TRAIL receptor.

BILE ACIDS AND APOPTOSIS RESISTANCE IN ESOPHAGEAL CANCER

Avoidance of apoptosis is one of the major characteristics of cancer^[25]. The normal squamous epithelium is exposed to low pH and/or hydrophobic bile acids during esophageal reflux. Although a short-term effect of bile acids is the induction of apoptosis, a long-term effect of repeated exposures to bile acids is a selection for cells resistant to apoptosis^[38]. These cells have a growth advantage in the presence of agents that ordinarily induce apoptosis and they proliferate to form a field of apoptosis resistant cells^[39].

Our data suggest that epithelial cells of Barrett's tissue are resistant to apoptosis induced by bile acids^[40]. These results are consistent with the reported increase in the expression of Mcl-1 and Bcl-xL in BE^[40] and studies suggesting that apoptosis resistance may lead to transformation from BE to adenocarcinoma^[41].

It is clear from many studies that bile acids activate both pro-survival and apoptotic pathways (Figure 2). The classic survival pathways induced by bile acids involve membrane perturbation, the activation of phospholipase A₂ and the synthesis of prostaglandins and leukotrienes, catalyzed by cyclooxygenase and lipoxygenase, with reactive oxygen species (ROS) as a byproduct^[42]. Bile acids also activate, in a ligand-independent manner, the epidermal growth factor receptor (EGFR) and receptors of the tumor necrosis factor superfamily (*e.g.*, FAS, TRAIL)^[43]. Activation of the EGFR pathway is generally pro-survival, whereas activation of the Fas and TRAIL pathways are pro-apoptotic.

Hydrophobic bile acids also generate ROS by activation of nicotinamide adenine dinucleotide phosphate-oxidase, phospholipase A₂, and by damaging mitochondria^[44]. Our studies showed that deoxycholic acid significantly increases levels of superoxide, hydrogen peroxide and peroxynitrite^[45]. Furthermore, we reported that human esophageal biopsies produce ROS after exposure to acidified medium containing bile acid cocktail^[46]. It was shown that in esophageal cells ROS produced by bile acids directly activate the redox sensitive transcriptional factor NF-κB^[47]. Consequently NF-κB upregulates production of different cytokines, such as IL-6, which leads to an increase in STAT3 signaling and expression of anti-apoptotic and prosurvival proteins. Indeed, a recent study showed that IL-6 and activated STAT3 were increased in transformed Barrett's cells (transfected with H-ras and p53 siRNA)^[17].

In addition, Quante *et al.*^[48] recently developed L2-IL-1b transgenic mouse model of BE/EAC. In this model human IL-1b is overexpressed in mouse esophagus and forestomach to mimic chronic esophageal inflammation. Furthermore, L2-IL-1b mice were fed 0.2% deoxycholic acid to accelerate the development of BE and EAC. Interestingly, when these transgenic mice were crossed with IL-6^{-/-} mice no metaplastic and/or dysplastic lesions were found^[48]. These studies confirm our previous results indicating the importance of IL-6 in BE/EAC development.

DISCUSSION

This review provides an evidence for a strong link between chronic inflammation, IL-6, STAT3 activation and esophageal carcinogenesis. IL-6 is a cytokine that is frequently increased in different cancers. Bile and gastric acid in the refluxate are two of the major factors involved in the pathogenesis of BE. We hypothesize that repeated exposure of esophageal tissue to bile acids leads to IL-6 upregulation, increased activation of STAT3, apoptosis resistance and cancer development. This hypothesis is consistent with the studies demonstrating that tissue biopsies from BE patients (1) secrete large amounts of IL-6; (2) are resistant to apoptosis induced by bile acids; and (3) express activated STAT3 and anti-apoptotic proteins regulated by IL-6/STAT3 signaling, Bcl-xL and Mcl-1^[11,16,40].

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Is it worth investigating splenic function in patients with celiac disease?

Antonio Di Sabatino, Laura Brunetti, Gabriella Carnevale Maffè, Paolo Giuffrida, Gino Roberto Corazza

Antonio Di Sabatino, Laura Brunetti, Gabriella Carnevale Maffè, Paolo Giuffrida, Gino Roberto Corazza, Department of Internal Medicine, Celiac Centre, S. Matteo Hospital Foundation, University of Pavia, 27100 Pavia, Italy

Author contributions: All authors synthesized ideas and wrote the paper.

Correspondence to: Dr. Antonio Di Sabatino, Department of Internal Medicine, Celiac Centre, S. Matteo Hospital Foundation, University of Pavia, Piazzale Golgi 5, 27100 Pavia, Italy. a.disabatino@smatteo.pv.it

Telephone: +39-382-501596 Fax: +39-382-502618

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Abstract

Celiac disease, an immune-mediated enteropathy induced in genetically susceptible individuals by the ingestion of gluten, is the most frequent disorder associated with splenic hypofunction or atrophy. Defective splenic function affects more than one-third of adult patients with celiac disease, and it may predispose to a higher risk of infections by encapsulated bacteria and thromboembolic and autoimmune complications, particularly when celiac patients have concomitant pre-malignant and malignant complications (refractory celiac disease, ulcerative jejunoileitis and enteropathy-associated T-cell lymphoma). However, the clinical management of patients with celiac disease does not take into account the evaluation of splenic function, and in patients with high degree of hyposplenism or splenic atrophy the prophylactic immunization with specific vaccines against the polysaccharide antigens of encapsulated bacteria is not currently recommended. We critically re-evaluate clinical and diagnostic aspects of spleen dysfunction in celiac disease, and highlight new perspectives in the prophylactic management of infections in this condition.

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Key words: Hyposplenism; Memory B cell; Pitted red cell; Pneumococcal vaccine; Splenic atrophy

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INTRODUCTION

Functional hyposplenism has been regarded as an acquired disorder, potentially associated with several diseases, sometimes accompanied by a reduction in spleen size, and burdened by the same complications occurring in surgical asplenia^[1,2]. The spleen, apart from acting as a phagocytic filter, thus removing ageing and damaged cells, is crucial in regulating immune homeostasis by linking innate and adaptive immunity, and in protecting against infections by encapsulated bacteria^[3-5].

Removal of encapsulated bacteria in the course of initial infection requires natural antibodies produced by immunoglobulin M (IgM)-memory B cells, a unique B cell population resident in the marginal zone of the spleen, which, unlike switched-memory, are responsible for a T-independent response against bacteria^[6-9]. The key role of the spleen in mounting an immune response against encapsulated bacteria is supported by the dramatic reduction of the IgM-memory B cell pool following removal of the spleen^[10,11]. An impairment of the immune function of the spleen results in (1) reduced number of IgM-memory B cells and defective activity of opsonizing molecules, *i.e.*, properdin and tuftsin, thus predisposing to infections caused by encapsulated bacteria (mainly *Streptococcus pneumoniae*, *Neisseria meningitidis* and *Haemophilus influenzae*); and (2) decreased number of marginal zone B cells which predisposes to the emergence of autoreactive T-cell clones as a consequence of T-regulatory cells depletion, with

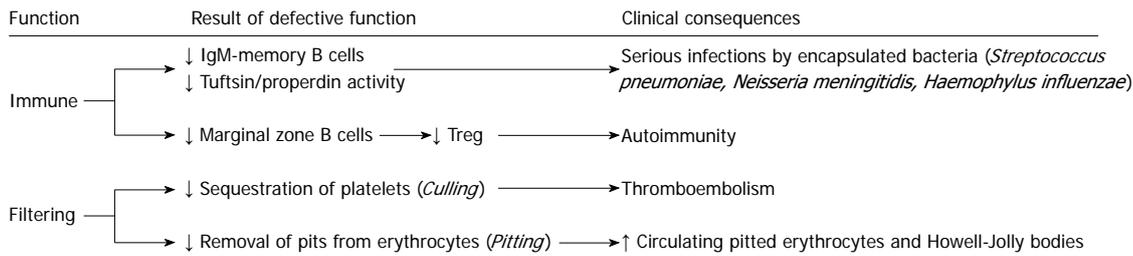


Figure 1 Schematic representation of immune and filtering function of the spleen. IgM: Immunoglobulin M; Treg: T-regulatory cells.

| Table 1 Case reports of hyposplenism-related infections in patients with celiac disease | | | |
|---|--------------|---|------------------------|
| Ref. | No. of cases | Type of infection | Supplementary findings |
| Corazza <i>et al</i> ^[23] | 1 | Pneumococcal pneumonia | Splenic atrophy |
| Matuchansky <i>et al</i> ^[24] | 2 | Pneumococcal pneumonia, infectious pericarditis | Splenic atrophy, MLNC |
| O'Donoghue ^[25] | 1 | Pneumococcal septicemia | Splenic atrophy |
| Logan <i>et al</i> ^[26] | 2 | Pneumococcal meningitis, septicemia by <i>Salmonella</i> | Splenic atrophy |
| Stevens <i>et al</i> ^[27] | 3 | Lung abscesses by <i>Staphylococcus</i> and <i>Klebsiella</i> | Splenic atrophy |
| Howat <i>et al</i> ^[28] | 2 | Fatal chest infection, septicemia | Splenic atrophy, MLNC |
| Harmon <i>et al</i> ^[29] | 1 | Septicemia by <i>Klebsiella</i> | Splenic atrophy |

MLNC: Mesenteric lymph node cavitation.

subsequent development of autoimmunity^[8,9,12]. On the other hand, an impairment of the filtering function results in (1) reduced platelet sequestration, which predisposes to thromboembolism; and (2) defective removal of pits from erythrocytes with consequent increase of circulating Howell-Jolly bodies and pitted red cells (Figure 1)^[1-3].

Detection of pitted red cells by phase-interference microscopy^[13] is considered an accurate method to assess splenic function, quite easy to perform, less expensive and invasive than radioisotopic methods, and more accurate than Howell-Jolly bodies detection^[14], particularly in the quantitation of mild degrees of hyposplenism^[3]. The inverse correlation observed in asplenic and hyposplenic patients between pitted red cells and IgM-memory B cells suggests the possible use in clinical practice of flow cytometric B cell analysis as a quantitative alternative test^[15]. The little we know about the natural history of hyposplenism leads us to believe that it evolves from a reversible mild impairment of splenic function - as occurs in responder Crohn's disease patients after anti-tumor necrosis factor treatment with infliximab^[16] - to an irreversible impairment of splenic function, up to severe splenic atrophy.

Among all the various diseases associated with hyposplenism, celiac disease is the most frequent^[2,17]. Hyposplenism, assessed by pitted red cell counting, affects more than one-third of celiac patients^[18]. Defective filtering function, measured by pitted red cell counting, is paralleled by a defect in the frequency of circulating IgM memory B cells and serum tuftsin activity, and both these parameters significantly correlate with the degree of splenic function in untreated celiac disease^[18,19]. Hyposplenism does not complicate celiac disease in infancy^[20]; in adults its incidence correlates with the duration of pre-exposure to gluten as shown by the correlation with age at diagnosis^[18], and a gluten-free diet is effective in restoring splenic func-

tion^[21]. When the data are split according to clinical severity, the prevalence of hyposplenism increases from 19% to 80% in celiacs with premalignant or malignant complications^[19]. Both splenic atrophy and mesenteric lymph node cavitation are recognised as poor prognostic factors in celiac disease^[19,22,23].

INFECTION

Major infections have been reported in a number of hyposplenic celiac patients in the last 25 years, variably associated with spleen atrophy and mesenteric lymph node cavitation (Table 1)^[23-29]. However, it was only in 2008 that two *ad hoc* studies highlighted the importance of this predisposition by showing a significantly higher relative risk of pneumococcal sepsis in adult celiacs, which is still significant when hospitalised patients are considered as reference individuals^[30,31]. The absolute risk of sepsis turned out to be even higher than that of hip fracture and lymphoma in the celiac cohort^[32]. These findings fit with the demonstration of an increased mortality due to infections (in particular septicemia) and respiratory diseases (mainly pneumonia) in the Swedish celiac cohort^[33].

Although anti-pneumococcal vaccination has been shown to reduce the prevalence of major infections in asplenic patients^[34-38], it is dramatically underused as shown by these data collected in England and Wales by examining 3584 patients with celiac disease or sickle cell anemia^[39]. Vaccines currently used in patients at risk of pneumococcal infections are the 23-valent pneumococcal polysaccharide vaccine^[40], whose protective action is based on the production of opsonising anti-capsular antibodies by means of a T-independent mechanism (it is actually recommended in asplenic/hyposplenic adults and children older than 5 years), and the 13-valent protein-

Table 2 Traditional polysaccharide and new conjugate anti-pneumococcal vaccines used in the prophylactic management of asplenic/hyposplenic patients

| Vaccine | Brand name | Structure | Mechanism | Serotype | Indication |
|---------|------------|------------------------------------|--------------------|---|---|
| PPV23 | Pneumovax® | Polysaccharide | T-cell independent | 1, 2, 3, 4, 5, 6B, 7F, 8, 9N, 9V, 10A, 11A, 12F, 14, 15B, 17F, 18C, 19A, 19F, 20, 22F, 23F, 33F | Asplenic or hyposplenic adults Asplenic or hyposplenic children > 5 yr |
| PCV13 | Prevnar® | Protein-conjugate (CRM197 protein) | T-cell dependent | 1, 3, 4, 5, 6A, 6B, 7F, 9V, 14, 18C, 19A, 19F, 23F | Asplenic or hyposplenic children < 5 yr |

PPV: Pneumococcal polysaccharide vaccine; PCV: Protein-conjugate pneumococcal vaccine.

conjugate pneumococcal vaccine (PCV-13, Pevnar)^[41], in which the CRM197 diphtheria protein changes the nature of the response from T-independent to T-dependent, making this vaccine particularly suitable in infants, especially below the age of 2 years, when the splenic IgM-memory B cell pool is still immature (Table 2)^[42-45]. Similarly, adult hyposplenic patients, in whom IgM-memory B cell are depleted, would benefit from PCV-13, as its T-dependent mechanism is supposed to bypass the immunological impairment due to the lack of IgM-memory B cells. Nevertheless, Pevnar is recommended by current guidelines only in infancy (Table 2)^[46].

AUTOIMMUNITY

Celiac disease is frequently associated with a number of autoimmune disorders, including Hashimoto's thyroiditis, insulin-dependent diabetes mellitus, Sjögren's syndrome, Addison disease, systemic lupus erythematosus, rheumatoid arthritis^[47,48]. The evidence that autoantibodies may develop within months of splenectomy^[49], together with the demonstration that celiac patients with blood film features of hyposplenism have a higher prevalence of autoantibodies^[50], have led to the hypothesis that defective splenic function might predispose the development of autoimmunity in celiac disease^[51,52].

The nature of the link between hyposplenism and autoimmune manifestations of celiac disease is not known, and it is not clear whether autoimmune disorders precede and cause splenic hypofunction or atrophy, or vice versa, or whether additional factors influence both conditions. The finding that hyposplenism in nonceliac patients with autoimmune disorders did not differ significantly from that of healthy controls supports the hypothesis that the higher risk for splenic hypofunction in celiac patients with autoimmune disorders might be related to celiac disease, rather than to autoimmunity *per se*^[19]. Of note, both hyposplenism and autoimmune disorders increase their prevalence with the length of pre-exposure to gluten in celiac disease^[18,53]. When we looked at the prevalence of celiac disease-associated hyposplenism, we found that it increases from 19% in uncomplicated patients to 59% in those with associated autoimmune diseases. Moreover, patients with celiac disease-associated autoimmune disorders have a significantly lower percentage of IgM memory B cells in comparison to uncomplicated celiac patients^[19]. This finding is quite interesting when considering that memory B cells resident in the marginal zone

of the spleen play a role in the tolerance of autoantigens through the B cell receptor^[54]. A similar role is exerted by marginal zone dendritic cells which internalise apoptotic leucocytes thus preventing autoantigens exposed on the surface of apoptotic bodies from causing autoantibody formation^[55]. Moreover, both marginal zone B cells and dendritic cells may favour the expansion of regulatory T cells which maintain tolerance through the up-regulation of anti-inflammatory cytokines, such as transforming growth factor- β and interleukin-10^[56]. The perturbation of these regulatory mechanisms have been shown to predispose to the development of autoimmunity in splenectomised or hyposplenic subjects^[19,49,57].

THROMBOEMBOLISM

Impaired spleen sequestration of circulating platelets and increased blood viscosity are supposed to be implicated in the development of thromboembolic events in splenectomised patients or in other hyposplenism-associated disorders^[58]. In the latter, the risk of thrombosis is difficult to assess as many of these disorders are associated with increased incidence of thrombosis *per se*. The hyperviscosity secondary to defective splenic function may be the result in part of the persistence of aged and damaged red cells in the circulation as well as intracellular inclusions, such as Howell-Jolly bodies, siderotic granules, and Heinz bodies, all of which promote decreased erythrocyte deformability^[59]. An increased risk of thromboembolism has been recently demonstrated in celiac disease, where it correlates with the duration of pre-exposure to gluten^[60]. However, in that study no data is available concerning the weight of the thromboembolic risk in the hyposplenic celiac patients, nor hyposplenism is mentioned among the possible factors predisposing to thromboembolism.

CONCLUSION

There is a number of critical issues that remain to be elucidated to define the optimal management of hyposplenic celiac patients and to clarify the pathogenic mechanisms underlying spleen hypofunction^[61,62]. We propose that splenic function is determined in patients with pre-malignant and malignant complications, concomitant autoimmune disorders, old age at diagnosis, previous history of major infections/sepsis or thromboembolism, mesenteric lymph node cavitation and/or spleen atrophy (Table 3). As a diagnostic tool, pitted red cell counting remains an accu-

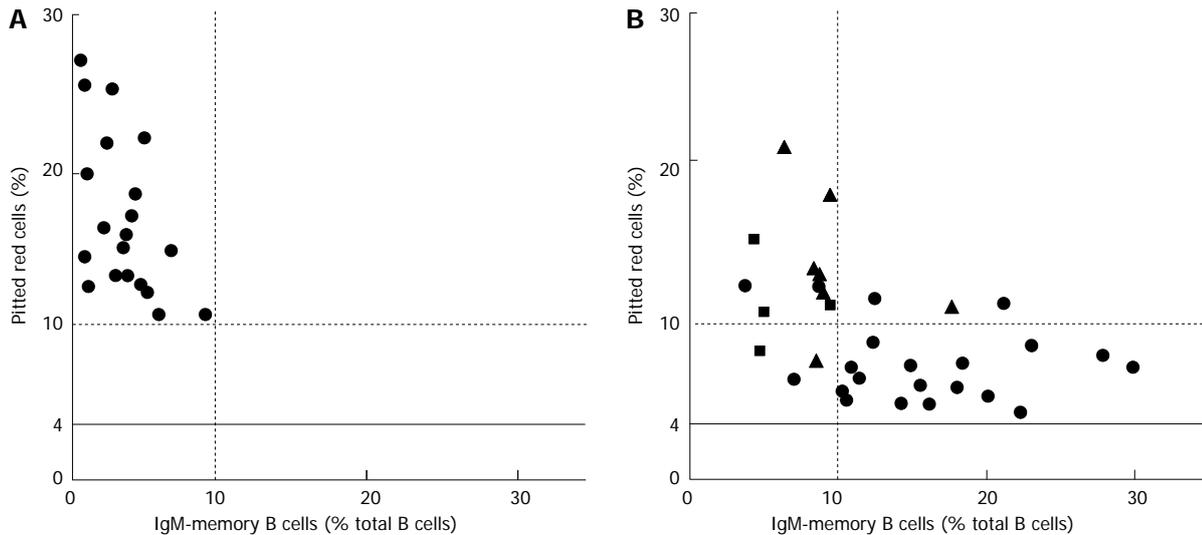


Figure 2 Correlation between circulating pitted red cells and immunoglobulin M memory B cells in splenectomised patients (A) and hyposplenic celiac patients (B). Among the latter, those affected by pre-malignant or malignant complications of celiac disease are indicated with a square, those affected by concomitant autoimmune disorders are indicated with a triangle. IgM: Immunoglobulin M.

| Table 3 Celiac patients in whom splenic function should be assessed |
|--|
| Patients with complications (RCD, UJI, EATL, collagenous sprue) |
| Patients with concomitant autoimmune disorders |
| Patients with old age at diagnosis |
| Patients with previous history of major infections/sepsis and/or thromboembolism |
| Patients with mesenteric lymph node cavitation and/or splenic atrophy |

EATL: Enteropathy-associated T-cell lymphoma; RCD: Refractory celiac disease; UJI: Ulcerative jejuno-ileitis.

rate, quantitative and inexpensive method, albeit observer-dependent^[63]. Flow cytometric analysis of memory B cells could be an alternative quantitative test, although studies assessing its sensitivity and specificity are lacking^[64]. We believe that protein-conjugate vaccines^[65-68] should be recommended in patients with major hyposplenism, defined -on the basis of data derived from asplenic patients- by a pitted red cells value higher than 10% and/or an IgM memory B cell frequency lower than 10%. Of note, most of the patients identified by these parameters are refractory or have concomitant autoimmune disorders (Figure 2). Understanding the pathogenic mechanisms underlying spleen dysfunction in celiac disease requires a greater knowledge of the connections between gut and spleen. The demonstration that spleen function is crucial for the presence of IgA-producing plasma cells in the gut of both asplenic mice and patients^[69], and that oral tolerance to gluten is predominantly mounted in the spleen^[70] represent preliminary attempts in this direction.

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Effect of biliary drainage on inducible nitric oxide synthase, CD14 and TGR5 expression in obstructive jaundice rats

Zi-Kai Wang, Jian-Guo Xiao, Xue-Fei Huang, Yi-Chun Gong, Wen Li

Zi-Kai Wang, Wen Li, Department of Gastroenterology and Hepatology, the General Hospital of the Chinese People's Liberation Army, Beijing 100853, China

Jian-Guo Xiao, Critical Care Medicine, the General Hospital of the Chinese People's Liberation Army, Beijing 100853, China

Xue-Fei Huang, Department of Cadre Health Care, the Navy General Hospital of the Chinese People's Liberation Army, Beijing 100048, China

Yi-Chun Gong, Intensive Care Unit, the 309th Hospital of the Chinese People's Liberation Army, Beijing 100091, China

Author contributions: Li W designed the research; Wang ZK, Xiao JG, Huang XF and Gong YC performed the majority of experiments and contributed equally to this work; Wang ZK and Li W wrote the paper.

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Correspondence to: Wen Li, MD, PhD, Department of Gastroenterology and Hepatology, the General Hospital of the Chinese People's Liberation Army, No. 28, Fuxing Road, Beijing 100853, China. liwencn2000@126.com

Telephone: +86-10-55499107 Fax: +86-10-88626386

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Abstract

AIM: To investigate the effect of biliary drainage on inducible nitric oxide synthase (iNOS), CD14 and TGR5 expression in rats with obstructive jaundice (OJ).

METHODS: Male adult Sprague-Dawley rats were randomly assigned to four groups: OJ, sham operation (SH), internal biliary drainage (ID) and external biliary drainage (ED). Rat models were successfully established by two operations and succumbed for extraction of Kupffer cells (KCs) and liver tissue collection on the 8th and 15th day. KCs were isolated by *in situ* hepatic perfusion and digested with collagen IV, density gradient centrifuged by percoll reagent and purified by cell culture attachment. The isolated KCs were cultured with the endo-

toxin lipopolysaccharide (LPS) with and without the addition of ursodeoxycholic acid (UDCA). The expression of iNOS, CD14 and bile acid receptor-TGR5 protein in rat liver tissues was determined by immunohistochemistry. The expression of iNOS and CD14 messenger RNA (mRNA) on the isolated KCs was detected by reverse transcription polymerase chain reaction (PCR) and the TGR5 mRNA level in KCs was measured by real-time quantitative PCR.

RESULTS: The iNOS protein was markedly expressed in the liver of OJ rats, but rare expressed in SH rats. After relief of OJ, the iNOS expression was decidedly suppressed in the ID group (ID *vs* OJ, $P < 0.01$), but obviously increased in rats of ED (ED *vs* OJ, $P = 0.004$). When interfered only with LPS, the expression of iNOS mRNA by KCs was increased in the OJ group compared with the SH group ($P = 0.004$). After relief of biliary obstruction, the iNOS mRNA expression showed slight changes in the ED group (ED *vs* OJ, $P = 0.71$), but dropped in the ID group (ID *vs* OJ, $P = 0.001$). Compared with the simple intervention with LPS, the expressions of iNOS mRNA were significantly inhibited in all four groups after interfered with both LPS and UDCA ($P < 0.01$, respectively). After bile duct ligation, the CD14 protein expression in rat liver was significantly strengthened (OJ *vs* SH, $P < 0.01$), but the CD14 mRNA level by KCs was not up-regulated (OJ *vs* SH, $P = 0.822$). After relieving the OJ, the expression of CD14 protein was reduced in the ID group (ID *vs* OJ, $P < 0.01$), but not reduced in ED group (ED *vs* OJ, $P = 0.591$). And then the CD14 mRNA expression was aggravated by ED (ED *vs* OJ, $P < 0.01$), but was not significantly different between the ID group and the SH and OJ groups (ID *vs* SH, $P = 0.944$; ID *vs* OJ, $P = 0.513$, respectively). The expression of TGR5 protein and mRNA increased significantly in OJ rats (OJ *vs* SH, $P = 0.001$, respectively). After relief of OJ, ID could reduce the expression of TGR5 protein and mRNA to the levels of SH group (ID *vs* SH, $P = 0.22$ and $P = 0.354$, respectively), but ED could not (ED *vs* SH, $P = 0.001$, respectively).

CONCLUSION: ID could be attributed to the regulatory function of activation of KCs and release of inflammatory mediators.

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Key words: Obstructive jaundice; Biliary drainage; Kupffer cells; CD14; TGR5; Ursodeoxycholic acid

Core tip: To date, there are still controversies over whether and how to perform preoperative biliary drainage in patients with malignant or benign obstructive jaundice (OJ), even though the complication-related mortality rate for OJ patients was high after surgery. Internal biliary drainage could reverse the raised expression of inducible nitric oxide synthase and CD14 both in protein and messenger RNA levels in obstructive jaundice rat models, but external drainage could not. The mechanism of internal biliary drainage superior to external drainage in relief of obstructive jaundice might be attributed to the regulatory function of activation of Kupffer cells and release of inflammatory mediators.

Wang ZK, Xiao JG, Huang XF, Gong YC, Li W. Effect of biliary drainage on inducible nitric oxide synthase, CD14 and TGR5 expression in obstructive jaundice rats. *World J Gastroenterol* 2013; 19(15): 2319-2330 Available from: URL: <http://www.wjg-net.com/1007-9327/full/v19/i15/2319.htm> DOI: <http://dx.doi.org/10.3748/wjg.v19.i15.2319>

INTRODUCTION

To date, there are still controversies over whether and how to perform preoperative biliary drainage in patients with malignant or benign obstructive jaundice (OJ), even though the complication-related mortality rate for OJ patients was high after surgery. One of the controversies is the necessity of relieving biliary obstruction before surgery^[1-4]. Some investigators suggested that preoperative biliary drainage should not be routinely performed in OJ patients planned for surgery due to the complications associated with the procedure itself, which might outweigh the potential benefit of it^[1,2]. On the contrary, other investigators confirmed the effect of preoperative biliary drainage in reducing the postoperative morbidity and mortality, complications of infection and hospital stay in patients with OJ^[3,4]. The second debate is which is the appropriate drainage method, internal biliary drainage (ID) or external biliary drainage (ED)^[5-8]? Some studies suggested that ID (*e.g.*, biliary stent endoprosthesis) could recover the enterohepatic circulation of bile acid, improve the intestinal barrier function and reduce endotoxin-related complications more obviously than ED, and therefore, ID may contribute to the early recovery and improve the patients' life quality compared with ED^[5,6]. On the contrary, some systematic reviews reported that

ED (*e.g.*, percutaneous transhepatic biliary drainage, endoscopic nasobiliary drainage and T-tube drainage) was superior to ID, because ID could increase the risk of retrograde cholangitis and had lower diagnostic value than ED^[7]. Moreover, ED is better than ID concerning the recovery of cellular immunity and liver inflammation in the short term after relief from biliary obstruction^[8].

Kupffer cells (KCs) as the resident liver macrophages, constitute a vital component of the reticuloendothelial system, and KCs are critically involved in the pathogenesis of OJ by acting as antigen presenting cells and producing many endotoxin-related inflammatory mediators. So we carried out a series of experimental studies based on the immune function of KC in OJ rat model to address those questions. Our previous experimental studies found that KC from rats with OJ could produce large amounts of endotoxin-mediated nitric oxide and ID was superior to ED in reversing the distorted nitric oxide due to the regulation of inducible nitric oxide synthase (iNOS), messenger RNA (mRNA)^[9,10]. ID could reverse the serum levels of endotoxin and proinflammatory cytokines, such as tumor necrosis factor- α (TNF- α) and interleukin-6 (IL-6), but ED could not^[10]. Although our earlier studies demonstrated the necessity of relieving obstruction preoperatively and the advantages of ID in relief of OJ in contrast with ED in animal models, but the mechanism is still unclear.

Lipopolysaccharide (LPS, endotoxin) as an abundant component of the cell wall of gram-negative bacteria, could provoke a generalized pro-inflammatory response and stimulate the production of pro-inflammatory mediators through the activation of KC in patients with OJ^[11]. Recent investigations showed that CD14, one of the most important LPS receptors, could play an important role in the activation of KC and LPS-mediated liver injury^[12,13]. We proposed a hypothesis that CD14 as a receptor of endotoxin might play an important role in the immune suppression in OJ, and related to the effects of biliary drainage.

Bile acids could successfully reduced endotoxin related complications following surgery in patients with OJ, and the immunomodulatory function of bile acids is obtaining more attention^[14,15]. Recently, the plasma membrane bound, G-protein coupled bile acid receptor TGR5 (Gpbar-1, M-Bar) has been first described by two separate research groups^[16,17]. TGR5 is highly expressed in CD14-positive monocytes/macrophages^[16]. Studies from Keitel *et al*^[18] demonstrated that TGR5 was localized in the plasma membrane of isolated KC, and bile acids could alter macrophage function by affecting phagocytic activity and inhibiting LPS-induced cytokines expression in KC *via* TGR5-cAMP dependent pathways. The immunoreactivity of TGR5 in KC was increased in rat livers following bile duct ligation, suggesting that TGR5 may play a protective role in OJ preventing excessive cytokine production, thereby reducing liver injury.

This study aims to detect the protein expression of iNOS, CD14 and TGR5 in liver and the mRNA expression in isolated KCs, and to investigate the immunomod-

ulatory effect of ursodeoxycholic acid (UDCA) in terms of the expression of iNOS mRNA after relief of OJ by ID and ED in rats, in order to further explore the mechanism whether ID is superior to ED in relief of OJ.

MATERIALS AND METHODS

Animals

Two hundred and forty adult male Sprague-Dawley rats weighing 270-350 g were used in the study. All animals were purchased from the Laboratory Animal Center of Academy of Military Medical Sciences [License: SCXK (Jun) 2007-004] and housed in the Experimental Animal Service Center of the Chinese People's Liberation Army General Hospital. This study was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The animal use protocol has been reviewed and approved by the Institutional Animal Care and Use Committee of the General Hospital of the Chinese People's Liberation Army. Rats were fed with a standard diet of commercial rat chow and tap water *ad libitum*. The experiment was approved by the Animal Research Ethics Committee of the General Hospital of the Chinese People's Liberation Army.

Animal models

Animal models were induced using a modified method in our previous study^[19]. In brief, rats were randomly assigned to four groups: OJ, sham operation (SH), ID and ED groups. All procedures were performed under anesthesia with 1.5% isoflurane and 98.5% oxygen using a delivery and scavenging system designed in our laboratory^[19]. Rats were subjected to laparotomy twice every seven days. OJ was induced by common bile duct ligation and SH was produced by separating bile duct locally but not dividing. ID was performed by implanting a drainage tube between the dilated end of common bile duct and duodenum, while ED was performed by exteriorizing a drainage tube at the nape of the rat. The rat models were succumbed for extraction of KC and liver tissue collection on the 8th and 15th day.

Hematoxylin and eosin staining

The caudate lobe of the liver was removed and the blood vessels were tied off for facilitating the isolation of KC in site perfusion. Liver tissues were fixed immediately in 10% neutral buffered formalin and paraffin-embedded blocks were made. Serial sections 5 μ m thick were stained with hematoxylin and eosin for evaluation of portal inflammation, hepatocellular necrosis and inflammatory cell infiltration. Sections were examined under a light microscope (CMS800, Olympus, Tokyo, Japan).

Immunohistochemistry and morphometry

The immunohistochemical staining was performed on sections of liver samples. Briefly, 5 μ m paraffin sections were deparaffinized with xylene and rehydrated in

a gradient of ethanol solutions. Antigen retrieval was carried out by microwave heating the sections for 20 min in citric acid buffer, and then cooling for 15 min at room temperature (RT). Endogenous peroxidase activity was quenched with 3% hydrogen peroxide at RT for 10 min. Nonspecific binding was blocked by incubating the sections for 10-15 min in the normal goat serum (5%, 100-150 μ L). The sections were incubated overnight at 4 °C with the anti-iNOS antibody (rabbit polyclonal antibody against iNOS; Santa Cruz, CA, United States) at a dilution of 1:200, the anti-CD14 antibody (rabbit polyclonal antibody against CD14, BA0719, Boster Biotechnology, Wuhan, China) at a dilution of 1:200 and the anti-TGR5 antibody (rabbit polyclonal antibody against TGR5, SC-98888, Santa Cruz, CA, United States) at a dilution of 1:100, respectively. Following several rinses in phosphate buffered solution, the sections were incubated with the biotinylated goat anti-rabbit immunoglobulin G antibody (Zhongshan Jinqiao Biotechnology, Beijing, China) for 30 min at 37 °C. Finally, the sections were colored with DAB at RT for 1-15 min, counterstained with hematoxylin for 30 s, dehydrated through gradient ethanol, cleared in xylene and then mounted with permount. Images were obtained using a light microscope (CMS800; Olympus, Tokyo, Japan). Immunoreactivity of iNOS, CD14 and TGR5 in rat liver was morphometrically identified by the Image Pro Plus 6.0 image analysis software system (Media Cybernetics, MD, United States).

Isolation and treatment of KCs

KCs were isolated and purified as previously described by a combination of Percoll gradient centrifuging and traditional attachment method^[9]. Briefly, non-parenchymal liver cells were dispersed by retrograde *in situ* collagenase perfusion from the inferior vena cava to the portal vein by Leffert's solution with collagenase IV (0.2 mg/mL; Sigma, NY, United States). KCs were purified by centrifugation through the two bands of Percoll gradients, and suspended in RPMI-1640 culture media (Gibco, Carlsbad, CA, United States) containing 1% penicillin/streptomycin and 10% heat-inactivated fetal bovine serum. Moreover, KCs were inoculated into the cell culture dish (Corning, NY, United States) in a humidified incubator with 5% CO₂ and 95% air at 37 °C. After 3 h cell culture, the non-adherent cells were washed away with a warm Hanks balanced salt solution, and then KCs attached to the bottom of the dish were used for TGR5 mRNA measurement immediately, cultured continuously for 18 h with LPS at a final concentration of 10 ng/mL for CD14 mRNA detection, and cultured with LPS (10 ng/mL) and LPS (10 ng/mL) + UDCA (0.1 mmol/L) respectively for iNOS mRNA detection^[20,21]. Viability of KCs was assessed with trypan blue exclusion test and purity was confirmed by peroxidase staining^[9].

Reverse transcription polymerase chain reaction

The iNOS mRNA expression was measured as described in our previous experiment^[10]. Briefly, after the isolated

KCs were cultured with LPS (10 ng/mL) or LPS (10 ng/mL) + UDCA (0.1 mmol/L) *in vitro* for 18 h, the expression levels of iNOS mRNA by KCs in these four groups were detected by reverse transcription polymerase chain reaction (RT-PCR).

Measurement of CD14 mRNA

Semiquantitative analysis of CD14 mRNA by KC was detected by RT-PCR after the isolated KCs were cultured with endotoxin (10 ng/mL) *in vitro* for 18 h. Total RNA was extracted by TRIzol reagent from Invitrogen and 1 µg RNA was primed with oligo (dT) using a reverse transcriptase kit from Promega according to the manufacturer's instructions. The sequences of CD14 primer were derived from the published *CD14* gene sequences: CD14 mRNA-sense: 5'-CTCAACCTAGAGCCGTTTCT-3', CD14 mRNA-antisense: 5'-CAGGA TTGTCAGA-CAGGTCT-3'; β-actin-sense: 5'-ATCATGTTGAGACCTTCAACA -3', β-actin-antisense: 5'-CATCTCTTGCTCGAAGTCCA-3^[11]. Two µL cDNA production from RT-PCR reaction system was amplified in an automated thermocycler from Eppendorf. The conditions for amplification were as follows: pre-denaturation for 5 min at 94 °C for 1 cycle; denaturation for 1 min at 94 °C, annealing for 1 min at 58 °C and extension for 1 min at 72 °C for a total of 35 cycles of PCR, followed by a final extension for 7 min at 72 °C for 1 cycle. The PCR products were electrophoresed in 1.5% agarose gels containing ethidium bromide and reviewed under the ultraviolet light with the gel documentation system (UVP, Cold-spring, Wilmington, DE, United States). Band intensity of each sample was determined using Glow Discharge Spectroscopy image analysis software (Cold-spring, Wilmington, DE, United States).

Measurement of TGR5 mRNA

Quantitative analysis of TGR5 mRNA was performed by RTQ-PCR. Total RNA from KCs was extracted using TRIzol reagent (Invitrogen, Carlsbad, CA, United States) according to the manufacturer's instructions. The quality and quantity of RNA were assessed using 1% agarose gel electrophoresis and spectrophotometric analysis of 260/280 ratios, and then RNA was stored at -70 °C prior to analysis. RNA was reversely transcribed with oligo (dT) primer using a reverse transcriptase kit (Promega, Madison, WI, United States). The resulting cDNA was detected using SYBR Green I dye (Qiagen GmbH, Hilden, Germany) and amplified using the BIO-ER Linegene-3320 system (Hangzhou Bioer Technology, China). Thermocycling conditions for RTQ-PCR were 1 cycle at 95 °C for 2 min and 45 cycles at 95 °C for 20 s, 60 °C for 25 s and 72 °C for 30 s. The primers were designed by Primer Premier 5.0 and Oligo 6.0 based on GeneBank and β-actin was used as an internal reference gene to normalize the transcript levels. The primer sequences were as follows: TGR5-sense: 5'-CCTG-GACCGCCACTTACG-3', TGR5-antisense: 5'-CCCT-GTGAGTAGCCCAGCTAGT-3'; β-actin-sense: 5'-C

Table 1 Changes of body weight after operation in the four groups (g) (mean ± SD)

| Day | Body weight | | | |
|-----|----------------|----------------|----------------|----------------|
| | OJ (n = 32) | SH (n = 30) | ID (n = 35) | ED (n = 29) |
| D1 | 297.65 ± 13.95 | 296.90 ± 20.04 | 300.60 ± 19.29 | 301.90 ± 20.65 |
| D8 | 299.70 ± 17.95 | 326.10 ± 30.16 | 307.60 ± 19.38 | 306.90 ± 29.89 |
| D15 | 312.10 ± 30.11 | 360.20 ± 20.27 | 333.65 ± 28.12 | 281.15 ± 29.99 |

D1: The 1st day; D8: The 8th day; D15: The 15th day; ED: External biliary drainage; ID: Internal biliary drainage; OJ: Obstructive jaundice; SH: Sham operation.

CCATCTATGAGGGTTACGC-3', β-actin-antisense: 5'-TTTAATGTCACG CACGATTTTC-3'. The relative mRNA levels of TGR5 were measured according to the 2^{-ΔΔCT} method.

Statistical analysis

Continuous data were expressed as mean ± SD. The one-way analysis of variance, Student Newman Keuls-*q* test or nonparametric test of K independent sample were used for the continuous data. A *P* value less than 0.05 was considered statistically significant. All statistical analyses of the experimental data were performed with SPSS 17.0 software (Chicago, IL, United States).

RESULTS

Morbidity and mortality

After bile duct ligation, the main cause of rat death was biliary leakage. During and after the biliary drainage procedures, hemorrhage and dehydration were the main death reasons. Finally, 126 rats were enrolled into this study and divided into four groups: OJ (*n* = 32), SH (*n* = 30), ID (*n* = 35) and ED (*n* = 29) groups. Rats were kept under veterinary care and body weights were measured on the 1st, 8th and 15th day before laparotomy (Table 1). After bile duct ligation, skin stained yellow and lethargy were observed in OJ rat models. OJ rats ate markedly less food and there was a slowly increasing trend of body weight between the 1st, 8th and 15th day (*P* = 0.155). On the contrary, weight gain was more significant in SH rats compared with that in OJ rats both on the 8th and 15th day (*P* < 0.01). After relief of OJ, the appetite of ID rats recovered and the body weight of ID rats on the 15th day was significantly higher than that on the 8th day (*P* < 0.01). On the contrary, weight loss was observed in ED rats and the body weight on day 15 was significantly lower than that on the 1st or 8th day (*P* < 0.05) (Figure 1). Moreover, the bile from ED could stimulate the neck wound and cause local skin inflammation.

Histopathological changes of liver tissues

After bile duct ligation for 7 d, the liver of OJ rats showed hepatocellular degeneration and mild bile duct proliferation with acute inflammatory cell infiltration in the portal and periportal areas. Focal necrosis and mild fibrosis were present in the cholestatic liver, but liver cirrhosis did not

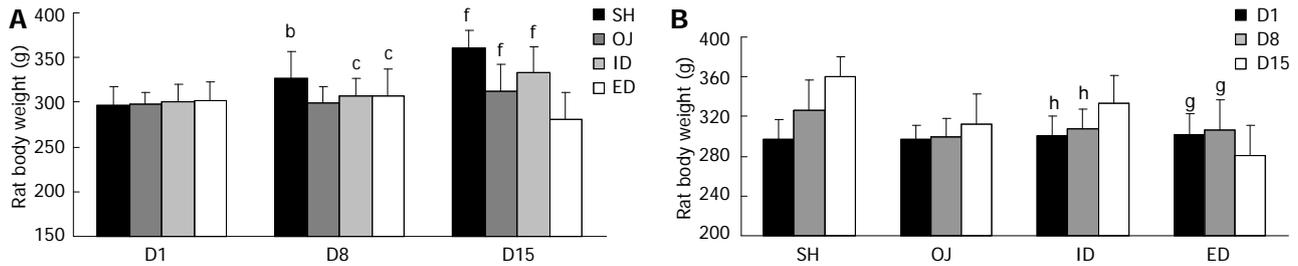


Figure 1 Changes of rat body weight with time following operations among the four groups. A: Obstructive jaundice (OJ) rats ate markedly less food and there was a slowly increasing trend of body weight between the 1st, 8th and 15th day ($P = 0.155$). On the contrary, weight gain was more significant in sham operation (SH) rats compared with that in OJ rats on the 8th and 15th day. After relief of OJ, the appetite of internal biliary drainage (ID) rats recovered and the body weight of ID rats on the 15th day became significantly higher than that on the 8th day. ^b $P < 0.01$ vs OJ; ^c $P < 0.05$ vs SH; ^f $P < 0.01$ vs external biliary drainage (ED); B: Weight loss was observed in ED rats and the body weight on day 15 was significantly lower than that on the 1st or 8th day. ^g $P < 0.05$, ^h $P < 0.01$ vs D15.

occur in OJ rats on the 8th day. However, following bile duct ligation for 14 d, the liver showed moderate to severe bile duct proliferation and fibrous expansion of the portal tracts with severe fibrosis and signs of early cirrhosis in the liver (Figure 2). After sham operation for 7 d, histologic study revealed a normal liver lobular architecture and no pathological changes in livers from SH rats. After 7 d of biliary drainage by ID and ED, the liver displayed normal morphological features of hepatocytes and preserved lobular architecture with only mild bile duct proliferation. Furthermore, the progression of fibrosis and cirrhosis stopped and reversal of the progression of cirrhosis and inflammation was observed (Figure 2).

Expression of iNOS in rat liver tissues

There was rare expression of iNOS protein in SH rats, but the liver of OJ rats markedly expressed iNOS protein in terms of the mean optical density (0.296 ± 0.055). After relief of OJ by ID, the expression of iNOS was noticeably suppressed (0.204 ± 0.029) when compared with the higher expression observed in OJ rat models (ID vs OJ, $P < 0.01$). However, the expression of liver iNOS obviously increased in rats of ED (0.399 ± 0.086) (ED vs OJ, $P = 0.004$) (Figure 3).

Expression of iNOS mRNA interfered with LPS and LPS + UDCA

When interfered only with LPS, the expression of iNOS mRNA by KC was stronger in the OJ group (0.58 ± 0.13) than in SH group (0.38 ± 0.07) (OJ vs SH, $P = 0.004$). After relief of biliary obstruction, iNOS mRNA expression showed slight changes in the ED group (0.59 ± 0.12) (ED vs OJ, $P = 0.71$), but dropped in the ID group (0.45 ± 0.12) as compared with ED and OJ groups (ID vs ED, $P = 0.004$; ID vs OJ, $P = 0.001$). When interfered with LPS and UDCA, inhibited iNOS mRNA expressions by KC were seen in all four groups (Figure 4).

Expression of CD14 in rat liver tissues

The immunoreactivity of CD14 protein was mainly detected in the membrane of KCs on the edge of liver sinusoid and portal areas. Moreover, in some sinusoidal liver endothelial cells and hepatic stellate cells, the expres-

sion of CD14 was also detected, and even a small quantity of positive expression was located on the surface of hepatocytes. Slight intrahepatic expression of CD14 was observed in SH rats (0.0014 ± 0.0008), but after bile duct ligation, the expression of CD14 protein in OJ rats was significantly stronger (0.0156 ± 0.0021) (OJ vs SH, $P < 0.01$). The expression of CD 14 protein in liver tissues reduced in ID rats (0.0015 ± 0.001) compared with OJ rats (ID vs OJ, $P < 0.01$), but not reduced in ED rats (0.0086 ± 0.0019) (ED vs OJ, $P = 0.591$) (Figure 5).

Expression of CD14 mRNA by KC

Under the stimulation of LPS, the expression of CD14 mRNA by KC was not strengthened in OJ group (1.998 ± 0.74) compared with that in SH group (1.388 ± 0.683) (OJ vs SH, $P = 0.822$). After relieving the OJ, the expression of CD14 mRNA was aggravated by ED (6.104 ± 2.171) (ED vs OJ, SH and ID, $P < 0.01$, respectively), but the expression of CD14 mRNA in ID group (1.018 ± 0.489) was not significantly different compared with that in SH and OJ groups (ID vs SH, $P = 0.944$; ID vs OJ, $P = 0.513$, respectively) (Figure 6).

Expression of TGR5 in rat liver tissues

TGR5 was mainly located in the plasma membrane of KCs, and in the cell wall of some sinusoidal endothelial cells and biliary epithelial cells. The expression of TGR5 protein in rat liver was significantly stronger in OJ group (0.513 ± 0.07) than in SH group (0.305 ± 0.01) ($P = 0.001$). ID could substantially down-regulate the expression level of TGR5 protein (0.356 ± 0.051) (ID vs OJ, $P = 0.001$) and there was no difference between ID and SH groups (ID vs SH, $P = 0.22$). On the contrary, the expression of TGR5 protein could not be inhibited by ED (0.439 ± 0.078) (ED vs OJ, $P = 0.062$) (Figure 7).

Expression of TGR5 mRNA by KCs

The expression of TGR5 mRNA by KCs was considerably stronger in OJ group (1.024 ± 0.325) ($2^{-\Delta\Delta CT}$) than in SH group (0.133 ± 0.045) ($P = 0.001$). There was no significant difference between ID group (0.320 ± 0.115) and SH group ($P = 0.354$). It was significantly stronger in ED group (0.632 ± 0.233) than in SH group ($P = 0.001$),

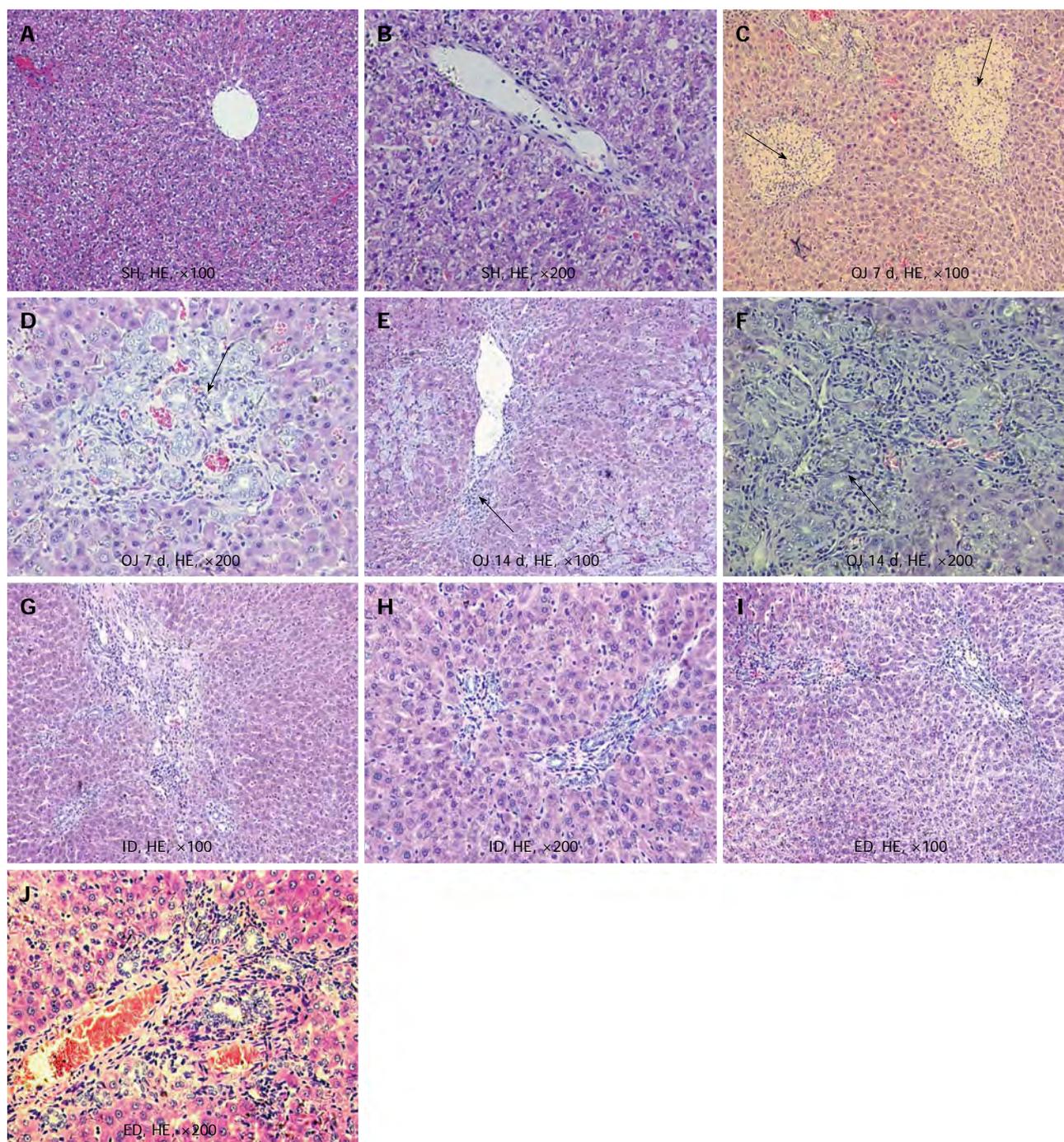


Figure 2 Micrograph of liver sections from rats of sham operation, obstructive jaundice, internal and external biliary drainage groups (the pathological changes were marked by black arrows). A, B: There was almost no pathological changes in the liver of sham operation (SH) rats, the liver lobular architecture was intact; C, D: After bile duct ligation for 7 d, the liver showed focal necrosis and mild bile duct proliferation with acute inflammatory cell infiltration in the portal and periportal areas, but the lobular architecture was still intact in the cholestatic liver; E, F: After bile duct ligation for 14 d, the liver showed striking liver fibrosis and prominent bile duct proliferation in the portal tracts; G, H: Following 7 d of internal biliary drainage (ID), the liver displayed preserved lobular architecture with only mild bile duct proliferation, and the progression of liver fibrosis and cirrhosis stopped; I, J: After external biliary drainage (ED), lobular architecture was preserved with only mild biliary proliferation, but the degree of liver fibrosis was still at a high level. HE: Hematoxylin and eosin; OJ: Obstructive jaundice.

and there was significant difference between ED and OJ group ($P = 0.003$) (Figure 8).

DISCUSSION

Patients with malignant or benign OJ carry an increased risk of postoperative complications and a high mortality

rate. Whether preoperative biliary drainage is still necessary for OJ patients planned for surgery is questioned by many experts^[1,4]. Another debate focuses on whether ID is superior to ED in terms of reducing the postoperative mortality and some associated complications^[5,6]. During the past decades, many clinical studies have been carried out to address the two controversies, but it is difficult to

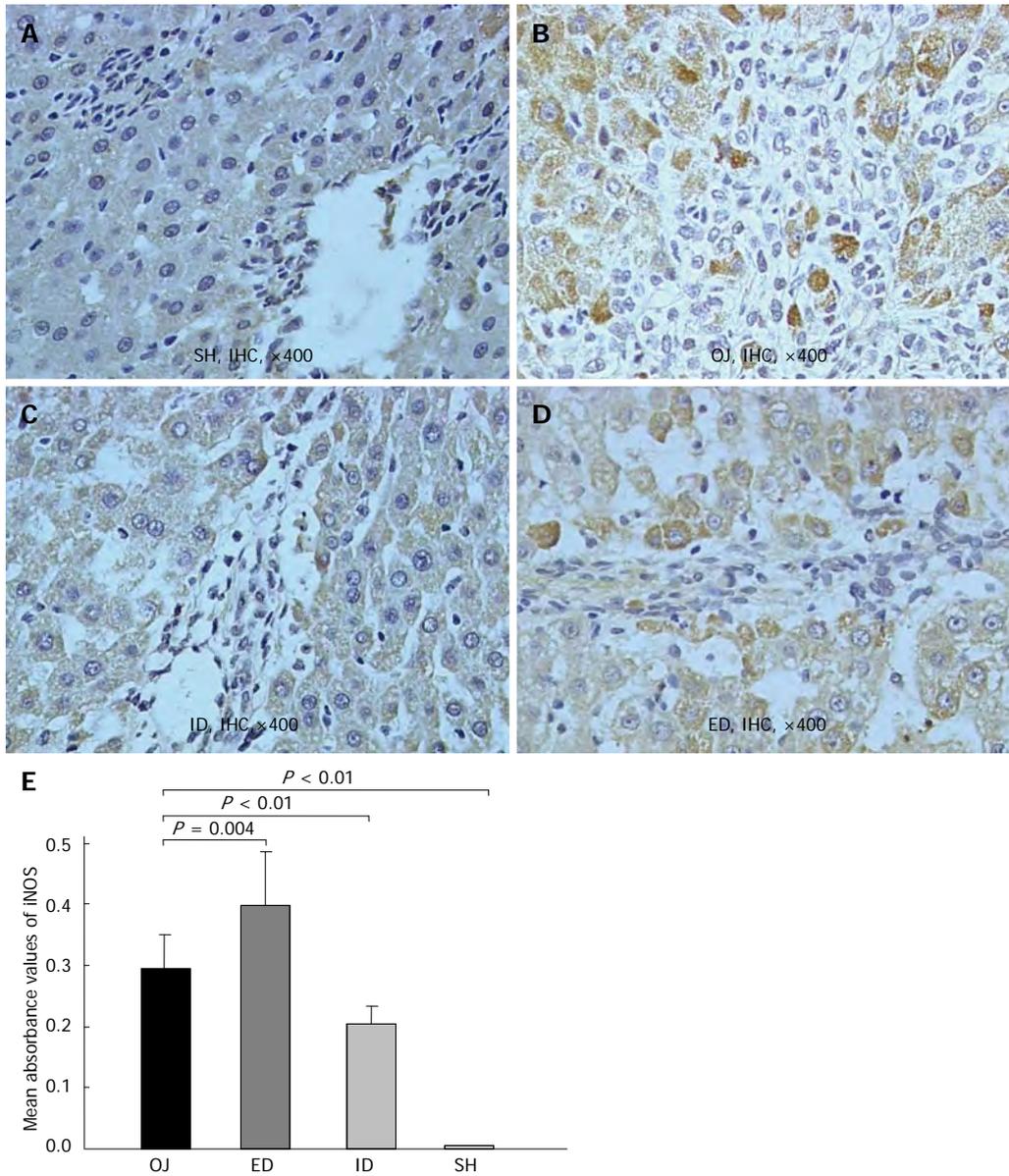


Figure 3 Representative immunohistochemical analysis of inducible nitric oxide synthase in rat liver of sham operation, obstructive jaundice, internal and external biliary drainage groups. A: There was rare expression of inducible nitric oxide synthase (iNOS) in sham operation (SH) group; B: 14 d after bile duct ligation, the iNOS-immunoreactivity became stronger as compared with SH group; C: After relief of obstructive jaundice (OJ) by internal biliary drainage (ID), the expression of iNOS was markedly suppressed; D: The expression of iNOS obviously increased in rat liver of external biliary drainage (ED); E: Comparison of mean absorbance values of iNOS in rat liver tissues among the four groups. IHC: Immunohistochemical.

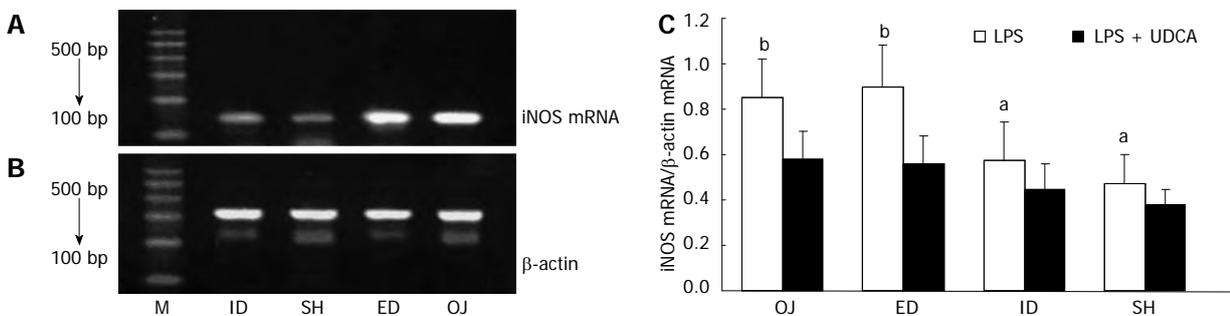


Figure 4 Ethidium bromide-stained agarose gel of inducible nitric oxide synthase reverse transcription polymerase chain reaction products, expression of inducible nitric oxide synthase messenger RNA on Kupffer cell interfered with lipopolysaccharide + ursodeoxycholic acid. A: Lane M, 100 bp molecular marker. The 138 bp-inducible nitric oxide synthase (iNOS) band; B: The 300 bp-β-actin products; C: Expression of iNOS messenger RNA (mRNA) interfered with lipopolysaccharide (LPS) as compared with LPS + ursodeoxycholic acid (UDCA). $^a P < 0.05$, $^b P < 0.01$ vs LPS + UDCA. M: Marker; ID: Internal biliary drainage; SH: Sham operation; ED: External biliary drainage; OJ: Obstructive jaundice.

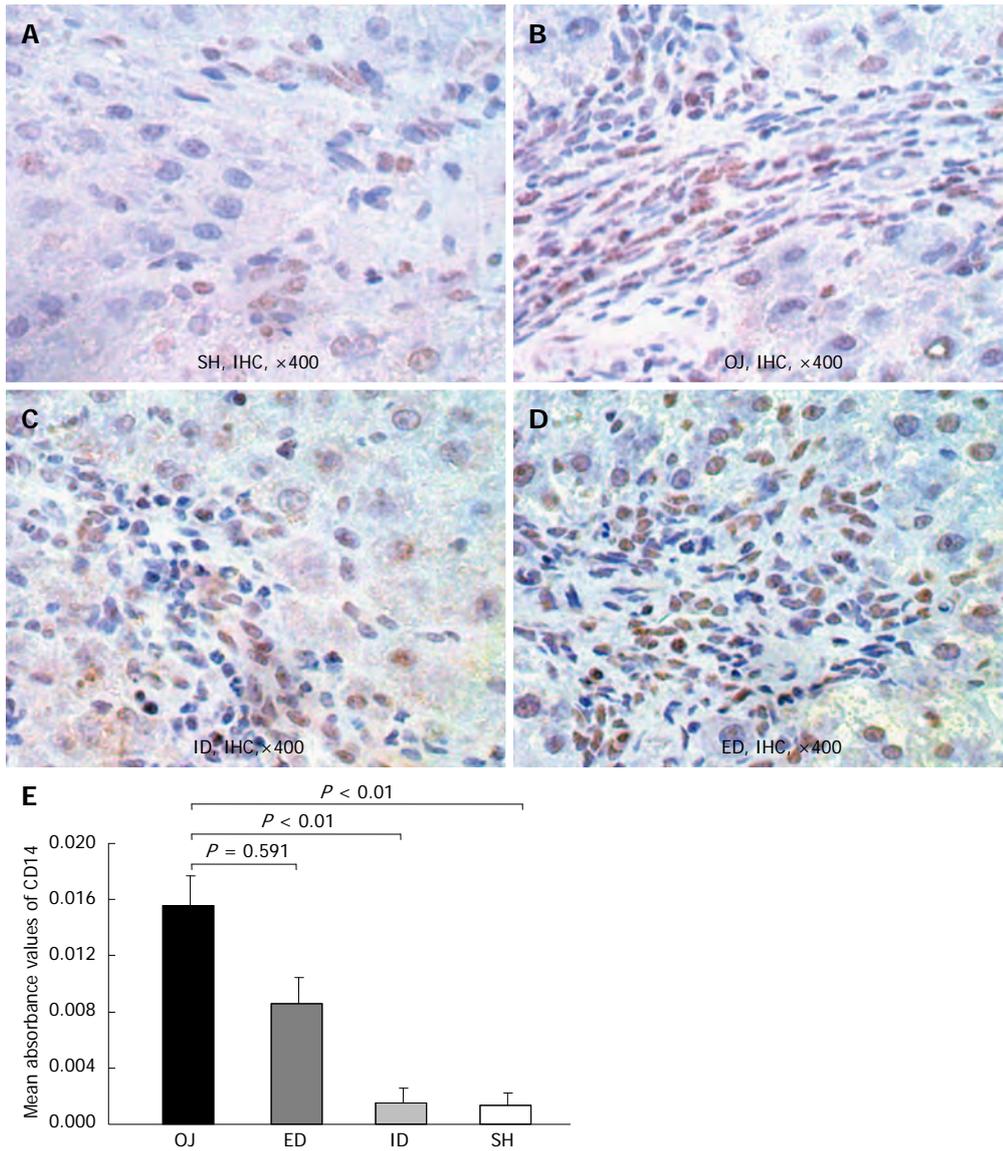


Figure 5 Representative immunohistochemical analysis of CD14 in rat liver tissues of sham operation, obstructive jaundice, internal and external biliary drainage groups. A: CD14-immunoreactivity was detected mainly in the membrane of Kupffer cell on the edge of liver sinusoid and portal areas. There was slight expression of CD14 in sham operation (SH) rats; B: 14 d after bile duct ligation, the CD14-immunoreactivity became stronger as compared to SH rats; C: After relief of obstructive jaundice (OJ) by internal biliary drainage (ID), the expression of CD14 was markedly suppressed; D: The expression of CD14 in external biliary drainage (ED) rats was still at a high level; E: Comparison of mean absorbance values of CD14 in liver tissues among the four groups. IHC: Immunohistochemical.

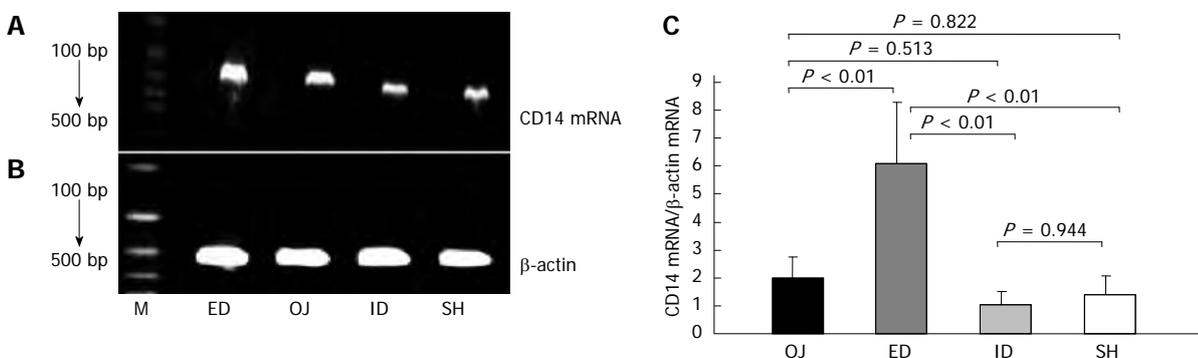


Figure 6 Ethidium bromide-stained agarose gel of CD14 reverse transcription polymerase chain reaction products products. A: Lane M, 100 bp molecular marker. The 267 bp-CD14 band; B: The 300 bp-β-actin reverse transcription polymerase chain reaction products products; C: Comparison of CD14 messenger RNA (mRNA) expression by Kupffer cell interfered with lipopolysaccharide. CD14 mRNA levels were standardized using β-actin mRNA. M: Marker; ID: Internal biliary drainage; SH: Sham operation; ED: External biliary drainage; OJ: Obstructive jaundice.

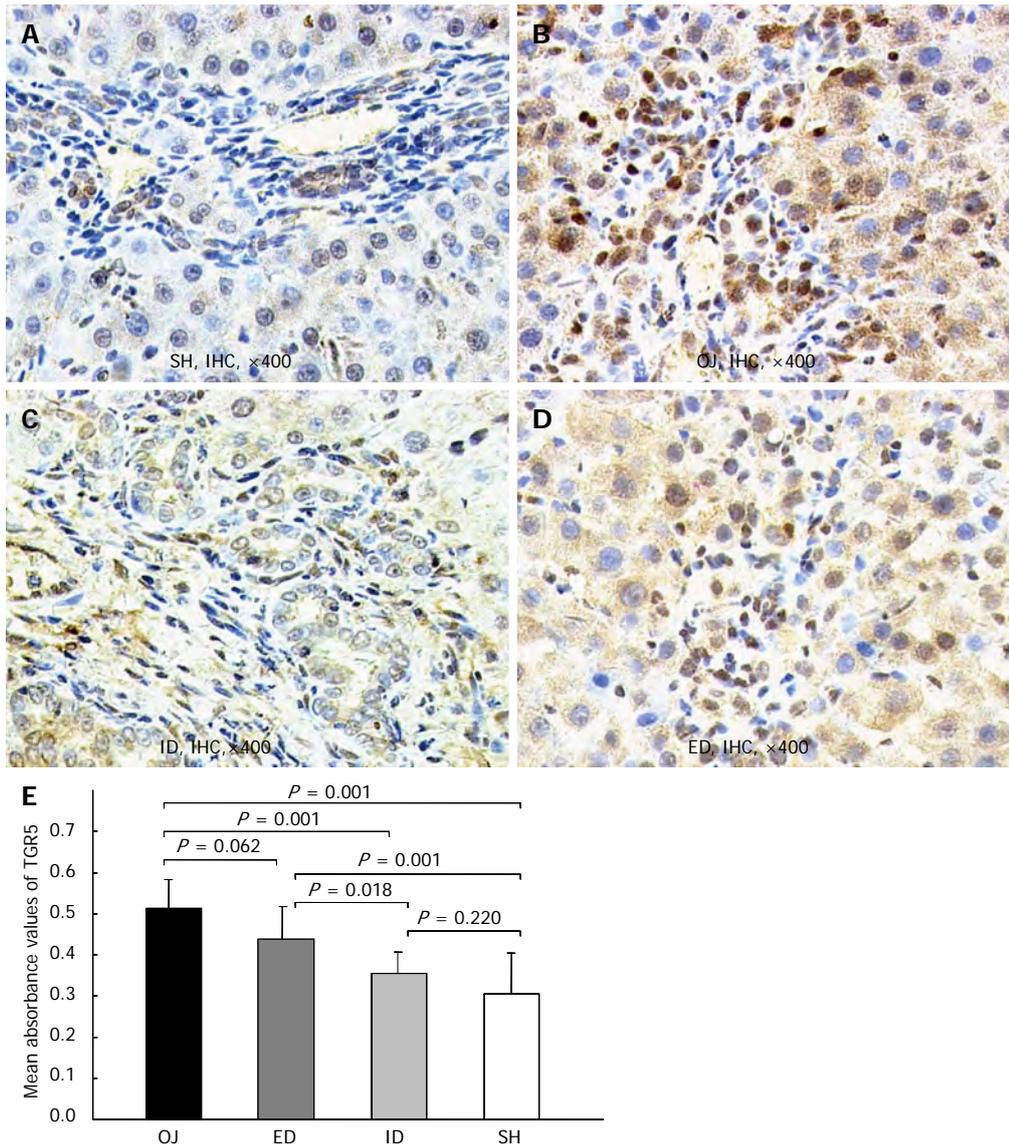


Figure 7 Representative immunohistochemical analysis of TGR5 in rat liver tissues of sham operation, obstructive jaundice, internal and external biliary drainage groups. TGR5-immunoreactivity was detected mainly in the plasma membrane of Kupffer cell and its intracellular compartments, and also localized in some sinusoidal endothelial cells and biliary epithelial cells, but merely in hepatocytes. A: There was slight expression of TGR5 in rats of sham operation (SH); B: After bile duct ligation, the TGR5-immunoreactivity became stronger as compared to SH rats; C: After relief of jaundice by internal biliary drainage (ID), the expression of TGR5 was markedly suppressed; D: The expression of TGR5 in external biliary drainage (ED) rats was still at a high level; E: Comparison of mean absorbance values of TGR5 in rat liver tissues among the four groups. OJ: Obstructive jaundice; IHC: Immunohistochemical.

reach a consensus. So we have performed a series of experimental studies based on the immune function of KCs to present some preliminary perspectives about these questions^[9,10,19].

In the present study, we observed that the expression of iNOS protein was markedly enhanced 14 d after bile duct ligation, but rarely expressed in SH rat models, which was in agreement with the results observed in other previous studies^[22,23]. Moreover, we found that ID could suppress the expression of iNOS while the expression of iNOS protein was promoted by ED. Our earlier studies have confirmed that KCs from rats with OJ produced large amounts of endotoxin-mediated NO^[9]. ID was better than ED in reversing the distorted NO production by KCs based on the activities of iNOS mRNA

under the stimulation of LPS^[10]. Altogether, these findings underlined that the production of NO in OJ rats could be induced by iNOS in both protein and mRNA levels, and ID was superior to ED in depressing the expression of iNOS.

The major pathogenic role of LPS in the progression of OJ has been supported in previous studies^[24,25]. LPS as a substantial component of the outer membrane of gram-negative bacteria, could stimulate the production of pro-inflammatory cytokines (*e.g.*, TNF- α , IL-6) and other mediators (*e.g.*, ROS, NO, iNOS) *via* CD14/toll-like receptor pathway^[23,26,27]. CD14 as one of the most important LPS recognition receptors is responsible for the activation of KCs by pathophysiological concentrations of LPS^[12,13]. The data presented here show that the CD14

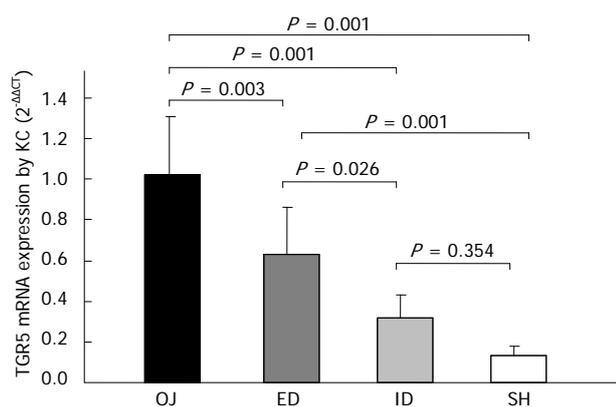


Figure 8 Effect of biliary drainages on TGR5 messenger RNA expression by Kupffer cell (2^{-ΔΔCT}). ID: Internal biliary drainage; SH: Sham operation; ED: External biliary drainage; OJ: Obstructive jaundice; KC: Kupffer cell; mRNA: Messenger RNA.

protein expression in rat liver was substantially up-regulated in the OJ group compared with the SH group. After relieving the OJ by ID, the expression of CD14 protein in liver tissues was significantly reduced, but could not be by ED. We investigated whether the induction of CD14 protein expression was correlated with the CD14 mRNA level, and found that the expression of CD14 mRNA by KC was not strengthened on OJ rats compared with SH rats, because the rat models receiving bile duct ligation for 14 d could develop severe fibrosis and cirrhosis in the liver, which influenced the isolation of KCs and the following analysis for CD14 mRNA. ID could decrease the expression of CD14 mRNA in rats with OJ and regulated the sensibility of *CD14* gene to endotoxin, but ED enhanced the expression of CD14 mRNA. In addition, our previous studies have already found that the levels of serum endotoxin, TNF- α and IL-6, the production of NO and the expression of iNOS by KCs were increased in OJ rats, and ID could entirely reverse the changes, but external drainage could not^[9,10]. Taken together, our results indicated that the LPS receptor-CD14 on the membrane of KCs could play an important role in the immune suppression in OJ, and related to the effects of biliary drainage.

The essential difference between ID and ED may be associated with the re-establishment of enterohepatic circulation of bile acids. Although ED could partially recover damaged liver function, it could not set up normal enterohepatic circulation^[19]. A large amount of bile lost through ED resulting in malabsorption of fat, loss of some immune substances as well as imbalance of water and electrolytes. Several studies have confirmed the immunomodulatory function of bile acids, for instance, oral or intravenous administration of UDCA could reduce endotoxin-related complications in OJ^[14,15,28]. The anti-inflammatory effect of UDCA is attributed to the inhibition of the production of endotoxin induced pro-inflammatory mediators^[20,21,29,30]. In the present study, we found that the expression level of iNOS mRNA by isolated KCs was lower under the stimulation with LPS +

UDCA compared with simple stimulation of LPS among the four groups. The consequence confirmed that UDCA had the suppressive effect on the endotoxin.

Recently, the bile acid receptor-TGR5 as a member of the G protein coupled receptors localized at the plasma membrane and internalized into the cytoplasm in response to its activation, has been identified as the first cell surface receptor for bile acids by two different groups respectively^[16,17]. TGR5 is highly expressed in CD14 positive monocytes and macrophages^[16-18]. KC as the CD14-positive and liver resident macrophages, has a higher expression level of TGR5 mRNA compared with other white blood cells^[17]. Several studies have reported that bile acids could inhibit LPS-induced pro-inflammatory cytokine expression in KC *via* TGR5-dependent pathway^[17,18]. The present study found that the immunoreactivity of TGR5 was mainly detected in the plasma membrane of KC and SEC, and also localized in some intracellular compartments, but merely in hepatocytes. There was slight expression of TGR5 in SH rats, but stronger expression in OJ rats. Keitel *et al.*^[31] have confirmed that TGR5 staining was not strong in SEC of OJ rats, so the high expression level of TGR5 in OJ rats was specific for KC. After relief of OJ, the TGR5-immunoreactivity could be reversed by ID, but not by ED. We also found that the induction of TGR5 protein expression was correlated with the expression level of TGR5 mRNA. Bile duct ligation could result in higher expression of TGR5 mRNA compared with sham operation. Likewise, ID could down-regulate the expression of TGR5 mRNA, but ED could not. Keitel *et al.*^[18] demonstrated the direct link between the protein and gene expression levels of TGR5 and the gene expression of pro-inflammatory cytokines by KCs. Based on our results, the variation of TGR5 was in accordance with the changes of serum endotoxin and pro-inflammatory cytokines among these four groups^[9,10]. Altogether, activation of TGR5 in KC could prevent excessive cytokine production, thereby alleviating liver injury, which indicated that the ID was superior to ED in relief of OJ, and the mechanism may be based on the regulation process of TGR5 in both protein and gene levels.

Besides the dysfunction of liver KC leading to the diminished clearance of endotoxin and the production of large amounts of pro-inflammatory cytokines, lack of bile acids in intestine through bile duct ligation could result in the disruption of the epithelial barrier, translocation of bacteria and endotoxin across the mucosa into lymph nodes and remote organ systems, and sometimes could cause lethal endotoxemia^[32]. Inagaki *et al.*^[33] have confirmed that the farnesoid X receptor (FXR), a nuclear receptor for bile acids, could induce genes involved in neuroprotection and inhibited bacterial overgrowth and mucosal injury in ileum caused by bile duct ligation. Bile acid receptors such as FXR and TGR5, mainly exerting in the lipid and cholesterol metabolism, are increasingly recognized as one of the new frontiers of immunology. These receptors expressed in liver and gut might play

an important role in the reaction to inflammation in enterohepatic tissues. Our study demonstrated that ID was superior to ED in relief of OJ, which might be based on the regulation process of TGR5 by KCs, further investigations derived from intestinal macrophages are necessary to elucidate the immunoregulatory role of bile acid receptor.

In conclusion, we found that internal biliary drainage could reverse the raised expression of iNOS and CD14 in both protein and mRNA levels in obstructive jaundice rat models, but external drainage could not. In addition, UDCA could protect KCs from the endotoxin of LPS related to the down-regulation of iNOS mRNA expression. The up-regulation of TGR5 in protein and gene levels in obstructive jaundice could play a protective role in alleviating the inflammatory reaction. The mechanism of internal biliary drainage superior to external drainage in relief of obstructive jaundice might be attributed to the regulatory function of activation of KCs and release of inflammatory mediators.

COMMENTS

Background

To date, there are still some controversies over whether and how to perform preoperative biliary drainage in patients with malignant or benign obstructive jaundice (OJ), even though the complication-related mortality rate is high for OJ patients followed by surgery.

Research frontiers

During the past decades, many clinical studies have been performed to address the two controversies, but it is difficult to reach a consensus. The authors performed a series of experimental studies based on the immune function of Kupffer cell (KC) in an attempt to offer some preliminary perspectives about these questions.

Innovations and breakthroughs

The authors found that internal biliary drainage could reverse the raised expression of inducible nitric oxide synthase (iNOS) and CD14 in both protein and messenger RNA levels in obstructive jaundice rat models, but external drainage could not. The mechanism of internal biliary drainage superior to external drainage in relief of obstructive jaundice might be attributed to the regulatory function of activation of KC and release of inflammatory mediators.

Peer review

This is an experimental study concerning superiority of internal biliary drainage to external biliary drainage. Internal biliary drainage could reverse the high expression of iNOS, CD14, and TGR5 in rats with obstructive jaundice, but external drainage could not. The mechanism might be attributed to the regulatory function of activation of KCs and release of inflammatory mediators. The manuscript itself is interesting and includes some new insights of biliary drainage.

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Special AT-rich sequence-binding protein 1 promotes cell growth and metastasis in colorectal cancer

Xue-Feng Fang, Zhi-Bo Hou, Xin-Zheng Dai, Cong Chen, Jing Ge, Hong Shen, Xiao-Feng Li, Li-Ke Yu, Ying Yuan

Xue-Feng Fang, Hong Shen, Xiao-Fang Li, Ying Yuan, Department of Medical Oncology, Second Affiliated Hospital, Zhejiang University College of Medicine, Hangzhou 310000, Zhejiang Province, China

Zhi-Bo Hou, Li-Ke Yu, First Department of Respiratory Medicine, Nanjing Chest Hospital, Nanjing 210029, Jiangsu Province, China

Xin-Zheng Dai, Liver Transplantation Center, First Affiliated Hospital of Nanjing Medical University, Key Laboratory of Living Donor Liver Transplantation, Ministry of Public Health, Nanjing 210029, Jiangsu Province, China

Cong Chen, Department of Gynecology of Traditional Chinese Medicine, Jiangsu Provincial Hospital of Traditional Chinese Medicine Affiliated to Nanjing University of Traditional Chinese Medicine, Nanjing 210029, Jiangsu Province, China

Jing Ge, Department of Endocrinology, Jiangsu Provincial Hospital of Traditional Chinese Medicine Affiliated to Nanjing University of Traditional Chinese Medicine, Nanjing 210029, Jiangsu Province, China

Author contributions: Fang XF, Hou ZB and Dai XZ contributed equally to this work; Hou ZB, Dai XZ and Chen C carried out the molecular genetic studies; Chen C, Ge J and Li XF participated in the animal study; Hou ZB, Chen C and Ge J analyzed final data; Fang XF, Hou ZB and Shen H drafted the manuscript; Yuan Y designed this research; all authors read and approved the final manuscript.

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Correspondence to: Dr. Ying Yuan, Department of Medical Oncology, Second Affiliated Hospital, Zhejiang University College of Medicine, 88 Jiefang Road, Hangzhou 310000, Zhejiang Province, China. yuanying1999@zju.edu.cn

Telephone: +86-571-87784795 Fax: +86-571-87767088

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METHODS: Immunohistochemistry was used to detect the protein expression of SATB1 in 30 colorectal cancer (CRC) tissue samples and pair-matched adjacent non-tumor samples. Cell growth was investigated after enhancing expression of SATB1. Wound-healing assay and Transwell assay were used to investigate the impact of SATB1 on migratory and invasive abilities of SW480 cells *in vitro*. Nude mice that received subcutaneous implantation or lateral tail vein were used to study the effects of SATB1 on tumor growth or metastasis *in vivo*.

RESULTS: SATB1 was over-expressed in CRC tissues and CRC cell lines. SATB1 promotes cell proliferation and cell cycle progression in CRC SW480 cells. SATB1 overexpression could promote cell growth *in vivo*. In addition, SATB1 could significantly raise the ability of cell migration and invasion *in vitro* and promote the ability of tumor metastasis *in vivo*. SATB1 could up-regulate matrix metalloproteases 2, 9, cyclin D1 and vimentin, meanwhile SATB1 could down-regulate E-cadherin in CRC.

CONCLUSION: SATB1 acts as a potential growth and metastasis promoter in CRC. SATB1 may be useful as a therapeutic target for CRC.

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Key words: Special AT-rich sequence-binding protein 1; Colorectal cancer; Proliferation; Migration; Invasion

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Abstract

AIM: To evaluate the expression of special AT-rich sequence-binding protein 1 (*SATB1*) gene in colorectal cancer and its role in colorectal cancer cell proliferation and invasion.

INTRODUCTION

The special AT-rich sequence-binding protein 1 (SATB1),

which locates at human chromosome 3p23, is a thymocyte-specific matrix association region-binding protein that links specific DNA elements to its unique cage-like network^[1]. Phosphorylation of SATB1 serves as a molecular switch in determining whether it acts as a transcriptional activator or repressor^[2]. SATB1 is predominantly expressed in thymocytes and regulates the spatiotemporal expression of numerous genes that involved in T cell proliferation, development, and differentiation^[3]. SATB1 has recently attracted considerable attention in cancer research and its overexpression is a frequent event in various cancers, such as breast cancer, laryngeal cancer, gastric cancer and liver cancer^[4-9]. Furthermore, accumulating evidence showed that SATB1 is also associated with tumor growth and metastasis^[4,6,8-10]. Han *et al.*^[5] found that SATB1 up-regulated the expression of matrix metalloproteases (MMP)2, MMP9 and down-regulated E-cadherin in breast cancer. On the other hand, SATB1 depletion blocks the up-regulation of E-cadherin and extracellular matrix (ECM) protein vimentin. Meng *et al.*^[11] showed that SATB1 plays a pivotal role in epithelial to mesenchymal transition (EMT) process and promotes liver cancer invasion. Only one study suggested that SATB1 is over-expressed in human rectal cancer and the expression of SATB1 is associated with clinicopathological parameters, including invasive depth and tumor-node-metastasis (TNM) stage in rectal cancer. Despite its importance, the roles and mechanisms of SATB1 in growth and metastasis of human colorectal cancer (CRC) remain poorly understood.

CRC is the third most common malignancy and the fourth cause of cancer mortality in the world^[12-14]. Although novel molecule-based therapies including monoclonal antibodies are currently widely used in the treatment of CRC, many patients with CRC still die from disease recurrence and metastasis^[15,16]. Consequently, further elucidation of the molecular mechanisms of CRC will be beneficial for developing novel therapeutic strategies to conquer this disease.

In this study, we analyzed the expression of SATB1 in CRC tissues and found that it was over-expressed in the cancerous tissue samples compared with the normal adjacent tissue samples. We also carried out *in vitro* and *in vivo* functional analysis of SATB1 by ectopic SATB1 expression in SW480 CRC cells. Further investigations focused on the regulation of SATB1 in potential downstream molecules MMPs (MMP2, MMP9), cyclin D1 (CCND1), E-cadherin and vimentin.

MATERIALS AND METHODS

Cell lines and plasmids

Human CRC cell lines SW480, SW620, RKO, HT29, HCT116 and Lovo were obtained from Shanghai Institute of Cell Biology (Shanghai, China) and were cultured in RPMI 1640 medium (Invitrogen, Carlsbad, CA, United States), supplemented with 10% fetal bovine serum. The pcDNA3.1 (Invitrogen, Carlsbad, CA, United States)

was used to construct a SATB1 over-expressing plasmid. DNA fragment with mature SATB1 or a negative control sequence was inserted to this vector. Stable transfection of the plasmids was carried out using Lipofectamine2000 (Invitrogen, Carlsbad, CA, United States) according to the manufacturer's instruction.

Immunohistochemistry and immunoblot analysis

Paraffin-embedded tumors and paired normal tissue samples were obtained from 30 CRC patients with the approval from the Ethics Committee of the Second Hospital of Zhejiang University Medical College. Immunohistochemical (IHC) analyses were performed on 3- μ m, formalin-fixed and paraffin-embedded sections. Primary antibodies for SATB1 were diluted at 1:250 (BD Biosciences, California, United States) for IHC^[17,18]. For immunoblot analysis, 20 g total cellular protein was loaded per lane, separated by 4%-12% SDS-polyacrylamide gel electrophoresis, and then transferred to nitrocellulose (Invitrogen, Carlsbad, CA, United States) by electroblotting. The membranes were incubated with either SATB1 antibody (diluted 1:1000; BD Biosciences, California, United States) or α -tubulin antibody (diluted 1:200; Santa Cruz Biotechnology) at 4 °C overnight^[19].

Cell proliferation assay and colony formation assay

Cell proliferation assay was determined by standard 3-(4,5-cimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assays. Briefly, the cells were seeded at a density of 2×10^3 cells per well in 96-well culture plates (Costar). Cell proliferation was assessed 24, 48 and 72 h later. One-tenth volume of 5 mg/mL MTT was added to each well, and the plate was further incubated at 37 °C for another 4 h; thereafter, the medium was replaced and the formazan crystals formed were dissolved in 150 μ L dimethyl sulfoxide with oscillation for 10 min. The optical density was determined with a multiwell spectrophotometer (BioTek, VT, United States) at 570 nm. Absorbance values were presented as percentages relative to untreated controls. The MTT assays were repeated at least three times^[20]. For colony formation assay, cells were trypsinized and counted. One hundred cells were seeded in six-well plates. After 2 wk of growth, colonies with a diameter greater than 4 mm were counted. Experiments were performed in quadruplicate^[21].

Scratch wound healing assay

Scratch wound healing assay was performed as previously described. Briefly, transfected cells in 6-well plates were cultured until cells reached confluence and starved overnight. Cell layers were wounded using a 200 μ L pipette tip and cultured for another 48 h. Photographs were taken at time 0, 48 and 72 h^[22].

Cell migration and invasion assay

A transwell cell migration and Matrigel invasion assay was used to investigate the impact of SATB1 on migratory and invasive ability of SW480 cells. For migration detec-

tion, transfected cells were placed in transwell Chamber at 2×10^4 cells/well. The lower transwell chamber contained 10% fetal bovine serum for use as a chemoattractant. For invasion assay, the bottom of the culture inserts (8-mm pores) were coated with 30 μ L of the mixture containing serum-free RPMI-1640 and Matrigel (1:8; BD Biosciences, Bedford, MA, United States). The Matrigel was allowed to solidify at 37 °C overnight. After solidification, cells (2×10^4 cells/well) were reseeded onto the upper chamber. Twenty-four hours later, the cells that had migrated or invaded through the membrane were fixed with 95% alcohol and stained with crystal violet. The number of migrated cells or invaded cells was quantified by counting 5 independent symmetrical visual fields under microscope^[23].

Xenograft studies

Cells of 2×10^4 were harvested, washed and resuspended in 200 mL phosphate-buffered saline, and was subcutaneously injected into the flanks of 5-wk-old female nude mice. Animal experimental procedures were performed strictly in accordance with the related ethics regulations of our university. Tumor sizes were measured in two dimensions with calipers every week. Tumor volumes (mm^3) were calculated using the following formula: $V = (\text{length} \times \text{width}^2)/2$ ^[24]. For *in vivo* metastasis assays, SW480-SATB1 cells or SW480-negative control (SW480-NC) cells were transplanted into nude mice (5-wk-old BALB/c-nu/nu, ten per group, 1×10^6 cells for each mice) through the lateral tail vein. Mice were killed after 10 wk. The lungs were dissected and subjected to hematoxylin and eosin staining. The numbers of metastases in the lungs were examined histologically.

Real-time polymerase chain reaction analysis

Total RNA was extracted from cells expressing SATB1 and negative control cells with Trizol (Invitrogen, Carlsbad, CA, United States). The expression of CCND1, E-cadherin, vimentin, MMP2 and MMP9 was detected by quantitative real-time polymerase chain reaction (PCR). The primers are as follows: CCND1, the forward primer 5'-TATT-GCGCTGCTACCGTTGA-3' and the reverse primer 5'-CCAATAGCAGCAAACAATGTGAAA-3'; MMP2, the forward primer TCTTCAAGGACCGGTTCAATTG and the reverse primer GATGCTTCCAAACTTCAC-GCTC; MMP9, the forward primer CACTGTC-CACCCCTCAGAGC and the reverse primer GCCACTT-GTCGGCGATAAGG; E-cadherin, the forward primer 5'-TGCCAGAAAATGAAAAAGG-3' and the reverse primer 5'-GTGTATGTGGCAATGCGTTC-3'; Vimentin, the forward primer 5'-TGGCCGACGCCATCAACACC-3' and the reverse primer 5'-CACCTCGACGCGGGCITT-GT-3'; β -actin was used as an internal control. The primers for β -actin were 5'-TGACGGGGTCACCCACACTGT-GCCCATCT-3' and 5'-GAAGTAGTAAGTGGGAACC-GTGT-3'. Real-time PCR was performed using the SYBR[®] Green (Invitrogen) dye detection method on ABI PRISM 7900 HT Sequence Detection System under default conditions: 95 °C for 10 min, and 35 cycles of 95 °C for 15 s

and 55 °C for 1 min. Comparative C_t method was used for quantification of the transcripts^[25].

Statistical analysis

Each experiment was repeated at least 3 times. All results were expressed as mean \pm SD. The difference between means was analyzed with Student's *t* test or the χ^2 test. All statistical analysis were performed using SPSS 16.0 software (Chicago, IL, United States). Differences were considered significant when $P < 0.05$ ^[26].

RESULTS

Expression of SATB1 increased in CRC tissue samples

To assess the role of SATB1 in CRC, we examined the protein expression of SATB1 in 30 human CRC tissue samples and pair-matched adjacent non-tumor tissue samples by IHC. We observed positive immunoreactivities in CRC in 53% (16 of 30) of cancer tissue samples, compared with only 10% (3 of 30) in the adjacent mucosa tissue cells. The representative examples of IHC staining results are shown in Figure 1A. Statistical analysis using Pearson χ^2 (df = 1, two-sided) indicates that the difference in SATB1 expression between cancer and adjacent tissues was significant ($P < 0.01$) (Table 1). Western blot analysis of SATB1 in established CRC cell lines showed that the expression level of SATB1 in SW620 was higher in the SW480 (Figure 1B). SW480 and SW620 were a matched pair of primary and metastatic population of cells from the same patient^[27]. SW620 cells were derived from the metastasis lymph node of Dukes' type C colorectal adenocarcinoma.

SATB1 expression promotes CRC cell proliferation *in vitro*

In order to investigate the role of SATB1 in CRC carcinogenesis, we tested the effect of SATB1 on the proliferation of SW480 cells. We established stable SATB1 expressing CRC cells. As shown in Figure 2A, SATB1 levels were higher in cells stably expressing SATB1 than the negative control cells. MTT assay showed that introduction of SATB1 caused a remarkable promotion of cell proliferation in SW480 cells ($P < 0.05$; Figure 2B). Furthermore, expression of SATB1 in SW480 cells significantly enhanced the numbers of colony formation. As shown in Figure 2C, the colony number for the negative control cells was 55.3 ± 5.0 , while that for the SATB1 overexpression was 23.7 ± 3.2 ($P = 0.018$). To clarify the mechanisms underlying growth promotion by SATB1 in CRC cell lines, we performed cell-cycle analysis using flow cytometry on the cells stained with propidium iodide. SW480-SATB1 cells showed a higher proportion of cells in S phase (17.59%), compared with the control cells (13.02% for SW480-NC cells) (Figure 2D).

SATB1 promotes tumorigenesis potential *in vivo*

In order to assess the role of SATB1 on CRC tumorigenesis *in vivo*, equal numbers of SW480-SATB1 cells and

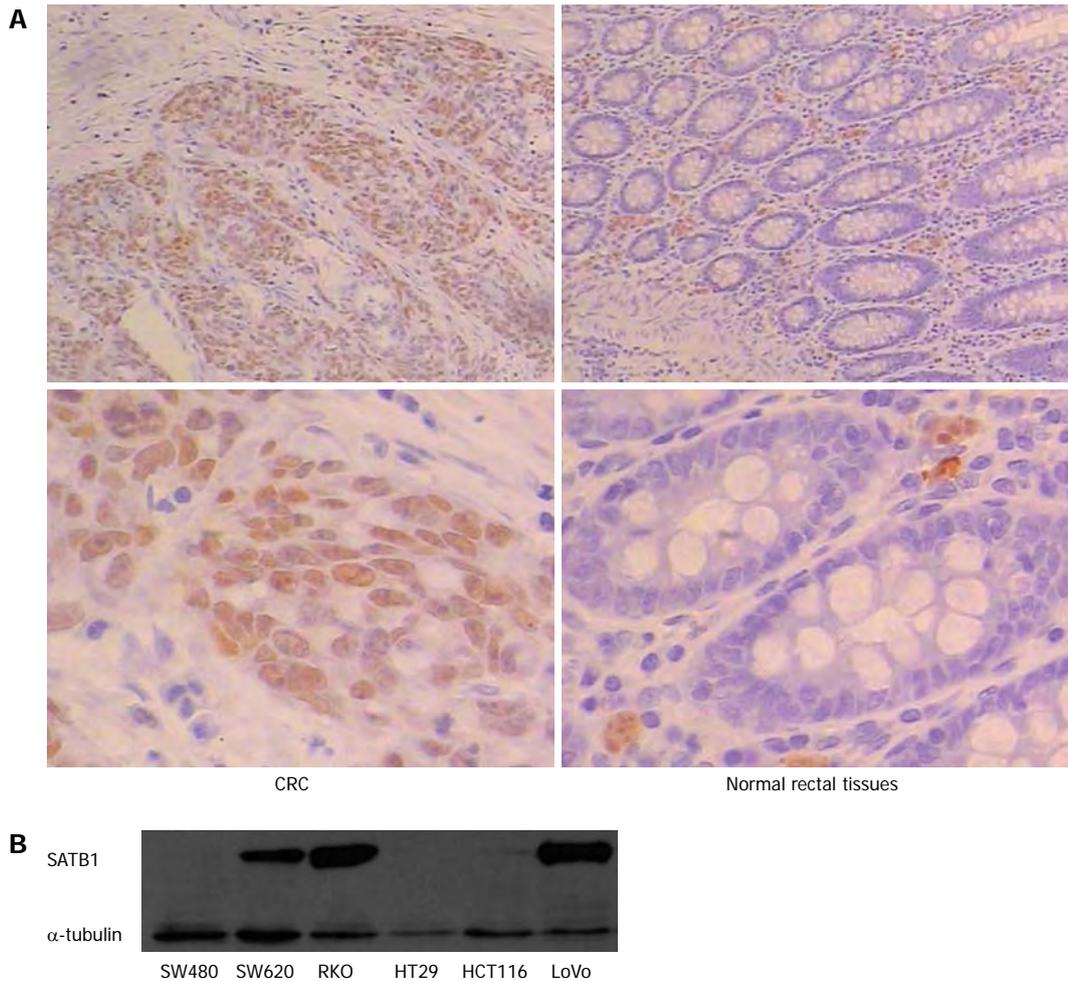


Figure 1 Upregulation of special AT-rich sequence-binding protein 1 in human colorectal cancer. A: Immunostaining for SATB1 protein in tissue of carcinomas and adjacent normal tissue mucosa. Top: Representative pictures of carcinomas (left) and normal tissue (right); B: Western blot detection of SATB1 protein in different colorectal cancer cell lines. SATB1: Special AT-rich sequence-binding protein 1; CRC: Colorectal cancer.

| Table 1 Summary of the immunohistochemistry findings | | |
|--|----------------|----------------|
| | Cancer tissues | Normal tissues |
| Total number of samples | 30 | 30 |
| Samples with SATB1 expression in nucleus | 16 | 3 |

SATB1: Special AT-rich sequence-binding protein 1.

SW480-NC cells were implanted onto flanks of 5-wk-old female nude mice, and the growth of the implanted tumors was measured at weeks 1-4. The results indicated that SW480 cells with enhanced SATB1 expression could promote the growth of subcutaneous tumors (Figure 2E) ($P < 0.01$).

SATB1 promotes CRC cell migration and invasion *in vitro* and *in vivo*

Wound-healing assay was performed to examine the effect of SATB1 expression on cell migration. We found that SW480-SATB1 cells healed the scratch wound earlier than negative controls cells (Figure 3A). We also estimated

the effects of SATB1 on the migration and invasion of SW480 cells using a Transwell cell migration and Matrigel invasion assay. The data demonstrated that the overexpression of SATB1 markedly promoted the migration and invasion of SW480 cells. The number of SW480-SATB1 cells (307 ± 20 , $P < 0.0001$) that had migrated through the membrane without Matrigel was significantly higher than that of SW480-NC cells (104 ± 20) (Figure 3B). A similar result was found with the invaded cells; the number of SW480-SATB1 cells (237 ± 19 , $P < 0.0001$) passing through the Matrigel was significantly higher than that of SW480-NC cells (82 ± 10) (Figure 3B). To further explore the effects of SATB1 on tumor metastasis *in vivo*, SW480-SATB1 cells or SW480-NC cells were transplanted into nude mice through the lateral tail vein. Histological analysis of the lung of mice confirmed that SATB1 could promote lung metastasis formation. Lung metastasis of SW480 cells was apparent in mice injected with SW480-SATB1 cells (Figure 3C). In contrast, few metastatic tumors were detected in mice injected with SW480-NC cells (Figure 3C). Our results indicate that SATB1 could promote CRC cell metastasis *in vivo*.

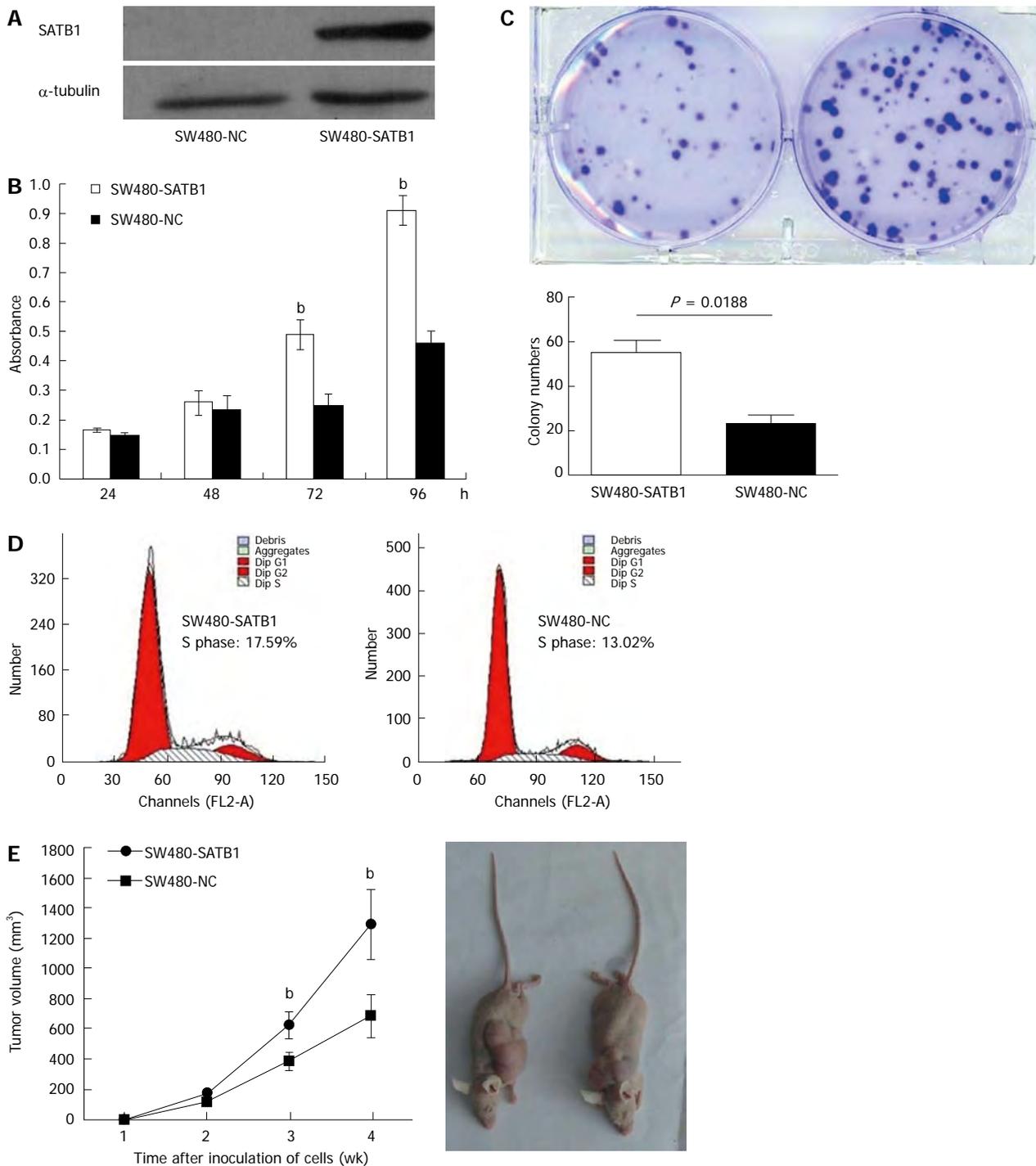


Figure 2 Special AT-rich sequence-binding protein 1 promotes cell growth and *in vivo* tumorigenesis potential in SW480 cells. **A:** Western blot analysis of special AT-rich sequence-binding protein 1 (SATB1) protein expression in colorectal cancer (CRC) cells that stably expressing SATB1 and SW480 negative control (NC) cells; **B:** Effect of SATB1 overexpression on cell proliferation by 3-(4,5-cimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide assay. ^b*P* < 0.01 vs NC cells; **C:** Colony formation assays for SW480-SATB1 cells and NC cells. Data are representatives of three independent experiments; **D:** Growth rates of SW480-SATB1 cells and negative control cells in an *in vivo* mouse model. Volumes of tumors were monitored every week. Bottom: Representative pictures of tumor samples. ^b*P* < 0.01 vs NC cells.

SATB1 induces proliferation and metastasis related gene expression change in SW480 cells

We detected the potential downstream molecules regulated by SATB1 *via* real-time PCR analysis to probe into the possible mechanism that SATB1 promotes CRC cell proliferation and metastasis. The results showed that the ex-

pression of CCND1 was up-regulated in SW480 cells with enhanced SATB1 expression. We also found that MMP2 and MMP9, the major MMPs that have a key role in the proteolytic cascade-leading ECM cleavage during metastasis in colon carcinoma, were up-regulated in SW480-SATB1 cells (Figure 3D). In addition, the expression of

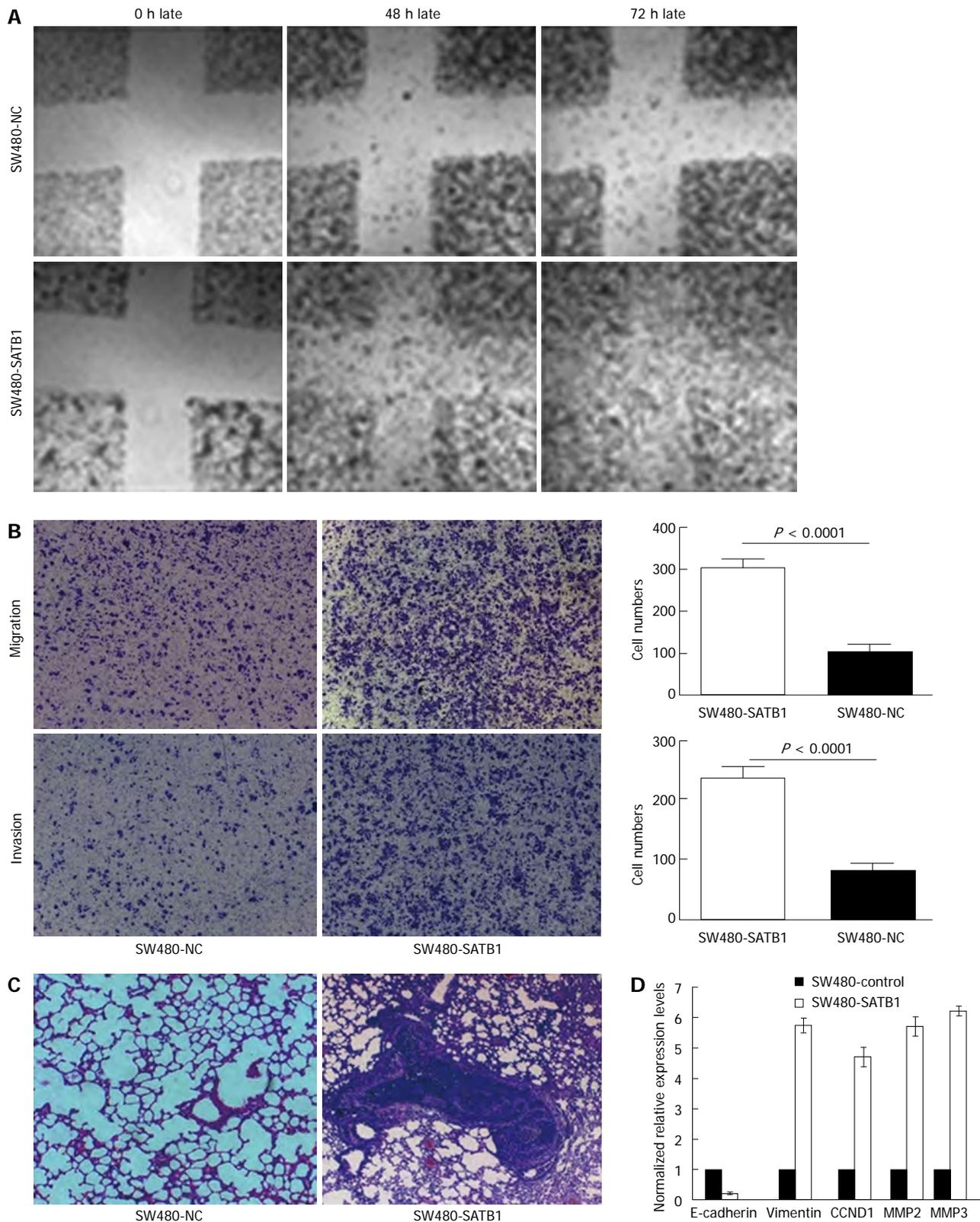


Figure 3 Special AT-rich sequence-binding protein 1 promotes migratory property of SW480 cell *in vitro* and *in vivo* metastasis potential. **A:** A wound-healing assay of SW480-special AT-rich sequence-binding protein 1 (SATB1) cells and negative control (NC) cells. Photographs were taken at the time of 0, 48 and 72 h. Representative photos from one of three replicate experiments are shown (40× original magnification); **B:** Representative photo-micrographs of Transwell results for SW480-SATB1 cells and NC cells were taken (40× original magnification). The number of SW480-SATB1 cells passing through the membrane with or without Matrigel was significantly lower than that of NCs; **C:** Representative hematoxylin and eosin stained sections of the lung tissues isolated from mice implanted with SW480-SATB1 cells and NC cells through the lateral tail vein. The data shown are the number of lung metastases from each group; **D:** A bar chart showing downstream molecules regulated by SATB1 using real-time polymerase chain reaction in SW480-SATB1 cells and NC cells. MMP: Matrix metalloproteases; CCND1: Cyclin D1.

EMT related gene vimentin was increased and E-cadherin was decreased in SW480-SATB1 cells (Figure 3D).

DISCUSSION

Previous studies have suggested the important role of SATB1 in tumor growth and metastasis. But there have been few researches on the relationship between SATB1 and CRC. More recently, Meng *et al*^[11] reported that high level of SATB1 expression was closely correlated with invasive depth and TNM stage in 93 paired samples of human rectal cancer. However, the effects of SATB1 on CRC remain poorly understood. In this study, we showed that overexpression of SATB1 in CRC tissue samples and cell lines could promote cell growth *in vitro* and *in vivo*. In addition, SATB1 could significantly increase the ability of cell migration and invasion *in vitro* and promote the ability of tumor metastasis *in vivo*. We further showed that SATB1 could up-regulate MMPs 2, 9 and vimentin, meanwhile SATB1 could down-regulate E-cadherin in CRC. The data from the current study suggested that SATB1 acts as a potential growth and metastasis promoter in CRC.

Immunohistochemical results showed that the SATB1 protein was overexpressed in CRC tissues and was localized in the nuclei of cancer cells. We also found that SATB1 was overexpressed in cell lines derived from CRC. Our finding is consistent with a recent report showing that the expression of SATB1 was increased in rectal cancer and cell lines^[11]. These data prompted us to analyze the functional effects of SATB1 in CRC cells. We found that SATB1 promotes cell proliferation and cell cycle progression in CRC SW480 cells. In addition, SATB1 expression could promote cell growth *in vivo*. These results suggested that SATB1 may play a tumor promoter role in CRC carcinogenesis.

Invasion and metastasis are the most influential factors for clinical outcome of CRC. Recent studies have shown that SATB1 contributes to tumor metastasis in many types of tumors, such as breast cancer, gastric cancer, and liver cancer^[5,7,9]. Up to date, there was only one report about SATB1 expression and clinical feature in rectal cancer which found that high levels of SATB1 expression were closely correlated with invasive depth and TNM stage in human rectal cancer samples^[11]. This report suggested that SATB1 may facilitate CRC metastasis. In this study, we found that ectopical SATB1 expression endows the non-aggressive SW480 cells with a capability of migration and invasion *in vitro* and metastasis *in vivo*. So we consider that SATB1 may play a crucial role in promoting cancer invasion and metastasis in CRC.

MMP2 and 9, which degrade ECM and promote tumor invasion^[28,29], were up-regulated in the SW480-SATB1 cells that ectopically expressed SATB1. We also found up-regulation of vimentin and down-regulation of E-cadherin in mRNA level in the SW480-SATB1 cells. As a genome organizer, SATB1 recruits chromatin remodeling factors and regulates the spatiotemporal expression of numer-

ous genes involving tumor growth and metastasis. SATB1 has been found to promote breast tumor metastasis and reprograms the genome to change the expression profiles consistent with invasive tumors^[5]. MMP2 and MMP9 are gelatinases that belong to multigene family of proteolytic enzymes^[30]. MMP2 and MMP9 are capable of degrading essentially all the ECM components and the basal membrane, both of which play an crucial role in preventing the migration of cancer cells^[31]. In this sense, MMP2 and MMP9 play an important role in the proteolytic cascade-leading ECM degradation during metastasis in colon carcinoma^[32,33]. E-cadherin is an adherent junction protein and tumor suppressor. Low E-cadherin and high vimentin are traditional markers currently accustomed to discern cells that have undergone a EMT process^[34]. EMT is a process that epithelial cells lose polarity, cell-to-cell contacts, and cytoskeletal integrity contributing to the dissemination of carcinoma cells from epithelial tumors^[35,36]. EMT is thought to be responsible for seeding distant dissemination, eventually leading to cancer-related mortality^[34]. Han *et al*^[5] firstly reported that SATB1 regulated EMT related gene such as E-cadherin, vimentin, fibronectin, N-cadherin, SNAIL and SIP1, and SATB1 depletion restores cell polarity and reduces aggressive phenotypes of breast cancer MDA-MB-231 cells *in vitro*. Another link between SATB1 and EMT was emphasized by Tu *et al*^[9], suggesting that SATB1 mainly induces EMT concomitant with increased expression of Snail1, Slug, Twist and vimentin and decreased expression of E-cadherin, tight junction protein ZO-1 and desmoplakin in liver cancer cell lines. The data from the current study suggest that SATB1 can promote CRC metastasis by degrading ECM and inducing EMT in part.

In conclusion, this study demonstrated that SATB1 is over-expressed in CRC and can promote the growth and metastasis of CRC cells *in vitro* and *in vivo*. We thus have found a new potential promoting factor for the development and progression of CRC.

COMMENTS

Background

Elucidation of the molecular mechanisms of colorectal cancer (CRC) is beneficial for developing novel therapeutic strategies to conquer this disease and in this study the authors analyzed the role of special AT-rich sequence-binding protein 1 (SATB1) in CRC carcinogenesis.

Research frontiers

Only one study suggested that the expression of SATB1 is associated with clinicopathological parameters in CRC. But the roles and mechanisms of SATB1 in growth and metastasis of human CRC remain poorly understood.

Innovations and breakthroughs

SATB1 promotes CRC cell growth, migration and invasion *in vitro* and the ability of tumor metastasis *in vivo*.

Applications

SATB1 acts as a potential growth and metastasis promoter in CRC. SATB1 may be useful as a therapeutic target for CRC.

Terminology

SATB1 gene locates at human chromosome 3p23, and is a thymocyte-specific matrix association region-binding protein. SATB1 is predominantly expressed in thymocytes and could regulate the spatiotemporal expression of numerous genes that involved in T cell proliferation, development, and differentiation.

SATB1 has recently attracted considerable attention in cancer research and its overexpression is a frequent event in various cancers. Furthermore, accumulating evidence showed that SATB1 is also associated with tumor growth and metastasis.

Peer review

The manuscript is well written and the findings are important in its field of investigation.

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Depletion of telomerase RNA inhibits growth of gastrointestinal tumors transplanted in mice

Xue-Cheng Sun, Jing-Yi Yan, Xiao-Lei Chen, Ying-Peng Huang, Xian Shen, Xiao-Hua Ye

Xue-Cheng Sun, Xiao-Hua Ye, Department of Gastroenterology and Hepatology, the First Affiliated Hospital of Wenzhou Medical College, Wenzhou 325000, Zhejiang Province, China
Jing-Yi Yan, Xiao-Lei Chen, Ying-Peng Huang, Xian Shen, Department of Gastroenterology and General Surgery, the First Affiliated Hospital of Wenzhou Medical College, Wenzhou 325000, Zhejiang Province, China

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Correspondence to: Jing-Yi Yan, Associate Chief Physician, Department of Gastroenterology and General Surgery, the First Affiliated Hospital of Wenzhou Medical College, No 2 Fuxue Road, Wenzhou 325000, Zhejiang Province, China. yjy751231@126.com

Telephone: +86-577-88069555 Fax: +86-577-88069555

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Abstract

AIM: To explore effects of telomerase RNA-targeting phosphorothioate antisense oligodeoxynucleotides (PS-ASODN) on growth of human gastrointestinal stromal tumors transplanted in mice.

METHODS: A SCID mouse model for transplantation of human gastrointestinal stromal tumors (GISTs) was established using tumor cells from a patient who was diagnosed with GIST and consequently had been treated with imatinib. GIST cells cultured for 10 passages were used for inoculation into mice. Transfection of PS-ASODN was carried out with Lipotap Liposomal Transfection Reagent. GISTs that subsequently developed in SCID mice were subjected to intratumoral injection once daily from day 7 to day 28 post-inoculation, and mice were divided into the following

four groups according to treatment: PS-ASODN group (5.00 $\mu\text{mol/L}$ of oligonucleotide, each mouse received 0.2 mL once daily); imatinib group (0.1 mg/g body weight); liposome negative control group (0.01 mL/g); and saline group (0.01 mL/g). On day 28, the mice were sacrificed, and tumor attributes including weight and longest and shortest diameters were measured. Tumor growth was compared between treatment groups, and telomerase activity was measured by enzyme-linked immunosorbent assay. Apoptosis was examined by flow cytometry. Real-time polymerase chain reaction was used to detect expression of the mRNA encoding the apoptosis inhibition B-cell leukemia/lymphoma 2 (*bcl-2*) gene.

RESULTS: In the PS-ASODN group, tumor growth was inhibited by 59.437%, which was markedly higher than in the imatinib group (11.071%) and liposome negative control group (2.759%) [tumor inhibition = (mean tumor weight of control group - mean tumor weight of treatment group)/(mean tumor weight of control group) \times 100%]. Telomerase activity was significantly lower ($P < 0.01$) in the PS-ASODN group (0.689 ± 0.158) compared with the imatinib group (1.838 ± 0.241), liposome negative control group (2.013 ± 0.273), and saline group (2.004 ± 0.163). Flow cytometry revealed that the apoptosis rate of tumor cells treated with PS-ASODN was $20.751\% \pm 0.789\%$, which was higher ($P < 0.01$) than that of the imatinib group ($1.163\% \pm 0.469\%$), liposome negative control group ($1.212\% \pm 0.310\%$), and saline group ($1.172\% \pm 0.403\%$). Expression of *bcl-2* mRNA in the transplanted GISTs was markedly downregulated ($P < 0.01$) in the PS-ASODN group (7.245 ± 0.739) compared with the imatinib group (14.153 ± 1.618) and liposome negative control group (16.396 ± 1.351).

CONCLUSION: PS-ASODN can repress GIST growth, mediated perhaps by inhibition of telomerase activity and downregulation of *bcl-2* expression.

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Key words: Gastrointestinal stromal tumor; Phosphorothioate antisense oligonucleotides; Imatinib; Tumor inhibitory rate; Telomerase activity

Core tip: Gastrointestinal stromal tumors (GISTs) are low-grade malignant mesenchymal tumors of the gastrointestinal tract. In our study, telomerase activity was repressed and the level of B-cell leukemia/lymphoma 2 mRNA markedly downregulated in SCID mice carrying transplanted human GISTs and treated with telomerase RNA-targeting phosphorothioate antisense oligodeoxynucleotides (PS-ASODN). Therefore, the therapeutic effect of PS-ASODN on GISTs is remarkable.

Sun XC, Yan JY, Chen XL, Huang YP, Shen X, Ye XH. Depletion of telomerase RNA inhibits growth of gastrointestinal tumors transplanted in mice. *World J Gastroenterol* 2013; 19(15): 2340-2347 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v19/i15/2340.htm> DOI: <http://dx.doi.org/10.3748/wjg.v19.i15.2340>

INTRODUCTION

Gastrointestinal stromal tumor (GIST) is a recently recognized tumor entity. It is now evident that GIST is a distinct tumor type and the most common sarcoma of the gastrointestinal tract in humans^[1]. GISTs account for 2.2% of morbidity associated with malignant tumors of the gastrointestinal tract^[2]. GISTs occur at a median age of 60 years, with a slight male predominance. Approximately 60% and 30% of GISTs arise in the stomach and small intestine, respectively. GISTs have a high propensity for metastatic relapse, specifically in the liver and peritoneum, after initial surgery for localized disease^[3,4]. GISTs are currently categorized based on cell morphology, namely spindle cell, epithelioid, or occasionally pleomorphic; the tumors generally arise in the gastrointestinal tract and usually express the protein KIT. GISTs are generally resistant to conventional cancer chemotherapy and are associated with poor outcome; in 2001, however, an adjuvant therapy with the tyrosine kinase inhibitor imatinib was found to be highly effective against GIST^[5]. Although imatinib has revolutionized the treatment of advanced GISTs, clinical resistance to this drug has proved to be a substantial problem requiring prolonged treatment^[6,7].

Zamecnik *et al*^[8] originally proposed the concept and therapeutic application of antisense nucleic acids. Antisense oligodeoxyribonucleotides are short DNA sequences that hybridize to complementary mRNA sequences by Watson-Crick base pairing. Antisense oligodeoxyribonucleotides do not form hybrids with noncomplementary RNAs encoded by other genes, and thus each individual oligodeoxyribonucleotide targets a unique RNA sequence, thereby effectively blocking the expression of the associated gene while transcription from other genes remains unaffected^[9]. The antisense approach has been

extensively applied in oncology research. Indeed, research has suggested that antisense oligodeoxyribonucleotides, which typically are approximately 20 nucleotides long, can preferentially penetrate tumor vessels because tumor vessels are leakier than normal vessels^[10,11]. Thus, antisense oligodeoxyribonucleotides show tremendous potential as drug candidates that can selectively downregulate and effectively block oncogene expression^[9]. In our present study, we used liposome-assisted transfection to investigate the therapeutic efficacy of delivering telomerase RNA-targeting phosphorothioate antisense oligodeoxynucleotides (PS-ASODN) to human GISTs transplanted into mice, with the goal of inhibiting tumor growth and enhancing tumor-cell apoptosis. Our results suggest a potential new therapeutic intervention for GISTs.

MATERIALS AND METHODS

Sample collection

GIST samples were obtained from a 51-year-old female patient upon admission to the First Affiliated Hospital of Wenzhou Medical College. Standard resection of GISTs in the small intestine was performed on the patient in June 2004, and treatment with a 400-mg daily dose of imatinib was applied for the postoperative period. The patient underwent surgery again in 2006 owing to GIST recurrence, and the daily dose of imatinib was increased to 800 mg postoperatively. A third surgical resection was carried out in 2009 owing again to GIST recurrence, and tumor tissues were resected and used to establish cell lines after obtaining informed consent.

Primary culture of GIST cells

GIST samples were washed twice with Hanks Balanced Salt Solution and cut into cubes of about 1-2 mm³ before 1 mL 0.1% collagenase type I (Gibco, Carlsbad, CA, United States) was added. Each sample was again incubated with another 5 mL of 0.1% collagenase type I in RPMI-1640 culture medium at 37 °C under sterile conditions. The tissues were pipetted 50 times with a slender-tip pipette, and then specimens were incubated for 1 h at 37 °C with gentle shaking every 20 min. The resultant cell suspensions were pipetted 20 times and centrifuged at 1000 × *g* for 5 min at room temperature, and the supernatant was discarded. Each pellet was resuspended in 5 mL RPMI-1640, and larger cell clumps were removed by filtration through a 200-μm mesh nylon gauze. Cells in the filtrate were placed in 4 mL RPMI-1640 complete medium containing 10% fetal bovine serum (Gibco), 100 U/mL penicillin, and 100 U/L streptomycin and incubated at 37 °C in an atmosphere of 5% CO₂ in air. The medium was renewed after 24 h and thereafter renewed every 2 d. After 10 d of culture, trypsin (Sigma, St. Louis, United States) at 0.25% was applied to partially digest the cells, and the cells were purified by differential adhesion. The cells that were most adherent were then subcultured twice a week. GIST cells of different generations were preserved in liquid nitrogen for subsequent experimental

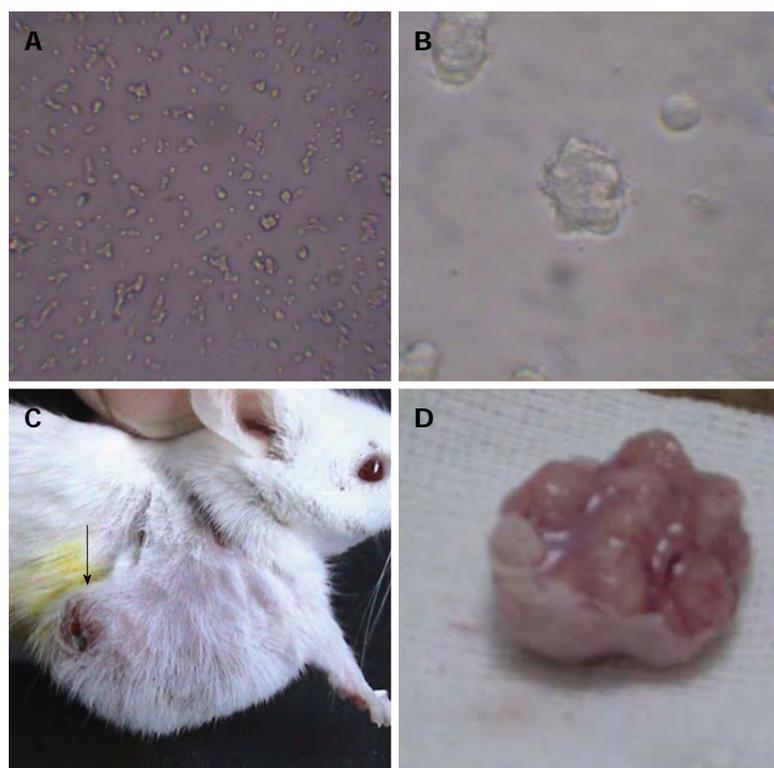


Figure 1 General characteristics of gastrointestinal stromal tumors. A, B: Morphological features of gastrointestinal stromal tumor cells as observed under inverted microscope; C: Tumors developed at the site of human tumor implantation in mice (arrow); D: Representative solid tumor removed from a mouse.

tion. This cell line was named GIST867 (Figure 1A and B).

Animals

Female SCID mice (7-wk old, 22 ± 2 g) were purchased from Shanghai Experimental Animal Center of the National Academy of Sciences, Shanghai, China. All mice were fed standard laboratory chow and water *ad libitum*. All procedures were performed in accordance with the Guidelines for Animal Experiments of Wenzhou Medical College, Wenzhou, China.

Synthesis and transfection of PS-ASODN

PS-ASODN with the sequence 5'-CTCAGTTAGGGT-TAGACA-3', which is a complementary region of the templating RNA of telomerase, was synthesized by Shanghai Biotechnology Engineering Company (Shanghai, China). Transfection of PS-ASODN was carried out with Lipotap Liposomal Transfection Reagent (Beyotime, Shanghai, China). This oligonucleotide was used directly without further purification, and all pipettes and tubes were autoclaved prior to use. The oligonucleotide was first diluted to a final concentration of 100 $\mu\text{mol/L}$ with 550 μL deionized H_2O and stored at -20°C . The Lipotap reagent was diluted with serum-free RPMI-1640 before transfection. PS-ASODN at a final concentration of 5.00 $\mu\text{mol/L}$ was then added and incubated for 15 min with diluted Lipotap reagent in Dulbecco's modified Eagle's medium without antibiotics or glutamine at various temperatures ranging from 15°C to 25°C .

Subcutaneous implantation of GIST867 cells and drug administration

For inoculation into SCID mice, GIST cells of the tenth

generation were digested with 0.25% trypsin and subcultured in RPMI-1640. Centrifugation yielded a single cell suspension having a density of 1.0×10^7 viable cells per 1 mL serum-free medium. A dose of 0.25 mL of single cell suspension was injected subcutaneously into the flank skin of each of two female SCID mice. The two mice were fed under sterile conditions, and at 28-d post-inoculation the diameter of the resultant tumor was 1-2 cm in each mouse. These two mice were anaesthetized and decapitated to obtain the tumors, which subsequently were cut into cubes of 1 mm^3 in 10% fetal bovine serum. The tumor cubes were placed subcutaneously into the left flank skin of 40 female SCID mice, and after 1 wk tumors were successfully induced in all mice (Figure 1C and D). The 40 tumor-bearing SCID mice were randomly divided into four groups (10 mice per group): the PS-ASODN group (5.00 $\mu\text{mol/L}$, each mouse received 0.2 mL by intra-tumor injection once daily); imatinib group (0.1 mg/g body weight, imatinib obtained from Novartis Pharma, Basel, Switzerland); liposome negative control group (0.01 mL/g); and saline group (0.01 mL/g). The mice in each group received the relevant treatment by intra-tumor injection once daily from day 7 to day 28 after implantation. After 28 d, the mice were sacrificed, and tumor weight and longest and shortest diameters were measured by electronic scale and vernier caliper, respectively. Inhibition of tumor growth was calculated as follows: inhibition of growth = (mean tumor weight of control group - mean tumor weight of treatment group) / (mean tumor weight of control group) $\times 100\%$. Aliquots of the tumor specimens were frozen in liquid nitrogen for further use.

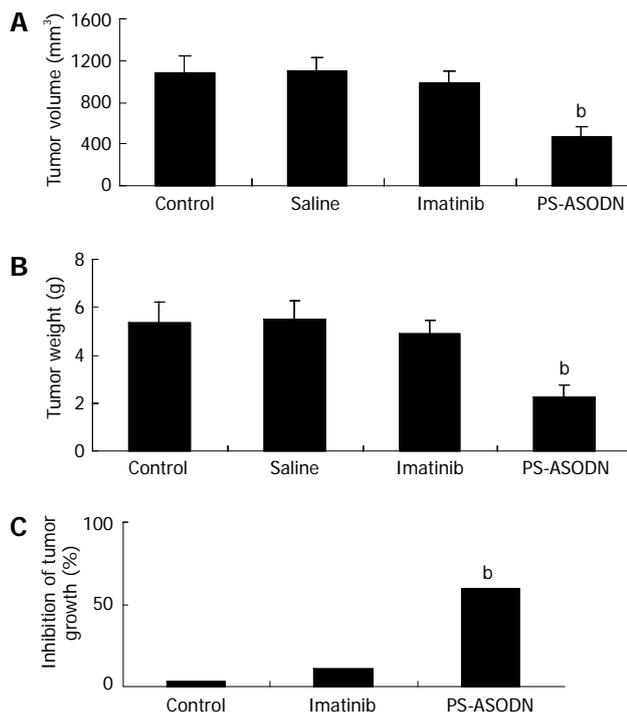


Figure 2 Phosphorothioate antisense oligodeoxynucleotides mediated inhibition of tumor growth in human gastrointestinal stromal tumors ($n = 10$ tumors per group). Daily intra-tumor injection of phosphorothioate antisense oligodeoxynucleotides (PS-ASODN) and other reagents commenced on post-inoculation day 7 and continued to day 28. A: Tumor volume; B: Tumor weight; C: Inhibition of tumor growth. ^b $P < 0.01$ vs liposome negative control and imatinib groups. Each bar represents the mean \pm SD.

Real-time polymerase chain reaction analysis

Total RNA was extracted using Trizol reagent (Beyotime), and the concentration and purity of RNA were determined by measuring the absorbance at 260 nm and 280 nm ($A_{260} > 2.0$, and $A_{280} > 1.7$). Real-time polymerase chain reaction (PCR) analysis was performed using an ABI PRISM 7500 Real-Time PCR System (Applied Biosystems Inc., Carlsbad, CA, United States). Each well (20 μ L volume) contained 10 μ L Power SYBR Green PCR master mix (Applied Biosystems), 1 μ L of each primer (5 μ mol/L) and 1 μ L template. Primer sequences were designed by PrimerExpress 5.0 and synthesized by the Shanghai Biotechnology Corporation (Shanghai, China); the sequences were (5'-3'): B-cell leukemia/lymphoma 2 (*bcl-2*) gene (235 bp): forward CAGCTGCACCTGACGCCCTT and reverse CCCAGCCTCCGTTATCCTGGA; β -actin (99 bp): forward CCACACTGTGCCATCTACG and reverse AGGATCTTCATGAGGTAGTCAGTCAG.

Telomerase activity assay

Telomerase activity was assayed by enzyme-linked immunosorbent assay following the procedure recommended by the manufacturer (Boehringer, Mannheim, Germany). The absorbance value in each well was read at 490 nm with a microtiter plate reader (BIO-TEK ELX800, Winooski, Vermont, CA, United States).

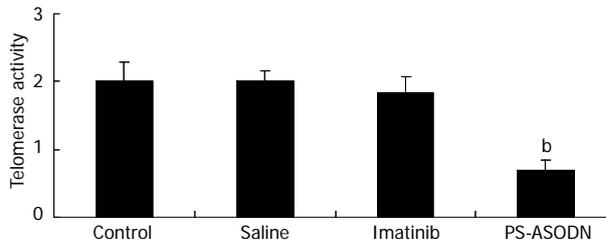


Figure 3 Effect of phosphorothioate antisense oligodeoxynucleotides and other reagents on telomerase activity in gastrointestinal stromal tumor tissues ($n = 10$ tumors per group). ^b $P < 0.01$ vs tumors treated with phosphorothioate antisense oligodeoxynucleotides (PS-ASODN) and imatinib.

Apoptosis as measured by flow cytometry

Apoptosis was assessed by flow cytometry. Tumor specimens were cut into cubes of 1 mm³, homogenized in 2 mL PBS, and filtered through 200- μ m mesh nylon gauze. The filtrate was left for 10 min in the dark and was then centrifuged at $2500 \times g$ for 7 min at room temperature. The pellet was resuspended in 200 μ L Binding Buffer (10 mmol/L HEPES, 140 mmol/L NaCl, 2.5 mmol/L CaCl₂, pH 7.4) and labeled with 10 μ L annexin V-FITC and 5 μ L propidium iodide from the annexin V-FITC Apoptosis Detection kit (eBioscience, San Diego, CA, United States). Apoptosis was assayed by flow cytometry (BD FACSCalibur CellSorting System, Becton Dickinson, Franklin Lakes, NJ, United States). The samples were tested in sextuplicate, and mean values were calculated.

Statistical analysis

All data are presented as the mean \pm SD deviation. Statistical analysis was carried out with SPSS 13.0 software (SPSS, Chicago, IL, United States). Statistically significant differences between groups were established using Fisher's least significant difference test. $P < 0.05$ was considered to be statistically significant.

RESULTS

Inhibition of tumor growth in PS-ASODN-treated mice

Tumor volume and weight were significantly lower in the PS-ASODN group compared with the liposome negative control and saline groups ($P < 0.01$, Figure 2A and B). Tumor volume and weight in the imatinib group were slightly lower than in the liposome negative control and saline groups, but the difference was not significant ($P > 0.05$). Inhibition of tumor growth in the PS-ASODN group (59.437%) was significantly greater than in the imatinib (11.071%) and liposome negative control groups (2.759%) (all relative to tumor growth observed in the saline control group, Figure 2C).

Effect of PS-ASODN on telomerase activity

Telomerase activity was significantly repressed in the PS-ASODN group compared with the imatinib and liposome negative control groups ($P < 0.01$, Figure 3). As expected, administration of imatinib did not significantly

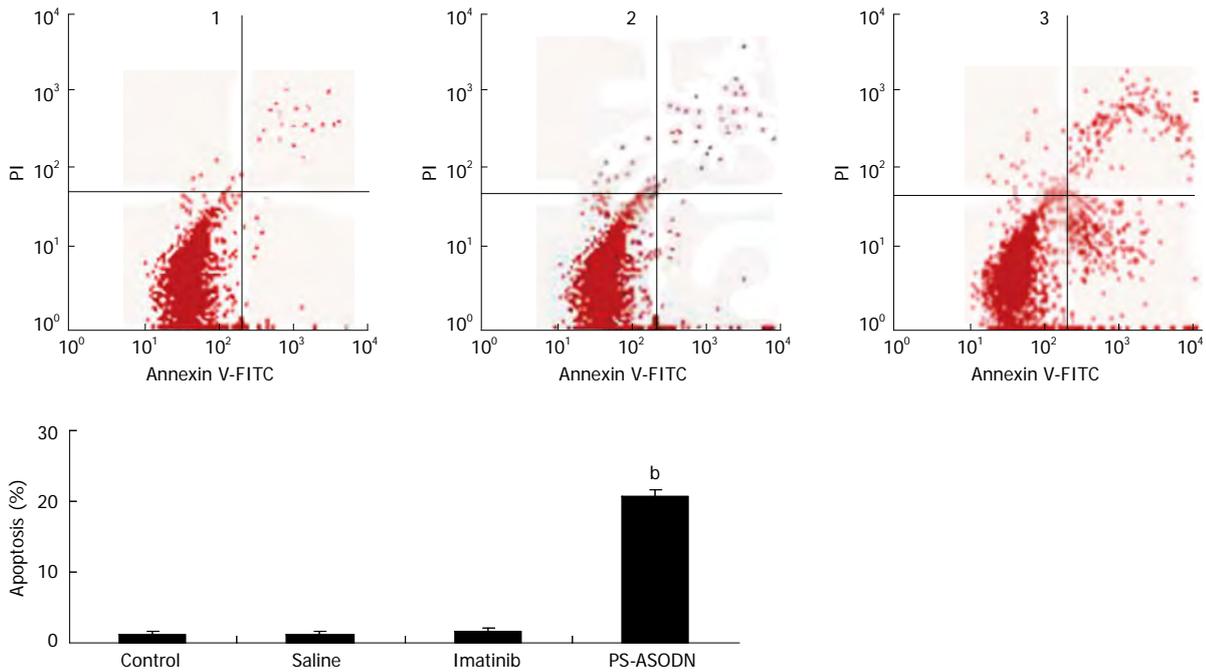


Figure 4 Effect of phosphorothioate antisense oligodeoxynucleotides and other reagents on tumor cell apoptosis ($n = 10$ tumors per group). ^b $P < 0.01$ vs liposome negative control and imatinib groups. 1: Liposome negative control group; 2: Imatinib group; 3: Phosphorothioate antisense oligodeoxynucleotides (PS-ASODN) group. PI: Propidium iodide; FITC: Fluorescein isothiocyanate.

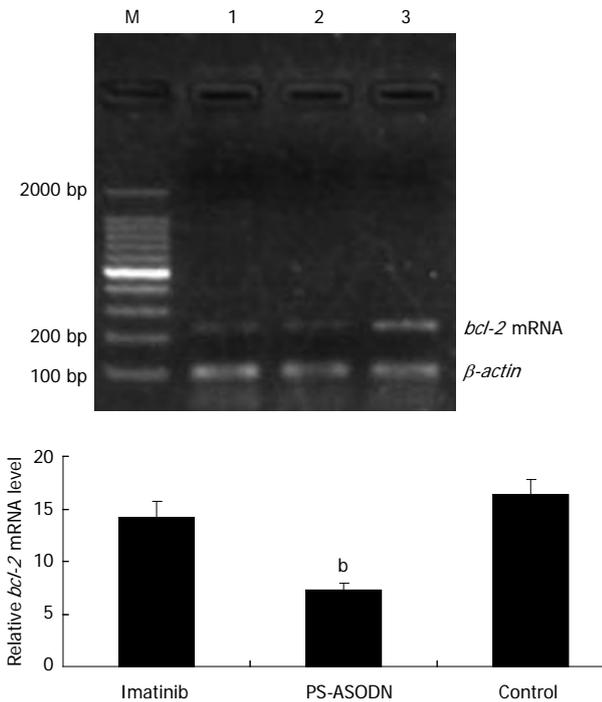


Figure 5 B-cell leukemia/lymphoma 2 mRNA expression in tumor samples as detected by real time-polymerase chain reaction ($n = 10$ mice per group). ^b $P < 0.01$ vs control and imatinib groups. Each bar represents the mean \pm SD. M: Marker, 100-2000 bp; 1: Imatinib-treated group; 2: Phosphorothioate antisense oligodeoxynucleotides (PS-ASODN)-treated group; Lane 3: Control group. bcl-2: B-cell leukemia/lymphoma 2.

affect telomerase activity compared with the liposome negative control group ($P > 0.05$).

Effect of PS-ASODN on tumor cell apoptosis

The percentage of apoptotic cells in tumors was determined by flow cytometry on day 28 after tumor implantation. Apoptosis was significantly higher in the PS-ASODN group (20.751% \pm 0.789%) compared with the imatinib (1.637% \pm 0.469%), liposome negative control, and saline groups ($P < 0.01$, Figure 4). There was no significant difference ($P > 0.05$) between the imatinib group and the liposome negative control and saline groups.

Effect of PS-ASODN on bcl-2 expression OR the level of bcl-2 mRNA

Agarose gel electrophoresis was used to verify the lengths of the PCR amplification fragments, namely 235 bp for bcl-2 (encoding B-cell lymphoma protein 2) and 99 bp for β -actin (Figure 5). The level of bcl-2 mRNA was significantly downregulated ($P < 0.01$) in the PS-ASODN group compared with the liposome negative control group (Figure 5).

DISCUSSION

GISTs are the most common mesenchymal neoplasms of the gastrointestinal tract, and the worldwide incidence of GISTs has been estimated to be 14-20 per million people. GISTs are low-grade malignant tumors that are believed to originate from neoplastic transformation of the interstitial cells of Cajal^[12-14]. The overall 5-year survival rate for GIST patients is about 45% in the United States^[15]. Nearly 50% of GISTs treated with imatinib ultimately demonstrate resistance in the first 2 years post-treatment,

and thus a new treatment strategy and/or more effective drug is needed.

There are at least two different mechanisms for the immortalization of tumor cells: reactivation of telomerase, and the inactivation of tumor suppressor genes such as *p53* and *pRB* that control cellular senescence^[16]. Human telomerase, which contains an RNA component, telomerase-associated protein and a catalytic subunit^[17-20], is activated in 80%-90% of carcinomas derived from various organs such as stomach, colon, lung and breast^[21-23]. The rate of telomere DNA shortening is regulated by telomerase expression and activity^[24-26]. In our study, we evaluated telomerase activity in GISTs and found that telomerase activity was markedly elevated, consistent with findings for other tumor types.

Controlling the levels of the anti-apoptotic bcl-2 family proteins is critical for regulating cell growth and apoptosis. bcl-2 localizes to cellular membranes, particularly in mitochondria, and can inhibit mitochondrial release of substances involved in signaling either the onset or execution of apoptosis^[27], and higher levels of bcl-2 promote the development and progression of many tumors^[28]. bcl-2 promotes cell survival by inhibiting adapters needed for activation of the proteases (caspases) that dismantle the cell. Therefore, bcl-2 and related cytoplasmic proteins are key inhibitory factors of apoptosis, which indeed is critical for development, tissue homeostasis, and protection against pathogens^[29-31]. Here, we found that the level of bcl-2 mRNA was significantly upregulated in GISTs, consistent with its established role in promoting tumorigenesis.

The drug resistance of a malignant tumor is an important issue for conventional clinical therapies such as chemotherapy, radiotherapy, and immunotherapy. If telomerase activity and/or expression is inhibited in cancer cells, the cells may become relatively more vulnerable to these conventional therapies^[32].

In this study, transfection with PS-ASODN significantly inhibited telomerase activity and induced apoptosis compared with the imatinib and control groups. Recently, research has shown that cells with long telomeres possess the ability to proliferate in the absence of telomerase, which demonstrates that telomerase activity does not require basic replicative functions of these cells; rather, maintaining a minimum telomere length seemingly requires telomerase activity^[33]. Other studies have shown that cells with high telomerase activity were more resistant to apoptosis than those with low telomerase activity^[34,35]. Kondo *et al.*^[32] found that inhibition of telomerase with an antisense telomerase expression vector not only decreased telomerase activity but also increased the susceptibility to cisplatin-induced apoptotic cell death in cisplatin-resistant U251-MG cells. Research suggests that bcl-2 is a regulator of programmed cell death, and its overexpression has been implicated in pathogenesis of some lymphomas. In our study, the SCID mice treated with PS-ASODN had significantly downregulated expression of bcl-2 mRNA compared with the liposome nega-

tive control and saline groups.

In conclusion, our study demonstrates that a synthetic antisense oligonucleotide can reduce both telomerase activity and bcl-2 mRNA expression and increase apoptosis of human GIST cells *in vivo*. The therapeutic effect of PS-ASODN on GISTs is remarkable, and the use of synthetic antisense oligonucleotides has the advantage of therapeutic convenience and flexibility. Our data clearly show the potential efficacy of antisense oligonucleotides for the treatment of human GISTs.

COMMENTS

Background

Gastrointestinal stromal tumor (GIST) is a distinct tumor type and the most common sarcoma of the gastrointestinal tract in humans, and it has a high propensity for metastatic relapse, specifically in the liver and peritoneum, after initial surgery for localized disease. Previous research has shown that antisense oligodeoxyribonucleotides, which can target a unique sequence of a single gene and block its expression while other genes are transcribed without interruption, have tremendous potential as promising drugs that can selectively downregulate oncogene expression.

Research frontiers

GISTs are low-grade malignant mesenchymal tumors of the gastrointestinal tract, and the overall 5-year survival rate for GIST patients is about 45% in the United States. The authors found telomerase activity was markedly elevated in GISTs. Therefore, telomerase may be reactivated at a certain stage in GIST progression, enabling cancer cells to escape telomere shortening and continue proliferating. B-cell leukemia/lymphoma 2 (bcl-2) is a key regulator of apoptosis and the level of bcl-2 mRNA is significantly upregulated in GISTs.

Innovations and breakthroughs

Drug resistance of a malignant tumor is an important challenge for conventional clinical therapies such as chemotherapy, radiotherapy, and immunotherapy. This study is the first to report a new viewpoint on GIST pathogenesis and the potential therapeutic effect of telomerase RNA-targeting phosphorothioate antisense oligodeoxynucleotides (PS-ASODN) on GIST.

Applications

The authors measured telomerase activity and the level of bcl-2 mRNA in mice carrying transplanted human GISTs. The results provide new insight into the pathogenesis of GIST and suggest an efficacious therapy for GIST.

Terminology

GISTs are low-grade malignant mesenchymal tumors of the gastrointestinal tract and are believed to originate from neoplastic transformation of the interstitial cells of Cajal. Antisense oligodeoxyribonucleotides are short DNA sequences that do not form hybrids with noncomplementary RNAs encoded by other genes, and thus each individual oligodeoxyribonucleotide targets a unique RNA sequence, thereby effectively blocking the expression of the associated gene while transcription from other genes remains unaffected.

Peer review

The authors of this study investigated the pathogenesis of GISTs. The results are interesting and suggest that telomerase activity was repressed and the level of bcl-2 mRNA significantly downregulated in SCID mice treated with PS-ASODN. They investigated the effect of PS-ASODN on proliferation, apoptosis, and telomerase activity of tumor cells in mouse transplanted GISTs, with the goal of attaining a new viewpoint on GIST pathogenesis and providing a new therapeutic intervention.

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United States-based practice patterns and resource utilization in advanced neuroendocrine tumor treatment

Jonathan Strosberg, Roman Casciano, Lee Stern, Rohan Parikh, Maruit Chulikavit, Jacob Willet, Zhimei Liu, Xufang Wang, Krzysztof J Grzegorzewski

Jonathan Strosberg, H Lee Moffitt Cancer Center and Research Institute, Gastrointestinal Tumor Department, Tampa, FL 33612, United States

Roman Casciano, Lee Stern, Rohan Parikh, Maruit Chulikavit, Jacob Willet, LA-SER Analytica, New York, NY 10018, United States

Zhimei Liu, Xufang Wang, Krzysztof J Grzegorzewski, Novartis Oncology, Florham Park, NJ 07932, United States

Author contributions: Strosberg J, Casciano R, Stern L, Parikh R, Chulikavit M, Liu Z, Wang X and Grzegorzewski KJ contributed to the study design and manuscript review; Willet J contributed to the writing, development, and editing of the manuscript.

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Correspondence to: Lee Stern, MS, LA-SER Analytica, 24 West 40th Street, Floor 8, New York, NY 10018, United States. lstern@la-ser.com

Telephone: +1-212-6864100 Fax: +1-212-6868601

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Abstract

AIM: To assess advanced neuroendocrine tumor (NET) treatment patterns and resource utilization by tumor progression stage and tumor site in the United States.

METHODS: United States Physicians meeting eligibility criteria were provided with online data extraction forms to collect patient chart data on recent NET patients. Resource utilization and treatment pattern data were collected over a baseline period (after diagnosis and before tumor progression), as well as initial and secondary progression periods, with progression defined according to measureable radiographic evidence of tumor progression. Resource categories used in the analysis include: Treatments (*e.g.*, surgery, chemo-

therapy, radiotherapy, targeted therapies), hospitalizations and physician visits, diagnostic tests (biomarkers, imaging, laboratory tests). Comparisons between categories of resource utilization and tumor progression status were examined using univariate (by tumor site) and multivariate analyses (across all tumor sites).

RESULTS: Fifty-five physicians were included in the study and completed online data extraction forms using the charts of 110 patients. The physician sample showed a relatively even distribution for those affiliated with academic versus community hospitals (46% vs 55%). Forty (36.3%) patients were reported to have pancreatic NET (pNET), while 70 (63.6%) patients had gastrointestinal tract (GI)/Lung as the primary NET site. Univariate analysis showed the proportion of patients hospitalized increased from 32.7% during baseline to 42.1% in the progression stages. While surgeries were performed at similar proportions overall at baseline and progression, pNET patients, were more likely than GI/Lung NET patients to have undergone surgery during the baseline (33.3% vs 25.0%) and any progression periods (26.7% vs 23.4%). While peptide-receptor radionuclide and targeted therapy utilization was low across NET types and tumor stages, GI/Lung types exhibited greater utilization of these technologies compared to pNET. Chemotherapy utilization was also greater among GI/Lung types. Multivariate analysis results demonstrated that patients in first progression period were over 3 times more likely to receive chemotherapy when compared to baseline (odds ratio: 3.31; 95%CI: 1.46-7.48, $P = 0.0041$). Further, progression was associated with a greater likelihood of having a study physician visit [relative risk (RR): 1.54; 95%CI: 1.10-2.17, $P = 0.0117$], and an increased frequency of other physician visits (RR: 1.84; 95%CI: 1.10-3.10, $P = 0.0211$).

CONCLUSION: Resource utilization in advanced NET in the United States is significant overall and data suggests progression has an impact on resource utilization

regardless of NET tumor site.

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Key words: Gastrointestinal cancers; Neuroendocrine tumors; Resource utilization; Health economics; Clinical practice patterns

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INTRODUCTION

Neuroendocrine tumors (NET) are a group of diverse neoplasms, commonly originating in the gastrointestinal tract (GI), lungs, and pancreas. The prevalence of NET is estimated to be 35.0 per 100000 in the United States, and the age-adjusted incidence of NET has increased from an estimated 1.1 per 100000 persons in 1973 to 5.3 per 100000 persons in 2004^[1]. The specific factors responsible for this rise in incidence are not known; however, improved classification of tumors and widespread use of endoscopy as a screening tool are likely contributors to this increase^[1].

Pancreatic NET (pNET) are often classified based on hormone produced (*e.g.*, gastrinoma, insulinoma)^[2]. GI and lung NET types have traditionally been classified by site of origin, and morphologic pattern. Newer classifications, however, have been formulated to reflect the considerable variability in histopathology and presentation within each site of origin^[3]. Patients with ileocecal NET typically present with carcinoid syndrome, which results from an over production of serotonin. Symptoms and complications include diarrhea, hot flashes, bronchoconstriction, and right-sided valvular heart disease^[3]. NET is commonly perceived as indolent^[1]; however, due to the variability and uncertainty of symptoms associated with the disease, NET is often diagnosed in an advanced stage whereby the prognosis is poor (65% 5-year mortality rate). This is particularly true of pNET, where the 5-year mortality rate has been reported to be as high as 73%^[1].

Patients with localized NETs and those with resectable oligometastases are often managed surgically. Advanced unresectable tumors are often treated with somatostatin analogs (SSA), either for control of symptoms or inhibition of tumor growth^[4]. Other treatment options include streptozocin or temozolomide-based chemotherapy, or targeted therapies such as sunitinib or everolimus^[5,6]. Platinum and etoposide-based regimens are typically used for poorly differentiated tumors. While guidelines to aid treatment decisions have been published^[5,6], little is known about how disease progression and tumor type influence NET treatment decisions among United States physicians

in a real-world setting. Therefore, the aim of the current study is to assess advanced NET treatment patterns and resource utilization by tumor progression stage and tumor site in the United States.

MATERIALS AND METHODS

Study design

In a United States-based sub-analysis of a global study (details of which have been published elsewhere^[7]) Physicians (gastroenterologists, endocrinologists, and oncologists) were contacted to take part in the research from December 2010 to January 2011. A total of 4100 physicians were identified in a market research database, and were recruited *via* an online invitation. A convenience sampling method was applied in order to achieve a final global study sample of 197 physicians, with a target sample of 55 physicians in the United States sub-study. Eligibility criteria included the following: practicing medicine for at least 3 years (but no more than 30 years) prior to the study date, spending at least 50% of one's working time on patient care, treating at least 3 NET patients in the past year, and specializing in gastroenterology, endocrinology, or oncology.

Physicians were instructed to complete internet-based data extraction forms, referring to clinical charts of patients. They were asked to refer only to charts on their most recent patients who were diagnosed with advanced NET of the GI tract, lung, or pancreas - at least one patient must have experienced tumor progression. Additionally, selected patients had to have confirmed well - to moderately-differentiated tumor histology, assessed as per the 2000 World Health Organization criteria^[8]. Data regarding patients with poorly differentiated tumors were not selected for this study. Proportions, frequencies, and means (respectively) were compared by NET progression stage and tumor site for the following measures:

NET progression: The main variable of interest was 3-level tumor progression stage. The baseline period, or first stage, was defined as the time between diagnosis of NET and diagnosis of tumor progression with measurable or radiographic evidence as reported by physicians. Initial progression, or second stage, was defined as the time period during which tumor progression was first diagnosed and treated. Secondary progression, or third stage, was defined as the time point when further measurable or radiographic evidence for progression was found, and the following period of treatment. Since not all patients had a second progression, physicians were asked to project resource use during this period, assuming a duration of 12 mo for all patients. The date of the last resource use served as a proxy for patients who had no recorded first progression or second progression date. Initial and secondary progression, in aggregate, were referred to as any progression (*i.e.*, the any progression period encompasses both the initial and secondary progression period, assessing resources accrued in both; Figure 1).

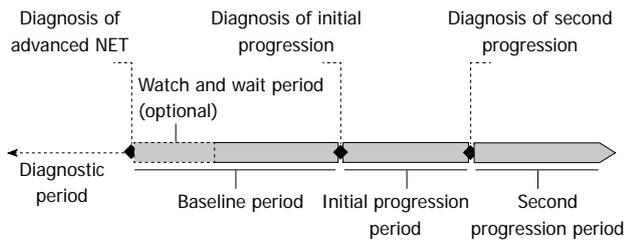


Figure 1 Advanced neuroendocrine tumor patient study timeline. Second progression period resource utilization data was derived from physician projections. NET: Neuroendocrine tumor.

NET type: The patient sample was further stratified into two groups, one with primary NET location as either the lung or GI, and the other with primary NET location as the pancreas. It should be noted that no pre-specified distribution among NET sub-types was implemented; physicians were asked only to report data on their most recent patients.

Patient baseline attributes: Include age, site of metastasis, ECOG performance status, tumor histology and grade, performance status, comorbidities, and tumor symptoms.

Physician attributes: Include primary specialty, whether they practiced in an academic or community hospital, number of years of training and practice, and proportion of time spent in direct patient care.

Medical resources: Chemotherapy, peptide-receptor radionuclide therapy (PRRT), SSA, and other pharmaceutical therapies including targeted therapies; as well as study-physician and other physician visits, hospitalizations, surgical procedures such as radiofrequency ablation, microwave ablation, cryoablation, radiotherapy, hepatic arterial embolization, and transplant; Biomarker tests including neuron specific enolase, chromogranin A, pancreatic polypeptide, neurotensin, plasma serotonin, vasoactive intestinal polypeptide, Ghrelin, human chorionic gonadotropin, Ki-67, 5-hydroxyindoleacetic acid, plasma substance P, total and free T4; other lab tests including CBC, BUN, serum glucose, Darkfield microscopy, serum creatinine, and lipid profile; and imaging test including ultrasounds, computed tomography (CT) scans, helical scans, and others.

Statistical analysis

Proportions and frequencies (for dichotomous) as well as means and medians (for count outcomes) were assessed for the study resource use categories (listed above) were compared according to NET progression stage and tumor site. Resources were assessed for any progression and any time. Any progression was measured at an event level, where first progression and second progression were considered as separate events. Thus, it included both first progression and second progression resource-use information, which may have resulted in multiple

events per patient. For any progression, utilization rates were reported as a proportion of patients per event for each specific resource. Any time included resources used at least once during baseline, first progression, or second progression. A given patient is counted only once if a particular resource is received in two or more time periods. Practice patterns for any time were analyzed as the proportion of patients utilizing each resource.

Post-hoc statistical analysis

Multivariable models were conducted to compare resource use across disease progression stages. As the study was not powered to ascertain statistically significant differences, these analyses were considered secondary. Some patients did not have baseline or first progression data (Measures), and repeated measurement of outcomes over tumor stage progression produced correlated outcomes. Therefore generalized estimating equations (GEE) were employed. GEE are generalized linear models which estimate the average response over the population, as opposed to predicting responses for individuals. Utilizing such an approach, models were computed for binary (yes/no) outcomes assuming a binomial distribution. For count outcomes a Poisson distribution was assumed. All models adjusted for the following covariates: primary NET location, age, country, physician specialty, tumor histology, ECOG performance status, and patient follow-up time. It should be noted that the resource use category study physician visits was assessed as a binary outcome (*i.e.*, did a patient have ≥ 1 visit or not); however, due to the issue of convergence, a Poisson distribution was assumed for this variable rather than a binomial distribution. A Bonferroni correction on *P* value was calculated to determine an appropriate alpha value for the statistical significance threshold ($0.05/9 = 0.0055$).

RESULTS

Patient and physician characteristics

Out of the total sample of physicians ($n = 55$), 13% ($n = 7$) primarily practiced medical oncology, 29% ($n = 16$) hematology/oncology, 29% ($n = 16$) gastroenterology, and 29% ($n = 16$) endocrinology. The physician sample showed a relatively even distribution for those affiliated with academic versus community hospitals (46% *vs* 55%). Each physician reported data on 2 patients ($n = 110$ total). Baseline data were not available for 6 United States patients because their date of diagnosis of advanced NET and first progression were the same; however, 61 patients had at least initial progression.

Forty (36.3%) patients were reported to have pNET, while 70 (63.6%) patients had GI/Lung as the primary NET site (GI/Lung NET). The mean duration of baseline period was found to be 14.0 mo for pNET, 13.3 mo for GI/Lung, and 13.6 mo for both tumor types. The mean duration of initial progression was 5.7 mo for pNET, 7.9 mo for GI/Lung, and 7.1 mo for both tumor types. Sixty three (57.3%) patients had well differentiated

Table 1 Any time resource utilization by neuroendocrine tumor type¹ n (%)

| | All NET (n = 110) | GI/lung NET (n = 70) | pNET (n = 40) |
|----------------------------------|----------------------|-------------------------|------------------|
| Treatments | | | |
| Surgery | 50 (45.45) | 30 (42.86) | 20 (50.00) |
| Chemotherapy ² | 42 (38.18) | 30 (42.86) | 12 (30.00) |
| PRRT | 6 (5.45) | 6 (8.57) | 0 (0.00) |
| Somatostatin | 86 (78.18) | 54 (77.14) | 32 (80.00) |
| Targeted therapy ³ | 7 (6.36) | 5 (7.14) | 2 (5.00) |
| Resources | | | |
| Hospitalizations | 62 (56.36) | 40 (57.14) | 22 (55.00) |
| Ultrasounds | 42 (38.18) | 22 (31.43) | 20 (50.00) |
| CT scans | 79 (71.82) | 53 (75.71) | 26 (65.00) |
| Helical scans | 42 (38.18) | 27 (38.57) | 15 (37.50) |
| Other imaging tests ⁴ | 64 (58.18) | 41 (58.57) | 23 (57.50) |
| Bio marker ⁵ | 73 (66.36) | 47 (67.14) | 26 (65.00) |
| Other lab tests ⁶ | 70 (63.64) | 41 (58.57) | 29 (72.50) |
| Physician visit | 109 (99.09) | 40 (100.00) | 69 (98.57) |

¹A patient was counted (once) if that individual utilized a resource at any stage of their disease; ²Chemotherapy includes 5-fluorouracil, actinomycin-D, capecitabine, carboplatin, cisplatin, cyclophosphamide, dacarbazine, doxorubicin, etoposide, gemcitabine, irinotecan, mitotane, oxaliplatin, streptozocin, temozolomide and vincristine; ³Targeted therapy includes everolimus, sunitinib, imatinib, and bevacizumab; the data collection form captured these therapies under the heading "other treatments"; ⁴Other imaging includes positron emission tomography, stereotactic radiosurgery, metaio benzylguanidine, magnetic resonance imaging, and chest X-ray; ⁵Biomarkers include neurotensin, chromogranin A, pancreatic polypeptide, neurotensin, plasma serotonin, vasoactive intestinal polypeptide, ghrelin, human chorionic gonadotropin, Ki-67, 5-hydroxyindoleacetic acid, plasma substance P, total and free T4; ⁶Other lab tests include serum glucose, complete blood count, blood urea nitrogen, serum creatinine, and lipid profile were captured. PRRT: Peptide-receptor radionuclide therapy; NET: Neuroendocrine tumor; pNET: Pancreatic NET; GI: Gastrointestinal tract; CT: Computed tomography.

tumor histology, while 47 (42.7%) patients had moderately differentiated tumors. Furthermore, 63 (57.3%) patients were found to have symptoms and 47 (42.7%) showed no symptoms. ECOG Performance varied as follows: 44 (40.0%) patients showed a score of 0-1, 24 showed a score of 2, 12 showed a score of 3, 5 showed a score of 4, and 25 patients had no recorded score.

Resource utilization and practice patterns

Resource utilization at any time and across all NET subtypes shows SSAs to be the treatment used in the highest proportion of patients (78.2%), followed by surgery and chemotherapy (used in 45.5% and 38.2% of patients, respectively). Any time resource utilization also indicates high proportions of patients undergoing hospitalization (56.4%) as well as diagnostics such as CT scans (71.8%), biomarkers (66.4%) and other lab tests (63.6%). pNET patients were proportionately less likely than GI/Lung NET patients to receive chemotherapy (30.0% *vs* 42.9%), CT scans (65.0% *vs* 75.7%), and PRRT (0.0% *vs* 8.6%). pNET patients were more likely to have received ultrasound (50.0% *vs* 31.4%) and other laboratory tests (72.5% *vs* 58.6%), and to have undergone surgery (50.0% *vs* 42.9%), when compared to GI/Lung NET patients. Utilization of other resources were found to be similar

between the two groups: SSA (80.0% *vs* 77.1%), targeted therapy (5.0% *vs* 7.1%), hospitalization (55.0% *vs* 57.1%), helical scans (37.5% *vs* 38.6%), other imaging tests (57.5% *vs* 58.6%), and biomarker tests (65.0% *vs* 67.1%; Table 1).

Resource utilization by progression state and by tumor site is summarized in Table 2. First, chemotherapy utilization followed markedly different patterns between pNET and GI/Lung NET patients. No chemotherapy use was observed among pNET patients at baseline; however, it increased to greater than 26.7% during progression. Doxorubicin, streptozocin, and temozolomide were the most frequently used chemotherapies. Among GI/Lung NET patients chemotherapy use gradually increased from baseline to second progression; cisplatin, 5-fluorouracil, etoposide and doxorubicin being the most frequently used chemotherapies. Second, targeted therapies were not widely used among pNET patients (2.8% at baseline; 1.7% during progression). GI/Lung NET patients showed marginally higher use of targeted therapies, with increased utilization during second progression (5.7% over baseline (2.9%) and initial progression (2.4%). Third, PRRT was not utilized among pNET patients; however, a small number of GI/Lung NET patients received PRRT (4.4% during baseline, 4.5% during progression).

Similar SSA use was observed among both pNET and GI/Lung NET patients. At baseline 63.5% of all NET patients received SSA. Utilization at first progression was marginally lower (62.3%), while second progression utilization decreased to 40.9%, with an average of 48.5% at any progression. The proportion of patients who were hospitalized increased between baseline and any progression periods (32.7%-42.1% overall). Proportions of patients utilizing CT Scans increased upon progression. Ultrasound, biomarker, laboratory tests and other imaging decreased upon progression. Lastly, surgeries were performed at similar proportions overall at baseline and progression. pNET patients, however, were more likely than GI/Lung NET patients to have undergone surgery during the baseline (33.3% *vs* 25.0%) and any progression periods (26.7% *vs* 23.4%).

Post-hoc statistical results across tumor progression stages

Patients in first progression were observed to be over 3 times more likely to receive chemotherapy when compared to baseline (odds ratio: 3.31; 95%CI: 1.46-7.48; Table 3). Patients in first progression were also more likely to have a study physician visit [relative risk (RR): 1.54; 95%CI: 1.10-2.17], as well as an increased frequency of other physician visits (RR: 1.84; 95%CI: 1.10-3.10; Table 4).

DISCUSSION

The aim of this study was to assess the resource utilization and treatment patterns of NET by tumor progression stage and by tumor site. The results suggest that there is significant resource utilization associated with

Table 2 Resource utilization by progression state *n* (%)

| | Baseline | | | Any progression | | |
|---------------------------------|-------------|------------|-------------|-----------------|-------------|------------|
| | All NET | GI/Lung | pNET | All NET | GI/Lung | pNET |
| Treatments | | | | | | |
| Chemotherapy ¹ | 17 (16.4) | 17 (25.0) | 0 (0.0) | 46 (26.9) | 30 (27.0) | 16 (26.7) |
| Targeted therapies ² | 3 (2.9) | 2 (2.9) | 1 (2.8) | 6 (3.5) | 5 (4.5) | 1 (1.7) |
| PRRT | 3 (2.9) | 3 (4.4) | 0 (0.0) | 39 (6.1) | 5 (4.5) | 0 (0.0) |
| Somatostatin analogs | 66 (63.5) | 43 (63.2) | 23 (63.9) | 83 (48.5) | 52 (46.9) | 31 (51.7) |
| Surgery | 29 (27.9) | 17 (25.0) | 12 (33.3) | 42 (24.6) | 26 (23.4) | 16 (26.7) |
| Resources | | | | | | |
| Hospitalizations | 34 (32.7) | 23 (33.8) | 11 (30.6) | 72 (42.1) | 44 (39.6) | 28 (46.7) |
| Ultrasound | 38 (36.5) | 20 (29.4) | 18 (50.0) | 34 (19.9) | 22 (19.8) | 12 (20.0) |
| CT scans (conventional) | 62 (59.6) | 43 (63.2) | 19 (52.8) | 109 (63.7) | 72 (64.9) | 37 (61.8) |
| CT scans (helical or spiral) | 23 (22.1) | 15 (22.1) | 8 (22.2) | 51 (29.8) | 33 (29.7) | 18 (30.0) |
| Other imaging ³ | 51 (49.0) | 31 (45.6) | 20 (55.6) | 52 (30.5) | 33 (29.7) | 19 (31.7) |
| Biomarkers ⁴ | 66 (63.4) | 44 (64.7) | 22 (61.1) | 84 (49.1) | 55 (49.6) | 29 (48.3) |
| Lab tests ⁵ | 61 (58.7) | 36 (52.9) | 25 (69.4) | 84 (49.1) | 50 (45.1) | 34 (56.7) |
| Study physician visit | 102 (98.08) | 66 (97.06) | 36 (100.00) | 165 (96.49) | 107 (96.40) | 58 (96.67) |
| Other physician visit | 77 (74.04) | 50 (73.53) | 27 (75.00) | 143 (83.62) | 92 (82.88) | 51 (85.00) |

¹Chemotherapy includes 5-fluorouracil, actinomycin-D, capecitabine, carboplatin, cisplatin, cyclophosphamide, dacarbazine, doxorubicin, etoposide, gemcitabine, irinotecan, mitotane, oxaliplatin, streptozocin, temozolomide and vincristine; ²Targeted Therapy includes everolimus, sunitinib, imatinib, and bevacizumab; the data collection form captured these therapies under the heading "other treatments"; ³Other imaging includes positron emission tomography, stereotactic radiosurgery, metaiobenzylguanidine, magnetic resonance imaging, and chest X-ray; ⁴Biomarkers include neurotensin, chromogranin A, pancreatic polypeptide, neurotensin, plasma serotonin, vasoactive intestinal polypeptide, ghrelin, human chorionic gonadotropin, Ki-67, 5-hydroxyndoleacetic acid, plasma substance P, total and free T4; ⁵Other lab tests include serum glucose, complete blood count, blood urea nitrogen, serum creatinine, and lipid profile were captured. PRRT: Peptide-receptor radionuclide therapy; NET: Neuroendocrine tumor; pNET: Pancreatic NET; GI: Gastrointestinal tract; CT: Computed tomography.

Table 3 Multivariate analysis for resource use prevalence among patients at first progression vs baseline¹

| Resource ¹ | Beta | Odds | 95%CI | | P |
|-----------------------|----------|--------|-------------|-------------|---------------------|
| | estimate | ratio | Lower bound | Upper bound | value |
| Chemotherapy | 1.1962 | 3.3074 | 1.4625 | 7.4795 | 0.0041 ² |
| Somatostatin analogs | 0.5298 | 1.6986 | 0.8038 | 3.5892 | 0.1652 |
| Surgery | 0.4551 | 1.5763 | 0.7632 | 3.2556 | 0.2188 |
| Hospitalization | 0.3227 | 1.3808 | 0.6920 | 2.7550 | 0.3599 |
| Other physician visit | 0.7336 | 2.0826 | 0.9185 | 4.7221 | 0.0790 |

¹Generalized estimating equations model (assuming binomial distribution and unstructured covariance structure) while controlling for primary neuroendocrine tumor location, age, Eastern Cooperative Oncology Group status, tumor histology, physician specialty and follow-up time; ²Value denotes statistical significance.

Table 4 Multivariate analysis for patient hospitalization and physician visit frequency at first progression vs baseline

| Resource | Beta | Rate | 95%CI | | P |
|-------------------------------------|----------|--------|-------------|-------------|--------|
| | estimate | ratio | Lower bound | Upper bound | value |
| Study physician visit ¹ | 0.4346 | 1.5444 | 1.1014 | 2.1656 | 0.0117 |
| Hospitalizations ² | -0.0617 | 0.9402 | 0.2648 | 3.3379 | 0.9240 |
| Study physician visits ² | 0.1321 | 1.1412 | 0.8330 | 1.5635 | 0.4108 |
| Other physician visits ² | 0.6116 | 1.8433 | 1.0961 | 3.0999 | 0.0211 |

¹Generalized estimating equations model (assuming binomial distribution and unstructured covariance structure) while controlling for primary neuroendocrine tumor location, age, Eastern Cooperative Oncology Group status, tumor histology, physician specialty and follow-up time; ²Value denotes statistical significance.

United States NET patients regardless of tumor site, particularly with respect to hospitalizations, surgeries, imaging and lab tests, chemotherapy, and SSA (Table 1). As may be expected, disease progression is associated with a decrease in utilization rates for certain diagnostics including ultrasound, biomarker, laboratory tests and other imaging. However, disease progression is associated with an increase in other resources, such as other (non-study) physician visits, hospitalizations, chemotherapy, and CT scans (Table 2). In keeping with these results, the multivariate analysis demonstrates that NET patients are more likely to receive chemotherapy and visit physicians when

disease progresses.

While overall resource utilization increases with disease progression irrespective of tumor site, there were variations in practice patterns depending on whether patients had GI/Lung or pNET. For instance, pNET patients were found to be less likely than GI/Lung patients to be administered targeted, chemo-, or peptide-receptor radionuclide therapies, and more likely to have undergone surgical procedures than the GI/Lung patients. Although the heterogeneous nature of NET makes inferences about whether physicians treated patients according to current guidelines difficult, some comparisons

can be made with caution^[5,6]. The high utilization of surgical procedures and SSA observed here is consistent with NCCN guidelines^[5]. It is possible that utilization of targeted therapies (everolimus and sunitinib) in pNET was low because of their novelty, limited availability, and restricted reimbursement by managed care organizations during the patient data collection time period (December 2010-January 2011). Furthermore, recent clinical trial data supporting the use of these therapies were not available when the study was conducted^[9,10]. Inferences regarding the observed differences in utilization of targeted and PRRT therapies between pNET and GI/Lung types are inconclusive.

The low use of targeted therapies overall should be considered more closely. Recent clinical trial data suggest sunitinib and everolimus improve progression-free survival by 6-7 mo compared to placebo plus best supportive care^[9,11]. While more research is necessary to elucidate differences in adverse event reporting (and quality of life more generally) between patients receiving chemotherapy versus targeted agents, these trials suggest that targeted therapies are associated with relatively low rates of adverse events, and may be more tolerable than chemotherapy^[9,11].

Rates of chemotherapy use are higher than one might expect (42.86% in GI/Lung NET and 30.00% in pNET); especially given that chemotherapy is approved only in well- to moderately-differentiated pNET. While the reasons for these relatively high rates cannot be known for certain, it is possible that physicians are using chemotherapy regimens off-label, as a result of having few alternative treatments available for the advanced GI/Lung and pancreatic NET populations.

Interestingly, we found similar baseline durations for GI/Lung and pNET types; with marginally shorter first progression duration in pNET (secondary progression duration could not be ascertained). As pNET has been reported to have a more aggressive disease course^[1], caution should be taken in interpreting these results. It is plausible that the operational definition of disease progression in the current study is not sensitive to changes in the tumor pathology which lead to the mean differences in survival and progression, generally observed elsewhere^[1].

In comparison to results from a global analysis^[7], this United States-specific sub-analysis shows several differences in resource utilization and practice patterns. Specifically, cross-sectional resource utilization is lower in the United States for certain categories including chemotherapy, PRRT, and notably hospitalizations. Multivariate analyses are in line with those from the global analysis, showing an increase in chemotherapy and physician visits associated with progression. However, while the global analysis also showed an increase in SSA use with progression, the United States sub-analysis data do not.

Limitations

Due to possible selection bias and the exclusion criteria used to identify physicians for study eligibility, the sample

of participating physicians may differ from the general population of physicians in ways that may differentially affect the study results. Some patients are missing baseline data ($n = 6$), and for those patients with initial progression data but not secondary progression data, hypothetical resource use projections were made by the study physicians. Because of the cross-sectional nature of the study, recall bias may have affected the observed results. Additionally, the self-reported nature of the data limited the ability to assess the variety of symptoms associated with NET clinical syndromes, ancillary treatments used to palliate hormonal symptoms. Targeted therapy utilization was likely under-reported due to the structure of the data collection form, which relied on open-ended responses for this treatment category. As noted above, the study findings show high rates of chemotherapy usage. While the reported results may represent off-label usage, it is also possible that patients with poorly-differentiated NET were included, as etoposide- and cisplatin- based regimens are approved in this indication.

Overall study results confirm that advanced NET in the United States is associated with significant resource use regardless of tumor site. Resource utilization follows a consistent pattern across NET tumor types as the disease progresses, suggesting progression has an impact on resource utilization regardless of tumor site. Targeted therapy use (everolimus and sunitinib) was reported to be relatively low compared to other treatments, likely due to pending regulatory approval at the time of the study. However, with the regulatory approvals in place, targeted therapy use is expected to increase in the future. Further research involving larger patient populations is warranted to fully depict the nature of NET resource utilization and related treatment patterns and to define the real world economic impact of NET disease progression.

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COMMENTS

Background

Neuroendocrine tumors (NET) are a group of diverse neoplasms, commonly originating in the gastrointestinal tract, lungs, and pancreas. The prevalence of NET is estimated to be 35.0 per 100000 in the United States.

Research frontiers

While guidelines to aid treatment decisions have been published, little is known about how disease progression and tumor type influence NET treatment decisions among United States physicians in a real-world setting.

Innovations and breakthroughs

This study assessed the resource utilization and treatment patterns of NET by tumor progression stage and by tumor site. Overall study results confirm that advanced NET in the United States is associated with significant resource use regardless of tumor site.

Applications

Further research involving larger patient populations is warranted to fully depict the nature of NET resource utilization and related treatment patterns and to

define the real world economic impact of NET disease progression.

Terminology

Resource categories used in the resource utilization analysis include: Treatments (e.g., surgery, chemotherapy, radiotherapy, targeted therapies), hospitalizations and physician visits, diagnostic tests (biomarkers, imaging, laboratory tests).

Peer review

This is a well-written article summarizing resource utilization of treatments for neuroendocrine tumors in the United States based on anonymous physician surveys. While such a study is inherently subject to recall bias, the manuscript lists this potential pitfall in the discussion section.

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Patient comfort and quality in colonoscopy

Vivian E Ekkelenkamp, Kevin Dowler, Roland M Valori, Paul Dunckley

Vivian E Ekkelenkamp, Kevin Dowler, Roland M Valori, Paul Dunckley, Department of Gastroenterology, Gloucestershire Hospitals NHS Trust, Gloucester GL1 3NN, United Kingdom
Vivian E Ekkelenkamp, Department of Gastroenterology and Hepatology, Erasmus University Medical Center, 3000 CA Rotterdam, The Netherlands

Author contributions: Ekkelenkamp VE and Dowler K contributed to conception and design, analysis and interpretation of the data, drafting of the article, final approval of the article; Valori RM contributed to conception and design, critical revision of the article for important intellectual content, final approval of the article; Dunckley P contributed to conception and design, analysis and interpretation of the data, critical revision of the article for important intellectual content, final approval of the article.

Correspondence to: Vivian E Ekkelenkamp, MD, Department of Gastroenterology and Hepatology, Erasmus University Medical Center, PO Box 2040, 3000 CA Rotterdam, The Netherlands. v.ekkelenkamp@erasmusmc.nl

Telephone: +31-10-7032983 Fax: +31-10-7034682

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RESULTS: A total of 17027 colonoscopies were performed by 23 independent endoscopists between 2008 and 2011. Caecal intubation rate varied from 79.0% to 97.8%, with 18 out of 23 endoscopists achieving a CIR of > 90%. The percentage of patients experiencing significant discomfort during their procedure (defined as NRCL of 4 or 5) ranged from 3.9% to 19.2% with an average of 7.7%. CIR was negatively correlated with NRCL-45 ($r = -0.61$, $P < 0.005$), and with poor patient experience ($r = -0.54$, $P < 0.01$). The average dose of midazolam (mean 1.9 mg, with a range of 1.1 to 3.5 mg) given by the endoscopist was negatively correlated with CIR ($r = -0.59$, $P < 0.01$). CIR was positively correlated with PDR ($r = 0.44$, $P < 0.05$), and with the numbers of procedures performed by the endoscopists ($r = 0.64$, $P < 0.01$).

CONCLUSION: The best colonoscopists have a higher CIR, use less sedation, cause less discomfort and find more polyps. Measuring patient comfort is valuable in monitoring performance.

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Key words: Endoscopy; Colonoscopy; Quality; Comfort; Performance

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Abstract

AIM: To explore the relationship of patient comfort and experience to commonly used performance indicators for colonoscopy.

METHODS: All colonoscopies performed in our four endoscopy centres are recorded in two reporting systems that log key performance indicators. From 2008 to 2011, all procedures performed by qualified endoscopists were evaluated; procedures performed by trainees were excluded. The following variables were measured: Caecal intubation rate (CIR), nurse-reported comfort levels (NRCL) on a scale from 1 to 5, polyp detection rate (PDR), patient experience of the procedure (worse than expected, as expected, better than expected), and use of sedation and analgesia. Pearson's correlation coefficient was used to identify relationships between performance indicators.

INTRODUCTION

Colonoscopy is a very common procedure performed to investigate colonic symptoms and screen for cancer and polyps^[1]. It has always been known that colonoscopy can cause harm and even death, but poor quality colonoscopy has only been linked to other important outcomes in the last decade. Back-to-back colonoscopies identified

important missed lesions^[2], fast withdrawal times were associated with lower adenoma detection rates^[3,4], and low adenoma detection rates are associated with higher rates of missed cancer^[5]. Several studies have shown that colonoscopy misses, and fails to “protect” individuals from, cancer^[6-10]. Thus there has been increasing attention on the quality of colonoscopy^[11,12], especially in the context of colorectal cancer screening where there is potential for causing harm to otherwise healthy people.

In order to assess quality, the British Society of Gastroenterology (BSG) has defined a set of indicators and auditable outcomes for colonoscopy^[13]. Important key performance indicators are an unadjusted caecal intubation rate (CIR) of > 90% and an adenoma detection rate of > 10%. CIR is globally recognised as the main measure of competence in colonoscopy in a non-screening setting and is one of the key measures used in a colorectal cancer screening. It is an absolute requirement for total colonoscopy, and poor completion rates may be one reason why colonoscopy does not prevent cancer in the right colon^[14-16]. However, there are several factors that can influence the CIR and thus the performance of an endoscopist^[17].

A possible consequence of having CIR as a prime indicator of quality is that individuals with poor technique may push harder and persist for longer to achieve the standard. This could lead to more pain and the administration of more sedation. Clearly this could cause unnecessary harm to patients, including more perforations and sedation related complications^[18].

To prevent this eventuality the BSG proposed that other key performance indicators should be sedation and comfort^[13]. Standards were set for sedation, particularly for older patients, but there is no standard for comfort so it was designated an essential “auditable outcome”: a standard that should be measured, reviewed and acted upon, but not one for which an absolute performance level could be defined.

Various studies have addressed patient pain or discomfort during colonoscopy, and identified predictive factors of pain^[19-22]. However, none have explored the use of sedation and patient comfort as measures of performance.

This study aims to analyse the different factors affecting an individual’s performance in diagnostic colonoscopy and to explore the use of patient comfort scores as performance indicators for colonoscopy.

MATERIALS AND METHODS

All colonoscopies performed in the four endoscopy units in one healthcare organisation are recorded on two electronic endoscopy reporting systems (SQL scope and Unisoft), which log the key performance indicators defined by the BSG: CIR; polyp detection rate (PDR) (adenomatous and hyperplastic); and sedation (invariably opiates and midazolam). Colonoscopies performed by all independently practicing endoscopists during the four

Table 1 Five-point scale of nurse-reported comfort levels

| Nurse-reported comfort levels | Descriptors |
|-------------------------------|---|
| No discomfort | Talking/comfortable throughout |
| Minimal discomfort | 1 or 2 episodes of mild discomfort with no distress |
| Mild discomfort | More than 2 episodes of discomfort without distress |
| Moderate discomfort | Significant discomfort experienced several times with some distress |
| Severe discomfort | Frequent discomfort with significant distress |

year period of 2008 to 2011 were included in the analysis. Throughout the United Kingdom (and in this study) an unadjusted CIR is used: the rate is not adjusted at all, even for obstructions and poor bowel preparation.

Comfort is assessed using nurse-reported comfort levels (NRCL) on a 5-point scale, which is shown in Table 1. The attending endoscopy nurses assess the comfort of the patient during the procedure without discussing it with the endoscopist, and record it immediately. For this study, significant discomfort was defined as a NRCL of either level 4 or 5 (NRCL-45).

The patient experience (PE) is captured by the recovery nurse before the patient leaves the unit. Patients are asked whether their experience was: better than expected, as expected, or worse than expected. Both the comfort scores and the PE are recorded on the hospital administration system. The colonoscopists are identified in the reporting system so that all data can be linked to individuals.

The influence of midazolam and opiate analgesia on NRCL and worse patient experience (PE-W) was also explored. A further variable used in this analysis was PDR. The dataset for PDR was less complete as our endoscopic reporting systems did not mandate the input of PDR until September 2010.

A complete dataset was not available for all variables. Table 2 lists the numbers of colonoscopies where data was not documented.

Statistical analysis

Relationships of CIR to comfort (NRCL-45), sedation and PE-W were explored using Pearson’s correlation coefficient. The relationship between the number of procedures performed per year and CIR was also studied using Pearson’s correlation coefficient. Only endoscopists performing colonoscopies for the full four year period were included in this analysis. A Mann-Whitney *U* test was used to assess whether there was a difference in the number of colonoscopies performed by those with a higher CIR.

RESULTS

During the four year period from 1 January 2008 to 28 December 2011, 17027 colonoscopies were performed by 23 colonoscopists; 88.8% of procedures were performed

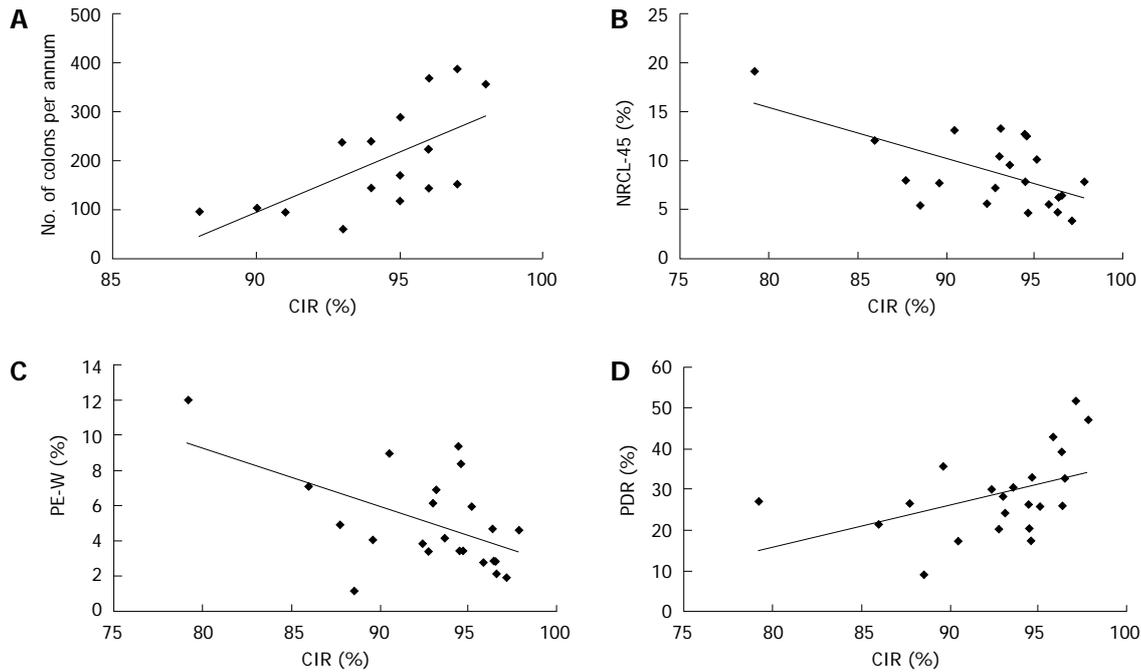


Figure 1 The figure shows correlations of caecal intubation rate with number of annual colonoscopies (A), nurse-reported comfort level of 4-5 (B), patient experience worse than expected (C) and polyp detection rate (D). CIR: Caecal intubation rate; NRCL: Nurse-reported comfort levels; PE-W: Worse patient experience; PDR: Polyp detection rate.

Table 2 Data completeness on colonoscopies performed from 2008-2011

| Variable | Total number of colonoscopies with missing data | Percent of colonoscopies with complete data |
|-----------------|---|---|
| CIR | 0 | 100% |
| NRCL | 520 | 95% |
| PE | 1647 | 84% |
| Midazolam | 62 | 99% |
| Opiates | 65 | 99% |
| Polyp detection | 3863 | 71% |

CIR: Caecal intubation rate; NRCL: Nurse-reported comfort levels; PE: Patient experience.

on service lists; 11.2% of procedures were performed on bowel cancer screening lists. Data is reported as performance data for these colonoscopists.

Colonoscopy completion

CIR varied from 79.0% to 97.8%, with 18 out of 23 endoscopists achieving > 90%. Four endoscopists completed colonoscopy in 85%-89% of the procedures and 1 locum endoscopist in 79%. The effect of the number of colonoscopies performed on CIR was studied. Only endoscopists performing colonoscopy during the whole period were included in this analysis alone ($n = 16$). CIR was positively correlated with the average number of procedures performed per annum ($r = 0.64$, $P < 0.01$) (Figure 1A). The average CIR for these 16 endoscopists was 94.3%. Endoscopists with a CIR of less than 94.3% performed an average of 139.9 colonoscopies per year whereas those with a CIR of greater than 94.3% per-

formed an average of 245.9 procedures ($P < 0.05$).

Patient comfort

The percentage of patients experiencing significant discomfort during their procedure (defined as NRCL of 4 or 5) ranged from 3.9% to 19.2% with an average of 7.7%. There was significant negative correlation between NRCL-45 and CIR ($r = -0.61$, $P < 0.005$) (Figure 1B).

Patient experience

A worse than expected patient experience (PE-W) was recorded in 4.3% of procedures (1.2%-12.0%). PE-W correlated negatively with CIR ($r = -0.54$, $P < 0.01$) (Figure 1C). There was strong correlation between NRCL-45 and PE-W ($r = 0.92$, $P < 0.0001$). Only 2% of patients with a NRCL of 1, 2 or 3 rated the procedure as worse than expected compared to 28% of patients with a NRCL of 4 or 5.

Sedation

The sedation used in our endoscopy units for colonoscopy is usually a combination of an opiate (either pethidine or fentanyl) and midazolam. An increasing proportion of procedures are done without sedation.

The average amount of midazolam used per procedure was 1.9 mg, varying from 1.1 mg to 3.5 mg. Average dose of midazolam was negatively correlated with CIR ($r = -0.59$, $P < 0.01$). To assess whether this was due to higher doses of midazolam being used by colonoscopists with worse CIRs or to a higher rate of no sedation being used by those with better CIRs, the analysis was repeated for the sedated colonoscopies only. In this sedated group

Table 3 Improvements in key performance indicators between 2008-2011

| Year | CIR | NRCL-45 | PE-W | Midazolam, mg (mean dose) | PDR |
|------|-------|---------|------|---------------------------|-------|
| 2008 | 93.3% | 10.0% | 5.6% | 2.3 | 29.6% |
| 2009 | 93.4% | 7.8% | 4.2% | 2.0 | 27.4% |
| 2010 | 94.6% | 7.6% | 4.1% | 1.8 | 31.9% |
| 2011 | 95.9% | 5.8% | 3.7% | 1.7 | 37.7% |

CIR: Caecal intubation rate; NRCL: Nurse-reported comfort levels; PE-W: Worse patient experience; PDR: Polyp detection rate.

($n = 14870$) there was a significant correlation between average midazolam usage and CIR ($r = -0.60$, $P < 0.005$). The percentage of colonoscopies performed without sedation was not significantly correlated with CIR ($r = 0.30$, $P = 0.13$). There was also a correlation between midazolam dose and NRCL-45 ($r = 0.54$, $P < 0.01$) but not for midazolam and PE-W ($r = 0.37$, $P = 0.08$). In unsedated patients, there was no correlation between CIR with either NRCL-45 ($r = -0.09$, $P > 0.05$) or PE-W ($r = -0.01$, $P > 0.05$). However, the numbers were smaller in this group, especially for colonoscopists who rarely performed colonoscopy without sedation. Furthermore, the more uncomfortable procedures would have led to patients being given sedation thereby introducing bias.

There were 4 endoscopists who used fentanyl and 19 who used pethidine as their opiate of preference. To ensure uniformity, the endoscopists using fentanyl were excluded from the analysis on analgesia. There was no significant correlation between average pethidine dose, and CIR ($r = -0.39$, $P > 0.05$), NRCL-45 ($r = 0.17$, $P > 0.05$) or PE-W ($r = 0.06$, $P > 0.05$).

Polyp detection

In this study, the average PDR (including both hyperplastic and adenomatous polyps) was 31.8% (range 9.2%-51.9%). There was a positive correlation between PDR and CIR ($r = 0.44$; $P < 0.05$) (Figure 1D).

Performance indicators over time

Table 3 shows data on the CIR, NRCL-45, PE-W, midazolam usage and PDR for each year. A consistent improvement is seen in all variables between 2008 and 2011.

DISCUSSION

In this study, we explored factors that predict high performance in colonoscopy. Ideally a colonoscopy should be safe, complete and comfortable. It should also detect and remove safely and completely all important lesions. The CIR has become the most universally recognised performance indicator. While striving to achieve and exceed target CIRs there is a potential danger that a colonoscopist will cause more discomfort, or put the patient at risk of perforation and excessive sedation. The results of this study indicate the reverse: those colonoscopists with the highest CIR use less sedation, cause less discomfort and achieve a better patient experience. Furthermore, it ap-

pears they are more vigilant, identifying more polyps than those with lower intubation rates. The results also show that better colonoscopists perform more colonoscopies. In this study, colonoscopists with a CIR of greater than 94.3% performed an average of 245.9 procedures per annum compared with 139.9 for the endoscopists with a CIR lower than 94.3%. This is consistent with previously published data^[23]. This study adds further weight to the argument that there should be a minimum number of procedures performed by an endoscopist per annum to maintain their skills.

There are very large variations in the use of sedation across the world ranging from virtually none in Scandinavian countries to increasing use of deep sedation with propofol in Australia, France, Germany and the United States. The use of sedation is still not as safe as we would like^[24]. In the United States, it is now common to perform a colonoscopy with propofol and it has been shown that patient satisfaction is higher than with other types of sedation^[25,26]. Conversely, a Scandinavian study showed that high sedation rates were not associated with less painful colonoscopies^[21]. Another Scandinavian group showed that sedation is not necessary for screening individuals, and an American group clearly believes unsedated colonoscopy has a place and has coined the phrase "sedation-risk-free colonoscopy"^[27].

In our study, the average midazolam dose used was negatively correlated with CIR: the more often the caecum was reached, the less midazolam was used and, furthermore, patients did not experience more discomfort. These findings demonstrate that colonoscopy can be performed without deep sedation and without significant discomfort in the majority of patients.

Sedation alters the perception and recollection of discomfort experienced during colonoscopy. Thus the patient cannot necessarily provide an accurate guide of pain during the procedure. An alternative to the patient assessing discomfort is for the endoscopist or endoscopy nurse to make the assessment. We ask the nurse to make this assessment because they are more likely to be objective and have the benefit of observing all colonoscopists perform colonoscopy. Our comfort scale has not been formally validated but it assesses three components of discomfort: severity, frequency and the extent to which it is distressing the patient. Interestingly there was strong correlation of this nurse-assessed scale with patient reports ($r = 0.92$, $P < 0.0001$). Only 2% of patients with a NRCL of 1, 2 or 3 rated the experience as worse than expected. It is likely that different nurses rate discomfort differently but that discrepancy would be applied to all colonoscopists. There are always two nurses in the procedure room during a colonoscopy and the nurses are encouraged to discuss the comfort score with each other before making a final decision.

The assessment of patient experience is different from that of discomfort by a health professional. Because of the effect of sedation on experience and recall, we chose not to ask patients to rate comfort but to rate their experience of the procedure compared to what they expected. This mea-

sure was chosen on the assumption that a worse experience than expected was unacceptable and a better or as expected experience was acceptable. Clearly a patient's rating will be affected by the way they are prepared for the procedure and hearsay. It is possible that the patients of a colonoscopist who routinely tells them that they will experience terrible pain will rarely report the experience worse than expected. We cannot control or assess this possibility. It seems very unlikely that the colonoscopists with high CIR tell their patients that they will have a bad experience when the nurses rate them as causing less pain than their colleagues.

Sedation practice varies but the majority use a combination of opiates and sedatives, and an increasing number use no sedation. It is therefore difficult to make meaningful comparisons. However, whichever way the data was examined the same conclusion was drawn: colonoscopists with high CIR use less sedation (midazolam). One argument against using CIR (especially an unadjusted rate) as a performance indicator is that endoscopists may use excessive force to ensure that the caecum is intubated. However, data from this study shows that comfort scores were better in colonoscopists with a higher CIR and there was no evidence that they were using more opiate analgesia.

A possible bias in this study is case mix. It is possible that the colonoscopists with the highest CIR were colonoscoping the easiest patients. Previous studies have identified factors that predict lower CIR: female sex, older patient and the presence of diverticular disease^[19,28]. Until recently our reporting system was not capturing diagnoses according to a recognised coding system so it is not possible to determine the proportion of patients with diverticular disease in each of the colonoscopist cohorts. About 30% of patients listed for colonoscopy are pooled and listed with the endoscopist that is first available. This sharing of patients reduces the likelihood that an individual will be scoping a particularly difficult group of patients. Furthermore, colonoscopists with a higher CIR are often asked to scope "difficult" patients meaning case mix is more likely to affect them adversely. Another possible source of case mix bias is bowel cancer screening (FOBT positive) patients because only accredited colonoscopists are allowed to colonoscope them. These patients are usually asymptomatic and may therefore be easier to colonoscope; there is however no data available on this topic. They certainly have more polyps than other patients, which may bias polyp detection data. Whilst only 10% of all colonoscopies are performed on screen positive patients, up to 50% of the procedures performed by the bowel cancer screening colonoscopists are on screened patients. However, only 2 of the 23 colonoscopists for the majority of the study period were screening accredited and several of the high performing (high CIR, low sedation, low discomfort) colonoscopists were not screening colonoscopists. Another possible confounder is the use of unadjusted CIR instead of the CIR being adjusted for poor bowel preparation or obstruction. CIR would invariably have been higher if adjusted. We chose to use unadjusted CIR as this is standard practice in the United King-

dom for quality assessment. The number of cases with poor bowel preparation or obstruction was probably low and there is no reason to believe that one endoscopist was exposed to all those cases especially as the bowel preparation was standardised across all four units. Therefore, we feel that it is unlikely that the use of adjusted CIR would influence the main findings in this study.

Adenoma detection rate is a key performance indicator and has been shown to be related to the chance of post colonoscopy colorectal cancer^[5]. Ideally, adenoma detection rate should be recorded but linking endoscopic with pathology databases is difficult, and late entry of pathology data into an endoscopic database is fraught with problems. In view of this difficulty, we have used polyp rather than adenoma detection in this study whilst recognising the limitations of this approach. However, recent studies have shown that PDR can be used as a marker for ADR because they are highly correlated^[29,30]. A recent study of colonoscopies performed on the United Kingdom Bowel Cancer Screening programme also found a positive correlation between adenoma detection rate and caecal intubation rate^[31].

In each of the endoscopy units included in this study there is a robust quality assurance process for colonoscopy. All colonoscopists are fed back their performance indicators on a quarterly basis. If any colonoscopist underperforms, the endoscopy lead will discuss this with them and, if appropriate, offer further support and training. Furthermore most of the colonoscopists in this study have completed a training the trainer course during which there is detailed discussion of colonoscopy technique and ways to improve it. These approaches are likely to have contributed to the consistent improvements in CIR, patient comfort/experience and PDR. One aspect of quality assurance we did not address in this study is occurrence of complications in colonoscopy. Our study explores the intubation performance, not performance of therapy. There were no diagnostic perforations during the period of this study and no procedure related deaths. Literature tells us that less than 1:1000 patients will suffer from a complication of colonoscopy without biopsies or polypectomy^[32]. A much larger sample size would be required to test the relationship of key performance indicators and complication rates.

In conclusion, this study demonstrates that the best colonoscopists are doing more colonoscopies per year, get to the caecum more often, use less sedation, cause less discomfort, achieve a better patient experience and find more polyps. We believe that measurement of patient comfort and experience, use of sedation, together with CIR, could provide a richer picture of a colonoscopist's performance, at least of intubation skills.

This study shows that the best colonoscopists, *i.e.*, the ones that have the highest CIR and PDR, also have the best comfort scores, despite using less sedation. Measurement of patient comfort during sedated or non sedated colonoscopy may provide useful information on endoscopist performance.

COMMENTS

Background

Caecal intubation rate (CIR), use of sedation and adenoma detection rate are key performance indicators for colonoscopy. CIR is the most widely recognised measure of performance. Patient comfort is not routinely assessed; it is unknown whether higher intubation rates are achieved at the expense of greater patient discomfort, deeper sedation and possibly higher risk.

Research frontiers

Quality in colonoscopy is an important topic, especially with the introduction of bowel cancer screening programs in different countries. Caecal intubation rate is a key performance indicator of quality. Patient comfort is an auditable outcome, but there are little data on the topic.

Innovations and breakthroughs

Measuring patient comfort through nurse assessment provides valuable information about performance of endoscopists. Performing colonoscopies under deep sedation is not necessary to achieve good patient comfort. The colonoscopists that get to the caecum most often and see and remove the most polyps, have the best patient comfort scores.

Applications

Measuring patient comfort through nurse assessment is a valuable addition in measuring performance. Nurse assessment correlates well with patient experience. People believe that, in the future, assessment of patient comfort, next to CIR and ADR, could be a good performance indicator.

Terminology

Nurse-reported comfort level: assessment of patient comfort during the procedure by endoscopy nurses. Patient experience: the patient's experience of the procedure, assessed by the patient himself directly after the colonoscopy.

Peer review

The paper about patient comfort and quality in colonoscopy is very interesting. Questions were raised on influence of using adjusted CIR on the results, and on the relationship of age, gender and previous surgical procedures with the nurse-reported comfort levels and patient experience.

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Colonoscopy can miss diverticula of the left colon identified by barium enema

Ryota Niikura, Naoyoshi Nagata, Takuro Shimbo, Junichi Akiyama, Naomi Uemura

Ryota Niikura, Naoyoshi Nagata, Takuro Shimbo, Junichi Akiyama, Naomi Uemura, Department of Gastroenterology and Hepatology, National Center for Global Health and Medicine, Shinjuku, Tokyo 162-8655, Japan

Takuro Shimbo, Department of Clinical Research and Informatics, National Center for Global Health and Medicine, Shinjuku, Tokyo 162-8655, Japan

Naomi Uemura, Department of Gastroenterology and Hepatology, Kohnodai Hospital, National Center for Global Health and Medicine, Ichikawa, Chiba 272-8516, Japan

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Correspondence to: Dr. Naoyoshi Nagata, Department of Gastroenterology and Hepatology, National Center for Global Health and Medicine, 1-21-1 Toyama, Shinjuku, Tokyo 162-8655, Japan. nnagata_ncg@yahoo.co.jp

Telephone: +81-3-32027181 Fax: +81-3-32071038

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Abstract

AIM: To identify the diagnostic value of colonoscopy for diverticulosis as determined by barium enema.

METHODS: A total of 65 patients with hematochezia who underwent colonoscopy and barium enema were analyzed, and the diagnostic value of colonoscopy for diverticula was assessed. The receiver operating characteristic area under the curve was compared in relation to age (< 70 or ≥ 70 years), sex, and colon location. The number of diverticula was counted, and the detection ratio was calculated.

RESULTS: Colonic diverticula were observed in 46 patients with barium enema. Colonoscopy had a sensitivity of 91% and specificity of 90%. No significant dif-

ferences were found in the receiver operating characteristic area under the curve (ROC-AUC) for age group or sex. The ROC-AUC of the left colon was significantly lower than that of the right colon (0.81 vs 0.96, $P = 0.02$). Colonoscopy identified 486 colonic diverticula, while barium enema identified 1186. The detection ratio for the entire colon was therefore 0.41 (486/1186). The detection ratio in the left colon (0.32, 189/588) was significantly lower than that of the right colon (0.50, 297/598) ($P < 0.01$).

CONCLUSION: Compared with barium enema, only half the number of colonic diverticula can be detected by colonoscopy in the entire colon and even less in the left colon.

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Key words: Colonoscopic diagnosis; Colonic diverticulosis; Colonic diverticular bleeding; Barium enema; Receiver operating characteristic area under the curve

Core tip: We identified the diagnostic value of colonoscopy for colonic diverticulosis as determined by barium enema. The only half the number of colonic diverticula can be detected in the entire colon and even less in the left colon. By revealing the diagnostic value of colonoscopy for colonic diverticula, it may contribute to further therapeutic interventions strategies for the treatment of colonic diverticular disease.

Niikura R, Nagata N, Shimbo T, Akiyama J, Uemura N. Colonoscopy can miss diverticula of the left colon identified by barium enema. *World J Gastroenterol* 2013; 19(15): 2362-2367 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v19/i15/2362.htm> DOI: <http://dx.doi.org/10.3748/wjg.v19.i15.2362>

INTRODUCTION

Colonic diverticulosis is a common disease that occurs

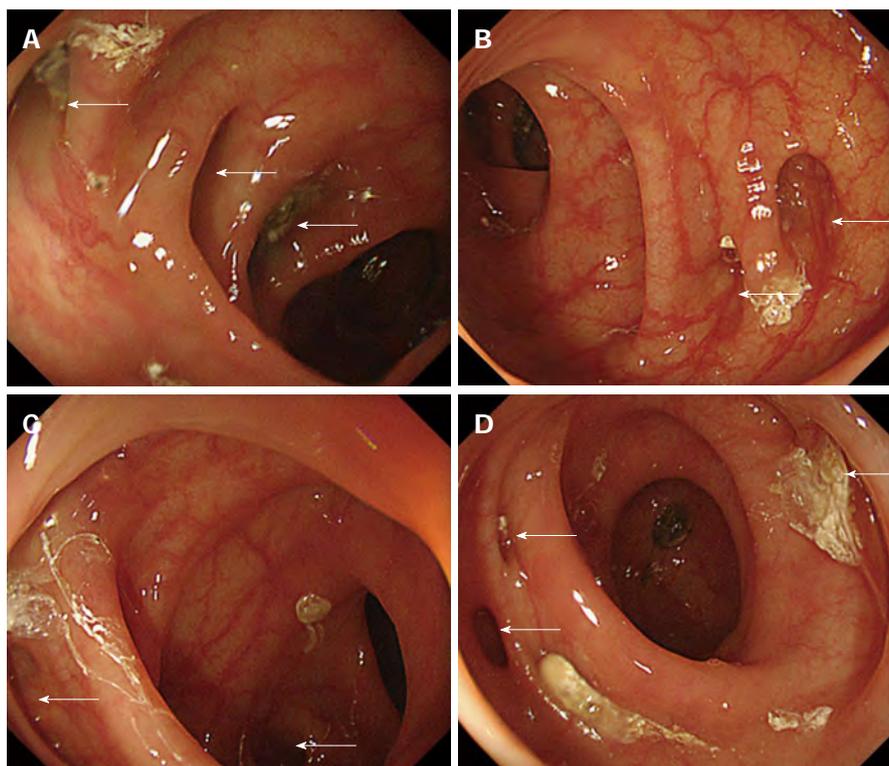


Figure 1 Colonic diverticula in the left colon on endoscopy. The colon location was classified as the right colon (cecum and ascending and transverse colon) or the left colon (descending colon and sigmoid colon). A: Sigmoid-descending colon junction; B: Proximal sigmoid colon; C: Sigmoid top; D: Distal sigmoid colon. Arrows show colonic diverticula determined by colonoscopy.

in approximately one third of the population older than 45 years and in up to two thirds of the population older than 85 years in the United States^[1,2]. In Asia, the prevalence of colonic diverticula is 28% and is increasing^[3]. The prevalence of diverticulitis and diverticular bleeding has also been increasing^[4].

Diverticulosis of the colon is often diagnosed during routine screening colonoscopy. In clinical practice, severe diverticulosis anatomically increases the risk of perforation because of fixed angulations, deep folds, and peristalsis of the colon^[5-7]. Therefore, the true lumen can sometimes be confused with diverticulosis when multiple large diverticular orifices are encountered. Moreover, circular muscular atrophy with severe diverticula can also create deep crevices in the colonic wall, making polyp detection more difficult^[8]. Colonoscopy is usually used to diagnose colonic diverticular bleeding^[9,10]. Identification of the bleeding site of colonic diverticula on colonoscopy enables endoscopic treatment with clips^[11], epinephrine injection, heat probing^[10], and ligation^[12]. These modalities can circumvent complications such as hemorrhagic shock and rebleeding^[10]. Therefore, identifying the diagnostic value of colonoscopy for colonic diverticula is important but has remained unclear.

By contrast, barium enema can clearly detect colonic diverticula^[5] because barium fills the entire colon in diverticulosis. Consequently, we evaluated the diagnostic value of colonoscopy for colonic diverticula in patients with hematochezia who underwent both colonoscopy and barium enema. In addition, differences in diagnostic value with regard to age, sex, and colonic location were assessed.

MATERIALS AND METHODS

Patients

We retrospectively selected 436 patients from the electronic endoscopic database who had undergone colonoscopy for hematochezia from 2008 to 2011 at the National Center for Global Health and Medicine. We excluded 355 patients who did not receive barium enema and 16 who did not undergo total colonoscopy. After exclusion, 65 patients were enrolled.

Colonoscopic assessment

Intestinal lavage for endoscopic examination was performed using 2 L of a solution containing polyethylene glycol. An electronic video endoscope (high-resolution scope, model CFH260; Olympus Optical, Tokyo, Japan) was used for the diagnosis of hematochezia by expert endoscopists. The results of the endoscopic examination were saved in the electronic database. Upon detecting diverticula, the location (cecum and ascending, transverse, descending, and sigmoid colon) and number were recorded (Figure 1).

Barium enema examination

Barium enema is a diagnostic imaging modality of the colon that has been used previously to evaluate diverticula^[13,14]. Barium enema was indicated for patients with colorectal cancer or to prevent recurrence of colonic diverticular bleeding^[6,15,16] and was performed within three days after colonoscopy. Intestinal lavage for the barium enema examination was performed using sodium picosulfate (1 mL) and magnesium citrate (250 mL) the day be-



Figure 2 Colonic diverticula in the left colon on radiography with barium enema. The colonic location was classified as the right colon (cecum and ascending and transverse colon) or the left colon (descending colon and sigmoid colon).

for the assessment and bisacodyl suppositories (10 mg) on the day of assessment. The barium solution (200-400 mL) was 70%-200%. Barium was injected from the anus to the cecum in all patients to visualize the entire colon tract in different positions. The presence, location, and number of colonic diverticula were determined by radiography (Figure 2).

Ethics

This study was approved by the institutional review board of the National Center for Global Health and Medicine (approval number: 765).

Statistical analysis

The gold standard for detecting colonic diverticula is barium enema radiography. To identify the diagnostic value of colonoscopy for colonic diverticula, we calculated the sensitivity, specificity, receiver operating characteristic area under the curve (ROC-AUC), positive likelihood ratio (PLR), and negative likelihood ratio (NLR). In a subgroup analysis, we assessed the diagnostic value of colonoscopy with regard to age, sex, and colonic location. Subjects were divided into two groups according to age: ≥ 70 years old and < 70 years old. The colon location was classified as the left colon (descending and sigmoid colon) and the right colon (cecum and ascending and transverse colon). Differences in the ROC-AUCs were compared in relation to age, sex, and location.

The receiver operating characteristic is a diagnostic test that presents its results as a plot of sensitivity vs 1-specificity (often called the false-positive rate). The ROC-AUC indicates the probability of a measure or predicted risk being higher for patients with disease than for those without disease^[17-19]. The detection ratio (colonoscopy/barium enema) of colonic diverticula was assessed and also compared between the left and right colon using the χ^2 test. A *P* value < 0.05 was considered significant. All statistical analyses were performed using Stata version 10 software (StataCorp, College Station, TX, United States).

RESULTS

Patient characteristics

Sufficient imaging by colonoscopy and barium enema

Table 1 Patient characteristics (*n* = 65)

| | |
|---|--------------|
| Sex (male/female) | 40/25 |
| Mean age (IQR) | 73 (66-78) |
| Median duration (IQR) between the onset of hematochezia and colonoscopy | 4 (2-8) |
| Cause of hematochezia | |
| Colonic diverticular bleeding | 30 |
| Colon cancer (cecum/ascending/transverse/sigmoid colon) | 19 (3/8/2/6) |
| Rectal cancer | 14 |
| Unknown | 2 |

IQR: Interquartile range.

was obtained for all patients due to adequate stool cleaning. No patients experienced perforation during air insufflation to expand the colon, and none showed a worsened condition such as abdominal pain or nausea after colonoscopy or barium enema. The median age was 73 years, and many patients were elderly men (Table 1). The causes of hematochezia included colonic diverticular bleeding, colonic cancer, and rectal cancer.

Diagnostic value of colonoscopy for colonic diverticula

Colonic diverticula were observed in 46 patients (71%) using barium enema. The number of colonic diverticula identified by colonoscopy was 486, whereas that by barium enema was 1186. Colonoscopy had a sensitivity of 91%, a specificity of 90%, a PLR of 8.7, and an NLR of 0.097 for the diagnosis of colonic diverticula (Table 2).

In a subgroup analysis, no significant differences were found in the ROC-AUCs between age groups and sex. However, the ROC-AUC of the left colon was significantly lower than that of the right colon (0.96 vs 0.81, *P* = 0.02).

Detection ratio (colonoscopy/barium enema) of colonic diverticula

The detection ratio for the entire colon was 0.41 (486/1186) (Figure 3). The detection ratio for the left colon (0.32, 189/588) was significantly lower than that of the right colon (0.50, 297/598) (*P* < 0.01).

DISCUSSION

No previous study has reported the diagnostic value of colonoscopy for colonic diverticulosis. Colonoscopy has been used worldwide as a standard tool for the screening of colonic cancer and the diagnosis of other lower gastrointestinal tract diseases^[20]. Colonic diverticulosis is a common disease in Asia, Europe, and the United States^[2], occurring in approximately one third of the population older than 45 years^[1]. The prevalence of colonic diverticulosis increases with age, and serious complications with diverticulitis and diverticular bleeding have been on the rise in recent years^[1]. To address these problems, we investigated the diagnostic value of colonoscopy in colonic diverticulosis.

While the detection rate for diverticula with colo-

Table 2 Diagnostic value of colonoscopy for colonic diverticula in 65 patients

| Number ¹ | Sens, % (95%CI) | Spec, % (95%CI) | LR (+) (95%CI) | LR (-) (95%CI) | ROC-AUC (95%CI) | P value |
|---------------------|-----------------|-----------------|----------------|--------------------|------------------|---------|
| All (44/21) | 91 (79-98) | 90 (67-99) | 8.7 (2.3-32) | 0.097 (0.038-0.52) | 0.90 (0.82-0.99) | |
| Age (yr) | | | | | | |
| < 70 (18/9) | 90 (67-99) | 88 (47-100) | 7.2 (1.1-45) | 0.12 (0.032-0.46) | 0.89 (0.74-1) | |
| ≥ 70 (26/12) | 93 (76-99) | 91 (59-100) | 10 (1.6-66) | 0.082 (0.021-0.31) | 0.92 (0.82-1) | 0.68 |
| Sex | | | | | | |
| Female (13/12) | 100 (72-100) | 86 (57-98) | 5.8 (1.8-18) | 0.05 (0.0033-0.76) | 0.93 (0.83-1) | |
| Male (31/9) | 89 (73-97) | 100 (48-100) | 11 (0.74-150) | 0.14 (0.056-0.33) | 0.94 (0.89-1) | 0.47 |
| Location | | | | | | |
| Right colon (40/25) | 95 (84-99) | 96 (79-100) | 23 (3.4-156) | 0.051 (0.013-0.20) | 0.96 (0.90-1) | |
| Left colon (26/39) | 66 (49-80) | 96 (81-100) | 18 (2.6-123) | 0.36 (0.23-0.56) | 0.81 (0.73-0.90) | 0.02 |

¹With/without diverticulosis. P values were calculated using the receiver operating characteristic curve area under the curve (ROC-AUC) for comparisons in this category. Sens: Sensitivity; Spec: Specificity; LR: Likelihood ratio.

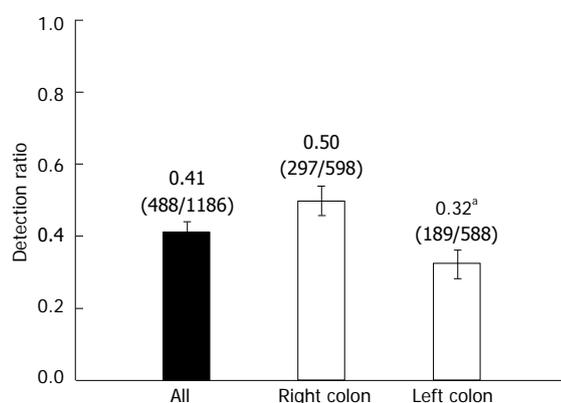


Figure 3 Detection ratio (colonoscopy/barium) of colonic diverticula in 65 patients. Right colon denotes the cecum, ascending colon, and transverse colon. Left colon denotes the descending colon and sigmoid colon. ^aP < 0.05 by χ^2 test. Error bars show the 95%CI of the detection ratio.

noscopy was acceptable, our results showed that the diagnostic value decreased significantly for detection in the left colon. In addition to detecting the presence of diverticula, we counted the number of diverticula in 65 patients and found that colonoscopy detected only one third of the diverticula in the left colon identified by barium enema. The detection rate for diverticula by colonoscopy was higher than that reported previously^[21], and this is presumably because a different group of patients was used in this study; Song *et al.*^[21] enrolled patients screened by colonoscopy, while the present study investigated patients with hematochezia.

We believe these results were influenced greatly by anatomical factors of the colon, and we thus propose two hypotheses for the poor diagnostic value of colonoscopy in the left colon. First, the diameters of the ascending and transverse colons on the right side of the body are reportedly 4.9 and 4.2 cm, respectively^[22]. However, the diameters of the descending and sigmoid colons constituting the left colon are both 3.3 cm, notably narrower than the right colon^[22]. Because of the narrower diameter, the field of view in colonoscopy is expected to be lower in the left colon, making the detection of diverticula more difficult.

In addition, the sigmoid colon, which accounts for one third of the left colon and is not supported by the mesentery, bends sharply^[22]. Intestinal bending not only creates blind spots for colonoscopy but also complicates distinguishing diverticula from the true lumen^[5,23], thus greatly affecting the accuracy of diagnosis with colonoscopy.

Although the present study also investigated other factors such as age and sex in addition to anatomical factors, no significant differences were observed. Sadahiro *et al.*^[22] investigated age- and sex-related differences in the surface area of the large intestine with barium enema. They found that the mean surface area of the large intestine in men aged ≥ 70 years was 1569.4 cm², while that in men aged ≤ 69 years was 1566 cm², with no significant difference. The mean surface area in women aged ≥ 70 years was 1575.4 cm², while that in women aged ≤ 69 years was 1628 cm². These results support our finding that the anatomy of the colon is rarely influenced by age or sex. In a previous study on the detection of colonic polyps, it was reported that the degree of bowel preparation^[24] and observation time^[25] were associated with missed colonic polyps. Although we were unable to evaluate this issue in the present study, we plan to investigate it in the future.

We believe the present findings will influence the diagnosis and treatment of colonic diverticulosis and complications including diverticular bleeding. Knowing the areas of the colon where it is easy to overlook the presence of diverticula or to confuse a diverticulum with a true lumen may help endoscopists perform proper screening colonoscopy and reduce the risk of perforation^[5,7,23]. It is important to identify the stigmata of recent hemorrhage (SRH) when diagnosing or treating diverticular bleeding^[23]. To date, diagnosis and treatment have been conducted under the assumption that colonoscopy will identify all colonic diverticula regardless of the anatomical factors of the colon. However, our study showed that colonoscopy detected only 32% of all diverticula in the left colon. This suggests that the SRH were overlooked, which is supported by a previous study reporting that colonoscopy identified the SRH in only one third of patients with colonic diverticular bleeding^[23].

Here, we evaluated colonic diverticula using static

images, but it proved difficult to determine the exact number of colonic diverticula using these images. We therefore plan to perform a prospective study of colonic diverticula using video of live endoscopy procedures.

In the present study, the diagnostic value of colonoscopy for the left colon was relatively low, revealing only one third of the diverticula observed by barium enema. The diagnosis and treatment of colonic diverticulosis and complications, such as diverticular bleeding, should be performed with consideration of the findings of this study.

COMMENTS

Background

Colonic diverticulosis is a common disease, and the prevalence of diverticulitis and diverticular bleeding has been increasing. Colonoscopy is useful for diagnosing colonic diverticula and colonic diverticular bleeding. However, the diagnostic value of colonoscopy has remained unclear.

Research frontiers

Colonoscopy is often used to diagnose colonic diverticular bleeding and therefore can be useful for subsequent endoscopic treatment if the bleeding site can be identified. Determining the diagnostic value of colonic diverticula is important. In this study, the authors revealed the diagnostic value of colonic diverticula by colonoscopy.

Innovations and breakthroughs

Previous reports have highlighted the prevalence of diverticula by barium enema. The authors identified the diagnostic value of colonoscopy for colonic diverticulosis as determined by barium enema. The only half the number of colonic diverticula can be detected in the entire colon and even less in the left colon.

Applications

By revealing the diagnostic value of colonoscopy for colonic diverticula, the results of this study may contribute to future therapeutic intervention strategies for the treatment of patients with colonic diverticular disease.

Terminology

The receiver operating characteristic is a diagnostic testing modality that presents its results as a plot of sensitivity vs 1-specificity (often called the false-positive rate). The receiver operating characteristic area under the curve indicates the probability of a measure or predicted risk being higher for patients with disease than for those without disease.

Peer review

The authors evaluated the diagnostic value of colonic diverticula by colonoscopy compared with barium enema. This study revealed that threefold more diverticula can be detected by barium enema than by colonoscopy.

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Ultrasound-guided vs endoscopic ultrasound-guided fine-needle aspiration for pancreatic cancer diagnosis

Masato Matsuyama, Hiroshi Ishii, Kensuke Kuraoka, Seigo Yukisawa, Akiyoshi Kasuga, Masato Ozaka, Sho Suzuki, Kouichi Takano, Yuko Sugiyama, Takao Itoi

Masato Matsuyama, Hiroshi Ishii, Kensuke Kuraoka, Seigo Yukisawa, Akiyoshi Kasuga, Masato Ozaka, Sho Suzuki, Kouichi Takano, Department of Gastroenterology, Cancer Institute Hospital, Tokyo 135-8550, Japan

Yuko Sugiyama, Department of Gynecology, Cancer Institute Hospital, Tokyo 135-8550, Japan

Takao Itoi, Department of Gastroenterology and Hepatology, Tokyo Medical University, Tokyo 160-0023, Japan

Author contributions: Matsuyama M and Ishii H performed most of the examinations; Kuraoka K, Yukisawa S, Kasuga A, Ozaka M, Suzuki S and Takano K managed the patients; Sugiyama Y supported the cytopathology; Matsuyama M, Ishii H and Itoi T wrote the paper.

Correspondence to: Masato Matsuyama, MD, PhD, Department of Gastroenterology, Cancer Institute Hospital, 3-8-31, Ariake, Koto-ku, Tokyo 135-8550,

Japan. mahsanmahsan2000@yahoo.co.jp

Telephone: +81-3-35200111 Fax: +81-3-35700111

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Abstract

AIM: To clarify the effectiveness and safety of endoscopic ultrasound-guided fine-needle aspiration (EUS-FNA) for the diagnosis of pancreatic cancer (PC).

METHODS: Patients who were diagnosed with unresectable, locally advanced or metastatic PC between February 2006 and September 2011 were selected for this retrospective study. FNA biopsy for pancreatic tumors had been performed percutaneously under extracorporeal ultrasound guidance until October 2009; then, beginning in November 2009, EUS-FNA has been performed. We reviewed the complete medical records of all patients who met the selection criteria for the following data: sex, age, location and size of the targeted tumor, histological and/or cytological findings, details

of puncture procedures, time from day of puncture until day of definitive diagnosis, and details of severe adverse events.

RESULTS: Of the 121 patients who met the selection criteria, 46 had a percutaneous biopsy (Group A) and 75 had an EUS-FNA biopsy (Group B). Adequate cytological specimens were obtained in 42 Group A patients (91.3%) and all 75 Group B patients ($P = 0.0192$), and histological specimens were obtained in 41 Group A patients (89.1%) and 65 Group B patients (86.7%). Diagnosis of malignancy by cytology was positive in 33 Group A patients (78.6%) and 72 Group B patients (94.6%) ($P = 0.0079$). Malignancy by both cytology and pathology was found in 43 Group A (93.5%) and 73 Group B (97.3%) patients. The mean period from the puncture until the cytological diagnosis in Group B was 1.7 d, which was significantly shorter than that in Group A (4.1 d) ($P < 0.0001$). Severe adverse events were experienced in two Group A patients (4.3%) and in one Group B patient (1.3%).

CONCLUSION: EUS-FNA, as well as percutaneous needle aspiration, is an effective modality to obtain cytopathological confirmation in patients with advanced PC.

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Key words: Endoscopic ultrasound-guided fine needle aspiration; Percutaneous needle aspiration; Pancreatic cancer

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INTRODUCTION

Pancreatic cancer (PC) is currently the fifth leading cause of cancer-related mortality in Japan. Although complete surgical removal of the tumor is the only chance of cure, almost all PC patients are initially diagnosed as having advanced unresectable disease despite recent improvements in diagnostic techniques. In recent decades, techniques were developed to obtain proof of cancer from the primary tumor in PC patients. Pancreatic juice cytology *via* endoscopic retrograde pancreatography was initially developed to meet this challenge; however, in practical settings the positive rate for cancer cells has remained low, indicating the presence of false-negative results^[1,2]. Ultrasonography-guided fine-needle aspiration (US-FNA) biopsy or computed tomography (CT)-guided FNA biopsy appears to provide a more definitive diagnosis of PC^[3,4]. US-FNA is convenient but its usefulness is limited for masses in the pancreatic tail. In contrast, CT-guided FNA is the biopsy procedure of choice to assess pancreatic lesions. However, this technique is time-consuming and is limited by a substantial false-negative rate of approximately 20%^[5]. In addition, there have been concerns about percutaneous cancer seeding^[6,7]. Recently, endoscopic ultrasound-guided fine needle aspiration (EUS-FNA) has been developed as a more feasible method to obtain definitive specimens for cytological and/or histological examinations for diagnosis of PC^[8-12]. Three years ago, we began to perform EUS-FNA although until that time US-FNA was the standard technique at our institute.

In the current study, we retrospectively examined the diagnostic ability of EUS-FNA for PC compared with US-FNA.

MATERIALS AND METHODS

Patients

The inclusion criteria were: (1) the patient underwent US-FNA between February 2006 and October 2009 or EUS-FNA between November 2009 and September 2011 at the Cancer Institute Hospital, Tokyo, Japan for suspected PC; and (2) the patient was subsequently diagnosed as having clinical stage III or IV PC. Unresectable PC, which was indicated by International Union Against Cancer clinical stage III (locally advanced disease: T4N0-1 and M0) or IV (metastatic disease: T1-4N0-1 and M1), was diagnosed by CT.

The exclusion criteria were: (1) a contraindication for EUS (esophageal stenosis, duodenal stenosis, ileus, or perforation of the digestive tract); and (2) a contraindication for EUS-FNA and US-FNA (severe cardiovascular disease or respiratory disease, poor performance status, difficulty in visualization of the target, bleeding tendency, or impossibility of ensuring the puncture route).

Patients who met the selection criteria were identified from the database in our division, which was updated daily.

US- and EUS-FNA procedures

A short admission, usually for one or two nights, was mandatory according to the protocol for FNA biopsy of a suspected pancreatic tumor in our division. FNA biopsy for pancreatic tumors had been performed percutaneously under extracorporeal ultrasound guidance (US-FNA) until October 2009; then, beginning in November 2009, FNA biopsies have been performed under EUS guidance (EUS-FNA). In general, FNA examinations were performed and managed by Ishii H until October 2009 and by Matsuyama M since November 2009. Written informed consent was obtained from each patient before the examination.

US-FNA was performed using SSA-550A (Toshiba, Tokyo, Japan) as the ultrasound device and SONOPSY C1 21G (Hakko, Osaka, Japan) as the ultrasound-guided biopsy needle. After systemic premedication and percutaneous local anesthesia, FNA was performed 1-3 times repeatedly until adequate material was obtained. Pathological examination of the obtained materials and cytological examination of the needle-washing water were done. There was no on-site cytotechnologist during the performance of US-FNA.

EUS-FNA was performed using EU-ME1 and UCT240-AL5 (Olympus, Tokyo, Japan) as the EUS system and the Echo-Tip ULTRA 22G (Wilson-Cook, Bloomington, IN, United States) as the ultrasound-guided biopsy needle. After systemic premedication and pharyngeal local anesthesia, FNA was performed endoscopically *via* the stomach or duodenum. Aspiration puncture was repeated until an on-site cytology screener confirmed that adequate materials had been obtained.

After the examination, patients stayed in the hospital overnight and were discharged the following morning if no problems were revealed by physical examination, complete blood count tests and biochemistry tests that included serum amylase level. Three to 7 d later, the patients came to the outpatient clinic for an explanation of the results of the biopsy and examination for late adverse events, and were then able to start chemotherapy.

The final diagnosis was based on pathology results or clinical follow-up of > 6 mo.

Statistical analysis

We reviewed the complete medical records of all patients who met the selection criteria for the following data: sex, age, location and size of the targeted tumor, histological and/or cytological findings of the obtained specimens, details of puncture procedures, time from day of puncture until the day of definitive diagnosis, and details of severe adverse events, if any. The tumor status (location and size) was determined by dynamic CT before puncture. Frequency analysis was performed with Fisher's exact test for 2 × 2 tables, χ^2 test for 3 × 2 tables, and Mann-Whitney test. All analysis were performed using the statistical software SPSS 11.0J for Windows. Statistical significance was defined as a two-sided *P* value ≤ 0.05.

Table 1 Characteristics of patients and comparison of results of percutaneous biopsy with those of endoscopic ultrasound-guided fine-needle aspiration

| | Percutaneous biopsy | | EUS-FNA | P value |
|--|---------------------|--------------------------|---------|----------|
| | Group A | Group B | | |
| Patients | 46 | 75 | | |
| Site of puncture | | | | |
| Pancreas | 46 | 74 | | > 0.9999 |
| Head/body/tail | 12/32/2 | 34/31/9 | | 0.0114 |
| Sex (male/female) | 25/21 | 39/36 | | > 0.8525 |
| Age, yr | | | | > 0.8466 |
| ≥ 65 | 28 | 48 | | |
| < 65 | 18 | 27 | | |
| Tumor diameter, mm (range) | 44.8 (18-111) | 25.5 (7-70) | | |
| ≥ 40 | 30 | 25 | | 0.0007 |
| < 40 | 16 | 50 | | |
| Passes (range) | 2.26 (1-4) | 2.85 (2-5) | | < 0.0001 |
| Adequate specimens obtained ¹ n (%) | | | | |
| Cytology | 42 (91.3) | 75 (100) | | 0.0192 |
| Histology | 41 (89.1) | 65 (86.7) | | 0.7812 |
| Positivity for cancer n (%) | | | | |
| Cytology | 33 (78.6) | 72 (94.6) | | 0.0079 |
| Histology | 33 (80.5) | 51 (78.4) | | > 0.9999 |
| Total n (%) | 43 (93.5) | 73 (97.3) | | 0.3672 |
| Complications n (%) | 2 (4.3) | 1 (1.3) | | > 0.5567 |
| Fever ¹ | | Peritonitis ¹ | | |
| Bleeding ¹ | | | | |
| Time from puncture to definitive diagnosis | | | | |
| Cytology, d (range) | 4.05 (0-8) | 1.65 (0-5) | | < 0.0001 |
| Histology, d (range) | 3.95 (2-7) | 3.18 (2-10) | | 0.7066 |

¹An on-site pathologist was available for endoscopic ultrasound-guided fine-needle aspiration (EUS-FNA) but not for ultrasonography-guided-FNA.

RESULTS

US-FNA was performed in 48 patients from February 2006 until October 2009. Two cases (renal cell carcinoma and malignant lymphoma) were excluded from the analysis of US-FNA because the patients did not have primary PC. EUS-FNA was attempted in 125 cases and was successfully performed in 123 cases from November 2009 until September 2011. Among these, 48 patients did not meet the selection criteria (lymph node metastasis, 34 cases; other pancreatic tumor, 10 cases; other abdominal tumor, three cases, and mediastinum tumor, one case). EUS-FNA could not be performed in two patients because of difficulty of visualization due to total gastrectomy in one case, and impossibility of ensuring the puncture route in the other. Thus, 46 patients who underwent US-FNA (Group A) and 75 who underwent EUS-FNA (Group B) were eligible for analysis.

Table 1 shows the characteristics of the study subjects. The distribution of the target tumor in the pancreas differed significantly between the two groups, with the tumor location more frequent in the pancreatic head/tail than in the pancreatic body in Group B. The maximum diameter of the target tumor ranged from 18 to 111 mm (median, 44.8 mm) in Group A and from 7 to 70 mm (median, 25.5 mm) in Group B. A significantly larger number of target tumors were < 40 mm in Group B than in Group A ($P = 0.0007$).

Table 1 shows a comparison of the results of percutaneous biopsy with those of EUS-FNA. Adequate cytological and histological specimens were obtained in 42 (91.3%) and 41 (89.1%) Group A patients ($n = 46$), respectively, and in 75 (100%) and 65 (86.7%) Group B patients ($n = 75$).

Results of cytology indicated the presence of cancer cells in 33 Group A patients (78.6%) and in 72 Group B patients (94.6%). Histological studies showed cancer tissue in 33 (80.5%) and 51 (78.4%) patients in Group A and Group B, respectively. In total, a cancer diagnosis was made in 43 Group A (93.5%) and 73 Group B (97.3%) patients by cytology and/or histology. These 116 patients were diagnosed with pancreatic adenocarcinoma by cytology/histology as well as by imaging and their subsequent clinical course. The final diagnosis of PC in the remaining five patients for whom there was no cytological or histological proof was confirmed by the clinical course until April 2012. The positive cytology/histology rate did not differ between the two groups.

Total puncture procedures per patient varied from one to five, with a median of 3. The frequency of multiple punctures, that is, > 2, was significantly higher in Group B than in Group A. Time from the day of puncture until the day of the final cytological diagnosis varied from 0 to 8 d (median, 4.1 d) in Group A and from 0 to 5 d (median, 1.7 d) in Group B. The period was significantly shorter in Group B than in Group A. The time from the day of puncture until the day of the final histological diagnosis varied from 2 to 7 d (median, 4.0 d) in Group A and 2 to 10 d (median, 3.2 d) in Group B, with no significant difference between the two groups.

Severe adverse events occurred in two Group A patients (4.3%) and in one Group B patient (1.3%). In Group A, one patient developed a high fever, which required hospitalization but resolved with only symptomatic treatment. The other Group A patient experienced upper gastrointestinal bleeding, which was confirmed by endoscopy to be related to the needle biopsy. This patient was treated by blood transfusion and antiulcer medication and was hospitalized for 1 wk without surgical intervention. The adverse event in Group B was an abdominal abscess that required surgical drainage. The patient experienced continuous abdominal pain one night after EUS-FNA, and dynamic CT demonstrated an abscess in front of the pancreatic body tumor, which was clearly related to the EUS-FNA puncture. Fortunately, she recovered after surgery and antibiotic therapy and could receive chemotherapy thereafter. There was no cancer seeding event up to 6 mo from the time of puncture in any patient in either group.

DISCUSSION

The aim of the current study was to investigate the results of two different approaches to obtain pancreatic biopsy specimens, which are a percutaneous approach and EUS-FNA, because this issue has seldom been ad-

dressed^[12]. Our results confirmed the usefulness of EUS-FNA, especially with regard to cytology. The National Comprehensive Cancer Network Guidelines (2012) require that cytological or histological confirmation is needed for the diagnosis of unresectable pancreatic carcinoma^[13]. In patients with stage IV PC, a biopsy of the metastatic lesion is preferred for proof of cancer. However, in those with stage III PC and some patients with stage IV PC in whom it is difficult to access metastatic sites for biopsy procedures, the primary tumor of the pancreas must be targeted to obtain proof of cancer. Pancreatic juice cytology was developed in the early 1980s and is still being performed; however, cancer cells cannot easily be observed by collection of pancreatic juice^[1,2,14]. Percutaneous needle biopsy was developed with the expectation of a more definitive method to obtain proof of cancer from the primary pancreatic tumor^[3,15,16]. Our institute then used percutaneous needle biopsy under extracorporeal US guidance as the standard for histological confirmation of the pancreatic primary tumor. Recently, EUS-FNA was introduced and was used mainly in high-volume cancer centers in Japan^[17-22]. As a result of the risk of cancer seeding as well as other risks with percutaneous biopsy, we adopted EUS-FNA beginning in November 2009 in place of percutaneous biopsy. We expected that EUS-FNA would have advantages over a percutaneous procedure with regard to efficacy in confirmation of cancer and avoiding adverse reactions before administering chemotherapy to patients with PC.

Our results demonstrated that EUS-FNA is effective and feasible for obtaining proof of cancer in candidates for PC chemotherapy. In fact, EUS-FNA might have merits with regard to obtaining specimens from small tumors or tumors in the pancreatic tail, for which performance of percutaneous biopsy is difficult^[2,23-27]. In this study, the location of the target tumor was most frequent at the body of the pancreas in Group A. In addition, the target tumors were larger in Group A than in Group B. These findings suggest that patients might have been excluded from Group A in which difficulty could be expected in making a puncture because the tumor was either small or difficult to delineate. In these cases, endoscopic retrograde cholangiopancreatography or liver biopsy might have been performed to obtain confirmation of malignancy, if possible.

Horwhat *et al.*^[12] have performed a randomized controlled trial of EUS-FNA and percutaneous biopsy of the pancreas (US- and CT-guided) in 2006. Although there was no statistically significant difference in accuracy between the two methods, the results showed that EUS-FNA had the advantage in the diagnosis of pancreatic malignancy. In our study, the diameters of the target tumors in the EUS-FNA group (Group B) were smaller than those in the US-FNA group (Group A) and the deviation of distribution around the puncture site was smaller in the EUS-FNA than the US-FNA group. Our results indicated high performance through the use of EUS-FNA and are not inconsistent with those of Hor-

what *et al.*^[12]. In the present study, there was no analysis of accuracy in the two groups, because our institution is an oncology hospital and we rarely perform biopsies of benign cases.

The benefits of EUS-FNA might be maximized to make a pathological diagnosis in patients with an abdominal tumor of an uncertain type. The definite merit of our EUS-FNA procedure was thought to be rapid cytological results, but perhaps success in this regard was mainly due to the contribution of an on-site cytotechnologist and not to the EUS-FNA procedure itself. Iglesias-Garcia *et al.*^[28] have claimed that on-site cytological evaluation improves the diagnostic yield of EUS-guided FNA for the cytological diagnosis of solid pancreatic masses. Savoy *et al.*^[29] have pointed out that even trained endosonographers have variable and, in some cases, inferior abilities in interpreting on-site cytology in comparison with cytotechnologists. In the present study, we had adequate specimens for all cases in the EUS-FNA group. This is natural because we continued the examination until we obtained a sufficient quantity of specimens that were checked by the on-site cytotechnologist. On the contrary, there was no difference in the rate of adequate specimens obtained for histological examination between the EUS-FNA and US-FNA groups, because the collected tissue was checked by the examiner's naked eye in both groups. The presence of an on-site cytotechnologist to accompany EUS-FNA is considered to be necessary, at least, in high-volume centers.

In the present study, the positivity rate for malignancy was higher for EUS-FNA cytology than for histology. Supporting the current results, another study has shown that the positivity rate for malignancy in EUS-FNA cytology of the pancreas was higher than that in histology^[30].

As previously reported, EUS-needle core biopsy is useful for histological and cytological diagnosis in terms of sample volume^[31]. In addition, the combined results of EUS-FNA cytology and EUS-needle core biopsy have been reported to improve diagnosis^[32-34]. However, to confirm the malignancy, EUS-FNA cytology is more useful than EUS-needle core biopsy^[35]. This result is similar to the results of our study, indicating that cytology might be more useful than histology for the diagnosis of malignancy.

In the current study, there was no cancer seeding in any patient in either group. As previously reported, there were rare cases of seeding among patients who underwent US-guided FNA^[36]. With regard to the puncture route, we suggest that there is less possibility of seeding in patients who undergo EUS-FNA than in patients who undergo US-FNA, although some recent studies have shown the possibility of seeding in patients who undergo EUS-FNA^[37-39]. We did inform patients who were scheduled to undergo EUS-FNA about the possibility of this complication.

The limitations of our study included its retrospective nature. Furthermore, there were no cases of benign pancreatic conditions to enable an evaluation of US and EUS-FNA for accurate differentiation between malignant

and benign diseases.

In conclusion, EUS-FNA, as well as percutaneous needle aspiration, is an effective modality to obtain cytopathological confirmation in patients with advanced PC. EUS-FNA cytology was able to detect malignancy at a high rate. We believe that EUS-FNA has advantages for smaller tumors located deeply and for tumors in which the diagnosis is uncertain by various other imaging modalities.

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COMMENTS

Background

Ultrasonography-guided fine-needle aspiration (US-FNA) biopsy or computed tomography (CT)-guided FNA biopsy was used for histological/cytological diagnosis of pancreatic cancer (PC). US-FNA is limited to masses in the pancreatic tail. CT-guided FNA is time-consuming and limited by a substantial false-negative rate. There have been concerns about percutaneous cancer seeding and difficulty in puncturing for small tumors. Endoscopic ultrasound (EUS)-guided FNA has been developed as a more feasible method of obtaining definitive specimens for the diagnosis of PC. Studies on the results of the two different approaches to obtain pancreatic biopsy specimens, which are the percutaneous approach and EUS-FNA, have rarely been conducted.

Research frontiers

The benefits of EUS-FNA might be maximized to be able to make a pathological diagnosis in patients with an abdominal tumor of an uncertain type.

Innovations and breakthroughs

EUS-FNA is effective and feasible for obtaining proof of cancer in PC chemotherapy candidates. In fact, EUS-FNA might have advantages with regard to obtaining specimens from small tumors or tumors in the pancreatic tail, for which performance of percutaneous biopsy is difficult.

Applications

The results suggest that EUS-FNA is the best method of obtaining cytological samples for diagnosis of unresectable PC. This method can be used for other types of cancer.

Terminology

On-site cytotechnologist: An on-site cytotechnologist should attend the puncture examination to confirm quickly the existence of atypical cells. The information of the cytotechnologist is more appropriate than that of the endoscopist.

Peer review

This is a good descriptive study in which EUS-FNA is a feasible and safe technique to acquire pancreatic specimens. The results are interesting in that the advantages of EUS-FNA over the percutaneous procedure are time between examination and diagnosis, the possibility of puncture of small tumors, and tumors in the tail of the pancreas.

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Seroprevalence of celiac disease among healthy adolescents in Saudi Arabia

Abdulrahman M Aljebreen, Majid A Almadi, Alwaleed Alhammad, Faleh Z Al Faleh

Abdulrahman M Aljebreen, Majid A Almadi, Faleh Z Al Faleh, Gastroenterology Division, King Khalid University Hospital, King Saud University, Riyadh 11461, Saudi Arabia
Majid A Almadi, Gastroenterology Division, McGill University Health Center, Montreal General Hospital, McGill University, Montreal H3G 1A4, Canada

Alwaleed Alhammad, Immunology Unit, Department of Pathology, King Saud University, Riyadh 11461, Saudi Arabia

Author contributions: Aljebreen AM designed the study, analyzed the data and wrote the paper; Almadi MA analyzed the data and wrote the paper; Alhammad A conducted the blood test and revised the paper; Al Faleh FZ designed the study and wrote the paper.

Correspondence to: Abdulrahman M Aljebreen, FRCPC, FACP, Associate Professor of Internal Medicine, Consultant of Gastroenterology, Gastroenterology Division, King Khalid University Hospital, King Saud University, PO Box 2925, Riyadh 11461, Saudi Arabia. amaljebreen@gmail.com

Telephone: +966-1-4671215 Fax: +966-1-4671217

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Abstract

AIM: To identify the seroprevalence of celiac disease among healthy Saudi adolescents.

METHODS: Between December 2007 and January 2008, healthy students from the 10th to 12th grades were randomly selected from three regions in Saudi Arabia. These regions included the following: (1) Aseer region, with a student population of 25512; (2) Madinah, with a student population of 23852; and (3) Al-Qaseem, with a student population of 16067. Demographic data were recorded, and a venous blood sample (5-10 mL) was taken from each student. The blood samples were tested for immunoglobulin A and immunoglobulin G endomysial antibodies (EMA) by indirect immunofluorescence.

RESULTS: In total, 1167 students (614 males and 553 females) from these three regions were randomly selected. The majority of the study population was classified as lower middle class (82.7%). There were 26 (2.2%) students who had a positive anti-EMA test, including 17 females (3.1%) and 9 males (1.5%). Al-Qaseem region had the highest celiac disease prevalence among the three studied regions in Saudi Arabia (3.1%). The prevalence by region was as follows: Aseer 2.1% (10/479), Madinah 1.8% (8/436), and Al-Qaseem 3.2% (8/252). The prevalence in Madinah was significantly lower than the prevalence in Aseer and Al-Qaseem ($P = 0.02$).

CONCLUSION: Our data suggest celiac disease prevalence might be one of the highest in the world. Further studies are needed to determine the real prevalence.

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Key words: Celiac disease; Saudi Arabia; Prevalence; Antiendomysial antibody; Epidemiology

Core tip: The celiac disease (CD) prevalence has progressively increased and, recently, it was proposed that it might be higher than 1 in 100. Until the 1990s, the prevalence of CD in Middle Eastern and North African countries was considered low. In this cohort of 1167 healthy young Saudi students who had anti-endomysial antibodies (EMA) test, the seroprevalence of celiac disease was 2.2% (1 in 45) and as high as 3.1% among females. Although intestinal biopsies were not available in our study, the high specificity of immunoglobulin A anti-EMA might indicate the celiac disease prevalence in Saudi Arabia might be one of the highest celiac disease prevalence rates in the world.

Aljebreen AM, Almadi MA, Alhammad A, Al Faleh FZ. Seroprevalence of celiac disease among healthy adolescents in Saudi

Arabia. *World J Gastroenterol* 2013; 19(15): 2374-2378 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v19/i15/2374.htm> DOI: <http://dx.doi.org/10.3748/wjg.v19.i15.2374>

INTRODUCTION

Celiac disease (CD) is a chronic systemic autoimmune disorder induced by gluten proteins present in wheat, barley, and rye. Genetically susceptible individuals develop autoimmune injury to the gut, skin, liver, joints, uterus, brain, heart, and other organs. The classical definition of CD includes gastrointestinal manifestations (chronic diarrhea, failure to thrive, weight loss, vomiting, abdominal pain, bloating, distention, and constipation) confirmed by a small bowel biopsy, with findings of villous atrophy, crypt hyperplasia, and normalization of the villous architecture in response to a gluten-free diet^[1]. Celiac disease has been classified into 4 phenotypes: classic, atypical, silent and latent. "Latent" celiac disease describes asymptomatic individuals with currently normal histological findings on a gluten-sufficient diet who subsequently develop celiac disease or those with a prior diagnosis of celiac disease who responded to a gluten free diet and retained normal mucosal histological findings despite the long-term ingestion of gluten^[2]. Until the 1980s, CD was considered to be a rare disease, but in the 1990s, it became clear that CD was a frequent condition. In 1995, it was suggested that the prevalence of CD in the general population could be approximately 1 in 250 individuals^[3]. This prevalence has progressively increased and, recently, it was proposed that it might be higher than 1 in 100. This increase is mainly due to the increased use of assays that detect celiac antibodies to identify affected individuals^[4,5]. Until the 1990s, the prevalence of CD in Middle Eastern and North African countries was considered low. However, with the introduction of antigliadin antibodies, anti-endomysial antibody (EMA), and tissue transglutaminase antibodies testing, CD has been more readily reported from these regions^[6], and its prevalence appears similar to that of North American and European countries^[6-9]. There are scarce data regarding the prevalence of celiac disease in Saudi Arabia^[10]. A recent study has shown a seroprevalence of 1.5% among 204 healthy blood donors^[11].

The aim of this study was to identify the seroprevalence of celiac disease among a healthy adolescent population in three regions of Saudi Arabia.

MATERIALS AND METHODS

Study population

Saudi Arabia is comprised of 13 regions. The first region in our study, Aseer, is located in the southwestern part of Saudi Arabia and is a mountainous area with mild weather throughout the year. The population of this region is estimated to be 1.75 million people. Madinah, where the second holy city is located, is in the western part of Saudi

Arabia and has a population of 1.61 million people. The third region, Al-Qaseem, is located in central Saudi Arabia, with a total population of approximately 1.07 million people. Al-Qaseem is considered an agricultural region. The three regions comprise approximately 18.5% of the Saudi population. These data were taken from the last population census in Saudi Arabia conducted in 2007.

To test for the prevalence rate of celiac disease using anti-EMA, we used blood samples that had been collected for a previous study^[12]. The samples had been collected from a population of students in the 10th to 12th grades (corresponding to the ages of 16 to 18 years) in three regions of Saudi Arabia. The composition of the student population was as follows: Aseer region, with a total school population of 25512 (13996 males and 11516 females); Madinah region, with a total school population of 23852 (12133 males and 11719 females); and Al-Qaseem region, with a total school population of 16067 (7974 males and 8093 females).

The sample was selected using a stratified random sampling technique, where the Kingdom was stratified into three strata. A proportional allocation method was used to determine the recruited number of students in each stratum.

Within each stratum, the sample was proportionally allocated according to sex. In every region, the schools served as the sampling units. It is worth noting that the schooling system in Saudi Arabia depends on segregating males and females in different schools, and this situation was taken in consideration for sampling. From the list of schools in the region, one or more male schools and one or more female schools that satisfied the required sample size were randomly selected. A total of 1358 students (679 males and 679 females) from these regions were randomly selected. The socioeconomic status (SES) of this population was stratified as lower, middle, and upper class. The SES of a student was taken to be representative of that of the father and/or mother and was classified from the socioeconomic score derived from the type of house, the number of rooms per house, the number of cohabiting family members, parents' educational levels, and parents' occupations. The SES of students was measured using a point scale of 1-21 as follows: housing, 3 points; education of parents, 6 points; type of work of parents, 6 points; number of family members, 3 points; and number of rooms in the house, 3 points. Students who scored 17-21 points were classified as upper class, 15-16 as upper middle class, 11-14 as lower middle class, and 10 or less as lower class^[12].

The protocol of this study has been approved previously by King Abdulaziz City for Science and Technology, and informed consent was obtained from the parents and the participating students. All participants were offered further medical evaluation by a gastroenterologist in case of a positive EMA test.

Data collection, blood sampling and testing

The fieldwork for this study was undertaken in December

2007 and January 2008. Demographic data were recorded, and a venous blood sample (5-10 mL) was taken from each student. The serum was separated by centrifugation, coded, and stored at -70°C . The blood samples were tested for immunoglobulin A (IgA) and immunoglobulin G anti-EMA by indirect immunofluorescence (IMMCO Diagnostics, Inc., Buffalo, NY, United States).

Statistical analysis

Data were entered into electronic databases and analyzed using Stata Version 10 (Stata Corporation, College Station, TX, United States). Descriptive statistics (proportional) were used to summarize categorical variables. The chi-square test, followed by an analysis of residuals, was used to calculate the statistical association between two categorical variables. The chi-square test for trends was used to calculate the significance of proportions of variables with three or more categories. A *P* value of < 0.05 was considered statistically significant. Based on a general prevalence of 1% in various populations and an estimated prevalence of 3% in the Saudi population, we determined the sample size at *P* value (alpha) = 0.05 and power = 0.70 and a standard deviation of 0.25, the estimated sample size would be 965 individuals.

RESULTS

Blood samples of 1167 students (614 males and 553 females) were available for testing, while 191 samples were either missing or insufficient for analysis. The mean age for the study population was 16.6 ± 0.6 years. There were no differences in the sex distribution among the three regions (*P* = 0.08). The majority of the study population was classified as lower middle class (82%).

There were 26 (2.2%) students who had a positive anti-EMA test, including 17 females (3.1%) and 9 males (1.5%). Al-Qaseem region had the highest CD prevalence among the three studied regions in Saudi Arabia (3.1%) (Table 1).

The prevalence by region was as follows: Aseer 2.1% (10/479), Madinah 1.8% (8/436), and Al-Qaseem 3.2% (8/252). The prevalence in Madinah was significantly lower than the prevalence in Aseer and Al-Qaseem (*P* = 0.02). There was no statistically significant difference in prevalence between Aseer and Al-Qaseem.

DISCUSSION

Two decades ago, celiac disease was considered a comparatively uncommon disorder, with prevalence rates of 1 in 1000 or lower^[13,14]. CD was even thought to be rare or nonexistent among native Africans, Japanese or Chinese populations^[14]. Several recent population based studies, however, have shown a much higher prevalence, and it is now estimated that celiac disease may affect between 1 in 100 to 200 individuals^[4,15].

The seroprevalence rate of 2.2% (1 in 45) found in our study might be one of the highest seroprevalence

Table 1 Seroprevalence according to regions and sex *n* (%)

| | Aseer (479) | Madinah (436) | Al-Qaseem (252) | Total (1167) |
|--------------|--------------|---------------|-----------------|---------------|
| Male (614) | 4/250 (1.6) | 1/244 (0.4) | 4/120 (3.4) | 9/614 (1.5) |
| Female (553) | 6/229 (2.6) | 7/192 (3.6) | 4/132 (3.0) | 17/553 (3.1) |
| Total (1167) | 10/479 (2.1) | 8/436 (1.8) | 8/252 (3.2) | 26/1167 (2.2) |

rates of celiac disease in the world. Although the prevalence of diagnosed CD varied widely, the estimates of combined undiagnosed and diagnosed (or silent and active) CD were remarkably similar at 0.7%-2.0% in most other populations, including the United States. The prevalence of childhood CD has been reported to be between 1:285 and 1:77 in Sweden^[16] and 1:230 and 1:106 in Italian school aged children^[17]. Generally, similar rates have been reported for non-European white populations, such as New Zealand^[18], Australia^[19], Brazil^[20] and Argentina^[21]. Recent epidemiological studies of CD prevalence rates for North Africa (reported as 0.53% in Egypt, 0.79% in Libya, and 0.6% in Tunisia), the Middle East (0.88% in Iran and 0.6% in Turkey), and India (0.7%) show prevalence rates that overlap with the European data^[22]. A recent study among 204 healthy Saudi blood donors showed a celiac seroprevalence of 1.5%^[11].

A recently published large international, multicenter study investigated a large population sample in four different European countries; on average, the overall prevalence of CD was 1%, with large variations among the studied countries (2.0% in Finland, 1.2% in Italy, 0.9% in Northern Ireland, and 0.3% in Germany). This study confirmed that many CD cases would remain undetected without active serological screening^[23].

Of 3654 students (age range, 7 to 16 years) from Finland who had been screened for anti-endomysial and tissue transglutaminase antibodies, Mäki *et al*^[15] found that 1 in 66 students (1.5%) had positive antibody tests. Of the 36 students from that study with positive antibody assays who agreed to undergo biopsy, 27 had evidence of celiac disease on biopsy. Thus, the estimated biopsy proved prevalence was 1 case in 99 (1%) children. In our study, we have only used a single serological marker (EMA) without duodenal biopsy. The diagnostic standard in celiac serologies remains the anti-endomysial IgA antibodies. These markers are highly specific for celiac disease, with nearly 100% accuracy, which is a crucial point when we use them to study populations at low risk of CD^[24]. Of 20190 Turkish students, Dalgic *et al*^[25] found 489 (2.4%) patients with positive antibodies (IgA-tTG and IgA-EMA). Among 215 patients who underwent an intestinal biopsy, there were only 95 children who were consistent with CD, with an estimated biopsy proven prevalence of 1:212 (0.47%) children. Hogen Esch *et al*^[26] demonstrated that mass screening unavoidably reveals some false-positive and/or false-negative test results, regardless of the type of celiac antibody test. The predictive value of a diagnostic test depends on the prevalence of the disease and the sensitivity and specificity of the test^[27]. In low-risk populations (such

as groups undergoing mass screening), the positive predictive value of the serological tests is always lower than in symptomatic patients or at-risk groups^[28]. However, IgA EMA has an approximately 100% specificity and is the best among all celiac serologies.

Another important finding of our study is the higher prevalence of celiac disease among female compared to male students, which is a finding that was observed in most of the celiac disease epidemiological studies. In addition, there was a significant variation of celiac disease seroprevalence from region to region in Saudi Arabia.

The high prevalence of celiac disease found in our study might be attributed to the high levels of consanguinity and the heavy gluten ingestion in our population. The Saharawi population of Arab-Berber origin living in Algeria has the highest prevalence of CD (5.6%) among all world populations^[29]. The reason for such a frequency of the “celiac trait” in this population is not clear but is likely to be related to their genetic background.

In conclusion, our study provides evidence of a high seroprevalence of CD in a group of school-aged children in 3 regions of Saudi Arabia. Although intestinal biopsies were not available in our study, the high specificity of IgA anti-EMA might indicate one of the highest celiac disease prevalence rates in the world. Further seroprevalence studies with larger samples combined with multiple duodenal biopsies are highly recommended to determine the true celiac disease prevalence in our country.

COMMENTS

Background

The celiac disease (CD) prevalence has progressively increased and, recently, it was proposed that it might be higher than 1 in 100. This increase is mainly due to the increased use of assays that detect celiac antibodies to identify affected individuals. Until the 1990s, the prevalence of CD in Middle Eastern and North African countries was considered low. However, with the introduction of celiac antibodies, CD has been more readily reported from these regions, and its prevalence appears similar to that of North American and European countries. A recent study has shown a seroprevalence of 1.5% among 204 healthy Saudi blood donors. The aim of this study was to identify the seroprevalence of celiac disease among a healthy adolescent population in three regions of Saudi Arabia.

Research frontiers

There are scarce data regarding the prevalence of celiac disease in Saudi Arabia. A recent study has shown a seroprevalence of 1.5% among 204 healthy blood donors.

Innovations and breakthroughs

The results suggest a very high seroprevalence of celiac disease among healthy young Saudi students. Although intestinal biopsies were not available in this study, the high specificity of immunoglobulin A anti-endomysial antibody might indicate one of the highest celiac disease prevalence rates in the world.

Applications

Further seroprevalence studies with larger samples combined with multiple duodenal biopsies are highly recommended to determine the true celiac disease prevalence in Saudi Arabia.

Peer review

The manuscript underlines the seroprevalence of the celiac disease in Saudi Arabia.

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Factors influencing clinical outcomes of Histoacryl[®] glue injection-treated gastric variceal hemorrhage

Varayu Prachayakul, Pitulak Aswakul, Tanyaporn Chantarojanasiri, Somchai Leelakusolvong

Varayu Prachayakul, Pitulak Aswakul, Tanyaporn Chantarojanasiri, Somchai Leelakusolvong, Division of Gastroenterology, Department of Internal Medicine, Faculty of Medicine, Mahidol University, Siriraj Hospital, Bangkok 10700, Thailand
Pitulak Aswakul, Liver and Digestive Institute, Samitivej Sukhumvit Hospital, Bangkok 10700, Thailand

Author contributions: Chantarojanasiri T acquired the data; Leelakusolvong S critically assessed the manuscript's intellectual content; Aswakul P conceptualized and designed the study, analyzed and interpreted the data, drafted and revised the manuscript; Prachayakul V conceptualized and designed the study, analyzed and interpreted the data, and critically assessed and revised the manuscript's intellectual content.

Correspondence to: Dr. Varayu Prachayakul, Division of Gastroenterology, Department of Internal Medicine, Faculty of Medicine, Mahidol University, Siriraj Hospital, 2 Prannok road, Siriraj, Bangkok Noi, Bangkok 10700, Thailand. kaiyjr@gmail.com

Telephone: +66-2-4121088 Fax: +66-2-4199610

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Abstract

AIM: To determine the factors associated with clinical outcomes and complications of Histoacryl[®] glue injection for acute gastric variceal hemorrhage.

METHODS: Patients who presented to the Siriraj Gastrointestinal Endoscopy Center with active gastric variceal bleeding and were admitted for treatment between April 2008 and October 2011 were selected retrospectively for study inclusion. All bleeding varices were treated by injection of Histoacryl[®] tissue glue (B. Braun Melsungen AG, Germany) through a 21G or 23G catheter primed with lipiodol to prevent premature glue solidification. Data recorded for each patient included demographic and clinical characteristics, endoscopic findings, clinical outcomes in terms of early and late re-bleeding, mortality, and procedure-related com-

plications. Data from admission (baseline) and post-treatment were comparatively analyzed using stepwise logistic regression analysis to determine the correlation between factors and clinical outcomes.

RESULTS: A total of 90 patients underwent Histoacryl[®] injection to treat bleeding gastric varices. The mean age was 55.9 ± 13.9 (range: 15-88) years old, and 74.4% of the patients were male. The most common presentations were hematemesis (71.1%), melena (12.2%), and coffee ground emesis (8.9%). Initial hemostasis was experienced in 97.8% of patients, while re-bleeding within 120 h occurred in 10.0%. The presence of ascites was the only factor associated with early and late re-bleeding [odds ratio (OR) = 10.67, 95%CI: 1.27-89.52, $P = 0.03$ and OR = 4.15, 95%CI: 1.34-12.86, $P = 0.01$, respectively]. Early procedure-related complications developed in 14.4% of patients, and were primarily infections and non-fatal systemic embolization. Late re-bleeding was significantly correlated with early procedure-related complications by univariate analysis (OR = 4.01, 95%CI: 1.25-12.87, $P = 0.04$), but no factors were significantly correlated by multivariate analysis. The overall mortality rate was 21.1%, the majority of which were related to infections. The factors showing strong association with higher mortality risk were elevated total bilirubin (OR = 16.71, 95%CI: 3.28-85.09, $P < 0.01$), a large amount of transfused fresh frozen plasma (OR = 1.001, 95%CI: 1.000-1.002, $P = 0.03$), and late re-bleeding (OR = 10.99, 95%CI: 2.15-56.35, $P = 0.02$).

CONCLUSION: Histoacryl[®] injection is a safe and effective hemostatic method for treating gastric variceal hemorrhage. Patients with compromised liver, including ascites, have a higher risk of re-bleeding.

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Key words: Histoacryl; Gastric varices; Clinical outcome; Complications; Hemorrhage

Core tip: Acute gastric variceal hemorrhage is associated with a high mortality rate which accounts for one third of the patients. Histoacryl[®] injection has been reported as one of the effective procedures for treating this condition. The present study investigated patients presenting with acute gastric variceal hemorrhage and found that Histoacryl[®] injection was a safe and highly effective hemostatic method for treating gastric variceal hemorrhage with 97.8% initial hemostasis; only a 10% early re-bleeding rate and a 14.4% procedure-related complication rate were found. The risk factors for re-bleeding were compromised liver status and presence of ascites.

Prachayakul V, Aswakul P, Chantarojanasiri T, Leelakusolvong S. Factors influencing clinical outcomes of Histoacryl[®] glue injection-treated gastric variceal hemorrhage. *World J Gastroenterol* 2013; 19(15): 2379-2387 Available from: URL: <http://www.wjg-net.com/1007-9327/full/v19/i15/2379.htm> DOI: <http://dx.doi.org/10.3748/wjg.v19.i15.2379>

INTRODUCTION

Bleeding from esophagogastric varices is the second most frequent etiology of upper gastrointestinal hemorrhage and is associated with high rates of mortality, even up to five years after the initial episode. In addition, the clinical management procedures used to resolve the bleeding can themselves cause complications, such as infection or tissue injury, that increase the patient's risk of re-bleeding episodes and mortality. The condition and its treatment can be further complicated by the presence of underlying or concomitant diseases. In fact, more than 80% of reported cases have concomitant liver disease, including portal hypertension and cirrhosis^[1-5].

Variceal band ligation is regarded as the most effective standard treatment for bleeding esophageal varices. However, this procedure has proven largely ineffective in treating gastric varices, producing a low rate of hemostasis (reports range from 26%-71%) but having a high rate of re-bleeding (from 60%-90%)^[1-5]. While bleeding gastric varices are substantially less common than those involving the esophageal tissues (accounting for only about 20% of cirrhotic patients), they are invariably related to massive hemorrhaging and significant complications. Moreover, the mortality rate for bleeding gastric varices is about 30%. Other treatment procedures, such as endoscopic sclerotherapy, have shown equally unsatisfactory outcomes; endoscopic band ligation is reported to produce hemostasis in only 45% of cases and to have a 31% re-bleeding rate^[1]. The available hemostatic modalities with the best rates of successful application are: Histoacryl[®] tissue adhesive (2-N-butyl-cyanoacrylate; B. Braun Dexon, Spangenberg, Germany) injection, transarterial intrahepatic portosystemic shunting (TIPS), and balloon-occluded retrograde transvenous obliteration (B-RTO). Of these three, Histoacryl[®] injection represents the most

commonly used treatment for initial acute gastric varices, with TIPS or B-RTO applied subsequent to Histoacryl[®] injection failure. The preference for Histoacryl[®] injection is largely due to liver status-related contraindications affecting TIPS and B-RTO, as well as their inconvenience for application in emergency care clinical settings^[1-10].

Since its introduction in 1984^[3], widespread use of Histoacryl[®] has shown that this agent can successfully resolve bleeding gastric varices. Recent evaluation of the accumulated reports of complications arising in Histoacryl[®]-treated patients, such as systemic embolization, end-organ infarction, visceral fistula, and bacteremia, have also indicated this adhesive is relatively safe and effective^[1,9-13]. A few small case series from Germany, the United Kingdom, Italy and Uruguay have shown 88%-98% initial hemostasis with only a 1% rate of severe complications, such as systemic embolization, and a re-bleeding rate of 10%-29%^[4-10]. Studies from China and Korea reported 95%-100% success rates but a similar range of re-bleeding rates at 1-year follow-up^[6-13]. However, no studies to date have identified the factors related to clinical outcome of gastric variceal hemorrhage following Histoacryl[®] treatment. Therefore, we performed the current retrospective analysis to evaluate the correlation of clinical and/or demographic characteristics to clinical outcome and procedure-related complications.

MATERIALS AND METHODS

Patient selection and clinical procedures

The medical records of the Siriraj Gastrointestinal Endoscopy Center were searched to identify all patients who presented with active gastric variceal bleeding and underwent Histoacryl[®] injection treatment between April 2008 and October 2011. Data recorded at admission (baseline) and during the subsequent hospitalization and follow-up examinations were recorded, including demographic and clinical characteristics, endoscopic procedures and findings, and clinical outcomes in terms of early and late re-bleeding, mortality, and procedure-related complications. The study was carried out with pre-approval by the Siriraj Institutional Review Board (No. Si001/2012).

Each gastric varices case was classified as GOV1, GOV2, IGV1 or IGV2, according to the strategy described by Marques *et al*^[13]. All of the cases were diagnosed as acute gastric variceal bleeding. The clinical setting was classified as emergency when the procedure was carried out within 24 h of the bleeding episode, and as urgent in the case of self-limited bleeding. All patients underwent the treatment procedure within the initial admission period, and no patient was released and re-admitted for the treatment. Achievement of initial hemostasis was defined by stable vital signs and absence of re-bleeding within 24 h. Re-bleeding was defined by the presence of active bleeding from the treated varices directly observed by endoscopy (using forward-viewing gastroscopes GIF 1T145, GIF XTQ 160, or GIF Q180; Olympus, Tokyo, Japan) or indicated by melena/or hematemesis with concurrent hemoglobin decrease of > 2 mg/dL. Re-bleeding was defined as early if it oc-

curred within 120 h after the index procedure, and as late if it occurred within two weeks after the index procedure.

A standardized Histoacryl[®] injection method was used according to the recommendations of Seewald *et al*¹⁴. A working solution of Histoacryl[®] was generated by mixing 0.5 mL with 0.8 mL of lipiodol (Guerbert, Roissy, France). The injection catheter (21G or 23G Interject[™]; Boston Scientific, Spencer, IN, United States) was first primed with 0.8-1.2 mL of lipiodol to prevent premature solidification of the Histoacryl[®]. Then, the bleeding gastric varix was punctured and the working solution was injected, followed immediately by 1.0-2.0 mL of sterile distilled water to ensure delivery of the entire working solution volume into the varix. The needle was retracted and immediately flushed with sterile distilled water to maintain patency. Then, the varix was probed with the injection catheter and if it was found to have remained soft, an additional 1.3 mL injection was initiated to achieve complete obliteration (defined as absolute firmness of the injected varix). All of these procedures were carried out without the aid of fluoroscopic monitoring. All procedures were carried out by experienced gastroenterologists or by second-year gastroenterologist trainees under the supervision of an experienced gastroenterologist.

Post-operative monitoring included clinical and laboratory examinations to identify development of complications. Most patients received continuous intravenous infusion of vasopressors (Octreotide, Sandostatin[®]) for three to five days following the procedure. Complications identified during routine examinations, and not based on clinical symptoms and signs, were classified as minor, and included abdominal pain, chest discomfort, or embolization. Complications identified upon examination in response to clinical signs and symptoms were classified as major, and included systemic embolization; major complications required further treatment and extended hospitalization by about three days. Furthermore, complications that occurred within 24 h of the procedure were classified as early, while those that occurred within two weeks of the procedure were classified as late.

Statistical analysis

Statistical analysis were carried out by the SPSS software, version 13.0 (SPSS, Inc., Chicago, IL, United States). Descriptive data are reported as mean \pm SD or as percentage. The Student's *t* test and the χ^2 test were used to assess differences between groups. Forward stepwise logistic regression analyses, both univariate and multivariate, and receiver operating characteristic curve (ROC) analysis were used to determine the correlation between factors and clinical outcomes. A two-tailed *P* value $>$ 0.05 was considered as statistically significant.

RESULTS

A total of 90 cases of gastric variceal hemorrhage treated by Histoacryl[®] injection were analyzed. The majority of the cases were male ($n = 62$, 74.4%). The average pa-

tient age was 55.9 ± 13.9 (range: 15-88) years old. The most frequent clinical presentations were hematemesis (71.1%), melena (12.2%), coffee ground vomiting (8.9%), and hematochezia (6.7%). One-third of the patients presented with concomitant hypotension, while one-fifth had clinical signs of hepatic encephalopathy and about one-third had concurrent hepatocellular carcinoma (HCC). According to Child-Pugh classification, 20.0% of patients had class A liver status, while 46.7% and 32.2% had class B and C liver status, respectively. According to scoring for model of end-stage liver disease (MELD), the median MELD score for all patients was 10 and the scores ranged from 6 to 28. Nearly all cases of portal hypertension were caused by cirrhosis related to various etiologies, including alcoholism (34.4%), chronic hepatitis B infection (28.9%), chronic hepatitis C infection (14.4%), non-alcoholic steatohepatitis (2.2%), cryptogenic factors (12.2%), and other factors (7.8%). Only one patient had non-cirrhotic portal hypertension.

Ninety percent of the total patients with bleeding gastric varices required blood transfusion prior to endoscopy or during hospital admission. Seventy-three percent of the procedures were carried out as emergency endoscopic treatments. The gastric varices cases represented GOV1 (44.4%), GOV2 (33.3%), IGV1 (21.2%) and IGV2 (1.1%). Two-thirds of the patients had concurrent esophageal varices, but no cases showed evidence of esophageal index bleeding.

The mean volume of Histoacryl[®] working solution delivered per procedure was 3.12 mL. Initial hemostasis was achieved in 97.8% of the procedures. The average hospital stay was nine days. Early re-bleeding occurred in 10.0% of the total patients, but 21.1% of patients experienced late re-bleeding. Early complications occurred in 14.4% of the total cases, and included subclinical systemic embolization (4.4%), aspiration pneumonia (5.5%), spontaneous bacterial peritonitis (1.1%), and other infection (3.3%). A total of 19 patients died during the follow-up period, and 80.0% of the deaths were attributed to HCC or advanced cirrhosis (all of which had been treated conservatively). The remaining deaths were related to the gastric varices re-bleeding.

The patients' baseline characteristics are shown in Table 1, and data related to the procedure and clinical outcome, including complications, are shown in Table 2. The first clinical outcome considered in univariate and multivariate analyses was re-bleeding, and both early and late episodes were analyzed. As shown in Tables 3 and 4, the factors associated with early re-bleeding by univariate analysis were presence of ascites [odds ratio (OR) = 10.90, 95%CI: 1.30-91.51, $P = 0.01$] and concurrent HCC along with a large volume of transfused packed red cells (PRC) (6.89 ± 3.85 units, $P < 0.01$) (OR = 4.95, 95%CI: 1.14-21.50, $P = 0.05$). The factors correlated with late re-bleeding by univariate analysis were presence of ascites (OR = 4.25, 95%CI: 1.37-13.17, $P = 0.01$) and concurrent HCC in general (OR = 2.98, 95%CI: 1.05-8.46, $P = 0.04$). However, multivariate analysis identified only the

Table 1 Patient baseline characteristics and clinical presentation *n* (%)

| Clinical factor | <i>n</i> = 90 | mean ± SD |
|---|---------------|----------------|
| Age, yr | | 55.9 ± 13.9 |
| Male sex | 67 (74.4) | |
| Clinical presentations | | |
| Hematemesis | 64 (71.1) | |
| Melena | 11 (12.2) | |
| Hematochezia | 6 (6.7) | |
| Coffee ground | 8 (8.9) | |
| Not reported | 1 (1.1) | |
| Vital signs at presentation | | |
| Normal | 49 (54.4) | |
| Tachycardia | 7 (7.8) | |
| Hypotension | 34 (37.8) | |
| Etiology of cirrhosis | | |
| Alcoholism | 31 (34.4) | |
| Chronic hepatitis B | 26 (28.9) | |
| Chronic hepatitis C | 13 (14.4) | |
| Nonalcoholic steatohepatitis | 2 (2.2) | |
| Cryptogenic | 11 (12.2) | |
| Other | 7 (7.8) | |
| Liver status by Child-Pugh classification | | |
| Class A | 18 (20.0) | |
| Class B | 42 (46.7) | |
| Class C | 29 (32.2) | |
| Concurrent hepatocellular carcinoma | | |
| Yes | 29 (32.2) | |
| Blood transfusion | | |
| Yes | 81 (90.0) | |
| Transfusion volume | | |
| Packed red cells, units | | 3.5 ± 2.8 |
| Fresh frozen plasma, mL | | 890.9 ± 1183.4 |
| Endoscopic setting | | |
| Emergency | 66 (73.3) | |
| Urgent | 24 (26.7) | |

presence of ascites as correlated with early re-bleeding (OR = 10.67, 95%CI: 1.27-89.52, $P = 0.03$) and late re-bleeding (OR = 4.15, 95%CI: 1.34-12.86, $P = 0.01$).

The second clinical outcome considered in univariate and multivariate analyses was mortality at the last follow-up. As shown in Table 5, the factors significantly correlated with mortality by univariate analysis were presence of ascites (OR = 3.09, 95%CI: 1.05-9.12, $P = 0.04$), elevated total bilirubin (8.50 ± 6.71 mg/dL, $P < 0.01$) (OR = 16.7, 95%CI: 3.28-85.09), concurrent HCC (OR = 2.98, 95%CI: 1.05-8.47, $P = 0.03$), high volume of transfused PRC (5.68 ± 3.32 units, $P < 0.01$) or fresh frozen plasma (1934.0 ± 1850.78 mL, $P < 0.01$), emergency endoscopic setting (OR = 0.17, 95%CI: 0.01-0.92, $P = 0.02$), high volume of Histoacryl® injection (4.13 ± 1.99 mL, $P < 0.01$) (OR = 2.28, 95%CI: 2.32-108.72, $P < 0.01$), early re-bleeding (OR = 20.12, 95%CI: 3.72-108.32, $P < 0.01$) and late re-bleeding (OR = 10.32, 95%CI: 3.35-34.91, $P < 0.01$). Multivariate analysis showed correlations with mortality only for total bilirubin (OR = 16.71, 95%CI: 3.28-85.09, $P < 0.01$), large volume of transfused fresh frozen plasma (OR = 1.001, 95%CI: 1.000-1.002, $P = 0.03$), and late re-bleeding (OR = 10.99, 95%CI: 2.15-56.35, $P = 0.02$).

The last clinical outcome considered in univariate and multivariate analyses was procedure-related complica-

Table 2 Procedures, clinical outcomes, and complications *n* (%)

| Procedures and clinical factors | <i>n</i> = 90 | mean ± SD |
|---|---------------|-----------|
| Type of gastric varix | | |
| GOV1 | 40 (44.4) | |
| GOV2 | 30 (33.3) | |
| IGV1 | 18 (20.0) | |
| IGV2 | 1 (1.1) | |
| Combination of variceal type | 1 (1.1) | |
| Size of gastric varices, cm | | 2.1 ± 0.9 |
| Concurrent esophageal varix | | |
| No | 28 (31.1) | |
| Yes | 62 (68.9) | |
| Bleeding stigmata observed by endoscopy | | |
| Yes | 71 (78.9) | |
| Initial hemostasis | | |
| Yes | 88 (97.8) | |
| Early re-bleeding | | |
| Yes | 9 (10.0) | |
| Late re-bleeding | | |
| Yes | 19 (21.1) | |
| Early complications | | |
| No | 77 (85.6) | |
| Non-significant systemic embolization | 4 (4.4) | |
| Pneumonia | 4 (4.4) | |
| Spontaneous bacterial peritonitis | 1 (1.1) | |
| Infection elsewhere | 3 (3.3) | |
| Late complications | | |
| No | 88 (97.8) | |
| Infection elsewhere | 1 (1.2) | |
| Mean aliquot used/procedure | | 3.0 ± 1.7 |
| Follow-up clinical status | | |
| Dead | 19 (21.1) | |
| Worsening | 3 (3.3) | |
| Stable | 8 (8.9) | |
| Improved | 60 (66.7) | |
| Causes of death (<i>n</i> = 19) | | |
| Bleeding-related | 4 (4.4) | |
| Infection | 9 (10.0) | |
| Liver failure | 1 (1.1) | |
| Cardiovascular diseases | 2 (2.2) | |
| Renal failure | 1 (1.1) | |
| Bowel gangrene | 2 (2.2) | |

tions. As shown in Table 6, univariate analysis identified only one factor as correlated with procedure-related complications: late re-bleeding (OR = 4.01, 95%CI: 1.25-12.87, $P = 0.04$). However, the multivariate analysis did not identify any factors as significantly correlated with this clinical outcome.

ROC analysis of total bilirubin correlation with mortality indicated that the cut-off level was > 4.5 mg/dL (area under the curve was 0.926). Classification of the patients into two groups according to this cut-off level followed by multivariate analysis identified total bilirubin > 4.5 mg/dL as significantly correlated with mortality (OR = 7.25, 95%CI: 2.39-22.02, $P < 0.01$).

DISCUSSION

In our study, the majority of patients with gastric variceal hemorrhage had underlying decompensated liver cirrhosis and presented with hematemesis. Surprisingly, only one-third of the patients presented with active bleeding

Table 3 Factors related to early re-bleeding

| Factors | Early re-bleeding | | | | | | | |
|-------------------------------|-------------------|------------------|---------------------|------------|------|-------------------|------------|-------|
| | Yes (n = 9) | No (n = 81) | Univariate | | | Multivariate | | |
| | | | P value | 95%CI | OR | P value | 95%CI | OR |
| Decompensated liver diseases | | | | | | | | |
| Yes | 9 | 62 | 0.19 | | | | | |
| MELD score > 12 | 3 | 19 | 0.69 | | | | | |
| Encephalopathy | 3 | 24 | 0.33 | | | | | |
| Ascites | 8 | 33 | 0.01 ¹ | 1.30-91.51 | 10.9 | 0.03 ¹ | 1.27-89.52 | 10.67 |
| Concurrent HCC | | | | | | | | |
| Yes | 6 | 23 | 0.05 | 1.14-21.50 | 4.95 | | | |
| Transfusion volume | | | | | | | | |
| PRC, unit | 6.89 ± 3.85 | 3.09 ± 2.56 | < 0.01 ¹ | | | 0.04 ¹ | 1.12-116.0 | 11.41 |
| FFP, mL | 1943.33 ± 1064.61 | 773.69 ± 1142.92 | < 0.01 ¹ | | | - | - | - |
| Type of gastric varix | | | | | | | | |
| GOV | 7 | 63 | | | | | | |
| IGV | 2 | 17 | 0.99 | | | | | |
| Mean GV size, cm | 2.16 ± 0.70 | 2.12 ± 0.88 | 0.88 | | | | | |
| Mean aliquot number/procedure | 3.88 ± 1.72 | 2.93 ± 1.72 | 0.146 | | | | | |
| Endoscopic red stigmata | | | | | | | | |
| Yes | 9 | 69 | 0.19 | | | | | |

¹Statistically significant difference. HCC: Hepatocellular carcinoma; MELD: Model of end-stage liver disease; PRC: Packed red cell; FFP: Fresh frozen plasma; OR: Odds ratio.

Table 4 Factors related to late re-bleeding

| Factors | Late re-bleeding | | | | | | | |
|-------------------------------|-------------------|-----------------|---------------------|------------|------|-------------------|------------|------|
| | Yes (n = 19) | No (n = 71) | Univariate | | | Multivariate | | |
| | | | P value | 95%CI | OR | P value | 95%CI | OR |
| Decompensated liver diseases | | | | | | | | |
| Yes | 18 | 53 | 0.19 | | | | | |
| MELD score > 12 | 5 | 17 | 0.92 | | | | | |
| Encephalopathy | 7 | 10 | 0.07 | | | | | |
| Ascites | 14 | 27 | 0.01 ¹ | 1.37-13.17 | 4.25 | 0.01 ¹ | 1.34-12.86 | 4.15 |
| Concurrent HCC | | | | | | | | |
| Yes | 10 | 19 | 0.04 ¹ | 1.05-8.46 | 2.98 | - | - | - |
| Transfusion volume | | | | | | | | |
| PRC, unit | 5.00 ± 3.59 | 3.06 ± 2.36 | < 0.01 ¹ | | | - | - | - |
| FFP, mL | 1648.68 ± 1720.18 | 688.11 ± 906.73 | 0.03 ¹ | | | - | - | - |
| Type of gastric varix | | | | | | | | |
| GOV | 14 | 56 | 0.89 | | | | | |
| IGV | 5 | 15 | | | | | | |
| Mean GV size, cm | 2.05 ± 0.76 | 2.15 ± 0.89 | 0.67 | | | | | |
| Mean aliquot number/procedure | 3.17 ± 1.75 | 2.98 ± 1.74 | 0.69 | | | | | |
| Endoscopic red stigmata | | | | | | | | |
| Yes | 17 | 54 | 0.55 | | | | | |

¹Statistically significant difference. HCC: Hepatocellular carcinoma; MELD: Model of end-stage liver disease; PRC: Packed red cell; FFP: Fresh frozen plasma; OR: Odds ratio.

and as hemodynamically unstable. This examination of 90 cases treated by Histoacryl[®] injection revealed that almost all patients required blood transfusion prior to the endoscopic procedure or during the subsequent hospital admission, and that the most common types of gastric varices were GOV1 and GOV2. Moreover, concurrent esophageal varices and HCC were frequently present in these patients. In 2005, Noophun *et al*^[15] reported a similar retrospective study of 24 Thai patients who presented with gastric variceal hemorrhage and were treated with Histoacryl[®] injection. In that study population, initial hemostasis was achieved in 58% of patients and 29% ex-

perienced re-bleeding; however, these findings were quite different from the other studies in the literature, which were reporting success rates as high as 90%-100%^[2-17]. In our present study population, initial hemostasis was achieved in 97.8%. However, the rates of early and late re-bleeding were lower than those reported in the previous studies (10% and 20%, respectively, *vs* 12%-54%)^[2-5]. One previous study by Wang *et al*^[7] had reported that about 10% of Histoacryl[®] extrusion occurs within the first week after injection, and suggested that this phenomenon might be related to re-bleeding of the gastric varices. Therefore, we hypothesize that the early re-

Table 5 Correlation analysis of factors associated with mortality at final follow-up

| Factors | Mortality | | | | | | | |
|-------------------------------|-------------------|-----------------|---------------------|------------|-------|---------------------|-------------|-------|
| | Yes (n = 19) | No (n = 71) | Univariate | | | Multivariate | | |
| | | | P value | 95%CI | OR | P value | 95%CI | OR |
| Age, yr | 56.4 ± 3.34 | 55.8 ± 1.65 | 0.95 | | | | | |
| Decompensated liver diseases | | | | | | | | |
| Yes | 18 | 53 | 0.10 | | | | | |
| MELD score > 12 | 8 | 14 | 0.06 | | | | | |
| Ascites | 13 | 28 | 0.04 ¹ | 1.05-9.12 | 3.09 | - | - | - |
| Encephalopathy | 6 | 11 | 0.29 | | | | | |
| Total bilirubin, mg/dL | 8.50 ± 6.71 | 2.73 ± 2.93 | < 0.01 ¹ | | | < 0.01 ¹ | 3.28-85.09 | 16.7 |
| Concurrent HCC | | | | | | | | |
| Yes | 10 | 19 | 0.03 ¹ | 1.05-8.47 | 2.98 | - | - | - |
| Transfusion volume | | | | | | | | |
| PRC, unit | 5.68 ± 3.32 | 2.87 ± 2.28 | < 0.01 ¹ | | | - | - | - |
| FFP, mL | 1934.00 ± 1850.78 | 611.76 ± 724.89 | < 0.01 ¹ | | | 0.03 ¹ | 1.000-1.002 | 1.001 |
| Type of gastric varix | | | | | | | | |
| GOV | 14 | 56 | 0.54 | | | | | |
| IGV | 5 | 14 | | | | | | |
| Mean GV size, cm | 1.97 ± 0.74 | 2.16 ± 0.89 | 0.38 | | | | | |
| Mean aliquot number/procedure | 4.13 ± 1.99 | 2.76 ± 1.57 | < 0.01 ¹ | | | - | - | - |
| Early re-bleeding | | | | | | | | |
| Yes | 7 | 2 | < 0.01 ¹ | 3.72-108.3 | 20.12 | - | - | - |
| Late re-bleeding | | | | | | | | |
| Yes | 8 | 11 | < 0.01 ¹ | 3.35-34.91 | 10.32 | < 0.01 ¹ | 2.15-56.35 | 10.99 |

¹Statistically significant difference. HCC: Hepatocellular carcinoma; MELD: Model of end-stage liver disease; PRC: Packed red cell; FFP: Fresh frozen plasma; OR: Odds ratio.

Table 6 Correlation analysis of factors associated with procedure-related complications

| Factors | Complications | | | | | | | |
|-------------------------------|---------------|---------------|-------------------|------------|------|--------------|-------|----|
| | Yes (n = 16) | No (n = 74) | Univariate | | | Multivariate | | |
| | | | P value | 95%CI | OR | P value | 95%CI | OR |
| Age, yr | 55.92 ± 12.56 | 56.06 ± 19.69 | 0.97 | | | | | |
| Decompensated liver diseases | | | | | | | | |
| Yes | 14 | 57 | 0.19 | | | | | |
| MELD score > 12 | 8 | 14 | 0.14 | | | | | |
| Encephalopathy | 1 | 16 | 0.06 | | | | | |
| Ascites | 8 | 33 | 0.79 | | | | | |
| Concurrent HCC | | | | | | | | |
| Yes | 5 | 24 | 0.90 | | | | | |
| Mean GV size, cm | 2.28 ± 0.99 | 2.09 ± 0.83 | 0.44 | | | | | |
| Mean aliquot number/procedure | 2.87 ± 1.50 | 3.06 ± 1.79 | 0.71 | | | | | |
| Early re-bleeding | | | | | | | | |
| Yes | 4 | 5 | 0.49 | | | | | |
| Late re-bleeding | | | | | | | | |
| Yes | 7 | 12 | 0.04 ¹ | 1.25-12.87 | 4.01 | | | |

¹Statistically significant difference. HCC: Hepatocellular carcinoma; MELD: Model of end-stage liver disease; OR: Odds ratio.

bleeding cases in the present study population might be associated with early glue extrusion.

Procedure-related complications following Histoacryl[®] injection developed in 13.9% of the current study's population. This rate is slightly lower than the rate of 15% reported by Fry *et al.*¹¹. One of the most concerning complications of endoscopy is fatal systemic embolization^[8-27]; fortunately, no cases of severe systemic embolization developed in the current study population, despite the average amount of Histoacryl[®] working solution used per case being about 3 mL. Most of the cases of embolization complications in the current study did not

manifest any significant clinical symptoms and/or signs, and were incidentally detected by lipiodal staining in chest X-ray or computed tomography scan. None of the fatal consequences of systemic embolization, which include organ infarction and abscess formation, developed in this study population. Thus, the collected data suggest that Histoacryl[®] injection is an effective and safe option for treating active or recent gastric variceal hemorrhage.

The overall post-procedure mortality in the current study's population was similar to that reported from previous studies^[2-11]. We noted that one-half of the patient deaths were associated with infections, with hospital-

acquired pneumonia or ventilator-associated pneumonia being predominant. Moreover, the infections occurred despite the use of prophylactic antibiotics. We believe that the pneumonic infections, in particular, might have resulted from incidental aspiration that occurred during the active bleeding condition or were secondary consequences of bacteremia^[25]. Thus, this complication might be prevented by extending the antibiotic prophylaxis schedule, by performing early endotracheal intubation to prevent aspiration, or by using a needle fitted with a covered-tip catheter to reduce contamination.

Previously, Chang *et al.*^[2] investigated the factors which might affect clinical outcomes of patients who underwent Histoacryl[®] injection for gastric variceal hemorrhage. Although only 9% of that study population was represented by patients with gastric variceal hemorrhage, the two predictive factors of re-bleeding identified were a large amount of PRC transfusion and high MELD scores. Another study of 118 Taiwanese patients with gastric variceal hemorrhage identified concomitant HCC as associated with early re-bleeding^[5]. In particular, advanced cancer stage, newly-developed HCC, active bleeding, and high MELD score were reported as being associated with poor outcome. To date, however, no study has reported predictive factors for early re-bleeding in cirrhotic patients with active gastric variceal hemorrhage. In the present study, the presence of ascites was the only factor associated with both early and late re-bleeding in patients with active gastric variceal hemorrhage treated by Histoacryl[®] injection. Therefore, we propose that these re-bleeding episodes may have been related to pre-existing defects in the liver status. Ascites are one of the items considered in the Child-Pugh scoring system of liver status, yet neither the Child-Pugh score nor the MELD score was found to be significantly correlated with re-bleeding in the current study population.

A large amount of transfused PRC was identified as another potential predictive factor of re-bleeding, which is logical since this factor corresponds to the severity of active bleeding at the initial presentation for which surgery is indicated. Yet another factor, concurrent HCC, was correlated with both early and late re-bleeding by univariate analysis only, and the correlation was lost in multivariate analysis. It is possible that our relatively small study population size limited our ability to detect the true correlation, and future study with a larger population might confirm the predictive nature of this factor. Surprisingly, the endoscopic finding of recent bleeding signs, such as red nipple or white nipple, or even the type and size of GV itself, including the amount of injected Histoacryl[®], could not be used as the predictors for re-bleeding in the present study. In addition, late re-bleeding was identified as a potential predictive factor of procedure-related complications, but again the significant correlation was lost in multivariate analysis. Because infections accounted for more than half of complications occurring in the current study population, we hypothesized that the processes of re-bleeding and infection may each represent both cause and effect; for example, the bleeding site might be a portal by which

pathogenic agents achieve more systemic distribution, or the infection itself might trigger a bleeding episode in already weakened tissues further damaged by the actions of inflammatory cytokines.

The mortality rate of patients with bleeding gastric varices has been previously shown to be related to the amount of blood transfusion and the patient's liver status (Child-Pugh score and MELD score). The present study showed that a larger amount of transfused fresh frozen plasma, elevated total bilirubin level (> 5 mg/dL), and late re-bleeding were significantly correlated with mortality. Therefore, we hypothesize that the risk of mortality for a patient with gastric variceal hemorrhage following treatment with Histoacryl[®] injection is associated with pre-existing liver conditions, severity of the index bleeding, and development of infectious complications. Concurrent HCC and MELD score may also influence mortality, but studies with larger populations are needed to confirm their role.

Some limitations exist in the present study design that may affect generalization of our findings. First, this was a retrospective study in which the decision making of treatment strategy depended on the endoscopist who was in charge on the day of the procedure. However, all of the endoscopists were trained in a standardized protocol for Histoacryl[®] injection. Second, some of the re-bleeding patients diagnosed with advanced HCC or decompensated liver disease were managed noninvasively. Conservative treatment can be associated with a higher mortality rate and may have impacted our mortality data. Lastly, the relatively small population size of the current study may have weakened the power of detecting true correlations; studies with larger populations are required to confirm our findings.

Histoacryl[®] injection is an effective and safe treatment option for gastric variceal hemorrhage. Neither the type or size of gastric varices, the amount of Histoacryl[®], nor the injection technique were associated with rates of re-bleeding, complications, or mortality. However, the severity of index bleeding and a pre-existing decompensated liver status, especially the presence of ascites or elevated total bilirubin, are associated with the rates of re-bleeding and mortality.

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COMMENTS

Background

Gastric variceal hemorrhage is an uncommon cause of upper gastrointestinal bleeding, and is mostly related to portal hypertension; however, this condition is associated with very high rates of morbidity and mortality. Injection of Histoacryl[®] tissue adhesive (N-2-butyl-cyanoacrylate) can achieve hemostasis, but is also associated with development of life-threatening procedure-related complications, such as fatal systemic embolization, and appreciable rates of re-bleeding. To date, there are limited data regarding the factors associated with the clinical outcomes of Histoacryl[®] injection to treat bleeding gastric varices.

Research frontiers

In this article, the authors evaluate the factors associated with clinical outcomes of gastric variceal hemorrhage treated by Histoacryl[®] injection. These data may help to identify patients at greater risk of experiencing re-bleeding episodes following treatment and those who will benefit from closer clinical monitoring for an extended period of time following the surgical procedure.

Innovations and breakthroughs

Initial hemostasis was achieved in 97.8% of bleeding gastric varices patients treated with Histoacryl[®] injection. The rate of early (within 120 h of procedure) re-bleeding was 10.0%, and the rate of late (within two weeks of procedure) re-bleeding was 21.1%. The overall complication rate was 13.9%, and the majority of cases that died were associated with infection. The factors associated with adverse clinical outcome involved the patients' liver status, and the procedure itself appeared to be much less involved.

Peer review

The authors performed a retrospective analysis of patients with acute gastric variceal hemorrhage to determine the factors associated with clinical outcomes and complications of Histoacryl[®] injection. The Histoacryl[®] injection procedure and compound were effective and safe for treating gastric variceal hemorrhage, achieving a high rate of hemostasis while producing low rates of re-bleeding and procedure-related complications. The clinical outcomes were mostly associated with liver status of the patients during the index bleeding episode. The results provide insights into the underlying etiologies of re-bleeding following treatment and may help to identify patients at higher risk of re-bleeding and mortality.

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Effect of *Helicobacter pylori* eradication on serum ghrelin and obestatin levels

Celal Ulasoglu, Banu Isbilen, Levent Doganay, Filiz Ozen, Safak Kiziltas, Ilyas Tuncer

Celal Ulasoglu, Levent Doganay, Safak Kiziltas, Ilyas Tuncer, Department of Gastroenterology, Istanbul Medeniyet University, Goztepe Education and Research Hospital, 34470 Istanbul, Turkey

Banu Isbilen, Department of Biochemistry, Istanbul Medeniyet University, Goztepe Education and Research Hospital, 34470 Istanbul, Turkey

Filiz Ozen, Medical Genetics, Istanbul Medeniyet University, Goztepe Education and Research Hospital, 34470 Istanbul, Turkey
Author contributions: Ulasoglu C designed the study, collected the materials and contributed to writing the manuscript; Kiziltas S and Tuncer I evaluated the data and contributed to writing the manuscript; Isbilen B and Ozen F performed laboratory procedures; Doganay L edited the manuscript and performed the statistical analysis.

Correspondence to: Dr. Celal Ulasoglu, Department of Gastroenterology, Istanbul Medeniyet University, Goztepe Education and Research Hospital, 34470 Istanbul,

Turkey. ulasoglu@gmail.com

Telephone: +90-216-5666600 Fax: +90-216-5666628

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Abstract

AIM: To investigate changes in serum ghrelin and obestatin levels before and after *Helicobacter pylori* (*H. pylori*) eradication.

METHODS: A total of 92 patients presenting with symptoms of dyspepsia were enrolled in the study. Upper endoscopy was performed on all patients and used to diagnose *H. pylori* infection according to the presence of characteristic histopathological findings; seventy patients were diagnosed with *H. pylori* infection and the remaining 22 non-infected patients were classified as healthy controls. *H. pylori* eradication was accomplished by administering the classical triple therapy drug regimen, consisting of lansoprazole 30 mg *bid*, amoxicillin 1 g *bid*, and clarithromycin 500 mg *tid* for 14 d. The eradication of *H. pylori* was assessed with C¹⁴-urea breath

test, which was performed at eight weeks after treatment. Levels of serum active ghrelin and obestatin were assessed at beginning of the study (prior to treatment) and after eight weeks. The levels were comparatively analyzed between the *H. pylori* negative control group, the *H. pylori* eradicated group, and the *H. pylori* non-eradicated group.

RESULTS: A total of 92 patients, 50 females and 42 males with a mean age of 38.2 ± 11.9 years (range: 19-64), were analyzed. *H. pylori* eradication success was achieved in 74.3% (52/70) of *H. pylori* positive patients. The initial levels of ghrelin in the *H. pylori* positive and control cases were 63.6 ± 19.8 pg/mL and 65.1 ± 19.2 pg/mL ($P = 0.78$), respectively, and initial obestatin levels were 771 ± 427 pg/mL and 830 ± 296 pg/mL ($P = 0.19$), respectively. The difference between the initial levels and the week 8 levels of ghrelin and obestatin in the control group was insignificant [4.5% ($P = 0.30$) and -0.9% ($P = 0.65$), respectively]. The difference between the initial and week 8 levels of ghrelin and obestatin in the *H. pylori* non-eradicated group were also insignificant [0.9% ($P = 0.64$) and 5.3% ($P = 0.32$), respectively]. The *H. pylori* eradicated group had a greater change in obestatin levels when compared to the control and the non-eradicated groups (148 ± 381 pg/mL *vs* -12 ± 138 pg/mL and -72.8 ± 203 pg/mL, respectively, $P = 0.015$), while decreases in ghrelin levels were insignificant (-7.2 pg/mL *vs* -1.4 pg/mL and -1.9 pg/mL, respectively, $P = 0.52$). The ghrelin/obestatin ratio for the initial and week 8 levels changed significantly in only the *H. pylori* eradicated group (0.11 *vs* 0.08 , respectively, $P = 0.015$). For overweight patients (as designated by body mass index), we observed significant increases in obestatin levels in the eradicated group as compared to non-eradicated group (201 ± 458 pg/mL *vs* -5 ± 81 pg/mL, respectively, $P = 0.02$). In the *H. pylori*-eradicated group, the levels did not differ between the sexes for ghrelin (-6.3 ± 26.9 pg/mL *vs* -8.0 ± 24.0 pg/mL, respectively, $P = 0.97$) or obestatin (210 ± 390 pg/mL *vs* 96 ± 372 pg/mL, respectively, $P = 0.23$).

CONCLUSION: Serum levels of ghrelin decreased while obestatin levels increased in *H. pylori* eradicated subjects, especially in overweight and male patients.

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Key words: Ghrelin; Obestatin; *Helicobacter pylori*; Gastric peptides; Appetite

Core tip: Ghrelin and obestatin are peptides that have opposing roles in the regulation of appetite and satiety. *Helicobacter pylori* (*H. pylori*), a common cause of gastric inflammation, may have important effects on these peptides and in turn be a potential target of anti-obesity strategies. While the interplay between *H. pylori* and these peptides are well studied, this study included two novel approaches. First, we collected serum samples at two separate time points for both the experimental and control groups, eliminating potential seasonal problems. Second, we focused on not only to *H. pylori* positive patients that responded to therapy, but also those who did not. This helped to distinguish the effects of antibiotherapy on ghrelin and obestatin regardless of the effectiveness of *H. pylori* treatment.

Ulasoglu C, Isbilen B, Doganay L, Ozen F, Kiziltas S, Tuncer I. Effect of *Helicobacter pylori* eradication on serum ghrelin and obestatin levels. *World J Gastroenterol* 2013; 19(15): 2388-2394 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v19/i15/2388.htm> DOI: <http://dx.doi.org/10.3748/wjg.v19.i15.2388>

INTRODUCTION

Ghrelin and obestatin are both important peptides that regulate appetite and play roles as orexigenic signals and in satiety pathways. Both are secreted mainly from gastric oxyntic mucosa and are thought to be influenced by *Helicobacter pylori* (*H. pylori*)^[1-4]. However, the influences of *H. pylori* on serum concentrations of ghrelin are contradictory, since multiple factors interfere with its serum level. Further, and only a limited number of studies have focused on interaction between obestatin and *H. pylori*^[5,6].

Ghrelin is a 28 amino acid (aa) peptide with a name derived from the root ghre-, which means “grow”. This peptide was discovered in 1999 and is secreted endocrine cells of the stomach and by the brain, bowel, testes, pancreatic islet cells, and kidney^[1,7]. Ghrelin has adipogenic properties and is an orexigenic peptide, acting as an appetite stimulant. Moreover, it is a somatotrophic peptide involved in regulating body weight, is controlled by the *GHRL* gene, is derived from preproghrelin (contains 117 aa), and acts as a growth hormone stimulator^[1,7]. It has a molecular weight of 3370.9 Da. Before release into the serum, an n-octanyl moiety is attached to a serine residue at position three, thus making the molecule hydrophobic and facilitating penetration into the hypothalamus and

hypophysis of the brain^[1,4,7].

Obestatin is a 23 aa peptide that is thought to be an appetite suppressant and named from the Latin “obedere”, meaning to devour, and “statin”, which denotes suppression^[2]. Both ghrelin and obestatin are controlled by ghrelin/obestatin prepropeptide gene (*GHRL*), is produced by the post-translational modification by addition of -NH₂ and splitting from the same protein precursor that also produces ghrelin (117 aa, preproghrelin), and is secreted mainly by the stomach^[2,8]. It has a molecular weight of 2516.84 Da and it activates a rhodopsin type G coupled receptor (GPR-39), which is a member of the ghrelin receptor superfamily^[2,3,5].

The underlying purpose for this mechanism that produces two hormones with opposite effects remains unclear, however, this may explain earlier findings that initially seemed ambiguous. For example, removing the ghrelin gene from mice does not significantly reduce appetite, and ghrelin may play a physiological role in the vagal control of gastric function in rats^[5,6]. Moreover, obestatin counteracts growth hormone secretion and food intake induced by ghrelin^[2]. Additionally, intracerebroventricular and systemic injections of obestatin suppress body weight gain in rats. Some gastrointestinal diseases, such as irritable bowel syndrome^[7,8], obesity, Prader-Willi syndrome (chromosome 15-related congenital obesity and hyperphagia)^[9], and type II diabetes mellitus^[10], may be related to the serum ghrelin/obestatin ratio.

H. pylori is a bacteria that is the main cause of gastric inflammation and peptic ulcer disease worldwide. The exact role of *H. pylori* on appetite hormones, such as ghrelin and obestatin, remains unclear^[11-13]. In this study, we compared the changes in these hormones after a successful *H. pylori* eradication.

MATERIALS AND METHODS

Human subjects

The sample population enrolled in this study consisted of ninety-two consecutive patients (50 female and 42 male patients, with ages between 19 and 65 years) who were treated for *H. pylori* infections based on histopathological diagnoses after upper endoscopies to investigate dyspepsia. *H. pylori* positive patients received classical anti-*Helicobacter* triple therapy as treatment (lansoprazole 30 mg *bid*, amoxicillin 1 g *bid*, and clarithromycin 500 mg *tid* for 14 d). Serum ghrelin and obestatin levels were assessed before treatment and eight weeks after the completion of the eradication therapy. Patients had no comorbidities, no chronic illnesses such as diabetes mellitus, no endocrinological disturbances, were not currently taking any medication, and had no history of gastrointestinal surgery. They were restricted from smoking and exercise on sampling day. The success of the *H. pylori* eradication therapy was assessed with C¹⁴-urea breath test (C-UBT) eight weeks after the cessation of the therapy. Before the initial endoscopy and C-UBT, patients did not use any antibiotic or proton pump inhibitors for one month prior.

Table 1 Demographic characteristics and laboratory values for the sample population and control group

| | Controls (n = 22) | <i>H. pylori</i> infected (n = 70) | <i>P</i> value ¹ | <i>H. pylori</i> eradicated (n = 52) | <i>H. pylori</i> non-eradicated (n = 18) | <i>P</i> value ¹ |
|-----------------------------|-------------------|------------------------------------|-----------------------------|--------------------------------------|--|-----------------------------|
| Sex, M/F | 10/12 | 32/38 | 0.98 | 24/28 | 8/10 | 0.99 |
| NW/OW | 9/13 | 31/39 | 0.89 | 23/29 | 8/10 | 0.99 |
| Age (yr) | 40 ± 13 | 38 ± 12 | 0.30 | 38 ± 12 | 37 ± 11 | 0.74 |
| Ghr initial, pg/mL | 65 ± 19 | 64 ± 20 | 0.78 | 63 ± 20 | 64 ± 20 | 0.82 |
| Ghr week 8, pg/mL | 64 ± 17 | 58 ± 20 | 0.16 | 56 ± 20 | 62 ± 19 | 0.35 |
| <i>P</i> value ² | 0.30 | 0.013 ^a | | 0.012 ^a | 0.64 | |
| Ob initial, pg/mL | 830 ± 296 | 771 ± 427 | 0.19 | 765 ± 461 | 787 ± 322 | 0.35 |
| Ob week 8, pg/mL | 818 ± 291 | 863 ± 462 | 0.70 | 914 ± 505 | 714 ± 269 | 0.22 |
| <i>P</i> value ² | 0.65 | 0.07 | | 0.01 ^a | 0.32 | |
| Ghr/Ob ratio initial | 0.089 | 0.107 | 0.420 | 0.113 | 0.092 | 0.460 |
| Ghr/Ob ratio week 8 | 0.092 | 0.086 | 0.480 | 0.081 | 0.099 | 0.140 |
| <i>P</i> value ² | 1.0 | 0.06 | | 0.01 ^a | 0.64 | |
| ΔGhr | -1.4 | -5.9 | 0.49 | -7.2 | -2.0 | 0.19 |
| ΔOb | -12.3 | 91.7 | 0.16 | 148.7 | -72.8 | 0.02 ^a |
| ΔGhr_% | 4.5 | -2.6 | 0.32 | -3.9 | 0.9 | 0.16 |
| ΔOb_% | -0.9 | 29.4 | 0.13 | 41.5 | -5.3 | 0.03 ^a |

¹Mann-Whitney *U* test; ²Wilcoxon-signed ranks test. ^a*P* < 0.05 is significant. Δ: Change in mean; M: Male; F: Female; Ghr: Ghrelin; Ob: Obestatin; *H. pylori*: *Helicobacter pylori*; NW: Body mass index (BMI) ≤ 25 (normal weight); OW: BMI > 25 (overweight).

The body mass index (BMI) for each of the patients was defined as their weight in kilograms divided by the square of height in meters (kg/m²). A cutoff of 25 kg/m² was used to define normal versus overweight participants. The study was done in accordance with the Declaration of Helsinki and using principles of the Good Clinical Practice. The Goztepe Education and Research Hospital approved these studies (18/H-2012). Each patient gave conscious, written, and informed consent before participating in the study.

Biochemical methods

Fasting blood samples (12 h fast) were collected on the day of upper endoscopy and 8 wk later. The blood was allowed to clot at room temperature without any chemical treatment with protease inhibitors. Within one hour of the blood draw, serum was obtained by centrifugation at 2000 × *g* for 10 min and stored at -80 °C until needed. ELISA kits were used for the measurement of active (acylated) serum ghrelin (EMD Millipore, Billerica, MA, United States) and serum obestatin (Peninsula Laboratories LLC, San Carlos, CA, United States). Serum levels of both active ghrelin and obestatin were measured and calculated according to the manufacturers' instructions. The analytic sensitivity of the active ghrelin test was 25 pg/mL. The intra- and inter-assay coefficients of variation (%CV) for the active ghrelin test when a mean concentration of 65.2 pg/mL was tested were 3.63% and 3.55%, respectively. The obestatin test kit measured human obestatin within the range of 0.412-100 ng/mL, the intra-assay CV was less than 5%, and the inter-assay CV was less than 15%.

The units of measure for these peptides were all converted to pg/mL in order to make comparisons. In some studies ghrelin levels were expressed in fmol/mL and were converted to pg/mL by multiplying by a conversion factor of 3.372. Obestatin levels expressed as pmol/L were converted to pg/mL by multiplying by a conversion

factor of 2.5 according to the following formula: pmol/L/0.397 = pg/mL.

Statistical analysis

Serum ghrelin levels, serum obestatin levels, the ghrelin/obestatin ratios, and the changes in levels as percentage were analyzed according to the patients' age, sex, BMI, and eradication of *H. pylori* infection. The Kolmogorov and Shapiro-Wilks tests were used to analyze the normality of the distribution depending on the number of cases (over or under 50, respectively). Independent continuous variables were analyzed using the Mann-Whitney *U* test. Repeated (paired) measures of serum ghrelin and obestatin levels were analyzed using the Wilcoxon signed-rank test. Alterations in serum ghrelin and obestatin levels after the anti-*Helicobacter* triple therapy were also calculated as numerical and percentage and then statistically analyzed. The results were given as mean ± SD. All statistics were done using SPSS 20 (Chicago, IL, United States). In all analyses, double sided *P* values were considered significant if the *P* value was lower than 0.05.

RESULTS

Successful *H. pylori* eradication occurred in 52 (74.3%) of 70 infected patients. The remaining 22 patients were not infected with *H. pylori* at the initial examination and served as the control group. Serum ghrelin and obestatin levels ranged from 28.5-101.4 pg/mL and 180-2230 pg/mL, respectively.

There was no significant difference between the levels of ghrelin or obestatin between the *H. pylori* infected and control groups. The initial levels of ghrelin in the *H. pylori* positive cases and the control group were 63.6 ± 19.8 pg/mL and 65.1 ± 19.2 pg/mL, respectively. Initial obestatin levels for these two groups were 771 ± 427 pg/mL and 830 ± 296 pg/mL, respectively (Table 1). Eighth week ghrelin levels were 56.0 ± 19.9 pg/mL, 62.4 ± 19.0

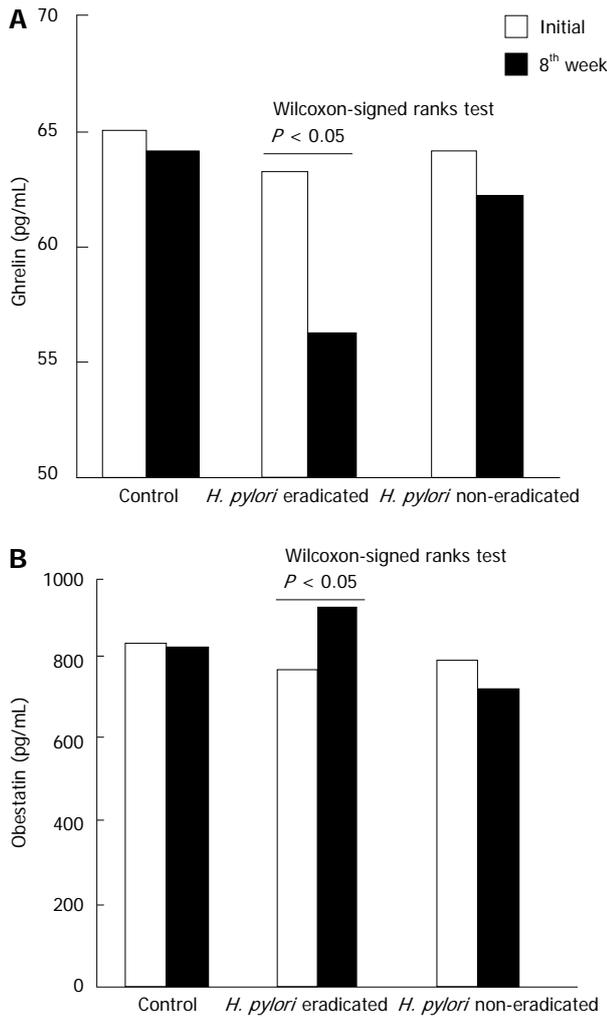


Figure 1 Ghrelin (A) and obestatin (B) levels at the initial and 8th week assessments. *H. pylori*: *Helicobacter pylori*.

pg/mL, and 63.7 ± 17.4 pg/mL for the eradicated, non-eradicated and the control groups, respectively. Following eight weeks of therapy, obestatin levels were 914 ± 505 pg/mL, 714 ± 269 pg/mL, and 818 ± 291 pg/mL for the eradicated, non-eradicated and the control groups, respectively (Table 1 and Figure 1).

No significant difference between the initial and 8th week measurements of ghrelin and obestatin was observed in the control group (Table 1). The *H. pylori* eradicated group demonstrated a significant increase in obestatin after eight weeks of treatment, while the control and non-eradicated groups showed only slight decreases. Similarly, ghrelin levels also slightly decreased in the control and non-eradicated groups. Interestingly however, ghrelin levels only showed an insignificant decrease in the eradicated group (Figure 2). The Mann-Whitney *U* test revealed significant differences in the ghrelin and obestatin levels in the *H. pylori* eradicated group as compared to the non-eradicated group (Table 1). The ghrelin/obestatin ratio at the initial assessment and the 8th week changed significantly only in the *H. pylori* eradicated group (Table

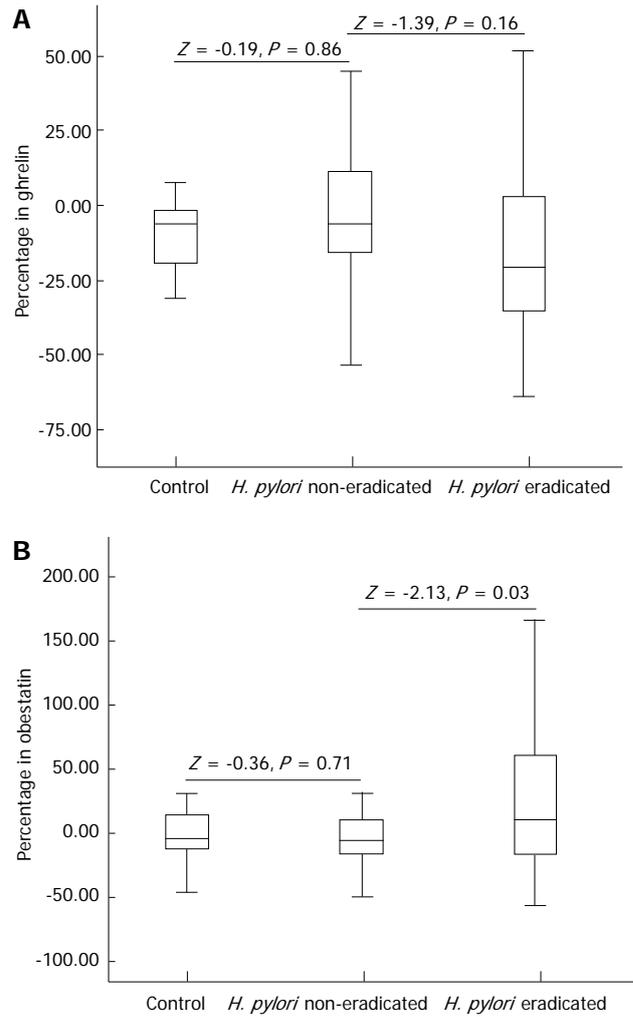


Figure 2 The mean change as percentage in ghrelin (A) and obestatin (B) levels between the initial and 8th week assessments. *H. pylori*: *Helicobacter pylori*.

1). Overweight patients in the eradicated group demonstrated a significant rise in obestatin levels as compared to that of the overweight patients in the non-eradicated group (Table 2). For all participants with a BMI over 25 kg/m², the Mann-Whitney *U* test revealed higher ghrelin and obestatin levels as compared to participants with normal BMIs (Table 2).

The differences in ghrelin and obestatin levels in males and females were insignificant in the *H. pylori*-eradicated group (-6.3 ± 26.9 pg/mL vs -8.0 ± 24.0 pg/mL, respectively, $P = 0.97$ for ghrelin and 210 ± 390 pg/mL vs 96 ± 372 pg/mL, respectively, $P = 0.23$ for obestatin). We observed no significant differences in ghrelin or obestatin levels between males and females in non-eradicated and control groups. In the *H. pylori*-eradicated group, males demonstrated an insignificantly higher percentage change in obestatin than females (51% vs 33%, respectively, $P = 0.90$), but the males in the *H. pylori*-eradicated group demonstrated a significantly higher percentage change in obestatin than males in the non-eradicated group (51% vs -8.5%, respectively, $P = 0.03$).

Table 2 Ghrelin and obestatin changes for normal weight and overweight patients after *Helicobacter pylori* eradication treatment

| | Ghrelin initial, pg/mL | Ghrelin week 8, pg/mL | <i>P</i> value ¹ | Obestatin initial, pg/mL | Obestatin week 8, pg/mL | <i>P</i> value ¹ |
|--|------------------------|-----------------------|-----------------------------|--------------------------|-------------------------|-----------------------------|
| <i>H. pylori</i> eradicated BMI-NW | 58.8 ± 18.6 | 57.5 ± 22.0 | 0.46 | 759 ± 452 | 865 ± 456 | 0.08 |
| <i>H. pylori</i> eradicated BMI-OW | 68.8 ± 20.3 | 54.1 ± 17.1 | 0.002 ^a | 774 ± 413 | 975 ± 565 | 0.05 ^a |
| <i>P</i> value ² | 0.07 | 0.67 | | 0.17 | 0.62 | |
| <i>H. pylori</i> non-eradicated BMI-NW | 68.7 ± 19.7 | 60.8 ± 22.0 | 0.57 | 780 ± 397 | 653 ± 258 | 0.20 |
| <i>H. pylori</i> non-eradicated BMI-OW | 69.0 ± 21.0 | 64.4 ± 15.6 | 0.88 | 796 ± 221 | 791 ± 280 | 0.77 |
| <i>P</i> value ² | 0.40 | 0.69 | | 0.76 | 0.46 | |

¹Mann-Whitney *U* test; ²Wilcoxon-signed ranks test. ^a*P* < 0.05 is significant; BMI: Body mass index (NW: ≤ 25, normal weight; OW: > 25, overweight); *H. pylori*: *Helicobacter pylori*.

DISCUSSION

This study compared the differences in serum acyl-ghrelin and obestatin levels according to *H. pylori* eradication status, sex, and BMI. The results revealed a significant decrease in ghrelin levels and increase in obestatin levels in the *H. pylori* eradicated group after treatment. Despite to reported in some studies, no significant sex differences in ghrelin and obestatin levels were observed between any of the subgroups, including the control group.

The normal levels of these peptides can vary widely; the reported ranges for ghrelin and obestatin are between 5.78 to 1732 pg/mL and 200 to 1156 pg/mL, respectively^[10,14-22]. In fact, the expected normal serum levels for the different forms of ghrelin are very different: 32.61-65.2 pg/mL for octanoylated ghrelin, 300-430 pg/mL for non-octanoylated ghrelin, and 326-489 pg/mL for total ghrelin, plasma inactive ghrelin (without the *n*-octanoyl modification) accounts for > 90% of total circulating ghrelin and the ratio of inactive to active ghrelin can be modified under some physiological or pathological conditions. These wide ranges may be due to several reasons including the methodological differences in measurement, the ethnic differences in the study populations, the commercial kit used, the differences in the pre-treatment procedures, the presence of cytotoxin-associated gene A protein positive *H. pylori*, the nutritional and eating habits of the sample population, and the presence of diabetes or other metabolic syndromes^[22]. Therefore, these wide ranges in values should be the subject of further evaluations. To acquire accurate data on ghrelin concentrations, this study recommends a standard procedure for the collection of blood samples: (1) the collection of blood samples with ethylenediaminetetraacetic acid-aptinin is preferred; (2) blood samples should be chilled and centrifuged as soon as possible, at least within 30 min after collection; and (3) because acidification is the best method for the preservation of plasma ghrelin, 1 mol/L HCl (10% of sample volume) can be added to the plasma sample for adjustment to pH 4^[23]. Ghrelin binds to almost 50% to the high density lipoprotein (HDL) in circulation, and the HDL level varies considerably in different ethnic groups^[24,25]. Thus, changes in the serum levels of HDL may also alter the ghrelin levels. All patients in our study were of Turkish descent and HDL was not considered, though it would be an interesting area of further study to

evaluate how these variables potentially affected the peptide levels.

Small intestine bacterial overgrowth (SIBO) is another possible factor that may explain the variation in ghrelin and obestatin levels, a variable that has been largely neglected to date. Antibiotics used in *H. pylori* eradication may alter the intestinal flora and may trigger any effects by SIBO on appetite hormones. Any comparison of the *H. pylori* eradicated and non-eradicated groups may need to overcome the possible effect of SIBO on these peptides^[26,27]. Moreover, the half-life of ghrelin is very short (about 60 min), and serum esterase easily breaks ghrelin down to des-octanoyl-ghrelin, the inactive form^[28,29]. The differences in these factors and the activity of ghrelin O-acyltransferase may also lead to different results. These technological disadvantages could partially be diminished by including compatible healthy control individuals in each assay^[13]. In a study, two popular commercial RIA kits were compared on the same sample set, and a 10-fold difference in the measured total ghrelin levels was seen^[30].

In our study, no difference was observed in the ghrelin/obestatin ratio of *H. pylori* negative and *H. pylori* positive patients. There are conflicting views of the role of ghrelin and obestatin in the literature. In some reports, the presence of *H. pylori* was associated with decreasing ghrelin, and its eradication was associated with increased ghrelin levels^[31,32]. In contrast, ghrelin decreased after *H. pylori* eradication in some studies; in fact, Osawa *et al.*^[32] reported ghrelin decrease in a majority of patients, while only 50 out of 134 patients demonstrated an increase in this hormone. In Chinese adults, a reduction in the ghrelin/obestatin ratio was associated with patients who were *H. pylori* positive as compared to uninfected controls^[5].

Our results showed that males had insignificantly higher ghrelin and obestatin levels than females (*P* = 0.66 and *P* = 0.73, respectively). Even though a number of studies have reported higher ghrelin levels in females^[33], fluctuating levels of estrogen related to the different phases of the menstrual cycle may influence serum ghrelin levels^[34].

Overweight participants in the *H. pylori* eradication group demonstrated significant changes in ghrelin and obestatin levels in this study. They showed a decrease in ghrelin levels, an increase in obestatin levels, and a decrease in the ghrelin/obestatin ratio. Thus, overweight cases demonstrated opposite changes that what was ex-

pected in terms of their serum ghrelin levels (Table 2).

This study revealed no significant sex and age group (cut-off of 40 years) differences in ghrelin levels, obestatin levels, ghrelin/obestatin ratios, and *H. pylori* eradication. Changes in the ghrelin/obestatin ratio were also insignificant in comparison to the *H. pylori* eradicated, non-eradicated, and control groups. According to our results, obestatin, not ghrelin, seems to be more influenced by *H. pylori* eradication; in fact, this was prominent in overweight and male patients.

The results of these study are valid since influencing factors, errors and procedures were identical for all cases. However, these results suggest a variety of useful, future studies. For example a larger study population would allow generalization of the results to be applicable more than just a small subset of ethnically similar subjects. In addition, it will be interesting to explore several additional variables that were not studied here, including studying subjects in a non-fasting state to allow a comparison of the postprandial or diurnal changes in these hormones. Moreover, including an assessment of the satiety threshold of the patients, taking into account the menstrual status of the female patients and testing for SIBO would also be of great interest.

H. pylori eradication was associated with an significant decrease in ghrelin levels and significant increase in obestatin levels in our study. Due to the contradictory results reported in the literature, the effect of *H. pylori* on these appetite hormones should be the subject of future studies, and the results may provide important insight for anti-obesity treatment strategies.

COMMENTS

Background

Helicobacter pylori (*H. pylori*) is a major cause of stomach inflammation also worldwide. The pathophysiology of some appetite and satiety peptides such as ghrelin and obestatin secreted from gastric mucosa may be altered by this infection.

Research frontiers

Overweight and obesity are major risk factors for a number of chronic diseases, including diabetes, cardiovascular diseases and cancer according to the World Health Organization reports. Worldwide, obesity has increased 82% in the last two decades. A number of studies report the relation of *H. pylori* with some appetite hormones as ghrelin and obestatin, but the results are not consistent. Some reveal increase and some no change of these peptides related with *H. pylori*. The reported levels of these hormones vary in a wide spectrum thus all studies need to be accompanied with a control group. In this study, the authors report the influence of *H. pylori* on these appetite peptides.

Innovations and breakthroughs

In this study, the change of appetite peptides were measured in in three sub-groups of human volunteers: *H. pylori* infected/eradicated, *H. pylori* infected/non-eradicated and non-infected control. The monitoring of the non-eradicated group eliminated of a number of factors allowing us to focus only on the effect of *H. pylori*. In this study, comparison of ghrelin and obestatin change between eradicated and non-eradicated group gave the advantage of discarding the effect of receiving antibiotherapy. Also, including the control group provided the exclusion of possible external factors as month, season and placebo effect of being under examination. Finally, as a novel proposal, the effect of small intestinal bacterial overgrowth, considering menstrual cycles and high density lipoprotein influence on these peptides, are also discussed.

Applications

The study results suggest that appetite hormones may be related with *H. pylori*

and this may be important in anti-obesity strategies.

Terminology

Appetite hormones ghrelin and obestatin, two opposite acting peptides involved in appetite and satiety and *H. pylori* infection and eradication were the main terminological parameters.

Peer review

The study intends to investigate the changes in serum ghrelin and obestatin levels before and after *H. pylori* eradication. The authors found that serum ghrelin and obestatin levels decreased and increased in *H. pylori* eradication groups compared to non-eradicated patients and controls, respectively. These changes were more prominent in overweight and male patients. The manuscript is well written. The methods are adequate. The results justify the conclusions drawn.

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Increased international normalized ratio level in hepatocellular carcinoma patients with diabetes mellitus

Hui Zhang, Chun Gao, Long Fang, Shu-Kun Yao

Hui Zhang, Chun Gao, Long Fang, Shu-Kun Yao, Department of Gastroenterology, China-Japan Friendship Hospital, Ministry of Health, Beijing 100029, China

Author contributions: Zhang H participated in the acquisition of data, statistical analysis and manuscript writing; Gao C and Fang L conceived the study, participated in the study design, acquisition of data, statistical analysis and manuscript writing; Yao SK participated in the study design and critical revision of manuscript for important intellectual content; all authors have read and approved the final manuscript.

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Correspondence to: Long Fang, MD, Department of Gastroenterology, China-Japan Friendship Hospital, Ministry of Health, No. 2 Yinghua East Road, Beijing 100029, China. longfang76@sohu.com

Telephone: +86-10-84205313 Fax: +86-10-84205313

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Abstract

AIM: To determine the association of diabetes mellitus (DM) and international normalized ratio (INR) level in hepatocellular carcinoma (HCC) patients.

METHODS: Our present study included 375 HCC patients who were treated at the China-Japan Friendship Hospital, Ministry of Health (Beijing, China), in the period from January 2003 to April 2012, and with a hospital discharge diagnosis of HCC. The demographic, clinical, laboratory, metabolic and instrumental features were analyzed. χ^2 test, Student's *t* test and Mann-Whitney *U* test were used to compare the differences between HCC patients with and without DM. Unconditional multivariable logistic regression analysis was used to determine the association of DM and INR level in HCC patients. A sub-group analysis was performed to assess the effect of liver cirrhosis or hepatitis B virus

(HBV) infection on the results. The Pearson correlation test was used to determine the relationship between INR level and fasting glucose. In addition, association between diabetes duration, and diabetes treatment and INR level was determined considering the potentially different effects.

RESULTS: Of the total, 63 (16.8%) patients were diabetic (diabetic group) and 312 (83.2%) patients were diagnosed without diabetes (non-diabetic group). Their mean age was 56.4 ± 11.0 years and 312 (83.2%) patients were male. Compared with patients without DM, the HCC patients with diabetes were older (59.5 ± 10.3 vs 55.8 ± 11.1 , $P = 0.015$), had a lower incidence of HBV infection (79.4% vs 89.1% , $P = 0.033$), had increased levels of systolic blood pressure (SBP) (133 ± 17 vs 129 ± 16 mmHg, $P = 0.048$) and INR (1.31 ± 0.44 vs 1.18 ± 0.21 , $P = 0.001$), had lower values of hemoglobin (124.4 ± 23.9 vs 134.2 ± 23.4 , $P = 0.003$) and had a platelet count (median/interquartile-range: $113/64-157$ vs $139/89-192$, $P = 0.020$). There was no statistically significant difference in the percentages of males, overweight or obesity, drinking, smoking, cirrhosis and Child classification. After controlling for the confounding effects of age, systolic blood pressure, hemoglobin, platelet count and HBV infection by logistic analyses, INR was shown as an independent variable [odds ratio (OR) = 3.650; 95%CI: 1.372-9.714, $P = 0.010$]. Considering the effect of liver cirrhosis on results, a sub-group analysis was performed and the study population was restricted to those patients with cirrhosis. Univariate analysis showed that diabetic patients had a higher INR than non-diabetic patients (1.43 ± 0.51 vs 1.25 ± 0.23 , $P = 0.041$). After controlling for confounding effect of age, SBP, hemoglobin, platelet count and HBV infection by logistic analyses, INR level remained as the sole independent variable (OR = 5.161; 95%CI: 1.618-16.455, $P = 0.006$). No significant difference in the relationship between INR level and fasting glucose was shown by Pearson test ($r = 0.070$, $P = 0.184$). Among the 63 diabetic patients, 35 (55.6%)

patients had been diagnosed with DM for more than 5 years, 23 (36.5%) received oral anti-diabetic regimens, 11 (17.5%) received insulin, and 30 (47.6%) reported relying on diet alone to control serum glucose levels. No significant differences were found for the association between DM duration/treatment and INR level, except for the age at diabetes diagnosis.

CONCLUSION: The INR level was increased in HCC patients with DM and these patients should be monitored for the coagulation function in clinical practice.

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Key words: International normalized ratio; Coagulation function; Diabetes mellitus; Hepatocellular carcinoma; Chinese patients

Core tip: This study showed that the international normalized ratio (INR) level was an independent variable associated with diabetes mellitus (DM) in hepatocellular carcinoma (HCC) patients compared with those HCC patients without DM, after controlling for the confounding effect of age, systolic blood pressure, hemoglobin, platelet and hepatitis B virus infection by logistic analyses. Considering the effect of liver cirrhosis on results, a sub-group analysis was performed and the study population was restricted to those HCC patients with cirrhosis. A similar result was obtained. These results indicated that INR level was increased in HCC patients with DM and this is independent of liver cirrhosis.

Zhang H, Gao C, Fang L, Yao SK. Increased international normalized ratio level in hepatocellular carcinoma patients with diabetes mellitus. *World J Gastroenterol* 2013; 19(15): 2395-2403 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v19/i15/2395.htm> DOI: <http://dx.doi.org/10.3748/wjg.v19.i15.2395>

INTRODUCTION

Hepatocellular carcinoma (HCC), with a mounting annual incidence of 4.9 per 100000 persons, is the third most common cause of cancer death worldwide^[1-3]. In China, there is a particularly high incidence of 40 per 100000 persons per year^[4,5]. Although many advances in treatment have been made, the prognosis of HCC is very poor and the total 5-year survival rate is as low as 10%, even in those developed countries like the United States^[6]. Risk factors of HCC that were identified include hepatitis B virus (HBV), hepatitis C virus (HCV), cirrhosis, heavy alcohol consumption, non-alcoholic steatohepatitis (NASH), aflatoxin exposure, increasing age, male sex, and positive family history^[7,8]; however, in 15%-50% of HCC patients no specific risk factor has been found^[4,5,9].

Recently, emerging evidence suggest that diabetes mellitus (DM) is a potential risk factor for HCC^[10-13], which has been strengthened by several meta-analyses^[13,14-17].

Generally, DM is associated with about two to three fold increased risk of HCC and diabetes may also increase the risk of death from HCC, which has been observed in a large cohort study conducted in Europe^[18]. In addition, DM can affect the prognosis of HCC after curative therapy and this prognostic impact is independent of the basic demographics, liver cirrhosis, and other comorbidities of HCC patients^[19-21].

Blood coagulation disorders are common findings in cancer patients^[22,23]. Cancer cells can activate blood coagulation through the expression of procoagulant molecules such as tissue factors and cancer procoagulant which consequently activate serine proteases factor VIIa, factor Xa and thrombin^[24,25]. However, HCC patients with impaired liver function have a more complex hemostatic disturbance, especially those with liver cirrhosis^[26,27]. Moreover, some preclinical and clinical studies have suggested that diabetes is associated with coagulation disorders, which are responsible for an increased thrombotic tendency and risk of cardiovascular disease^[28-30]. Therefore, the mechanisms for these coagulation alterations may be complex; however, no information was available for the association between DM and coagulation disorders in HCC patients, to our best knowledge.

Prothrombin time (PT), which is used to measure the coagulation factors of the “extrinsic pathway”, is the most frequently used coagulation test in routine laboratories. International normalized ratio (INR), which was introduced to overcome the problem of marked variation in PT results among laboratories, has been used to standardize PT value in liver diseases and been included in some prognostic models of HCC and liver cirrhosis, such as Child-Turcotte-Pugh (CTP) score and the model for end stage liver disease (MELD)^[31]. Considering that no information was available for the effect of DM on the INR level in HCC patients, our study was designed to determine the association of DM and INR in our Chinese HCC patients.

MATERIALS AND METHODS

Study population

A cohort of patients who were treated at our hospital in the period from January 2003 to April 2012, and with a hospital discharge diagnosis of HCC, were included in our present study. Chronic HBV infection was defined as serum HBsAg-positive for at least 6 mo or at diagnosis of HCC. Patients who followed these criteria would be excluded: (1) those who had been treated by any method at inclusion or with confirmed diagnosis of HCC for more than 15 d; (2) those who had other malignancies, including leukemia and lymphoma; (3) those who were non-Chinese; (4) those who had autoimmune hepatitis, schistosomiasis, primary biliary cirrhosis, Budd-Chiari syndrome, primary sclerosing cholangitis, hemochromatosis, Wilson's disease, rheumatic diseases or allergic disorder; and (5) those who had serious diseases of other organs or systems, such as severe heart failure, uremia, or acute exacerbations of chronic obstructive pulmonary disease. The study was approved by the Human Research

Ethics Committee of the China-Japan Friendship hospital and it was in accordance with the principles of the Declaration of Helsinki.

Subject determinations

HCC diagnosis was based on the histological findings of needle biopsy/surgery or typical radiological features shown by at least two image examinations including ultrasound (US), contrast-enhanced dynamic computed tomography (CT) and magnetic resonance imaging (MRI) and hepatic angiography or by a single positive imaging with a serum alpha fetoprotein level > 400 ng/mL^[32]. DM was characterized by fasting plasma glucose of 126 mg/dL or greater on at least two occasions, plasma glucose of 200 mg/dL or greater at 2-h oral glucose tolerance test, or the need for an oral hypoglycemic drug or insulin to control glucose^[33].

Clinical and laboratory parameters

The demographic, metabolic, biochemical, radiological, and pathological features of the patients with HCC were recorded. The data were obtained at the diagnosis of HCC, but excluded those obtained 15 d before or after the diagnosis. Patients who had missing values which may affect statistical results were excluded. Body mass index (BMI) was computed as body weight (in kilograms) divided by the square of the height (in meters). Overweight was defined as $BMI \geq 23$ kg/m² and obesity $BMI \geq 25$ kg/m², according to the Asian and Chinese criteria^[4,5]. The diagnosis of hypertension was defined as systolic blood pressure (SBP) ≥ 140 mmHg and/or diastolic blood pressure (DBP) ≥ 90 mmHg, and mean arterial pressure (MAP) was computed as $1/3$ SBP plus $2/3$ DBP. Blood pressure was measured in a quiet room at a comfortable temperature. Physical activity, smoking, coffee drinking, or having eaten within the past 30 min were to be avoided. Five associated parameters, including total bilirubin, albumin, INR, ascites and hepatic encephalopathy, were used to calculate the CTP score.

Venous blood samples were taken in the morning after a 12 h overnight fast. Standard methods were used to measure the laboratory parameters, including INR. Chronic HCV infection was defined as anti-HCV seropositivity and/or having detectable HCV RNA. Based on the recorded results, the findings of physical examinations and imaging techniques including US, CT, MRI and hepatic angiography were re-assessed carefully by at least two authors independently for tumor-node-metastasis (TNM) stage and clinical classification. We classified tumor stages according to the 7th TNM staging system recommended by International Union Against Cancer. In clinical classification, massive HCC was defined as a diameter of ≥ 5 cm, nodular HCC as a diameter of < 5 cm, and small HCC as a diameter of < 3 cm for single or two nodules.

Statistical analysis

The statistical analysis was performed using SPSS for Windows, version 17.0 (SPSS, Chicago, IL, United

States). For the categorical variables, the numbers and proportions were described, and Pearson χ^2 test, continuity correction χ^2 tests or Fisher's exact test were used. For the continuous variables, mean \pm SD deviation was described and Independent-Samples *t* test was used. If the continuous variable had a skewed distribution, it would be described using the median value and inter-quartile range and analyzed by Mann-Whitney non-parametric *U* test. Unconditional multivariable logistic regression analysis was used to determine the association of DM and INR level in HCC patients. According to the results of univariate analysis, six variables were included, including age, SBP, HBV infection, hemoglobin, platelet count and INR level. To assess the effect of liver cirrhosis on our results, a sub-group analysis was performed and the study population was restricted to those HCC patients with and without cirrhosis. The Pearson correlation test was used to determine the relationship between INR level and fasting glucose. Stepwise multiple regression analysis (Backward: Wald; Entry: 0.05, Removal: 0.10) was used. We expressed results as odds ratio (OR) and their 95%CI. For all tests, $P < 0.05$ was considered statistically significant and all *P* values quoted are two-sided.

RESULTS

Baseline characteristics of the study population

Our present study included 375 HCC patients based on the diagnostic, inclusion and exclusion criteria. Of the total, 63 (16.8%) patients were diabetic (diabetic group) and 312 (83.2%) patients were diagnosed without diabetes (non-diabetic group). Their baseline characteristics were shown in Table 1. Their mean age was 56.4 ± 11.0 years and 312 (83.2%) patients were male. Of these patients, 328 (87.5%) had HBV infection and 22 (5.9%) patients had HCV infection. One hundred and ninety-nine (53.1%) patients were diagnosed with liver cirrhosis, 88 (23.5%) patients had a past history of hypertension, 159 (159/281, 56.6%) patients were overweight or obese, 104 (27.7%) patients were alcohol drinkers, and 154 (41.4%) patients were smokers. TNM stage and clinical classification of our study population were shown in Table 2. For the clinical classification, massive-type HCC (213, 56.8%) and nodular-type HCC (117, 31.2%) were the two major types, which accounted for nearly 90% of the total patients. For M stage, 269 (71.7%) were diagnosed with M0.

Univariable analysis: Comparison of HCC patients with and without DM

Of the 63 diabetic patients, the mean age was 59.5 ± 10.3 years, 51 (81.0%) were male, the mean BMI was 23.69 ± 3.51 kg/m², and 17 (27.0%) patients had a past history of hypertension. Twenty (31.7%) patients were smokers, and 14 (22.2%) were drinkers (Table 1). The duration and treatment of diabetes is shown in a later section.

χ^2 test, Student's *t* test and Mann-Whitney *U* test were used to compare the differences between HCC patients with and without diabetes. As shown in Table 1, compared

Table 1 Baseline characteristics of the study population and univariate analysis of comparison of hepatocellular carcinoma patients with and without diabetes *n* (%)

| Variable | Total patients (<i>n</i> = 375) | HCC patients with diabetes (<i>n</i> = 63) | HCC patients without diabetes (<i>n</i> = 312) | <i>P</i> value |
|--|-------------------------------------|--|--|----------------|
| Male sex | 312 (83.2) | 51 (81.0) | 261 (83.7) | 0.601 |
| Mean age, yr (mean ± SD) | 56.4 ± 11.0 | 59.5 ± 10.3 | 55.8 ± 11.1 | 0.015 |
| Body weight ¹ , kg (mean ± SD) | 68.1 ± 10.9 | 69.3 ± 13.0 | 67.8 ± 10.5 | 0.346 |
| Body height ² , cm (mean ± SD) | 168.8 ± 7.0 | 168.6 ± 8.0 | 168.9 ± 6.8 | 0.810 |
| BMI ³ , kg/m ² (mean ± SD) | 23.77 ± 3.39 | 23.69 ± 3.51 | 23.68 ± 3.71 | 0.991 |
| Overweight or obesity ³ | 159 (56.6) | 27 (57.4) | 132 (56.4) | 0.896 |
| History of hypertension | 88 (23.5) | 17 (27.0) | 71 (22.8) | 0.470 |
| SBP, mmHg (mean ± SD) | 130 ± 17 | 133 ± 17 | 129 ± 16 | 0.048 |
| DBP, mmHg (mean ± SD) | 79 ± 10 | 78 ± 10 | 79 ± 10 | 0.455 |
| Smoking | 154 (41.4) | 20 (31.7) | 134 (42.9) | 0.099 |
| Alcohol intake | 104 (27.7) | 14 (22.2) | 90 (28.8) | 0.284 |
| HBV infection | 328 (87.5) | 50 (79.4) | 278 (89.1) | 0.033 |
| HCV infection | 22 (5.9) | 3 (4.8) | 19 (6.1) | 0.908 |
| Liver cirrhosis | 199 (53.1) | 38 (60.3) | 161 (51.6) | 0.206 |
| Fatty liver | 11 (2.9) | 2 (3.2) | 9 (2.9) | 1.000 |
| Child-Turcotte-Pugh classification ⁴ | | | | |
| Child A | 243 (65.5) | 35 (56.5) | 208 (67.3) | 0.101 |
| Child B | 92 (24.8) | 17 (27.4) | 75 (24.3) | 0.600 |
| Child C | 36 (9.7) | 10 (16.1) | 26 (8.4) | 0.061 |
| AFP > 400 ng/mL ⁵ | 167 (45.8) | 27 (43.5) | 140 (46.2) | 0.702 |
| Neutrophil, × 10 ⁹ /L (mean ± SD) | 4.13 ± 2.49 | 3.88 ± 2.52 | 4.18 ± 2.49 | 0.389 |
| Hemoglobin, g/L (mean ± SD) | 132.5 ± 23.8 | 124.4 ± 23.9 | 134.2 ± 23.4 | 0.003 |
| Platelet count, × 10 ⁹ /L | 130 (85-189) | 113 (64-157) | 139 (89-192) | 0.020 |
| ALT, U/L | 45 (29-81) | 44 (27-91) | 45 (30-79) | 0.943 |
| AST, U/L | 62 (38-117) | 52 (34-97) | 66 (39-119) | 0.124 |
| ALP, U/L | 111 (82-176) | 111 (84-171) | 111 (81-177) | 0.907 |
| GGT, U/L | 106 (55-233) | 96 (51-190) | 109 (57-239) | 0.406 |
| INR ⁶ (mean ± SD) | 1.20 ± 0.27 | 1.31 ± 0.44 | 1.18 ± 0.21 | 0.001 |
| Total bilirubin, mg/L | 16 (10-27) | 17 (10-31) | 16 (10-25) | 0.561 |
| Albumin, g/L (mean ± SD) | 37.4 ± 6.0 | 36.4 ± 5.9 | 37.6 ± 6.0 | 0.148 |
| Total cholesterol, mmol/L (mean ± SD) | 4.26 ± 1.34 | 4.18 ± 1.12 | 4.28 ± 1.39 | 0.662 |
| BUN, mmol/L | 5.14 (4.00-6.36) | 5.12 (3.92-6.46) | 5.18 (4.04-6.34) | 0.738 |
| Creatinine, μmol/L | 80 (71-88) | 80 (70-97) | 80 (71-88) | 0.860 |

Data were available in ¹366 (60 + 306), ²286 (49 + 237), ³281 (47 + 234), ⁴371 (62 + 309), ⁵365 (62 + 303) and ⁶367 (62 + 305) patients. The numbers before the brackets indicate the total available cases in the two groups. BMI: Body mass index; BUN: Blood urea nitrogen; DBP: Diastolic blood pressure; HBV: Hepatitis B virus; HCC: Hepatocellular carcinoma; HCV: Hepatitis C virus; INR: International normalized ratio; SBP: Systolic blood pressure; ALT: Aminolucine transferase; AST: Aspartate aminotransferase; ALP: Alkaline phosphatase; GGT: Galactosylhydroxylslyl glucosyltransferase.

with patients without DM, the HCC patients with diabetes had: an older age (59.5 ± 10.3 *vs* 55.8 ± 11.1, *P* = 0.015); a lower incidence of HBV infection (79.4% *vs* 89.1%, *P* = 0.033); increased levels of SBP (133 ± 17 *vs* 129 ± 16, *P* = 0.048) and INR (1.31 ± 0.44 *vs* 1.18 ± 0.21, *P* = 0.001); lower values of hemoglobin (124.4 ± 23.9 *vs* 134.2 ± 23.4, *P* = 0.003); and platelet count (median/interquartile-range: 113/64-157 *vs* 139/89-192, *P* = 0.020). There was no statistically significant difference in the percentages of males, overweight or obesity, drinking, smoking, cirrhosis and Child classification. Results of univariable analysis for the TNM stage and clinical classification were shown in Table 2. No significant differences were demonstrated for T stage, N stage, M stage and the clinical classification.

Multivariable analysis: Increased INR levels in HCC patients with DM

Unconditional multivariable logistic regression analysis was used to determine the association of DM and INR level in HCC patients. According to the results of univar-

iate analysis, six variables were included (Table 3), including age, SBP, HBV infection, hemoglobin, platelet count and INR level. Statistical differences were shown for three variables (Table 3): INR level (OR = 3.650; 95%CI: 1.372-9.714; *P* = 0.010), SBP (OR = 1.019; 95%CI: 1.002-1.036; *P* = 0.029), and hemoglobin value (OR = 0.987; 95%CI: 0.975-0.999; *P* = 0.038).

Considering that some factors may play possible roles in HCC based on the published literature and our current knowledge, for example male gender, alcohol drinking, HCV infection, liver cirrhosis, and CTP classification. To control the effect of these variables, they were included in the multivariable analysis, although no statistical significance were shown by univariate analysis (Table 1). For this purpose, more potentially confounding factors were controlled and logistic regression was repeated, including age, sex, HBV infection, HCV infection, alcohol drinking, liver cirrhosis, CTP classification and INR (Table 3). Results showed that INR level remained statistically significant (OR = 4.487; 95%CI: 1.713-11.754; *P* = 0.002).

Table 2 Tumor-node-metastasis stage and clinical type of the study population *n* (%)

| Variable | Total patients (<i>n</i> = 375) | HCC patients with diabetes (<i>n</i> = 63) | HCC patients without diabetes (<i>n</i> = 312) | <i>P</i> value |
|-------------------------|-------------------------------------|--|--|----------------|
| T stage | | | | |
| T1 | 82 (21.9) | 15 (23.8) | 67 (21.5) | 0.683 |
| T2 | 90 (24.0) | 19 (30.2) | 71 (22.8) | 0.210 |
| T3a | 157 (41.9) | 22 (34.9) | 135 (43.3) | 0.221 |
| T3b | 37 (9.9) | 6 (9.5) | 31 (9.9) | 0.920 |
| T4 | 9 (2.4) | 1 (1.6) | 8 (2.6) | 0.991 |
| N stage | | | | |
| N0 | 334 (89.1) | 57 (90.5) | 277 (88.8) | 0.694 |
| N1 | 41 (10.9) | 6 (9.5) | 35 (11.2) | - |
| M stage | | | | |
| M0 | 269 (71.7) | 49 (77.8) | 220 (70.5) | 0.243 |
| M1 | 106 (28.3) | 14 (22.2) | 92 (29.5) | - |
| Clinical classification | | | | |
| Massive | 213 (56.8) | 29 (46.0) | 184 (59.0) | 0.059 |
| Nodular | 117 (31.2) | 22 (34.9) | 95 (30.4) | 0.485 |
| Small-cancer | 28 (7.5) | 8 (12.7) | 20 (6.4) | 0.142 |
| Diffuse | 17 (4.5) | 4 (6.3) | 13 (4.2) | 0.503 |

HCC: Hepatocellular carcinoma; T: Tumor; N: Node; M: Metastasis.

Table 3 Multivariable analysis: Increased international normalized ratio level in hepatocellular carcinoma patients with diabetes mellitus

| Variable | AOR | 95%CI | <i>P</i> value |
|------------|-------|--------------|----------------|
| Model 1 | | | |
| INR | 3.650 | 1.372-9.714 | 0.010 |
| SBP | 1.019 | 1.002-1.036 | 0.029 |
| Hemoglobin | 0.987 | 0.975-0.999 | 0.038 |
| Model 2 | | | |
| INR | 4.487 | 1.713-11.754 | 0.002 |
| Age | 1.032 | 1.005-1.059 | 0.020 |

Model 1: Based on the results of univariate analysis, unconditional multivariable logistic regression analysis was performed. Six variables were included, including age, systolic blood pressure (SBP), hepatitis B virus (HBV) infection, hemoglobin, platelet count and international normalized ratio (INR). Statistical differences were shown for 3 variables (shown in Table), and other four variables were omitted because no significant differences were found; Model 2: According to the published literatures and our current knowledge, more potential confounding factors were controlled, including age, sex, HBV infection, hepatitis C virus infection, alcohol drinking, liver cirrhosis, Child-Turcotte-Pugh classification and INR. AOR: Adjusted odds ratio.

In addition, one significant difference was found for age (OR = 1.032; 95%CI: 1.005-1.059; *P* = 0.020).

Sub-group analysis

As demonstrated in Table 1, among the six variables which were shown as statistically significant in univariate analysis, 5 were associated with liver cirrhosis, including age, HBV infection, hemoglobin, platelet count and INR level. To assess the effect of liver cirrhosis on our results, sub-group analysis was performed although liver cirrhosis had been controlled in the multivariable logistic analysis. We restricted our study population to those HCC patients with and without cirrhosis.

In the 199 HCC patients with liver cirrhosis, data were not available in 7 patients for INR level and 192 were included in the sub-group analysis, including 37 pa-

Table 4 Results of multivariable analysis for sub-group analysis

| Variable ¹ | AOR ² | 95%CI | <i>P</i> value |
|--------------------------------------|------------------|--------------|----------------|
| HCC patients with liver cirrhosis | | | |
| INR | 5.161 | 1.618-16.455 | 0.006 |
| HCC patients without liver cirrhosis | | | |
| INR | 2.082 | 0.130-33.333 | 0.604 |
| Age | 1.060 | 1.015-1.107 | 0.008 |
| HCC patients with HBV infection | | | |
| INR | 2.508 | 0.860-7.315 | 0.092 |
| Hemoglobin | 0.984 | 0.970-0.998 | 0.024 |

¹International normalized ratio (INR) and variables with significant difference were shown; ²Adjusted odds ratio (AOR) for the confounding effect of age, systolic blood pressure, hemoglobin, platelet count, and hepatitis B virus (HBV) infection using unconditional multivariable logistic regression analyses, based on the results of univariate analysis shown in Table 1. HCC: Hepatocellular carcinoma.

tients in the diabetic group and 155 in the non-diabetic group. Univariate analysis showed that diabetic patients had a higher level of INR than non-diabetic patients (1.43 ± 0.51 vs 1.25 ± 0.23 , *P* = 0.041). After controlling for the confounding effects of age, SBP, hemoglobin, platelet count and HBV infection by logistic analyses (Table 4), INR level remained as the sole independent variable (OR = 5.161; 95%CI: 1.618-16.455, *P* = 0.006).

When the study population was restricted to those patients without liver cirrhosis, no significant difference was found for INR value (1.12 ± 0.19 vs 1.10 ± 0.16 , *P* = 0.721). A similar result was gained in logistic analyses (OR = 2.082; 95%CI: 0.130-33.333; *P* = 0.604). In addition, considering that more than 80% of HCC has been attributed to HBV infection in China (this number was 87.5% in our present study), we performed a sub-group analysis in those HCC patients with HBV infection. Unfortunately, no statistically significant differences were found in univariate analysis (1.28 ± 0.43 vs 1.18 ± 0.21 , *P* = 0.091) and multivariable analysis (OR = 2.508; 95%CI: 0.860-7.315, *P* = 0.092).

Table 5 Association between diabetes duration/treatment and International normalized ratio level *n* (%)

| Variable | INR < 1.20 (<i>n</i> = 36) | INR ≥ 1.20 (<i>n</i> = 27) | <i>P</i> value | INR < 1.50 (<i>n</i> = 52) | INR ≥ 1.50 (<i>n</i> = 11) | <i>P</i> value |
|-------------------------------|--------------------------------|--------------------------------|----------------|--------------------------------|--------------------------------|----------------|
| Duration of diabetes, yr | | | | | | |
| < 5 | 15 (41.70) | 13 (48.10) | 0.608 | 23 (44.20) | 5 (45.50) | 1.000 |
| ≥ 5 | 21 (58.30) | 14 (51.90) | - | 29 (55.80) | 6 (54.50) | - |
| Age at diabetes diagnosis, yr | | | | | | |
| < 50 | 11 (30.60) | 12 (44.40) | 0.257 | 15 (28.80) | 8 (72.70) | 0.016 |
| ≥ 50 | 25 (69.40) | 15 (55.60) | - | 37 (71.20) | 3 (27.30) | - |
| Diabetes treatment | | | | | | |
| Oral treatment | | | | | | |
| Non-users | 21 (58.30) | 19 (70.40) | 0.326 | 31 (59.60) | 9 (81.80) | 0.296 |
| Users | 15 (41.70) | 8 (29.60) | - | 21 (40.40) | 2 (18.20) | - |
| Insulin treatment | | | | | | |
| Non-users | 27 (75.00) | 25 (92.60) | 0.138 | 43 (82.70) | 9 (81.80) | 1.000 |
| Users | 9 (25.00) | 2 (7.40) | - | 9 (17.30) | 2 (18.20) | - |
| Diet only | | | | | | |
| Non-users | 16 (44.40) | 17 (63.00) | 0.145 | 26 (50.00) | 7 (63.60) | 0.411 |
| Users | 20 (55.60) | 10 (37.00) | - | 26 (50.00) | 4 (36.40) | - |
| Type of oral treatment | | | | | | |
| Biguanide | | | | | | |
| Non-users | 32 (88.90) | 22 (81.50) | 0.640 | 43 (82.70) | 11 (100) | 0.310 |
| Users | 4 (11.10) | 5 (18.50) | - | 9 (17.30) | 0 (0) | - |
| Sulfonylureas | | | | | | |
| Non-users | 29 (80.60) | 23 (85.20) | 0.886 | 42 (80.80) | 10 (90.9) | 0.713 |
| Users | 7 (19.40) | 4 (14.80) | - | 10 (19.20) | 1 (9.1) | - |
| α-glucosidase inhibitor | | | | | | |
| Non-users | 29 (80.60) | 25 (92.60) | 0.323 | 44 (84.60) | 10 (90.9) | 0.946 |
| Users | 7 (19.40) | 2 (7.40) | - | 8 (15.40) | 1 (9.1) | - |

INR: International normalized ratio.

Association of INR and fasting glucose level

A Pearson correlation test was used to determine the relationship between INR level and fasting glucose. In the entire study population of 367 patients (data were not available in 8 patients for INR), the mean value of fasting glucose was 8.83 ± 3.12 mmol/L for diabetic patients whereas the value was 5.21 ± 1.07 mmol/L for non-diabetic patients ($P < 0.001$). However, no significant difference was shown by Pearson test ($r = 0.070$, $P = 0.184$). Even after the analysis was restricted to diabetics only, the same result was obtained.

Association between diabetes duration/treatment and INR level

Considering the potentially different effects of diabetes duration and anti-diabetic agents, we studied the association between DM duration/treatment and INR level. Among the 63 diabetic patients (Table 5), 35 (55.6%) patients had been diagnosed with DM for more than 5 years, 23 (36.5%) received oral anti-diabetic regimens, 11 (17.5%) received insulin, and 30 (47.6%) reported relying on diet alone to control serum glucose level. The cutoff values of 1.20 and 1.50 were determined based on the mean value and range of the normal value. χ^2 test, continuity correction χ^2 tests or Fisher's exact test were used to determine the association. As shown in Table 5, no statistically significant differences were found for the association between DM duration/treatment and INR level, except for the age at diabetes diagnosis.

DISCUSSION

Our study showed that the INR level was an independent variable associated with DM in HCC patients (OR = 3.650; 95%CI: 1.372-9.714; $P = 0.010$), compared with those HCC patients without DM, after controlling for the confounding effect of age, SBP, hemoglobin, platelet and HBV infection by logistic analyses. Considering the effect of liver cirrhosis on results, a sub-group analysis was performed and the study population was restricted to those HCC patients with cirrhosis. A similar result was obtained (OR = 5.161; 95%CI: 1.618-16.455, $P = 0.006$). These results indicated that INR level was increased in HCC patients with DM and this is independent of liver cirrhosis.

No information was available for the association between DM and coagulation disorders in HCC patients, to the best of our knowledge. For the first time, our study was designed to determine the association of DM and INR in HCC patients. INR, developed by the World Health Organization to standardize PT reporting in the early 1980s, is used worldwide to monitor oral anticoagulation therapy^[22,23]. The INR level is used to measure the extrinsic pathway of the coagulation cascade and influenced by coagulation factors I (fibrinogen), II (prothrombin), V, VII, and X. This index has been also recommended to evaluate the survival of patients with severe liver disease, and been included in some prognostic models, such as CTP score and MELD score.

Previous studies have reported that DM has a role in the activation of coagulation factors and subsequently increases thrombotic tendency and cardiovascular risk^[28-30]. However, no information was available in HCC patients. Our study included 375 HCC patients based on the diagnostic, inclusion and exclusion criteria, and the unconditional multivariable logistic regression analysis used to determine the association. Our results indicated that coagulation disorders could also be found in HCC with DM, but the effect may be different from previously published literature.

Patients with DM have higher levels of circulating tissue factor (TF), which initiate the extrinsic pathway of the coagulation cascade by binding and activating factor VII^[28,29]. The levels of TF are directly modulated by glucose and insulin, as well as by nuclear factor kappa B which is activated by the formation of advanced glycation end products and reactive oxygen species^[34,35]. Levels of factor VII are influenced by triglyceride levels, which often increase in poorly controlled diabetic patients. Plasma levels of other coagulation factors, such as fibrinogen and prothrombin, are also elevated in diabetic patients. In addition to the changes in levels of coagulation factors, DM induces quantitative modifications of those factors, which also increases thrombosis risk.

However, our study showed that the INR level was increased in HCC patients with DM. The exact mechanism remains as yet unclearly understood and it was deduced as follows. The first was due to the liver itself. Diabetes plays its role in increasing the levels of coagulation factors in the circulation mainly through increased synthesis of the liver. When liver function is impaired, the synthesis of coagulation factors would also be impaired. In addition, our study found that a similar result was obtained when the population was restricted to those HCC patients with liver cirrhosis, whereas no significant difference was observed for those patients without cirrhosis. Increased INR levels in HCC patients with DM may be partly associated with liver cirrhosis.

Secondly, activation of inflammatory and coagulation pathways is important in the pathogenesis of coronary artery disease that worsens the prognosis of HCC patients^[36]. There is ample evidence that extensive cross-talk between these two systems exists, whereby inflammation not only leads to activation of coagulation, but coagulation also markedly affects inflammatory activity. The main interfaces linking coagulation and inflammation are beyond the tissue factor pathway and thrombin, the protein C system and the fibrinolytic (or plasminogen-plasmin) system. Proinflammatory cytokines (mainly IL-6) and chemokines can affect all these coagulation mechanisms, and vice versa, activated coagulation proteases and physiological anticoagulants or components of the plasminogen-plasmin system can modulate inflammation by specific cell receptors. The intricate relationship between inflammation and coagulation is extremely clear in nonalcoholic fatty liver disease (NAFLD)^[36].

Thirdly, angiogenesis is required for tumor growth

and metastasis. Activation of the coagulation pathway also enhances tumor growth and metastasis^[37]. Procoagulants involved in angiogenesis include TF and thrombin. Vascular endothelial growth factor, the most potent pro-angiogenic factor, is an indirect procoagulant; it is capable of inducing vascular hyperpermeability and of increasing TF expression on endothelial cells. Vascular hyperpermeability results in leakage of plasma proteins, including prothrombin and fibrinogen, into the extracellular matrix. Prothrombin converted into thrombin by the activated coagulation pathway may result in platelet activation and the production of fibrin from fibrinogen^[37].

In our study, we found that, compared with patients without DM, HCC patients with diabetes were older. The reason remains as yet unclearly understood. It was deduced to be possibly related to the duration, treatment and monitoring of diabetes. Some limitations should also be acknowledged. The first limitation is the case-control design, which could not provide definite evidence to clarify the causal association. To clarify the causal relationship, well-designed prospective studies are required. The second is that most of the HCC and cirrhotic patients were diagnosed clinically rather than by biopsy, and the diagnosis of most diabetics was dependent on their self-reported history or fasting serum glucose, not on an oral glucose tolerance test. Thus the role of DM could be underestimated. However, we followed strictly the diagnostic criteria recommended by the authorized institutes and used them widely in clinical practice. We believe that our results are more likely applicable in clinical practice.

The third limitation was due to the nature of our case-control study, which meant that some data could not be obtained and some possible factors could not be adjusted. For example, NAFLD and NASH have been regarded as risk factors for HCC, but we could not assess these changes. However, biopsy was unnecessary for these confirmed HCC patients and was not recommended. In addition, activated partial thromboplastin time is another commonly used screening test for evaluating coagulation disorders. Unfortunately, data were not available for some patients and the analysis could not be performed. However, in clinical practice, this parameter is not frequently used in liver disease, compared with PT and the INR.

Our present study also raises several questions for future research. Firstly, how DM affects the coagulation system in HCC patients; secondly, whether increased INR in diabetic patients has a clinical meaning or can affect the prognosis of HCC patients; and last but not least, whether antidiabetic treatment can improve coagulation function in HCC. To answer these questions, further pre-clinical and clinical studies are needed.

In conclusion, our study showed that INR level was increased in HCC patients with DM and these patients should be monitored for coagulation function in clinical practice. More studies are required for a better understanding of this change.

COMMENTS

Background

Diabetes mellitus (DM) is a potential risk factor for hepatocellular carcinoma (HCC). HCC patients with impaired liver function have complex coagulation disorders, especially in those with liver cirrhosis. However, no information was available for the association between DM and coagulation disorders in HCC patients.

Research frontiers

International normalized ratio (INR) has been included in some prognostic models of HCC and liver cirrhosis, such as Child-Turcotte-Pugh score and the model for end stage liver disease. This study was designed to determine the association of DM and INR in HCC patients, considering that no information was available for the effect of DM on INR in HCC.

Innovations and breakthroughs

The authors found that the INR level was increased in HCC patients with DM which is independent of liver cirrhosis. This is the first time that information was available for the effect of DM on the INR level in HCC.

Applications

HCC patients with DM should be monitored for coagulation function in clinical practice.

Peer review

The effect of DM in INR levels in HCC is quite interesting with clinical therapeutic implications. Nice paper to be published after minor revision.

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Twist2 is a valuable prognostic biomarker for colorectal cancer

Hao Yu, Guang-Zhi Jin, Kai Liu, Hui Dong, Hua Yu, Ji-Cheng Duan, Zhe Li, Wei Dong, Wen-Ming Cong, Jia-He Yang

Hao Yu, Kai Liu, Ji-Cheng Duan, Zhe Li, Jia-He Yang, Department of Laparoscopy, Eastern Hepatobiliary Surgery Hospital, the Second Military Medical University, Shanghai 200438, China

Guang-Zhi Jin, Hui Dong, Hua Yu, Wei Dong, Wen-Ming Cong, Department of Pathology, Eastern Hepatobiliary Surgery Hospital, the Second Military Medical University, Shanghai 200438, China

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Correspondence to: Jia-He Yang, MD, PhD, Department of Laparoscopy, Eastern Hepatobiliary Surgery Hospital, the Second Military Medical University, No. 225, Changhai Road, Shanghai 200438, China. ehbhjhyang@163.com

Telephone: +86-21-81875262 Fax: +86-21-81875262

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Abstract

AIM: To investigate the significance of Twist2 for colorectal cancer (CRC).

METHODS: In this study, 93 CRC patients were included who received curative surgery in Eastern Hepatobiliary Surgery Hospital from January 1999 to December 2010. Records of patients' clinicopathological characteristics and follow up data were reviewed. Formalin-fixed, paraffin-embedded tissue blocks were used to observe the protein expression of Twist2 and E-cadherin by immunohistochemistry. Two independent pathologists who were blinded to the clinical information performed semi-quantitative scoring of immunostaining. A total score of 3-6 (sum of extent + intensity) was considered as Twist2-positive expression. The expression of E-cadherin

was divided into two levels (preserved and reduced). An exploratory statistical analysis was conducted to determine the association between Twist2 expression and clinicopathological parameters, as well as E-cadherin expression. Furthermore, the variables associated with prognosis were analyzed by Cox's proportional hazards model. Kaplan-Meier analysis was used to plot survival curves according to different expression levels of Twist2.

RESULTS: Twist2-positive expression was observed in 66 (71.0%) samples and mainly located in the cytoplasm. Forty-three (46.2%) samples showed reduced expression of E-cadherin. There were no significant correlations between Twist2 expression and any of the clinicopathological parameters. However, Twist2-positive expression was significantly associated with reduced expression of E-cadherin ($P = 0.040$). Multivariate analysis revealed that bad M-stage [hazard ratio (HR) = 7.694, 95%CI: 2.927-20.224, $P < 0.001$] and Twist2-positive (HR = 5.744, 95%CI: 1.347-24.298, $P = 0.018$) were the independent risk factors for poor overall survival (OS), while Twist2-positive (HR = 3.264, 95%CI: 1.455-7.375, $P = 0.004$), bad N-stage (HR = 2.149, 95%CI: 1.226-3.767, $P = 0.008$) and bad M-stage (HR = 10.907, 95%CI: 4.937-24.096, $P < 0.001$) were independently associated with poor disease-free survival (DFS). Survival curves showed a definite trend for Twist2-negative patients to have longer OS and DFS than Twist2-positive patients, not only overall, but also for patients in different stages, especially for DFS of patients in stage III ($P = 0.033$) and IV ($P = 0.026$).

CONCLUSION: Our data suggests, for the first time, that Twist2 is a valuable prognostic biomarker for CRC, particularly for patients in stage III and IV.

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Key words: Colorectal cancer; Prognostic biomarker; Twist2; Epithelial-mesenchymal transition; Immunohistochemistry

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INTRODUCTION

Colorectal cancer (CRC) is one of the most common malignant tumors and continues to be one of the leading causes of cancer-related death worldwide^[1]. Traditionally, the prognosis of patients with CRC is mainly evaluated by the tumor (T) node (N) metastasis (M) stage^[2]. However, patients in the same stage frequently had different outcomes, despite similar postoperative treatments. There must be some unknown mechanisms affecting patients' outcome beyond the clinical stage. Although many efforts have been made to find biomarkers to predict CRC, truly effective clinical biomarkers are rare. Therefore, new and more effective biomarkers are still needed.

Recently, Twist2 (Dermo1), a highly homologous protein of Twist1^[3,4], has attracted our attention. Koh *et al*^[5] reported that Twist2 could increase resistance to galectin-1-mediated-apoptosis, which facilitated the progression of some T-cells into tumors. Gasparotto *et al*^[6] found over-expression of Twist2 correlated with the poor prognosis of head and neck squamous cell carcinomas. Zhou *et al*^[7] suggested that Twist2 is associated with the invasion and metastasis of salivary adenoid cystic carcinoma. Li *et al*^[8] found that Twist2 is involved in the cervical malignant conversion and tumor metastasis. Twist2 is also considered an inducer of epithelial-mesenchymal transition (EMT)^[9-11], a well-known progression involved in embryogenesis^[12,13], tumor invasion and metastasis^[14-18], and drug resistance^[19]. Evidently Twist2 is a significant biomarker for human tumors. However, until now, the relationship between Twist2 and CRC has remained unknown.

Therefore, we undertook the present investigation to determine the significance of Twist2 for CRC and to verify its function as an EMT inducer.

MATERIALS AND METHODS

Patients and tumor samples

Ninety-three CRC patients were included who underwent curative surgery in Eastern Hepatobiliary Surgery Hospital, the Second Military Medical University of China, from January 1999 to December 2010. The patients met the following criteria: no anti-cancer treatments were given before surgery; all the visible tumor nodules were resected (including the distant metastatic nodules); patients who died during surgery or from serious surgical complications were excluded; the resected nodules were identified as primary CRC or metastasis of CRC and the surgical margin was free of tumor cells by pathological examination; patients with lymphatic metastasis or/and

distant metastasis had received postoperative adjuvant chemotherapy, patients who died from non-CRC diseases or accidents were excluded; and the clinicopathological and follow-up data were available. All the formalin-fixed and paraffin-embedded primary CRC samples were obtained from the Department of Pathology of Eastern Hepatobiliary Surgery Hospital. All patients in this study gave written informed consent.

Follow-up and postoperative treatment

Patients were followed up until death or until June 15, 2011. All patients were monitored by physical examination, routine blood tests [including serum carcinoembryonic antigen (CEA) concentration], chest X-ray and abdominal ultrasonography every 2 mo in the first year after surgery, and every 3-6 mo thereafter. A computed tomography scan (CT) or magnetic resonance imaging was performed every 6 mo or immediately when a recurrence/metastasis was suspected. If needed, a whole-body fludeoxyglucose positron emission tomography/CT was performed. The follow-up data were recorded during the postoperative examination in our hospital, while patients who were examined in another hospital were followed up by telephone or letter. Recurrence was determined by at least two imaging examination results. Once recurrent tumors were confirmed, further treatment was implemented, such as a second surgery and palliative chemotherapy. Disease-free survival (DFS) was defined as the period from the tumor resection until the tumor recurrence or the last observation. The overall survival (OS) was the interval between the surgery and death or the last follow-up examination.

Immunohistochemistry

Immunohistochemistry was carried out as described previously^[20]. Representative 4- μ m serial sections were prepared from 10% formalin-fixed, paraffin-embedded tissue blocks. To increase the immunoreactivity, microwave antigen retrieval was performed in citrate buffer (pH 6.0) for 5 min, then cooled the sections at room temperature for at least 30 min. Subsequently 3% hydrogen peroxide was used for 10 min to block endogenous peroxidase activity. After nonspecific binding sites were blocked for 30 min with goat serum, a monoclonal antibody against Twist2 (1:300, H00117581-M01, Abnova) and polyclonal antibodies against E-cadherin (1:100, BS1098, Bioworld Technology) were used to incubate the sections in a humid chamber at 4 °C overnight. Next, an EnVision Detection kit (GK500705, Gene Tech, China) was used to visualize tissue antigens. Sections were counterstained with hematoxylin for 5 min. Negative control sections were incubated with phosphate buffered solution instead of the primary antibody.

Evaluation of immunohistochemistry

Two independent pathologists (Dong H and Cong WM), who were blinded to clinical information, assessed the expression of Twist2 and E-cadherin semiquantitatively.

Table 1 Relationship between Twist2 expression and clinicopathological characteristics *n* (%)

| Characteristics | Total | Twist2 expression | | <i>P</i> value |
|-----------------------|-----------|-------------------|-----------|--------------------|
| | | Negative | Positive | |
| Gender | | | | |
| Female | 52 (55.9) | 18 (66.7) | 34 (51.5) | 0.182 ¹ |
| Male | 41 (44.1) | 9 (33.3) | 32 (48.5) | |
| Age | | | | |
| < 59 | 41 (44.1) | 13 (48.1) | 28 (42.4) | 0.614 ¹ |
| ≥ 59 | 52 (55.9) | 14 (51.9) | 38 (57.6) | |
| T-stage | | | | |
| T1-2 | 17 (18.3) | 5 (18.5) | 12 (18.2) | 1.000 ² |
| T3-4 | 76 (81.7) | 22 (81.5) | 54 (81.8) | |
| N-stage | | | | |
| N0 | 52 (55.9) | 17 (63.0) | 35 (53.0) | 0.381 ¹ |
| N1-2 | 41 (44.1) | 10 (37.0) | 31 (47.0) | |
| M-stage | | | | |
| M0 | 52 (55.9) | 17 (63.0) | 35 (53.0) | 0.381 ¹ |
| M1 | 41 (44.1) | 10 (37.0) | 31 (47.0) | |
| Tumor differentiation | | | | |
| Moderate/good | 83 (89.2) | 26 (96.3) | 57 (86.4) | 0.271 ² |
| Poor | 10 (10.8) | 1 (3.7) | 9 (13.6) | |
| Vascular invasion | | | | |
| No | 56 (60.2) | 17 (63.0) | 39 (59.1) | 0.729 ¹ |
| Yes | 37 (39.8) | 10 (37.0) | 27 (40.9) | |
| Tumor location | | | | |
| Rectum | 19 (20.4) | 4 (14.8) | 15 (22.7) | 0.390 ¹ |
| Colon | 74 (79.6) | 23 (85.2) | 51 (77.3) | |
| CEA level (ng/mL) | | | | |
| ≤ 5 | 48 (51.6) | 12 (4.4) | 36 (54.5) | 0.376 ¹ |
| > 5 | 45 (48.4) | 15 (55.6) | 30 (45.5) | |

¹Pearson's χ^2 test; ²Fisher's exact test. T-, N-, M-stage are tumor, node, and metastasis stage (6th edition), performed according to the American Joint Committee on Cancer; CEA: Serum carcinoembryonic antigen.

Twist2 staining was observed only in the cytoplasm of CRC tumor cells (described in the results); therefore, the nucleolus staining was not evaluated. Cytoplasmic staining of Twist2 was scored according to its extent and intensity (extent + intensity), similar to the methods described previously^[21-24]. The extent of staining was graded as follows: 0 for < 15% positive cells, 1 for 15%-30%, 2 for 30%-60% and 3 for more than 60% positive cells. The intensity of staining was scored on the following scale: 0, no staining; 1, weak staining; 2, moderate staining; 3, strong staining. The total score was 0 to 6 when summed (extent + intensity) together. Subsequently, a total score of 0-2 was considered to be a negative/low expression, while a score of 3-6 was considered as positive/high expression. For E-cadherin, the scoring was determined as previous studies^[25,26]. Preserved expression of E-cadherin was defined where tumor cells were stained as strongly and homogeneously as normal epithelial cells. Heterogeneous staining, weaker staining or completely negative staining of E-cadherin was considered as reduced expression.

Statistical analysis

Pearson's χ^2 test and Fisher's exact test (wherever was applicable) were performed to determine the relationship between Twist2 expression and clinicopathological parameters and E-cadherin expression. The prognostic

factors for OS and DFS were examined by both univariate and multivariate analyses (Cox's proportional hazards model). Survival curves were plotted by Kaplan-Meier analysis and by a log rank test. A *P* value < 0.05 (two-sided) was considered statistically significant. All statistical analysis were performed using SPSS version 19 (SPSS Inc., Chicago, IL, United States).

RESULTS

Patients' clinicopathological characteristics are shown in Table 1. The mean age was 58.9 years, ranging from 16 to 81. Forty-one patients had distant metastasis (M1); all the metastatic nodes were in the liver. The median follow-up period was 32 mo (range 6-144 mo). At the last follow-up, 55 patients had tumor recurrence, including one in rectal anastomotic, two with pelvic metastasis, three in the lung, one in both the liver and the lung and the other 48 only in the liver. Thirty patients had died. The OS and DFS rates were 82.2% and 61.0% at 1 year, 71.3% and 42.4% at 3 years, and 66.2% and 30.3% at 5 years, respectively.

Twist2 and E-cadherin expression in CRC

Although some previous investigations found Twist2 was expressed in both the cytoplasm and the nucleus in several tumors^[7,8,11,27], we found Twist2 was mainly expressed in the cytoplasm in CRC, not in the nucleus (Figure 1). A similar expression pattern of Twist2 was found in hepatocellular carcinoma (HCC)^[28]. By semiquantitative analysis, 66 (71.0%) of the 93 primary CRC tissue samples were positive for Twist2 expression, while the other 27 (39.0%) were negative. Twist2 expression was generally low in normal colon mucosa compared with the cancer tissues. For E-cadherin, as described previously^[29,30], normal epithelial cells were strongly and homogeneously stained in the membrane, while tumor cells were stained mainly in the membrane and occasionally in the cytoplasm (Figure 2). E-cadherin was considered as reduced in 43 (46.2%) patients. The other 50 (53.8%) patients were preserved.

Relationship between Twist2 expression and clinicopathological parameters and E-cadherin expression

As shown in Table 1, we did not find that Twist2 expression correlated with any of the clinicopathological parameters (gender, age, T-stage, N-stage, M-stage, tumor differentiation, vascular invasion, Tumor location and serum CEA level, all *P* > 0.05). When the relationship between Twist2 and E-cadherin expression was analyzed, we found a significant correlation: Twist2-positive patients showed a higher percentage of reduced E-cadherin than Twist2-negative ones (53.0% *vs* 29.6%, *P* = 0.040, Table 2).

Prognostic analysis

For OS on univariate analysis, bad N-stage (lymph node metastasis), bad M-stage (distant metastasis), vascular invasion, serum CEA level (> 5 ng/mL) and Twist2-positive

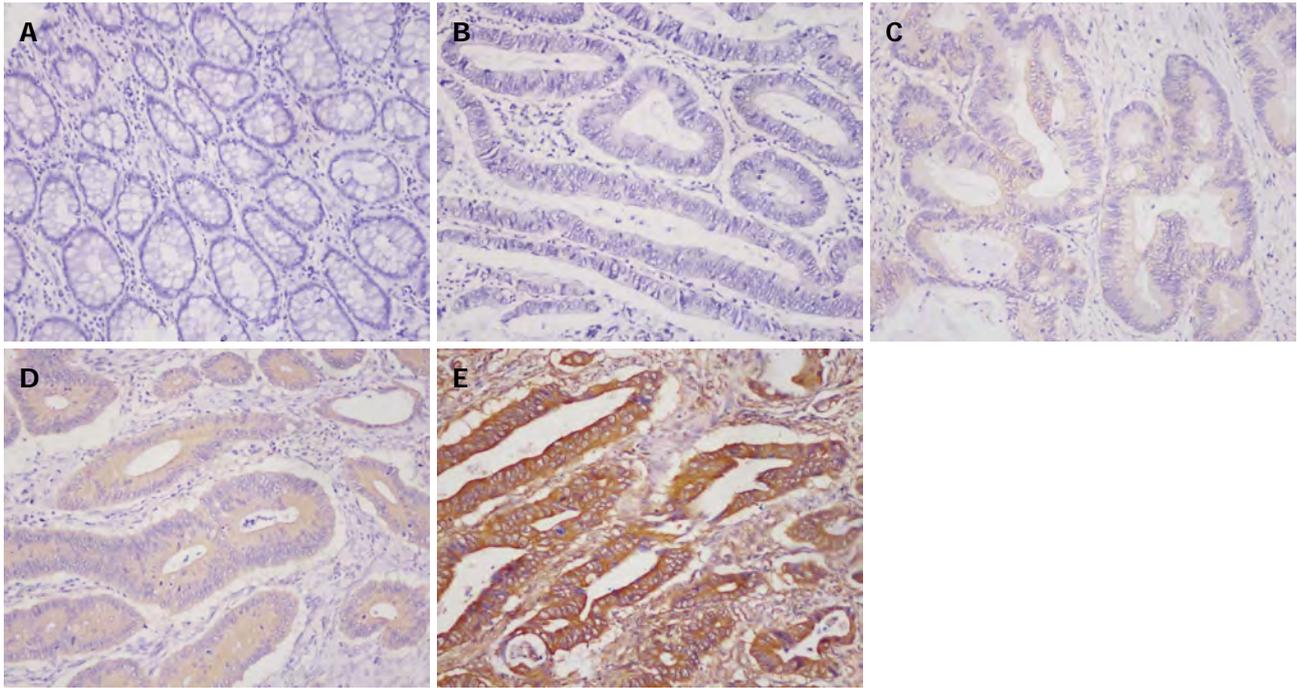


Figure 1 Immunohistochemical images of Twist2. A: Negative staining in the normal mucosa; B: Negative; C: Weak; D: Moderate; E: Strong cytoplasmic staining in colorectal cancer (200× magnification).

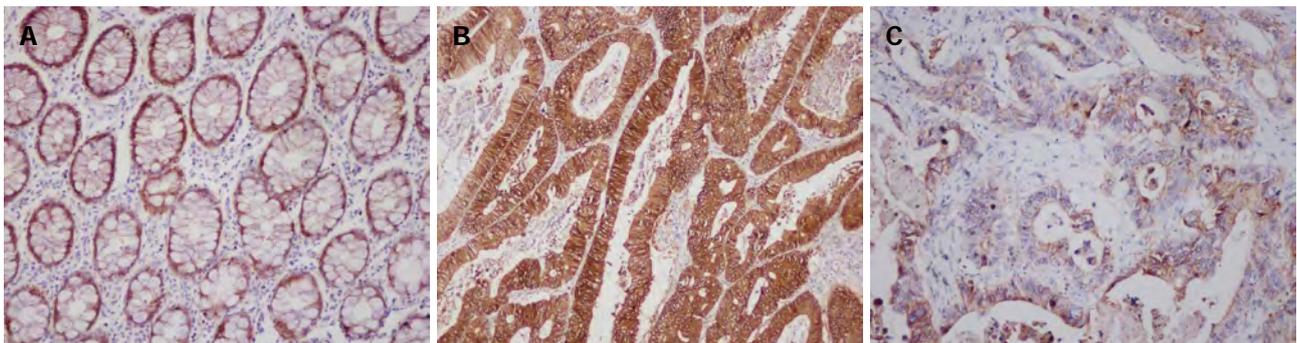


Figure 2 Immunohistochemical images of E-cadherin. A: Strong and homogeneous staining in normal mucosa; B: Preserved expression in colorectal cancer (CRC); C: Reduced expression in CRC (200× magnification).

Table 2 Relationship between Twist2 and E-cadherin expression *n* (%)

| Twist2 expression | E-cadherin expression | | <i>P</i> value |
|---------------------------|----------------------------|--------------------------|----------------|
| | Preserved (<i>n</i> = 50) | Reduced (<i>n</i> = 43) | |
| Positive (<i>n</i> = 66) | 31 (47.0) | 35 (53.0) | 0.040 |
| Negative (<i>n</i> = 27) | 19 (70.4) | 8 (29.6) | |

were significantly associated with poor survival (all *P* < 0.05, Table 3). When adjusted by multivariate analysis by Cox's proportional hazard model, bad M-stage [hazard ratio (HR) = 7.694, 95%CI: 2.927-20.224, *P* < 0.001] and Twist2-positive (HR = 5.744, 95%CI: 1.347-24.298, *P* = 0.018) were considered to independent risk factors for poor OS.

We also analyzed the risk factors for DFS (Table 4). The result of univariate analysis was similar to OS: bad N-stage, bad M-stage, vascular invasion, serum CEA

level (> 5 ng/mL) and Twist2-positive were risk factors for poor DFS (all *P* < 0.05). After adjustment, multivariate analysis revealed bad N-stage (HR = 2.149, 95%CI: 1.226-3.767, *P* = 0.008), bad M-stage (HR = 10.907, 95%CI: 4.937-24.096, *P* < 0.001) and Twist2-positive (HR = 3.264, 95%CI: 1.455-7.375, *P* = 0.004) were independent risk factors for poor DFS, while vascular invasion and serum CEA level were not.

Survival curves plotted according to different expression levels of Twist2 are shown in Figure 3. Significantly, Twist2-negative patients had a higher 5-year OS (86.2% *vs* 59.6%, *P* = 0.015, Figure 3A) and 5-year DFS (55.4% *vs* 24.8%, *P* = 0.012, Figure 3B) than the Twist2-positive patients. Interestingly, further analysis of the value of Twist2 for CRC patients in different stages showed that for patients in stage I - II (*n* = 34), there were no differences in OS or DFS (both *P* > 0.05, Figure 3C and D). For patients in stage III (*n* = 18) and IV (*n* = 41), Kaplan-

Table 3 Univariate and multivariate analysis of the prognostic factors for overall survival

| Prognostic factors | Univariate analysis | | | Multivariate analysis | | |
|-----------------------|---------------------|--------------|---------|-----------------------|--------------|---------|
| | HR | 95%CI | P value | HR | 95%CI | P value |
| Gender | | | | | | |
| Female | 1 | | | | | |
| Male | 1.011 | 0.490-2.085 | 0.977 | NA | NA | NA |
| Age | | | | | | |
| < 59 | 1 | | | | | |
| ≥ 59 | 1.579 | 0.750-3.326 | 0.229 | NA | NA | NA |
| T-stage | | | | | | |
| T1-2 | 1 | | | | | |
| T3-4 | 1.118 | 0.493-2.534 | 0.789 | NA | NA | NA |
| N-stage | | | | | | |
| N0 | 1 | | | | | |
| N1-2 | 2.172 | 1.056-4.468 | 0.035 | NS | NS | NS |
| M-stage | | | | | | |
| M0 | 1 | | | 1 | | |
| M1 | 6.324 | 2.659-15.041 | < 0.001 | 7.694 | 2.927-20.224 | < 0.001 |
| Tumor differentiation | | | | | | |
| Moderate/good | 1 | | | | | |
| Poor | 1.290 | 0.491-3.384 | 0.229 | NA | NA | NA |
| Vascular invasion | | | | | | |
| No | 1 | | | | | |
| Yes | 3.398 | 1.601-7.211 | 0.001 | NS | NS | NS |
| Tumor location | | | | | | |
| Rectum | 1 | | | | | |
| Colon | 1.188 | 0.517-2.729 | 0.685 | NA | NA | NA |
| CEA level (ng/mL) | | | | | | |
| ≤ 5 | 1 | | | | | |
| > 5 | 3.173 | 1.475-6.827 | 0.003 | NS | NS | NS |
| Twist2 expression | | | | | | |
| Negative | 1 | | | 1 | | |
| Positive | 4.964 | 1.181-20.863 | 0.029 | 5.744 | 1.347-24.298 | 0.018 |

Multivariate analysis included adjustment for N-stage, M-stage, vascular invasion, serum carcinoembryonic antigen level and Twist2 expression. T: Tumor; N: Node; M: Metastasis; HR: Hazard ratio; NA: Not available; NS: Not significant.

Meier curves showed a clear trend that Twist2-negative patients had a more favorable outcome. Although the differences in OS were not statistically significant (both $P > 0.05$, Figure 3E and G), we found significant differences in DFS for both stage III and IV ($P = 0.033$ and $P = 0.026$ respectively, Figure 3F and H).

DISCUSSION

This study, which investigated the significance of Twist2 protein expression in CRC, identified some variables that affected the patients' prognosis. Bad N-stage, bad M-stage (liver metastasis in our study), vascular invasion, serum CEA level (> 5 ng/mL) and Twist2-positive were valuable predictors for both OS and DFS by univariate analysis. After adjustment by multivariate analysis, bad M-stage and Twist2-positive remained as independent risk factors for poor OS, while bad N-stage, bad M-stage and Twist2-positive were the independent risk factors for poor DFS. Twist2-positive was identified to be an independent risk factor for both poor OS and DFS. Kaplan-Meier analysis showed that patients with Twist2-negative expression had significantly longer OS and DFS than the positive pa-

Table 4 Univariate and multivariate analysis of the prognostic factors for disease-free survival

| Prognostic factors | Univariate analysis | | | Multivariate analysis | | |
|-----------------------|---------------------|--------------|---------|-----------------------|--------------|---------|
| | HR | 95%CI | P value | HR | 95%CI | P value |
| Gender | | | | | | |
| Female | 1 | | | | | |
| Male | 1.001 | 0.587-1.706 | 0.998 | NA | NA | NA |
| Age | | | | | | |
| < 59 | 1 | | | | | |
| ≥ 59 | 1.060 | 0.621-1.809 | 0.831 | NA | NA | NA |
| T-stage | | | | | | |
| T1-2 | 1 | | | | | |
| T3-4 | 1.258 | 0.833-1.899 | 0.276 | NA | NA | NA |
| N-stage | | | | | | |
| N0 | 1 | | | 1 | | |
| N1-2 | 2.511 | 1.468-4.295 | 0.001 | 2.149 | 1.226-3.767 | 0.008 |
| M-stage | | | | | | |
| M0 | 1 | | | 1 | | |
| M1 | 11.737 | 5.442-25.313 | < 0.001 | 10.907 | 4.937-24.096 | < 0.001 |
| Tumor differentiation | | | | | | |
| Moderate/good | 1 | | | | | |
| Poor | 1.140 | 0.537-2.418 | 0.733 | NA | NA | NA |
| Vascular invasion | | | | | | |
| No | 1 | | | | | |
| Yes | 3.246 | 1.874-5.625 | < 0.001 | NS | NS | NS |
| Tumor location | | | | | | |
| Rectum | 1 | | | | | |
| Colon | 1.557 | 0.810-2.992 | 0.184 | NA | NA | NA |
| CEA level (ng/mL) | | | | | | |
| ≤ 5 | 1 | | | | | |
| > 5 | 2.958 | 1.692-5.172 | < 0.001 | NS | NS | NS |
| Twist2 expression | | | | | | |
| Negative | 1 | | | 1 | | |
| Positive | 2.632 | 1.184-5.809 | 0.017 | 3.264 | 1.455-7.375 | 0.004 |

Multivariate analysis included adjustment for N-stage, M-stage, vascular invasion, serum carcinoembryonic antigen level and Twist2 expression. T: Tumor; N: Node; M: Metastasis; HR: Hazard ratio; NA: Not available; NS: Not significant.

tients. When considering the prognostic value of Twist2 for CRC patients in different stages, we observed a trend for Twist2-negative patients to have a more favorable prognosis compared with Twist2-positive patients, especially for the patients in stage III and IV. Although the P values for OS in stage III and IV didn't reach significance, the P values for DFS in stage III and IV were statistically significant.

To the best of our knowledge, our study is the first report on the prognostic value of Twist2, based on the protein level, for human CRC. Currently, there is a lack of clinical biomarkers for effectively and routinely predicting CRC, especially for the patients in stage IV. Therefore, the findings of this study are very useful, as we found that Twist2 could be an effective biomarker for predicting the prognosis of CRC, even for patients in stage IV. Furthermore, the expression of Twist2 protein is easily detected by immunohistochemistry. For these reasons, Twist2 is potentially an extremely useful clinical biomarker for predicting the prognosis of CRC patients.

Reduced expression of E-cadherin, which is a hallmark of EMT^[14] and plays a significant role in multi-stage carcinogenesis^[31], generally represents a common

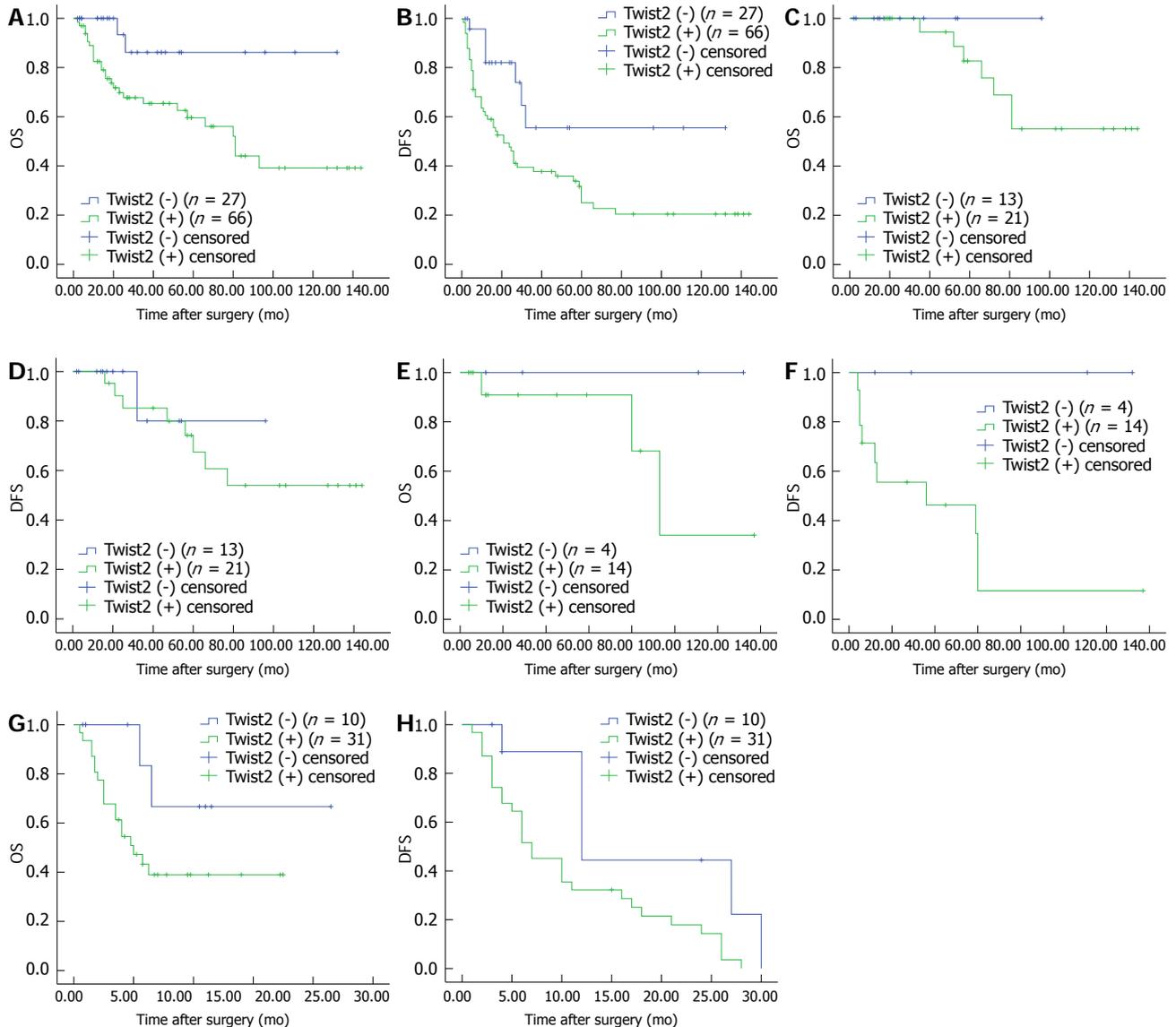


Figure 3 Kaplan-Meier analysis of overall survival and disease-free survival, according to the expression levels of Twist2. A, B: All patients (A, OS, $P = 0.015$ and B, DFS, $P = 0.012$); C, D: Patients in stage I - II (C, OS, $P = 0.351$ and D, DFS, $P = 0.652$); E, F: Patients in stage III (E, OS, $P = 0.178$ and F, DFS, $P = 0.033$); G, H: Patients in stage IV (G, OS, $P = 0.101$ and H, DFS, $P = 0.026$). OS: Overall survival; DFS: Disease-free survival.

feature of EMT inducers if these biomarkers were also upregulated^[8]. In this study, Twist2-positive expression was significantly associated with reduced expression of E-cadherin, which supports the view that Twist2 is an EMT inducer in CRC. Unfortunately, we did not find a significant correlation between Twist2 expression and the adverse biological behaviors of CRC (bad T, N, M-stage, poor differentiation and vascular invasion). Thus, the mechanism remains unclear. However, other prognostic biomarkers share similar features with Twist2, such as vimentin^[32], a-smooth muscle actin^[33] and S100A4^[29] for CRC, and osteopontin for HCC^[34].

Considering the previous reports and the present study, several mechanisms probably contribute to the function of Twist2. Crucially, as an inducer of EMT, Twist2 can activate the EMT program, which is frequently involved in tumor progression and correlates with acquisi-

tion of therapeutic resistance^[14-19]. In addition, hypoxia may participate in Twist2 function, as Zhou *et al*^[7] found that positive expression of hypoxia-inducible factor-2 α was significantly associated with Twist2 overexpression in salivary adenoid cystic carcinoma. Furthermore, Twist2 also correlates with methylation^[35,36] and cancer stem cell self-renewal^[11], as well as drug resistance^[37], which may explain the different outcomes of patients in the same stage. As EMT, cancer stem cells and drug resistance together comprise an axis of evil during tumor progression^[19], we speculate that Twist2 is a key component of this axis. In summary, the mechanism of Twist2's function in CRC is likely to be complex rather than simple.

In conclusion, the results of this study suggest that Twist2 is an independent prognostic factor for CRC. In particular, Twist2 exhibits a prognostic value for CRC in stage III and IV. Future studies with larger samples and

functional experiments are needed to confirm the function of Twist2 in CRC.

COMMENTS

Background

Colorectal cancer (CRC) is one of the most common malignant tumors and continues to be one of the most common causes of cancer death worldwide. It is important to identify biomarkers to predict patients' outcomes. Twist2 is a potential prognostic biomarker, but its value for CRC is unknown.

Research frontiers

Twist2 is a regulatory factor of epithelial-mesenchymal transition, a well-known progression involved in embryogenesis, tumor invasion, metastatic dissemination and acquisition of therapeutic resistance. Hypoxia, methylation, cancer stem cell self-renewal and drug resistance correlate with Twist2 function. Therefore, Twist2 is a potential prognostic biomarker for tumors, and its prognostic value has also been identified for head and neck squamous cell carcinomas.

Innovations and breakthroughs

This study revealed that Twist2 was overexpressed in CRC at the protein level. Twist2-positive expression correlated with the poor prognosis of CRC, particularly for patients in stage III and IV (tumor-node-metastasis stage).

Applications

These results suggest that overexpression of Twist2 can probably serve as a prognostic factor for patients with CRC.

Terminology

EMT is an important change in cell phenotype, which allows the escape of epithelial cells from the structural constraints imposed by tissue architecture, and was first recognized as a central process in early embryonic morphogenesis. Over recent decades, a series of studies have identified the involvement of EMT in solid tissue epithelial cancers' invasiveness and metastasis.

Peer review

This study investigated Twist2 expression in 93 CRC patients and evaluated its value as a prognostic biomarker based on relapse and survival data of patients. The results indicate that Twist2 could be used as an effective prognostic biomarker for CRC. This paper is generally well designed and the result looks reliable.

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Elevated serum levels of human relaxin-2 in patients with esophageal squamous cell carcinoma

Peng Ren, Zhen-Tao Yu, Li Xiu, Mei Wang, Hua-Min Liu

Peng Ren, Zhen-Tao Yu, Li Xiu, Mei Wang, Hua-Min Liu, Department of Esophageal Cancer, Tianjin Medical University Cancer Institute and Hospital and Key Laboratory of Cancer Prevention and Therapy, Tianjin 300060, China

Author contributions: Ren P and Liu HM performed the majority of experiments; Yu ZT and Wang M collected all the human materials; Yu ZT designed the study and wrote the manuscript; Ren P and Xiu L revised the manuscript.

Correspondence to: Zhen-Tao Yu, MD, Department of Esophageal Cancer, Tianjin Medical University Cancer Institute and Hospital and Key Laboratory of Cancer Prevention and Therapy, Huanhu West Road, Tianjin 300060, China. renpengtj@126.com
Telephone: +86-22-23340123 Fax: +86-22-23340123

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Abstract

AIM: To assess the prognostic value of serum human relaxin 2 (H2 RLN) level in patients with esophageal squamous cell carcinoma (ESCC).

METHODS: From October 1998 to September 2009, 146 patients with histopathologically confirmed ESCC were enrolled in this study. One hundred patients underwent *en bloc* esophagectomy, and 46 patients with unresectable tumors underwent palliative surgery. Five of the 146 patients died of surgical complications. Serum levels of H2 RLN were measured by enzyme linked immunosorbent assay. The relationship between serum H2 RLN level and each of the clinicopathological parameters was analyzed using the χ^2 test. Patients were classified into two groups according to their H2 RLN level (< 0.462 ng/mL vs ≥ 0.462 ng/mL). When any analysis cell had fewer than five cases, the Fisher's exact test was used. The statistical difference between groups A and B in each clinicopathological category was determined by the Student's *t* test (two-tailed) or analysis of variance. Survival curves were plotted using the Kaplan-Meier method. The statistical difference in

survival between the different groups was compared using the log-rank test. Survival correlation with the prognostic factors was further investigated by multivariate analysis using the Cox proportional hazards model with backward stepwise likelihood ratio.

RESULTS: ESCC patients tended to have significantly higher serum H2 RLN concentrations (0.48 ± 0.17 ng/mL, $n = 141$) compared with the healthy control group (0.342 ± 0.12 ng/mL, $n = 112$). There was a significant difference between patients with lymph node involvement (0.74 ± 0.15 ng/mL, $n = 90$), distant metastasis (0.90 ± 0.19 ng/mL, $n = 32$) and those without lymph node involvement (0.45 ± 0.12 ng/mL, $n = 51$), and distant metastasis (0.43 ± 0.14 ng/mL, $n = 109$), respectively ($P < 0.01$). Patients with high H2 RLN levels (≥ 0.462 ng/mL) had a poorer prognosis than patients with low serum H2 RLN levels (< 0.462 ng/mL; $P = 0.0056$). The H2 RLN level was also correlated with survival and tumor-node-metastasis staging, but not with age, tumor size, gender, lymphovascular invasion or the histological grade of tumors. Cox regression analysis showed that H2 RLN was an independent variable.

CONCLUSION: Serum concentrations of H2 RLN are frequently elevated in ESCC patients and are correlated with disease metastasis and survival. Serum concentrations of H2 RLN may be an important prognostic marker in ESCC patients.

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Key words: Esophageal squamous cell carcinoma; Relaxin; Tumor markers; Metastasis

Core tip: Esophageal squamous cell carcinoma (ESCC) is one of the most aggressive carcinomas of the gastrointestinal tract. Despite improvements in detection, surgical resection, and (neo-) adjuvant therapy, the overall survival of ESCC patients remains lower than

that of patients with other solid tumors due to distant metastasis. Therefore, it is important to detect disease progression and metastasis as early as possible to improve timely treatment and improve survival. In this study, the authors assessed the prognostic value of serum human relaxin 2 (H2 RLN) level in patients with ESCC, and found that serum concentrations of H2 RLN were elevated in ESCC patients and were correlated with disease metastasis and survival. Serum concentrations of H2 RLN may be an important prognostic marker in ESCC patients.

Ren P, Yu ZT, Xiu L, Wang M, Liu HM. Elevated serum levels of human relaxin-2 in patients with esophageal squamous cell carcinoma. *World J Gastroenterol* 2013; 19(15): 2412-2418 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v19/i15/2412.htm> DOI: <http://dx.doi.org/10.3748/wjg.v19.i15.2412>

INTRODUCTION

Esophageal squamous cell carcinoma (ESCC) is one of the most aggressive carcinomas of the gastrointestinal tract. Despite improvements in detection, surgical resection, and (neo-) adjuvant therapy, the overall survival of ESCC patients remains lower than that of patients with other solid tumors due to distant metastasis^[1-4]. Therefore, it is important to detect disease progression and metastasis as early as possible to improve timely treatment and improve survival.

Several recent studies have shown that tumor-node-metastasis (TNM) stage and the number of diseased lymph nodes are two important factors associated with the prognosis of ESCC^[2-4]. Although these two factors can only be assessed during surgery, they are not applicable for monitoring disease advancement and the potential of metastasis. On the other hand, serum biomarkers are often associated with the biological behavior of cancer cells. The prognostic measure of a serum biomarker that can reflect the concerted interaction between the tumor and the host immune system may provide scientific insight to improve the therapeutic strategy. However, there are few serum biomarkers that can be used as complementary prognostic factors for patients with ESCC.

Relaxin (RLN) is a short circulating peptide hormone. Two highly homologous genes on human chromosome 9 encode relaxin gene 1 (RLN1) and relaxin gene 2 (RLN2) peptides with a predicted 82% identity at the amino acid level^[5,6]. Despite having two peptide-coding genes, RLN1 and RLN2, the major stored and circulatory form of relaxin in humans is RLN2. RLN2 is produced in the prostate by males^[7] and in the corpus lutea in females^[8], and RLN1 is a pseudogene, which does not translate into a functional peptide in rodents, humans and other non-human species.

RLN plays an important role in the remodeling of extracellular matrix (ECM) in several reproductive tract tissues^[9]. There is growing evidence that implicates

RLN in supporting tumor cell growth and metastasis^[10]. RLN is known to regulate the expression of a variety of genes, including collagens and matrix metalloproteinase (MMP)-1^[11], vascular endothelial growth factor^[9] and cyclooxygenase-2^[10]. In addition, the expression and catalytic activities of MMP-1, 2, 3 and 9 are increased by RLN^[12,13]. Moreover, RLN regulates the complex interactions of the plasminogen activator and MMPs/tissue inhibitors of MMP systems on the ECM, thus facilitating tumor cell attachment, migration and invasion. RLN has been shown to enhance *in vitro* invasiveness of breast cancer cells by upregulating the MMP-2, -7, -9, -13 and -14^[14]. Similarly, adenovirus-mediated expression of RLN promotes the invasive potential of breast cancer cells^[15].

A previous study showed that RLN concentrations in cancer patients were significantly higher than in a control population of healthy blood donors and patients with various other diseases. There was a significant difference between patients with and without metastases. Overall survival was shorter in RLN-positive than in RLN-negative patients. Cox regression analysis showed that RLN was not an independent variable, in contrast to metastatic disease and primary lymph node involvement.

In this study, we measured the serum levels of human relaxin 2 (H2 RLN) in ESCC patients and healthy controls using an enzyme linked immunosorbent assay (ELISA), and analyzed the association between clinical parameters of ESCC and serum H2 RLN levels.

MATERIALS AND METHODS

Ethics statement

This study was approved by the Medical Ethics Committee of the Cancer Center at Tianjin University.

Clinical specimen collection and preparation

From October 1998 to October 2009, 146 consecutive patients with histopathologically proven ESCC were enrolled in this study. The average age was 62.7 ± 11.3 years, and the male: female ratio was 40:3 (male 136, female 10). Tumor stage was classified according to the TNM system^[16]. Extensive preoperative examinations including esophagoscopy with biopsy, esophagogram, chest radiography, sonograms of abdomen and neck, computed tomography of the chest, and radionuclide bone scanning were performed to determine the need for surgery. Patients with resectable tumor ($n = 100$) underwent *en bloc* esophagectomy with locoregional lymphadenectomy through a right thoracotomy, laparotomy with reconstruction using the stomach through a retrosternal route, and cervical esophagogastrostomy. For patients at stage II b or beyond, concurrent chemoradiotherapy was administered after surgery. Patients with unresectable tumor ($n = 46$) received chemoradiotherapy after the installation of a feeding jejunostomy or bypass procedure. None of these patients received neoadjuvant therapy. After treatment, all patients were followed regularly. Five patients died of cardiopulmonary or chronic obstructive pulmonary disease

complications after surgery and were excluded from the prognosis analysis. Serum samples were obtained from each patient at the time of diagnosis. Serum samples from 112 healthy individuals with equivalent age and sex distribution were used as normal controls. The Medical Ethics Committee (Tianjin University) approved the protocol, and written informed consent was obtained from each healthy individual (normal controls). The healthy individuals were negative for hepatitis B virus, hepatitis C virus, and human immunodeficiency virus. Abdominal and breast (female) ultrasound examination, chest X-ray, routine blood tests, and biochemistry tests were performed for the healthy controls, and the results were within normal ranges. After centrifugation of the peripheral blood, serum samples were stored at -20 °C until assayed.

ELISA assays

The serum level of H2 RLN was measured with a commercially available ELISA kit (Santa Cruz, Shanghai, China) following the manufacturers' instructions. Briefly, 96-well ELISA microplates were coated overnight with 100 µL H2 RLN antibody (Santa Cruz, Shanghai, China) at a final concentration of 0.25 mg/L in phosphate buffered saline (PBS). After washing with PBS/0.05% (w/v) Tween-20 (PBST, pH 7.4), the wells were blocked with blocking buffer at room temperature for 1 h. Then, 100 µL diluted serum samples (at 1:30 dilution) were added and incubated at room temperature for 2 h. Similarly, 100 µL PBS with 0.04% Tween 80 (PBST), PBST lacking antibody was used as a negative control. Following three washes with PBST, 100 µL antibody diluted to a concentration of 0.25 mg/L was added. After incubation at room temperature for 2 h, 100 µL avidin-horseradish peroxidase conjugated secondary antibody (at 1:2000 dilution) was added, and the plates were incubated at room temperature for 30 min. Excess conjugate was removed by washing the plates three times with PBST. The amount of bound conjugate was determined by adding phosphate buffer and 3-ethylbenzothiazoline-6-sulfonic acid. Liquid substrate solution to each well, and plates were incubated at room temperature for color development. The absorbance was measured at 405 nm using a Model 680 microplate reader (Bio-Rad Lab. Inc., Hercules, CA, United States). All analyses were performed in triplicate. The coefficient of variation was lower than 15% between analyses. Concentrations of H2 RLN were presented in ng/mL. Patients were classified into two groups according to their H2 RLN level (< 0.462 ng/mL vs ≥ 0.462 ng/mL).

Statistical analysis

The results were expressed as mean \pm SD. The relationship between serum H2 RLN level and each of the clinicopathological parameters (age, size, lymph node involvement, distant metastasis, cell differentiation, lymphovascular invasion, and tumor stage) was analyzed using the χ^2 test. When any analysis cell had fewer than five cases, Fisher's exact test was used. The statistical difference between groups A and B in each clinicopathological category

was determined by Student's *t* test (two-tailed) or analysis of variance. Survival curves were plotted using the Kaplan-Meier method. The statistical difference in survival between the different groups was compared using the log-rank test. Survival correlation with the prognostic factors was further investigated by multivariate analysis using the Cox proportional hazards model with backward stepwise likelihood ratio. Statistical analysis was performed using SPSS statistical software (Chicago, IL, United States). Statistical significance was assumed for $P < 0.05$.

RESULTS

Serum H2 RLN level and clinicopathological features in ESCC patients

Serum H2 RLN was 0.342 ± 0.12 ng/mL in normal healthy controls (range, 0.26-0.41 ng/mL; $n = 112$) and was significantly higher in patients with ESCC (0.48 ± 0.17 ng/mL; range, 0.43-0.58 ng/mL, $n = 141$; $P < 0.05$). This was above the normal range in 69.5% (98 of 141) of ESCC patients before surgery, and 30.5% (43 of 141) of these patients had levels < 0.462 ng/mL, the mean plus 1 SD as determined from the control. Using 0.462 ng/mL as the cutoff value, these ESCC patients were then divided into group A ($n = 43$) as those with the lower level (< 0.462 ng/mL; mean, 0.45 ng/mL; range, 0.164-0.618 ng/mL) and group B ($n = 98$) as those with the higher level (> 0.462 ng/mL; mean, 0.71 ng/mL; range, 0.624-0.93 ng/mL). Of the 146 ESCC patients, 136 male patients had a serum H2 RLN level ranging from 0.26-0.40 ng/mL and 10 female patients had a serum H2 RLN level ranging from 0.253-0.41 ng/mL. χ^2 analysis showed that the preoperative serum H2 RLN levels correlated well with lymph node involvement (N status) and distant metastasis (M status, Table 1). Higher H2 RLN levels were related to disease progression (Table 2). Serum levels of H2 RLN were significantly higher in patients with lymph node metastasis or distant metastasis than in those without lymph node involvement or distant metastasis. No relationship was found between gender and H2 RLN levels. However, although the patients were grouped by their histopathological findings (pathological grade or lymphovascular invasion), no more differences were found in their serum H2 RLN levels (Tables 1 and 2). Although ESCC was most often found in male patients, there were only a limited number of female patients ($n = 10$), and we were unable to study the correlations between H2 RLN levels and sex hormones (ER, PR and Her-2).

Correlation of serum H2 RLN level with the prognosis of ESCC patients

The associations between median serum H2 RLN levels and clinicopathological parameters are presented in Table 2. H2 RLN levels were not associated with age, tumor size and gender (data not shown), cell differentiation, lymphovascular invasion and tumor status, however, elevated median H2 RLN levels were significant for patients with

Table 1 Relationship between serum levels of human relaxin 2 and clinicopathological factors

| Clinicopathological factors | Groups ¹ | | P value ² |
|-----------------------------|---------------------|-------|----------------------|
| | A (n) | B (n) | |
| Age (yr) | | | 0.144 |
| < 65 (n = 72) | 21 | 51 | |
| ≥ 65 (n = 69) | 22 | 47 | |
| Tumor status | | | 0.089 |
| T1 (n = 16) | 2 | 14 | |
| T2 (n = 14) | 4 | 10 | |
| T3 (n = 75) | 24 | 51 | |
| T4 (n = 36) | 13 | 23 | |
| Lymph node involvement | | | 0.003 |
| Positive (n = 90) | 32 | 58 | |
| Negative (n = 51) | 11 | 40 | |
| Distant metastasis | | | 0.027 |
| Positive (n = 32) | 21 | 11 | |
| Negative (n = 109) | 22 | 87 | |
| Lymphovascular invasion | | | 0.186 |
| Positive (n = 42) | 18 | 24 | |
| Negative (n = 99) | 25 | 74 | |
| Stage (TNM) | | | 0.035 |
| I (n = 17) | 7 | 10 | |
| II (n = 37) | 8 | 29 | |
| III (n = 53) | 10 | 43 | |
| IV (n = 34) | 18 | 16 | |
| Cell differentiation | | | 0.267 |
| Well (n = 23) | 9 | 14 | |
| Moderate (n = 90) | 23 | 56 | |
| Poor (n = 28) | 9 | 19 | |

¹Patients were grouped by preoperative serum levels of human relaxin 2 (H2 RLN). In group B (n = 43), serum H2 RLN levels were ≥ 0.462 ng/mL, and in group A (n = 98), H2 RLN levels were < 0.462 ng/mL; ²P values were determined by χ^2 test. TNM: Tumor-node-metastasis.

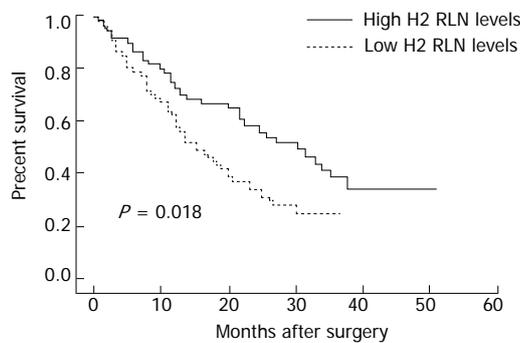


Figure 1 Survival of patients with esophageal squamous cell carcinoma in relation to their serum human relaxin 2 levels. The cutoff value of serum human relaxin 2 (H2 RLN) concentration for dividing patients into groups A and B was defined as 0.462 ng/mL which represents the mean plus 1 SD measured from the healthy control subjects. Survival analysis between group A (H2 RLN, ≥ 0.462 ng/mL) and group B (H2 RLN, < 0.462 ng/mL) was assessed using the Kaplan-Meier method, and the survival difference between the groups was compared using the log rank test ($P = 0.018$).

distant metastasis and lymph node involvement ($P < 0.05$). We also noted that the median levels of H2 RLN significantly increased with increasing T classification ($P < 0.05$) of the malignancy. Next, patients were classified into two groups according to their H2 RLN level (< 0.462 ng/mL *vs* ≥ 0.462 ng/mL); the relationships between H2 RLN levels and clinicopathological parameters were

Table 2 Levels of serum human relaxin 2 in patients with esophageal squamous cell carcinoma

| Category | H2 RLN (ng/mL) | P value |
|--------------------------|----------------|--------------------|
| Normal control (n = 112) | 0.34 ± 0.12 | 0.026 ¹ |
| ESCC (n = 141) | 0.48 ± 0.17 | |
| Tumor status | | 0.142 ² |
| T1 (n = 16) | 0.32 ± 0.08 | |
| T2 (n = 14) | 0.53 ± 0.13 | |
| T3 (n = 75) | 0.49 ± 0.16 | |
| T4 (n = 36) | 0.47 ± 0.19 | |
| Lymph node involvement | | 0.014 ¹ |
| Positive (n = 90) | 0.74 ± 0.15 | |
| Negative (n = 51) | 0.45 ± 0.12 | |
| Distant metastasis | | 0.016 ¹ |
| Positive (n = 32) | 0.90 ± 0.19 | |
| Negative (n = 109) | 0.43 ± 0.14 | |
| Lymphovascular invasion | | 0.342 ² |
| Positive (n = 42) | 0.51 ± 0.18 | |
| Negative (n = 99) | 0.46 ± 0.16 | |
| Stage (TNM) | | 0.002 ² |
| I (n = 17) | 0.42 ± 0.09 | |
| II (n = 37) | 0.46 ± 0.12 | |
| III (n = 53) | 0.47 ± 0.14 | |
| IV (n = 34) | 0.82 ± 0.23 | |
| Cell differentiation | | 0.542 ¹ |
| Well (n = 23) | 0.46 ± 0.21 | |
| Moderate (n = 90) | 0.48 ± 0.13 | |
| Poor (n = 28) | 0.52 ± 0.12 | |

P value was determined by: ¹Student's *t* test (two-tailed) or ²one way analysis of variance test. ESCC: Esophageal squamous cell carcinoma; H2 RLN: Human relaxin 2; TNM: Tumor-node-metastasis.

assessed. No significant difference in age, gender, tumor size (data not shown), tumor status, lymphovascular invasion and cell differentiation was found between the two groups; however, patients with lymph node metastasis, distant metastasis and higher clinical stage were more frequently observed in the elevated H2 RLN group (≥ 0.462 ng/mL) than in the non-elevated H2 RLN group (< 0.462 ng/mL). The overall cumulative survival rates of our patients were 42.4% at 2 years and 18.2% at 5 years. In view of the serum H2 RLN level, group A patients seemed to have a much worse prognosis than group B patients ($P = 0.018$; Figure 1). Figures 2 and 3 show the comparison of survival time between different disease stages. The cumulative 2-year survival rate for group A patients was 27.3% and for group B patients was 48.2%. The median survival for group A was 7.8 mo, and for group B was 22.4 mo. Among patients who had persistently high H2 RLN levels or a marked increase in H2 RLN level within a short interval (1-6 mo) after esophagectomy, distant metastasis of cancer was frequently found. On the other hand, patients with a low preoperative level of H2 RLN or H2 RLN levels which decreased after esophagectomy could remain disease-free for 12-23 mo. As previously mentioned, the depth of tumor invasion, lymph node metastasis, distant nodal or organ metastasis correlated well with H2 RLN level.

Further univariate and multivariate analyses also showed these parameters to be independent factors for patient survival (lymph node metastasis, $P < 0.001$; distant

Table 3 Univariate and multivariate survival analysis in patients with esophageal squamous cell carcinoma

| Characteristic | Univariate analysis | | | Multivariate analysis | | |
|--------------------------------|---------------------|-------------|-----------------------------|-----------------------|-------------|-----------------------------|
| | HR | 95%CI | <i>P</i> value ¹ | HR | 95%CI | <i>P</i> value ¹ |
| Age (yr) | | | | | | |
| < 65 vs ≥ 65 | 1.321 | 0.864-1.743 | 0.185 | 1.268 | 0.747-1.826 | 0.174 |
| Gender | | | | | | |
| Male vs female | 0.942 | 0.621-1.563 | 0.342 | 0.852 | 0.732-1.472 | 0.296 |
| Lymph node involvement | | | | | | |
| Yes vs no | 2.136 | 1.142-2.687 | < 0.001 | 2.830 | 1.384-2.982 | 0.001 |
| Distant metastasis | | | | | | |
| Yes vs no | 3.784 | 2.543-5.894 | < 0.001 | 3.549 | 1.302-3.108 | 0.001 |
| pTNM | | | | | | |
| I - II vs III-IV | 3.120 | 2.148-5.143 | < 0.001 | 1.632 | 1.130-2.740 | 0.028 |
| Lymphovascular invasion | | | | | | |
| Yes vs no | 1.121 | 0.765-1.574 | 0.129 | 1.290 | 0.862-1.420 | 0.172 |
| H2 RLN level | | | | | | |
| < 0.462 ng/mL vs ≥ 0.462 ng/mL | 2.469 | 1.362-2.836 | 0.015 | 2.530 | 1.424-2.732 | 0.003 |

¹Cox hazard regression model. HR: Hazard ratio; TNM: Tumor-node-metastasis; H2 RLN: Human relaxin 2.

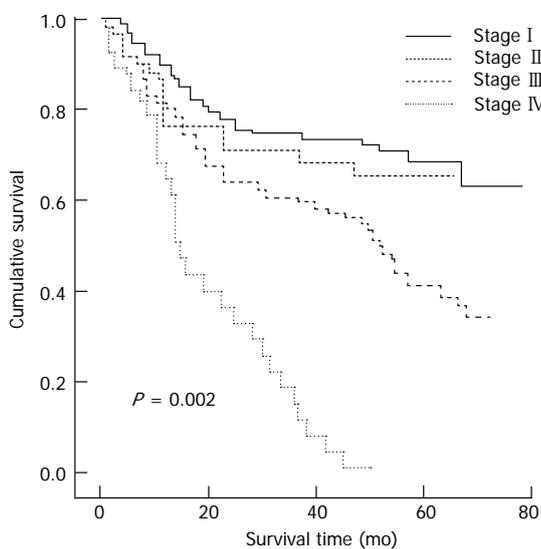


Figure 2 Comparison of overall survival between different disease stages. We also analyzed the prognostic value of human relaxin 2 (H2 RLN) levels in selected patient subgroups stratified according to disease stage. Esophageal squamous cell carcinoma patients with elevated H2 RLN levels had a significantly shorter overall survival rate compared to patients with non-elevated H2 RLN levels in the clinical stage I - II subgroup ($n = 54$; log-rank $P < 0.001$; Figure 3A), and the clinical stage III-IV subgroup ($n = 87$; log-rank $P < 0.001$; Figure 3B).

organ metastasis, $P < 0.001$; H2 RLN, $P < 0.05$ (Table 3).

DISCUSSION

Identification of targets for early detection of ESCC is important to improve the prognosis of patients with this pernicious disease. Currently, carcinoembryonic antigen^[17], cytokeratin-19 fragments^[18], and squamous cell carcinoma-associated antigen^[18], are routinely used as serum markers for the detection of ESCC. Due to the low sensitivity and specificity of detection of these markers^[19], additional serum markers must be established for early detection and diagnosis of ESCC.

It has been previously shown that the polypeptide

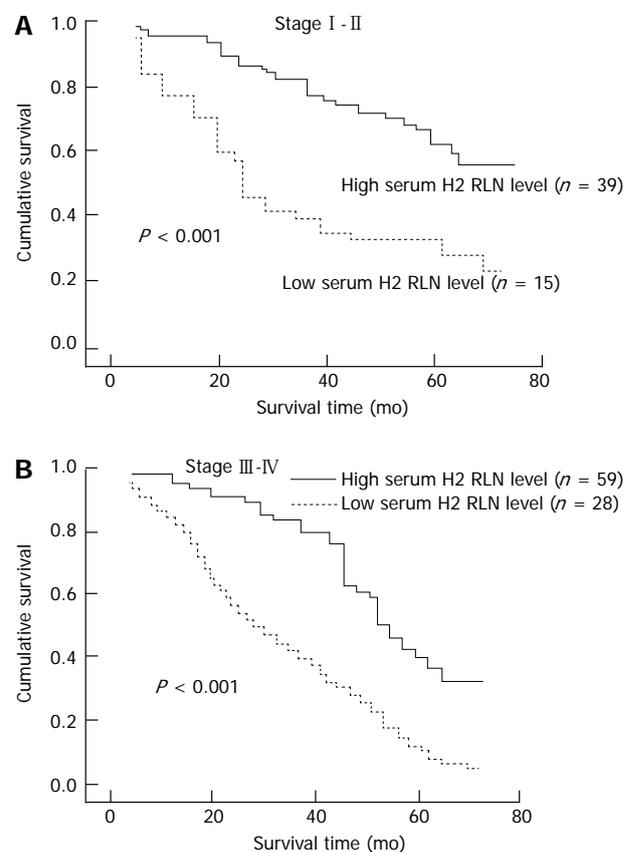


Figure 3 Overall survival curves for patients with esophageal squamous cell carcinoma after curative resection. The patients were categorized with elevated (≥ 0.462 ng/mL) or non-elevated (< 0.462 ng/mL) levels of relaxin. *P* values were determined using the log rank test. A: Clinical stage I - II subgroup; B: Clinical stage III-IV subgroup.

hormone relaxin is expressed exclusively in human breast cancer^[20], and relaxin confers increased carcinoma cell growth, motility, adhesion and *in vitro* invasiveness in human breast cancer cells^[14,21]. Furthermore, adenoviral-mediated delivery of the prorelaxin 2 gene increases the invasiveness of canine breast cancer cells^[10]. It was reported

that serum RLN concentrations were significantly higher in breast cancer patients than in a control population of healthy blood donors^[22]. Notably, serum RLN levels were higher in patients with metastatic disease compared to those without known metastases^[22].

Immunostaining with antibodies to human relaxin (H2) suggests the presence of a relaxin-like peptide in the gastrointestinal tract and its tumors^[23]. In our preliminary experiment, we found that H2 RLN was overexpressed in ESCC tissues using an immunohistochemistry assay (data not shown). In this study, we evaluated the H2 RLN serum concentrations in ESCC patients. Our results demonstrate that median H2 RLN serum concentrations in a population of ESCC patients were significantly higher than those in a control group of healthy blood donors. H2 RLN concentrations were particularly elevated in patients with lymph node metastasis and distant metastasis. However, no significant differences were found in the serum levels of H2 RLN in ESCC patients according to different histological tumor grades, age, tumor size and tumor status. Thus, H2 RLN concentration may be a suitable routine serum marker for the detection of metastatic disease in ESCC. Because our study involved a relatively small number of ESCC patients and the sensitivity and specificity of our assay which measured serum H2 RLN were not sufficiently high, further confirmation of these findings in a larger sample size is warranted.

Evaluation of survival data showed that survival was significantly shorter in patients with H2 RLN concentrations > 0.462 ng/mL, and high H2 RLN concentrations predicted a poor prognosis in patients with ESCC, especially in those presenting with distant and lymph node metastasis. H2 RLN was not an independent variable in ESCC. However, in breast cancer^[22], although elevated RLN serum concentrations were a significant discriminator between metastatic and non-metastatic patients, the predictive power of individual RLN values in the investigated population was rather low, as there was a broad overlap of concentrations in the two groups with or without metastases, suggesting cell type-specific effects of RLN.

In conclusion, we found that serum H2 RLN levels were increased in ESCC patients and correlated positively with TNM stage and both lymph node and distant metastasis, but not with gender, tumor size or the histological grade of ESCC. H2 RLN was an independent variable in ESCC. Examining and monitoring serum H2 RLN levels may be useful in estimating the prognosis of patients with ESCC.

ACKNOWLEDGMENTS

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COMMENTS

Background

Although the serum level of human relaxin-2 (H2 RLN) has been shown to cor-

relate with progression and prognosis of several cancers, data to support its clinical significance in esophageal squamous cell carcinoma (ESCC) are limited. This study was conducted to assess the prognostic value of serum human H2 RLN level in patients with ESCC.

Research frontiers

Serum levels of H2 RLN were measured by ELISA in 146 patients with histopathologically confirmed ESCC. The authors also assessed the prognostic value of serum human H2 RLN level in these patients with ESCC.

Innovations and breakthroughs

Serum concentrations of H2 RLN are frequently elevated in ESCC patients and are correlated with disease metastasis and survival.

Applications

Serum concentrations of H2 RLN may be an important prognostic marker in ESCC patients.

Peer review

The authors assessed the prognostic value of serum H2 RLN level in patients with ESCC, and found that serum concentrations of H2 RLN were elevated in ESCC patients and were correlated with disease metastasis and survival. This is an interesting report.

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Exposure to gastric juice may not cause adenocarcinogenesis of the esophagus

Peng Cheng, Jian-Sheng Li, Lian-Feng Zhang, Yong-Zhong Chen, Jun Gong

Peng Cheng, Jian-Sheng Li, Lian-Feng Zhang, Yong-Zhong Chen, Department of Gastroenterology, the First Hospital of Zhengzhou University, Zhengzhou 450052, Henan Province, China

Jun Gong, Department of Gastroenterology, the Second Hospital of Xi'an Jiao Tong University, Xi'an 710004, Shaanxi Province, China

Author contributions: Cheng P designed the study, wrote the manuscript and performed the majority of experiments; Li JS, Zhang LF and Chen YZ provided vital reagents and analytical tools and were also involved in revising the manuscript; Gong J designed the study and provided financial support for this work.

Correspondence to: Peng Cheng, MD, Associate Professor, Department of Gastroenterology, the First Hospital of Zhengzhou University, No. 1, Jianshe Donglu, Zhengzhou 450052, Henan Province, China. cpzxczcc2004@yahoo.com.cn

Telephone: +86-371-66862062 Fax: +86-371-66964992

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Abstract

AIM: To determine the effects of gastric juice on the development of esophageal adenocarcinoma (EAC).

METHODS: A animal model of duodenogastroesophageal reflux was established in Sprague-Dawley rats undergoing esophagoduodenostomy. The development of EAC and forestomach adenocarcinoma was investigated 40 wk after the treatment. Intraluminal pH and bile of the forestomach were measured.

RESULTS: There were no significant differences in pH ($t = 0.117, P = 0.925$) or bile ($\chi^2 = 0.036, P = 0.85$) in the forestomach before and 40 wk after esophagoduodenostomy. There were also no significant differences between the model and controls during esophagoduodenostomy or 40 wk after esophagoduodenostomy. The incidence of intestinal metaplasia (88%) and in-

testinal metaplasia with dysplasia and adenocarcinoma (28%) in the esophagus in the model was higher than in the controls 40 wk after surgery ($\chi^2 = 43.06, P < 0.001$ and $\chi^2 = 9.33, P = 0.002$, respectively) and in the forestomach in the model ($\chi^2 = 32.05, P < 0.001$ and $\chi^2 = 8.14, P = 0.004$, respectively). The incidence rates of inflammation in the esophagus and forestomach were 100% and 96%, respectively ($\chi^2 = 1.02, P = 0.31$) in the model, which was higher than in the esophageal control (6.8%) ($\chi^2 = 42.70, P < 0.001$).

CONCLUSION: Gastric juice exposure may not cause intestinal metaplasia with dysplasia or adenocarcinoma of the forestomach and may not be related to EAC.

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Key words: Intestinal metaplasia; Gastric juice; Pathogenesis; Esophageal adenocarcinoma; Gastroesophageal reflux

Core tip: The incidence of esophageal adenocarcinoma (EAC) has rapidly increased, which may be related to the increased incidence of gastroesophageal reflux disease. A better understanding of how refluxate contributes to development of EAC will help decrease the incidence of cancer. We surgically developed a rat model of duodenogastroesophageal reflux and found that although exposure of the forestomach to gastric juice may induce inflammation and mild metaplasia, it does not lead to the development of metaplasia with dysplasia or adenocarcinoma. It is concluded that gastric juice may not be related to the development of EAC.

Cheng P, Li JS, Zhang LF, Chen YZ, Gong J. Exposure to gastric juice may not cause adenocarcinogenesis of the esophagus. *World J Gastroenterol* 2013; 19(15): 2419-2424 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v19/i15/2419.htm> DOI: <http://dx.doi.org/10.3748/wjg.v19.i15.2419>

INTRODUCTION

The incidence rate of esophageal adenocarcinoma (EAC) has recently increased more quickly than that of any other malignancies, which has attracted attention^[1]. The rapid increase in the incidence of EAC might be related to the increase in that of gastroesophageal reflux disease (GERD) and Barrett's esophagus^[2,3]. The presence of Barrett's metaplasia with specialized intestinal epithelium is the main risk factor for these tumors. This epithelium is an acquired condition after a particular type of healing from esophageal mucosal injury resulting from reflux disease^[4]. Reflux of gastric acid and duodenal juice is the main cause of GERD, and gastric acid has always been regarded as the major risk element in GERD; the main clinical treatment of which is acid suppression^[5]. However, because of the rapid increase in the incidence of EAC, the role of gastric acid in the development of GERD remains controversial.

Gastric juice that has refluxed into the esophagus in patients with GERD also contains biliary and pancreatic secretions that have refluxed into the stomach from the duodenum. Early studies have shown that reflux of combined duodenal and gastric juices into the esophagus causes severe esophagitis^[6]. Reflux of duodenal juice results in the same degree of esophageal injury in gastrectomized animals^[7]. Evidence from both animal models^[8-11] and human clinical studies^[12] has implicated esophageal exposure to duodenal juice as a key factor in the genesis of specialized intestinal metaplasia and the development of adenocarcinoma. Some researchers believe that acid resistance may be related to the obvious increase in the incidence of EAC^[13]. With the development of the dynamic surveying system of duodenal juice, the role of duodenal juice reflux in the pathological process has attracted increasing attention. One study has even confirmed that duodenal juice reflux could induce Barrett's esophagus and EAC in rats^[10].

Therefore, the roles of gastric juice and of bile and pancreatic juice regurgitation in duodenal juice reflux in the development of EAC without exogenous carcinogens should be studied in an animal model of duodenogastroesophageal reflux. The aim of the current study was to investigate the role of gastric juice in the genesis of intestinal metaplasia and EAC in this rat model.

MATERIALS AND METHODS

Animals

Sixty healthy 8-wk-old Sprague-Dawley rats weighing 200-250 g were purchased from the Experimental Animal Center of Xi'an Jiao Tong University. The paired male and female rats were randomly divided into two groups: sham-operated control ($n = 30$) and model ($n = 30$) groups.

Experimental animal model

A Sprague-Dawley rat model of duodenogastroesophageal reflux was created in accordance with the method of

Zhang *et al*^[14] and a sham-operated group was used as the control group. Surgical diversion of duodenal secretions into the esophagus was induced by end-to-side esophago-duodenostomy in the experimental group. All operated rats underwent esophagoduodenostomy. The esophagus was separated from the posterior vagal trunk and left gastric vessels, tied with silk at the gastroesophageal junction, and divided 2 mm proximal to the tie. The anterior vagus nerve was divided when the esophagus was cut and sutured with 16 interrupted stitches of 7-0 polypropylene.

Esophagoduodenostomy was the only procedure performed in 30 animals. The purpose of the anastomosis was to induce reflux of both gastric and duodenal juice into the esophagus. The anterolateral wall of the distal duodenum was opened longitudinally 1 cm from the pylorus, and the broken ends of the esophagus were anastomosed to the duodenal incision.

The sham-operated group included 30 rats. After the rats were paunched, only the lower esophagus and first portion of the duodenum were dissociated.

Operations were performed after an acclimatization period of 4 d. Rats were kept in hanging cages on a 12 h light-dark cycle at a temperature of 21 °C and humidity of 60%. Water and standard chow were given *ad libitum*. Food was discontinued in the evening before surgery or sacrifice, and water was discontinued in the morning of surgery. Rats were anesthetized with an intramuscular injection of xylazine hydrochloride (18 mg/kg) and ketamine (72 mg/kg), with further doses administered intraperitoneally during surgery as required. Before closure, 0.5 mL-1.5 mL 0.9% sodium chloride was instilled into the peritoneal cavity. Water was permitted when the rats awoke, and chow was provided on the next day. The rats were housed in cages at 22 °C-25 °C with free access to standard rat pellet food and water for 40 wk. Rats were treated following the Guidelines for the Care and Use of Laboratory Animals of the National Animal Welfare Committee.

Intraluminal pH and bile of the forestomach were measured during esophagoduodenostomy with a portable glass electrode pH monitor (Digitrapper MK; Medtronic Synectics, Stockholm, Sweden) and a portable bile monitor (Bilitec 2000; Medtronic Synectics). These parameters were also measured after rats were sacrificed 40 wk after the operation. For duodenal gastric reflux, the 2-min period was considered reflux positive if the bilirubin optical density was > 0.14 and lasted 5 s. An absorbance > 0.14 was used as the Bilitec threshold value^[15].

Tissues and specimens

The rats were sacrificed 40 wk after surgery. The esophagus and forestomach were opened longitudinally, and gross pathological changes were examined macroscopically. The samples of the esophagus and forestomach were then fixed in formalin, made into paraffin sections after numbering, and stained with hematoxylin-eosin. The characteristics of the pathological tissues were then observed under a light microscope.

Table 1 Intraluminal pH and bile positive in the forestomach between the time of esophagoduodenostomy and 40 wk after esophagoduodenostomy

| | Model (<i>n</i> = 25) | | Control (<i>n</i> = 29) | |
|-------------------------------------|------------------------|----------------------------|--------------------------|----------------------------|
| | pH (mean ± SD) | Bile positive (<i>n</i>) | pH (mean ± SD) | Bile positive (<i>n</i>) |
| At the time of esophagoduodenostomy | 3.22 ± 0.29 | 2 | 3.23 ± 0.29 | 3 |
| 40 wk after esophagoduodenostomy | 3.24 ± 0.31 | 2 | 3.25 ± 0.25 | 4 |

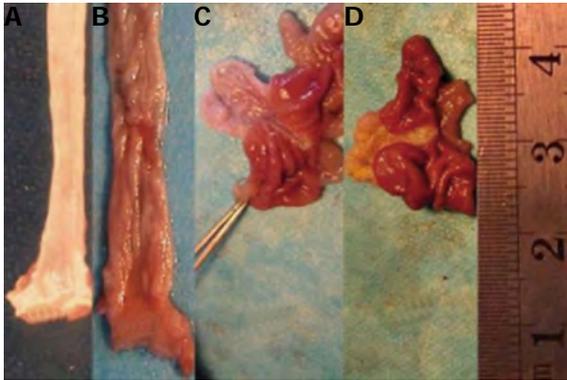


Figure 1 Gross specimens changes in the esophagus and forestomach mucosa in the sham-operated and model groups. A, B: Gross esophageal specimens of the sham-operated group (A) and animal model group (B); C, D: Gross forestomach specimens of the sham-operated group (C) and animal model group (D).

Statistical analysis

The incidence rates of inflammation, intestinal metaplasia, intestinal metaplasia with dysplasia, and adenocarcinoma in the esophagus and forestomach were analyzed and compared using χ^2 tests with SPSS software. Intraluminal pH of the forestomach was compared using *t* tests. Intraluminal bile of the forestomach was compared using χ^2 tests. The level of significance was set at $P < 0.05$.

RESULTS

Twenty-five model and 29 control rats survived. Six rats died, and the mortality rate was 10%.

pH and bile in the forestomach

There were no significant differences in intraluminal pH ($t = 0.117$, $P = 0.925$) (Table 1) or bile ($\chi^2 = 0.036$, $P = 0.85$) (Table 1) in the forestomach between the time of esophagoduodenostomy and 40 wk after the operation in the model rats. There were also no significant differences in pH ($t = 0.006$, $P = 0.99$ and $t = 0.20$, $P = 0.87$) (Table 1) or bile ($\chi^2 = 0.218$, $P = 0.64$ and $\chi^2 = 0.466$, $P = 0.495$) (Table 1) in the forestomach between model and control rats at the time of esophagoduodenostomy and 40 wk after the operation.

Gross specimens

In the sham-operated group, the esophagus and forestomach walls were thin, the mucosa was smooth, and the blood vessels below the mucous membrane were visible with occasional changes consistent with congestive

inflammation. In the animal models, inflammation and intestinal metaplasia differed in the esophagus and forestomach. Inflammation appeared as mucosal hyperplasia characterized by a thickened, rough surface with both small and large kernels or mild erosion and ulceration. Intestinal metaplasia appeared as a smooth and velvet-like surface. Adenocarcinoma in the forestomach had not developed. However, adenocarcinoma in the esophagus had developed and was characterized by nodular hyperplasia, ulceration, and a fish-like appearance (Figure 1).

Histological characteristics

Normal forestomach and esophageal epithelia appeared as stratification of squamous epithelium in neat rows, and some showed keratinization. Inflammation in the forestomach and esophagus appeared as hyperplasia of scaly epithelial basal cells, excessive keratinization of papillomatosis, visible neutrophilic granulocytes, infiltration of lymphoepithelioid cells, and mucosal erosion and edema of the submucosa and lower layer of the mucosa. Intestinal metaplasia was characterized by replacement of the squamous mucosa with simple columnar epithelium. EAC was characterized by severe intestinal metaplasia with dysplasia, pathological invasion of the basilar membrane, and some invasion of the blood or lymphatic vessels (Figure 2).

Incidence rates of inflammation, intestinal metaplasia, and adenocarcinoma in the forestomach and esophagus 40 wk after surgery

The incidence of intestinal metaplasia (88%) or intestinal metaplasia with dysplasia and adenocarcinoma (28%) in the esophagus in model rats was higher than in the control rats 40 wk after surgery ($\chi^2 = 43.06$, $P < 0.001$ and $\chi^2 = 9.33$, $P = 0.002$, respectively) (Table 2). In model rats, the incidence of inflammation in the esophagus and forestomach was 100% and 96%, respectively ($\chi^2 = 1.02$, $P = 0.31$). However, the rates of intestinal metaplasia (8%) and intestinal metaplasia with dysplasia and adenocarcinoma (0%) in the forestomach were lower than those in the esophagus ($\chi^2 = 32.05$, $P < 0.001$ and $\chi^2 = 8.14$, $P = 0.004$, respectively) (Table 2). In a comparison of model and control rats 40 wk after creating the models, the incidence of inflammation in the forestomach was 96% and 6.8%, respectively ($\chi^2 = 42.70$, $P < 0.001$), and the incidence of intestinal metaplasia was 12% and 3.4%, respectively ($\chi^2 = 1.43$, $P = 0.32$).

DISCUSSION

Rat stomach has a nonglandular forestomach and glandular

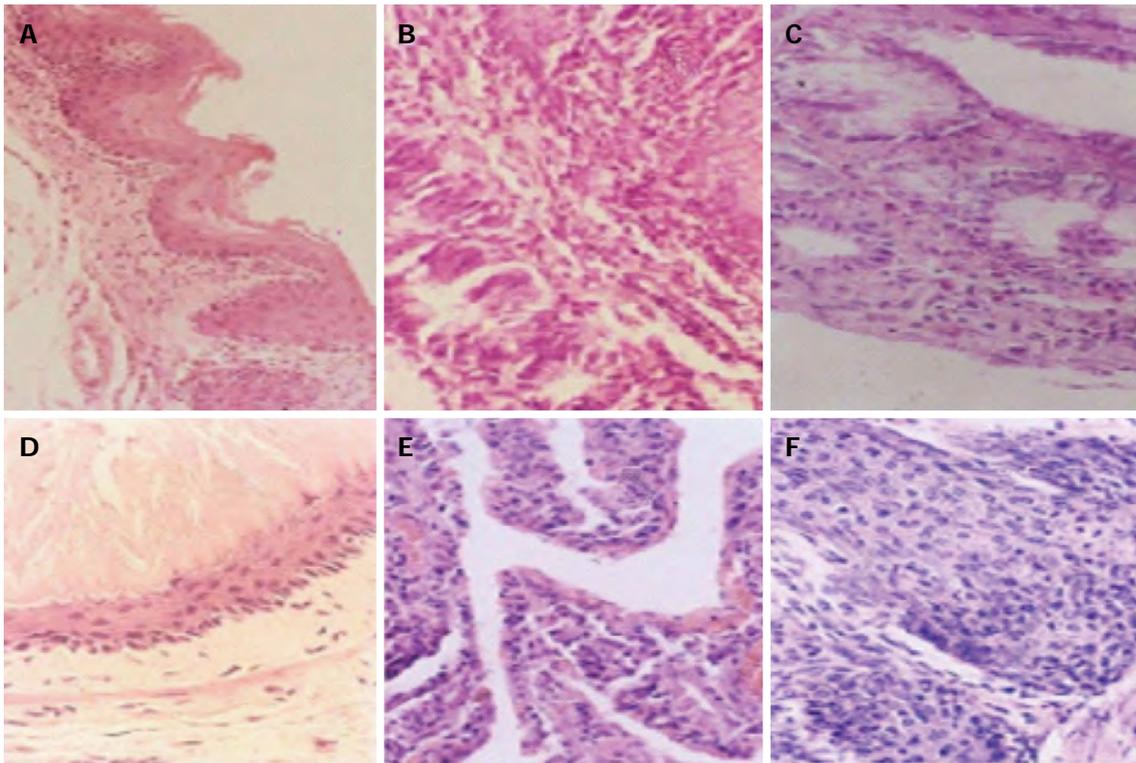


Figure 2 Changes in the esophagus and forestomach mucosa in the sham-operated and model groups under light microscope (200×). A: Normal esophagus in the sham-operated group; B: Esophagitis and Barrett's esophagus in the model group; C: Intestinal metaplasia with dysplasia and esophageal adenocarcinoma in the model group; D: Normal forestomach in the sham-operated group; E, F: Inflammation in the forestomach of the model group.

Table 2 Incidence of inflammation, intestinal metaplasia, intestinal metaplasia with dysplasia, and adenocarcinoma in the esophagus and forestomach 40 wk after surgery *n* (%)

| | Inflammation | Intestinal metaplasia | Intestinal metaplasia with dysplasia | Adenocarcinoma |
|--------------------------------------|--------------|-----------------------|--------------------------------------|----------------|
| Control esophagus (<i>n</i> = 29) | 2 (6.8) | 1 (3.4) | 0 | 0 |
| Model esophagus (<i>n</i> = 25) | 24 (96) | 22 (88) | 5 (20) | 2 (8) |
| Control forestomach (<i>n</i> = 29) | 2 (6.8) | 1 (3.4) | 0 | 0 |
| Model forestomach (<i>n</i> = 25) | 25 (100) | 2 (8) | 0 | 0 |

dular portions separated by the limiting ridge. The forestomach, which is the proximal compartment of the stomach in many animal species, is especially well developed in rats. Its function is storage and predigestion of food, and histologically it is covered with esophageal-type mucosa; thus, the rodent forestomach is considered to be a dilation of the lower esophagus. In small laboratory rodents that are commonly used for carcinogenicity studies (rats, mice and hamsters), the forestomach comprises about 50% of the gastric surface^[16].

The issues discussed above raise obvious questions about the predictive value of forestomach carcinogenesis. Indeed, the chronic animal study is regarded as the most predictive test for carcinogenicity in humans, and anatomical or physiological interspecies differences that might result in different tumor patterns are generally tolerated without seriously affecting the weight of evidence^[17]. It would be provocative to consider the forestomach as a model for the human esophagus from

anatomical and histological points of view; especially for studying the mechanism of action of human esophageal cancer^[18]. However, several attempts to demonstrate similar reactivity for the esophageal and forestomach mucosa in various species have been unsuccessful.

GERD refers to conditions in which gastric and duodenal contents are regurgitated into the esophagus, which causes pathological mucosal lesions and esophageal changes^[19]. Gastroesophageal reflux could cause EAC. The incidence rate of the latter has increased significantly in recent years and has taken the lead among all tumors^[20]. The yearly increase in the incidence of GERD has been accompanied with an increasing trend in the incidence of EAC. Clinical epidemiology has shown that gastroesophageal reflux correlates closely with EAC^[21].

The mechanism of induction of EAC by gastroesophageal reflux has been a hot research topic^[22]. Recent studies have shown that reflux of both gastric and duodenal juice can damage the esophageal mucosa^[23]. How-

ever, which contents are related to induction of EAC by gastroesophageal reflux is still controversial^[24].

Gastric acid is considered to be an important factor in GERD^[25]. Gastric acid and duodenal juice reflux is the main cause of GERD, and gastric acid has always been regarded as the major risk element in GERD; the main clinical treatment of which is acid suppression^[5]. However, because of the rapid increase in the incidence of EAC, the role of gastric acid in the development of GERD remains controversial. Proton-pump inhibitors (PPIs) have not prevented recent increases in EAC^[26]. Three large studies have examined PPI usage and EAC risk in Barrett's esophagus patients; each reporting a strong inverse correlation. Two studies have shown a decreased risk with longer duration of PPIs, and one an increased risk with delayed PPI use^[27].

We investigated the effects of gastric acid on intestinal metaplasia with dysplasia and malignant transformation of stratified squamous epithelium in the forestomach to study the specific factors involved in the induction of EAC through the surgical establishment of an animal model of duodenogastric reflux.

Surgical establishment of a duodenogastroesophageal reflux rat model showed that the forestomach developed abnormal changes. Most of the lesions were inflammatory (including mucosal damage); very few had intestinal metaplasia, and none had intestinal metaplasia with dysplasia or adenocarcinoma. As a result of surgical retention of the vagus nerve to maintain gastric acid secretion, the pH value in the forestomach was unchanged after the operation. The absence of bile detection explained why there was no obvious duodenal juice reflux in the forestomach. These results indicate that the simple lack of food and long-term stimulation of gastric juice might not cause the stratified squamous epithelium of intestinal metaplasia with dysplasia or adenocarcinoma. The histological structure of the forestomach and esophagus of the rat is the same: both comprise stratified squamous epithelial cells. It can be concluded that long-term stimulation by gastric juice of esophageal stratified squamous epithelial cells may only cause inflammation, mucosal damage, and mild intestinal metaplasia, but no induction of intestinal metaplasia with dysplasia or adenocarcinoma.

However, due to the small sample size of the study, the observation time was short, and there may be some limitations to the results. Nevertheless, the result provides new ideas and methods for the pathogenesis of EAC.

COMMENTS

Background

The incidence of esophageal adenocarcinoma (EAC) is currently rising faster than any other cancers in the Western world, although the cause of this increase is largely unknown. However, the relationship between the specific reflux components and the induction of EAC remains unclear.

Research frontiers

Gastroesophageal reflux can cause EAC, and the mechanisms have been the subject of extensive research. The specific gastroesophageal reflux components responsible for EAC remain largely unknown. In this study, the authors demonstrated that stimulation of long-term gastric juice on esophageal stratified

squamous epithelial cells may only cause inflammation, mucosal damage, and mild intestinal metaplasia, but no induction of intestinal metaplasia with dysplasia or adenocarcinoma.

Innovations and breakthroughs

Recent reports have highlighted the importance of duodenal juice in the pathogenesis of EAC. This study indicates that forestomach gastric juice exposure does not cause adenocarcinogenesis. The results of this study therefore suggest that gastric juice plays no role in the pathogenesis of EAC.

Applications

By understanding of the roles of gastric juice in the pathogenesis of EAC, this study may represent a future strategy for therapeutic intervention in the treatment of patients with EAC.

Terminology

Metaplasia is the reversible replacement of one differentiated cell type with another mature differentiated cell type. Dysplasia is an expansion of immature cells, with a decrease in the number and location of mature cells. Duodenogastric reflux is esophagus exposure to gastric and duodenal juice.

Peer review

The manuscript proposes interesting aspects of the development of EAC (Barrett's esophagus and carcinoma), although contradictory to the current and past literature.

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Endoscopic papillary balloon intermittent dilatation and endoscopic sphincterotomy for bile duct stones

Bai-Qing Fu, Ya-Ping Xu, Li-Sheng Tao, Jun Yao, Chun-Suo Zhou

Bai-Qing Fu, Ya-Ping Xu, Li-Sheng Tao, Jun Yao, Chun-Suo Zhou, Department of Gastroenterology, the People's Hospital, Affiliated to Jiangsu University, Zhenjiang 212002, Jiangsu Province, China

Author contributions: Xu YP designed the research; Yao J and Zhou CS performed the research; Tao LS analyzed the data; Fu BQ wrote the paper.

Correspondence to: Ya-Ping Xu, Chief Physician, Department of Gastroenterology, the People's Hospital, Affiliated to Jiangsu University, 8 Dianli Road, Zhenjiang 212002, Jiangsu Province, China. yapingxu@yeah.net

Telephone: +86-511-88915641 Fax: +86-511-85234387

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Abstract

AIM: To compare the effectiveness and safety of endoscopic papillary balloon intermittent dilatation (EPBID) and endoscopic sphincterotomy (EST) in the treatment of common bile duct stones.

METHODS: From March 2011 to May 2012, endoscopic retrograde cholangiopancreatography was performed in 560 patients, 262 with common bile duct stones. A total of 206 patients with common bile duct stones were enrolled in the study and randomized to receive either EPBID with a 10-12 mm dilated balloon or EST (103 patients in each group). For both groups a conventional reticular basket or balloon was used to remove the stones. After the procedure, routine endoscopic nasobiliary drainage was performed.

RESULTS: First-time stone removal was successfully performed in 94 patients in the EPBID group (91.3%) and 75 patients in the EST group (72.8%). There was no statistically significant difference in terms of operation time between the two groups. The overall incidence of early complications in the EPBID and EST groups was 2.9% and 13.6%, respectively, with no deaths reported during the course of the study and

follow-up. Multiple regression analysis showed that the success rate of stone removal was associated with stone removal method [odds ratio (OR): 5.35; 95%CI: 2.24-12.77; $P = 0.00$], the transverse diameter of the stone (OR: 2.63; 95%CI: 1.19-5.80; $P = 0.02$) and the presence or absence of diverticulum (OR: 2.35; 95%CI: 1.03-5.37; $P = 0.04$). Postoperative pancreatitis was associated with the EST method of stone removal (OR: 5.00; 95%CI: 1.23-20.28; $P = 0.02$) and whether or not pancreatography was performed (OR: 0.10; 95%CI: 0.03-0.35; $P = 0.00$).

CONCLUSION: The EPBID group had a higher success rate of stone removal with a lower incidence of pancreatitis compared with the EST group.

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Key words: Endoscopic papillary balloon dilatation; Endoscopic retrograde cholangiopancreatography; Endoscopic sphincterotomy; Common bile duct stones; Success rate

Core tip: Previous studies have shown that endoscopic papillary balloon dilatation with a 8 mm dilated balloon and endoscopic sphincterotomy (EST) have similar success rates in terms of stone removal. The incidence of postoperative pancreatitis with these procedures is high, so its application is limited. We compared the safety and efficacy of endoscopic papillary balloon intermittent dilatation, with an increase in dilated balloon diameter (10-12 mm) and extended dilatation time, and EST in the treatment of common bile duct stones (transverse diameter ≤ 12 mm).

Fu BQ, Xu YP, Tao LS, Yao J, Zhou CS. Endoscopic papillary balloon intermittent dilatation and endoscopic sphincterotomy for bile duct stones. *World J Gastroenterol* 2013; 19(15): 2425-2432 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v19/i15/2425.htm> DOI: <http://dx.doi.org/10.3748/wjg.v19.i15.2425>

INTRODUCTION

Endoscopic retrograde cholangiopancreatography (ERCP) has gradually replaced a surgical operation and become the preferred method for the treatment of common bile duct stones because of its minimal invasiveness and low cost. There are two main ERCP methods for treating choledocholithiasis: endoscopic papillary balloon dilatation (EPBD) and endoscopic sphincterotomy (EST). Previous studies have shown that EPBD with an 8 mm dilated balloon and EST have similar success rates in terms of stone removal. The incidence of postoperative pancreatitis with these procedures is high^[1], so their application is limited. In recent years, some studies showed that the incidence of postoperative pancreatitis was reduced when the dilatation diameter was larger and the dilatation time was extended during EPBD^[2,3]. However, few studies have compared EST and EPBD which had an increased dilatation diameter and extended dilatation time. We compared the safety and efficacy of endoscopic papillary balloon intermittent dilatation (EPBID) and EST in the treatment of common bile duct stones (transverse diameter ≤ 12 mm) after increasing the dilated balloon diameter (10-12 mm) and extending the dilatation time. This study was conducted at the People's Hospital affiliated to Jiangsu University. The study was approved by the hospital's ethics committee.

MATERIALS AND METHODS

Clinical data

From March 2011 to May 2012, ERCP was performed at our hospital on common bile duct stones and a medical X-ray gauge (Philips EasyDiagnost 4.0) was used to measure the transverse diameter of stones. During this period, 206 consecutive patients (97 male, 109 female) with a stone transverse diameter of ≤ 12 mm were identified. Patient age ranged from 15 to 93 years (median: 61 years). Patients who had previously undergone EPBD or EST for stone removal, distal common bile duct stenosis, stones with transverse diameters greater than 12 mm, severe coagulation dysfunction, hepatobiliary and/or pancreatic duct malignant tumor, or calculus incarceration in the duodenal papilla were excluded from the study.

According to the requirement of randomized controlled trials, the treatment schemes were randomly generated and put into sealed capsules. After the bile duct cannula was successfully performed, the researchers randomly chose a capsule to allocate patients into the EPBID or EST group. Neither the patients nor the endoscopy doctors were aware of the treatment option the patient would receive. There were specialized researchers observing and recording the experimental procedures to ensure that the experiment was conducted according to the appropriate procedures.

EPBID and EST procedures

Equipment included an Olympus JF-240/TJF-240 elec-

tronic duodenoscope, standard radiographic catheters, smart-type pulling papillotome, Boston 30 mm \times 10 mm dilating balloon (balloon length 30 mm, maximum dilated diameter 12 mm), ERBE200 high-frequency electrosurgical generator, mechanical lithotripsy basket, reticular basket and balloon catheter.

Preoperative preparations: all patients were asked to fast for 12 h prior to the start of the procedure. Scopolamine butylbromide (10 mg), dolantin (50 mg) and valium (10 mg) were injected intramuscularly 20 min before the start of the procedure to suppress intestinal peristalsis, alleviate pain and provide sedation. Lidocaine hydrochloride mucilage was used to provide local anesthesia of the throat.

EPBID procedure: The presence of common bile duct stones (maximum diameter less than or equal to 12 mm) was confirmed by cholangiography. Based on the stone size, the balloon was dilated to 10-12 mm using a pressure pump. The pressure was maintained for about 1 min and removed after 30 s. One minute pressure followed by 30 s relaxation was repeated two more times (total dilatation time: 3 min).

EST procedure: The presence of common bile duct stones (maximum diameter less than or equal to 12 mm) was confirmed by cholangiography. Endoscopic sphincterotomy was performed at an 11/12 o'clock position. The incision length (medium-large incision) was determined according to the stone size.

Steps performed similarly within the two groups: Use of a reticular basket or balloon catheter to remove the common bile duct stones, use of mechanical lithotripsy basket to break the stones or expand the incision length or increase the dilated balloon diameter if stone removal failed. If another failure occurred, the application of an indwelling nasobiliary tube and scheduling for ERCP on a different day, or a referral for a surgical operation was given. The nasobiliary tube was inserted into all patients after the procedure, and 3 d later nasobiliary duct radiography was performed to check for residual stones.

Observation indices

Operation time: The time taken from the beginning of papilla incision or dilatation until the end of stone removal.

Lithotomy success or failure: Complete removal of the stones using a conventional reticular basket or balloon was deemed a success. The following scenarios were all considered as failed lithotomy: removal of the stones through mechanical lithotripsy, expanding endoscopic incision or increasing the balloon dilatation diameter, referral for surgery, or identifying residual stones in postoperative nasobiliary drainage radiography.

Postoperative clinical symptoms and laboratory parameters: Postoperative clinical symptoms and labora-

Table 1 Comparison of characteristics of the two groups of patients

| Group | EPBID | EST | P value |
|-----------------------------------|---------------|---------------|---------|
| Male/female | 52/51 | 45/58 | 0.33 |
| Age (yr) | 61.83 ± 17.36 | 60.48 ± 14.69 | 0.55 |
| Common bile duct diameter (mm) | 12.74 ± 2.79 | 12.55 ± 3.05 | 0.65 |
| Transverse diameter of stone (mm) | 8.38 ± 2.67 | 7.71 ± 2.35 | 0.06 |
| No. of stones | 2.17 ± 1.43 | 1.89 ± 1.37 | 0.15 |

EPBID: Endoscopic papillary balloon intermittent dilatation; EST: Endoscopic sphincterotomy.

Table 2 Comparison of success rate and stone removal time between the two stone removal methods

| Group | EPBID | EST | P value |
|--------------------------------------|-------------|-------------|---------|
| Success rate of stone removal | 94/103 | 75/103 | 0.00 |
| Transverse diameter of stone < 10 mm | 53/54 | 55/71 | 0.00 |
| Transverse diameter of stone ≥ 10 mm | 41/49 | 20/32 | 0.03 |
| Operation time (min) | 9.86 ± 5.21 | 9.03 ± 4.92 | 0.29 |

EPBID: Endoscopic papillary balloon intermittent dilatation; EST: Endoscopic sphincterotomy.

tory parameters including abdominal pain, tenderness, nausea, vomiting, fever and melena 4, 12 and 24 h after the procedure, and blood amylase were recorded.

Follow up: One month after operation, the patients were followed up by telephone to check for any symptoms.

Assessment of complications

ERCP postoperative pancreatitis was diagnosed using the following criteria: No pancreatitis existed before the procedure; 4 h after the procedure, the blood amylase level was increased to over 3 times higher than the upper normal limit accompanied by abdominal pain, nausea and vomiting, fever or signs of peritoneal irritation and other clinical symptoms; or morphological changes of the pancreas were detected *via* imaging techniques.

Endoscopic bleeding observed: ERCP postoperative bleeding was defined as having hematemesis, melena and other clinical manifestations accompanied by a decrease in hemoglobin levels (at least 2 g/dL)^[4-7], after the exclusion of other possible causes for upper gastrointestinal bleeding. Endoscopic bleeding observed during the procedure was not considered as ERCP postoperative bleeding.

Other complications: Patients were considered to have biliary infections if they had a body temperature above 38 °C, right upper abdominal pain, and increased total leukocyte and neutrophil differential counts. Gastrointestinal perforation was diagnosed in the presence of abdominal pain and radiographic evidence.

Statistical analysis

Stata7.0 was used for statistical analysis. Measurement data

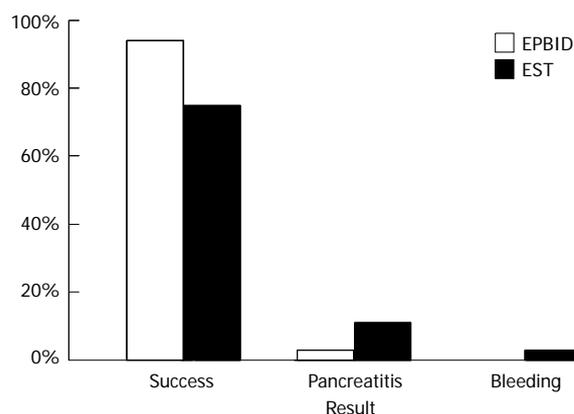


Figure 1 Comparison of case numbers of successful lithotomy, postoperative pancreatitis, and postoperative bleeding of the two groups. EPBID: Endoscopic papillary balloon intermittent dilatation; EST: Endoscopic sphincterotomy.

were expressed as mean ± SD and compared using the Student *t* test. Numerical data were compared using the χ^2 test. Logistic regression analysis was applied to evaluate the success rate of the procedures and the incidence rates of complications. Regression analysis was performed to determine any correlations of success rate of the procedures, postoperative pancreatitis and postoperative bleeding with respect to sex, age (< 60 and ≥ 60 group), presence of a diverticulum near papilla, diameter of the common bile duct (< 12 mm and ≥ 12 mm), maximum transverse diameter of the stone (< 10 mm and 10-12 mm), number of stones (1 or ≥ 2), application of pre-cut, stone removal methods, and use of pancreatography (if the guide wire was inserted into the bile duct more than 4 times it was considered as pancreatography). Statistical significance for all tests was set at $P < 0.05$.

RESULTS

Comparison between general data of the two groups of patients

The differences in sex, age, common bile duct diameter, transverse diameter of the stone, and number of stones between the two groups were not statistically significant ($P > 0.05$, Table 1).

Comparison between success rates of stone removal by the two methods

First-time stone removal was successfully carried out in 94 cases in the EPBID group (91.3%) and in 75 cases in the EST group (72.8%). The success rate of stone removal in the EPBID group was significantly higher than that of the EST group ($P < 0.05$, Figure 1; Table 2). For those with a transverse stone diameter of < 10 mm, the success rate of stone removal in the EPBID and EST groups was 53 out of 54 (98.1%), and 55 out of 71 (77.5%), respectively. The difference between the two groups was statistically significant ($P < 0.05$, Table 2). For the group of patients with stone transverse diameters of 10-12 mm, the stone-removal success rate of the EPBID

Table 3 Comparison of postoperative complications

| Group | EPBID | EST | P value |
|--------------------------------------|-------|-------|---------|
| Pancreatitis | 3/88 | 11/88 | 0.03 |
| Transverse diameter of stone < 10 mm | 0/43 | 7/61 | 0.04 |
| Transverse diameter of stone ≥ 10 mm | 3/45 | 4/27 | 0.41 |
| Bleeding | 0/103 | 3/103 | 0.25 |

EPBID: Endoscopic papillary balloon intermittent dilatation; EST: Endoscopic sphincterotomy.

and EST groups was 41 out of 49 (83.7%) and 20 out of 32 (62.5%), respectively, with statistical significance ($P < 0.05$, Table 2) favoring the EPBD group.

Comparison between the operation times of the two successful stone removal methods

The stone removal time for the EPBD and EST groups were 9.86 ± 5.21 min and 9.03 ± 4.92 min, respectively, and showed no significant difference ($P > 0.05$, Table 2).

Outcome after failure of the two stone removal methods

In the EPBID group a total of 9 cases failed of which 3 were transferred for surgical operations; 3 were referred for mechanical lithotripsy, and 2 underwent an increase in dilated balloon diameter. Radiography found residual stones in one patient in the EPBID group who subsequently underwent ERCP a second time.

In the EST group a total of 28 cases failed, of which 2 were transferred to surgical operations, 3 underwent enlargement of the incision, 9 were transferred to receive balloon dilatation, and 4 received mechanical lithotripsy. Through radiography, residual stones in 10 patients of the EST group were found, for which the ERCP procedure was performed a second time.

Comparison of postoperative complications

In the EPBID and EST groups there were 88 cases without pancreatitis before the operation. Three patients in the EPBID group reported postoperative pancreatitis while 11 patients in the EST group reported postoperative pancreatitis. All the cases of pancreatitis were mild. The incidence of pancreatitis in the EPBID group was statistically significantly lower than in the EST group (3.4% *vs* 12.5%; $P < 0.05$; Figure 1; Table 3). For the group of patients who had stones with a transverse diameter < 10 mm, the incidence of pancreatitis in the EPBID and EST groups was 0% (0 out of 43) and 11.5% (7 out of 61), respectively, and showed a clinically significant difference favoring the EPBID group ($P < 0.05$, Table 2). For the group of patients with stones of a transverse diameter of 10-12 mm, the incidence of pancreatitis in the EPBID group was 6.7% (3 out of 45) and the incidence of pancreatitis in the EST group was 14.8% (4 out of 27), with no significant difference ($P > 0.05$, Table 2). Also, no significant difference was seen between the 2 groups in terms of the incidence of postoperative bleeding (0% in EPBID group and 2.9% in EST group; $P > 0.05$, Figure 1; Table

3). Overall, no perforations and postoperative cholangitis was observed in any patient. The doctors evaluating the postoperative complications of the patients did not know which treatment methods had been used for the treatment of each particular patient.

The success rate of stone removal was related to the stone removal method, the presence or absence of papilla diverticulum, and the maximum transverse diameter of the common bile duct stones (< 10 mm *vs* 10-12 mm group) ($P < 0.05$, Tables 4 and 5). Postoperative pancreatitis was related to whether pancreatography was performed, and the method used for stone removal ($P < 0.05$, Tables 6 and 7). This study did not identify risk factors related to postoperative bleeding.

DISCUSSION

The ERCP procedure has become the main method for the treatment of common bile duct stones because it is less invasive and has a lower cost than EST. Kawai *et al.*^[8] first reported on EST; since then, EST has been widely accepted as an option in the clinical management of the disease and has gradually replaced surgical operations. However, since EST causes damage to the papilla sphincter, it is generally believed to have a negative lasting impact on papilla sphincter function. In 1983, Staritz *et al.*^[9] introduced EPBD for the first time. Since EPBD does not require sphincterotomy, it is believed that EPBD can better protect sphincter function compared with EST, whereas EST results in a permanent loss of sphincter function^[10,11]. However, Takezawa *et al.*^[12] suggested that the difference between the two methods in protecting papilla sphincter function is not statistically significant. Both domestic and international research studies showed that, compared with EPBD, EST had a higher stone recurrence rate^[13-15]. Most recently, a method that uses larger dilated balloons (EPLBD) that requires a small incision has been performed for larger stones^[16-21]. Kim *et al.*^[18] showed that for the stones with a transverse diameter greater than 10 mm, the first-time success rate of stone removal by EPLBD is higher than that of simple sphincterotomy. In addition, EPBLD can reduce the probability of mechanical lithotripsy with no additional incidence of complications.

Yu *et al.*^[22] indicated that the difference in the success rate for stone removal between EST and EPBD was not statistically significant. Their overall success rate of stone removal for EST and EPBD was 97.5% and 98.1% respectively (first-time success rate of 70% and 65%, respectively). In our study however, the success rate of stone removal of the EPBID group (91.3%) was significantly higher than that of the EST group (72.8%). Conventional EPBD usually uses smaller dilated balloon diameters (around 8 mm) and a short dilatation time. However, in this investigation, the dilatation diameter was larger, and the dilatation time was extended making the pathway of stone removal wider and leading to a higher success rate of stone removal. Multiple regression analysis showed that the success rate of stone removal was

Table 4 Clinical factors and success rate of stone removal

| Factor | Sample size | Success | Sample size | Failure | OR | P value |
|--------------------------------------|-------------|---------------|-------------|---------------|------|---------|
| Age (yr) | 169 | 60.30 ± 16.62 | 37 | 64.60 ± 12.81 | 1.31 | 0.14 |
| Sex | | | | | 1.59 | 0.21 |
| Male | 83 | 49.11% | 14 | 37.84% | | |
| Female | 86 | 50.89% | 23 | 62.16% | | |
| Papilla diverticulum | | | | | 2.25 | 0.03 |
| Yes | 36 | 21.30% | 14 | 37.84% | | |
| No | 133 | 78.70% | 23 | 62.16% | | |
| Papilla pre-cut | | | | | 0.91 | 0.93 |
| No | 164 | 97.04% | 36 | 97.30% | | |
| Yes | 5 | 2.96% | 1 | 2.70% | | |
| Performance of pancreatography | | | | | 0.50 | 0.18 |
| Yes | 15 | 8.88% | 6 | 16.22% | | |
| No | 154 | 91.12% | 31 | 83.78% | | |
| Stone removal method | | | | | 3.90 | 0.00 |
| EPBD | 94 | 55.62% | 9 | 24.32% | | |
| EST | 75 | 44.38% | 28 | 75.68% | | |
| Common bile duct diameter | 169 | 12.38 ± 2.49 | 37 | 13.86 ± 4.21 | 3.26 | 0.00 |
| Common bile duct stone number | 169 | 2.02 ± 1.43 | 37 | 2.08 ± 1.32 | 1.65 | 0.82 |
| Maximum transverse diameter of stone | 169 | 7.81 ± 2.53 | 37 | 9.11 ± 2.28 | 2.08 | 0.00 |

EPBD: Endoscopic papillary balloon intermittent dilatation; EST: Endoscopic sphincterotomy; OR: Odds ratio.

Table 5 Multiple regression model to predict the success rate of stone removal

| Factors | β | SE | P value | OR | 95%CI |
|-------------------------------|---------|------|---------|------|------------|
| Stone removal method | 1.68 | 0.44 | 0.00 | 5.35 | 2.24-12.77 |
| Whether there is diverticulum | 0.85 | 0.42 | 0.04 | 2.35 | 1.03-5.37 |
| Stone transverse diameter | 0.97 | 0.40 | 0.02 | 2.63 | 1.19-5.80 |
| Constant term | -3.22 | 0.48 | 0.00 | | |

OR: Odds ratio.

related to the method of stone removal, the transverse diameter of the stones and the presence or absence of diverticulum. Often, when the transverse diameter of a stone is larger it is more difficult to remove. In these cases, mechanical lithotripsy, enlargement of the incision or increasing the dilated balloon diameter may be required. In this investigation, no significant difference between the two groups was seen in terms of stone removal time.

Liu *et al*^[1] indicated that EPBD had a much lower incidence of postoperative bleeding than EST, but its incidence of postoperative pancreatitis was significantly higher than that of EST. The mechanism of the development of EPBD-induced postoperative pancreatitis is thought to be due to compartment syndrome. Two hours after EPBD procedures, the papilla develops inflammatory edema. The papillary sphincter does not relax sufficiently, which restricts the expansion of the internal contents and results in compartment syndrome. This finally leads to poor drainage of pancreatic fluid and postoperative pancreatitis^[2,23]. We believe that if the balloon diameter is small in EPBD, bile will not be easily discharged and would flow back to the pancreatic duct, thus increasing the risk of pancreatitis; in addition, the smaller the dilatation diameter, the slower the removal of the stone. The reticular basket or balloon is required to

be repeatedly moved forward and backward through the papilla passage, which could induce further tissue edema. In our investigation, by increasing the dilated balloon diameter, and the time of intermittent dilatation (the total time of dilatation was three minutes), the papilla sphincter was torn apart and fully relaxed, alleviating papillary edema and reducing the obstruction of the pancreatic duct. Therefore, postoperative bile excretion was easier and reduced the risk of postoperative pancreatitis.

The results of this study also suggest that the risk of postoperative pancreatitis is related to whether pancreatography was performed as well as the method of stone removal. In the course of pancreatography, contrast medium bubbles enter the pancreatic duct, and the internal pressure of the pancreatic duct increases making the pancreas prone to inflammation. If the guidewire repeatedly comes into the pancreatic duct it is highly likely to cause edema around the pancreatic duct orifice and would then contribute to postoperative pancreatitis. Ueki *et al*^[24] showed that postoperative pancreatitis was related to pre-cut sphincterotomy (PST) because cannulation was often difficult in patients who received PST, and their operation time was longer, causing papillary edema. In this investigation, there were a few cases of PST and the difference in the incidence of pancreatitis between the groups had no statistical significance. The results of this study showed that in the EPBD group, 3 cases of postoperative pancreatitis occurred in those patients who had stones of < 10 mm in diameter. The development of pancreatitis was more common in the patients of the EST group who had stones of over 10 mm in diameter. However, because the sample size was small, statistical significance could not be shown. Previous studies suggest that the possibility of EST postoperative bleeding was between 2.5% and 5%^[6]. In this investigation the EST group had 3 patients who suffered from complicated postoperative bleeding

Table 6 Clinical factors and incidence of postoperative pancreatitis

| Factor | Sample size | No pancreatitis | Sample size | Pancreatitis | OR | P value |
|--------------------------------|-------------|-----------------|-------------|---------------|------|---------|
| Age | 162 | 60.33 ± 16.17 | 14 | 64.29 ± 14.58 | 1.09 | 0.38 |
| Sex | | | | | 1.51 | 0.47 |
| Male | 74 | 45.68% | 5 | 35.71% | | |
| Female | 88 | 54.32% | 9 | 64.29% | | |
| Papilla diverticulum | | | | | 1.75 | 0.34 |
| Yes | 39 | 24.07% | 5 | 35.71% | | |
| No | 123 | 75.93% | 9 | 64.29% | | |
| Papilla pre-cut | | | | | 8.83 | 0.05 |
| No | 159 | 98.15% | 12 | 85.71% | | |
| Yes | 3 | 1.85% | 2 | 14.29% | | |
| Performance of pancreatography | | | | | 0.12 | 0.00 |
| Yes | 13 | 8.02% | 6 | 42.86% | | |
| No | 149 | 91.98% | 8 | 57.14% | | |
| Stone removal method | | | | | 4.05 | 0.03 |
| EPBD | 85 | 52.47% | 3 | 21.43% | | |
| EST | 77 | 47.53% | 11 | 78.57% | | |
| Common bile duct diameter | 162 | 12.64 ± 2.98 | 14 | 13.86 ± 2.98 | 2.53 | 0.15 |
| Common bile duct stone number | 162 | 2.09 ± 1.46 | 14 | 1.43 ± 0.85 | 0.46 | 0.10 |
| Transverse diameter of stone | 162 | 8.17 ± 2.45 | 14 | 8.29 ± 2.64 | 1.49 | 0.86 |

EPBD: Endoscopic papillary balloon intermittent dilatation; EST: Endoscopic sphincterotomy; OR: Odds ratio.

Table 7 Multiple regression model to predict the incidence of postoperative pancreatitis

| Factor | β | SE | P value | OR | 95%CI |
|--------------------------------|---------|------|---------|------|------------|
| Performance of pancreatography | -2.35 | 0.66 | 0.00 | 0.10 | 0.03-0.35 |
| Stone removal method | 1.61 | 0.71 | 0.02 | 5.00 | 1.23-20.28 |
| Constant term | -1.66 | 0.69 | 0.02 | | |

OR: Odds ratio.

while no patient in the EPBD group experienced complicated postoperative bleeding; however, due to the small sample size there was no statistical significance. There were no cases of duodenal perforations, cardiovascular accidents or other complications.

This study was different because of the increase in the dilated balloon diameter in EPBD and of the extended time of intermittent dilatation. The first 1-min balloon dilatation tore apart the papilla sphincter; the second and the third 1-min balloon dilatations compressed the bleeding caused by the first dilatation tear and further tore the papilla sphincter, while avoiding long-time compression of the pancreatic duct orifice. This was intended to remove the stone and reduce the incidence of postoperative pancreatitis. Results of this study indicate that by increasing the balloon dilatation diameter and extending the dilatation time in EPBD, bile duct stones with transverse diameters smaller than 12 mm could be removed in a quicker, more effective way compared with EST. The use of EPBD can also reduce significantly the incidence of postoperative pancreatitis. Bang *et al.*^[25] showed that continuous endoscopic dilatations for 20 s and 60 s were not significantly different in the success rate of stone removal and in the incidence of postoperative pancreatitis. We believe that extending the papillary dilatation time to 60 s is still insufficient to fully clear the passage. Liao *et al.*^[3]

showed that continuous endoscopic dilatation for 5 min, rather than 1 min, resulted in a higher success rate of stone removal, and significantly decreased the incidence of postoperative pancreatitis. The results of this study showed that the EPBD group had a success rate of up to 98.1% for the removal of stones of a maximum transverse diameter of 10 mm. Therefore, properly increasing the dilatation diameter and extending the dilatation time can contribute to a higher success rate in stone removal and lower the risk of postoperative pancreatitis. However, it should be noted that an excessive increase in the diameter of balloon dilatation could increase the chance of perforation. Theoretically speaking, the balloon dilatation diameter determines the size of the stones removed, and the method of using a 10 mm-diameter balloon for continuous dilatation for 5 min adopted by Liao *et al.*^[3] applies to stones with a size of 15 mm. Therefore, further research is needed to provide more details on the optimal dilatation time and diameter as well as their relationship to the transverse diameter of the stone.

COMMENTS

Background

Cholelithiasis is a common disease in the gastroenterology clinic, and can cause biliary obstruction, acute obstructive suppurative cholangitis, acute pancreatitis, hepatic failure, gallstone shock, even threaten patient's life. Endoscopic retrograde cholangiopancreatography (ERCP) has gradually replaced surgery and become the preferred method for the treatment of common bile duct stones because of its minimal invasiveness and low cost. There are two main ERCP methods for treating cholelithiasis: endoscopic papillary balloon dilatation (EPBD) and endoscopic sphincterotomy (EST). Since EPBD does not require sphincterotomy, it is believed that EPBD can protect sphincter function better than EST.

Research frontiers

Previous studies have shown that EPBD with a 8 mm dilated balloon and EST have similar success rates in terms of stone removal. The incidence of postoperative pancreatitis with these procedures is high, so its application is

limited. In recent years, some studies showed that the incidence of postoperative pancreatitis could be reduced when the dilatation diameter was larger and the dilatation time was extended during EPBD. Recent studies indicated that a small incision plus endoscopic papillary balloon intermittent dilatation (EPBID) had more advantages over EST in treating larger diameter (> 15 mm) common bile duct stones.

Innovations and breakthroughs

There are few studies focusing on the comparison of EST and EPBID with increased dilatation diameter and extended dilatation time. We compared the safety and efficacy of EPBID and EST in the treatment of common bile duct stones (transverse diameter \leq 12 mm) after increasing the dilated balloon diameter (10-12 mm) and extending the dilatation time. The advantages of EPBID are larger dilatation diameter, longer dilatation time, less bleeding.

Applications

EPBD have several advantages over EST, such as no incision, lower operational difficulty, less bleeding and less perforation, therefore EPBD would be suitable for novices to remove the common bile duct stones.

Terminology

EPBID: Duodenal side mirrors was inserted into the duodenal papilla, then the guide wire was inserted into bile duct, and balloon catheter was put in common bile duct, the balloon was dilated 10 to 12 mm using a pressure pump. The pressure was maintained for about 1 min and removed for 30 s. One-minute pressure followed by the 30 s relaxation was repeated two more times (total dilatation time: 3 min). Then the common bile duct stones were removed by using the reticular basket or the balloon catheter.

Peer review

This is an interesting manuscript comparing EPBID and EST for normal size stones. It has an important impact on the further expansion of EPBID as a technology for remove of bile duct stones.

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Dysphagia lusoria: A late onset presentation

Alice Louise Bennett, Charles Cock, Richard Heddle, Russell Kym Morcom

Alice Louise Bennett, Department of Gastroenterology and Hepatology, Flinders Medical Centre, South Australia 5042, Australia
Charles Cock, Richard Heddle, Department of Gastroenterology and Hepatology, Repatriation General Hospital, South Australia 5042, Australia

Russell Kym Morcom, Department of Radiology, Repatriation General Hospital, South Australia 5042, Australia

Author contributions: Bennett AL and Cock C were involved in the conception of the paper, data acquisition and analysis and coordinated the writing of the manuscript; Heddle R participated in the data acquisition and analysis, and contributed to the writing of the manuscript; Morcom RK participated in the data acquisition; Bennett AL, Cock C and Heddle R read and approved the final version of the manuscript.

Correspondence to: Dr. Alice Louise Bennett, Department of Gastroenterology and Hepatology, Flinders Medical Centre, Flinders Drive Bedford Park, South Australia 5042, Australia. alicebennett14@hotmail.com

Telephone: +61-8-82044964 Fax: +61-8-82042943

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Core tip: Dysphagia lusoria is a term used to describe dysphagia as a consequence of vascular compression of the oesophagus. Our case describes a rare anatomical variant of a right-sided aortic arch with aberrant left subclavian artery with late onset dysphagia. Manometric studies were abnormal with solid bolus, likely contributing to the worsening of the patient's symptoms over time. The patient is managing to maintain weight and nutrition through dietary modification and no operative intervention is currently planned.

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Abstract

Dysphagia lusoria is a term used to describe dysphagia secondary to vascular compression of the oesophagus. The various embryologic anomalies of the arterial brachial arch system often remain unrecognised and asymptomatic, but in 30%-40% of cases can result in tracheo-oesophageal symptoms, which in the majority of cases manifest as dysphagia. Diagnosis of dysphagia lusoria is *via* barium swallow and chest Computed tomography scan. Manometric abnormalities are variable, but age-related manometric changes may contribute to clinically relevant dysphagia lusoria in patients who present later in life. Our report describes a case of late-onset dysphagia secondary to a right aortic arch with an aberrant left subclavian artery, which represents a rare variant of dysphagia lusoria. The patient had proven additional oesophageal dysmotility with solid bolus only and a clinical response to dietary modification.

INTRODUCTION

Dysphagia lusoria is a term used to describe dysphagia as a consequence of vascular compression of the oesophagus. Bayford coined the term itself meaning "freak or jest of nature" in 1761 in describing a case of longstanding dysphagia leading to emaciation and eventual death of a 62-year old female patient. On autopsy the patient was found to have an aberrant right subclavian artery (ARSA) running anterior to and causing compression of her oesophagus^[1]. The majority of cases of dysphagia lusoria are due to ARSA causing posterior oesophageal compression; yet only 20%-40% of aberrant arteries are thought to cause tracheo-oesophageal symptoms including dysphagia^[2,3]. In patients who present at an advanced age, decreased vascular compliance is thought to be the most predominant factor; however the additional contribution

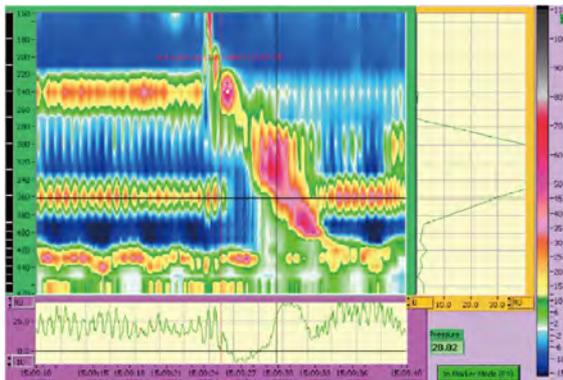


Figure 1 Manometry study demonstrating a high pressure band in the lower oesophagus (at the 360 mm mark/36 cm) consistent with arterial pulsations. This pressure band does not manometrically cause obstruction with a clear relaxation across the region of interest during the swallow.

of age-related oesophageal dysmotility to their symptoms needs to be considered. Our report describes a case of late-onset dysphagia secondary to a right aortic arch with an aberrant left subclavian artery, which represents a rare variant of dysphagia lusoria.

CASE REPORT

A 68-year-old male presented to the Gastroenterology Outpatient Department with a five-year history of dysphagia. The initial presenting symptom was that of intermittent dysphagia to solids, which had recently worsened and become more progressive in nature. The patient also reported a sensation of food sticking in the left side of his chest. There was no weight loss reported. Past medical history was noteworthy for well-controlled gastro-oesophageal reflux and cardiovascular disease including both ischaemic heart disease and peripheral vascular disease. Physical examination was unremarkable.

Initial investigations some 5 years ago included routine laboratory blood tests and chest X-ray, which were within normal limits. A barium swallow and modified barium swallow (MBS) were obtained suggestive of oesophageal dysmotility, which was treated with several empirical oesophageal dilations over the subsequent years with transient improvement. Throughout the MBS assessment the patient repeatedly cleared his throat and complained of perceived left-sided food residue in the absence of the actual presence of pharyngeal or oesophageal residue. Oesophageal manometry was normal for liquid swallows by Chicago criteria. Solid bolus swallows demonstrated shortened distal latency and a rapid contractile front velocity. The lower oesophageal sphincter relaxed appropriately with all swallows during manometry (Figure 1). A non-contrast computed tomography (CT) scan of the abdomen and chest was not reported as showing any abnormal pathology. A therapeutic trial of a proton pump inhibitor and domperidone was commenced, but failed to improve his symptoms.

Due to the progression of his symptoms, a repeat video-fluoroscopic barium swallow was performed. This



Figure 2 Barium oesophogram demonstrating the extrinsic impression on the oesophagus secondary to the aberrant left subclavian artery superiorly and right aortic arch distally.



Figure 3 Computed tomography chest demonstrating the right aortic arch and dilatation at the origin of the aberrant left subclavian artery (Kommerell's diverticulum).

demonstrated passage of barium freely through the pharynx and upper oesophagus (Figure 2). An extrinsic impression running obliquely across the upper oesophagus just below the level of the aortic arch and a right-sided aortic arch was noted. A contrast CT scan of the chest demonstrated an aberrant subclavian artery running posteriorly to the oesophagus, as well as a Kommerell's diverticulum at the origin of the attenuated vessel (Figure 3).

Given the patient's co-morbidities, age and considering the ability to maintain weight and nutrition, it was decided that the patient would not be a suitable candidate for vascular revision of his aberrant vessel and he was managed conservatively. Lifestyle and diet issues were addressed in conjunction with modification of atherosclerotic risk factors. It was thought that the progressive symptoms were likely due to a combination of the oesophageal dysmotility demonstrated with solids and atherosclerotic progression with vascular non-compliance.

DISCUSSION

ARSA is a common variant of embryonic aortic arch involution within the general population^[3]. This occurs as a consequence of a persistent 7th intersegmental artery with involution of the 4th vascular arch with the right

dorsal aorta^[4,5]. In the majority of cases the aberrant artery has a course posterior to the oesophagus, but in a small percentage may run anterior to the oesophagus, as in the original description of the syndrome by Bayford^[1]. At times a broad base, known as Kommerell's diverticulum, accompanies ARSA^[3]. In rare cases Kommerell's diverticulum may become aneurysmal with subsequent oesophageal compression and dysphagia^[6,7].

The prevalence in the general population of an aberrant subclavian artery is estimated at 0.4% to 0.7% in the majority of the published literature. A study by Haesemeyer *et al*^[8] found 29 cases in 7174 chest CT scans performed in trauma patients. Abhaichand *et al*^[9] found 14 in 3730 patients undergoing transradial coronary angiography. Fockens *et al*^[10] found 6 cases out of 1629 examined *via* endoscopic ultrasound. Kelly^[11] found a single case in 223 patients undergoing upper gastrointestinal endoscopy for dysphagia. Molz *et al*^[12] described a prevalence of 0.7% during autopsies. Aberrant subclavian arteries are present on routine endoscopy as a pulsatile posterior indentation in the upper oesophagus. However, endoscopic diagnosis is rare.

The majority of lesions are a right subclavian artery originating from the left-sided aortic arch. A similar abnormality can occur as a consequence of a left-sided aberrant subclavian artery with a right-sided aortic arch, although this arterial abnormality is much rarer^[13,14]. This anomaly develops when the right dorsal artery remains patent and either the left 4th arch or left dorsal aorta regress abnormally. A complete vascular ring is formed when the anomaly is associated with a left sided ductus arteriosus passing from the left subclavian artery to the proximal left pulmonary artery. 30%-40% of patients with vascular anomalies have dysphagia as a consequence, with the majority presenting with solid bolus dysphagia^[3]. Patients may at times report the dysphagia to be one-sided, such as in our case. However, these anomalies largely remain asymptomatic and are often an incidental finding on imaging.

Dynamic barium swallow studies, including assessment of solid bolus swallow, serve as diagnostic screening for dysphagia lusoria. CT chest or magnetic resonance imaging with vascular reconstruction are used to define the vascular lesion and plan surgical interventions. Manometry may show variable abnormalities and is not helpful in diagnosis. Manometric studies on six patients with dysphagia lusoria by Janssen *et al*^[2] showed abnormalities in five out of six patients with two studies showing diminished amplitude contractions, two showing a high pressure zone (increase in intrabolus pressure) above the aberrant artery and one a hypocontractile zone proximal to the aberrant artery. When dysphagia occurs with ageing it seems probable that non-specific age-related manometric abnormalities (such as an increased prevalence of peristaltic failure)^[2,15,16] may be contributory to dysphagia in these patients.

Several explanations exist for patients who present with late onset of symptoms. Motility abnormalities and

oesophageal stiffening, which occur as a consequence of ageing represent one possibility^[2]. Several additional mechanisms have been proposed, including atherosclerosis induced vascular changes leading to stiffening of the obstructing artery, aortic elongation with increased traction on the obstructing artery or aneurysmal dilatation in the presence of Kommerell's diverticulum^[2].

The management of patients with dysphagia lusoria is dependent on the degree of symptoms and impact on the ability of the patients to maintain their weight and nutrition. It would appear approximately half of patients can be managed through dietary modification and through eating slower and chewing well. Severe symptoms, not amenable to interventional dietary and swallowing strategies may warrant surgical treatment.

Gross^[17] first reported surgical management of this condition, describing the division and ligation of an aberrant right subclavian artery in a 4-mo old infant *via* left thoracotomy. Lichter^[18] described surgery on an adult patient. The most common approach to repair of a right sided aortic arch and aberrant left subclavian artery is a left postero-lateral thoracotomy followed by division of the ligamentum with dissection. This allows the mediastinal structures to be freed in order to assume a less constricting position^[19]. The decision to ligate or re-implant the aberrant vessel to avoid steal syndrome remains an intra-operative one.

Our case describes a rare anatomical variant of a right-sided aortic arch with aberrant left subclavian artery with late onset dysphagia. Manometric studies were abnormal with solid bolus, likely contributing to the worsening of the patient's symptoms over time. The patient is managing to maintain weight and nutrition through dietary modification and no operative intervention is currently planned.

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Fifteen-year-old colon cancer patient with a 10-year history of ulcerative colitis

Seung Yeon Noh, Seung Young Oh, Soo-Hong Kim, Hyun-Young Kim, Sung-Eun Jung, Kwi-Won Park

Seung Yeon Noh, Seung Young Oh, Department of Surgery, Seoul National University Hospital, Seoul 110-744, South Korea
Soo-Hong Kim, Hyun-Young Kim, Sung-Eun Jung, Kwi-Won Park, Department of Pediatric Surgery, Seoul National University Children's Hospital, Seoul 110-744, South Korea

Author contributions: Noh SY, Oh SY, Kim SH, Kim HY, Jung SE and Park KW contributed equally to this work; Noh SY, Oh SY, Kim SH, Kim HY, Jung SE and Park KW designed the research; Noh SY, Oh SY, Kim SH, Kim HY and Park KW performed the research; Noh SY and Oh SY wrote the paper.

Correspondence to: Kwi-Won Park, MD, PhD, Department of Pediatric Surgery, Seoul National University Children's Hospital, 101 Daehak-Ro Jongno-Gu, Seoul 110-744,

South Korea. pedsurg@snu.ac.kr

Telephone: +82-2-20723635 Fax: +82-2-7475130

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Core tip: Inflammatory bowel disease (IBD) is regarded as one of the important risk factors of colorectal cancer. Several cases of colorectal cancer with pediatric IBD have been reported. However, this case is noticeable in that the onset of disease in the patient was at a relatively young age and the duration of illness was rather short, even though the patient was given continuous medication and regular follow-ups. Therefore, this case highlights the importance the early diagnosis of the disease with a high level of awareness in children with a history of predisposing factors.

Noh SY, Oh SY, Kim SH, Kim HY, Jung SE, Park KW. Fifteen-year-old colon cancer patient with a 10-year history of ulcerative colitis. *World J Gastroenterol* 2013; 19(15): 2437-2440 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v19/i15/2437.htm> DOI: <http://dx.doi.org/10.3748/wjg.v19.i15.2437>

Abstract

Inflammatory bowel disease (IBD) is regarded as one of the risk factors for colorectal cancer, and early detection of cancer in these patients may be difficult, especially in pediatric patients. Prognosis of pediatric colorectal cancer is known to be poor, because of delayed diagnosis and unfavorable differentiation. We report a case of a pediatric patient with a 10-year history of ulcerative colitis who was diagnosed with sigmoid colon cancer when he was 15 years old. He underwent proctocolectomy with ileal pouch anal anastomosis. Postoperative pathological examination of the tumor revealed adenocarcinoma. The pericolonic tissue layer was infiltrated, but metastases were not found in either of the two lymph nodes. Children with a long history of predisposing factors such as IBD need particular attention to the possibility of colorectal cancer. Early diagnosis through regular screening with colonoscopy is one of the most important critical factors for a good prognosis.

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INTRODUCTION

Pediatric colorectal cancer has a poor prognosis compared with adult colorectal cancer because of delayed diagnoses at an advanced stage. Thus, early diagnosis based on a high degree of suspicion could be the most important factor in a more favorable prognosis, especially in patients with predisposing factors. Here, we report a 15-year-old boy with a 10-year history of ulcerative colitis (UC) who developed sigmoid colon cancer.

Pediatric colorectal cancer is very rare. The reported incidence is 0.3 to 2 cases per million, accounting for 0.4% of all fatal malignancies in patients younger than 15 years of age^[1-6]. According to some studies, most cases occur in the second decade of life^[1,7,8]. The sex distribution is equal in adults, whereas in children, a notable preponderance of boys has been reported^[2,7].

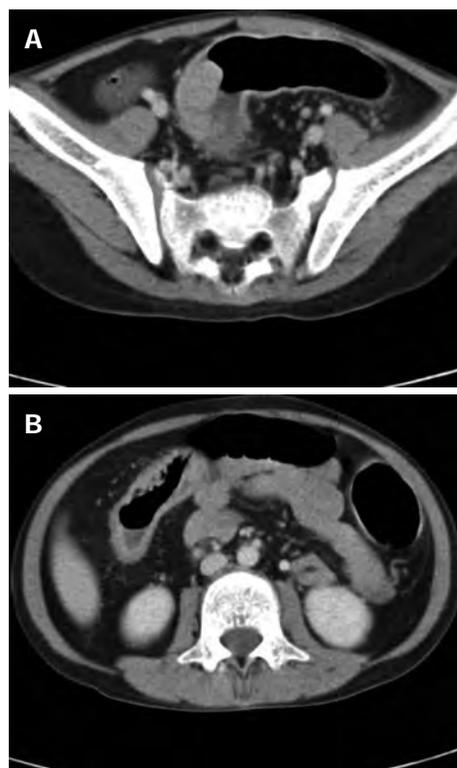


Figure 1 Preoperative computed tomography. A: Enhanced protruding polypoid mass at the sigmoid colon; B: diffuse colonic wall thickening with loss of haustra (“lead pipe appearance”).

Although colorectal cancer has a relatively good prognosis in adults, the overall reported survival of pediatric colorectal cancer is poorer than in adults^[5,9,10]. The most likely reason is delayed diagnoses with advanced stages of colorectal cancer and high potential for dissemination^[2,8,11]. The other reason for the poor prognosis of pediatric colorectal cancer is a high proportion of mucinous histology, accounting for more than 50% of the cases^[2,7,12].

CASE REPORT

A 15-year-old boy was transferred from another hospital because of abnormal computed tomography (CT) findings. He presented with abdominal pain, vomiting and poor oral intake for 1 wk before admission.

He had a very long and complicated past medical history. When he was 3 years old, he was treated for an anal fissure. Additionally, when he was 5 years old, he had bilateral knee joint pain with swelling, and a laboratory test was positive for antinuclear cytoplasmic antibody. Through a colon study and colonoscopic biopsy, he was finally diagnosed with UC. After the diagnosis, he was maintained on the combined medications of mesalazine, azathioprine and prednisolone for 5 years.

When he was 10 years old, he moved to another hospital which was in his home town. He had follow-ups through the hospital for 5 years. Then, he was admitted to that hospital for abdominal pain and vomiting, and his

CT scan showed suspected cancer lesions. Therefore, he was referred back to our institute for further evaluation.

Several clinical tests were performed after admission to evaluate the patient. The esophagogastroscopy did not show any abnormal findings. The abdominal CT showed a segmental polypoid mass at the sigmoid colon, which was consistent with cancer (Figure 1A). The finding of diffuse colonic wall thickening with a loss of haustra, called “lead pipe appearance”, which was consistent with UC, was also observed (Figure 1B).

The colonoscopy showed diffuse granular lesions in the ascending colon, multiple ulcerations from the transverse colon to rectum, and two polypoid masses without ulcerations in the descending and sigmoid colon (Figure 2).

The patient underwent surgery. In a digital rectal examination, we found a 6 cm nodular lesion at the level of the anal verge which was not found in colonoscopy. At the level of the sigmoid colon, a 3 cm-sized mass involving one-third of the lumen was found. Based on these findings, a total proctocolectomy with ileal pouch anal anastomosis was performed.

Grossly, there were two lesions suspicious of malignancy. A 4.0 cm × 2.0 cm × 1.0 cm-sized ulceroinfiltrative mass was located at the ascending colon. The other lesion, a 5.0 cm × 3.0 cm × 1.1 cm-sized polypoid mass, was located at the sigmoid colon (Figure 3). Microscopically, the tumors were adenocarcinomas and were T3 and T1 stage, respectively. Of the 127 lymph nodes, none were positive for metastatic carcinoma. Postoperative pathological examination of the tumor revealed adenocarcinoma. The pericolonic tissue layer was infiltrated, but metastases were not found in either of the two lymph nodes.

DISCUSSION

Many studies have reported risk factors for colorectal cancer. Known genetic factors that can increase the risk of colorectal cancer are familial polyposis of the colon, Gardner’s syndrome, Turcot’s syndrome, Peutz-Jegher’s syndrome, UC, familial occurrence of colorectal cancer, and Bloom’s syndrome^[9,13,14]. According to most studies, 10% of pediatric colorectal cancers have predisposing factors^[9,13].

The rate of adenocarcinoma in childhood-onset UC patients is higher than that in adult-onset UC patients^[15,16]. Eaden *et al*^[16] investigated the long-term incidence of colorectal cancer among patients with childhood-onset UC through a meta-analysis. They reported that the cumulative probabilities of developing colorectal cancer were 5.5% at 10 years after onset of UC, 10.8% at 20 years and 15.7% at 3 years.

Diagnosis of inflammatory bowel disease at a young age is a well-known factor for an increased risk of colorectal cancer^[15]. Ekbom *et al*^[17] reported that the average incidence of colorectal cancer among patients with UC between the ages of 0 and 14 was 118.3 times that of the control population.

Considering the relatively high incidence of colorectal

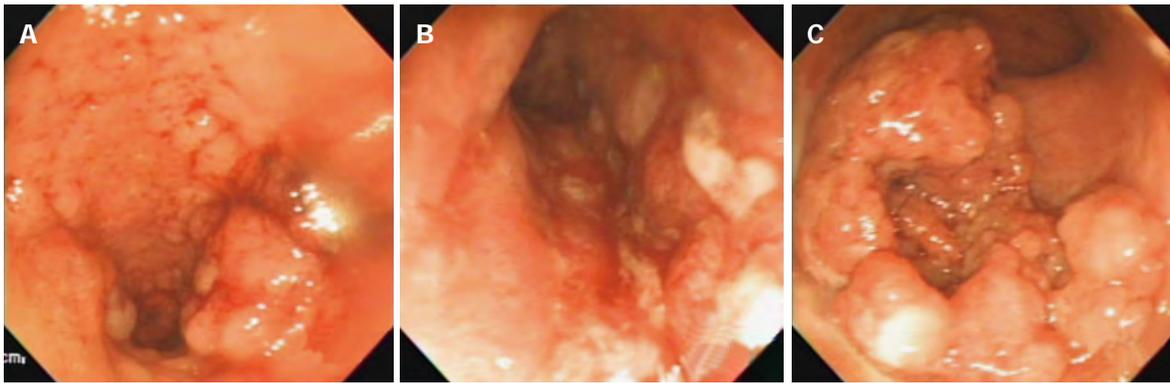


Figure 2 Colonoscopy findings. A: Diffuse granular lesions; B: Multiple ulcerations from the transverse colon to the rectum; C: A polypoid mass at the sigmoid colon.

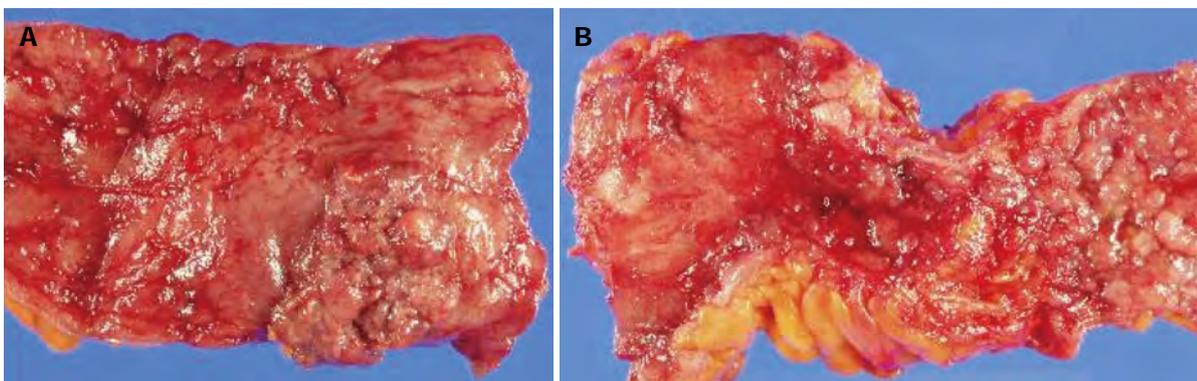


Figure 3 Gross appearance of the colon. A: There was a 5 cm × 3 cm-sized polypoid mass at the sigmoid colon; B: There was severe nodularity with fibrosis in the whole colon.

cancer and the poor prognosis in patients with childhood-onset UC, early diagnosis through regular screening with colonoscopy can increase the resectability and improve the prognosis.

In summary, pediatric surgeons always have to keep in mind the possibility of colorectal cancer in children with a long history of predisposing factors such as UC. Early diagnosis through regular screening with colonoscopy is one of the most important critical factors for a good prognosis.

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Duct-to-duct biliary reconstruction after radical resection of Bismuth IIIa hilar cholangiocarcinoma

Wen-Guang Wu, Jun Gu, Ping Dong, Jian-Hua Lu, Mao-Lan Li, Xiang-Song Wu, Jia-Hua Yang, Lin Zhang, Qi-Chen Ding, Hao Weng, Qian Ding, Ying-Bin Liu

Wen-Guang Wu, Jun Gu, Ping Dong, Jian-Hua Lu, Mao-Lan Li, Xiang-Song Wu, Jia-Hua Yang, Lin Zhang, Qi-Chen Ding, Hao Weng, Qian Ding, Ying-Bin Liu, Department of General Surgery, Xinhua Hospital, Affiliated to School of Medicine, Shanghai Jiaotong University, Shanghai 200092, China

Author contributions: Wu WG, Gu J and Liu YB designed the research; Dong P, Lu JH, Li ML, Wu XS and Yang JH performed the research; Zhang L, Ding QC, Weng H and Ding Q contributed new reagents or analytic tools; Dong P, Lu JH, Li ML, Wu XS and Yang JH analyzed data; Wu WG, Gu J and Liu YB wrote the paper; Wu WG and Gu J contributed equally to this work.

Correspondence to: Ying-Bin Liu, PhD, MD, Department of General Surgery, Xinhua Hospital, Affiliated to School of Medicine, Shanghai Jiaotong University, 1665 Kongjiang Road, Shanghai 200092, China. liuybphd@126.com

Telephone: +86-21-25077880 Fax: +86-21-25077880

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Abstract

At present, radical resection remains the only effective treatment for patients with hilar cholangiocarcinoma. The surgical approach for R0 resection of hilar cholangiocarcinoma is complex and diverse, but for the biliary reconstruction after resection, almost all surgeons use Roux-en-Y hepaticojejunostomy. A viable alternative to Roux-en-Y reconstruction after radical resection of hilar cholangiocarcinoma has not yet been proposed. We report a case of performing duct-to-duct biliary reconstruction after radical resection of Bismuth IIIa hilar cholangiocarcinoma. End-to-end anastomosis between the left hepatic duct and the distal common bile duct was used for the biliary reconstruction, and a single-layer continuous suture was performed along the bile duct using 5-0 prolene. The patient was discharged favorably without biliary fistula 2 wk later. Evidence for tumor recurrence was not found after an 18 mo follow-up. Performing bile duct end-to-end anastomosis in

hilar cholangiocarcinoma can simplify the complex digestive tract reconstruction process.

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Key words: Hilar cholangiocarcinoma; Biliary reconstruction; Duct-to-duct; Radical resection; Digestive tract reconstruction; Hepaticojejunostomy; Bile duct anastomosis

Core tip: Roux-en-Y anastomosis is the standard of care for biliary reconstruction after radical resection of hilar cholangiocarcinoma. However, a direct duct-to-duct biliary reconstruction preserves the normal sphincter mechanism and endoscopic access to the biliary tree for diagnostic and therapeutic purposes. Duct-to-duct biliary reconstruction is widely used in liver transplantation and hepatic resection. The objective of this study was to determine the feasibility of duct-to-duct biliary reconstruction in the setting of Bismuth IIIa hilar cholangiocarcinoma with limited biliary confluence involvement.

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INTRODUCTION

Surgical outcomes for treating hilar cholangiocarcinoma have gradually improved due to advances in surgical procedures and the accumulation of anatomic knowledge concerning the hepatic hilum^[1-5]. As cancer-free margins

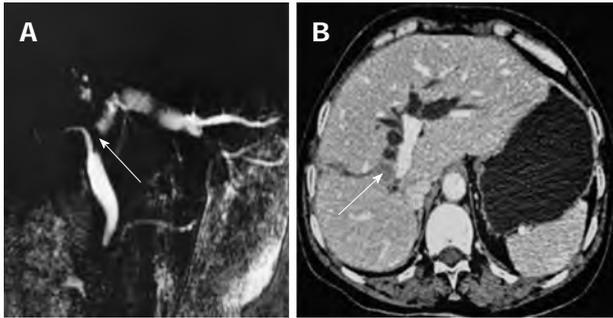


Figure 1 Magnetic resonance cholangiopancreatograph (A)/abdominal enhanced computed tomography (B) showing the hepatic portal soft tissue signal intensity (arrow in B), which is approximately 1.6 cm in diameter, and the common bile duct proximal locally shows truncated change (arrow in A). The extrahepatic bile duct is widened and the left hepatic intrahepatic bile duct is dilated. Hepatic cirrhosis is present. Images indicate hilar cholangiocarcinoma with intrahepatic bile duct dilatation.

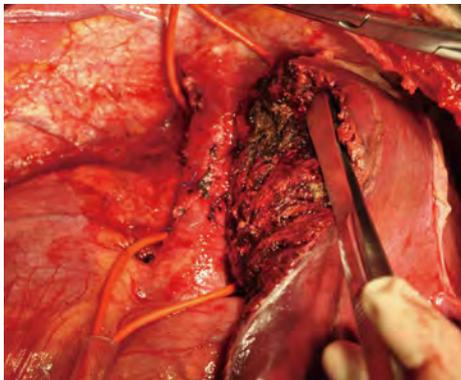


Figure 2 Right hemihepatectomy with caudate process lobectomy and systematic lymphadenectomy of the nodes.

are considered to be particularly important for curative resection of hilar cholangiocarcinoma^[6], bile ducts should be dissected longitudinally as far from the tumor as possible to ensure curative resection^[7]. For biliary reconstruction after resection, almost all surgeons use Roux-en-Y hepaticojejunostomy^[11-17]. Following curative resection of a tumor, if there is still sufficient tissue available for remodeling the normal biliary structure, should hepatic duct and common bile duct one stage anastomosis be considered? We report a case of performing duct-to-duct biliary one stage reconstruction for biliary reconstruction after radical resection of Bismuth IIIa hilar cholangiocarcinoma. Evidence for tumor recurrence was not found after an 18 mo follow-up.

CASE REPORT

The patient, a 58-year old female, was admitted to the hospital on February 15, 2011 because of “paroxysmal right upper quadrant pain for one week”. Admission examination: body skin and sclera are slightly yellow; the whole abdomen is soft, with no tenderness, no rebound tenderness and no mass; and no other symptoms are noted. Liver function: Glutamic-pyruvic-transaminase 213



Figure 3 Performing hepatoduodenal ligament lymphadenectomy in hilar cholangiocarcinoma and right hemihepatectomy with caudate process lobectomy. The left hepatic duct and common bile duct stump are shown (arrows).

U/L, Glutamic-oxalacetic-transaminase 177 U/L, alkaline phosphatase 1171 U/L, gamma-glutamyltransferase 1262 U/L, total bilirubin 21.3 $\mu\text{mol/L}$, direct bilirubin 8.8 $\mu\text{mol/L}$. Tumor markers: carbohydrate antigen 19-9 336.10 U/mL, carbohydrate antigen-50 38.46 U/mL, alpha fetoprotein 1.88 ng/mL. Magnetic resonance cholangiopancreatograph (MRCP)/abdominal enhanced computed tomography (CT): hepatic portal soft tissue signal intensity, approximately 1.6 cm in diameter, and the common bile duct proximal locally shows truncated change. The extrahepatic bile duct widened, and the left hepatic intrahepatic bile duct dilatation staggered. Hepatic cirrhosis was noted. A diagnosis of hilar cholangiocarcinoma (Bismuth IIIa type) was made (Figure 1).

The patient underwent right hemihepatectomy with caudate process lobectomy on February 18 and systematic lymphadenectomy of the nodes (Figure 2). The lymph node groups resected en bloc included the anterior pancreaticoduodenal lymph nodes (lymph node station 17 in the Japanese system), the posterior pancreaticoduodenal lymph nodes (station 13), nodes in the hepatoduodenal ligament (stations 12a, 12b and 12c), nodes along the common hepatic artery (station 8a), and the superior pyloric node (station 5). Intraoperative frozen pathological examination indicated duct cell carcinoma in the right hepatic duct and common bile duct. Negative margins were found on the left hepatic duct and common bile duct (Figure 3). After removal of the right lobe and the caudate process, the left hepatic duct and distal common bile duct end-to-end anastomoses were used for the biliary reconstruction and a biliary stent was placed in the bile duct. The specific method of the bile duct reconstruction was as follows: (1) ensure blood supply of the left hepatic duct and common bile duct resection margin; (2) mobilization of the left liver was performed from the left side followed by the Kocher maneuver to release the duodenal descending portion to reduce anastomosis tension; and (3) single-layer continuous suture was performed for bile duct reconstruction with 5-0 prolene (Figure 4). Ten days after the operation, liver function had generally returned to normal. The patient was discharged favorably without

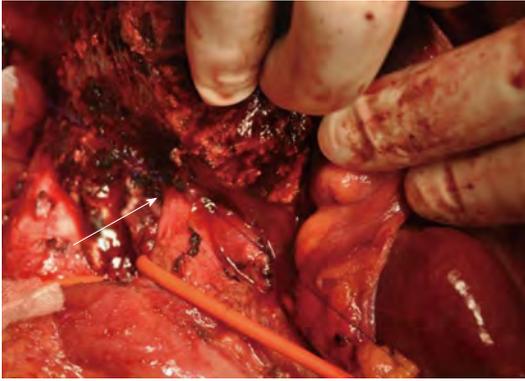


Figure 4 After the radical resection of hilar cholangiocarcinoma, duct-to-duct anastomosis was performed using continuous 5-0 polydioxanone sutures (arrow), with a biliary stent in the bile duct.

biliary fistula 2 wk later. Postoperative pathology indicated hilar bile duct adenocarcinoma grade II involving the right hepatic duct. A 2 cm diameter tumor was found with invasion to the fibrous muscular layer and outer connective tissue. The common bile duct and the left hepatic duct resection margin was negative. The tumor-free margin of the left duct was approximately 5 mm in the final postoperative pathological assessment. No regional lymph node metastasis was observed in a total of 19 dissected nodes. Consequently, the tumor was staged according to the American Joint Commission on Cancer staging as T2N0M0. No evidence of tumor recurrence was found using MRCP scans 18 mo post-operation (Figure 5).

DISCUSSION

Surgical radical resection currently remains the only effective method that increases long-term survival for treating patients with hilar cholangiocarcinoma^[8]. The hilar cholangiocarcinoma surgical approach is complex and diverse; however, for biliary reconstruction after resection, almost all surgeons use the biliary-enteric Roux-en-Y anastomosis method. The Roux-en-Y hepaticojejunostomy is primarily preferred for the following reasons: (1) it is imperative to remove as much of the bile duct as possible to ensure that the bile duct resection margin is negative; (2) removal of the common bile duct simplifies the hepatoduodenal ligament lymphadenectomy, reduces the difficulty of lymphadenectomy and generally improves its quality; and (3) the lower tension of Roux-en-Y hepaticojejunostomy reduces the occurrence of postoperative biliary fistula. However, performing duct-to-duct biliary reconstruction in hilar cholangiocarcinoma can simplify the complex digestive tract reconstruction process required in the traditional Roux-en-Y hepaticojejunostomy because it requires less radical alteration to normal gastrointestinal physiology, reduces some postoperative complications, and can simplify the treatment of complications. The faster anastomotic procedure in duct-to-duct may be another advantage over Roux-en-Y hepaticojejunostomy.

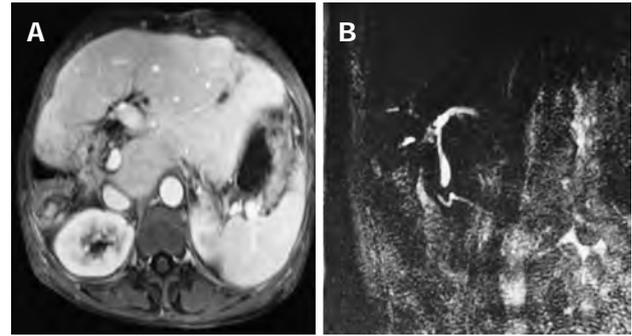


Figure 5 Evidence for tumor recurrence was not found in an magnetic resonance cholangiopancreatograph scan (A, B) 18 mo after operation.

Studies suggest that the invasion longitude of hilar cholangiocarcinoma along the bile duct varies significantly, with distances ranging from a few millimeters to several centimeters^[9], with variations related to bile duct cancer type, degree of differentiation, and other factors. A positive bile duct resection margin not only correlates with higher local recurrence rate after surgery but is also an independent risk factor for poor prognosis of hilar cholangiocarcinoma; furthermore, its role is similar to a positive lymph node^[10]. Unfortunately, preoperative procedures such as CT or MRCP, and even intraoperative exploration, cannot conclusively determine bile duct involvement. At present, intraoperative frozen pathological examination of bile duct resection margins is an important method to determine if a clean bile duct resection margin was achieved. For cases with intraoperative local excision of bile duct and a frozen pathological examination indicating a negative resection, it may be unnecessary to expand the scope of the bile duct resection and perform the routine Roux-en-Y hepaticojejunostomy. If there is still sufficient tissue available for remodeling the normal biliary structure after the tumor R0 resection, should hepatic duct and common bile duct one stage anastomosis be considered? In addition, performing the skeletonization of the hepatoduodenal ligament in carcinoma of the gallbladder or intrahepatic cholangiocarcinoma does not require the expense of the extrahepatic bile duct to reduce the difficulty of operation and improve the quality of skeletonization. The current practice of unconditional selection of the Roux-en-Y hepaticojejunostomy to reconstruct the biliary tract for hilar cholangiocarcinoma requires further reflection and research. Duct-to-duct anastomosis is currently a favorable method to reconstruct the biliary tract, even in live donor liver transplantation^[11,12]. Technically easier manipulation and the preservation of physiologic biliary-enteric continuity are two main advantages of duct-to-duct anastomosis over Roux-en-Y hepaticojejunostomy. Furthermore, following Roux-en-Y hepaticojejunostomy, the loss of the normal biliary tract and the digestive tract anatomical structures makes anastomotic stenosis or stone formation relatively complicated to address using minimally invasive endoscopic retrograde cholangiopancreatography and other treatments. These complications represent clinical issues that need to be considered.

Therefore, we believe that in the case of intraoperative frozen pathology indicating a negative bile duct resection margin, it is unnecessary to expand the removal of bile duct. For cases with a lesser degree of bile duct resection, duct-to-duct anastomosis of the bile duct should be considered. Provided that the blood supply of the bile duct stump is adequate, performing end-to-end tension-free anastomosis is the most effective way to ensure the anastomosis is secure, which can reduce the occurrence of the postoperative biliary fistula.

In conclusion, duct-to-duct biliary reconstruction may be a better option for bile duct reconstruction after R0 resection of hilar cholangiocarcinoma when sufficient bile duct remains for remodeling the normal biliary structure. However, the precise candidates for duct-to-duct anastomosis are difficult to define and still require further investigation.

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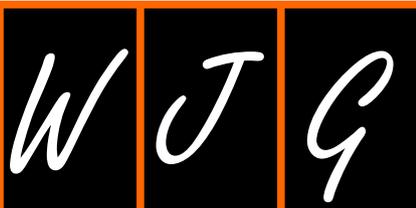
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E-mail: wjg@wjgnet.com
<http://www.wjgnet.com>

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Surgery for inflammatory bowel disease in the era of laparoscopy

Giuseppe S Sica, Livia Biancone

Giuseppe S Sica, Livia Biancone, GastroIntestinal Surgical Unit, Tor Vergata University of Rome, 00133 Rome, Italy
Author contributions: Sica GS and Biancone L gave substantial contributions to conception and design, acquisition, analysis and interpretation of data; Sica GS wrote the manuscript and Biancone L revised critically for important intellectual content; both authors gave their final approval of the version to be published.

Correspondence to: Giuseppe S Sica, MD, PhD, Gastrointestinal Surgical Unit, Tor Vergata University of Rome, Viale Oxford 81, 00133 Rome, Italy. sigisica@gmail.com
Telephone: +39-620-9083596 Fax: +39-620-902926
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Abstract

During the course of inflammatory bowel disease (IBD), surgery may be needed. Approximately 20% of patients with ulcerative colitis (UC) will require surgery, whereas up to 80% of Crohn's disease (CD) patients will undergo an operation during their lifetime. For UC patients requiring surgery, total proctocolectomy and ileoanal pouch anastomosis (IPAA) is the operation of choice as it provides a permanent cure and good quality of life. Nevertheless a permanent stoma is a good option in selected patients, especially the elderly. Minimally invasive surgery has replaced the conventional open approach in many specialized centres worldwide. Laparoscopic colectomy and restorative IPAA is rapidly becoming the standard of care in the treatment of UC requiring surgery, whilst laparoscopic ileo-cecal resection is already the new gold standard in the treatment of complicated CD of terminal ileum. Short term advantages of laparoscopic surgery includes faster recovery time and reduced requirement for analgesics. It is, however, in the long term that minimally invasive surgery has demonstrated its superiority over the open approach. A better cosmesis, a reduced number of incisional hernias and fewer adhesions are the long term advantages of laparoscopy in IBD surgery. A reduction

in abdominal adhesions is of great benefit when a second operation is needed in CD and this influences positively the pregnancy rate in young women undergoing restorative IPAA. In developing the therapeutic plan for IBD patients it should be recognized that the surgical approach to the abdomen has changed and that surgical treatment of complicated IBD can be safely performed with a true minimally invasive approach with great patient satisfaction.

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Key words: Laparoscopy; Ulcerative colitis; Surgery; Inflammatory bowel disease; Laparoscopic surgery; Proctocolectomy; Ileoanal pouch anastomosis

Core tip: The clinical management of inflammatory bowel disease (IBD) patients has dramatically changed in the last decades and primary, secondary and even tertiary levels of medical treatment are available to treat both Crohn's disease or ulcerative colitis. However, it should be recognized that surgical approaches have also changed, for the better, in the last few years and that minimally invasive surgery is now available in most centers. The timing of surgery is a key issue for proper management of IBD patients. Laparoscopic surgery should be seen as less aggressive than the standard surgical approach and could lower the threshold for surgical intervention.

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INTRODUCTION

During the course of inflammatory bowel disease (IBD),

surgery may be needed. Approximately 20% of patients with ulcerative colitis (UC) will require surgery, whereas up to 80% of Crohn's disease (CD) patients will undergo an operation during their lifetime^[1]. For UC patients requiring surgery, total proctocolectomy is the operation of choice as it provides a permanent cure and ileoanal pouch anastomosis (IPAA) has replaced the classic permanent ileostomy as the procedure of choice to accompany a proctocolectomy. Partial colectomy is rarely performed because of the high probability that the disease will recur in the remaining colon. Nevertheless partial colectomy and ileo-rectal anastomosis as well as proctocolectomy and permanent ileostomy are still good options in selected patients, especially the elderly.

For CD, surgery is not a definitive cure. Therefore, intestinal resection is indicated for patients who are refractory to the therapy or who are intolerant to medical treatments. In addition, patients that show severe complications of the disease will require surgery for obstruction, recurrent sub-obstructions, abdominal abscesses, perforation, massive bleeding or even cancer. The most common surgical procedure is ileo-cecal resection and primary reconstruction, which is indicated in patients with CD of distal ileum and/or ileo-colon. Strictureplasty is less frequently indicated in patients with limited proximal small bowel strictures. Endoscopic dilatations of jejunum and ileum and more limited resections are also employed in a minority of cases. Endoscopic recurrence 1 year after ileo-colonic resection is observed in up to 80% of patients, while clinical recurrence is observed in about 20% of patients at 2 years and in up to 80% at 20 years^[2].

There is little question that the timing of surgical intervention is a key issue for proper management of IBD patients. Indication and timing of surgery in IBD requires a joint evaluation by dedicated gastroenterologists and surgeons.

IBD surgery can be regarded as a cure for UC whilst it is undertaken to improve symptoms and to ameliorate the quality of life for CD patients. In both UC and CD, surgery can be a salvage procedure for acute, severe disease.

Although gastroenterologists are familiar with the potential complications associated with acute severe disease, most are reassured by statistics consistently showing an overall standardized mortality ratio for IBD patients near to that of general population^[3]. A large scale analysis performed in the Oxford region (United Kingdom) using record linkage studies, showed that 3-year mortality was significantly lower among people who underwent elective colectomy for IBD than among those who were admitted to hospital without colectomy or who had had emergency colectomy^[4]. The most worrying finding of the study by Roberts and colleagues is that in patients admitted for UC and CD who had no surgery (13.6% and 10.1% respectively) most deaths occur between 6 mo and 36 mo after admission. The decision to operate is an important one and should be made after a careful evaluation of all the clinical variables in each individual patient at the time of the diagnosis of the disease. Undoubtedly, many people

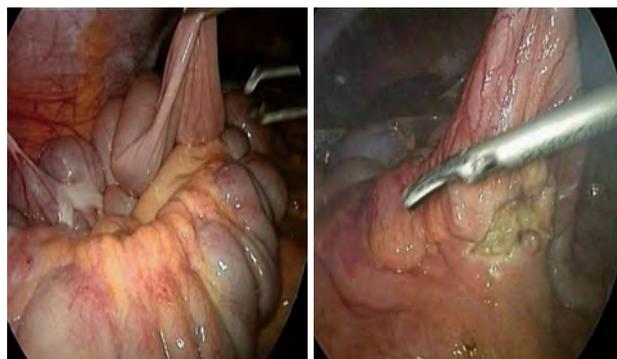


Figure 1 Laparoscopic ileo-cecal resection.

suffer needlessly because they try to avoid surgery. Surgical delay does not only put the patient through unnecessary periods of pain and suffering, but it can also increase the risks of operative complications and ultimately lead to a worse outcome. On the other hand, it is clear that avoiding or delaying surgery may be the better choice for IBD patients, particularly in young CD patients, because of the almost certain recurrence of the lesions.

The general belief that surgery for IBD should be the last resort is flawed by reports on outcomes after colectomy in IBD patients, showing a relatively good prognosis^[2,5-7]. However, these reports are mainly small, short term studies from specialist centers, not taking into account data from district hospitals where operations are performed mainly in emergency situations or with less positive results.

Furthermore the development of the therapeutic plan for IBD patients should also take into account the fact that the surgical approach to the abdomen has changed in recent years. Colectomies can be safely performed using a minimally invasive approach^[8] to the great satisfaction of the patient (Figure 1). Most CD patients who have undergone laparoscopic ileo-cecal resection have been reported to choose a laparoscopic operation should the disease recur^[9].

This is in agreement with data from the bariatric surgery where a steep increase in the request for surgical treatment of morbid obesity was observed after the development of minimally invasive procedures.

Surgery is certainly not the cure for CD, but is a viable therapeutic option and, given the potential advantages of the minimally invasive surgery, it shouldn't be always put at the top of the pyramid of treatment. In selected subgroups of patients, early surgery is correlated with a more favorable surgical outcome and a laparoscopic ileo-cecal resection together with a fast track recovery protocol^[10] may represent an appealing alternative to several years of medication. There is currently enough evidence to suggest a laparoscopic ileo-cecal resection as the gold standard in the management of CD patients with obstructive symptoms, but no significant evidence of active inflammation^[11]. In fact, this group of patients (less than 40 cm affected bowel and appreciable symptoms but no imminent obstruction) respond well to medical treatment



Figure 2 Totally laparoscopic proctocolectomy and restaurative ileoanal pouch anastomosis.

but will almost always require surgery during the course of their disease. A delayed surgical approach may not only increase the risk of septic complications but it may also reduce the possibility of performing a minimally invasive operation. CD patients who have undergone years of medication, and who present with a severely thickened bowel and mesentery due to marked inflammation and fibrosis, are candidates for more extensive resection with little chance of undergoing a minimally invasive procedure.

The quality of life for patients with UC is improved after colectomy^[2]. Surgery is indicated in UC when medical therapy is ineffective, intractability being one of the most common reasons for proctocolectomy. Alternatively, steroid dependence which is not responsive to immunomodulatory drugs (including biologics), low compliance, multifocal dysplasia or high grade dysplasia, may represent indications for surgery. As for CD, delaying surgery may increase mortality and morbidity in UC. In patients who have undergone an emergency colectomy for UC, the risk of death increases substantially in the four to six months after surgery^[4]. It has been recommended that about 85% of patients who do not respond to conventional steroid treatment within 6 d of hospitalization should undergo colectomy. A subgroup of these patients could alternatively be treated with anti-tumor necrosis factor “rescue” therapy, in accordance with the European Crohn and Colitis Organization guidelines^[12]. Nevertheless, all of these criteria need to be considered in the light of the clinical characteristics of each individual patient, and a possible decision for surgery needs to be jointly assessed by an experienced gastroenterologist and surgeon on a daily basis. Furthermore, in UC the timing of surgery influences the surgical approach and vice versa: an emergency colectomy usually ends with a terminal ileostomy. This procedure is followed, generally after several months, by a complete proctectomy, restaurative pouch and lateral ileostomy. The third and last operation, the ileostomy closure, will be performed after a few months, provided the good condition of the ileo-anal pouch. However, in cases of planned elective surgery it will be possible to avoid one operation by performing a totally laparoscopic procto-colectomy and

IPAA at the same time, followed by the closure of the lateral ileostomy (Figure 2).

Optimal care of patients with IBD continues to involve a great deal of judgment. Avoiding mortality and achieving a good quality of life are the guiding principles in the care of IBD patients. The decision to operate remains a difficult one and should take into account all the pros and cons of a planned “nice” laparoscopic resection compared to the symptomatic relief that may be achieved by primary, secondary or even tertiary medical therapy. Randomized controlled trials that include a large number of patients are required to establish the optimal timing for surgery in IBD.

Finally, but most importantly, unless severe complications indicate the need for emergency surgery, a decision for elective surgery must take into account not only the clinical characteristics of each patient but also the view of the patient. Indeed, the timing of surgery needs to be extensively discussed and approved not only by the gastroenterologist and surgeon, but also by the patient, who should be clearly informed of the risks and benefits of both medical and surgical therapy.

Laparoscopic surgery in IBD is safe and feasible. It offers both cosmetic advantages and some short term advantages, such as a possible reduction in perioperative complications^[13]. Long term advantages include fewer incisional hernias and fewer adhesions^[14] with a significant impact on female fertility in UC patients^[15]. The minimally invasive procedure is the approach that is preferred in specialized centres. Considering its proven advantages and popularity amongst patients, it should be seen as a new strategic option when considering therapeutic alternatives in complicated IBD patients.

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Genetic association of interleukin-6 polymorphism (-174 G/C) with chronic liver diseases and hepatocellular carcinoma

Lydia Giannitrapani, Maurizio Soresi, Daniele Balasus, Anna Licata, Giuseppe Montalto

Lydia Giannitrapani, Maurizio Soresi, Daniele Balasus, Anna Licata, Giuseppe Montalto, Unit of Internal Medicine, Biomedical Department of Internal Medicine and Specialties DiBiMIS, University of Palermo, 90127 Palermo, Italy

Author contributions: Giannitrapani L and Soresi M collected and analyzed literature data; Balasus D and Licata A contributed analytic tools; Montalto G supervised the paper and Giannitrapani L wrote the paper.

Correspondence to: Dr. Lydia Giannitrapani, Unit of Internal Medicine, Biomedical Department of Internal Medicine and Specialties, University of Palermo, Via del Vespro 141, 90127 Palermo, Italy. lydia.giannitrapani@unipa.it

Telephone: +39-91-6552916 Fax: +39-91-6552977

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Abstract

Interleukin-6 (IL-6) is a pleiotropic cytokine which is expressed in many inflammatory cells in response to different types of stimuli, regulating a number of biological processes. The *IL-6* gene is polymorphic in both the 5' and 3' flanking regions and more than 150 single nucleotide polymorphisms have been identified so far. Genetic polymorphisms of *IL-6* may affect the outcomes of several diseases, where the presence of high levels of circulating IL-6 have been correlated to the stage and/or the progression of the disease itself. The -174 G/C polymorphism is a frequent polymorphism, that is located in the upstream regulatory region of the *IL-6* gene and affects IL-6 production. However, the data in the literature on the genetic association between the -174 G/C polymorphism and some specific liver diseases characterized by different etiologies are still controversial. In particular, most of the studies are quite unanimous in describing a correlation between the presence of the high-producer genotype and a worse evolution of the chronic liver disease. This is valid for patients with hepatitis C virus (HCV)-related chronic hepatitis and liver cirrhosis and hepatocellular

carcinoma (HCC) whatever the etiology. Studies in hepatitis B virus-related chronic liver diseases are not conclusive, while specific populations like non alcoholic fatty liver disease/non-alcoholic steatohepatitis, autoimmune and human immunodeficiency virus/HCV co-infected patients show a higher prevalence of the low-producer genotype, probably due to the complexity of these clinical pictures. In this direction, a systematic revision of these data should shed more light on the role of this polymorphism in chronic liver diseases and HCC.

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Key words: Single nucleotide polymorphisms; Interleukin-6; Chronic hepatitis; Liver cirrhosis; Hepatocellular carcinoma

Core tip: Several studies suggested the possibility of an association between -174 interleukin-6 gene G/C polymorphism and some liver diseases however, the data in the literature are still controversial. This work aims to review the literature data on the role of this polymorphism and its possible biological function in chronic liver diseases and hepatocellular carcinoma.

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INTRODUCTION

In recent decades chronic liver diseases (chronic hepatitis, liver cirrhosis) and hepatocellular carcinoma have become more and more diffuse both in Western and in

Eastern countries, representing an important problem for health systems worldwide^[1-5]. Whatever the etiology, these diseases share a common pathogenetic mechanism which is linked to chronic inflammation^[6]. Hepatotropic viruses, toxins and alcohol, metabolic liver disease or autoimmunity can be the triggers which, acting chronically in the liver, ultimately activate cellular pathways involving transcription factors of the nuclear factor kappa-light-chain-enhancer of activated B cells (NF- κ B) family and signal transducer and activator of transcription 3 (STAT3), as well as cytokines such as interleukin-6 (IL-6) and IL-1 α , *etc.*

In particular, IL-6 is a cytokine involved in the regulation of several cellular processes including proliferation and differentiation and plays a pivotal role in acute phase response and in the control of the balance between pro-inflammatory and anti-inflammatory pathways. The *IL-6* gene is located on chromosome 7p21^[7]. A number of studies indicated that the presence of a G/C single nucleotide polymorphism (SNP) at the promoter -174 of the *IL-6* gene, one of the numerous known polymorphisms in the *IL-6* gene, is related to the *IL-6* gene transcription rate and, as a consequence, to the control of circulating IL-6 levels^[8,9].

Subsequently, two phenotypes for this polymorphism were identified: the high-producer phenotype, including the -174 G/G and -174 G/C genotypes, characterized by higher circulating IL-6 levels; and the low-producer phenotype, including the -174 C/C genotype^[8]. Genetic population studies have shown that there are ethnic differences in the frequency of the -174 G allele, with higher frequencies in non-Caucasian than in Caucasian populations^[10,11].

High circulating levels of IL-6 have been documented in several clinical conditions (inflammatory, neoplastic diseases) and in particular in various liver diseases such as viral chronic hepatitis^[12], alcoholic liver disease^[13], liver cirrhosis and hepatocellular carcinoma (HCC)^[14]. A small number of studies have investigated a possible correlation between the presence of the -174 G/C polymorphism, IL-6 circulating levels and the stage of disease^[15]. However, the results of these studies are quite controversial. This work aims to review the literature data on the role of G/C base exchange at position -174 of the *IL-6* gene and its possible biological function in chronic liver diseases and HCC.

IL-6 POLYMORPHISM (-174 G/C) AND HEPATITIS C VIRUS AND HEPATITIS B VIRUS INFECTION

Produced by a variety of cells such as macrophages, B and T cells and fibroblasts, IL-6 plays a central role in the inflammatory response associated with the course of chronic hepatitis due to hepatitis C virus (HCV)- and hepatitis B virus (HBV)-related infection^[16,17].

To mediate its biological effects it interacts with a receptor complex consisting of a specific ligand-binding

protein (IL-6R, gp80) and a signal transduction protein (gp130) (Figure 1A). When IL-6 binds its cell surface receptor (IL-6R) on the hepatocyte a homodimer of the signal transduction receptor gp130 is recruited to the complex and it activates a janus kinase 1 which in turn triggers two main signaling pathways: the gp130 Tyr759-derived Src homology 2 domain-containing protein tyrosine phosphatase-2/extracellular-signal-regulated kinase/mitogen-activated protein kinase pathway and the gp130 YXXQ-mediated Janus associated kinase/signal transducer and activator of transcription pathway (Figure 1B). Interestingly, sIL-6R (soluble form of IL-6R) also binds with IL-6, and the IL-6-sIL-6R complex can then form a complex with gp130^[18,19]. Through this receptor system IL-6 can influence various cell types and exert its multiple biological activities regulating immune response, acute phase response and inflammation.

During HCV infection, an altered production of cytokines seems to be related to viral persistence and to affect response to therapy. Barret *et al.*^[20] comparing various cytokine polymorphisms (including -174 G/C *IL-6*) in individuals with spontaneous viral clearance after HCV infection and in those with persistent viremia, reported that the CC genotype with low IL-6 production was associated with spontaneous viral clearance, while an association between the high IL-6 producer genotypes and persistent infection only became apparent when both genotypes (GG and GC) were combined. As regards the influence of the genetic background in individuals with HCV infection and response to the antiviral therapy, the most recent literature data have investigated the role of *IL-28B* polymorphisms as a predictor of the outcome of the commonly-used treatments^[21]. However, because of the central importance of IL-6 as a mediator of the immune response to infectious agents, and considering that host genetic variation, and in particular haplotypes, may affect IL-6 expression, Yee *et al.*^[22] examined the contribution of haplotypes in the *IL-6* gene to therapy for chronic HCV infection on sustained viral response (SVR). Among the SNPs genotyped and included in haplotype construction, the authors found some SNPs (including -174G/C, and in particular genotypes GG and GC) showing significant associations with a reduced likelihood of SVR.

These results are in contrast with previously reported ones published by Nattermanne *et al.*^[23] which, however, were obtained in another specific population, *i.e.*, patients co-infected with both acute and chronic HCV and human immunodeficiency virus (HIV). The aim of this study was to evaluate whether *IL-6* -174 G/C polymorphism could affect response to antiviral treatment in HCV-infected HIV-positive patients. The study group was compared to a group of HCV- and a group of HIV-monoinfected patients, as well as to a group of healthy individuals and no significant difference was found in the distribution of *IL-6* genotypes between the study groups. However, the authors concluded that carriers of high-producer genotypes (genotypes *IL-6* 174 GG and 174 GC) had significantly higher SVR rates than patients with an IL-6 low-

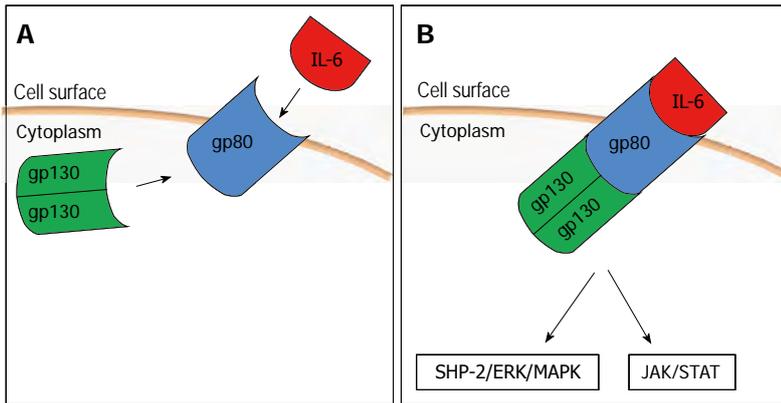


Figure 1 Interleukin-6 interaction with its receptor complex. A: Mechanism of action of the interleukin-6 (IL-6); B: Activation of the two pathways triggered by the IL-6 action. SHP-2: Src homology 2 domain-containing protein tyrosine phosphatase-2; ERK/MAPK: Extracellular-signal-regulated kinase/mitogen-activated protein kinase; JAK/STAT: Janus associated kinase/signal transducer and activator of transcription.

producer genotype (genotype 174 CC).

Another particular subgroup of HCV patients is the one with persistently normal or near normal alanine aminotransferases levels (PNALT), which for several years was supposed to have a milder course of disease, whereas it is now well known that in a few cases it can evolve to cirrhosis^[24]. Among the studies evaluating genetic polymorphisms in chronic HCV carriers with PNALT, Falletti *et al.*^[25] evaluated the role of five *IL-6* polymorphisms (among them -174 G/C) in modulating fibrosis progression in PNALT patients with chronic HCV infection. The principal point of interest in this study were the associations found between *IL-6* polymorphisms and grading and staging increase during the follow-up of the patients with chronic viral hepatitis C and PNALT. In particular, grading increase appeared to be related to the presence of the G allele of the *IL-6* -174G/C polymorphism, while the C allele seemed to be protective.

As cytokines also play a fundamental role in the immune response to HBV and HBV infection may have different forms of evolution (self-limited or persistent and progressive), *IL-6* polymorphisms have also been studied to investigate a possible correlation between *IL-6* promoter variants and chronic hepatitis B progression, infection evolution in adult patients and risk of HCC development. Unfortunately, the data reported by Park *et al.*^[26] are not conclusive because in their attempt to analyze additional polymorphisms in variants of genes implicated in chronic hepatitis B progression they found that Koreans and Caucasians had different genetic backgrounds in terms of the allele frequencies of the *IL-6* promoter SNPs. In particular, in their study the allele frequencies reported in Caucasians (range: 0.40-0.45) were much higher than those found in Koreans (allele frequencies 0.002). The authors concluded that at least in their population, although *IL-6* may have important functions in the progression of chronic HBV infection, its genetic variants probably do not influence the development of LC and HCC from chronic HBV infection, due to too low frequencies of *IL-6* 174 G/C.

Another attempt to correlate cytokine genetic polymorphism with hepatitis B infection evolution was made

in a Brazilian population, but the study found no significant differences in the polymorphism of *IL-6* -174 between the chronic HBV patient group and the self-limited infection group as regards alleles, genotypes or phenotypic expression^[27].

Similarly, the study of a Japanese population by Migita *et al.*^[28] with the aim of characterizing cytokine gene polymorphisms in chronic HBV infection and their associations with HCC, was unable to show conclusive data about the role of *IL-6* -174 because no polymorphisms were found at that position (Table 1).

***IL-6* POLYMORPHISM (-174 G/C) AND NON-VIRAL CHRONIC LIVER DISEASES**

Non-alcoholic steatohepatitis

Non-alcoholic fatty liver disease (NAFLD) includes a broad spectrum of clinic-pathological entities, including simple steatosis and non-alcoholic steatohepatitis (NASH), which can progress to advanced liver diseases^[29]. Its pathogenesis is strictly linked to insulin resistance and to all the mechanisms described for the development of metabolic syndrome, and in this perspective an important role is played by genetic background^[30,31]. It is well known that the balance between pro- and anti-inflammatory acting cytokines is fundamental in the control of hepatic and systemic insulin action, and as a consequence, in the development of NAFLD. In particular, serum levels of this cytokine correlate remarkably well with the presence of insulin resistance, and adipose tissue-derived *IL-6* has been shown to regulate hepatic insulin resistance *via* up-regulation of suppressor of cytokine signaling 3^[32]. However, the role of -174 G/C polymorphism in this population raises some questions. In fact, a study by Carulli *et al.*^[33] found that the *IL-6* -174C variant, is significantly more prevalent in NAFLD than in healthy subjects, is associated with increased fasting insulin and homeostasis model assessment of insulin resistance, and is an independent predictor of NAFLD and NASH. This finding is in contrast with other studies which showed that the *IL-6* -174G variant was as-

sociated with lipid abnormalities^[34] and with diabetes in Caucasians as well as Pima Indians^[35-38] and that the C allele at -174 position was unlikely to play a role in the development of type 2 diabetes mellitus in a Taiwanese population^[39]. One possible explanation for these contradictory results can be found in the conclusion of a study on an experimental mouse model of ASH and NAFLD: IL-10-/- mice were prone to liver inflammatory response but resistant to steatosis and hepatocellular damage induced by ethanol or high-fat diet feeding, thanks to the elevation of inflammation-associated hepatic IL-6/STAT3 activation that subsequently down-regulated lipogenic genes, but up-regulated fatty acid oxidation-associated genes in the liver^[40].

Alcoholic liver diseases

In an attempt to explain why only a minority of heavy drinkers develop alcoholic liver cirrhosis or alcohol use disorders, some genetic factors have been considered^[41,42], such as polymorphisms of genes encoding cytokines. Several studies support the hypothesis of a pivotal role of ethanol-induced cytokine changes in contributing to alcohol pathogenesis in a number of tissues, including the liver^[43-45]. Moreover, elevated serum concentrations of pro-inflammatory cytokines such as tumor necrosis factor- α , IL-1, IL-6 and IL-8 and decreased levels of anti-inflammatory cytokines like IL-10 have been shown in patients with this disease^[43,46,47]. However, the only study on common polymorphisms in interleukin genes (including -174G/C *IL-6*) in a population of Spanish alcoholic patients did not find any statistically significant associations between any of the studied polymorphisms or the combinations of pro-inflammatory polymorphisms and the risk of alcoholic liver cirrhosis or alcohol abuse or dependence^[48].

Autoimmune liver diseases

Autoimmune hepatitis (AIH), primary biliary cirrhosis (PBC), and primary sclerosing cholangitis represent the three main categories of autoimmune liver diseases. However, their etiology and possible environmental triggers still remain obscure even if it is well established that a complex genetic background contributes to disease susceptibility and severity. Several studies have established that genetic factors are involved in the pathogenesis of autoimmune liver diseases^[49-52]. Among these studies, one in a Chinese population of patients with AIH and PBC found that frequency of *IL-6* -174C was high and significantly increased in PBC patients compared with controls. This result supports the hypothesis that the *IL-6* -174G/C polymorphism could contribute to the change in susceptibility to PBC in some subjects^[53] (Table 2).

IL-6 POLYMORPHISM (-174 G/C) AND HCC

An interrelation between chronic inflammation and cancer has been suspected for a long time^[54]. Many tumors occur in association with chronic infectious diseases and persistent inflammation increases the risk and accelerates

the development of cancer^[55-59]. HCC is one of the most clear examples of inflammation-related cancer^[60,61]. It is a tumor that slowly progresses through a chronic inflammation state, triggered by exposure to various agents. The molecular links that connect inflammation and cancer are not completely known, although there is a consistent body of evidence pointing to the role of transcription factors such as NF- κ B^[62] and STAT3^[63] and cytokines like IL-6^[64] as well as other inflammatory mediators in HCC development. A first attempt to study the potential role of cytokine polymorphisms in determining the risk of HBV-related HCC was made in 2005 by Nieters *et al*^[65], who examined the correlation between polymorphisms in Th1 and Th2 cytokine genes in a group of 250 patients with incident HCC and a group of 250 matched hospitalized controls in China: however, none of the study participants presented the C allele of the *IL-6* -174 G/C polymorphism, therefore this polymorphism was not further investigated. Subsequently, a population-based case-control study of HCC, including 120 HCC patients and 230 matched control subjects, was conducted in non-Asian residents of Los Angeles County, California, into genetic polymorphisms in the cytokine genes and risk of HCC. The authors demonstrated that the GG *IL-6* genotype showed the strongest influence on HCC risk among all the cytokine polymorphisms studied^[66]. In a more recent study Falletti *et al*^[25] investigated whether *IL-6* polymorphisms could be associated with the occurrence of HCC in patients with liver cirrhosis, analyzing 219 consecutive patients who underwent liver transplantation for liver cirrhosis. They found a significant association between the presence of the low-producer genotype (-174 CC) and absence of HCC^[67]. Finally, our group performed a study which aimed to evaluate the frequency of SNPs in the *IL-6* promoter region at position -174 and IL-6 serum levels in a group of patients with HCC and underlying liver cirrhosis compared with a group of LC patients without HCC. We found that IL-6 serum levels were higher in G/G compared to C/C genotypes only in HCC; IL-6 serum levels in G carriers were higher in HCC versus LC patients while there were no differences for the C allele. IL-6 serum levels in HCC correlated with G carriers^[15] (Table 3).

CONCLUSION

The possibility of a genetic association between -174 G/C polymorphism and some specific liver diseases has been suggested by several studies which are quite unanimous in observing a correlation between the presence of the high-producer genotype (GG) and a worse evolution of the chronic disease. This has been observed in patients with HCV-related chronic hepatitis even with PNALT and in patients with liver cirrhosis and HCC whatever the etiology. Studies on HBV-related chronic hepatitis have not been conclusive because they were performed in populations (generally Asiatic) which have much lower frequencies of the -174 C allele than Caucasian populations. Finally, specific populations like NAFLD/NASH, autoim-

Table 1 Studies examining the role of interleukin-6 polymorphism (-174 G/C) in hepatitis C virus and hepatitis B virus infection

| Ref. | Country | Ethnicity | Cases | Controls | Genotyping method | Association with chronic hepatitis/ response to therapy |
|---|-------------|-----------------------|-------|----------|--------------------------|--|
| Barrett <i>et al</i> ^[20] | Ireland | Caucasian | 158 | - | PCR-SSP | Positive significant/- |
| Nattermann <i>et al</i> ^[23] | Germany | Caucasian | 210 | 100 | Cytokine genotyping tray | -/Uncertain |
| Falletti <i>et al</i> ^[25] | Italy | Caucasian | 121 | - | PCR-RFLP | Positive significant /- |
| Park <i>et al</i> ^[26] | South Korea | Asian | 1046 | - | PCR-SBE | NS/- |
| Ribeiro <i>et al</i> ^[27] | Brazil | American ¹ | 26 | 41 | PCR-SSP | NS/- |

¹White, black and hispanic. PCR-SSP: Polymerase chain reaction - single specific primer; PCR-RFLP: Polymerase chain reaction - restriction fragment length polymorphism; PCR-SBE: Polymerase chain reaction - single base primer extension assay; NS: Not significant.

Table 2 Studies examining the role of interleukin-6 polymorphism (-174 G/C) in alcoholic and autoimmune liver diseases

| Ref. | Country | Ethnicity | Cases | Controls | Genotyping method | Association |
|---|---------|-----------|-------|----------|-------------------|---|
| Carulli <i>et al</i> ^[33] | Italy | Caucasian | 79 | 114 | PCR-RFLP | Positive significant (NAFLD) |
| Fernández-Real <i>et al</i> ^[34] | Spain | Caucasian | 32 | - | PCR-RFLP | Positive significant (diabetes and lipid abnormalities) (G allele) |
| Marcos <i>et al</i> ^[48] | Spain | Caucasian | 258 | 101 | TaqMan genotyping | NS (alcoholic liver disease) |
| Fan <i>et al</i> ^[53] | China | Asian | 77 | - | PCR-RFLP | Positive significant (PBC) |

PCR-RFLP: Polymerase chain reaction - restriction fragment length polymorphism; NAFLD: Non-alcoholic fatty liver disease; PBC: Primary biliary cirrhosis.

Table 3 Studies examining the role of interleukin-6 polymorphism (-174 G/C) in hepatocellular carcinoma

| Ref. | Country | Ethnicity | Cases | Controls | Genotyping method | Association with HCC |
|--|---------------|-----------------------|-------|----------|--|----------------------|
| Nieters <i>et al</i> ^[65] | China | Asian | 250 | 250 | PCR-RFLP | NS |
| Ognjanovic <i>et al</i> ^[66] | United States | American ¹ | 120 | 230 | 5'nuclease Taqman allelic discrimination assay | Positive significant |
| Falletti <i>et al</i> ^[67] | Italy | Caucasian | 219 | - | PCR-RFLP | Positive significant |
| Giannitrapani <i>et al</i> ^[15] | Italy | Caucasian | 105 | - | PCR-RFLP | Positive significant |

¹White, black and hispanic. PCR-RFLP: Polymerase chain reaction - restriction fragment length polymorphism; HCC: Hepatocellular carcinoma; NS: Not significant.

immune and HIV/HCV co-infected patients not achieving SVR showed a higher prevalence of the CC genotype, probably as a result of many other complex immunological, virological and host-related interrelations that cannot be explained by the presence of a unique SNP.

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Annexin A1: A new immunohistological marker of cholangiocarcinoma

Nuttanan Hongsrichan, Rucksak Rucksaken, Yaovalux Chamgramol, Porntip Pinlaor, Anchalee Techasen, Puangrat Yongvanit, Narong Khuntikeo, Chawalit Pairojkul, Somchai Pinlaor

Nuttanan Hongsrichan, Rucksak Rucksaken, Somchai Pinlaor, Department of Parasitology, Liver Fluke and Cholangiocarcinoma Research Center, Faculty of Medicine, Khon Kaen University, Khon Kaen 40002, Thailand

Yaovalux Chamgramol, Chawalit Pairojkul, Department of Pathology, Liver Fluke and Cholangiocarcinoma Research Center, Faculty of Medicine, Khon Kaen University, Khon Kaen 40002, Thailand

Porntip Pinlaor, Centre for Research and Development in Medical Diagnostic Laboratory, Faculty of Associated Medical Sciences, Liver Fluke and Cholangiocarcinoma Research Center, Faculty of Medicine, Khon Kaen University, Khon Kaen 40002, Thailand

Anchalee Techasen, Puangrat Yongvanit, Department of Biochemistry, Liver Fluke and Cholangiocarcinoma Research Center, Faculty of Medicine, Khon Kaen University, Khon Kaen 40002, Thailand

Narong Khuntikeo, Department of Surgery, Liver Fluke and Cholangiocarcinoma Research Center, Faculty of Medicine, Khon Kaen University, Khon Kaen 40002, Thailand

Author contributions: Hongsrichan N performed experiments, analyzed the data and wrote the manuscript; Rucksaken R analyzed the data; Chamgramol Y and Pairojkul C constructed tissue arrays and advised for immunohistochemical data; Pinlaor P revised the manuscript; Techasen A and Yongvanit P advised on siRNA experiment; Khuntikeo N provided specimens and advised clinical data; Pinlaor S designed the experiments and wrote the manuscript.

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Correspondence to: Somchai Pinlaor, PhD, Associate Professor, Department of Parasitology, Liver Fluke and Cholangiocarcinoma Research Center, Faculty of Medicine, Khon Kaen University, Khon Kaen 40002, Thailand. psomec@kku.ac.th
Telephone: +66-43-348387 Fax: +66-43-202475

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Abstract

AIM: To evaluate a new immunohistological marker, annexin A1 (ANXA1), in cholangiocarcinoma (CCA) and hepatocellular carcinoma (HCC).

METHODS: Expression of ANXA1 protein was investigated in liver tissues from patients with CCA and HCC by immunohistochemistry. Its expression on differences stages of tumor development was investigated in hamster CCA tissues induced by *Opisthorchis viverrini* and *N*-nitrosodimethylamine. Moreover, mRNA expression of ANXA1 was assessed in CCA cell lines by quantitative real-time polymerase chain reaction and silencing of *ANXA1* gene expression using small interfering RNA.

RESULTS: In human CCA tissue arrays, immunohistochemical analysis revealed that the positive expression of ANXA1 was 94.1% (64/68 cases) consisting of a high expression (66.2%, 45/68 cases) and a low expression (33.8%, 23/68 cases). However, expression of ANXA1 protein was negative in all histologic patterns for HCC (46/46 cases) and healthy individuals (6/6 cases). In hamster with opisthorchiasis-associated CCA, the expression of ANXA1 was observed in the cytoplasm of inflammatory cells, bile duct epithelia and tumor cells. Grading scores of ANXA1 expression were significantly increased with tumor progression. In addition, mRNA expression of ANXA1 significantly increased in all of the various CCA cell lines tested compared to an immortalized human cholangiocyte cell line (MMNK1). Suppressing the *ANXA1* gene significantly reduced the matrix metalloproteinase (MMP) 2 and MMP9, and transforming growth factor- β genes, but increased nuclear factor- κ B gene expression.

CONCLUSION: ANXA1 is highly expressed in CCA, but low in HCC, suggesting it may serve as a new immunohistochemical marker of CCA. ANXA1 may play a role in opisthorchiasis-associated cholangiocarcinogenesis.

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Key words: Cholangiocarcinoma; *Opisthorchis viverrini*; Hepatocellular carcinoma; Annexin A1; Biomarker

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INTRODUCTION

Cholangiocarcinoma (CCA) is the leading cancer cause of death in northeastern Thailand. The incidence of CCA, a bile duct cancer, from the Khon Kaen Cancer Registry, Thailand, between 1985 and 2009 was 44.3 per 100000 in the males and 17.6 per 100000 in the females^[1] which was the highest incidence rate in Southeast Asia^[2,3], while its incidence is quite low in European countries^[4]. The high incidence of CCA is found mainly in persons over 35 years of age and varies from 93.8 to 317.6 per 100000 person-years^[5]. The incidence of mainly the intrahepatic type of CCA most typical in the northeastern part of Thailand is associated with the high prevalence of opisthorchiasis caused by *Opisthorchis viverrini* (*O. viverrini*) infection^[5,6]. Because CCA is lacking specific symptoms and with no early diagnostic markers, the patients often present at late onset when the disease is in the advanced stage and the patients end up with a poor prognosis^[7]. After surgery, no effective drug treatment is available^[8] resulting in short survival outcomes^[8,9].

The histologic distinction between CCA and hepatocellular carcinoma (HCC) is difficult due to heterogeneity and similarities in morphology^[10-13]. Although several immunohistochemical markers such as cytokeratin (CK) 7, CK20, and hepatocyte paraffin 1 (HepPar1) are widely used to distinguish CCA and HCC, these markers can be expressed by both cancers^[14,15]. Therefore, novel diagnostic markers for diagnostic differentiation of primary liver tumors are required.

Since the liver fluke associated with intrahepatic CCA is believed to have an immunopathological effect^[6,16], a proteomics based approach was used to search for protein alterations during the host-parasite interaction in an *in vitro* study. A candidate molecule namely annexin A1 (ANXA1) was found which significantly upregulated persistently during the long-term host-parasite interaction. ANXA1 has diverse functions including the regulation of cell division, proliferation, apoptosis and cell growth. Its function participates in stimulation of epithelial cell motility which is crucial in the development of metastasis by disruption of cell morphology^[17]. To date, however, the expression of ANXA1 in CCA and its relationship with clinicopathologic factors is unclear.

Up-regulation^[18] and down-regulation^[19] expressions of ANXA1 have been found in sporadic CCA. Similar to in HCC, its expression is controversial, both up-regulation^[20,21] and down-regulation^[22] have been reported.

In the present study, the expression of ANXA1 in tumor tissues of intrahepatic CCA and HCC patients and its relationship with the clinicopathologic factors was investigated by immunohistochemical staining. In addition, opisthorchiasis-associated CCA in hamsters at the different stages of tumor development was assessed. The expression and regulation in CCA cell lines of ANXA1 was also determined *in vitro* using small interfering RNA (siRNA) to suppress the *ANXA1* gene.

MATERIALS AND METHODS

Patient tissue samples

Tissue microarrays (TMAs) were constructed from archival paraffin embedded tissue samples of 68 intrahepatic CCA patients, 46 HCC patients and tissues from 6 normal healthy livers. These patients underwent liver resection at Srinagarind Hospital, Khon Kaen University, Thailand during 1999-2010. Diagnosis of both CCA and HCC patients were evaluated by clinical data, imaging analysis, tumor markers, and pathology. Immunohistochemical studies for pathological diagnosis included antibodies to CK7, cancer antigen or carbohydrate antigen 19-9 (CA19-9), HepPar1 and alpha-fetoprotein (AFP). The tumor tissues were verified based on the following criteria: as CCA when either CK7⁺ or HepPar1⁺, with or without CA19-9⁺, were found; or as HCC when either HepPar1⁺ or CK7⁺, with or without AFP⁺, were found. The study protocol was approved by The Human Research Ethics Committee, Khon Kaen University, Thailand (HE551407).

TMA and immunohistochemistry

TMAs of 68 *O. viverrini*-associated CCA cases and 46 HCC cases were generated manually from the paraffin-embedded tissues. In brief, four randomly selected regions from each paraffin block were identified on a hematoxylin-eosin (HE)-stained slide, after which the slide was aligned with the surface of the original paraffin block to locate the sampling areas. The designated areas in the paraffin block were punched with a 1-mm-diameter needle before each punched tissue was then manually transferred to a new recipient paraffin block to generate a TMA block. Five-micrometer-thick sections were cut from the TMA block, and applied on silane-coated slides (Sigma, St. Louis, MO, United States). After TMA construction, to confirm the presence of intact tumor tissue, a HE stained section of the TMA block was prepared and reviewed by two independent pathologists.

An immunohistochemical reaction was performed on 5 μ m-thick sections of TMA on silane-coated slides (Sigma) by an immunoperoxidase method. Tissue samples were deparaffinized and dehydrated before the endogenous peroxidase activity was blocked by adding 3% H₂O₂

in phosphate buffer saline (PBS) for 30 min. After washing with PBS, pH 7.4, and blocking with 2% skim milk in PBS, pH 7.4, for 30 min, the samples were incubated with rabbit polyclonal anti-ANXA1 antibody (1:100, Santa Cruz, Heidelberg, Germany) diluted in 2% nonfat dried milk, followed by a horseradish peroxidase (HRP)-conjugated secondary antibody (1:400, GE healthcare, Piscataway, NJ, United States). The appearance of the brown color corresponding to the peroxidase activity was developed using 3,3'-diaminobenzidine tetrahydrochloride as a chromogen and counterstained with Mayer's hematoxylin. Negative controls were performed in a similar manner but omitting the primary antibody. The ANXA1 staining was scored based on signal intensity and positive area as follows: negative, < 10%; weak (+), 10%-25%; moderate (++), 26%-75%; and strong (+++), > 75%. Consensus evaluation from at least two of the three investigators was considered acceptable.

Animal tissue samples

The Animal Ethics Committee of Khon Kaen University, Thailand approved the study protocols for the animal experiments (AEKKU 17/2552). Thirty male Syrian golden hamsters (*Mesocricetus auratus*) aged between 4 and 6 wk were obtained from the Animal Unit, Faculty of Medicine, Khon Kaen University, Khon Kaen, Thailand. Animals were divided into two groups; normal control group and infection with *O. viverrini* plus *N*-nitrosodimethylamine (NDMA) (*O. viverrini* + NDMA). In the treated group, hamsters induced by infection with *O. viverrini* metacercariae isolated from naturally infected cyprinid fish by pepsin (Wako, Japan) digestion and subsequently treated with 12.5 ppm NDMA at the same time point as described previously^[23]. NDMA was given in drinking water for 2 mo, and withdrawn thereafter until the animals were sacrificed at 21 d, and then at 3 and 6 mo post-treatment ($n = 5$ for each sub-group). Animals were anaesthetized and killed with an overdose of diethyl ether. The liver was dissected and was placed in 10% buffered formalin and used to evaluate histopathological changes and immunohistochemical studies^[23].

Cell lines and cell culture

Four human CCA cell lines, namely M156, M055, M213 and M214 were isolated from intrahepatic CCA patients from northeastern Thailand. CCA tissues were characterized as M156, M055 and M214 moderately differentiated CCA and M213 adenosquamous cell carcinoma. Those CCA cell lines were used to assess *ANXA1* gene expression and compared to an immortalized human cholangiocyte cell line (MMNK1). In addition, the M214 CCA cell line was used to verify ANXA1-related molecule regulation. These CCA cell lines were kindly provided by Associate Professor Banchop Sripa. All cell culture materials (media, serum and antibiotics) were purchased from Gibco, Invitrogen (Auckland, New Zealand). The CCA cells were cultured in HAM's F-12 medium supplemented with 10% heat-inactivated fetal bovine serum, 1% Penicillin-

streptomycin, 1.176 g/L sodium bicarbonate and adjusted to pH 7.1 with 1 mol/L HCl. The cells were incubated at 37 °C under a humidified 5% CO₂ atmosphere.

Western blot analysis

Protein was extracted from the CCA cell lines and the concentration was measured by the Bradford assay (Bio-Rad, Hercules, United States) according to the manufacturer's instructions. Twenty micrograms of protein were separated on a 12% sodium dodecyl sulfate polyacrylamide gel electrophoresis gel and transferred to a polyvinylidene difluoride membrane (Amersham Bioscience, Piscataway, NJ, United States) for 2 h at 60 V. The membrane was incubated overnight at 4 °C with rabbit polyclonal anti-ANXA1 antibody (1:1000, Santa Cruz, Heidelberg, Germany) diluted in 2% nonfat dried milk/phosphate buffered saline with Tween 20 (PBS-T). Subsequently, the membrane was incubated with an appropriate HRP-conjugated secondary antibody (1:3000, GE healthcare) diluted in 2% nonfat dried milk/PBS-T. The immunoreactive materials were developed by enhanced chemiluminescence using the ECL Western blotting Detection Reagent (GE Healthcare).

Quantitative real-time reverse transcription-polymerase chain reaction

Total RNA extraction was performed from various CCA cell lines (3×10^5 cells) of each experiment using the TRIzol reagent (Invitrogen, Carlsbad, CA, United States). An aliquot of total RNA was reverse transcribed into cDNA using reverse transcriptase (Invitrogen) following the manufacturer's protocol. Polymerase chain reaction (PCR) was carried out in duplicate in a 20- μ L volume using Faststart Universal SYBR Green Master (ROX, Roche Applied Science, Penzberg, Germany) using the following sets of primers: matrix metalloproteinase 2 (MMP2) (5'-TTGATGGCATCGCTCAGATC-3' and 5'-CTGCCAAGAACACAGCCTTC-3'), MMP9 (5'-CATTGTCATCCAGTTTGGT G-3' and 5'-AC-CACAACTCGT CGTCGTC-3'), transforming growth factor (TGF)- β (5'-ACATCGACTTTCGCAAGGAC-3' and 5'-TGGTTGTAG AGGGCAAGGAC-3'), nuclear factor- κ B (NF- κ B) (5'-GCITTGCAAACCTGGGAA-TA-3' and 5'-CAAGG TCAGAATGCACCAGA-3'), and GAPDH (5'-AGAAGACTGTGGATGGCCCC-3' and 5'-TGACCTTGCCCACAGCCTT-3'). Reactions were performed in the ABI 7500 thermal cycler (Applied Biosystems, Foster City, CA, United States). All data were analyzed using 7500 system software with a cycle threshold (Ct) in the linear range of amplification and then processed by the $2^{-\Delta\Delta C_t}$ method.

Knockdown of ANXA1 using siRNA against the ANXA1 gene

The M214 CCA cells were transiently transfected with siRNA against the *ANXA1* gene (Silencer® Select siRNA; siRNA ID s1380, Ambion, TX, United States) using the lipofectamine™ 2000 transfection reagent (Invitrogen) following the manufacturer's protocol with some modi-

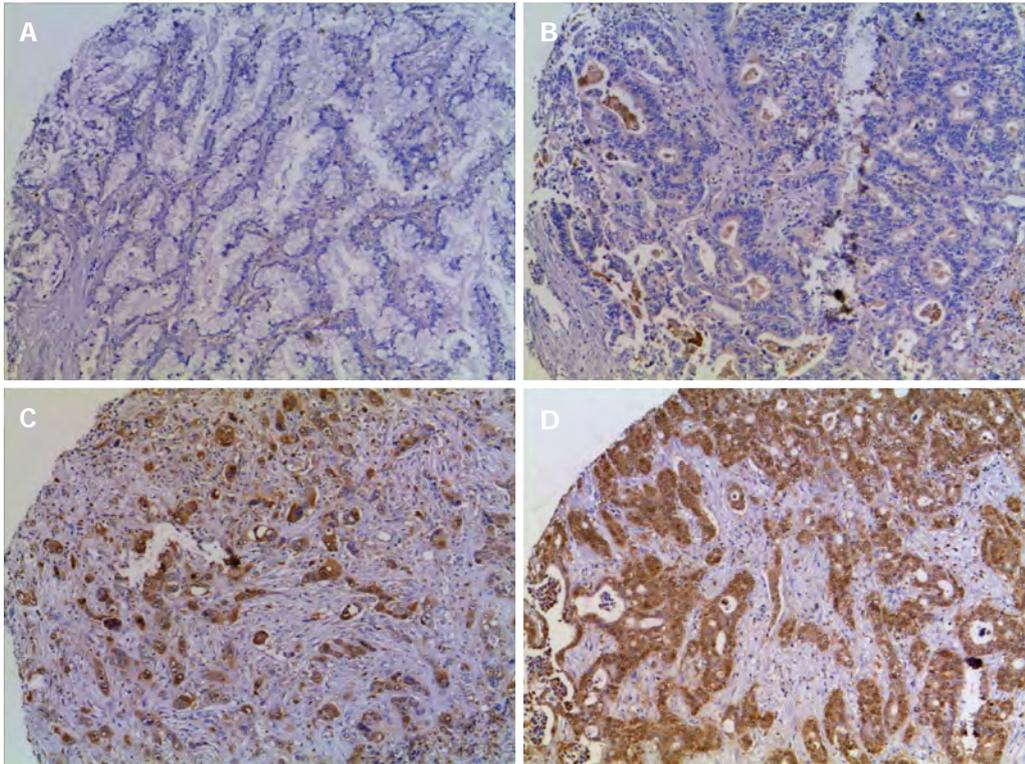


Figure 1 Immunostaining of annexin A1 protein expression in cholangiocarcinoma tissue microarrays. A-D: Tissue microarray of cholangiocarcinoma samples was stained with anti-annexin A1 antibody and counter stained with Mayer's hematoxylin and represented by A for negative, B for +, C for ++, and D for +++ when expression was < 10%, 10%-25%, 26%-75% and > 75%, respectively (magnification, × 200).

fications. In brief, 5 μ L of 5 μ mol/L stock concentration siRNA or scrambled siRNA and 5 μ L lipofectamine™ 2000 transfection reagent were separately diluted in 250 μ L OPTI-MEM I medium (Invitrogen). The diluted siRNA solution was mixed with the diluted transfection reagent and incubated for 20 min at room temperature before being added to a six-well plate seeded with 1×10^5 CCA cells in 2 mL transfection medium. The level of ANXA1 mRNA was accessed at 48 and 72 h after transfection. The negative controls were performed by using Silencer® Negative Control siRNA No. 1 (Ambion), a non-targeted sequence.

Statistical analysis

The data were expressed as mean \pm SD. To compare the data between groups, statistical significance was determined by Student's *t*-test. The χ^2 test was used to analyze the correlation between ANXA1 expression and the categorical variables regarding clinicopathological parameters. Survival analysis was done by Kaplan-Meier and log-rank tests. Statistical analyses were performed using SPSS version 15 (SPSS, Inc, Chicago, IL). A *P* value of less than 0.05 was considered statistically significant.

RESULTS

Expression of ANXA1 as a potential diagnostic marker for CCA but not HCC

ANXA1 expression in TMA obtained from 68 CCA and

46 HCC patients was examined using immunohistochemistry. Expression of ANXA1 was observed in the cytoplasm of epithelial bile duct tumor cells and some of inflammatory cells but was seen faintly or with no staining in tumor stroma and in hepatocytes (Figure 1). Sixty-four cases were positive (94.1%, 64/68) for ANXA1 expression. A high expression was coded in 21 cases for ++ and 24 cases for +++ or 66.2% (45/68 cases) and low expression where 4 cases were negative and 19 cases for + or 33.8% (23/68 cases) (Table 1; Figure 1). The histological feature in the tubular type showed that high expression (78.8%, 26/33) was significantly higher than low expression (21.2%, 7/33) (*P* < 0.05) (Table 1).

To determine ANXA1 expression distinguishing CCA from HCC, ANXA1 expression was determined in TMA of 46 HCC patients. Slight expression of ANXA1 was observed in the cytoplasm of some inflammatory cells, but was seen faintly or with no staining in tumor cells and hepatocytes. All histologic patterns of HCC samples 100% (46/46) were negative or of low expression (44 for negative and 2 for +) as shown in Figure 2. In addition, no staining or faintly stained immunoreactivity in normal liver tissues of healthy individuals (6/6) was also found. ANXA1 protein as an immunohistological marker could detect CCA and differential CCA from HCC in the primary liver cancer similar to CK7 and CA19-9 (Figure 3). ANXA1 immunohistochemistry showed high sensitivity (94%) and specificity (100%) and the positive prediction value was 100%.

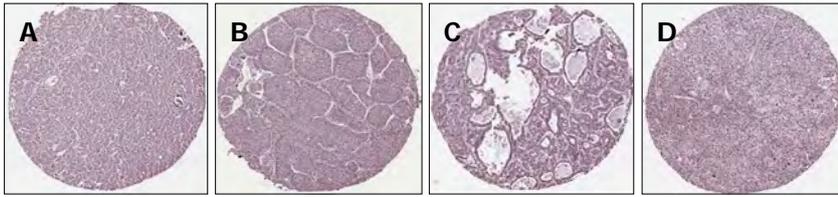


Figure 2 Immunostaining of annexin A1 in hepatocellular carcinoma tissue microarrays. Tissue microarray of hepatocellular carcinoma (HCC) samples was stained with anti-annexin A1 (ANXA1) antibody and counter stained with Mayer's Hematoxylin. ANXA1 shows low expression in all histologic patterns of HCC. A: Trabecular pattern; B: Broad trabecular pattern; C: Trabecular with pseudoacinar pattern; D: Solid growth pattern (magnification, × 40).

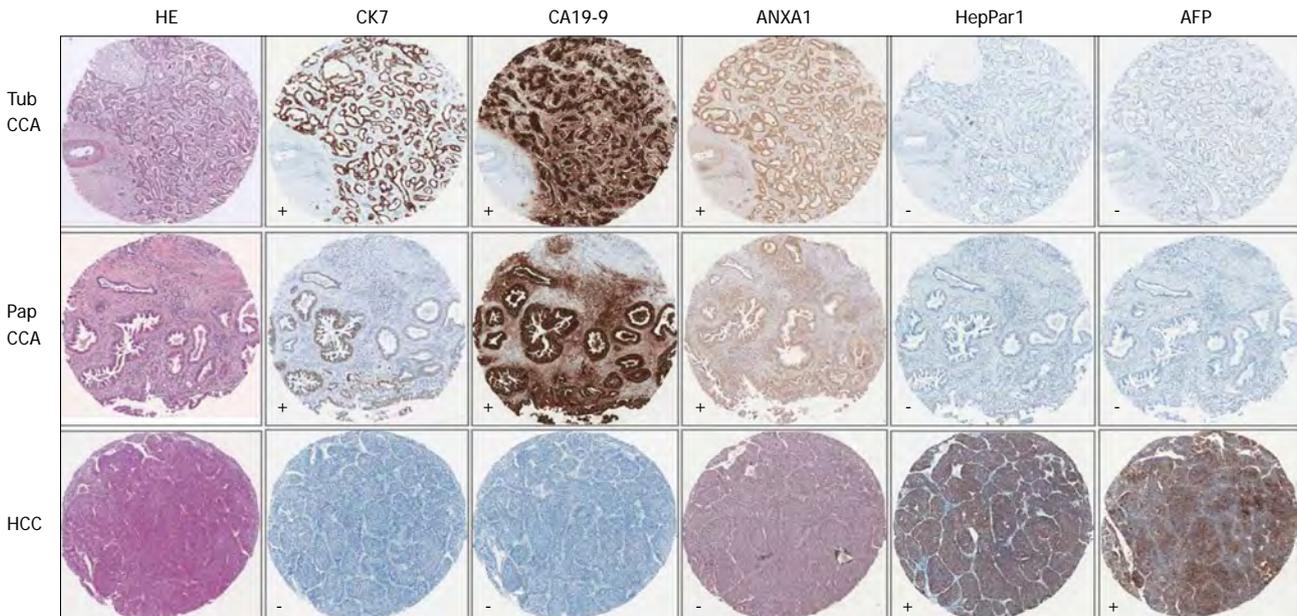


Figure 3 Illustration of the comparative immunohistochemical stains in 3 representative cases. The 3 representative cases include tubular type cholangiocarcinoma (CCA) (Tub CCA), papillary type CCA (Pap CCA) and hepatocellular carcinoma (HCC). Immunopositivity of cytokeratin 7 (CK7), annexin A1 (ANXA1), hepatocyte paraffin 1 (HepPar1) and alpha-fetoprotein (AFP) appeared as brown cytoplasmic staining of tumor cells, while positivity to carbohydrate antigen 19-9 (CA19-9) appeared as cytoplasmic and luminal staining of tumor (magnification, × 40). Noted that CCA are CK7⁺/CA19-9⁺/ANXA1⁺ and HCC is HepPar1⁺/AFP⁺/ANXA1⁻. -: Negative result; +: Positive result.

The correlation between ANXA1 expression and clinicopathological parameters was analyzed as shown in Table 1. There were no correlations between ANXA1 expression level and age, sex, tumor location, tumor size, gross type, lymph node metastasis (Table 1) and patients' survival outcome (data not shown). Notably, high ANXA1 expression levels were positively correlated with the histological features of the tubular type ($P = 0.03$) but not the papillary type. All 26 cases of high expression in the tubular type had positive lymph nodes.

Expression of ANXA1 in hamsters CCA tissues

To evaluate the expression of ANXA1 in the different stages of CCA tissues in *O. viverrini* + NDMA-induced CCA in the hamster model, immunohistochemistry was used to evaluate the inflamed tissues at 21 d, when the tumors began at 3 mo, and tumor progression at 6 mo post-treatment. The percentage of CCA 60% (3/5) at 3 mo, and 100% (5/5) at 6 mo post-treatment were described previously^[23]. Here, it was shown that the expression of ANXA1 was observed mainly in the cytoplasm of the

epithelial bile ducts, some inflammatory cells (large cell, macrophage-like cell), and tumor cells which increased with time as bile duct proliferation and CCA development progressed (Figure 4). The level of its expression at 21 d was +, 3 mo was ++, and 6 mo was +++ for all five animals per group. In addition, low or a little expression was found in the normal livers with no changes in each time-sacrificed group.

Expression of ANXA1 and the effect of its gene inhibition in CCA cell lines

Since ANXA1 was positive in CCA tissue but not in HCC tissue, the expression was further confirmed in various intrahepatic human CCA cell lines compared to MMNK1. Real-time reverse transcription (RT)-PCR revealed that a significantly increased expression of the *ANXA1* gene was observed in all four CCA cell lines including M156, M055, M213 and M214 compared to in MMNK1 ($P < 0.01$) (Figure 5).

In addition, ANXA1 was also investigated whether it was involved in MMPs in CCA metastasis by silenc-

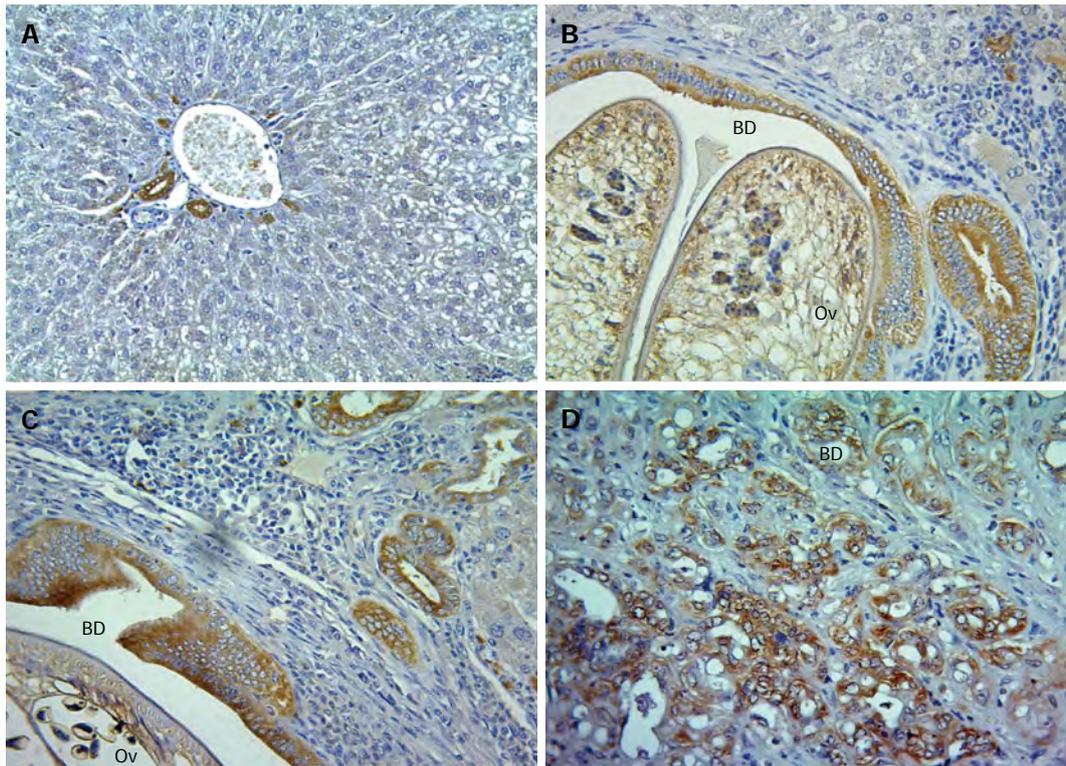


Figure 4 Immunostaining of annexin A1 protein expression in hamster tissues. A-D: Hamster tissues were stained with anti-annexin A1 antibody and counterstained with Mayer's hematoxylin represented by negative for A (normal at 6 mo post-treatment), grade + for B (21 d post-treatment), grade ++ for C (3 mo post-treatment) and grade +++ for D (6 mo post-treatment) (magnification, × 200). Ov: *Opisthorchis viverrini*; BD: Bile duct lumen.

Table 1 Clinical-pathological variables and the expression status of annexin A1 in cholangiocarcinoma tissues

| Variables | Annexin A1 | | | P value |
|---------------------------|------------|------|-------|---------|
| | Low | High | Total | |
| Age (yr) | | | | 0.328 |
| ≤ 56 | 11 | 16 | 27 | |
| > 56 | 12 | 29 | 41 | |
| Gender | | | | 0.487 |
| Male | 15 | 33 | 48 | |
| Female | 8 | 12 | 20 | |
| Histopathologic feature | | | | 0.033 |
| Tubular type | 7 | 26 | 33 | |
| Papillary type | 16 | 19 | 35 | |
| The intrahepatic location | | | | 0.197 |
| Peripheral | 11 | 16 | 27 | |
| Hilar | 11 | 29 | 40 | |
| Gallbladder bed | 1 | 0 | 1 | |
| Tumor size (cm) | | | | 0.889 |
| < 3 | 5 | 10 | 15 | |
| 3-6 | 10 | 17 | 27 | |
| > 6 | 8 | 18 | 26 | |
| Gross type | | | | 0.244 |
| Mass forming | 10 | 28 | 38 | |
| Periductal infiltrating | 6 | 9 | 15 | |
| Intra ductal | 7 | 7 | 14 | |
| Lymph node metastasis | | | | 0.382 |
| Absent | 7 | 21 | 28 | |
| Present | 11 | 20 | 31 | |

When the sum of subset numbers does not match patient totals, data were missing or unavailable.

ing ANXA1 using siRNA to target of *ANXA1* gene expression in M214 CCA cells. Real-time RT-PCR and Western blotting analysis showed that the mRNA level of cells transfected with siRNA at 48 h was suppressed by 87.3% and the protein level was decreased by 80% compared to that of controls (Figure 6). *In vitro* proliferation of the ANXA1 silenced cells was also reduced (data not shown). Suppression of the *ANXA1* gene significantly inhibited the mRNA expression of MMP2 by 50% and MMP9 by 45% ($P < 0.05$), and TGF- β by 15%, but induced NF- κ B by 70% (Figure 7).

DISCUSSION

The most frequent malignancy among Thais from north-eastern Thailand registered between 1985 and 2009 in liver cancers was 42% HCC and 58% CCA^[1]. Distinguishing CCA from HCC can be problematic due to heterogeneous morphological. Here, ANXA1, a candidate marker obtained from proteomics was used to investigate CCA in humans and hamsters by immunohistochemistry and tested its regulation expression in an *in vitro* study. In human TMAs of intrahepatic CCA, expression of ANXA1 protein was highly expressed (94.1%, 64/68), similar to a previous study (83.3%, 10/12) in sporadic intrahepatic CCA^[18]. An increased expression of the *ANXA1* gene was verified in all of four CCA cell lines, supporting its over-expression in CCA tissue. In contrast, a decreased

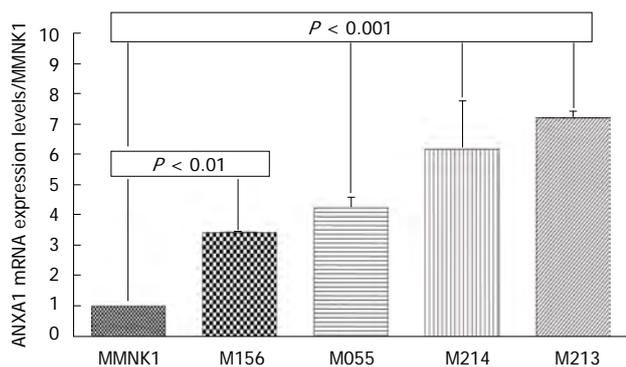


Figure 5 Expression of the annexin A1 gene in various cholangiocarcinoma cell lines. The mRNA expression level of annexin A1 (ANXA1) was evaluated by real-time reverse transcription-polymerase chain reaction in various cholangiocarcinoma (CCA) cell lines including M156, M055 and M214 moderately differentiated CCA, M213 adenocarcinoma and compared to an immortalized human cholangiocyte cell line (MMNK1). Data are derived from duplicate independent experiments and presented as mean \pm SD, $P < 0.01$, $P < 0.001$ vs MMNK1 is significantly different by the Student *t*-test.

expression of ANXA1 is a common event in extrahepatic CCA and is significantly correlated with a poorer outcome in Chinese patients^[19], indicating that its expression is varied according to tumor location. Moreover, down-regulation of ANXA1 expression was found in the HCC tissue array reported by Xue *et al*^[22] but opposite from the previous report in transgenic mice with HCC^[20] and in a human HCC cell line^[21]. Therefore, expression of ANXA1 has different expression in primary liver cancer. With the current results and previous study as evidence, this indicates that ANXA1 is a new immunohistochemical marker to distinguish between CCA and HCC. Nevertheless, more studies in sporadic CCA are warranted.

ANXA1 is bound to cellular membranes in a Ca^{2+} -dependent manner. It is a glucocorticoid-induced protein with multiple actions in the regulation of inflammatory cell activation, cellular processes and involved in carcinogenesis^[24]. Recently, its expression has been proposed for the regeneration of skeletal muscle tissue^[25] and in liver regeneration and transformation^[20]. In hamster CCA, ANXA1 expression has been observed mainly in bile duct epithelia and increasing with bile duct proliferation and bile duct cancer progression in this current study. It may imply that ANXA1 may contribute to regenerate bile duct injury triggered by the fluke and NDMA-mediated CCA. Likewise, in mice after hepatectomy, regeneration of hepatocytes and bile ducts may lead to activate ANXA1 expression in the liver^[20].

In addition, although its expression was not positively correlated with patients' survival, and lymph node invasion, most cases of a high expression were found in the tubular type having positive lymph nodes, implying that it may be involved in tumor invasion^[26] and specific for the tubular type but not in the papillary type. ANXA1 may regulate MMPs expression for CCA progression. The *in vitro* study revealed that ANXA1 in CCA cell lines of intrahepatic CCA patients was positively correlated with TGF- β and MMP2 and MMP9, but had a negative

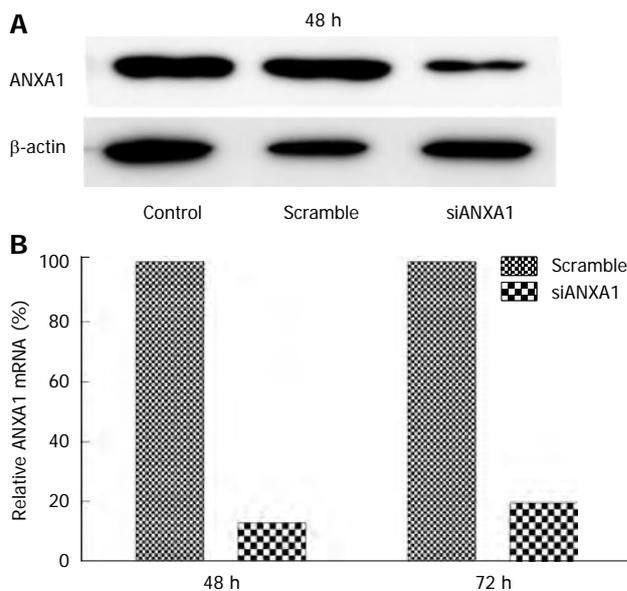


Figure 6 Effect of annexin A1 knockdown on the M214 cholangiocarcinoma cell line. A, B: Suppression of annexin A1 (ANXA1) expression at the translational level (at 48 h) and transcriptional level (at 48 and 72 h) in knock-down M214 cells was evaluated by Western blotting (A) and real-time reverse transcription-polymerase chain reaction (B).

relationship with NF- κ B. ANXA1 inhibits NF- κ B, a key regulator of inflammation, the common pathophysiological mechanism of inflammatory bowel diseases^[27]. These results indicated that ANXA1 functions as a positive regulator of TGF- β , MMP2 and MMP9 expression and invasion of cancer cells through specific activation of the NF- κ B signaling pathway^[28]. ANXA1 may regulate TGF- β signaling and promote metastasis^[29] leading to up-regulation of MMP2 and MMP9^[30] to degrade extracellular matrix (ECM) for tumor development and metastasis. The chronological expression of ANXA1 was shown to have different expression levels according to tumor development. An increased expression of ANXA1 with time is likely to be positively correlated with an increased accumulation of fibrosis and ECM, MMP9 expression^[23] for maintenance of cytoskeleton and ECM integrity, and differentiation^[25] for tumor onset^[31] and tumor progression^[32].

Recently, expression of ANXA1 was reported in inflamed tissue such as in ulcerative colitis^[33] and in chronic granulomatous inflammation which might become activated at different stages of this chronic inflammatory response^[34]. Moreover, expression of ANXA1 was correlated with the tumor staging in adenocarcinoma of the esophagus and the esophagogastric junction^[35] and bladder cancer^[32]. The results of the current study revealed that expression of the ANXA1 level in the inflamed tissues on 21 d was lower than when the tumor began at 3 mo and lower than in the tumor progression at 6 mo post-treatment in the hamster model, this finding may be supported by previous findings. In contrast, in benign breast tissue, myoepithelial cells showed stronger expression of ANXA1 than in tumorous tissue of breast cancer. A decreased expression of ANXA1 is correlated

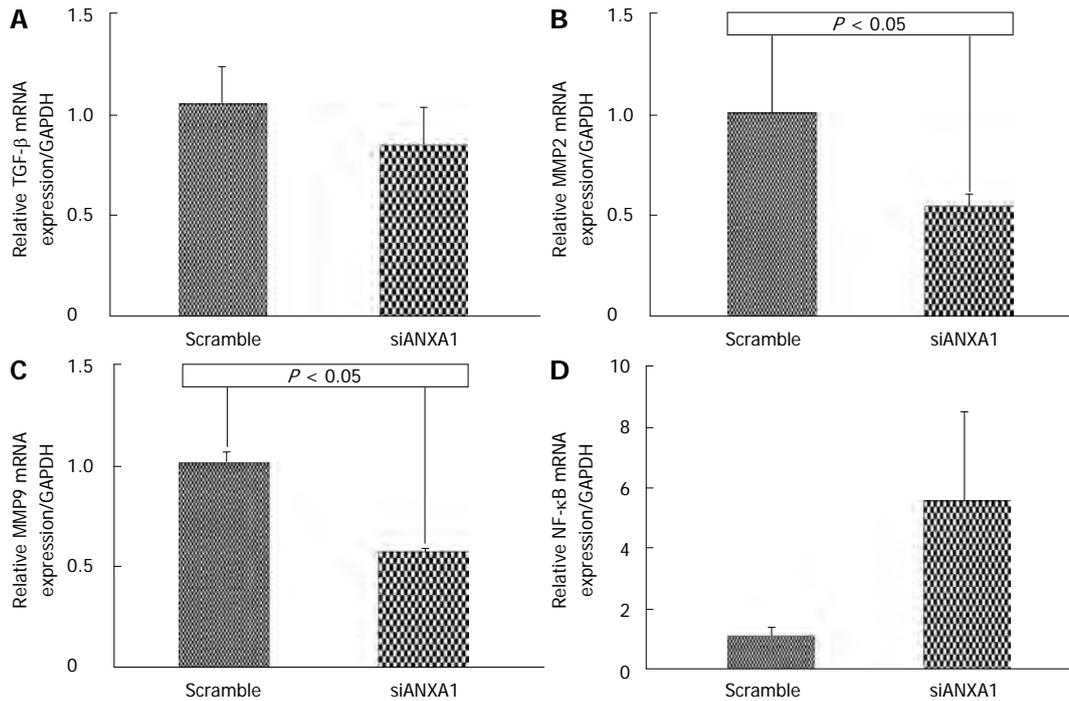


Figure 7 Effect of gene expression in the knockdown of the annexin A1 gene in the M214 cholangiocarcinoma cell line. A: Relative transforming growth factor-β (TGF-β) mRNA expression; B: Relative matrix metalloproteinase (MMP) 2 mRNA expression; C: Relative MMP9 mRNA expression; D: Relative nuclear factor (NF)-κB mRNA expression. Real-time reverse transcription-polymerase chain reaction was used to confirm the expression of TGF-β, MMP2, MMP9 and NF-κB in the knockdown of the annexin A1 (*ANXA1*) gene in the M214 cholangiocarcinoma cell line relative to glyceraldehyde-3-phosphate dehydrogenase (GAPDH). Data are derived from duplicate independent experiments and presented as mean ± SD, and $P < 0.05$ vs scramble is significantly different by the Student *t*-test.

with breast cancer development and progression^[36].

In conclusion, strong expression of ANXA1 was observed in CCA, but was low in HCC. In diagnosis of primary liver cancer, ANXA1 could be a new immunohistochemical marker for differential diagnosis of CCA from HCC. ANXA1 expression increased along with cholangiocarcinogenesis in hamsters induced by *O. viverrini* infection and NDMA administration. Its expression was positively correlated with TGF-β and MMPs but negatively correlated with the NF-κB signaling pathway, suggesting that ANXA1 is involved in inflammation-associated CCA, and a potential for therapeutic drug targeting.

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COMMENTS

Background

Cholangiocarcinoma (CCA) is associated with late presentation, has high mortality rate, and poses challenges for diagnostic. The histological CCA is

difficult distinguish from hepatocellular carcinoma (HCC). New diagnostic markers with better diagnostic differentiation are required. Annexin A1 (ANXA1) is a multipotent protein involved in several functions including regulation of cell differentiation, apoptosis, and carcinogenesis. ANXA1 involved in this process is frequently overexpressed in many types of cancers. Moreover, its expression in the same cancer is controversial.

Research frontiers

Although the expression of ANXA1 has been demonstrated in many types of cancers, its expression in CCA and HCC is controversial. Moreover, its expression at different stages of CCA development and its regulation in CCA has not been reported.

Innovations and breakthroughs

In this study, strong expression of ANXA1 was observed in CCA, but was low in HCC, which was similar to the available markers for CCA (cytokeratin 7 and carbohydrate antigen 19-9), implying its expression could present as a new marker for differential diagnosis of primary liver cancer. ANXA1 increased expression along with cholangiocarcinogenesis in hamsters induced by *Opisthorchis viverrini* infection and *N*-nitrosodimethylamine administration. Its expression was positively correlated with transforming growth factor-β (TGF-β) and matrix metalloproteinase (MMP) but negatively with the nuclear factor κB (NF-κB) signaling pathway. ANXA1 is involved in carcinogenesis of chronic inflammation related-CCA. ANXA1 is a promising biomarker and a potential for a therapeutic target in this aggressive cancer.

Applications

This study demonstrated that the ANXA1 protein was expressed in CCA, but had low expression in HCC and therefore it may provide a new diagnostic marker in CCA by the immunohistochemistry technique. By silencing of ANXA1 gene expression in the *in vitro* study the results demonstrate that ANXA1 represents a potential therapeutic target for the future intervention in CCA patients.

Terminology

Annexin A1 (also named macrocortin, renocortin, lipomodulin and lipocortin 1) is a 37 kDa protein member of an annexin superfamily which has a binding or annexing property to acidic phospholipid in a calcium dependent manner. It also has been shown to play a critical role in the regulation of inflammatory cell activation, cellular processes and involved in carcinogenesis.

Peer review

This is a good paper with sensible use of different experimental techniques. The authors examined the expression of ANXA1 in CCA and HCC tissues microarrays; ANXA1 protein had high sensitivity and specificity for immunohistochemistry diagnosis in CCA. Suppression of ANXA1 gene expression showed it inhibiting of TGF- β and MMPs but induction NF- κ B expression. The results are interesting, represent a new immunohistochemistry analysis of CCA and ANXA1 is a promising therapeutic potential of drugs targeting in CCA.

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Hepatocellular carcinoma in patients with chronic kidney disease

Chern-Horng Lee, Sen-Yung Hsieh, Ja-Liang Lin, Maw-Sen Liu, Tzung-Hai Yen

Chern-Horng Lee, Sen-Yung Hsieh, Ja-Liang Lin, Maw-Sen Liu, Tzung-Hai Yen, College of Medicine, Chang Gung University, Taoyuan 333, Taiwan

Chern-Horng Lee, Maw-Sen Liu, Department of General Internal Medicine and Geriatrics, Chang Gung Memorial Hospital, Linkou 333, Taiwan

Sen-Yung Hsieh, Liver Research Unit, Chang Gung Memorial Hospital, Taoyuan 333, Taiwan

Ja-Liang Lin, Tzung-Hai Yen, Department of Nephrology, Chang Gung Memorial Hospital, Taipei 105, Taiwan

Author contributions: Lee CH contributed to data analysis, write the paper and perform the study; Hsieh SY and Lin JL contributed to supervise the study; Lee CH, Hsieh SY, Lin JL, Liu MS and Yen TH contributed to patient care and management; Yen TH contributed to conceive and design the study, help in data analysis and manuscript writing.

Correspondence to: Tzung-Hai Yen, MD, PhD, Department of Nephrology, Chang Gung Memorial Hospital, 199 Tung Hwa North Road, Taipei 105, Taiwan. m19570@adm.cgmh.org.tw
Telephone: +886-3-3281200 Fax: +886-3-3282173

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Abstract

AIM: To investigate outcomes of hepatocellular carcinomas (HCCs) in patients with chronic kidney disease (CKD).

METHODS: Four hundred and forty patients referred between 2000 and 2002 for management of HCCs were categorized according to their CKD stage, *i.e.*, estimated glomerular filtration rate (eGFR) > 90 (stage 1), 60-90 (stage 2), 30-60 (stage 3), 15-30 (stage 4), and < 15 (stage 5) mL/min per 1.73 m², respectively. Demographic, clinical and laboratory data were collected and mortality rates and cause of mortality were analyzed. The mortality data were examined with Kaplan-meier method and the significance was tested using a log-rank test. An initial univariate Cox regres-

sion analysis was performed to compare the frequency of possible risk factors associated with mortality. To control for possible confounding factors, a multivariate Cox regression analysis (stepwise backward approach) was performed to analyze those factors that were significant in univariate models ($P < 0.05$) and met the assumptions of a proportional hazard model.

RESULTS: Most HCC patients with CKD were elderly, with mean age of diagnosis of 60.6 ± 11.9 years, and mostly male (74.8%). Hepatitis B, C and B and C co-infection virus were positive in 61.6%, 45.7% and 14.1% of the patients, respectively. It was found that patients with stages 4 and 5 CKD were not only older ($P = 0.001$), but also had higher hepatitis C virus carrier rate ($P = 0.001$), lower serum albumin level ($P = 0.001$), lower platelet count ($P = 0.037$), longer prothrombin time ($P = 0.001$) as well as higher proportions of advanced cirrhosis ($P = 0.002$) and HCCs ($P = 0.001$) than patients with stages 1 and 2 CKD. At the end of analysis, 162 (36.9%) patients had died. Kaplan-Meier analysis revealed that patients with stages 4 and 5 CKD suffered lower cumulative survival than stages 1 and 2 CKD (log-rank test, $\chi^2 = 11.764$, $P = 0.003$). In a multivariate Cox-regression model, it was confirmed that CKD stage [odds ratio (OR) = 1.988, 95%CI: 1.012-3.906, $P = 0.046$], liver cirrhosis stage (OR = 3.571, 95%CI: 1.590-8.000, $P = 0.002$) and serum albumin level (OR = 0.657, 95%CI: 0.491-0.878, $P = 0.005$) were significant predictors for mortality in this population.

CONCLUSION: HCC patients with stages 4 and 5 CKD had inferior survival than stages 1 and 2 CKD. This warrants further studies.

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Key words: Hepatocellular carcinoma; Hepatitis B virus; Hepatitis C virus; Chronic kidney disease; End-stage renal disease

Core tip: There is a paucity of data regarding outcomes of hepatocellular carcinoma (HCC) in patients with chronic kidney disease (CKD), even though both hepatitis B virus and CKD are endemic in Taiwan. In a large-scale study, a total of 440 patients with HCC were categorized according to their CKD stage. At the end of analysis, it was found that HCC patients with stages 4 and 5 CKD suffered poorer survival than stages 1 and 2 CKD, which might be explained by inferior liver reserve. Interestingly, tumor stage was not a significant predictor for mortality according to a multivariate Cox regression model.

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INTRODUCTION

Taiwan has the highest prevalence and second highest incidence of end-stage renal disease (ESRD) in the world^[1,2]. According to the 2012 Renal Data System of the United States^[3], the United States, Taiwan and Japan continue to have some of the highest rates of incident ESRD, at 369, 361 and 288 per million population in 2010. In Taiwan, the prevalence of ESRD reached 2584 per million in 2010, while rates of 2260 and 1870 were reported in Japan and the United States. Similarly, hepatitis B virus (HBV) and hepatocellular carcinoma (HCC) are endemic in Taiwan^[4-6]. The carrier rate of hepatitis B surface antigen (HBsAg) in the general population is 15%-20%^[4]. Consequently, many Taiwanese patients with CKD or ESRD are also chronic HBV carriers^[7].

There is strong evidence of increased cancer risk in patients with CKD^[8], in patients with ESRD being treated with chronic dialysis^[9-11], and in recipients of renal transplant^[12]. The CKD was associated with malignancy and worse prognosis^[13-16]. Moderately reduced kidney function may be an independent risk factor for cancer in older men. The risk begins when estimated glomerular filtration rate (eGFR) falls to approximately 55 mL/min and progressively increases as eGFR declines to 40 mL/min, similar to the risk of patients on dialysis or of transplant recipients^[8]. Because survival time after the confirmation of recurrence was significantly shorter in the CKD group, their cumulative survival rate was significantly lower compared to that in the non-CKD group, although the difference in disease-free survival was not significant^[17].

Almost all cancers in the renal parenchyma are renal cell carcinoma, whereas cancers in the urinary tract are urothelial carcinoma. These two cancers differ markedly in terms of carcinogenesis and basic biology. Renal cell carcinoma is the most common urologic cancer in Western patients on dialysis, whereas urothelial carcinoma is

the most common urologic cancer in Asian patients on chronic dialysis^[11]. This is a unique and distinguishing epidemiologic characteristic of the Taiwanese population^[11]. On the other hand, very few data^[17-22] are available regarding the outcome of patients with HCC and CKD, even though both are endemic in Taiwan.

Therefore, this study analyzed the registry of the Chang Gung Memorial Hospital to examine the epidemiology of HCC in CKD populations in Taiwan.

MATERIALS AND METHODS

This retrospective observational study complied with the guidelines of the Declaration of Helsinki and was approved by the Medical Ethics Committee of Chang Gung Memorial Hospital, a tertiary referral center in northern Taiwan. Because of the retrospective nature of this study, Institutional Review Board approval was obtained and the informed consent of risk of HCC and all treatment modalities of all patients on their initial admission was used. Moreover, all individual information was securely protected (by delinking identifying information from the main dataset) and available only to the investigators. All the data were analyzed anonymously and all primary data were collected according to epidemiologic guidelines. This policy was based on previous publications^[23,24].

Patients

Four hundred and forty patients referred between 2000 and 2002 for management of HCCs were categorized according to their CKD stage, *i.e.*, eGFR > 90 (stage 1), 60-90 (stage 2), 30-60 (stage 3), 15-30 (stage 4), and < 15 (stage 5) mL/min per 1.73 m², respectively. Demographic, clinical, laboratory and mortality data were obtained for analysis.

Diagnosis of HCC

HCC was diagnosed by alpha-fetoprotein, imaging studies such as ultrasonography, radio-contrast enhanced triphasic dynamic computed tomography, magnetic resonance imaging, angiography, and/or documented tissue histopathology^[25].

Diagnosis of liver cirrhosis

Cirrhosis was diagnosed by histopathology or by laboratory tests, hepatic ultrasonography, and clinical manifestations of chronic hepatitis with portal hypertension (*i.e.*, varices, thrombocytopenia, or splenomegaly), and/or hepatic decompensation (*i.e.*, jaundice, prolonged prothrombin time, and ascites)^[26].

Diagnosis of CKD

The eGFR was computed using the four-variable modification of diet in renal disease (MDRD) study equation^[27]. The CKD stage was defined as 1, 2, 3, 4 and 5 according to eGFR; > 90, 60-90, 30-60, 15-30, and < 15 mL/min per 1.73 m², respectively^[27].

Table 1 Baseline characteristics of patients with hepatocellular carcinoma, stratified according to the stage of chronic kidney disease *n* (%)

| Variable | Total (<i>n</i> = 440) | Stages 1 and 2 (<i>n</i> = 132) | Stage 3 (<i>n</i> = 263) | Stages 4 and 5 (<i>n</i> = 45) | <i>P</i> value |
|--------------------------------------|-------------------------|----------------------------------|---------------------------|---------------------------------|----------------|
| eGFR ¹ | 53.7 ± 27.1 | 73.5 ± 13.5 | 50.0 ± 26.4 | 17.6 ± 8.7 | 0.000 |
| Age ¹ (yr) | 60.6 ± 11.9 | 54.4 ± 12.3 | 63.1 ± 10.5 | 64.0 ± 11.7 | 0.000 |
| Male | 329 (74.8) | 115 (87.1) | 182 (69.2) | 32 (71.1) | 0.000 |
| HBV carrier | 271 (61.6) | 97 (73.5) | 152 (57.8) | 22 (48.9) | 0.002 |
| HCV carrier | 201 (45.7) | 42 (31.8) | 131 (49.8) | 28 (62.2) | 0.000 |
| HBV and HCV carrier | 62 (14.1) | 15 (11.4) | 39 (14.8) | 8 (17.8) | 0.488 |
| Follow-up duration ¹ (mo) | 37.6 ± 37.1 | 37.0 ± 37.6 | 40.7 ± 37.6 | 20.9 ± 27.4 | 0.013 |

¹Data are presented as mean ± SD. The *P* value represents comparison between patients with stages 4 and 5 and patients with stages 1 and 2. eGFR: Estimated glomerular filtration rate; HBV: Hepatitis B virus; HCV: Hepatitis C virus.

Table 2 Liver function tests of patients with hepatocellular carcinoma, stratified according to the stage of chronic kidney disease (mean ± SD)

| Variable | Total (<i>n</i> = 440) | Stage 1 and 2 (<i>n</i> = 132) | Stage 3 (<i>n</i> = 263) | Stages 4 and 5 (<i>n</i> = 45) | <i>P</i> value |
|--|-------------------------|---------------------------------|---------------------------|---------------------------------|----------------|
| AFP (ng/mL) | 6202.1 ± 83392.3 | 3564.6 ± 11906.0 | 8176.6 ± 106579.9 | 1866.3 ± 4866.0 | 0.911 |
| AST (U/L) | 84.8 ± 77.9 | 94.3 ± 76.1 | 77.4 ± 68.6 | 104.3 ± 126.5 | 0.495 |
| ALT (U/L) | 73.0 ± 90.4 | 79.4 ± 79.1 | 64.8 ± 49.2 | 103.0 ± 218.9 | 0.150 |
| T-bilirubin (mg/dL) | 1.8 ± 3.7 | 1.5 ± 1.6 | 1.8 ± 4.3 | 2.8 ± 4.4 | 0.198 |
| ALP (U/L) | 122.4 ± 92.1 | 124.0 ± 76.6 | 114.6 ± 79.7 | 164.5 ± 170.2 | 0.072 |
| Albumin (g/dL) | 3.5 ± 0.7 | 3.6 ± 0.6 | 3.5 ± 0.7 | 3.2 ± 0.7 | 0.001 |
| Prolonged PT (s) | 1.9 ± 4.3 | 1.4 ± 1.2 | 1.6 ± 2.1 | 5.1 ± 12.4 | 0.000 |
| Platelet count (x 10 ³ /μL) | 138.2 ± 90.7 | 163.7 ± 109.7 | 126.5 ± 80.1 | 131.4 ± 73.0 | 0.037 |

The *P* value represents comparison between patients with stages 4 and 5 and patients with stages 1 and 2. AFP: Alpha-fetoprotein; AST: Aspartate aminotransferase; ALT: Alanine aminotransferase; T-bilirubin: Total bilirubin; ALP: Alkaline phosphatase; PT: Prothrombin time.

Management of HCC

The HCC patients in the stages 1 and 2 groups underwent tumor resection, liver transplantation, or percutaneous interventional therapies for local tumor ablation, including radio-frequency ablation, pure ethanol injection therapy, pure acetic acid injection therapy, and transcatheter arterial chemo-embolization^[28]. Patients in the stage 3 group received trans-catheter arterial chemo-embolization and radio-frequency ablation^[28]. Those in stage 4 received palliative chemotherapy, trans-catheter arterial chemo-embolization, or radiotherapy, and medical care^[28].

Statistical analysis

Continuous variables are expressed as means and standard deviations and categorical variables as numbers with percentages in brackets. All data were tested for normality of distribution and equality of standard deviations before analysis. For comparisons between patient groups, we used General Linear Model (Least Significant Difference test) for quantitative variables and Cross-tabulation (χ^2 or Fisher's exact tests) for categorical variables. Mortality data were compared using the Kaplan-Meier method and the significance was tested using a log-rank test. An initial univariate Cox regression analysis was performed to compare the frequency of possible risk factors associated with mortality. To control for possible confounding factors, a multivariate Cox regression analysis (stepwise backward approach) was performed to analyze those factors that were significant in univariate

models ($P < 0.05$) and met the assumptions of a proportional hazard model. We considered results that rejected the null hypothesis with 95% confidence to be significant. All analyses were performed using IBM SPSS Statistics Version 20.

RESULTS

Baseline characteristics

Most HCC patients with CKD were elderly, with mean age of diagnosis of 60.6 ± 11.9 years (Table 1), and mostly male (74.8%). Hepatitis B, C and B and C co-infection virus were positive in 61.6%, 45.7% and 14.1% of the patients, respectively. It was found that patients with stages 4 and 5 CKD were older ($P = 0.001$) and had higher hepatitis C virus (HCV) carrier rates ($P = 0.001$) than patients with stages 1 and 2 CKD. On the other hand, there were more male ($P = 0.000$) and HBV carrier rate ($P = 0.002$) in stages 1 and 2 than stages 4 and 5 CKD.

Comparison of liver biochemistry test

Patients with stages 4 and 5 CKD not only had lower serum albumin level ($P = 0.001$) and platelet count ($P = 0.037$), but also had longer prothrombin time ($P = 0.001$) than stages 1 and 2 CKD (Table 2).

Comparison of liver reserve

Patients with stages 4 and 5 CKD had higher incidences of advanced cirrhosis than stages 1 and 2 CKD (Table 3, $P = 0.002$).

Table 3 Liver cirrhosis classification, tumor staging, cause of mortality of patients with hepatocellular carcinoma stratified according to the stage of chronic kidney disease *n* (%)

| Variable | Total (<i>n</i> = 440) | Stage 1 and 2 (<i>n</i> = 132) | Stage 3 (<i>n</i> = 263) | Stages 4 and 5 (<i>n</i> = 45) | <i>P</i> value |
|---|-------------------------|---------------------------------|---------------------------|---------------------------------|----------------|
| Liver cirrhosis classification (Child-Pugh score) | | | | | 0.002 |
| No | 53 (12.0) | 27 (20.5) | 23 (8.7) | 3 (6.7) | |
| Class A | 236 (53.6) | 67 (50.8) | 151 (57.4) | 18 (40.0) | |
| Class B | 110 (25.0) | 30 (22.7) | 64 (24.3) | 16 (35.6) | |
| Class C | 41 (9.3) | 8 (6.1) | 25 (9.5) | 8 (17.8) | |
| Tumor stage | | | | | 0.001 |
| Stage 1 | 213 (48.4) | 60 (45.5) | 133 (50.6) | 20 (44.4) | |
| Stage 2 | 113 (25.7) | 27 (20.5) | 76 (28.9) | 10 (22.2) | |
| Stage 3 | 84 (19.1) | 40 (30.3) | 36 (13.7) | 8 (17.8) | |
| Stage 4 | 30 (6.8) | 5 (3.8) | 18 (6.8) | 7 (15.6) | |
| Mortality | 162 (36.9) | 47 (35.6) | 94 (35.9) | 21 (46.7) | |
| Cause of mortality | | | | | 0.050 |
| Liver failure | 27 (6.1) | 10 (7.6) | 16 (6.1) | 1 (2.2) | |
| Tumor death | 35 (8.0) | 8 (6.1) | 22 (8.4) | 5 (11.1) | |
| Gastrointestinal bleeding | 27 (6.1) | 11 (8.3) | 16 (6.1) | 0 (0) | |
| Sepsis | 69 (15.7) | 18 (13.6) | 36 (13.7) | 15 (33.3) | |
| Others | 5 (1.1) | 1 (0.8) | 4 (1.5) | 0 (0) | |

The *P* value represents comparison between patients with stages 4 and 5 and patients with stages 1 and 2.

Table 4 Cox regression analysis of mortality in patients with hepatocellular carcinoma and chronic kidney disease

| Variable | Univariate analysis | | Multivariate analysis | |
|--|---------------------|----------------|-----------------------|----------------|
| | Odds ratio (95%CI) | <i>P</i> value | Odds ratio (95%CI) | <i>P</i> value |
| Chronic kidney disease stage | 2.237 (1.376-3.636) | 0.001 | 1.988 (1.012-3.906) | 0.046 |
| Liver cirrhosis stage | 4.566 (2.331-8.929) | 0.000 | 3.571 (1.590-8.000) | 0.002 |
| Tumor stage | 3.509 (1.789-6.849) | 0.000 | 2.169 (0.997-4.717) | 0.051 |
| Albumin, each increase of 1 mg/dL | 0.503 (0.399-0.633) | 0.000 | 0.657 (0.491-0.878) | 0.005 |
| Prolonged prothrombin time, each increase of 1 s | 1.062 (1.036-1.089) | 0.000 | 0.977 (0.942-1.013) | 0.215 |

Comparison of tumor staging

Most of the HCCs were diagnosed in the early stages (Table 3). Patients with stages 4 and 5 CKD had higher proportions of advanced HCCs than stages 1 and 2 CKD ($P = 0.001$).

Comparison of mortality

At the end of analysis, 162 (36.9%) patients had died (Table 3). It was revealed that patients with stages 4 and 5 CKD suffered higher fatal septic complications than stages 1 and 2 CKD ($P = 0.050$). The one-, three-, and five-year survival rates were 86.2%, 71.3% and 55.9%, respectively (Figure 1). In addition, patients with stages 4 and 5 CKD suffered lower cumulative survival than stages 1 and 2 CKD (Figure 2, log-rank test, $\chi^2 = 11.764$, $P = 0.003$).

Mortality analysis

In a multivariate Cox regression model (Table 4), it was confirmed that CKD stage [odds ratio (OR) = 1.988, 95%CI: 1.012-3.906, $P = 0.046$], liver cirrhosis stage (OR = 3.571, 95%CI: 1.590-8.000, $P = 0.002$) and serum albumin level (OR = 0.657, 95%CI: 0.491-0.878, $P = 0.005$) were significant predictors for mortality.

DISCUSSION

The analytical data demonstrated that HCC patients with stages 4 and 5 CKD had inferior survival than stages 1 and 2 CKD. The reason is unclear but inferior liver reserve in this subgroup should be considered. In a large-scale study in Taiwan^[16], there was a higher risk for overall cancer mortality in CKD patients compared to non-CKD patients (adjusted hazard ratio 1.2). Moreover, CKD was associated with increased mortality from liver, kidney, and urinary tract cancers, with adjusted hazard ratios of 1.74, 3.3, and 7.3, respectively. Most importantly, patients with stage 4 CKD had poorer prognosis than the other groups^[16], but the reason was also unclear.

In the present study, patients with HCC were stratified according to the stage of CKD. Kaplan-Meier analysis revealed that patients with stages 4 and 5 CKD suffered lower cumulative survival than stages 1 and 2 CKD ($P = 0.003$). A multivariate Cox regression model confirmed that CKD stage ($P = 0.046$), liver cirrhosis stage ($P = 0.002$) and serum albumin level ($P = 0.005$) were significant predictors of mortality. Tumor stage was a significant predictor for mortality in the univariate model ($P = 0.000$), but not after multivariate analysis ($P = 0.051$).

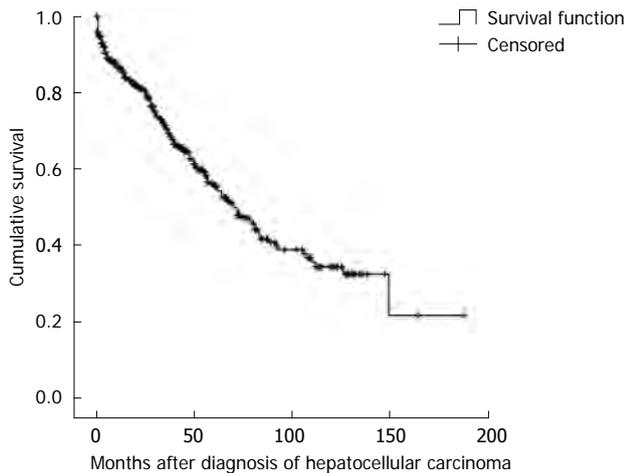


Figure 1 Kaplan-Meier survival analysis. The one-, three-, and five-year survival rate was 86.2%, 71.3% and 55.9%, respectively.

Previous study^[29] also reported that eGFR, as determined by MDRD equation, might provide better prognostic accuracy than the CKD-epidemiology collaboration equations independent of liver functional reserve and tumor staging, and is a more feasible renal surrogate for outcome prediction in patients with HCC and CKD stages 1-3 receiving TACE. Notably, the TNM classification did not accurately predict survival in HCC patients with CKD.

In this study, most HCC patients with CKD are male (74.8%). Moreover, there were more male in stages 1 and 2 than stages 4 and 5 CKD ($P = 0.000$). In 1981, Zevin *et al*^[30] reported a hemodialysis patient who developed HCC after long-term therapy with androgenic anabolic steroids. The tumor progressed very rapidly, with no evidence of regression despite discontinuation of the drug. The increased risk of malignancy in patients with chronic uremia and hemodialysis and the higher frequency of HCC associated with the use of anabolic steroids may explain the male predominance in CKD populations^[30]. Since CKD is associated with hypogonadism, the protective effect of estrogen on liver cancer may be reduced^[16]. Nevertheless, male predominance in HCC has been reported due to other than androgenic anabolic steroid use^[31].

In this study, hepatitis B, C and B and C co-infection virus were positive in 61.6%, 45.7% and 14.1% of the patients, respectively. Notably, there were more HCV ($P = 0.001$) in stages 4 and 5 CKD than stages 1 and 2 CKD, but more HBV ($P = 0.002$) in stages 1 and 2 than stages 4 and 5 CKD. Hence, the incidence of HBV (61.6%) is only a little higher than that of HCV (45.7%) in this study, although the national prevalence rates among adults in Taiwan is 1%-3% and 15%-20% for HCV and HBV, respectively^[32]. Notably, after implementation of national hepatitis B vaccination in Taiwan, the prevalence of HBsAg among persons younger than 15 years of age has decreased from 9.8% in 1984 to 0.7% in 1999^[33]. Higher incidences of HBV and HCV infection were noted in uremic patients, possibly because of cross-infection during hemodialysis. It was found that compared to those

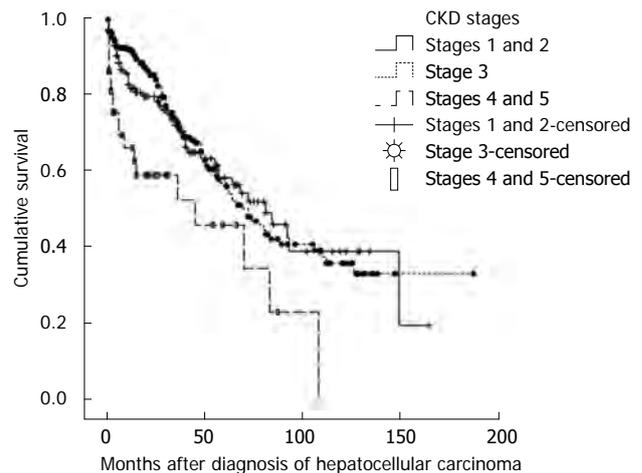


Figure 2 Survival analysis after stratification according to the stage of chronic kidney disease. Patients with stages 4 and 5 chronic kidney disease (CKD) suffered lower cumulative survival than stages 1 and 2 CKD (log-rank test, $\chi^2 = 11.764$, $P = 0.003$).

who were negative for both markers, patients with both HBsAg and anti-HCV had increased incidence of chronicity^[34]. Earlier study^[35] also found that HCV infection, but not HBV infection, is significantly associated with prevalence and disease severity of CKD in chronic dialysis patients. However, another population-based study^[36] found no association between HCV and risk of development of CKD.

Very few data are available regarding the outcome of HCC in CKD populations. Nevertheless, previous evidences^[17-22] suggested that we should be more aggressive on the principle for management for HCC in CKD patients, because the prognosis was not different between patients with and without CKD. For example, Huo *et al*^[18] compared the survival of 172 HCC patients with and without CKD who underwent percutaneous injection therapy. After a mean follow-up period of 24 ± 9 mo, there was no significant survival difference in patients with and without CKD^[18]. Kondo *et al*^[19] also reported that radiofrequency ablation was a safe and effective option for small HCCs in ESRD patients undergoing chronic hemodialysis. Orii *et al*^[17] compared the outcomes of 17 patients with CKD who had undergone hepatectomy for HCC with 51 non-CKD patients subjected to hepatectomy for HCC. The operative and pathologic findings were comparable between the two groups. Post-operative circulatory insufficiency occurred more frequently in the CKD group ($P = 0.013$). Although the disease-free survival rates were comparable between the two groups, the overall survival rates were significantly lower in the CKD group than in the non-CKD group ($P = 0.031$). Orii *et al*^[17] therefore concluded that hepatectomy for HCC should be considered even for CKD patients if careful peri-operative management and suitable multi-disciplinary treatment for recurrent disease are provided. In the study by Yeh *et al*^[20], the outcomes of 26 ESRD-HCC patients who underwent hepatic resection were reviewed and compared to 1198

HCC patients without ESRD who underwent hepatic resection. There were more associated disease, more physical signs of anemia and post-operative complications, lower hemoglobin, platelet, and alpha-fetoprotein levels, elevated blood urea nitrogen and creatinine levels, smaller tumors, lower HBsAg positivity, higher HCV positivity, and longer hospital stays in the ESRD-HCC group compared to the HCC group. Nonetheless, the overall and disease-free survival rates were similar between the two groups^[20]. Cheng *et al*^[21] also demonstrated that the operative morbidity and mortality between ESRD and non-ESRD groups were similar. The five-year disease-free survival rates for ESRD and non-ESRD groups were 35.0% and 34.2%, respectively ($P = 0.31$), while the five-year actual survival rates were 67.8% and 53.3%, respectively ($P = 0.54$). The study suggested that liver resection for HCC was justified in selected patients with ESRD^[21]. In the study by Sawada *et al*^[22], 91 patients who underwent hepatectomy were retrospectively divided into two groups based on their creatinine clearance (Ccr) values: a group with Ccr values ≥ 50 to < 100 mL/min ($n = 77$) and a group with Ccr values of ≥ 20 but < 50 mL/min ($n = 14$). There were no statistically significant differences between the two groups in terms of intra-operative blood loss or intra-operative urine volume. The difference between the two groups in post-operative complications was not statistically significant. Thus, the team concluded that adequate indications, appropriate operative procedures, and peri-operative management might allow hepatectomy to be performed safely in patients with non-uremic minimal renal failure^[22].

In conclusion, our results showed that HCC patients with stages 4 and 5 CKD had inferior survival than stages 1 and 2 CKD, which might be explained by poorer liver reserve. Nevertheless, the retrospective nature of the study, the small patient population, and the short follow-up duration are limitations that warrant further investigations to validate the conclusion drawn here.

COMMENTS

Background

There is a paucity of data regarding outcomes of hepatocellular carcinomas (HCCs) in patients with chronic kidney disease (CKD), even though both hepatitis B virus and CKD are endemic in Taiwan.

Research frontiers

Previous reports found a strong evidence of increased cancer risk in patients with CKD, and the CKD was associated with malignancy and worse prognosis.

Innovations and breakthroughs

The authors analyzed the database of Chang Gung Memorial Hospital to examine the epidemiology of HCC in CKD populations in Taiwan. It was found that HCC patients with stages 4 and 5 CKD had inferior survival than stages 1 and 2 CKD. On the other hand, the TNM classification (or tumor stage) did not accurately predict survival in HCC patients with CKD.

Applications

The data is important to understand the outcome of HCC in CKD population in Taiwan, an area with highest prevalence and second highest incidence of end-stage renal disease in the world.

Terminology

HCC is the most common type of liver cancer. Most cases of HCC are secondary to either a viral hepatitis infection or cirrhosis. CKD is the slow loss of

kidney function over time. There are five stages of CKD, but kidney function is normal in stage 1, and minimally reduced in stage 2, moderately reduced in stage 3, severely reduced in stage 4, and very severely reduced (or called end-stage renal disease) in stage 5.

Peer review

The epidemiology of HCC in CKD population has not been extensively investigated. Therefore, this data is particularly important.

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Overexpression of carbonic anhydrase II and Ki-67 proteins in prognosis of gastrointestinal stromal tumors

Li-Cheng Liu, Wen-Tong Xu, Xin Wu, Po Zhao, Ya-Li Lv, Lin Chen

Li-Cheng Liu, Wen-Tong Xu, Xin Wu, Lin Chen, Department of General Surgery, General Hospital of PLA, Beijing 100853, China

Po Zhao, Ya-Li Lv, Department of Pathology, General Hospital of PLA, Beijing 100853, China

Author contributions: Liu LC performed the majority of experiments, collected data and wrote the manuscript; Xu WT was in charge of this project, and revised the manuscript and provided financial support for this work; Zhao P and Lv YL provided vital reagents and analytical tools; Wu X and Chen L reviewed the manuscript.

Correspondence to: Wen-Tong Xu, MD, Department of General Surgery, General Hospital of PLA, 28 Fuxing Road, Beijing 100853, China. xuwentong@medmail.com.cn

Telephone: +86-10-66938328 Fax: +86-10-66938327

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Abstract

AIM: To investigate the expression and prognostic value of carbonic anhydrase II (CA II) and Ki-67 in gastrointestinal stromal tumors (GISTs).

METHODS: One hundred and thirteen GIST patients admitted to Chinese People's Liberation Army General Hospital from January 2004 to December 2010 were retrospectively followed up, and immunohistochemistry was used to detect CA II, Ki-67 and CD117 expression in tumor samples. The survival rates of the patients were analyzed using the Kaplan-Meier method. Log-rank test, χ^2 test and Cox proportional hazards model were used to determine the relationships between CA II, Ki-67 and CD117 expression and prognostic value in GISTs.

RESULTS: The survival rates at 1, 3 and 5 years were 90.0%, 82.0% and 72.0% in all patients. However, in patients with positive CA II or Ki-67, the survival rates were 92.0%, 83.0% and 77.0% or 83.0%, 66.6% and 53.0%, respectively. Compared with the negative

groups, the survival rates in the positive groups were significantly lower (CA II log-rank $P = 0.000$; Ki-67 log-rank $P = 0.004$). Multivariate Cox analysis revealed that CA II, CD117 and Ki-67 were considerable immune factors in prognosis of GIST patients (CA II $P = 0.043$; CD117 $P = 0.042$; Ki-67 $P = 0.007$). Besides, tumor diameter, mitotic rate, tumor site, depth of invasion, complete resection, intraoperative rupture, and adjuvant therapy were important prognosis predictive factors. Our study indicated that CA II had strong expression in GISTs and the prognosis of GISTs with high CA II expression was better than that of GISTs with low or no expression, suggesting that CA II is both a diagnostic and prognostic biomarker for GIST.

CONCLUSION: CA II and Ki-67 are significant prognostic factors for GISTs. CA II associated with neovascular endothelia could serve as a potential target for cancer therapy.

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Key words: Gastrointestinal stromal tumors; Carbonic anhydrase; CD117; Ki-67; Prognostic factor

Core tip: Gastrointestinal stromal tumors (GISTs) are mesenchymal tumors with a wide spectrum of clinical behavior. This is the first study showing the prognostic significance of carbonic anhydrase II (CA II) and Ki-67. The 1-, 3- and 5-year survival rates were 90.0%, 82.0% and 72.0%. However, in patients with positive CA II or Ki-67, the survival rates were 92.0%, 83.0% and 77.0% or 83.0%, 66.6% and 53.0%, respectively. Our study indicates that CA II has strong expression in GISTs and prognosis with high CA II expression is better than that with low or no expression, suggesting that CA II is both a diagnostic and prognostic biomarker for GIST.

Liu LC, Xu WT, Wu X, Zhao P, Lv YL, Chen L. Overexpression of carbonic anhydrase II and Ki-67 proteins in prognosis

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INTRODUCTION

Gastrointestinal stromal tumors (GISTs) are mesenchymal tumors that arise from the gastrointestinal tract. They show differentiation towards the interstitial cells of Cajal and account for < 1% of all gastrointestinal neoplasms^[1]. GISTs positively express discovered on GIST-1 (98%) and CD117 (95%) immune globulin. The estimated incidence of GISTs is 10-20 per million people annually worldwide. The majority of GISTs arise in the stomach (60%), small bowel (30%), esophagus and rectum (10%)^[2] and the remainder outside the gastrointestinal tract, comprising a wide spectrum from a curable disorder to highly malignant disease. As far as the molecular markers are concerned, previous studies have revealed that p53, CD147, monocarboxylate transporter 1 (MCT1), DEAD (Asp-Glu-Ala-Asp) box polypeptide 39 (DDX39) and natural killer cell p30 (NKp30) are related to the prognosis of GISTs^[3-7]. However, considering that these markers are different from tumor size, mitotic rate or tumor site, and due to their weak correlation, they are often mentioned in recurrence risk of GIST or prediction of patient prognosis.

Carbonic anhydrases (CAs) are a group of zinc-containing metalloenzymes that catalyze the reversible hydration of carbon dioxide, $\text{CO}_2 + \text{H}_2\text{O} \rightleftharpoons \text{HCO}_3^- + \text{H}^+$ and participate in various physiological processes, including respiration, gluconeogenesis, bone resorption, renal acidification, and formation of cerebrospinal fluid and gastric acid^[8]. To date, 15 isoforms of human (h) α -CA, 15 enzymatically active α CAs have been identified and characterized in mammals, including five cytoplasmic (CA I, CA II, CA III, CA VII and CA XIII), two mitochondrial (CA VA and CA VB), one secreted (CA VI), four membrane-associated (CA IV, CA IX, CA XII, and CA XIV) forms and three CA-related proteins (CA-RP VIII, CA-RP X and CA-RP XI). These functionally active CA isozymes, having been identified in mammals^[9-11], differ in their tissue distribution and enzymatic activity. Furthermore, in the present study, GIST cells demonstrated strong expression of CA-RPs VIII and XI. Overexpressed CA-RP XI is possibly substituted for CA-RP VIII in the cytoplasm and enhances the proliferation and invasion of GIST cells^[12]. These enzymes are commonly expressed in malignant tumor cells in which they promote tumor growth by contributing to intracellular alkalization and extracellular acidification^[13]. However, because of its wide distribution, high catalytic efficiency, and an important physiological role, CA II has become one of the hot research topics. CA II expression in the cytoplasm is a single polypeptide chain of molecular weight 29 kDa and its gene is located on chromosome 8, 8q22, 760 bp.

It is present in most tissues with high enzyme activity, including gastric cancer, liver and bile duct cancer, colon cancer, renal cell carcinoma, melanoma, brain astrocyte tumors, pancreatitis cells in mice^[14], and cardiomyocyte hypertrophy^[15]. In a recent study, high CA II expression was associated with a better disease-specific survival rate than low or no expression in GIST^[16]. Although CA II has been reported to represent potential diagnostic and therapeutic targets in the above cancers, there have been fewer reports discussing the predictive value of prognosis in GIST patients in Asia from a clinicopathological aspect.

Ki-67 is a nuclear marker that is closely related to tumor cell proliferation. It has been found to have a positive correlation with prognosis of various malignant tumors including GIST. One recent study has suggested that Ki-67 is a strong prognostic indicator even though it is less valuable than mitotic rate in GIST^[17]. A study by Nakamura *et al*^[18] supported the hypothesis that Ki-67 and risk grade are useful for predicting the aggressive biological behavior of GIST.

The aim of our study was to reveal the relationship between the above two molecular markers (CA II and Ki-67) and prognosis of GIST. As a diagnostic index, CD117 is located in the tumor cell membrane and cytoplasm^[19] and has a positive rate as high as 95% in GISTs. The predictive value of CD117 in prognosis was also explored.

MATERIALS AND METHODS

Study population and follow-up

We retrospectively followed up GIST patients who were admitted and operated upon in Chinese People's Liberation Army General Hospital between January 2004 and December 2010. Clinical follow-up was completed in February 2011. Inclusion criteria were: (1) age \geq 18 years; (2) GISTs diagnosed by the histopathological and immunohistochemical methods; and (3) not receiving any previous treatment. Exclusion criteria were: (1) female patients with pregnancy or lactation; (2) patients developing other malignancies during the past 5 years; and (3) patients with other serious diseases.

Pathological examination of tumor samples

Paraffin wax sections (5 μm thick) of GIST specimens were dewaxed in xylene and transferred to alcohol. Endogenous peroxidase activity was blocked with 0.5% hydrogen peroxide in methanol and the sections were subjected to heat-induced antigen retrieval using a microwave oven. Sections were incubated overnight at 4 °C with polyclonal antibodies for CA II, CD117 and Ki-67 (CD117, rabbit anti-human polyclonal antibody, 1:100, Abcam, Cambridge, United Kingdom; Ki67, rabbit polyclonal antibody to proliferation marker, 1:1000, Abcam; CA II, rabbit anti-human polyclonal antibody, 1:100, Abcam). Polyperoxidase-anti-mouse/rabbit immunoglobulin G was applied to the sections for 30 min at 37 °C, followed by

detection with 3,3'-diaminobenzidine (Bioss, Beijing, China). The reactions were developed with hematoxylin and were mounted with glue^[20,21]. The immunohistochemical reactions were visualized under high-power magnification ($\times 400$) using an Olympus BH2 microscope (Tokyo, Japan). Positive expression for CA II, Ki-67 and CD117 was defined by the percentage of positively stained cells (1 positive; 0 negative). The following scoring assessments for CA II, Ki-67 and CD117 were used. Score 0 was assigned for $\leq 10\%$, and 1 for $> 10\%$ staining positive cells.

Ethics

Approval for the use of clinical material for research was obtained from the hospital ethics committee, along with patient consent.

Statistical analysis

SPSS version 17.0 (SPSS Inc. Chicago, IL, United States) was used for statistical analysis. Analysis was performed assuming a nonparametric distribution using the χ^2 test. Actuarial survival rates were evaluated by Kaplan-Meier analysis and log-rank test. Multivariate survival analysis was performed by Cox proportional hazards model. All tests were two-tailed and statistical significance was set at $P < 0.05$.

RESULTS

Clinical characteristics

A total of 113 GIST patients (61 male, 52 female) with a median age of 60 years were included. Twenty-five patients died from GIST. Median follow-up time was 35.5 mo (1-90 mo).

Expression of CA II, Ki-67 and CD117 in tumor samples

Immunohistochemistry showed that the positive rate for CA II, CD117 and Ki-67 was 87.6% (99/113), 85.8% (97/113) and 65.5% (74/113) in all patients, respectively. CA II protein was strongly expressed in the cytoplasm of GIST cells (Figure 1B). Ki-67 protein was expressed in the nuclei of GIST cells (Figure 1D). In the control group, CA II was negatively expressed in GIST cells (Figure 1C), and only partially expressed in neural astrocytoma, schwannoma, leiomyoma of the stomach, and malignant solitary fibrous tumors (Figure 1E-H). The histopathological type (spindle cell, epithelioid or mixed type) was noted and mitoses were counted using a $\times 40$ objective for 50 high-power fields, as recommended.

Relationship between expression of CA II, Ki-67 and CD117 and clinicopathological characteristics of GISTs

The survival analysis for all GIST patients showed that the 1-, 3- and 5-year survival rates were 90.0%, 82.0% and 72.0%. The recurrence rate was 10.6% with a recurrence time of 6-20 mo. The highest survival rate was found in those patients who received complete tumor resection and took imatinib (400 mg/d) postoperatively. However, in those patients who did not undergo

complete tumor resection and were not treated with imatinib postoperatively, the survival rate was the lowest. However, in patients with positive CA II or Ki-67 expression, the survival rates were 92.0%, 83.0% and 77.0% or 83.0%, 66.6% and 53.0%, respectively. Considering molecular markers, the survival rates in the CA II-negative group or CD117-negative group were significantly lower than in the positive groups (CA II, log-rank $P = 0.000$; CD117, log-rank $P = 0.000$). However, it was higher in the Ki-67-positive group compared to the Ki-67-negative group (log-rank $P = 0.004$) (Figure 2).

According to the National Institutes of Health (NIH) risk grade^[22] in GIST, there were five cases of extremely low risk, 15 of low risk, 16 of medium risk, and 77 of high risk. Comparing these parameters (tumor diameter, tumor site, mitotic rate, NIH risk, and depth of invasion), the differences were significant between the Ki-67-positive and -negative group, while for CD117 marker, there was no difference. For CA II, significant differences were found between positive and negative groups only when they were compared by tumor diameter, mitotic rate and NIH risk. In the CA II-positive group, high NIH risk accounted for 66.6% (66/99) of the cases (Figure 3). As for the other pathological characteristics, CD34, SMA and desmin protein positive rates were 79.4%, 46.8% and 5%, respectively.

When comparing tumor site, mitotic rate, NIH risk, and depth of invasion, the differences were significant between Ki-67-positive and -negative groups ($P < 0.05$), whereas for CD117, significant differences were found for age, tumor site and depth of invasion ($P < 0.05$). For CA II, significant differences were not found between the positive and negative groups ($P > 0.05$) (Table 1).

Multivariate Cox model analysis suggested that CA II, Ki-67 and CD117, along with tumor site, tumor diameter, mitotic rate, depth of invasion, complete resection, intraoperative rupture, and adjuvant therapy were important prognosis predictive factors ($P < 0.05$). However, age, sex, mucosal erosion, biopsy and CD34 were not important prognosis predictive factors (Table 2).

DISCUSSION

It has been proved that tumor size, mitotic index, tumor location, and intraoperative tumor rupture are related to prognosis and recurrence of GIST^[23-25]. For instance, although p53, CD147, MCT1, DDX39 and Nkp30 are related to prognosis of GIST, they have never been mentioned as prognostic predictors due to their weak correlation.

Multivariate analysis showed that CA II provided additional information on patient survival as compared to age, sex, NIH risk classification and mutational status. Based upon the comprehensive recognition that CA II-positive tumor cells have oxidative activity, it is safe to suggest that CA II plays an important role in occurrence and development of GIST. By contrast, various studies have included only the membrane-bound isoforms,

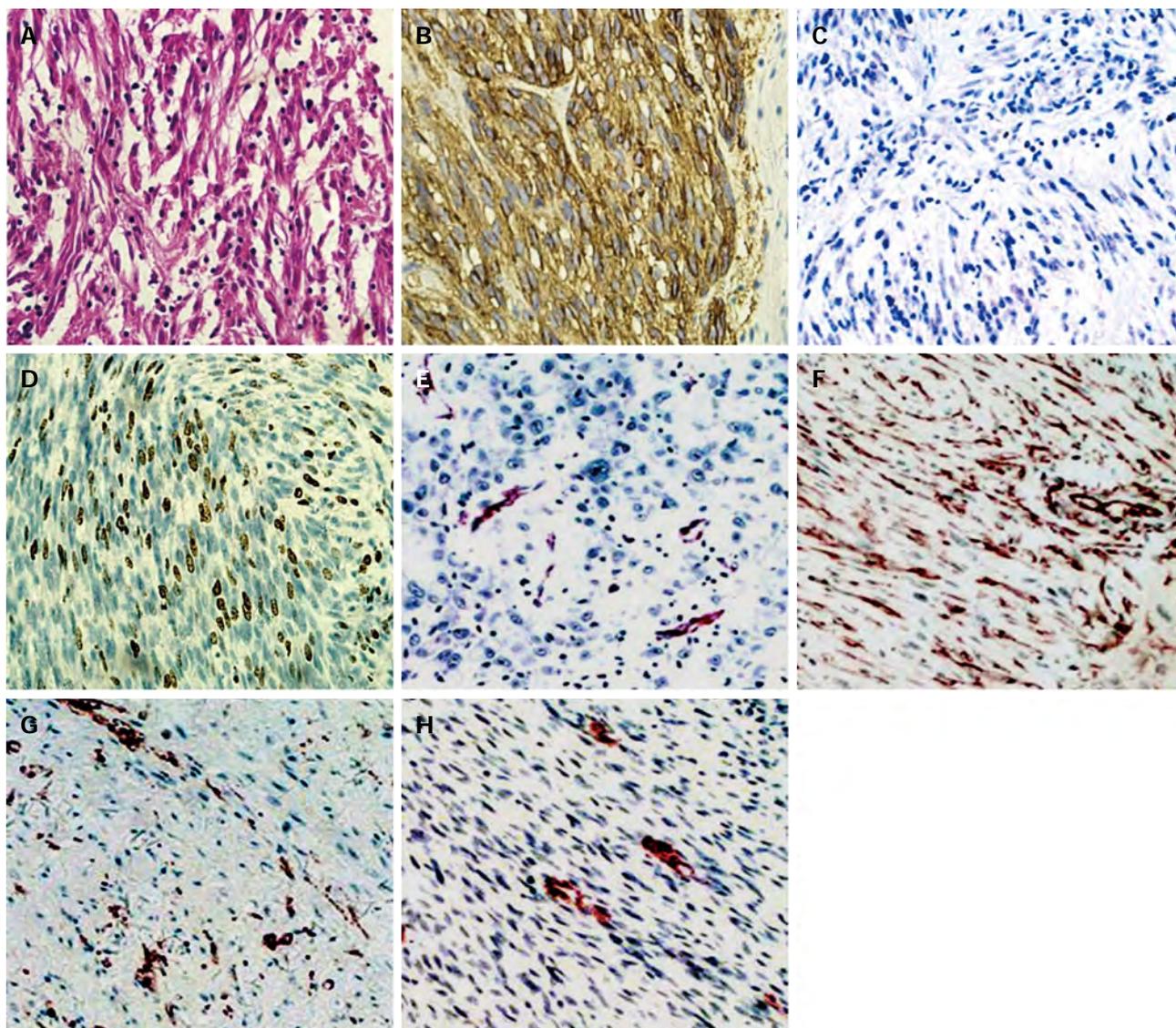


Figure 1 The scale is 5 μm using a $\times 400$ objective as recommended. Carbonic anhydrase II (CA II) and Ki-67 proteins expressed in gastrointestinal stromal tumors (GISTs) and other malignant tumors. A: Hematoxylin and eosin in GISTs; B: CA II positive protein in the GISTs; C: CA II and Ki-67 negative protein in GISTs; D: Ki-67 positive protein in the GISTs; E: CA II positive protein in neural astrocytoma; F: CA II positive protein in schwannoma; G: CA II positive protein in leiomyoma of the stomach; H: CA II positive protein in malignant solitary fibrous tumor.

CA IX and XII, which are overexpressed in several types of cancers^[26-29]. There has been only scattered evidence that CA II is expressed to some extent in malignant cells such as leukemic blast cells, and brain, colorectal and pancreatic cancers^[30-32]. A more recent study has indicated that CA II expression is induced in neovascular endothelial cells of malignant melanoma and in esophageal, renal and lung cancers. It has been suggested that CA II associated with the neovascular endothelia could serve as a potential target for cancer therapy. It has also been proposed that the presence of CA II in the endothelium could contribute to generation of autoantibodies that could, in turn, be a desired outcome in immunotherapy of cancer. Combined with our present results, a new therapeutic approach targeting CA II, as well as CA II to predict prognosis of GIST, might be promising. However, more studies are necessary.

Ki-67 protein exists in actively proliferating cells (G1,

S and G2 phase), which is a proliferation-related nuclear marker of tumor cell^[33]. Some studies have shown that Ki-67 expression is closely related to aggressive biological behavior of tumor cells in GISTs^[18] and represents a good prognostic predictor for GIST^[34]. However, the significance of Ki-67 in predicting prognosis is still in dispute.

Wong *et al*^[17] have found that Ki-67 was less reliable than mitotic count, even though it was useful in assessing the proliferation rate of tumor cells in GIST. We believe that the prognostic predictive value of Ki-67 in GIST might have been evaluated more objectively in a large survival study, with the various prognostic factors being taken into account. This was one of the aims of our present study. We found that the 1-, 3- and 5-year survival rates of patients with Ki-67-positive GIST were lower than in the Ki-67-negative group. Our survival analysis further indicated that the Ki-67 expression was

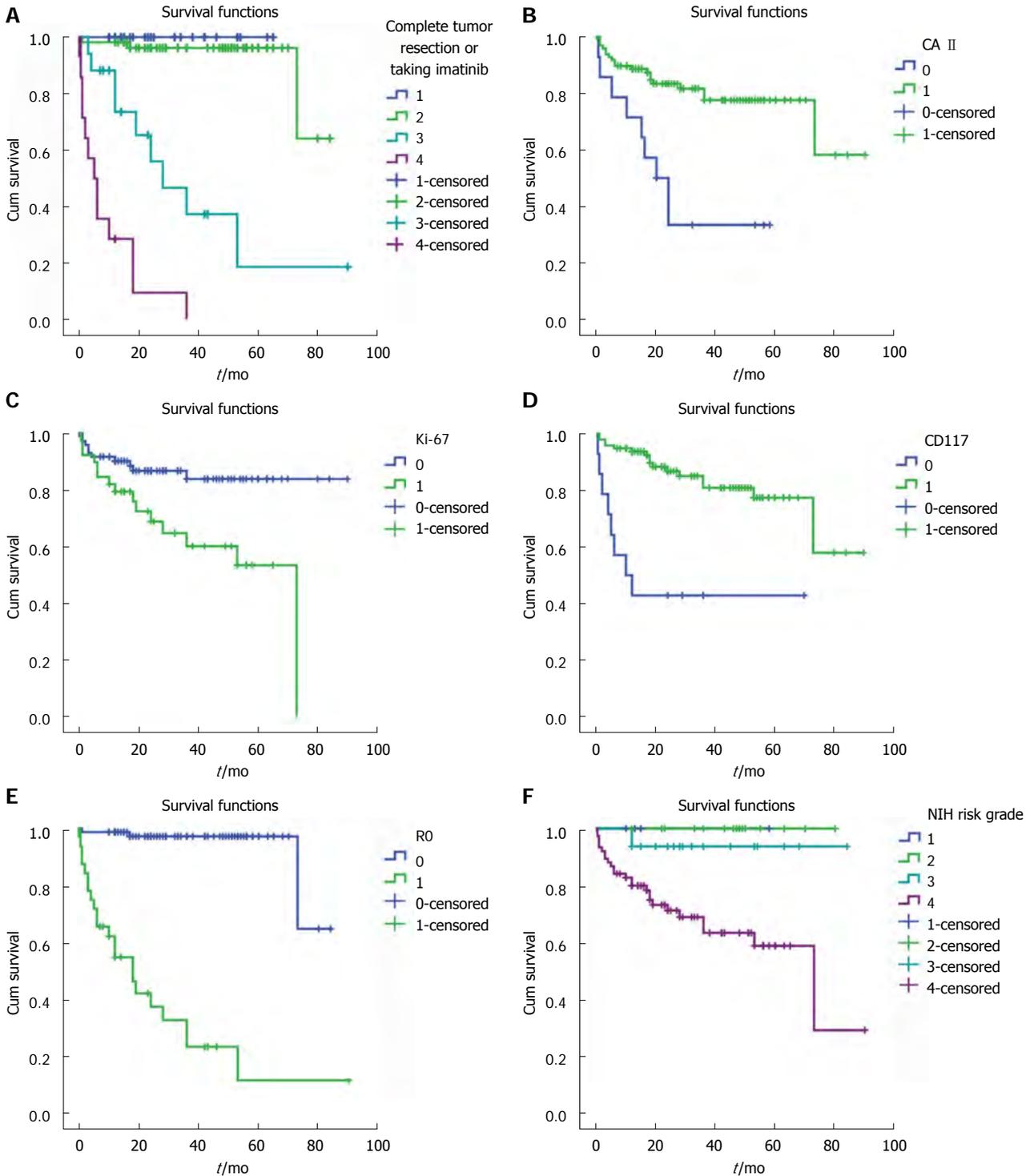


Figure 2 Analysis of survival rates (Kaplan-Meier) and comparison of survival rates in gastrointestinal stromal tumors groups (log-rank test). A: The highest survival rate was found in those patients that received complete tumor resection and postoperatively took imatinib (400 mg/d), while in those patients not receiving complete tumor resection and postoperative imatinib, the survival rate was lowest (log-rank $P = 0.000$). 1: Resected completely with imatinib administrated postoperatively, 2: Resected completely without imatinib administrated postoperatively, 3: Resected incompletely with imatinib administrated postoperatively, 4: Resected incompletely without imatinib administrated postoperatively; B: The survival rates in carbonic anhydrase (CA) II-positive group were significantly higher than those in negative groups (CA II, log-rank $P = 0.000$); C: The survival rates in Ki-67-positive group were higher than those in negative groups (Ki-67, log-rank $P = 0.004$); D: The survival rates in CD117-positive group were significantly higher than those in negative groups (CD117, log-rank $P = 0.000$); E: The higher survival rates were found in those patients that received complete tumor resection (R0) (R0, log-rank $P = 0.000$); F: The higher survival rates were found in those patients with National Institutes of Health (NIH) high risk (NIH risk, log-rank $P = 0.006$).

also an important prognostic predictor for GIST. The Wald indexes of Ki-67, diameter, mitotic rate and tumors site were all > 1 (7.282, 4.974, 11.081 and 15.581,

respectively), which indicated that the Ki-67 was another useful molecular marker in predicting the prognosis of GISTs.

Table 1 Pathological parameters of gastrointestinal stromal tumors in CD117, carbonic anhydrase II and Ki-67 proteins

| Variable | Total | CD117 | | P value | CA II | | P value | Ki-67 | | P value |
|-------------------|-------|-------|----|---------|-------|----|---------|-------|----|---------|
| | | + | - | | + | - | | + | - | |
| Sex | | | | | | | | | | |
| Male | 61 | 56 | 5 | 0.317 | 53 | 8 | 0.515 | 25 | 36 | 0.171 |
| Female | 52 | 41 | 11 | | 46 | 6 | | 14 | 38 | |
| Age (yr) | | | | | | | | | | |
| ≤ 60 | 61 | 48 | 13 | 0.036 | 54 | 7 | 0.485 | 22 | 39 | 0.859 |
| > 60 | 52 | 49 | 3 | | 45 | 7 | | 17 | 35 | |
| Diameter | | | | | | | | | | |
| ≤ 5 cm | 30 | 27 | 3 | 0.647 | 25 | 5 | 0.297 | 8 | 22 | 0.406 |
| > 5 cm | 83 | 70 | 13 | | 74 | 9 | | 31 | 52 | |
| Site | | | | | | | | | | |
| Stomach | 45 | 40 | 5 | | 41 | 4 | | 13 | 32 | |
| Small bowel | 35 | 34 | 1 | 0.004 | 31 | 4 | 0.459 | 8 | 27 | 0.014 |
| Others | 33 | 23 | 10 | | 27 | 6 | | 18 | 15 | |
| Mitotic rate | | | | | | | | | | |
| ≤ 5 MF/50 HPFs | 52 | 45 | 7 | 0.941 | 46 | 6 | 0.515 | 11 | 41 | 0.009 |
| > 5 MF/50 HPFs | 61 | 52 | 9 | | 53 | 8 | | 28 | 33 | |
| NIH risk | | | | | | | | | | |
| Very low | 5 | 4 | 1 | | 4 | 1 | | 2 | 3 | |
| Low | 15 | 13 | 2 | 0.980 | 13 | 2 | 0.424 | 2 | 13 | 0.031 |
| Medium | 16 | 14 | 2 | | 16 | 0 | | 2 | 14 | |
| High | 77 | 66 | 11 | | 66 | 11 | | 33 | 44 | |
| Depth of invasion | | | | | | | | | | |
| Mucosa | 24 | 21 | 3 | | 21 | 3 | | 6 | 18 | |
| Muscular | 41 | 33 | 8 | | 38 | 3 | | 10 | 31 | |
| Serous | 30 | 30 | 0 | 0.033 | 27 | 3 | 0.168 | 13 | 17 | 0.013 |
| Adjacent tissue | 18 | 13 | 5 | | 13 | 5 | | 10 | 8 | |

This table shows that Being compared by tumor site, mitotic rate, National Institutes of Health (NIH) risk, and depth of invasion of tumor cells, the differences were significant between the Ki-67-positive and -negative groups, while for CD117, significant differences were found for age, tumor site and depth of invasion of tumor cells. For carbonic anhydrase (CA) II, no significant differences were found between positive and negative groups. MF: Mitotic figures; HPFs: High-power fields.

Table 2 Multivariate survival analysis (Cox proportional hazards model) in gastrointestinal stromal tumors

| Variable | B | Wald | df | P value | HR | 95%CI for HR | |
|---------------------------|--------|--------|----|---------|--------|--------------|--------|
| | | | | | | Lower | Upper |
| Age | 0.023 | 1.696 | 1 | 0.193 | 1.024 | 0.988 | 1.060 |
| Sex | -0.239 | 0.342 | 1 | 0.559 | 0.788 | 0.354 | 1.754 |
| Mucosal erosion or not | 1.485 | 2.115 | 1 | 0.146 | 4.416 | 0.597 | 32.684 |
| Biopsy or not | -0.404 | 0.901 | 1 | 0.340 | 0.668 | 0.291 | 1.531 |
| CD34 | -0.071 | 0.086 | 1 | 0.769 | 0.932 | 0.582 | 1.493 |
| CA II | -0.319 | 4.113 | 1 | 0.043 | 0.727 | 0.543 | 0.989 |
| CD117 | -0.609 | 4.114 | 1 | 0.042 | 0.544 | 0.303 | 0.978 |
| Ki-67 | 1.103 | 7.282 | 1 | 0.007 | 3.014 | 1.352 | 6.717 |
| Diameter | 1.645 | 4.974 | 1 | 0.026 | 5.182 | 1.216 | 22.085 |
| Mitotic rate | 0.972 | 11.081 | 1 | 0.001 | 2.644 | 1.491 | 4.686 |
| Site | 1.334 | 15.581 | 1 | 0.000 | 3.796 | 1.955 | 7.371 |
| Depth of invasion | 0.559 | 7.445 | 1 | 0.006 | 1.748 | 1.170 | 2.612 |
| Complete resection or not | 2.807 | 24.674 | 1 | 0.000 | 16.555 | 5.470 | 50.104 |
| Intraoperative rupture | 1.937 | 17.997 | 1 | 0.000 | 0.144 | 0.059 | 0.353 |
| Adjuvant therapy | 1.757 | 35.579 | 1 | 0.000 | 5.796 | 3.254 | 10.325 |

This table shows that carbonic anhydrase (CA) II, CD117 and Ki-67 expression, tumor diameter, mitotic rate, tumor site, depth of invasion, complete resection, intraoperative rupture, and adjuvant therapy were important prognosis predictive factors. HR: Hazard ratios.

As for molecular markers, the negative expression of CD117 is believed to be associated with early postoperative recurrence of GIST^[35]. This was confirmed once again by our study. At the same time, our study indicated that positive expression of Ki-67 or negative expression of CA II and CD117 was a cue for poor prognosis

in GIST. However, there was a limitation to our study, namely, its small sample size. Our results could promote the clinical application of these two markers and provide clues to a novel therapeutic target for GIST in the future.

In conclusion, our study showed CA II expression in GIST. The prognosis of GIST with high CA II expres-

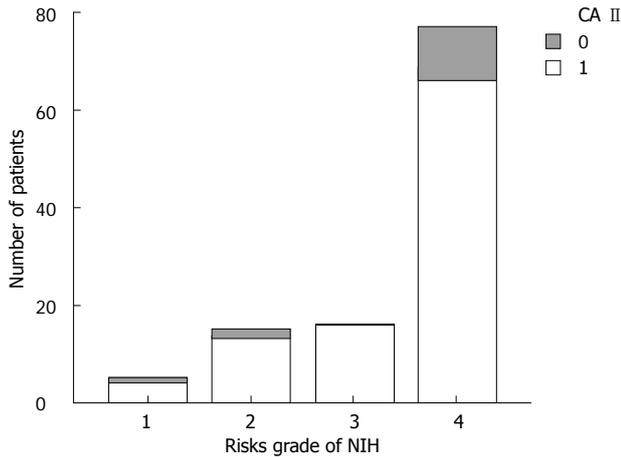


Figure 3 Carbonic anhydrase II positive cases in gastrointestinal stromal tumors according to National Institutes of Health risk grade. There were five cases with extremely low risk, 15 with low risk, 16 with medium risk, and 77 with high risk. Carbonic anhydrase (CA) II expressed in 113 gastrointestinal stromal tumors: "0" represents the negative cases, and "1" the positive cases. NIH: National Institutes of Health.

sion was better than that of GIST with low or no expression, suggesting that CA II is both a diagnostic and prognostic biomarker. Further validation studies with other CA antibodies should be undertaken to characterize CA II expression in a larger cohort of patients with GIST and other mesenchymal tumors of the gastrointestinal tract.

COMMENTS

Background

Gastrointestinal stromal tumors (GISTs) are mesenchymal tumors that arise from the gastrointestinal tract with a wide spectrum of clinical behavior. Thus, it is of great importance to define prognostic factors that could indicate survival rates. Carbonic anhydrase (CA) II is expressed in many malignant tumors, and higher expression is associated with a better disease-specific survival rate. Ki-67 is related to cell proliferation in various tumors and has a markedly positive correlation with prognosis.

Research frontiers

According to National Institutes of Health risk classification, some prognostic factors are well documented as prognostic factors of further tumor behavior, such as mitotic count and tumor size, along with tumor primary localization. However, the value of immunohistochemistry index in GISTs has not been clearly indicated. Furthermore, there have been a limited number of studies investigating the relationship between the prognosis of GISTs and CA II and Ki-67.

Innovations and breakthroughs

This is believed to be the first study showing so clearly the significance of CA II and Ki-67 as prognostic factors. The 1-, 3- and 5-year survival rates were 90.0%, 82.0% and 72.0% in all patients. However, in patients with positive CA II or Ki-67, the survival rates were 92.0%, 83.0% and 77.0% or 83.0%, 66.6% and 53.0%, respectively. This study indicated that CA II has strong expression in GISTs and the prognosis of GISTs with high CA II expression was better than that of GISTs with low or no expression, suggesting that CA II is both a diagnostic and a prognostic biomarker for GIST.

Applications

CA II and Ki-67 were useful for predicting the aggressive biological behavior of GISTs. CA II associated with neovascular endothelia could serve as a potential target for cancer therapy.

Terminology

CAs are a group of zinc-containing metalloenzymes that catalyze the reversible

hydration of CO₂ and participate in various physiological processes, including respiration, gluconeogenesis, bone resorption, renal acidification, and formation of cerebrospinal fluid and gastric acid. Ki-67 is a protein that is encoded by the *MKI-67* gene in humans. Ki-67 antigen is associated, and probably necessary, for cellular proliferation, and is associated with rRNA transcription.

Peer review

This study revealed that CA II is highly expressed in GIST cell lines and 87.6% of GISTs selectively. Until now, there have been few reports of CA II expression in GISTs. The result of this study showed that high CA II expression was associated with a better disease-specific survival rate than low or no expression, therefore, it might be a useful biomarker in diagnosis and prognosis of GISTs. This is a retrospective study and the results should be helpful for clinical practice and a potential therapeutic target.

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Emodin regulating excision repair cross-complementation group 1 through fibroblast growth factor receptor 2 signaling

Gang Chen, Hong Qiu, Shan-Dong Ke, Shao-Ming Hu, Shi-Ying Yu, Sheng-Quan Zou

Gang Chen, Shan-Dong Ke, Shao-Ming Hu, Integration Traditional Chinese Medicine and Western Medicine Department, TongJi Hospital, Huazhong University of Science and Technology, Wuhan 430030, Hubei Province, China

Hong Qiu, Shi-Ying Yu, Department of Oncology, TongJi Hospital, Huazhong University of Science and Technology, Wuhan 430030, Hubei Province, China

Sheng-Quan Zou, Department of Surgery, TongJi Hospital, Huazhong University of Science and Technology, Wuhan 430030, Hubei Province, China

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Correspondence to: Dr. Shao-Ming Hu, Integration Traditional Chinese Medicine and Western Medicine Department, TongJi Hospital, Huazhong University of Science and Technology, 1095 Jiefang Avenue, Qiaokou District, Wuhan 430030, Hubei Province, China. smhu@tjh.tjmu.edu.cn

Telephone: +86-27-83663532 Fax: +86-27-83663445

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Abstract

AIM: To investigate the molecular mechanisms underlying the reversal effect of emodin on platinum resistance in hepatocellular carcinoma.

METHODS: After the addition of 10 $\mu\text{mol/L}$ emodin to HepG2/oxaliplatin (OXA) cells, the inhibition rate (IR), 50% inhibitory concentration (IC_{50}) and reversal index (IC_{50} in experimental group/ IC_{50} in control group) were calculated. For HepG2, HepG2/OXA, HepG2/OXA/T, each cell line was divided into a control group, OXA group, OXA + fibroblast growth factor 7 (FGF7) group

and OXA + emodin group, and the final concentrations of FGF7, emodin and OXA in each group were 5 ng/mL, 10 $\mu\text{g/mL}$ and 10 $\mu\text{mol/L}$, respectively. Single-cell gel electrophoresis was conducted to detect DNA damage, and the fibroblast growth factor receptor 2 (FGFR2), phosphorylated extracellular signal-regulated kinase 1/2 (p-ERK1/2) and excision repair cross-complementing gene 1 (ERCC1) protein expression levels in each group were examined by Western blotting.

RESULTS: Compared with the IC_{50} of 120.78 $\mu\text{mol/L}$ in HepG2/OXA cells, the IC_{50} decreased to 39.65 $\mu\text{mol/L}$ after treatment with 10 $\mu\text{mol/L}$ emodin; thus, the reversal index was 3.05. Compared with the control group, the tail length and Olive tail length in the OXA group, OXA + FGF7 group and OXA + emodin group were significantly increased, and the differences were statistically significant ($P < 0.01$). The tail length and Olive tail length were lower in the OXA + FGF7 group than in the OXA group, and this difference was also statistically significant. Compared with the OXA + FGF7 group, the tail extent, the Olive tail moment and the percentage of tail DNA were significantly increased in the OXA + emodin group, and these differences were statistically significant ($P < 0.01$). In comparison with its parental cell line HepG2, the HepG2/OXA cells demonstrated significantly increased FGFR2, p-ERK1/2 and ERCC1 expression levels, whereas the expression of all three molecules was significantly inhibited in HepG2/OXA/T cells, in which FGFR2 was silenced by FGFR2 shRNA. In the examined HepG2 cells, the FGFR2, p-ERK1/2 and ERCC1 expression levels demonstrated increasing trends in the OXA group and OXA + FGF7 group. Compared with the OXA group and OXA + FGF7 group, the FGFR2, p-ERK1/2, and ERCC1 expression levels were significantly lower in the OXA + emodin group, and these differences were statistically significant. In the HepG2/OXA/T cell line that was transfected with FGFR2 shRNA, the FGFR2, p-ERK1/2 and ERCC1 expression levels were significantly inhibited, but there were no significant differences in these

expression levels among the OXA, OXA + FGF7 and OXA + emodin groups.

CONCLUSION: Emodin markedly reversed OXA resistance by enhancing OXA DNA damage in HepG2/OXA cells, and the molecular mechanism was related to the inhibitory effect on ERCC1 expression being mediated by the FGFR2/ERK1/2 signaling pathway.

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Key words: Hepatocellular carcinoma; Emodin; Fibroblast growth factor receptor 2; Excision repair cross-complementation group 1; Platinum resistance; Extracellular signal-regulated kinase

Core tip: In this study, our results indicated that emodin could significantly enhance the DNA damage caused by oxaliplatin (OXA) and induce OXA resistance reversal in HepG2/OXA cells. The molecular mechanism for this phenomenon is mediated by the inhibition of excision repair cross-complementing gene 1 expression by the fibroblast growth factor receptor 2/phosphorylated extracellular signal-regulated kinase 1/2 signaling pathway. The results for the reversal of platinum resistance by emodin and the emodin-based enhancement of the efficacy of platinum-based chemotherapy in hepatocellular carcinoma may provide an experimental basis for the further development and application of emodin in the reversal of platinum drug resistance in other types of malignant tumors.

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INTRODUCTION

For up to 85%-95% of the cases of liver cirrhosis in patients suffering from hepatocellular carcinoma (HCC), the therapeutic effects of chemotherapy have not been proven to provide survival benefits^[1]. For the patient and the oncologist, sorafenib, which is a unique targeting drug that generates proven survival benefits, does not produce many unexpectedly positive outcomes because of its limited cost-effectiveness^[2]. In 2010, at the annual meeting of the American Society of Clinical Oncology, the results of an international multi-center randomized phase III clinical study provided the first evidence demonstrating that platinum-based chemotherapy produces survival benefits for patients with advanced HCC. This discovery has triggered widespread interest into the use of oxaliplatin-based chemotherapy for HCC^[3]. Thus, the identification and characterization of a drug that strengthens platinum-based chemotherapy effects and

protects normal liver cells could provide clinical benefits for the treatment of HCC in China.

Emodin (1,3,8-trihydroxy-6-methylantraquinone) is a member of the family of anthraquinone alkaloids, which are the main active ingredients of rhubarb, *Polygonum cuspidatum*, *Polygonum multiflorum*, *Amomum*, lilies and other plants that are widely used in traditional Chinese medicine^[4]. Modern studies have demonstrated that emodin produces anti-tumor biological effects on a variety of malignancies, including HCC^[5]. In HCC, the primary anti-cancer mechanisms of emodin involve the induction of apoptosis and the inhibition of cell growth. Emodin can cause G2/M cell cycle arrest by regulating an assortment of cell cycle related genes, such as *cyclin A*, *cyclin B*, *Chk2*, *CDK2* and *p27*, in diverse human hepatoma cell lines, including Huh7, Hep3B and HepG2^[6]. Moreover, emodin can enhance the cytotoxicity of chemotherapy drugs, such as platinum-based compounds [cisplatin, carboplatin, and oxaliplatin (OXA)] in various types of malignant tumors, including liver cancer, gallbladder cancer^[7,8], non-small cell lung cancer^[9,10], and prostate cancer^[11]. However, the mechanism underlying the synergistic effects of combinations of emodin and platinum-based drugs requires further elucidation.

The DNA damage that is caused by cisplatin, OXA and other platinum chemotherapy drugs is the root cause of the cytotoxicity of these compounds^[12]. Nucleotide excision repair (NER) is the main pathway for repairing this damage, and excision repair cross-complementing gene 1 (*ERCC1*), which is the limiting enzyme in the NER pathway, plays an important role in this process^[13,14]. Compared with normal liver tissue, fibrous tissue in the liver displays significantly increased levels of ERCC1 expression^[15]. ERCC1 protein concentrations were significantly greater in liver cancers that were accompanied by hepatic fibrosis tissue than in liver cancers without hepatic fibrosis. In addition, high expression levels of ERCC1 are closely correlated with cisplatin resistance; thus, ERCC1 could be used as a predictor of sensitivity to platinum-based chemotherapy in cases of HCC^[16]. The relationship between synergistic effects and the regulation of ERCC1 expression is worthy of further study in HCC treatments that combine emodin with platinum drugs.

Fibroblast growth factor receptor 2 (FGFR2), which is a member of a transmembrane tyrosine kinase receptor family (FGFRs), is an expression product of the *bek* oncogene that plays an important role in the differentiation of HCC, the clinical staging of tumors, the incidence of tumor thrombosis and the determination of alpha-fetoprotein levels^[17]. As a molecular marker, FGFR2 can effectively predict the overall survival and progression-free survival of patients with HCC. Interestingly, *ERCC1* is a downstream target gene of FGFR2^[18], whereas emodin is a tyrosine kinase inhibitor^[10,19]. Previously published research has indicated that emodin down-regulates ERCC1 expression in non-small cell lung cancer, and its effects may be relevant to the ERK1/2 signaling pathway^[20]; however, the exact mechanism

through which emodin produces these effects has not been well established. The ERK signaling pathway is one of the downstream components of the FGFR2 pathway^[21,22]. Thus, we hypothesize that emodin reverses tumor drug resistance by increasing the DNA damage that is induced by platinum chemotherapy drugs, and we speculate that the molecular mechanism of this effect is related to the ERK1/2 pathway, which is mediated by FGFR2 signaling in hepatoma cells.

The primary aim of this study was to determine the molecular mechanisms underlying the reversal effect of emodin on platinum resistance in HCC.

MATERIALS AND METHODS

Oxaliplatin and emodin were purchased from Sigma (St. Louis, MO, United States). Dulbecco's modified Eagle's medium/high glucose (DMEM/H) and fetal bovine serum were produced by Invitrogen (Carlsbad, CA, United States), trypsin and dimethyl sulfoxide was purchased from Guge Biology CO (Wuhan, China). FGF7, puromycin dihydrochloride (sc-108071), ERCC1 mouse anti-monoclonal antibody and p-ERK1/2 rabbit anti-monoclonal antibody (sc-16982-R) were purchased from Santa Cruz Biotechnology (United States). FGFR2 mouse anti-monoclonal antibody was produced (MAB684) by R-D Systems, Inc. (United States). Dylight™ 800-labeled anti-mouse immunoglobulin G (IgG) antibody and Dylight™ 800-labeled anti-rabbit IgG antibody were bought from Gaithersburg Biotechnology (MD, United States).

Cell lines and culture conditions

The human hepatoma cell line HepG2 was used in the present study. HepG2 cells were obtained from the China Center for Type Culture Collection. The HepG2 cells were cultured in DMEM/H supplemented with 10% (v/v) fetal bovine serum, 200 IU/mL penicillin (ICN Biomedical, Costa Mesa, CA, United States), 100 mg/mL streptomycin (ICN Biomedical) and 0.5 mmol/L sodium pyruvate (Cambrex, Walkersville, MD, United States). The cells were cultured at 37 °C in a humidified atmosphere of 5% CO₂ in air. An OXA-resistant subline was established by discontinuously exposing parental HepG2 cells to high OXA concentration (25 μmol/L) medium over the course of one year until the resulting cells could grow exponentially in medium containing 1 μmol/L OXA. The HepG2/OXA cells were digested and subcultured three times prior to their use for this experiment.

Reversal effect of emodin on HepG2/OXA

Cells from the resistant cell line HepG2/OXA in logarithmic growth phase were grown in 96-well plates. In particular, 100 μL of cell suspension was inoculated into 8 mL of medium. Medium containing different concentrations of the chemotherapy drug OXA (3.125, 6.25, 12.5, 25, 50 or 100 μmol/L) was added after 12 h of culture. The experimental group (EG) also received a final concentration of 10 μmol/L emodin in medium. In addition,

a control group (CG) was established. The medium was aspirated after 24 h of culture; subsequently, 110 μL of a mixture of DMEM/H and cell counting kit-8 (CCK-8) (at a ratio of 10 μL CCK-8:100 μL DMEM/H) was added, after which the samples were incubated for 2 h. A cell-free blank group (BG) was established. The optical density (OD) of each well was determined at 450 nm. The formula for calculating the inhibition rate (IR) at different concentrations was $1 - (\text{OD}_{\text{EG}} - \text{OD}_{\text{BG}}) / (\text{OD}_{\text{CG}} - \text{OD}_{\text{BG}})$. Based on the IR at different concentrations of the anti-cancer drug, the concentration at which the inhibition rate was 50% (IC₅₀) was calculated using the SPSS 13.0 software (SPSS Inc., Chicago, IL, United States). The reversal index was calculated using the formula (IC_{50EG}/IC_{50CG}). Five wells were established at different experimental concentrations, and each experiment was repeated three times.

Short hairpin RNA for bek gene cell transfection in HepG2/OXA

Bek shRNA cell transfection was conducted in accordance with the protocol for the bek shRNA plasmid (h): sc-29218-S (Santa Cruz Biotechnology, Inc.). HepG2/OXA cells were cultured to 60%-80% adherence in 6-well cell culture plates by adding antibiotic-free fetal bovine serum growth medium. The shRNA plasmid DNA solution (Solution A) was added directly to the dilute shRNA plasmid transfection reagent (Solution B) using a pipette. The solution was mixed gently by pipetting up and down and was incubated for 30 min at room temperature. Subsequently, the cells were washed twice with 2 mL of shRNA transfection medium, after which the medium was aspirated. Immediately afterward, 200 μL shRNA plasmid DNA/shRNA plasmid transfection reagent was added. The cells were incubated for 6 h at 37 °C in a CO₂ incubator. Following this incubation, 1 mL of normal growth medium containing 2 times the normal serum and antibiotics concentration was added to each well, and the cells were incubated for an additional 24 h under normal conditions. Forty-eight hours after the transfection, the medium was aspirated and replaced with fresh medium containing 5 μg/mL puromycin. Every 2 d afterward, the medium was aspirated and replaced with freshly prepared selective medium. Finally, an aliquot of the cells was washed once with phosphate buffer saline (PBS). The cell sample was lysed in 300 μL 1 × electrophoresis sample buffer by gently rocking the 6-well plate and was subjected to sodium dodecyl sulfate (SDS) gel electrophoresis to confirm the silencing of the *FGFR2* gene. Thus, a Bek-silenced HepG2/OXA cell line (HepG2/OXA/T) was established.

Experimental groups and sample preparation

HepG2, HepG2/OXA and HepG2/OXA/T cells were seeded in culture flasks. Each cell type was divided into four groups: a CG, an OXA group, an OXA + FGF7 group and an OXA + emodin group. A final concentration of 10 μmol/L OXA was added to the OXA group,

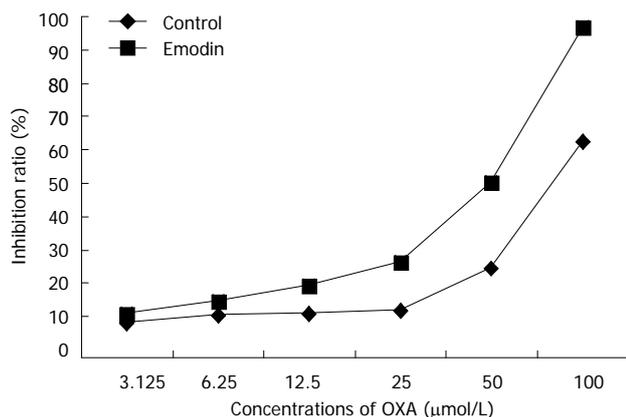


Figure 1 Inhibition ratio of different concentrations ($\mu\text{mol/L}$) of oxaliplatin in HepG2/oxaliplatin cells. The value of inhibition ratio increased steadily in the control group and the emodin group, which accompanied the elevated concentration of oxaliplatin (OXA) in the HepG2/OXA cells. However, the value of inhibition ratio in the emodin group treated with $10 \mu\text{mol/L}$ emodin was significantly higher in comparison with the control group.

the OXA + FGF7 group and the OXA + emodin group. and the final concentrations of FGF7, emodin and OXA in each group were 5 ng/mL , $10 \mu\text{g/mL}$ and $10 \mu\text{mol/L}$, respectively. After being incubated for 24 h and digested with trypsin, the cells were divided into two portions, one for total protein preparation for Western blotting, and one for single-cell gel electrophoresis to assay the DNA damage.

Single-cell gel electrophoresis to detect DNA damage

After counting the cells and adjusting them to a concentration of $2000 \text{ cells}/\mu\text{L}$, the cells in each group were gently suspended into single-cell suspension. Subsequently, $110 \mu\text{L}$ of 0.5% normal melting point agarose (NMA) at 45°C was poured onto Dakin slides, avoiding the production of air bubbles. The agarose solidified at room temperature. Subsequently, $5 \mu\text{L}$ PBS containing 10000 cells in each group was mixed well with $75 \mu\text{L}$ of 0.5% low melting point agarose (LMA). The upper cover-slip was carefully removed, the mixture was quickly added to the 0.5% NMA, the cells were spread evenly, and the slide was placed in a 4°C refrigerator for 5 min until the agarose solidified. The cover-slip was removed, and $75 \mu\text{L}$ of 0.5% LMA was added, after which the slide was placed in the refrigerator at 4°C again to solidify the agarose. The slide was slowly immersed in freshly prepared 4°C pre-cooling cell lysate and then set in a 4°C refrigerator for at least 1 h. The slide was then placed in a horizontal gel electrophoresis tank and incubated in the dark for 45 min, after which electrophoresis was performed for 30 min at 25 V at room temperature, and the height of the electrophoresis buffer liquid was adjusted to maintain a continuous 300 mA current. After the electrophoresis, each slide was dipped two times in a buffer in a darkroom for 5 min each. Following this treatment, $0.5 \mu\text{L}$ ethidium bromide dye was added to stain the DNA, the cover-slip was stamped, and the slide was observed and analyzed under a fluorescence microscope with a camera.

Protein expression of FGFR2, ERCC1, p-ERK1/2 and β -actin by Western blotting

Total protein was collected from the different groups of the cultured HepG2, HepG2/OXA and HepG2/OXA/T cells. The protein concentration was measured by a bicinchoninic acid protein assay kit (Beyotime Institute of Biotechnology, Jiangsu, China). Before electrophoresis, the protein was denatured in lithium dodecyl sulfate (LDS) sample buffer (106 mmol/L Tris-HCl, 141 mmol/L Tris base pH 8.5, 0.51 mmol/L ethylenediaminetetraacetic acid, 10% glycerol, 2% LDS, 0.22 mmol/L Serva blue G250, 0.175 mmol/L phenol red, and 0.1 mmol/L 2-mercaptoethanol) for 10 min at 95°C . The total protein ($20 \mu\text{g}$ per lane) was electrophoresed on a 8% SDS polyacrylamide gel electrophoresis gel and transferred onto a $0.45 \mu\text{m}$ nitrocellulose filter membrane (Roche, Indianapolis, IN, United States). The membranes were blocked with 5% (w/v) nonfat dry milk in PBS containing 0.05% Tween-20 (PBST) for 2 h at room temperature and incubated overnight at 4°C with antibodies against FGFR2 (1:250), p-ERK1/2 (1:250) or ERCC1 (1:100) (Santa Cruz, CA, United States). Next, the membranes were incubated with a Dylight™ 800-labeled antibody for 1 h after being washed 4 times for 5 min in PBST. Finally, the immunoblot signals were scanned and analyzed using an Odyssey Infrared Imaging System (Li-Cor Biosciences, Nebraska, United States).

Statistical analysis

All of the digital results are displayed as the means \pm SD. The quantitative ratios of different groups were compared using Student's t-test with the SPSS 13.0 software. Probability values of $P < 0.05$ were regarded as statistically significant. All of the statistical tests were two-sided.

RESULTS

The reversal effect of emodin on platinum resistance in the HepG2/OXA cell line

Compared with the CG, in which HepG2/OXA was treated with OXA alone, the inhibition ratio was significantly increased in the emodin group, in which HepG2/OXA cells were treated with a combination of OXA and emodin (Figure 1). Based on the inhibition ratios of different concentrations ($\mu\text{mol/L}$) of OXA, the IC_{50} of the OXA-resistant HepG2/OXA cells was $120.78 \mu\text{mol/L}$; however, in the OXA-resistant HepG2/OXA cells that were treated with $10 \mu\text{mol/L}$ emodin, the IC_{50} was reduced to $39.65 \mu\text{mol/L}$. This result indicated that the reversal index of $10 \mu\text{mol/L}$ emodin in HepG2/OXA cells was 3.05 (Table 1).

DNA damage detected by single-cell gel electrophoresis

Compared with the CG of HepG2 cells, the tail extent (TE) and the Olive tail moment (OTM) were considerably increased in the OXA group, OXA + FGF7 group, and OXA + emodin group; these differences were statistically significant ($P < 0.01$). Compared with the OXA group,

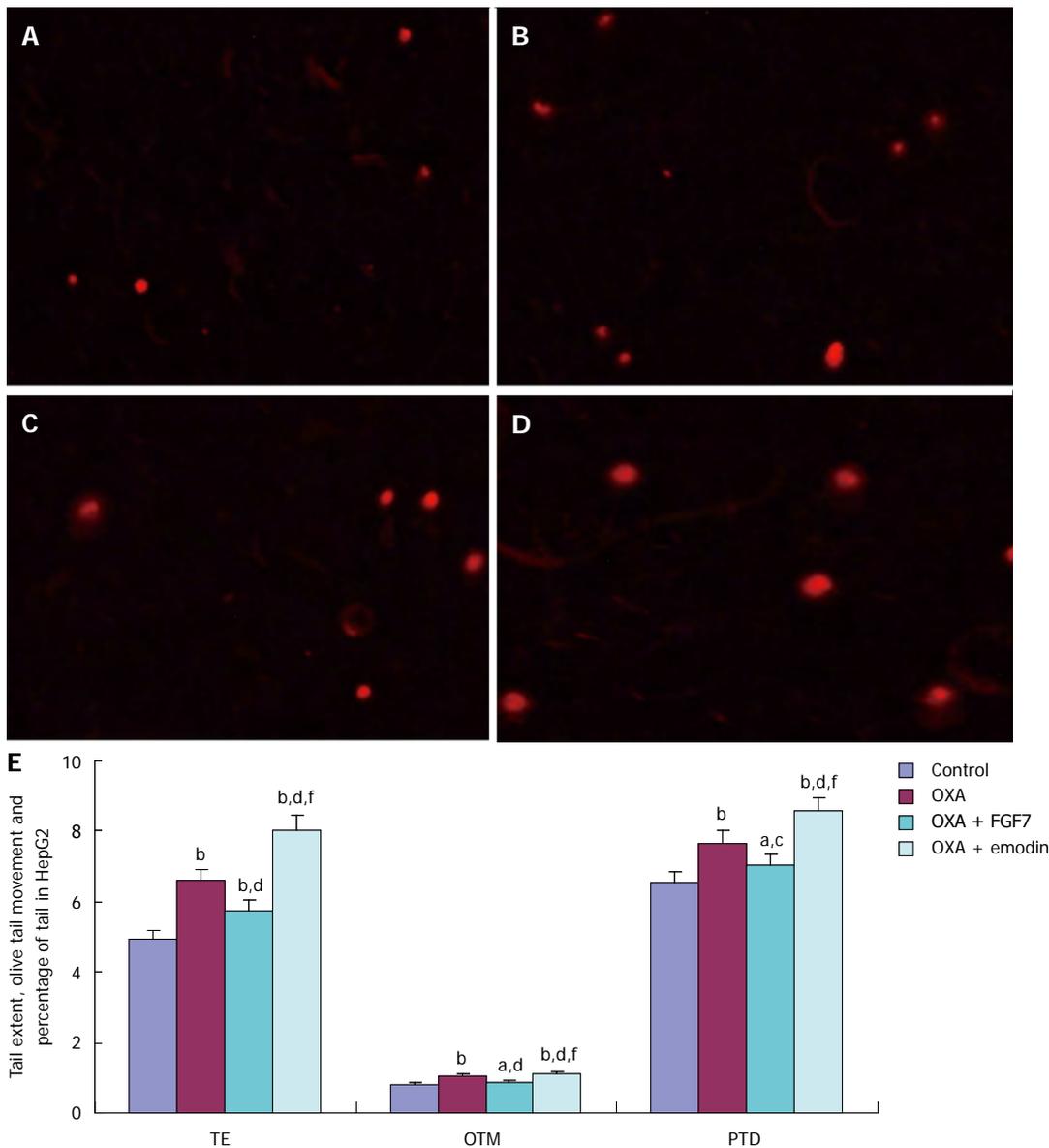


Figure 2 DNA damage detected by single cell gel electrophoresis in HepG2. A-D: Ethidium bromide stain (magnification × 200). Control group (A); Oxaliplatin (OXA) group (B); OXA + fibroblast growth factor 7 (FGF7) group (C); OXA + emodin group (D); E: The tail extent (TE), the Olive tail moment (OTM) and the percentage of tail DNA (PTD) were considerably increased in the OXA + emodin group in comparison with the OXA group and the OXA + FGF7 group, respectively, and these differences were statistically significant. TE, OTM and PTD were significantly lower in the OXA + FGF7 group compared with OXA + emodin group and OXA group; however, these values were higher than those of the control group (^a $P < 0.05$, ^b $P < 0.01$ vs control group; ^c $P < 0.05$, ^d $P < 0.01$ vs OXA group; ^f $P < 0.01$ vs OXA + FGF7 group).

TE and OTM in the OXA + FGF7 group were considerably decreased, and these differences were statistically significant ($P < 0.01$). TE, OTM and the percentage of tail DNA (PTD) were significantly greater ($P < 0.01$) in the OXA + emodin group than in the OXA + FGF7 group. PTD in the CG, OXA group, and OXA + FGF7 group did not significantly differ ($P > 0.05$) (Figure 2).

In HepG2/OXA cells, compared with the CG, TE, PTD and OTM in the OXA group, OXA + FGF7 group and OXA + emodin group were significantly increased; the differences were statistically significant ($P < 0.01$). Compared with the OXA group, OTM and PTD in the OXA + FGF7 group were reduced with statistically significant difference ($P < 0.01$). In the OXA + emodin group, TE, OTM and PTD were significantly increased; this dif-

ference was statistically significant ($P < 0.01$). Compared with the OXA + FGF7 group, TE, OTM and PTD in the OXA + emodin group were significantly increased ($P < 0.01$). These results are presented in Figure 3.

In HepG2/OXA/T cells, compared with the CG, TE, OTM and PTD in the OXA group, OXA + FGF7 group, and OXA + emodin group were significantly higher; the differences were statistically significant. Compared with the OXA group, OTM and PTD in the OXA + FGF7 group were reduced, and there was a significant difference; however, TE, OTM and PTD were significantly increased in the OXA + emodin group, with a statistically significant difference ($P < 0.01$). In comparison with the OXA + FGF7 group, TE, OTM and PTD were significantly increased in the OXA + emodin group, and the

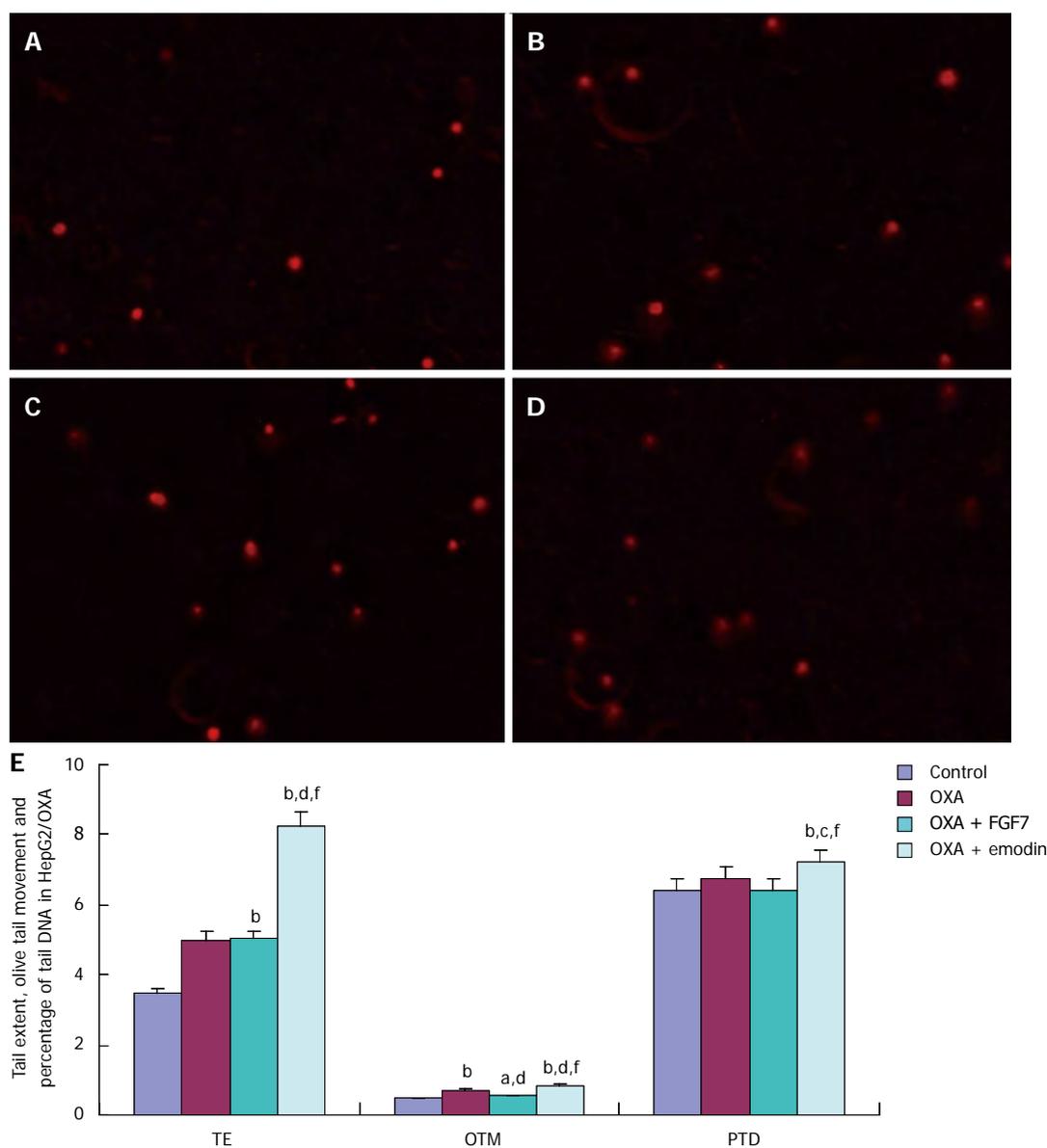


Figure 3 DNA damage detected by single cell gel electrophoresis in HepG2/oxaliplatin. A-D: Ethidium bromide stain (magnification × 200). Control group (A); Oxaliplatin (OXA) group (B); OXA + fibroblast growth factor 7 (FGF7) group (C); OXA + emodin group (D); E: The tail extent (TE), the Olive tail moment (OTM) and the percentage of tail DNA (PTD) were considerably increased in the OXA + emodin group in comparison with the OXA group and the OXA + FGF7 group, respectively, and these differences were statistically significant. As for PTD there was no significant difference between the OXA group and the OXA + FGF7 group (^a*P* < 0.05, ^b*P* < 0.01 vs control group; ^c*P* < 0.05, ^d*P* < 0.01 vs OXA group; ^e*P* < 0.01 vs OXA + FGF7 group).

Table 1 Inhibition ratio and 50% inhibitory concentration, and reversal index of 10 μmol/L emodin in HepG2/oxaliplatin (mean ± SD) (n = 5)

| OXA concentration (μmol/L) | 3.125 | 6.25 | 12.5 | 25 | 50 | 100 | IC ₅₀ (μmol/L) | Reversal index |
|----------------------------|--------------------------|--------------------------|--------------------------|---------------------------|---------------------------|---------------------------|---------------------------|----------------|
| IR in control (%) | 9.76 ± 1.18 | 11.94 ± 1.30 | 13.95 ± 1.11 | 14.58 ± 1.02 | 28.06 ± 2.01 | 63.95 ± 4.71 | 120.78 ± 9.68 | 3.05 |
| IR in emodin (%) | 12.35 ± 1.3 ^a | 16.76 ± 1.3 ^a | 21.69 ± 1.6 ^a | 29.87 ± 1.55 ^b | 48.14 ± 2.09 ^b | 80.34 ± 3.00 ^b | 39.65 ± 5.43 | |

^a*P* < 0.05, ^b*P* < 0.01 vs control group. IR: Inhibition ratio; OXA: Oxaliplatin; IC₅₀: 50% inhibitory concentration.

difference was statistically significant (*P* < 0.01), as shown in Figure 4.

FGFR2, pERK1/2, and ERCC1 protein expression

In comparison with the expression levels in the parental HepG2 cell line, the FGFR2, pERK1/2 and ERCC1

expression levels in the resistant cell line HepG2/OXA were significantly increased, whereas the pERK1/2 and ERCC1 expression levels in the shRNA-transfected cell line HepG2/OXA/T were significantly inhibited, as FGFR2 expression was silenced. Compared with the CG in HepG2 cells, FGFR2 expression was increased

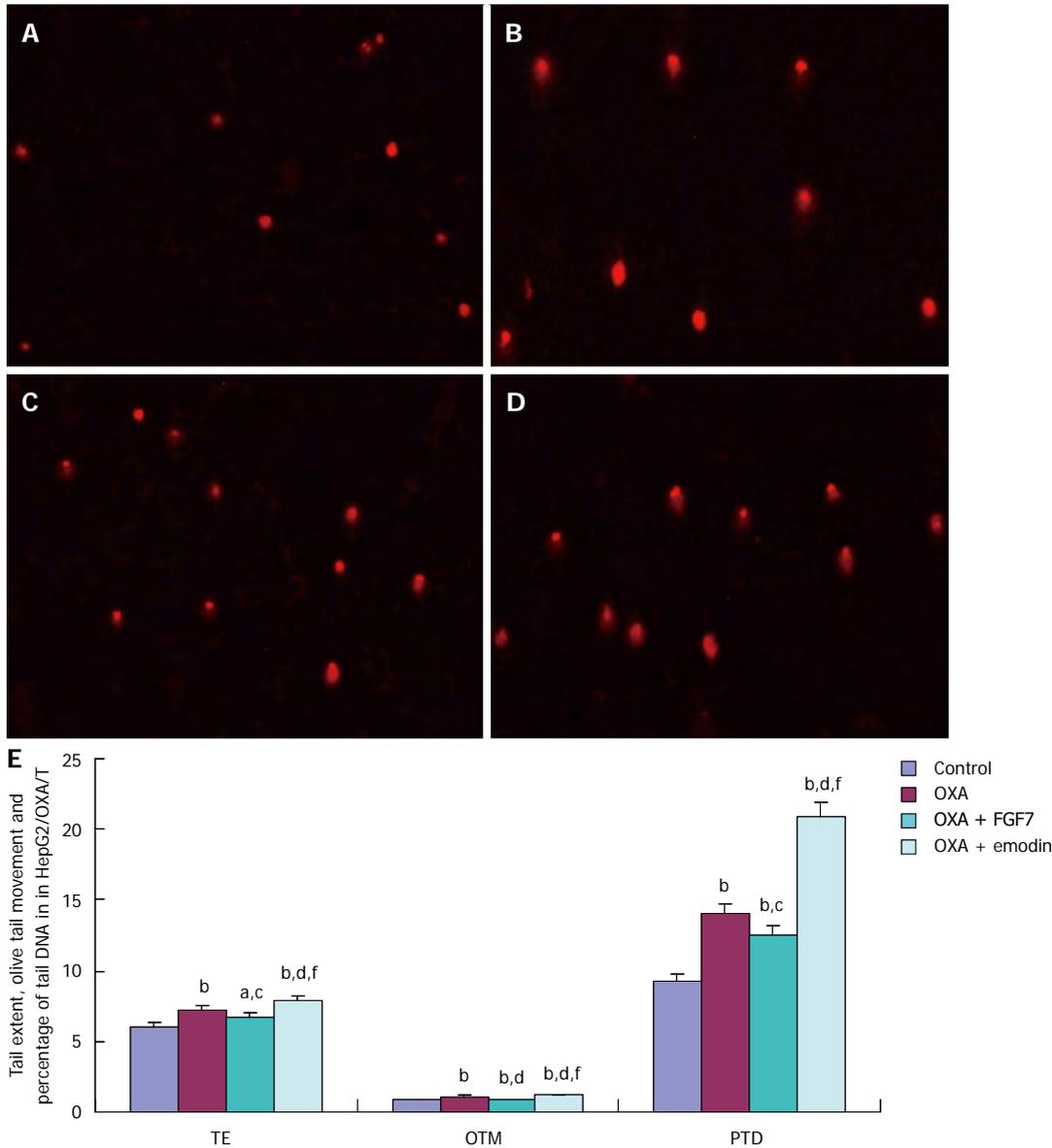


Figure 4 DNA damage detected by single cell gel electrophoresis in HepG2/oxaliplatin/T. A-D: Ethidium bromide stain (magnification $\times 200$). Control group (A), Oxaliplatin (OXA) group (B), OXA + fibroblast growth factor 7 (FGF7) group (C), OXA + emodin group (D); E: The tail extent (TE), the Olive tail moment (OTM) and the percentage of tail DNA (PTD) were considerably increased in the OXA + emodin group in comparison with the OXA group and the OXA + FGF7 group, respectively, and these differences were statistically significant. TE, OTM and PTD were significantly decreased in the OXA + FGF7 group in comparison to those in the OXA group and the OXA + FGF7 group, but were greater than those in the control group (^a $P < 0.05$, ^b $P < 0.01$ vs control group; ^c $P < 0.05$, ^d $P < 0.01$ vs OXA group; ^f $P < 0.01$ vs OXA + FGF7 group).

with statistical significance in the OXA + FGF7 treatment group; moreover, there was a significant difference between the OXA + emodin group and the OXA + FGF7 group. The same trend was observed in the HepG2 and HepG2/OXA cells with respect to the expression of pERK1/2 and ERCC1 among the different treatment groups. Compared with the pERK1/2 and ERCC1 expression in CG, the levels in the OXA group were significantly up-regulated. Compared with the CG, pERK1/2 and ERCC1 expression were increased in the OXA + FGF7 group and reduced in the OXA + emodin group. However, the expression of FGFR2, pERK1/2 and ERCC1 exhibited no significant differences among the treatment groups in the shRNA-transfected cell line HepG2/OXA/T (Figure 5).

DISCUSSION

Rhubarb and polygonum cuspidatum have been widely used in various heat syndromes to clear heat and detoxify in the body in accordance with the theories of traditional Chinese medicine. Emodin (1,3,8-trihydroxy-6-methylanthraquinone), the primary active ingredient in these traditional medicines, was identified by modern pharmacological studies as having a wide range of pharmacological effects, such as protecting the function of the liver and the kidney^[23], producing anti-inflammatory effects^[24] and regulating lipid metabolism^[25,26]. In recent years, its anti-cancer function has been revealed in a variety of malignancies, including HCC^[27]. In addition, combined with chemotherapy drugs such as platinum^[7-11,16], emodin

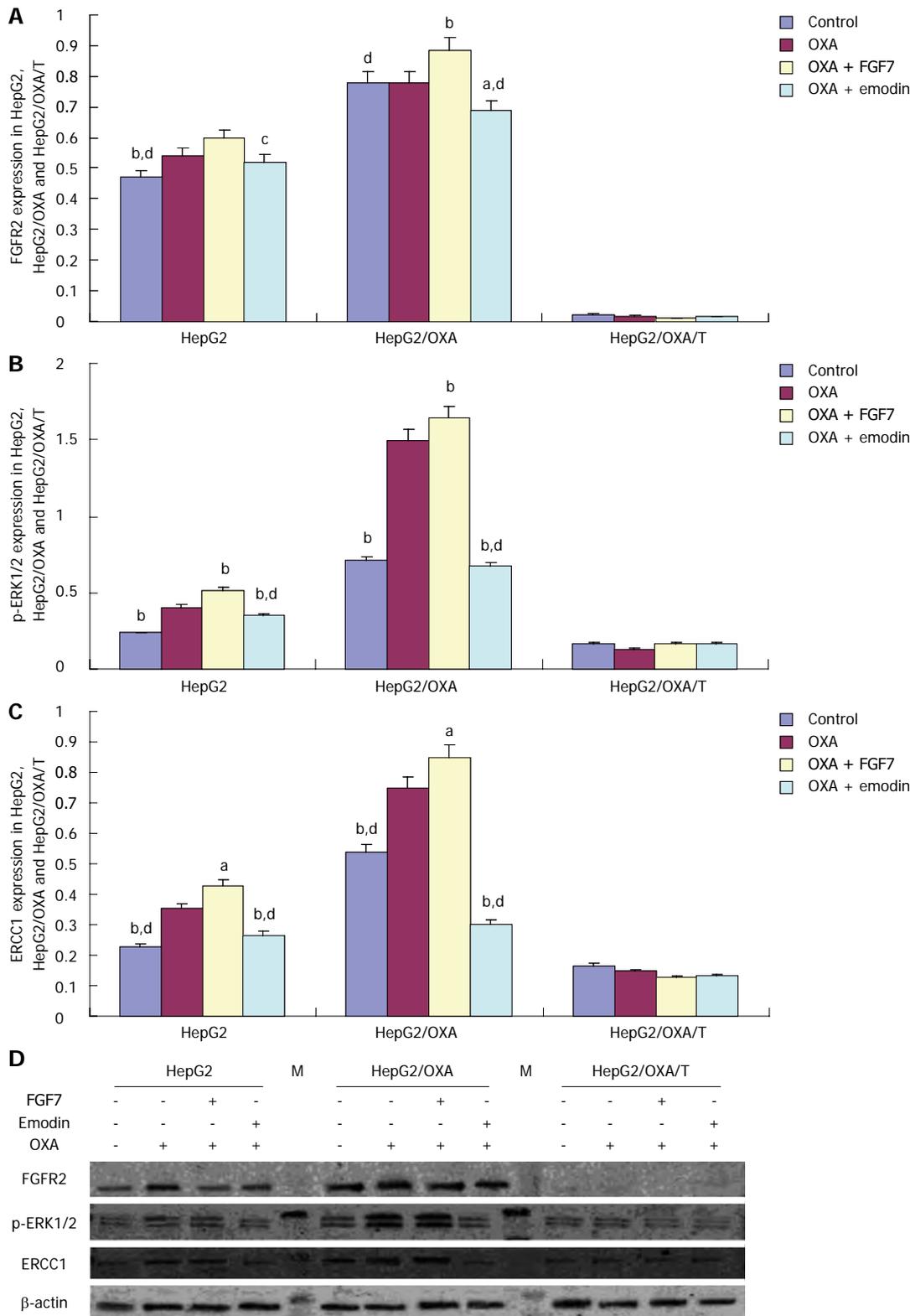


Figure 5 Comparison of fibroblast growth factor receptor 2, phosphorylated extracellular signal-regulated kinase 1/2, excision repair cross-complementing gene 1 expression in HepG2, HepG2/oxaliplatin and HepG2/oxaliplatin/T detected by Western blotting. A-C: In comparison with the parental HepG2 cell line, the expression levels of the fibroblast growth factor receptor 2 (FGFR2), phosphorylated extracellular signal-regulated kinase 1/2 (pERK1/2) and excision repair cross-complementing gene 1 (ERCC1) in the resistant cell line HepG2/oxaliplatin (OXA) were significantly increased, however, the pERK1/2 and ERCC1 expression levels in HepG2/OXA/T cells were significantly inhibited, as FGFR2 expression was silenced. The expression levels of pERK1/2 and ERCC1 in the HepG2 cells and that of FGFR2, pERK1/2 and ERCC1 in HepG2/OXA cells, was increased in the OXA + fibroblast growth factor 7 (FGF7) group and decreased in the OXA + emodin group with statistical significance compared with the OXA group, but the trend of those significant differences disappeared in HepG2/OXA/T cells (^a*P* < 0.05, ^b*P* < 0.01 vs OXA group; ^c*P* < 0.05, ^d*P* < 0.01 vs OXA + FGF7 group); D: Western blotting of FGFR2, pERK1/2, ERCC1 in HepG2, HepG2/oxaliplatin and HepG2/oxaliplatin/T. M: Marker.

demonstrates a synergistic effect and reverses platinum resistance. In the human multidrug-resistant breast cancer cell line MCF-7/Adr, the reversal index of emodin was 2.86 and 1.79 in combination with doxorubicin and cisplatin, respectively^[28]. Our study results are similar, yielding a value of 3.05 in resistance reversal effect for 10 $\mu\text{mol/L}$ in HepG2/OXA cells.

DNA interstrand cross-linking or chain cross-linking caused by platinum drugs induces apoptosis in tumor cells^[29]. Although the capacity to repair DNA damage by NER, which removes the chain of platinum drug-induced DNA adducts, is considered to be the main mechanism of tumor cell resistance to platinum, the synergistic effect and reversal of platinum resistance induced by emodin appears to function by enhancing the DNA damage^[30]. This study showed that TE, OTM and PTD were significantly higher in HepG2 cells after OXA and OXA + emodin treatment; moreover, differences are also statistically significant between the OXA group and the OXA + emodin group. A similar trend was observed in the resistant cell line HepG2/OXA. Our results suggest that emodin promotes the DNA damage that is induced by OXA. As the rate-limiting enzyme of the NER process, ERCC1 and XPF form heterodimers that exhibit damage recognition and nucleic acid cutting activity at the 5' end of the DNA in platinum-based chemotherapy. ERCC1 plays an important role in HCC, which was expected given that this gene is an effective predictor of the sensitivity of tumor cells to platinum-based chemotherapy^[15,16]. This study observed that an increase in the DNA damage level occurred in the resistant cell line HepG2/OXA and their parental cell line HepG2 after being treated with emodin combined with OXA. Moreover, ERCC1 expression levels were significantly decreased. The study results suggested that emodin-mediated down-regulation of the expression of ERCC1 plays an important role in enhancing the DNA damage induced by OXA. Zhou *et al.*^[28] also found that ERCC1 protein expression decreases gradually in MCF-7/Adr cells in proportion to the duration of emodin treatment and that the effect of 20 $\mu\text{g/mL}$ of emodin was greater than that of 10 $\mu\text{g/mL}$.

In the multidrug-resistant gastric cancer cell lines, our previous results indicated that *ERCC1* was a target gene in the FGFR2 signaling pathway^[18]. The same result was observed in HCC drug-resistant cell lines in this study. Accompanied with Bek shRNA silencing of *FGFR2* gene expression, the expression of ERCC1 was significantly reduced in the drug-resistant cell line HepG2/OXA, whereas after FGF7 stimulation of the FGFR2 signaling pathway, ERCC1 expression was significantly increased. These results support the idea that ERCC1 is a downstream gene of the FGFR2 signaling pathway. FGFR2, a member of the transmembrane tyrosine kinase receptor family (FGFRs) and the expression product of the *bek* oncogene, plays an important role in the cell differentiation of stomach cancer^[31,32] and HCC^[17], which effectively predicts overall survival and progression-free survival as a molecular marker. The protein kinase C,

Ras/Raf/MEK/ERK, janus kinase/signal transducer and activator of transcription, and PI3K signaling pathways are downstream cascades in the FGF-induced signaling pathways^[33]. ERCC1 expression can be inhibited by the ERK inhibitor U0126, suggesting that *ERCC1* is one of the target genes in the downstream of the ERK signaling pathway^[8]. Our results suggested that ERCC1 expression was inhibited by *bek* gene silencing; additionally, ERCC1 expression was significantly increased by the positive stimulus of FGF7. In addition, p-ERK1/2, the key molecule in the Ras/Raf/MEK/ERK pathway, was increased or reduced in conjunction with FGF7 stimulus or *bek* gene silencing, suggesting that the Ras/Raf/MEK/ERK pathway may be an important pathway in the FGFR2 regulation of ERCC1 expression. The most interesting result of this study was that FGFR2 protein expression disappeared accompanied with *bek* gene being silenced, whereas ERCC1 and p-ERK1/2 expression were not completely inhibited, suggesting that other signaling pathways may be involved in the pathway by which FGFR2 regulates ERCC1 expression.

By contrast to FGF7 stimulation of the FGFR2 signal pathway in the resistant hepatic cancer cell line HepG2/OXA and the parental cell line HepG2, the p-ERK1/2 phosphorylation level was significantly inhibited by emodin treatment; meanwhile ERCC1 expression levels were significantly decreased. These data were consistent with the results of Ko *et al.*^[9], who observed that emodin could significantly enhance the cytotoxicity of platinum drugs in lung cancer cell lines, and its mechanism is closely related to the inhibition of ERCC1 expression, and the downward effect of ERCC1 expression was achieved through the inactivation of the ERK1/2 pathway. Emodin, a tyrosine kinase inhibitor^[19,34] and FGF7, a positive stimulator, had no effect on the expression of ERCC1 and p-ERK1/2 if FGFR2 expression was inhibited by shRNA silencing, which suggested that the emodin regulation of ERCC1 expression by the ERK1/2 pathway was closely related to the inhibition of FGFR2 tyrosine kinase activity.

In summary, the results of this study indicated that emodin could significantly enhance the DNA damage that was caused by OXA and induce OXA resistance reversal in HepG2/OXA cells. The molecular mechanism for this phenomenon is mediated by the inhibition of ERCC1 expression by the FGFR2/ERK1/2 signaling pathway.

COMMENTS

Background

As the effect of platinum-based chemotherapy on advanced hepatocellular carcinoma (HCC) was re-proved in 2010, the drugs which could strengthen chemotherapy effects and protect normal liver cells were the hot research area. Emodin, as the main active ingredient in many Chinese herbs, interested us for its low toxicity and synergistic effect combined with platinum in HCC cells. Excision repair cross-complementing gene 1 (*ERCC1*), which was the limiting enzyme in the nucleotide excision repair pathway, plays an important role in the process of platinum drug resistance. The relationship between synergistic effects and the regulation of ERCC1 expression is worthy of further study in HCC treatments that combine emodin with platinum drugs.

Research frontiers

Fibroblast growth factor receptor 2 (FGFR2), as a transmembrane tyrosine kinase, plays an important role in the differentiation of HCC, the clinical staging of tumors, the incidence of tumor thrombosis and the determination of alpha-fetoprotein levels. Interestingly, ERCC1 is a downstream target gene of *FGFR2*, whereas emodin is a tyrosine kinase inhibitor. Current research has indicated that emodin down-regulates ERCC1 expression in non-small cell lung cancer, and its effects may be relevant to the extracellular signal-regulated kinase 1/2 (ERK1/2) signaling pathway, which is one of the downstream components of the FGFR2 pathway. However, the exact mechanism through which emodin produces these effects has not been well established.

Innovations and breakthroughs

In this study, the resistance reversal effect for 10 $\mu\text{mol/L}$ emodin was 3.05 in HepG2/oxaliplatin (OXA) cells. Meanwhile, the tail length, the olive tail length and the percentage of tail DNA were significantly higher after treatment combined with emodin, which suggest that emodin promotes the DNA damage induced by OXA. Accompanied with *shRNA* silencing and fibroblast growth factor 7 (FGF7) stimulation of *FGFR2* gene expression, the expression of phosphorylated extracellular signal-regulated kinase 1/2 (p-ERK1/2) and ERCC1 was significantly reduced and increased in the drug-resistant cell line HepG2/OXA, which supports the idea that ERCC1 is a downstream gene of the FGFR2/p-ERK1/2 signaling pathway. Furthermore, ERCC1 expression levels in HepG2/OXA and HepG2 cells were significantly decreased after emodin treatment with significant inhibition of the p-ERK1/2 phosphorylation level. However, if FGFR2 expression was inhibited by *shRNA* silencing, the inhibition effect of emodin and the stimulation effect of FGF7 on the expression of ERCC1 and p-ERK1/2 disappeared, which suggested that emodin regulation of ERCC1 expression by the ERK1/2 pathway was closely related to the inhibition of FGFR2 tyrosine kinase activity.

Applications

The results of emodin enhancing the DNA damage caused by OXA and its molecular mechanism associated with the inhibition of ERCC1 expression by the FGFR2/ERK1/2 signaling pathway may provide an experimental basis for the further development and application of emodin in the reversal of platinum drug resistance in HCC and other types of malignant tumors.

Terminology

Platinum drug resistance: Platinum-based compounds, including cisplatin, carboplatin and OXA, are widely used in a number of carcinomas, and compose a mainstay of chemotherapeutic treatment. The cytotoxicity of platinum is attributed to apoptosis induced by DNA damage through the formation of platinum crosslinks on DNA. Cancer cells have the capacity to decrease the platinum concentration and repair DNA damage, which is associated with platinum drug resistance. The capacity to remove the chain of platinum drug-induced DNA adducts, is considered to be the main mechanism of tumor cell resistance to platinum.

Peer review

This is a good descriptive study in which authors proved emodin could significantly enhance the DNA damage caused by OXA and induce OXA resistance reversal in HepG2/OXA cells, and investigated the molecular mechanism for this effect from the point of the inhibition of ERCC1 expression mediated by the FGFR2/ERK1/2 signaling pathway. The results are interesting and suggest that emodin is a potential therapeutic substance that could be used in reversing platinum drug resistance in the HCC and other types of malignant tumors.

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Correlation of fibrinogen-like protein 2 with progression of acute pancreatitis in rats

Xiao-Hua Ye, Tan-Zhou Chen, Jia-Ping Huai, Guang-Rong Lu, Xiao-Ju Zhuge, Ren-Pin Chen, Wu-Jie Chen, Chen Wang, Zhi-Ming Huang

Xiao-Hua Ye, Tan-Zhou Chen, Jia-Ping Huai, Guang-Rong Lu, Xiao-Ju Zhuge, Ren-Pin Chen, Wu-Jie Chen, Chen Wang, Zhi-Ming Huang, Department of Gastroenterology and Hepatology, First Affiliated Hospital of Wenzhou Medical College, Wenzhou 325000, Zhejiang Province, China

Author contributions: Ye XH and Chen TZ contributed equally to this work; Ye XH, Chen TZ and Huang ZM designed the research; Ye XH, Huai JP, Lu GR and Zhuge XJ performed the research; Chen RP, Chen WJ and Wang C provided analytic tools; Ye XH and Chen TZ wrote the paper.

Correspondence to: Zhi-Ming Huang, Professor of Medicine, Department of Gastroenterology and Hepatology, First Affiliated Hospital of Wenzhou Medical College, Fuxue Lane No. 2, Lucheng District, Wenzhou 325000, Zhejiang Province, China. wzhzm123@126.com

Telephone: +86-577-88069257 Fax: +86-577-88068257

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Abstract

AIM: To examine fibrinogen-like protein 2 (fgl2) expression during taurocholate-induced acute pancreatitis progression in rats and its correlation with pancreatic injury severity.

METHODS: Forty-eight male Sprague-Dawley rats were randomly divided into the severe acute pancreatitis (SAP) group ($n = 24$) and the sham operation (SO) group ($n = 24$). Sodium taurocholate (4% at doses of 1 mL/kg body weight) was retrogradely injected into the biliopancreatic ducts of the rats to induce SAP. Pancreatic tissues were prepared immediately after sacrifice. At the time of sacrifice, blood was obtained for determination of serum amylase activity and isolation of peripheral blood mononuclear cells (PBMCs). Pancreatic tissue specimens were obtained for routine light microscopy including hematoxylin and eosin staining, and the severity of pancreatic injury was

evaluated 1, 4 and 8 h after induction. Expression of fgl2 mRNA was measured in the pancreas and PBMCs using reverse transcription polymerase chain reaction. Expression of fgl2 protein was evaluated in pancreatic tissues using Western blotting and immunohistochemical staining. Masson staining was also performed to observe microthrombosis.

RESULTS: At each time point, levels of fgl2 mRNAs in pancreatic tissues and PBMCs were higher ($P < 0.05$) in the SAP group than in the SO group. For pancreatic tissue in SAP vs SO, the levels were: after 1 h, 3.911 ± 1.277 vs 1.000 ± 0.673 ; after 4 h, 9.850 ± 3.095 vs 1.136 ± 0.609 ; and after 8 h, 12.870 ± 3.046 vs 1.177 ± 0.458 . For PBMCs in SAP vs SO, the levels were: after 1 h, 2.678 ± 1.509 vs 1.000 ± 0.965 ; after 4 h, 6.922 ± 1.984 vs 1.051 ± 0.781 ; and after 8 h, 13.533 ± 6.575 vs 1.306 ± 1.179 . Levels of fgl2 protein expression as determined by Western blotting and immunohistochemical staining were markedly up-regulated ($P < 0.001$) in the SAP group compared with those in the SO group. For Western blotting in SAP vs SO, the results were: after 1 h, 2.183 ± 0.115 vs 1.110 ± 0.158 ; after 4 h, 2.697 ± 0.090 vs 0.947 ± 0.361 ; and after 8 h, 3.258 ± 0.094 vs 1.208 ± 0.082 . For immunohistochemical staining in SAP vs SO, the results were: after 1 h, 1.793 ± 0.463 vs 0.808 ± 0.252 ; after 4 h, 4.535 ± 0.550 vs 0.871 ± 0.318 ; and after 8 h, 6.071 ± 0.941 vs 1.020 ± 0.406 . Moreover, we observed a positive correlation in the pancreas ($r = 0.852$, $P < 0.001$) and PBMCs ($r = 0.735$, $P < 0.001$) between fgl2 expression and the severity of pancreatic injury. Masson staining showed that microthrombosis (%) in rats with SAP was increased ($P < 0.001$) compared with that in the SO group and it was closely correlated with fgl2 expression in the pancreas ($r = 0.842$, $P < 0.001$). For Masson staining in SAP vs SO, the results were: after 1 h, 26.880 ± 9.031 vs 8.630 ± 3.739 ; after 4 h, 53.750 ± 19.039 vs 8.500 ± 4.472 ; and after 8 h, 80.250 ± 12.915 vs 10.630 ± 7.003 .

CONCLUSION: Microthrombosis due to fgl2 overexpression contributes to pancreatic impairment in rats with SAP, and fgl2 level may serve as a biomarker during early stages of disease.

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Key words: Fibrinogen-like protein 2; Microthrombosis; Fibrin; Severe acute pancreatitis; Peripheral blood mononuclear cell

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INTRODUCTION

Severe acute pancreatitis (SAP) is a pathogenic condition that progresses rapidly and has a high mortality^[1-3], but the underlying pathophysiological mechanisms remain incompletely defined. SAP is currently considered to be complicated by microcirculatory disturbances and coagulation abnormalities^[4,5]. Inflammatory mediators such as interleukin (IL)-6, IL-1 β , and tumor necrosis factor α (TNF- α) released during acute inflammatory reactions are not just involved in the inflammatory process but may also be responsible for the systemic activation of hemostasis in patients with SAP^[6,7]. Intravascular coagulation and thromboembolism are believed to play an important role in the pathogenesis of SAP and are related to its severity^[8,9]. Acute inflammatory events during disease progression can lead to dysregulation of the coagulation cascade^[10]. In SAP patients, thrombin and platelets are deposited not only in the local pancreatic blood vessels but also in the connective tissue and intercellular spaces^[4]. Studies suggest that biochemical variables such as prothrombin time, D-dimer, and clotting time may have prognostic value, and direct anticoagulant therapy has been shown to be helpful in the treatment of SAP^[4,10-12]. These facts suggest that coagulation and inflammation in SAP are correlated, thus microthrombosis plays a crucial role in SAP^[10]. However, the exact pathophysiological mechanism remains unknown.

Fibrinogen-like protein 2 (fgl2)/fibroleukin (also termed fgl2 prothrombinase) was determined to be a new member of the fibrinogen-related protein superfamily (fibrinogen-related domain), which includes fibrinogen, tenascin, ficolin, and angiopoietin^[13-15]. fgl2 is a direct prothrombinase with serine protease activity. fgl2 can cleave prothrombin to thrombin *via* a noncanonical pathway, resulting in fibrin deposition^[16,17]. fgl2 leads to histopathological lesions and ischemic injury by mediating “immune coagulation”, fibrin deposition, and microthrombosis^[18-21]. Microvascular disturbances are caused by microthrombi that are activated and produced as a

consequence of fgl2 action^[18,21-23]. Nevertheless, whether fgl2 contributes to the pathogenesis of SAP is unclear.

In the present study, we used 4% sodium taurocholate to induce SAP in rats. We then investigated the expression and localization of fgl2 in pancreatic tissues. We also assessed fgl2 expression and its correlation with severity of pancreatic injury and microthrombi in rats with SAP to provide new insight into the pathogenesis of this disease.

MATERIALS AND METHODS

Animals

Forty-eight male Sprague-Dawley rats, weighing 200-250 g, were obtained from the Experimental Animal Center of Wenzhou Medical College, Wenzhou, China. All animals were fed standard rat chow, had free access to water, and were housed at a constant room temperature of 25 °C and a 12-h day/night cycle. All animals were acclimated for at least one week before the experiments were initiated. All procedures were performed in accordance with the Guidelines for Animal Experiments of Wenzhou Medical College.

Induction of SAP

All rats received intraperitoneal injection of 10% chloralhydrate (2 mL/kg body weight; Solarbio, Beijing, China) for anesthesia. The rats were divided into the SAP group ($n = 24$) and the sham operation (SO) group ($n = 24$). In the SAP group, a laparotomy was performed through a midline incision. Sodium taurocholate (4%; 1 mL/kg body weight; Sigma, St. Louis, MO, United States) was retrogradely injected into the biliopancreatic duct through the papilla using a segmental epidural catheter *via* a microinjection pump at a speed of 0.2 mL/min. A microclip was placed in the hepatic portion of the biliopancreatic duct to avoid reflux before the injection. SO rats underwent surgery but without infusion. After each operation, the abdomen was closed in two layers. All procedures were carried out using sterile techniques.

Sample collection and determination of serum amylase

At defined time points (1, 4 and 8 h; $n = 8$ per time point) after SAP induction, rats were anesthetized with 10% chloralhydrate (2 mL/kg body weight) and euthanized by exsanguination. Pancreatic tissues were harvested immediately and divided into two pieces. Portions of the tissues were fixed in 4% paraformaldehyde for immunohistochemical staining and microscopic observation, and other portions were removed and stored in liquid nitrogen until use. Blood samples (5 mL) were obtained *via* postcava puncture, and 4 mL of each sample was collected and stored in 5-mL tubes without anticoagulants (Generay, Shanghai, China). The blood samples were centrifuged at 1200 $\times g$ for 20 min, and the serum was collected for determination of amylase activity (U/L) with a fully automatic biochemical analyzer (Hitachi, Tokyo, Japan). The remaining 1 mL blood was stored in

ethylene diamine tetraacetic acid-containing tubes (Gen-eray) and used to isolate peripheral blood mononuclear cells (PBMCs).

Isolation of PBMCs

PBMC isolation was performed with density gradient centrifugation. The blood sample (1 mL) was diluted with 1 mL 0.9% saline. Subsequently, the diluted cell suspension was carefully laid over 2 mL Bandicoot percoll (Solarbio) and centrifuged at $2000 \times g$ for 20 min at 20 °C. The PBMC layer was carefully transferred into a new tube, and the volume was brought to 5 mL with 0.9% saline and centrifuged ($2000 \times g$, 5 min, 20 °C). This step was repeated. Finally, the supernatant was carefully removed, reserving the PBMCs at the bottom of the tube. PBMCs were immediately stored in liquid nitrogen until use.

Histological analysis and assessment of pancreatic tissue injury

Pancreatic tissue samples were fixed in 4% paraformaldehyde for histological analysis. The samples were dehydrated and embedded in paraffin. Pancreas sections were stained with hematoxylin and eosin (HE) for routine light microscopy. Slides were examined in a blinded fashion by two pathologists who were unaware of the treatment protocol according to the modified method of Schmidt *et al.*^[24] and Eşrefoğlu *et al.*^[25]. Variables of edema, hemorrhage, acinar cell degeneration, and interstitial inflammation were scored in 10 random fields of each slide to assess the severity of pancreatic injury under a light microscope (CX31, Tokyo, Japan; HE staining, $\times 200$). Each variable was scored as follows: (1) edema: 0 = absent, 1 = focally in the interlobular space, 2 = increased in the intralobular space, 3 = isolated-island appearance of pancreatic acinus; (2) hemorrhage: 0 = absent, 1 = slight, 2 = moderate, 3 = severe; (3) acinar cell degeneration: 0 = absent, 1 = focal ($< 5\%$); 2 = and/or sublobular ($< 20\%$), 3 = and/or lobular ($> 20\%$); and (4) inflammation: 0 = absent, 1 = slight, 2 = moderate, 3 = severe. The sum of these four variables for each pancreas section could have a maximum score of 12.

Pancreatic tissue sections were also stained with Masson stain to observe microthrombi in microvessels. One hundred microvessels on each slide were randomly selected, and the percent of microthrombus-positive vessels was calculated.

Real-time fluorescence-based quantitative polymerase chain reaction

Total RNA was extracted from pancreatic tissues and PBMCs using Trizol reagent (Invitrogen, Carlsbad, CA, United States), and cDNA was synthesized with the First Strand cDNA synthesis kit (MBI Fermentas, Burlington, Canada) according to the manufacturer's protocols. The samples were subsequently amplified using Moloney murine leukemia virus reverse transcriptase and Taq DNA polymerase (Invitrogen) using an ABI 7500 Sequence De-

tection System (Applied Biosystems Inc., Carlsbad, CA, United States). The sequences of the primers (Gen-eray) were as follows: fgl2 (153 bp): 5'-CCTGGAGATTGTG-GTTTCGT-3' (forward) and 5'-TACCATGCCTTTCTC-CAAGG-3' (reverse), β -actin (153 bp): 5'-TGTCAC-CAACTGGGACGATA-3' (forward) and 5'-GGGGT-GTTGAAGGTCTCAAA-3' (reverse). The cDNA was denatured at 95 °C for 5 min and amplified for 40 cycles of 95 °C (15 s), 60 °C (45 s), and 72 °C (60 s), followed by a final extension at 72 °C (5 min). The samples were tested in triplicate, and the results were calculated using the $2^{-\Delta\Delta CT}$ method. The expression of fgl2 mRNA was shown as relative to β -actin.

Western blotting

Pancreatic proteins were separated with 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis and transferred to a nitrocellulose membrane (Millipore, Billerica, MA, United States). The membrane was blocked with 5% skim milk in Tris-buffered saline and then incubated with a polyclonal antibody against fgl2 (Biosynthesis Biotechnology, Beijing, China; 1:200) at 4 °C overnight. The membrane was washed three times with Tris-buffered saline and incubated with secondary antibody (Santa Cruz Biotechnology, Santa Cruz, CA, United States) conjugated to horseradish peroxidase for 2 h at room temperature. The immunoreactive bands were visualized with an enhanced chemiluminescence reagent (Pierce, Rockford, IL, United States). Protein expression levels were normalized to β -actin.

Immunohistochemical staining

Immunohistochemical staining was performed to assess fgl2 protein expression in the pancreas using EnVision reagents (Dako, Glostrup, Denmark). Four- μ m-thick paraffin sections were routinely cut. Microwave antigen retrieval was conducted for 20 min in citrate buffer (pH 6.0) to activate antigens before quenching endogenous peroxidase activity in 0.3% H₂O₂ for 10 min. After three times of washing with phosphate-buffered saline (Gen-eray) and then the primary reaction solution, the sections were incubated with rabbit polyclonal anti-rat fgl2 (Bio-synthesis Biotechnology; 1:100) for 2 h at 37 °C. Following the same washing procedure, EnVision reagents were applied and incubated for 30 min at 37 °C. Finally, the reaction was developed with 0.05% diaminobenzidine and counterstained with hematoxylin for microscopy. Phosphate-buffered saline was used as a negative control instead of the primary antibody. To measure fgl2 protein expression, 10 randomly selected fields across each section were evaluated at $\times 200$ magnification.

Statistical analysis

All data represent the mean \pm SD. SPSS 15.0 software (SPSS, Chicago, IL, United States) was used for statistical analysis. Differences between the SAP and SO groups were analyzed with the Student's *t* test. One-way analysis of variance was used to check for statistical significance

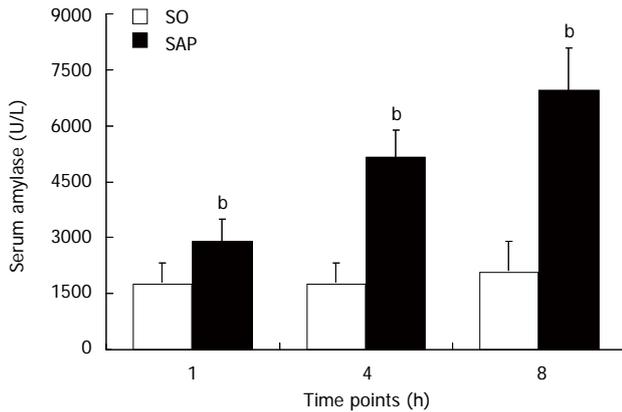


Figure 1 Levels of serum amylase in severe acute pancreatitis and sham-operated rats at each time point. Each time point (h) after operation consisted of 8 rats. There was no difference among the three time points in the sham operation (SO) group ($P > 0.05$). The data are expressed as the mean \pm SD. ^b $P < 0.01$ vs SO group. SAP: Severe acute pancreatitis.

among the three time points in the same group. Pearson's correlation coefficient was calculated to determine the strength of the association between two continuous variables. $P < 0.05$ was considered statistically significant.

RESULTS

Levels of serum amylase are elevated in rats with SAP

Serum amylase is the most commonly used biochemical indicator of acute pancreatitis. Levels of serum amylase were markedly elevated ($P < 0.01$) in the SAP group compared with the SO group at each time point. There was no significant difference in the levels of serum amylase among the three time points in the SO group (Figure 1).

Histopathology and pathological scoring of pancreatic tissues

Compared with the SO group, the pancreatic tissues in the SAP group (Figure 2A-C) at 1 h (A), 4 h (B), and 8 h (C) appeared much more severely damaged. The pancreatic tissue of the SO group (Figure 2D) appeared morphologically normal at 8 h. Microscopic examination of the pancreas in the SAP group showed edema, hemorrhage complicated by microthrombosis, acinar cell degeneration, and inflammation (Figure 2A-C). The mean pathological score of each rat with SAP was higher ($P < 0.01$) than that of control rats at each time point. Pancreatitis worsened over time, as demonstrated by the increasing pathological score ($P < 0.01$, Figure 2E).

Masson staining was used to observe microthrombosis, which was seen as very bright red regions under light microscope. Microthrombi were localized and tightly combined with the microvascular endothelium of the pancreatic tissues, which suggested that the microthrombi formed *in situ* with the formation of fibrin. The percent of Masson staining-positive microvessels in the pancreas of the SAP group was higher ($P < 0.01$) than that in control rats at all time points and tended to increase ($P < 0.01$, Figure 3).

Up-regulation of fgl2 mRNA and protein expression in rats with SAP

fgl2 expression was evaluated with real-time polymerase chain reaction, Western blotting, and immunohistochemical staining. The level of fgl2 mRNA was elevated in both pancreatic tissues and PBMCs ($P < 0.05$) beginning at 1 h after injection of 4% sodium taurocholate compared to the SO group. fgl2 mRNA increased over time in the SAP group ($P < 0.01$, Figure 4A and B). Western blot analysis revealed that fgl2 protein expression in the pancreas was higher ($P < 0.01$) in the SAP group than in the SO group and showed a tendency to increase over time ($P < 0.01$, Figure 4C and D). Immunohistochemical staining demonstrated that fgl2 was strongly expressed and localized in microvascular endothelial cells in pancreatic tissues in rats with SAP (Figure 5A-C). Only low levels of fgl2 expression were found in control rats (Figure 5D). In accordance with fgl2 mRNA level, fgl2 protein level was elevated as indicated by the mean absorbance value ($P < 0.01$) in the SAP group compared to the control rats and tended to increase ($P < 0.01$, Figure 5E). Pearson's correlation coefficient analysis was used to compare fgl2 expression and the proportion of Masson staining-positive microvessels, and the results showed a correlation ($r = 0.842$, $P < 0.01$). This result suggested that elevated fgl2 expression may contribute to microthrombosis.

fgl2 expression is relevant to the severity of pancreatic injury in rats with SAP

fgl2 expression and the severity of pancreatic injury of rats with SAP (as indicated by the pathological score) were higher compared with control rats, indicating a correlation between fgl2 expression and disease severity upon induction of SAP. Moreover, fgl2 expression in the pancreas ($r = 0.852$, $P < 0.01$) and PBMCs ($r = 0.735$, $P < 0.01$) correlated with the severity of pancreatic injury.

DISCUSSION

SAP is an inflammatory disorder mediated by up-regulated expression of proinflammatory cytokines such as TNF- α ^[4,10]. Inflammation and coagulation are interactive events during SAP. Microthrombosis is found in the early stages of the SAP rat model^[1]. The "immune coagulation" hypothesized by Levy means that fgl2 could be transcribed and the mRNA translated following the induction of cytokines such as IL-2 and TNF- α , resulting in immediate activation of coagulation^[17,23,26,27]. fgl2 functions as a bridge molecule between immune and coagulation reactions. fgl2 is highly expressed in endothelial cells due to the action of TNF- α ^[27,28]. Otherwise, interferon- γ is necessary for macrophage induction of fgl2^[27]. In the present study, fgl2 was clearly up-regulated and localized in inflammatory regions of the pancreas sections, suggesting that fgl2 as an effector molecule may contribute to SAP pathogenesis by initiating and promoting coagulation through the induction of proinflammatory cytokines such as TNF- α .

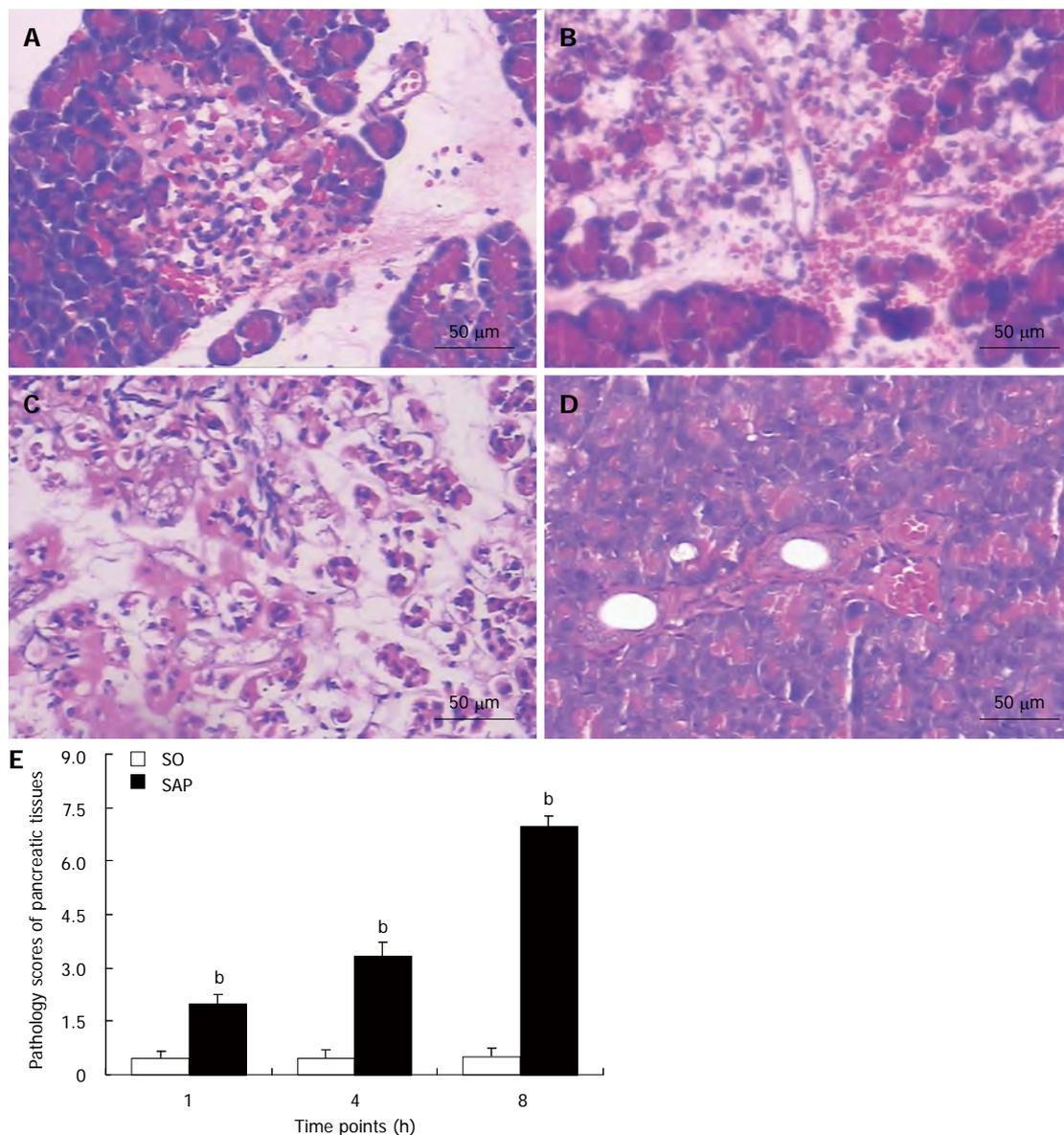


Figure 2 Histology of pancreatic tissues and pathological scores of pancreatic tissues from severe acute pancreatitis and sham-operated rats. A-C: Histological changes in pancreatic tissues at 1 h (A), 4 h (B), and 8 h (C) in the severe acute pancreatitis (SAP) group; D: Histological changes in pancreatic tissues at 8 h in the sham operation (SO) group (hematoxylin and eosin staining; $\times 200$); E: Pathological scores of pancreatic tissues. Each time point (h) after operation consisted of 8 rats. The data are expressed as the mean \pm SD. ^b $P < 0.01$ vs SO group.

fgl2/fibrinogen is a new procoagulant that belongs to the fibrinogen-related protein superfamily, which has a potent capability of inducing microthrombosis^[16,17,29]. fgl2 is expressed in activated macrophages, T cells, and endothelial cells^[30]. fgl2 expression and the subsequent fibrin deposition account for microthrombus formation *in situ*^[18], which occurs *via* a novel way by directly producing thrombin in addition to the classic extrinsic and intrinsic coagulant pathway^[16,17]. Researches suggest that microcirculatory disturbance is an important aspect of the mechanism of SAP^[4,8]. Our data show that microthrombi generated during pancreatitis (due to increased fgl2 expression) led to ischemia/hemorrhage injury and consequently resulted in necrosis and dysfunction of the pancreas. Moreover, we found that fgl2 plays a contributing role in pancreatic microthrombus formation in rats with

SAP. Our study also shows that fgl2 has procoagulant activity in the pancreatic endothelial cells of microvessels in rats with SAP, and fgl2 expression correlates strongly with the severity of pancreatic injury.

We observed that both fgl2 mRNA and protein levels were higher in rats with SAP and that the levels gradually increased in parallel with the progression of SAP. We also observed that fgl2 expression was associated with microthrombus formation and that microthrombus formation *in situ* may be caused by fgl2, leading to partial impairment of the pancreatic tissues and the functions involved. We propose that fgl2 functions similarly as in other diseases^[26,31-33]: microthrombi form as a consequence of fgl2 expression in the pancreas, leading to microcirculatory disturbance and consequent hemorrhage/ischemia injury in rats with SAP, thus aggravating the pancreatic injury.

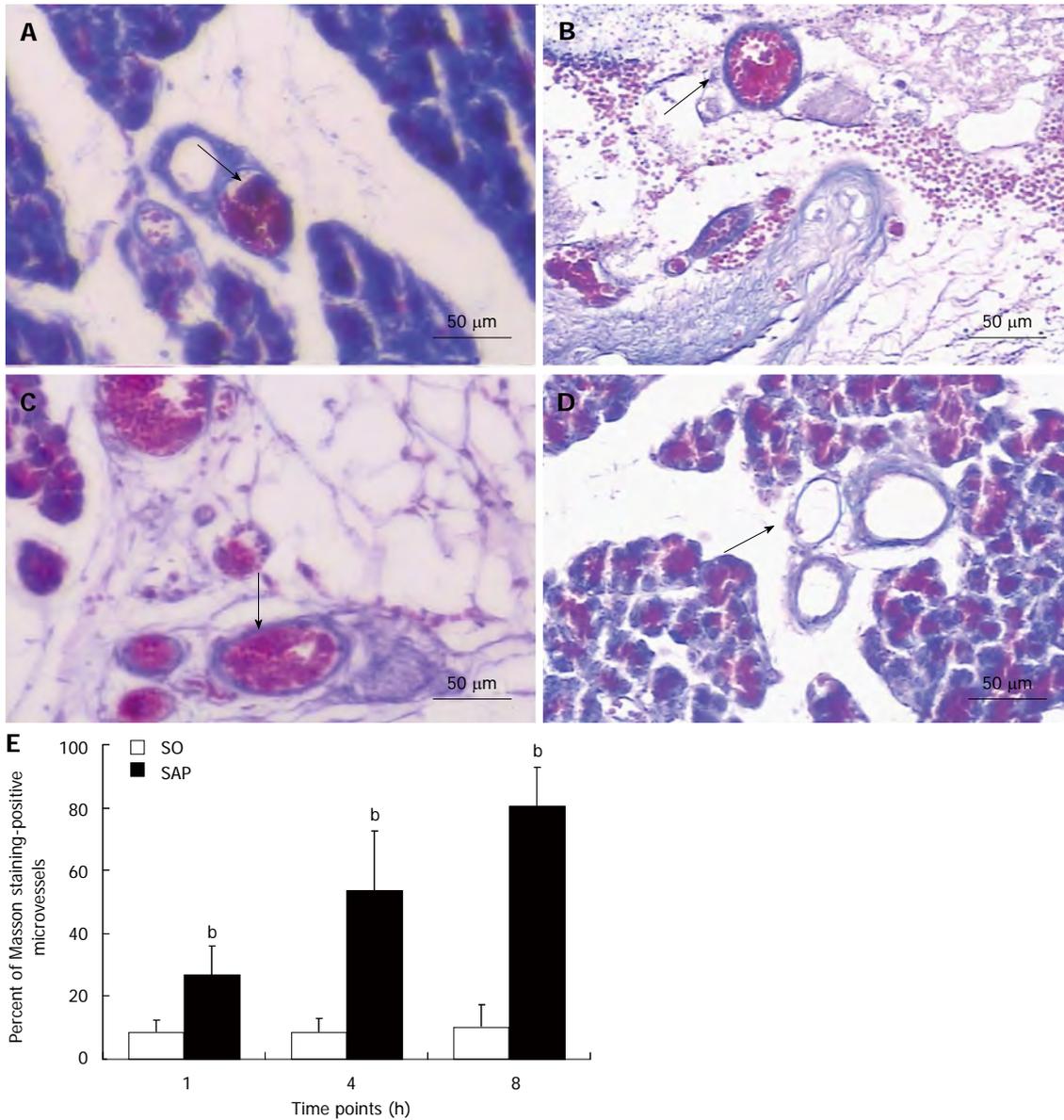
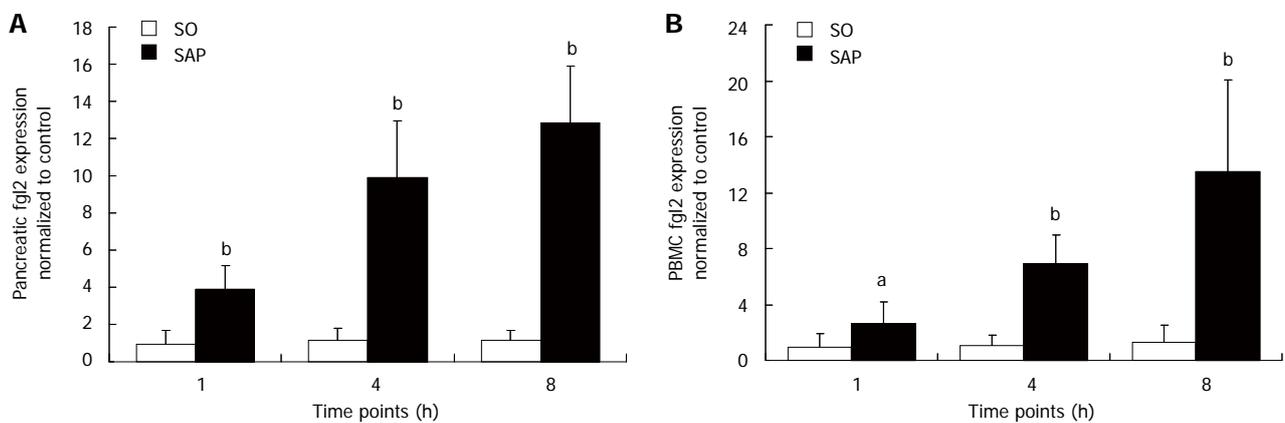


Figure 3 Masson staining of microthrombosis in pancreatic microvessels from severe acute pancreatitis and sham-operated rats ($\times 200$). A-C: Microthrombosis *in situ* in pancreatic microvessels of rats with severe acute pancreatitis (SAP) (arrows) at 1 h (A), 4 h (B), and 8 h (C) in the SAP group; D: No microthrombi were detected in the sham operation (SO) group (arrow); E: The percent of Masson staining-positive microvessels. Each time point (h) after operation consisted of 8 rats. The data are expressed as the mean \pm SD. ^b $P < 0.01$ vs SO group.



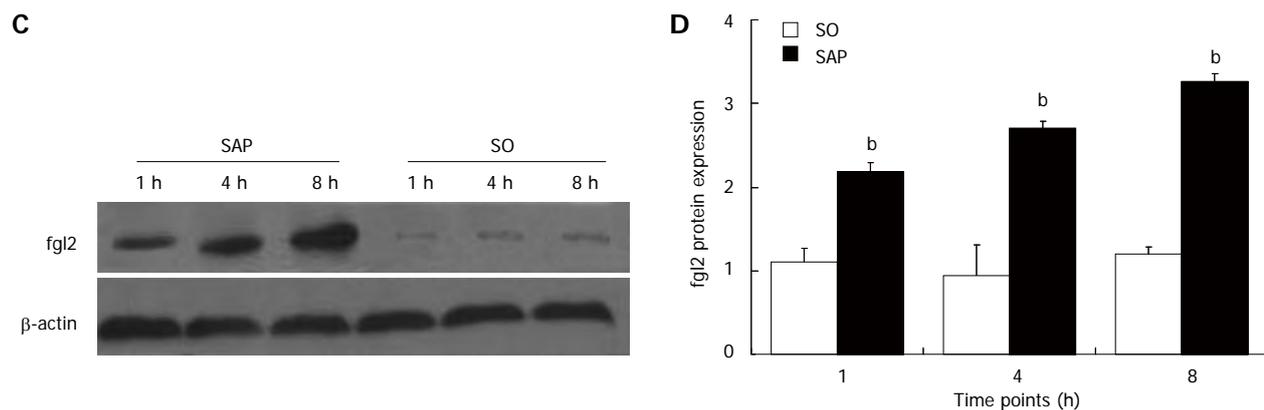


Figure 4 Levels of fibrinogen-like protein 2 mRNA and protein in severe acute pancreatitis and sham-operated rats. A, B: The calculated levels of fibrinogen-like protein 2 (fg12) mRNA in the pancreas and peripheral blood mononuclear cells (PBMCs). The expression of fg12 mRNA is relative to β -actin; C, D: fg12 protein expression revealed by Western blotting. Protein levels were normalized to β -actin. Each time point (h) after operation consisted of 8 rats. The data are expressed as the mean \pm SD. ^a $P < 0.05$, ^b $P < 0.01$ vs sham operation (SO) group. SAP: Severe acute pancreatitis.

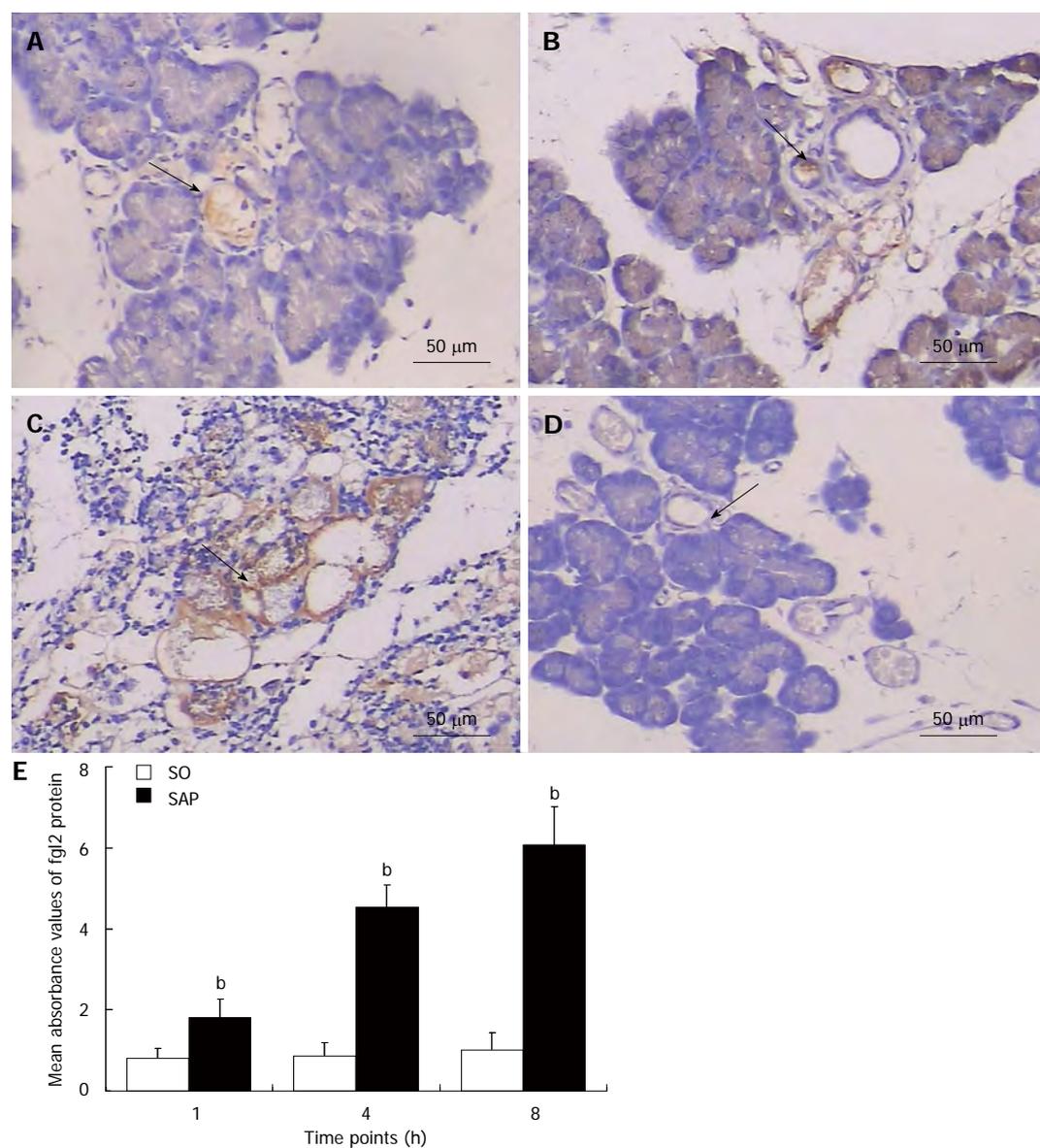


Figure 5 Fibrinogen-like protein 2 expression in pancreatic tissues of severe acute pancreatitis and sham-operated rats with immunohistochemical staining ($\times 200$). A-D: Fibrinogen-like protein 2 (fg12) proteins were expressed in microvascular endothelial cells of rats with severe acute pancreatitis (SAP) (arrows in A-C) and control rats (arrow in D) at 1 h (A), 4 h (B), and 8 h (C, D) after initiation of SAP; E: fg12 protein expression is indicated by the mean absorbance value. The data are expressed as the mean \pm SD. ^b $P < 0.01$ vs sham operation (SO) group.

To evaluate the relevance of fgl2 expression and the severity of pancreatic disease, a Pearson's correlation coefficient was calculated. Evaluation of fgl2 expression levels in both the pancreas and PBMCs (containing lymphocytes, monocytes, dendritic cells, and other cell types) revealed a strong correlation with the severity of pancreatic disease as illustrated by the pathological score. Thus, fgl2 expression may serve as a promising marker for predicting the occurrence of SAP in early stages.

Injection of a neutralizing antibody or genetic therapy against fgl2 in diseases involving fgl2, such as murine hepatitis virus 3-induced hepatitis and graft rejection, has been beneficial in terms of attenuating fibrin deposition and pathology and preventing death in mice^[18,34-36]. Thus, we will perform an in-depth investigation to see whether inhibiting fgl2 activity or applying fgl2 antibodies will delay or ameliorate the disease course of SAP.

In summary, fgl2 functions as a novel prothrombinase and may initiate the coagulation reaction, finally leading to microthrombosis in microvessels of pancreatic tissues in an experimental model of rats with SAP, and resulting in ischemia/hemorrhage injury as well as necrosis and dysfunction of the pancreas. fgl2 expression is closely correlated with the severity of pancreatic disease, and thus fgl2 may serve as a useful biomarker for predicting SAP at the onset of disease. Whether inhibiting fgl2 or using antibodies against fgl2 will delay or ameliorate SAP requires further exploration.

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COMMENTS

Background

Fibrinogen-like protein 2 (fgl2)/fibrinogen (also termed fgl2 prothrombinase) is a recently discovered member of the fibrinogen-related protein superfamily. fgl2 is a direct prothrombinase with serine protease activity and can cleave prothrombin to thrombin via a noncanonical pathway, resulting in fibrin deposition. Several studies have demonstrated that fgl2 leads to pathology by mediating "immune coagulation", fibrin deposition, and microthrombosis in murine hepatitis virus 3-induced fulminant hepatitis, spontaneous abortion, xenograft rejection, and type 2 diabetic nephropathy.

Research frontiers

Intravascular coagulation and thromboembolism play a pivotal role in the pathogenesis of severe acute pancreatitis (SAP) and are related to its severity. Acute inflammatory events during SAP may result in dysregulation of the coagulation cascade. However, whether fgl2 is involved in the pathogenesis of SAP has not been studied. In this study, the authors demonstrate that increased expression of fgl2 contributes to pancreatic impairment in rats with SAP by mediating microthrombosis. Thus, fgl2 level may serve as a useful biomarker at early stages of disease.

Innovations and breakthroughs

The mechanism(s) responsible for the microcirculatory disturbances and coagulation abnormalities during the SAP process remains to be elucidated. This is the first study to report that fgl2 is highly expressed in microvascular endothelial cells of pancreatic tissues in rats with SAP. Furthermore, microthrombosis due to fgl2 contributes to pancreatic impairment in rats with SAP, and fgl2 level may

serve as a useful biomarker at disease onset.

Applications

In the present study, the authors investigated fgl2 expression and localization in the pancreas of rats with SAP, which will provide new insight into the pathogenesis of SAP and efficacious anticoagulant therapy for SAP treatment.

Terminology

SAP is principally caused by autodigestion of the pancreas and is a potentially fatal pathogenic condition characterized by rapid progression and high mortality. fgl2 is a new member of the fibrinogen-related protein superfamily, which includes fibrinogen, tenascin, ficolin, and angiotensin.

Peer review

The authors of this study investigated the pathogenesis of acute pancreatitis. Their results provide insight into the contribution of microthrombosis to the development of pathological changes during the progression of acute pancreatitis. The authors have demonstrated increased expression of fgl2 mRNA in the pancreas and peripheral blood mononuclear cells with a subsequent increase in the levels of the corresponding proteins. The anticipated pathological changes were further substantiated by Masson staining. Stringency of the proposed hypothesis was validated, revealing a strong correlation between induced coagulation disorders and pathological changes in pancreatic tissues. It is an interesting subject, and the results are clearly described.

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How do we manage post-OLT redundant bile duct?

Victor Torres, Nicholas Martinez, Gabriel Lee, Jose Almeda, Glenn Gross, Sandeep Patel, Laura Rosenkranz

Victor Torres, Nicholas Martinez, Gabriel Lee, Glenn Gross, Sandeep Patel, Laura Rosenkranz, Division of Gastroenterology and Nutrition, Department of Medicine, University of Texas Health Science Center at San Antonio, San Antonio, TX 78229, United States

Jose Almeda, Department of Pancreatic and Hepatobiliary Surgery, University of Texas Health Science Center at San Antonio, San Antonio, TX 78229, United States

Author contributions: Torres V, Martinez N and Lee G wrote this paper, performed acquisition and analysis of data; Gross G Patel S and Rosenkranz L designed the research and were the primary endoscopist; Almeda J assisted in the scientific writing of the paper.

Correspondence to: Victor Torres, MD, Division of Gastroenterology and Nutrition, Department of Medicine, University of Texas Health Science Center at San Antonio, 7703 Floyd Curl Dr No. 209, San Antonio, TX 78229, United States. torresv5@uthscsa.edu

Telephone: +1-210-5674611 Fax: +1-210-5671976

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Abstract

AIM: To address endoscopic outcomes of post-Orthotopic liver transplantation (OLT) patients diagnosed with a "redundant bile duct" (RBD).

METHODS: Medical records of patients who underwent OLT at the Liver Transplant Center, University Texas Health Science Center at San Antonio Texas were retrospectively analyzed. Patients with suspected biliary tract complications (BTC) underwent endoscopic retrograde cholangiopancreatography (ERCP). All ERCP were performed by experienced biliary endoscopist. RBD was defined as a looped, sigmoid-shaped bile duct on cholangiogram with associated cholestatic liver biomarkers. Patients with biliary T-tube placement, biliary anastomotic strictures, bile leaks, bile-duct stones-sludge and suspected sphincter of oddi dysfunction were excluded. Therapy included single or multiple

biliary stents with or without sphincterotomy. The incidence of RBD, the number of ERCP corrective sessions, and the type of endoscopic interventions were recorded. Successful response to endoscopic therapy was defined as resolution of RBD with normalization of associated cholestasis. Laboratory data and pertinent radiographic imaging noted included the pre-ERCP period and a follow up period of 6-12 mo after the last ERCP intervention.

RESULTS: One thousand two hundred and eighty-two patient records who received OLT from 1992 through 2011 were reviewed. Two hundred and twenty-four patients underwent ERCP for suspected BTC. RBD was reported in each of the initial cholangiograms. Twenty-one out of 1282 (1.6%) were identified as having RBD. There were 12 men and 9 women, average age of 59.6 years. Primary indication for ERCP was cholestatic pattern of liver associated biomarkers. Nineteen out of 21 patients underwent endoscopic therapy and 2/21 required immediate surgical intervention. In the endoscopically managed group: 65 ERCP procedures were performed with an average of 3.4 per patient and 1.1 stent per session. Fifteen out of 19 (78.9%) patients were successfully managed with biliary stenting. All stents were plastic. Selection of stent size and length were based upon endoscopist preference. Stent size ranged from 7 to 11.5 Fr (average stent size 10 Fr); Stent length ranged from 6 to 15 cm (average length 9 cm). Concurrent biliary sphincterotomy was performed in 10/19 patients. Single ERCP session was sufficient in 6/15 (40.0%) patients, whereas 4/15 (26.7%) patients needed two ERCP sessions and 5/15 (33.3%) patients required more than two (average of 5.4 ERCP procedures). Single biliary stent was sufficient in 5 patients; the remaining patients required an average of 4.9 stents. Four out of 19 (21.1%) patients failed endotherapy (lack of resolution of RBD and recurrent cholestasis in the absence of biliary stent) and required either choledocojejunostomy (2/4) or percutaneous biliary drainage (2/4). Endoscopic complications included: 2/65 (3%) post-ERCP pancreatitis and 2/10 (20%)

non-complicated post-sphincterotomy bleeding. No endoscopic related mortality was found. The medical records of the 15 successful endoscopically managed patients were reviewed for a period of one year after removal of all biliary stents. Eleven patients had continued resolution of cholestatic biomarkers (73%). One patient had recurrent hepatitis C, 2 patients suffered septic shock which was not associated with ERCP and 1 patient was transferred care to an outside provider and records were not available for our review.

CONCLUSION: Although surgical biliary reconstruction techniques have improved, RBD represents a post-OLT complication. This entity is rare however, endoscopic management of RBD represents a reasonable initial approach.

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Key words: Redundant bile duct; Orthotopic liver transplantation; Biliary complications; Biliary stent; Endoscopic retrograde cholangiopancreatography

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INTRODUCTION

Despite the dramatic improvements in surgical techniques, biliary tract complications (BTC) are still a significant source of morbidity and mortality after orthotopic liver transplantation (OLT)^[1,2]. Since the beginning of liver transplantation, the biliary reconstruction has been a sensitive area regarding graft and recipient complications.

Presently, clinical evidence supports the choledochojejunostomy over the T-tube stent placement or Roux-en-Y choledocho-jejunostomy, as the preferred method of biliary reconstruction^[3,4]. It is postulated that several factors (*e.g.*, donor and recipient biliary ductal anatomy, duct-duct anastomosis technique, and blood supply to the bile ducts) can affect the final post-surgical bile duct configuration and may result in its ultimate successful function^[5,6]. Surgical management used to represent the initial standard of care for BTC; however, the advancement in endoscopic therapeutic interventions has replaced prompt surgical intervention in most of the immediate and delayed complications^[7-12].

Endoscopic therapy has been successful in the management of BTC. During the performance of the endoscopic retrograde cholangiopancreatography (ERCP), interventions such as: endoprosthesis (biliary stent) placement with or without concurrent sphincterotomy, balloon dilatation of anastomotic strictures, can be included^[12].

Bile duct stones, bile leaks and anastomotic strictures are among the most common post-transplant complications reported^[13-19]. The reported incidence of such complications among different centers has been variable^[8,12,17]. Our institution has previously reported the endoscopic experience with BTC in the post-OLT patient, however data did not include management of a “redundant bile duct” (RBD) (Figure 1)^[8].

We define the “RBD” a surgically reconstructed donor-recipient extrahepatic bile duct, which due to its length (longer than the native recipient duct), in the absence of anastomotic stricture, creates a looped, sigmoid-shaped (“S”, “Z”) appearance, which leads to delayed bile flow into the duodenum, functionally translating into cholestasis and abnormal pattern of the liver associated tests.

The term was described as an analogy to the “redundant colon”, which describes a large intestine (colon) that is longer than normal and as a result has repetitive, overlapping loops. Typically, the “redundant colon” is a normal anatomic variation.

From our large transplanted data we present our endoscopic experience with the RBD treatment in the post-OLT patient. To our best knowledge, this is the first presentation of successful endoscopic management of the RBD in the post-OLT patient.

MATERIALS AND METHODS

We performed a retrospective analysis of records from the Transplant Clinic, Endoscopy and radiology of patients who underwent OLT at the Liver Transplant Center, University Health Science Center at San Antonio.

One thousand two hundred and eighty-two patient records who received OLT from 1992 through 2011 were reviewed. Patients with biliary T-tube placement, biliary anastomotic strictures, bile leaks, bile-duct stones-sludge and suspected sphincter of oddi dysfunction were excluded.

Patients who underwent ERCP in the post-transplant period, indication and number of procedures per patient were reviewed. Laboratory data and pertinent radiographic imaging noted included the pre-ERCP period and a follow-up period of 6-12 mo after the last ERCP intervention.

RBD was identified as a sigmoid-shaped bile duct on cholangiogram (Figure 1) with associated cholestatic liver biomarkers. Endoscopic intervention included biliary stent placement with or without sphincterotomy. All ERCP were performed by experienced biliary endoscopists.

The incidence of RBD, the number of ERCP corrective sessions, and the type of endoscopic interventions were recorded. Successful response to endoscopic therapy (resolution of RBD) was defined as normalization of cholestatic liver profile up to one year after last endoscopic intervention and resolution of cholangiographic abnormalities (Figure 2).

Table 1 Patient data demographics

| | |
|-----------------------------------|-------|
| Men | 12 |
| Women | 9 |
| Average age (yr) | 59.6 |
| Indication for OLT | |
| Hepatitis C | 15 |
| Cryptogenic | 2 |
| Steatohepatitis | 1 |
| Medication induced failure | 1 |
| Alcoholic cirrhosis | 1 |
| Autoimmune hepatitis | 1 |
| Average time (d) from OLT to ERCP | 88.1 |
| Indication for ERCP | |
| Cholestatic LFT | 21/21 |

LFT: liver function test; OLT: Orthotopic liver transplantation; ERCP: Endoscopic retrograde cholangiopancreatography.

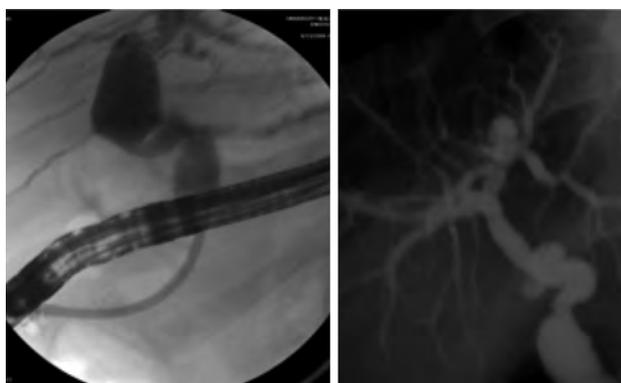


Figure 1 Cholangiogram of a redundant common bile duct.

Statistical analysis

Statistical analyses were performed with the SAS statistical software (version 9.2, SAS Institute Inc. Cary, NC). We used the χ^2 test to test whether categorical variables differed between individuals whose RBD resolved with ERCP and counterparts that failed ERCP intervention. Comparisons between the 2 groups for continuous variables were performed by using the Mann-Whitney *U* test (a nonparametric test). Results are reported as median and range or percentage as appropriate. Significance was assumed for $P < 0.05$ (2 sided).

RESULTS

Two hundred and twenty-four patients underwent ERCP for suspected BTC. RBD was reported in each of the initial cholangiograms by three individual experienced endoscopist (Patel S, Gross G, Rosenkranz L) and reviewed by the authors of the manuscript. Twenty-one out of 1282 (1.6%) of liver transplanted patients were identified as having RBD. Patient demographics are listed in Table 1. There were 12 men and 9 women, average age of 59.6 years. Primary indication for liver transplantation was end stage liver disease secondary to hepatitis C (71.4%). Primary indication for ERCP was cholestatic

Table 2 Interventions and results in 21 patients with redundant bile duct

| Results | Resolved (<i>n</i> = 15) | Failure (<i>n</i> = 6) | <i>P</i> value |
|--|------------------------------|----------------------------|-------------------|
| Men <i>n</i> (%) | 8/15 (53.3) | 4/6 (66.7) | 0.577 |
| Age ¹ , yr | 59.0 (39.0-70.0) | 64.5 (50-75) | 0.094 |
| Hepatitis C indication <i>n</i> (%) | 11/15 (73.3) | 4/6 (66.7) | 0.760 |
| Time from OLT to ERCP ¹ , d | 14 (4-1059) | 225 (8-865) | 0.086 |
| Total ERCP | 3 (2-10) | 3 (1-4) | 0.492 |
| Total biliary stents placed | | | |
| Average stent per patient ¹ | 3 (0-15) | 2 (0-4) | 0.475 |
| Average stent per session ¹ | 1.0 (0-1.5) | 0.9 (0-1) | 0.602 |
| ERCP sessions for resolution | | | - |
| Single session | 6/15 | - | |
| Two sessions | 4/15 | - | |
| > Two sessions | 5/15 | - | |
| Percutaneous biliary drainage | | 2 | - |
| Choldocojejunostomy | | 2 | - |
| T bili ¹ , mg/dL | 5.0 (0.3-37.3) | 6.1 (1.2-34.9) | 0.586 |
| AST ¹ | 122 (34-444) | 190 (40-1131) | 0.392 |
| ALT ¹ | 248 (42-668) | 262 (58-1579) | 0.846 |
| Alk phos ¹ | 460 (109-1066) | 345 (243-936) | 0.907 |

¹Median (range). OLT: Orthotopic liver transplantation; ERCP: Endoscopic retrograde cholangiopancreatography; AST: Aspartate aminotransferase; ALT: Alanine aminotransferase.

pattern of liver associated biochemical markers. Nineteen out of 21 patients underwent endoscopic therapy and 2/21 required immediate surgical intervention, for failure to stenting the bile duct. In the endoscopically managed group: 65 ERCP procedures were performed with an average of 3.4 per patient and 1.1 stent per session. Fifteen out of 19 (78.9%) patients were successfully managed with biliary stenting. Interventions and results are listed in Table 2. All stents were plastic. Selection of stent size and length were based upon endoscopist preference. Stent size ranged from 7 to 11.5 Fr (average stent size 10 Fr); Stent length ranged from 6 to 15 cm (average length 9 cm). Each stent remained in place for an average of 93 d. Concurrent biliary sphincterotomy was performed in 10/19 patients. Single ERCP session was sufficient in 6/15 (40.0%) patients, whereas 4/15 (26.7%) patients needed two ERCP sessions and 5/15 (33.3%) patients required more than two (average of 5.4 ERCP procedures). Single biliary stent was sufficient in 5 patients; the remaining patients required an average of 4.9 stents. Figure 3 represents a cholangiogram with multiple stents placed in a redundant bile duct. Four out of 19 (21.1%) patients failed endotherapy (lack of resolution of RBD and recurrent cholestasis in the absence of biliary stent) and required either choledocojejunostomy (2/4) or percutaneous biliary drainage (2/4). The medical records of the 15 successful endoscopically managed patients were reviewed for a period of one year after removal of all biliary stents. Eleven patients had continued resolution of cholestatic biomarkers (73%). One patient had recurrent hepatitis C, 2 patients suffered septic shock which was not associated with ERCP and 1 patient was transferred care to an outside provider and records

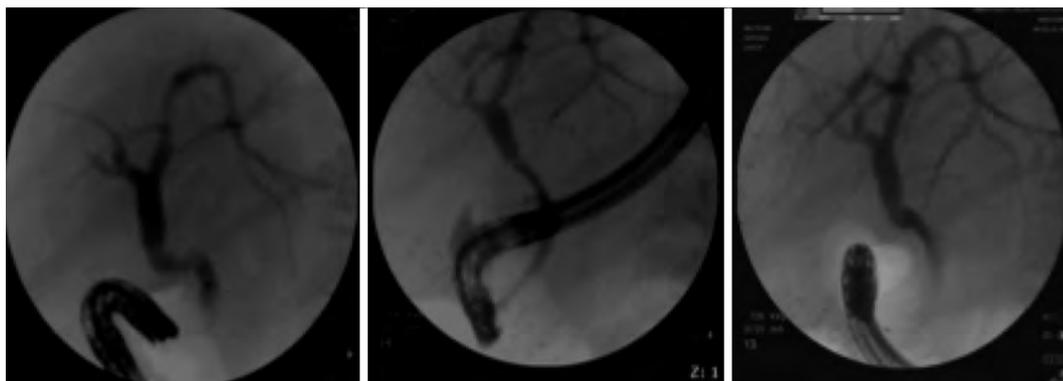


Figure 2 Single patient series of successful endoscopic management. Cholangiogram with redundant anastomosis. Placement of a 10 Fr by 9 cm plastic stent. Cholangiogram after stent removal with improvement in redundancy and normalization of cholestatic liver profile.

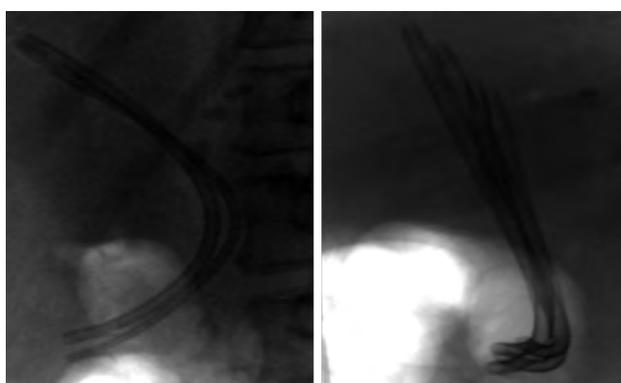


Figure 3 Multiple stent placement in a redundant bile duct.

were not available for our review. Endoscopic complications (ERCP-related) recorded included: 2/65 (3%) post-ERCP pancreatitis and 2/10 (20%) non-complicated post-sphincterotomy bleeding. No endoscopic related mortality was found.

DISCUSSION

Since their initial description, BTC remain a significant source of morbidity and mortality after OLT. Complication rates have been reported as high as 20% in some series^[18]. During organ procurement, the surgeon attempts to minimize any disruption of the donor bile duct blood supply using a variety of techniques^[20-24]. During transplantation, surgeons approximate the donor liver and bile duct to the native bile duct stump with caution. A laparotomy pad is placed above the liver, in order to maintain proper positioning during anastomosis and once completed, the pad is removed and the liver allowed to retract cephalad into its natural position. The bile duct is anastomosed with a gentle tension in order to reduce the risk of ischemia and bile leaks. Additionally, torsion of the liver during the transplant may lead to tension and leaks. It should be known that the surgeons do not make special attempts to avoid redundancy. Clearly overt discrepancies are addressed, but this aspect of the

operation is quick and concise.

These techniques are performed to preserve blood supply and may theoretically lead to less ischemic bile duct complications. The successful endoscopic management of biliary leaks, bile duct strictures and sphincter dysfunction has previously been reported however, to our best knowledge, this is the first report of successful endoscopic management of a RBD in the post-OLT patient. Although post-OLT RBD represents an uncommon complication with an incidence of 1.6%, endoscopic management appears to be a reasonable initial approach as 78.9% of patients with a RBD post-OLT can be successfully managed with a combination of biliary stenting and sphincterotomy. Endoprosthesis selection is based on the endoscopist preference and comprises plastic biliary stents of variable width (7-11.5 Fr) and length, therefore it is difficult to comment in a non-randomized retrospective study if stent size or length impacted the overall outcome. The exact mechanism of resolution remains unclear, however, we suspect that stent placement alters the configuration of duct anatomy thereby leading to a resolution of the redundant duct. Hepatobiliary biopsies pre and post stent placement would aid in the further evaluation of the histochemical changes associated with this entity^[25,26]. However this was not the main endpoint but does represent an avenue of further research. One year follow up of bilirubin and liver associated enzymes also suggest that endoscopic treatment is a viable option as 73% had continued resolution of cholestatic of liver profile.

COMMENTS

Background

Since their initial description, biliary tract complications (BTC) remain a significant source of morbidity and mortality after orthotopic liver transplantation (OLT). Despite improvement in surgical techniques, the biliary reconstruction remains a sensitive area regarding graft and recipient complications. Endoscopic therapies have been effective in the management of BTC. Authors present their experience with "redundant bile duct" (RBD) in the post-OLT setting.

Research frontiers

Management of BTC in the post-OLT setting has previously been reported; however, endotherapy and outcomes in the management of the RBD has not

been described until present. The surgical management of the RBD has been published. Authors' group is the first to propose endoscopic management via a combination of biliary stenting and sphincterotomy as an initial approach to the RBD.

Innovations and breakthroughs

This is the first to demonstrate that a RBD can be successfully managed with a combination of biliary stenting and sphincterotomy with a 78.9% success rate at our institution. One year follow up data also suggests that endoscopic management confers a sustained response.

Applications

Although post-OLT RBD an uncommon complication, endoscopic management appears to be a reasonable initial approach.

Terminology

BTC include: leaks, strictures, retained stones and sphincter of oddi dysfunction. RBD is a surgically reconstructed donor-recipient extrahepatic bile duct which creates a looped, sigmoid-shaped ("S", "Z") appearance thereby resulting in delayed bile flow into the duodenum, OLT, Endoscopic Retrograde Cholangiopancreatography.

Peer review

This manuscript reports on an unusual problem which they have termed the RBD. They reference their own prior study which suggests that such an entity may not be widely known or even accepted. Given that this could represent a real entity, publication may be appropriate.

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Human leukocyte antigen *DQ2/8* prevalence in non-celiac patients with gastrointestinal diseases

Daniel DiGiacomo, Antonella Santonicola, Fabiana Zingone, Edoardo Troncone, Maria Cristina Caria, Patrizia Borgheresi, Gianpaolo Parrilli, Carolina Ciacci

Daniel DiGiacomo, Antonella Santonicola, Fabiana Zingone, Edoardo Troncone, Gastrointestinal Unit, University Federico II Naples, 80131 Naples, Italy

Daniel DiGiacomo, Department of Medicine, Celiac Disease Center, Columbia University, New York, NY 10032, United States
Maria Cristina Caria, Celiac Center, Loreto Crispi Hospital, 80131 Naples, Italy

Patrizia Borgheresi, Gianpaolo Parrilli, Carolina Ciacci, Celiac Center, Gastrointestinal Unit, San Giovanni di Dio e Ruggi d'Aragona Hospital, University of Salerno, 84081 Salerno, Italy
Carolina Ciacci, Department of Medicine and Surgery, Campus di Baronissi, University of Salerno Medical School, 84084 Baronissi, Italy

Author contributions: DiGiacomo D designed the study, ran the statistical analyses and wrote the manuscript; Santonicola A, Zingone F, Troncone E, Caria MC, Borgheresi P and Parrilli G selected the patients in the outpatient clinics involved in the study and provided the collection of all human materials; and Ciacci C designed the study, provided financial support for the study and edited the manuscript.

Correspondence to: Carolina Ciacci, Professor, Department of Medicine and Surgery, Campus di Baronissi, University of Salerno Medical School, Via Salvador Allende 34, 84084 Baronissi, Italy. cciacci@unisa.it

Telephone: +39-89-965032 Fax: +39-89-672452

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[pre-post transplant liver disease, esophageal/gastric organic and functional diseases, irritable bowel syndrome (IBS) and inflammatory bowel disease (IBD)] and *DQ2/8* alleles, which correspond to a celiac disease genetic risk gradient. Subject allele frequencies were compared to healthy Italian controls.

RESULTS: One hundred and ninety-six out of four hundred and forty-three (44.2%) subjects, median age 56 years and 42.6% female, were *DQ2/8* positive. When stratifying by disease we found that 86/188 (45.7%) patients with liver disease were HLA *DQ2/8* positive, 39/73 (53.4%) with functional upper GI diseases and 19/41 (46.3%) with organic upper GI diseases were positive. Furthermore, 38/105 (36.2%) patients with IBS and 14/36 (38.9%) with IBD were HLA *DQ2/8* positive ($P = 0.21$). Compared to healthy controls those with functional upper GI diseases disorders had a 1.8 times higher odds of *DQ2/8* positivity. Those with liver disease had 1.3 times the odds, albeit not statistically significant, of *DQ2/8* positivity. Both those with IBS and IBD had a lower odds of *DQ2/8* positivity compared to healthy controls.

CONCLUSION: The proportion of individuals HLA *DQ2/8* positive is higher in those with liver/upper functional GI disease and lower in IBS/IBD as compared to general population estimates.

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Key words: Human leukocyte antigen *DQ2/8*; Gastrointestinal and liver disease; Celiac disease

Abstract

AIM: To investigate the prevalence of human leukocyte antigen (HLA) *DQ2/8* alleles in Southern Italians with liver and gastrointestinal (GI) diseases outside of celiac disease.

METHODS: HLA *DQ2/8* status was assessed in 443 patients from three ambulatory gastroenterology clinics in Southern Italy (University of Federico II, Naples, Loreto Crispi Hospital, Ruggi D'Aragona Hospital, Salerno). Patients were grouped based on disease status

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INTRODUCTION

The human leukocyte antigen (HLA) class II genes comprise a highly polymorphic region in the short arm of chromosome 6 and are responsible for the creation of molecules involved in exogenous antigen presentation to T cells^[1,2]. A subset of class II genes, encoding the *DQ2* and *DQ8* serotypes, have been frequently implicated in autoimmune disease pathogenesis. Prevalent in 30%-40% of healthy individuals, *DQ2* and *DQ8* are associated with diseases such as insulin-dependent diabetes mellitus and Hashimoto's Thyroiditis^[3,4]. These haplotypes may be best characterized through the gluten dependent relationship with celiac disease, an autoimmune mediated enteropathy affecting approximately 1% of Europeans and North Americans^[5-7]. Consequently, many studies have attempted to estimate or infer the proportion of celiac disease risk due to particular *DQ2/8* isoforms. For this reason, a genetic risk gradient has been recently characterized for *DQ2/8* allele variants^[8]. The risk associated with celiac disease compared to those healthy depends, incrementally, on the number/type of HLA alleles possessed by an individual. Those with one or both of the *DQ2/8* alleles have a risk ranging from 1:7-1:35, while those lacking all potential immunogenic loci have a near zero chance of contracting celiac disease^[8,9]. Beyond celiac disease risk, disease severity and anti-tTg antibody levels are thought to be further tied to this disease/genese-dose relationship^[10].

There are several reasons why it may be prudent to study *DQ2/8* alleles in liver/gastrointestinal (GI) disease outside of celiac disease. First, evidence suggests that celiac disease may modify the risk of developing irritable bowel syndrome (IBS), inflammatory bowel disease (IBD), eosinophilic esophagitis, or certain liver diseases^[11-14]. Recent research has also shown the presence of HLA *DQ2/8* alleles by themselves, outside of celiac disease, to be associated with GI disease^[15-17]. This suggests that *DQ2/8* haplotypes may act as a common factor in liver/GI disease pathogenesis; possibly through a similar mechanism to that of celiac disease. Furthermore, as *DQ2/8* haplotypes contain myriad genes involved in inflammatory processes, such as tumor necrosis factor- α , causal mechanisms between these genes and GI disease may exist^[18]. Comparisons of *DQ2/8* prevalence in non-celiac GI diseases, however have not been directly studied.

DQ2/8 associated disease risk is known to be modified across individuals or populations varying in ethnic background, geography or gender^[19-23]. Moreover, *DQ2/8* prevalence in Southern Italians has not been characterized. Thus, in this study we sought to first define the prevalence of HLA *DQ2/8* alleles in a Southern Italy non-celiac GI tertiary ambulatory clinic population. Subsequently, we desired to determine what HLA *DQ2/8* haplotypes, if any, were associated with specific liver/GI diseases.

MATERIALS AND METHODS

Subject population

Patients ($n = 463$) from the gastroenterology ambulatory clinics of three hospitals were recruited over a period of three months. Three hundred and twenty-two subjects were recruited from University of Federico II, Naples, Italy, 85 from Loreto Crispi Hospital, Naples, Italy and 56 from Ruggi D'Aragona Hospital, Salerno, Italy. During consultation patient's demographics and disease history were recorded. Disease status was classified according to the nature of presenting problem. Separate categories were attributed generally to pre- or post-liver transplant treatment for chronic viral hepatitis, upper functional and organic GI (gastritis, esophagitis) diseases, lower functional (IBS) and lower inflammatory GI (IBD) diseases. Those with IBS were diagnosed *via* Rome III criteria. Overall population characteristics are described in Table 1. Participants were excluded from this study if they had missing data on disease status, multiple upper GI diseases or a prior diagnosis of celiac disease ($n = 20$).

Informed consent was obtained from each patient or patient guardian prior to study enrollment. The study was approved by ethics review board of the University of Naples "Federico II" and complied with the Helsinki II declaration.

Sample collection and analysis

Peripheral blood was collected in ethylene-diamino-tetra-acetic tubes and stored at 4 °C. Genomic DNA was isolated and polymerase chain reaction with sequence-specific primer was then performed to test solely for the presence/absence of *DQ2/8* genes (Celiac Gene Screen, BioDiagene, Palermo, Italy). If patients were "susceptible to celiac disease", further analysis was performed to discern specific alleles known to be associated with celiac disease risk (Celiac Gene Alleles, BioDiagene, Palermo, Italy). Fluorescence detection of *DQ2/8* was performed using BioRun Reader (Celiac Gene Alleles, BioDiagene, Palermo, Italy). Patients "susceptible to celiac disease" are generally understood to have at least one of the HLA *DQ2/8* alpha or beta alleles.

Using fluoro-immuno-assay, with human recombinant tTg as an antigen, patients positive for HLA *DQ2/DQ8* alleles were tested for anti-tTg antibodies (a-tTg) and adequate immunoglobulin A (IgA) levels (CeliKey IgA, EliA, Phadia Freiburg, Germany). Anti-tissue transglutaminase levels greater than 10 (EliA U/mL) were considered positive. Those values between 7 and 10 (EliA U/mL) were considered equivocal and those less than 7 (EliA U/mL) negative. In later analysis both positive and equivocal groups were combined to increase power. None of the patients tested for total IgA were found to be deficient. Due to laboratory error several ($n = 46$) patients' a-tTg levels were unattainable.

Healthy controls

In order to compare the distribution of *DQ2/8* alleles

Table 1 General demographic attributes of study population

| | Overall | Liver | Upper functional | Upper organic | IBS | IBD |
|--------------|---------|-------|------------------|---------------|-------|-------|
| Total number | 443 | 188 | 73 | 41 | 105 | 36 |
| Gender | | | | | | |
| Male | 44.9% | 62.8% | 27.4% | 34.2% | 27.6% | 50% |
| Female | 55.1% | 37.2% | 72.6% | 65.8% | 72.4% | 50% |
| Age (yr) | | | | | | |
| Median | 56 | 61 | 50 | 57 | 43 | 32 |
| Range | 72 | 66 | 71 | 66 | 62 | 60 |
| Quartiles | | | | | | |
| 14-41 | - | 4.81% | 30.1% | 24.4% | 45.6% | 55.6% |
| 42-56 | - | 23.5% | 28.8% | 24.4% | 28.2% | 19.4% |
| 57-65 | - | 38.5% | 21.9% | 12.2% | 10.7% | 8.3% |
| 66-86 | - | 33.2% | 19.2% | 39% | 15.5% | 16.7% |

IBS: Irritable bowel syndrome; IBD: Inflammatory bowel disease.

in study participants to the general Italian population we incorporated data from a prior published study by Megiorni *et al*^[21]. Healthy participants consisted of 292 healthy and 259 family based controls from Rome, Italy. The prevalence of *DQ2.5/8* in healthy controls was 29%. This increased to 39% after incorporating the less common *DQ2* isoforms.

HLA classification

DQ2 and *DQ8* serotypes, if indicated, were tested for the following alleles: *DQA1*0201*, *DQA1*03*, *DQA1*05*, *DQB1*02*, *DQB1*0301/0304* and *DQB1*0302/0305*. The following *DR* alleles were typed in order to determine the presence of *DQ/DR* haplotypes: *DRB1*03*, *DRB1*04*, *DRB1*07*, *DRB1*11*, *DRB1*12*.

DQ2/8 haplotypes were classified by Megiorni *et al*^[8]. *DQ2* positivity was defined as *DQA1*05* in cohort with *DQB2*02* (*DQ2.5*), or *DQA1*0201* (*DQ2.2*)/*DQA1*03* (*DQ2.3*) with *B1*02*. *DQ8* positivity was defined as *DQA1*03* with *DQB1*0302*.

Statistical analysis

The Pearson χ^2 test was performed on categorical data regarding demographics and the overall relationship of prevalence data. Fisher's exact test was used for analysis of data with cell counts $n < 5$. Basic tabular analysis was also performed to obtain odds ratios. A cut-off of $P = 0.05$ was considered significant; all intervals were reported at 95% confidence. The analysis was performed using SAS 9.2 and SPSS 19.

RESULTS

We performed a cross-sectional analysis of HLA *DQ2/8* allele prevalence in a Southern Italian population of patients afflicted with either liver or other digestive diseases outside of celiac disease. *DQ2/8* haplotypes were stratified using a prior defined risk gradient relevant to celiac disease and prevalence in our disease population was compared to estimates in healthy controls.

Table 2 Prevalence of human leukocyte antigen *DQ2/8* by age, gender and gastrointestinal disease

| | Proportion of positive subjects | Prevalence | P value |
|---------------------|---------------------------------|------------|---------|
| Overall | 196/443 | 44.2% | 0.02 |
| Gender ¹ | | | |
| Male | 92/199 | 46.2% | |
| Female | 104/244 | 42.6% | 0.45 |
| Age (yr) | | | |
| 14-41 | 48/108 | 44.4% | |
| 42-56 | 42/111 | 37.8% | |
| 57-65 | 53/107 | 49.5% | |
| 66-85 | 52/114 | 45.6% | 0.37 |
| Disease groups | | | |
| Liver | 86/188 | 45.7% | |
| Upper functional | 39/73 | 53.4% | |
| Upper organic | 19/41 | 46.3% | |
| IBS | 38/105 | 36.2% | |
| IBD | 14/36 | 38.9% | 0.21 |

¹ $P > 0.05$ for differences between genders in each disease group except for those with upper functional disorders. In these patients significantly more males were positive than females ($P = 0.05$). IBS: Irritable bowel syndrome; IBD: Inflammatory bowel disease.

Prevalence of HLA alleles in study population

From the patients included in our analysis, 196/443 (44.2%; 95%CI: 39.6%-48.9%) were considered to be HLA *DQ2/8* positive, regardless of disease status. Within those who were positive 144/197 (73.1%) had *DQ2.5* and/or *DQ8*. Table 2 details the prevalence of HLA *DQ2/8* by age, gender and GI disease in the study's participants. The overall difference in *DQ2/8* prevalence between these disease groups was not statistically significant ($P = 0.21$).

Comparison of *DQ2/8* prevalence in study population to healthy controls

Subjects *DQ2/8* alleles were organized highest to lowest genetic risk of celiac disease, as described in Megiorni *et al*^[8] and compared to healthy controls. No statistically significant difference in HLA *DQ2/8* prevalence between our subject population and healthy controls was found ($P = 0.16$). As with healthy controls, study subjects clustered towards lower celiac disease risk *DQ2/8* alleles with *DQ2.5* heterozygotes lying in the majority. Odds ratio calculations revealed that those with functional gastric/esophageal disorders had a 1.8 fold higher odds of being HLA *DQ2/8* positive as compared to healthy controls. Patients with organic gastric/esophageal disorders had 1.3 higher odds of *DQ2/8* positivity as compared to healthy controls. The odds were also increased in the liver disease group and decreased in IBS/IBD groups although these values were not significant (Table 3).

α -tTg

Out of the patients with α -tTg data available no α -tTg positive patients were found in the liver disease/transplant and inflammatory bowel group. One out of sixty-

Table 3 Magnitude of associations between human leukocyte antigen positivity and specific gastrointestinal disease

| Disease group | Odds ratio | 95%CI |
|------------------|------------|----------|
| Overall | 1.2 | 0.96-1.6 |
| Liver | 1.3 | 0.94-1.8 |
| Upper functional | 1.8 | 1.1-2.9 |
| Upper organic | 1.3 | 0.71-2.7 |
| IBS | 0.89 | 0.57-1.4 |
| IBD | 0.99 | 0.49-1.9 |

IBS: Irritable bowel syndrome; IBD: Inflammatory bowel disease.

Table 4 Positive anti-tTg subject stratified by disease group

| Disease group | n | Prevalence |
|------------------|-------|------------|
| Liver | 0/173 | 0% |
| Upper functional | 1/64 | 1.54% |
| Upper organic | 0/35 | 0% |
| IBS | 4/90 | 4.26% |
| IBD | 0/36 | 0% |

$P = 0.04$, for any difference in prevalence of anti-tTg positive between disease groupings. IBS: Irritable bowel syndrome; IBD: Inflammatory bowel disease.

four (1.54%) subjects with functional gastric/esophageal issues and 4/90 (4.26%) with lower functional syndrome were found to be positive ($P = 0.04$; Table 4).

DISCUSSION

The clinical importance of HLA genetic testing has been established in several diseases^[3,24]. In this study we performed a cross-sectional analysis on a Southern Italian population with the goal of investigating the prevalence of several HLA *DQ2/8* serotypes in those with GI issues outside of celiac disease.

HLA *DQ2/8* prevalence in Italy is thought to be between 30% and 40%, although this estimate may vary by geographic subpopulation^[8,24]. Nearly half of the subjects in this study (44%) were considered HLA *DQ2/8* positive. A lesser proportion of those with IBS/IBD were HLA *DQ2/8* positive although these differences were not significant ($P = 0.21$) (Table 2). Within those who were HLA *DQ2/8* positive the majority possessed low risk celiac disease alleles (Table 5). Thus, our results suggest that *DQ2/8* haplotypes may play a role in liver/digestive disease through pathological mechanisms different from those of celiac disease.

Several studies have established significant associations between *DQ2*, primary sclerosing cholangitis and hepatitis C virus recurrence after transplant^[25,26]. The large proportion (46%) of *DQ2/8* positive viral hepatitis patients in our study population agrees with the hypothesis that these haplotypes may be involved in certain liver disease pathogenesis.

Differentiating between functional and organic GI disease can be difficult yet is important due to the im-

Table 5 Prevalence of specific human leukocyte antigen *DQ2/8* alleles between gastrointestinal and control populations n (%)

| Overall | Gastrointestinal | Controls | Risk |
|------------------------------------|------------------|------------|-------|
| <i>DQ2</i> and <i>DQ8</i> | 2 (0.45) | 1 (0.2) | 1:45 |
| <i>DQ2</i> , $\beta1^*02/\alpha02$ | 17 (3.8) | 13 (2.4) | 1:63 |
| <i>DQ8</i> , $\beta1^*02/\alpha02$ | 8 (1.8) | 4 (0.7) | 1:39 |
| $\beta2$, $\beta1^*02/\alpha02$ | 11 (2.5) | 2 (0.4) | 1:16 |
| <i>DQ2</i> , $\beta1^*02/X$ | 85 (19.2) | 106 (19.2) | 1:100 |
| <i>DQ8</i> , $\beta1^*02$ negative | 32 (7.2) | 36 (6.5) | 1:90 |
| $\beta2$, $\beta1^*02/X$ | 41 (9.3) | 53 (9.7) | 1:104 |
| $\alpha5$ + other | 247 (55.8) | 336 (60.9) | 1:101 |
| Total | 443 | 551 | |

Omnibus *DQ2/8* positive vs *DQ2/8* negative χ^2 ; $P = 0.16$. IBS: Irritable bowel syndrome; IBD: Inflammatory bowel disease.

pacts on clinical decision-making. In this study we stratified our patient population based on upper and lower functional or organic GI disorders. Several studies have directly compared *DQ2/8* haplotype prevalence in upper organic GI disease. Lucendo *et al*^[14] previously demonstrated a null association between *DQ2/8* and eosinophilic esophagitis. Interestingly, *DR3* and *DR4* have been significantly linked with atrophic gastritis in a similar Italian population^[27]. The positive yet non-significant relationship between our organic gastric/esophageal patients and *DQ2/8* may be a consequence of the various upper GI organic diseases captured in our patient population. Unfortunately, due to sample size restrictions, we were not able to stratify by specific disease. Ultimately these findings suggest that it may be inappropriate to generalize upper organic GI disease as one confluent group because it is unknown whether the lack of a significant association was truly due to causal or confounding disease factors.

Functional GI disorders represent the majority of GI cases yet many have etiologies, which are poorly understood. Whether it is genetic abnormalities, psychological factors or other environmental variables, functional disorders can represent complex, difficult to solve cases^[28]. The strongest evidence of an association with *DQ2/8* in this study was found in patients with functional upper GI disorders. Patients in this study had 1.8 higher odds of *DQ2/8* positivity if they had an upper functional GI disorder as compared to healthy controls. This may signify that the risk of functional upper GI onset or recurrence is modified by the presence of particular *DQ2/8* haplotypes. Currently, the only published data, which could be used to comment on these findings, relates to celiac disease and *DQ2/8*. For example, Ford *et al*^[29] conducted a meta-analysis, which found no association between celiac disease and functional dyspepsia. Overall, it is too early to decide whether *DQ2/8* could be used to differentiate functional vs organic disease or at least be incorporated into a clinical algorithm that dictates likelihood of disease.

Known immunological associations between IBD and *DR7*, which is linked to both *DQ2* and *DQ8* hap-

lotypes have been established^[17,30]. Prior prevalence data though suggests that IBD, particularly Crohn's disease, is lower in individuals with the *DQ2/8* linked celiac disease^[12]. The relative modest prevalence of IBD (39%) in our study supports this notion. IBS has also been linked to HLA *DQ2/8* haplotypes and bowel transit speeds^[16,31]. Additionally several studies have demonstrated that those with IBS and *DQ2/8* positivity tend to present with symptoms indicative of gluten sensitivity and are responsive to a gluten-free diet^[31,32]. Out of all of the disease groupings those with IBS had the lowest prevalence of *DQ2/8* positivity (36%). As those on a gluten-free diet were excluded from this study this may account for the low prevalence *DQ2/8* positivity in those with IBS.

A small part of this study wished to obtain a baseline level of a-tTg positive patients in a previously diagnosed GI population (Table 4). If these patients were assumed to have celiac disease, these results correspond with known prevalence estimates of the disease^[5].

Those who had prior diagnosed celiac disease or were adhering to a gluten-free diet were excluded from the study. It is thought to be common for patients with general abdominal pain, diarrhea and/or nausea to experiment with a gluten-free diet in an attempt to ameliorate symptoms^[33]. As such, removing individuals who may have experimented with this type of diet eliminated a potentially large source of bias. An additional strength of this study was the precision with which HLA haplotypes and disease types were measured. Barring laboratory error, the HLA typing assays in this study have been shown to have near perfect sensitivity and specificity^[34]. During blood collection patient's disease status was recorded. Thus, it was also unlikely that the classification of disease was subject to recall bias.

This study aimed to generally define HLA *DQ2/8* prevalence in diseases/disorders that may be linked to celiac disease. As such there were several limitations, which could have potentially biased the results. The cross-sectional nature limited the collection of subject's lifestyle and disease history. Therefore, data such as age of disease onset and severity were unavailable. The Southern Italian population is typically generically and environmentally homogenous thus unmeasured confounders would not significantly influence the results. Controls from the study were described as "healthy". We know from Mejiorni *et al*^[8] that these participants did not have celiac disease but it is possible they were afflicted with a liver or GI disease. This type of bias would have most likely pushed the magnitude of our estimates towards the null, masking potential associations. The small sample size in our study also limited our ability to make statistically significant conclusions and investigate specific *DQ2/8* allele associations.

Due to the limitations of the present study it may be difficult to make truly suggestive conclusions regarding the relationship between HLA *DQ2/8* positive patients and liver/GI conditions. This study though has taken the first step through implying a potential association between specific HLA *DQ2/8* alleles and GI disease patho-

genesis. Reproducibility of these results may eventually lead to the creation of clinical markers of elusive disease onset, such as in IBS or other clinically ambiguous disorders^[11,16,28,35,36]. Future studies should involve expanding the number of study participants in order to look at specific *DQ2* alleles or investigating the shared influence of non-HLA celiac disease risk alleles.

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COMMENTS

Background

Human leukocyte antigen (HLA) *DQ2/8* alleles and associated haplotypes are important players in the pathogenesis of several autoimmune diseases, particularly celiac disease. The distribution of *DQ2/8* alleles in gastrointestinal (GI) disease outside of celiac disease though has been poorly established.

Research frontiers

HLA *DQ2/8* alleles in patients can be easily tested with currently laboratory capabilities. Knowledge of HLA *DQ2/8* frequencies related to autoimmune disease is being used to identify high risk populations and drive clinical recommendations regarding *DQ2/8* gene testing.

Innovations and breakthroughs

Similar studies have described HLA *DQ2/8* risk alleles in diseases such as type 1 diabetes or celiac disease. For example, in celiac disease those with one or more of the *DQ2.5/8* alleles are at highest risk of disease onset. To date no studies have directly compared *DQ2/8* prevalence in GI disease outside of celiac disease. By comparing the prevalence of HLA *DQ2/8* to that of healthy controls we demonstrated that, like celiac disease, those with liver disease and esophageal/gastric disorders (both organic and functional) are more likely to be *DQ2/8* positive.

Applications

Although this study is preliminary in nature, the results suggest that the development of novel clinical susceptibility markers of GI disease may exist. Particular *DQ2/8* polymorphisms may point to increased risk of certain liver or esophageal/gastric disease.

Peer review

The study investigated the prevalence of HLA *DQ2/8* alleles in Southern Italians with liver and GI diseases outside of celiac disease. The proportion of individuals HLA *DQ2/8* positive resulted higher in those with liver/gastric or esophageal GI disease and lower in irritable bowel syndrome/inflammatory bowel disease as compared to general population estimates. The study is well designed and conducted.

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Gastroesophageal reflux disease after diagnostic endoscopy in the clinical setting

Nora B Zschau, Jane M Andrews, Richard H Holloway, Mark N Schoeman, Kylie Lange, William CE Tam, Gerald J Holtmann

Nora B Zschau, Jane M Andrews, Richard H Holloway, Mark N Schoeman, William CE Tam, Gerald J Holtmann, Department of Gastroenterology and Hepatology, Royal Adelaide Hospital, Adelaide, SA 5000, Australia

William CE Tam, Department of Gastroenterology and Hepatology, Lyell McEwin Hospital, Adelaide, SA 5112, Australia

Jane M Andrews, Richard H Holloway, Kylie Lange, Gerald J Holtmann, Faculty of Health Sciences, University of Adelaide, Adelaide, SA 5005, Australia

Gerald J Holtmann, Department of Gastroenterology and Hepatology, University of Queensland School of Medicine, Princess Alexandra Hospital, Brisbane, Woolloongabba, QLD 4102, Australia

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Correspondence to: Gerald J Holtmann, MD, PhD, MBA, FRACP, FRCP, Professor, Department of Gastroenterology and Hepatology, University of Queensland School of Medicine, Princess Alexandra Hospital, Brisbane, Ipswich Road, Woolloongabba, QLD 4102, Australia. g.holtmann@uq.edu.au

Telephone: +61-424-956000 Fax: +61-7-31762701

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Abstract

AIM: To investigate the outcome of patients with symptoms of gastroesophageal reflux disease (GERD) referred for endoscopy at 2 and 6 mo post endoscopy.

METHODS: Consecutive patients referred for upper endoscopy for assessment of GERD symptoms at two large metropolitan hospitals were invited to participate in a 6-mo non-interventional (observational) study.

The two institutions are situated in geographically and socially disparate areas. Data collection was by self-completion of questionnaires including the patient assessment of upper gastrointestinal disorders symptoms severity and from hospital records. Endoscopic finding using the Los-Angeles classification, symptom severity and its clinically relevant improvement as change of at least 25%, therapy and socio-demographic factors were assessed.

RESULTS: Baseline data were available for 266 patients and 2-mo and 6-mo follow-up data for 128 and 108 patients respectively. At baseline, 128 patients had erosive and 138 non-erosive reflux disease. Almost all patient had proton pump inhibitor (PPI) therapy in the past. Overall, patients with non-erosive GERD at the index endoscopy had significantly more severe symptoms as compared to patients with erosive or even complicated GERD while there was no difference with regard to medication. After 2 and 6 mo there was a small, but statistically significant improvement in symptom severity (7.02 ± 5.5 vs 5.9 ± 5.4 and 5.5 ± 5.4 respectively); however, the majority of patients continued to have symptoms (*i.e.*, after 6 mo 81% with GERD symptoms). Advantaged socioeconomic status as well as being unemployed was associated with greater improvement.

CONCLUSION: The majority of GORD patients receive PPI therapy before being referred for endoscopy even though many have symptoms that do not sufficiently respond to PPI therapy.

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Key words: Gastroesophageal reflux disease; Epidemiology; Proton pump inhibitor; Acid suppressive therapy; Endoscopy; Barrett's esophagus; Functional gastrointestinal disorders

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INTRODUCTION

Heartburn and/or acid regurgitation occurring at least weekly, is very common in the general population^[1]. Gastroesophageal reflux disease (GERD) is defined as typical symptoms occurring 2 or more times weekly, or symptoms perceived as problematic to patients, or resulting in complications^[2,3].

Many clinical trials have shown that proton pump inhibitors (PPIs) are highly effective for healing of erosive GERD, and controlling symptoms^[4]. Reflux symptoms are common, and treatment is readily available and regarded as highly efficacious. Thus, consistent with current guidelines many sufferers have therapy first, and are only referred for investigation (*i.e.*, endoscopy) if treatment fails or symptoms relapse. PPI are currently the most effective therapy for GERD, although cost effectiveness^[5], risks in long term treatment^[6,7] and their role in endoscopy-negative reflux disease are open to discussion^[8].

In the highly controlled clinical trial environment patients who do not respond to PPI therapy are typically excluded. Thus clinical trials may not mirror routine clinical care when patients are referred for endoscopy because symptoms are not controlled and clinicians might be left with an unrealistic expectation of treatment efficacy. Moreover, in routine clinical care settings, there are a number of confounders that may interfere with, or modulate, the response to therapy for reflux symptoms. While changes in lifestyle habits such as weight loss, smoking cessation and reduction of alcohol consumption are often advised^[9] very little is known about the role of body mass index, alcohol and smoking or socio-economic status (SES)^[10] on the response to therapy in real life. Whilst lifestyle factors have been related to the risk of having reflux^[1], it is unclear whether they affect the response to therapy. There are now sufficient data to show that less than 50% of patients with typical GERD symptoms have mucosal lesions. The remainder are referred to as patients with non erosive gastroesophageal reflux disease (NERD). While some of these patients may have an increased acid exposure without mucosal lesions, other patients may not have an increase esophageal acid exposure and moreover, their heartburn symptoms might not be associated with episodes of esophageal acid exposure^[11].

We therefore sought to determine and quantitate in a routine clinical setting in patients with GERD symptoms referred for endoscopy: (1) the symptom intensity and the improvement of symptoms to therapy (with PPIs); (2) the relation between symptoms and treatment response in relation to underlying structural lesions; and (3)

whether lifestyle factors or socio-demographic variables affect this response in patients presenting for endoscopy because of reflux symptoms.

MATERIALS AND METHODS

Study design/overall approach

During a 24 mo period, patients referred, for endoscopic assessment of reflux symptoms at two large metropolitan hospitals were invited to participate in this observational study. The two institutions, the Royal Adelaide Hospital (RAH) and the Lyell McEwin Hospital (LMH) were both located in a single metropolitan Area Health Service (Central Northern Adelaide Health System, CNAHS), however they are located within geographically (approximately 25 km apart) and socially disparate areas^[12]. The CNAHS serves a metropolitan and semi-rural population of more than 760000 residents. Both endoscopy units accept direct referrals from primary care doctors for upper gastrointestinal endoscopy (UGIE). All endoscopists were board certified with more extensive experience in diagnostic and therapeutic endoscopy. State of the art (Olympus) equipment was used.

This study did not include interventions other than obtaining informed consent and assessing symptoms and other parameters at baseline and during the follow-up. In particular there was no interference with normal care provided by general practitioners and specialists or interactions of the study staff that could shift attention towards symptoms or enhance compliance. Patients referred for UGIE with the primary complaints of typical reflux symptoms (heartburn +/- acid regurgitation) recorded as the indication on the referral for UGIE, between 18 and 65 years of age and capable of completing questionnaires were invited to participate. Exclusion criteria included significant medical co-morbidities (American Society of Anesthesiologists III or IV or reduced life expectancy), pregnancy and unstable psychiatric disorder and inability to read and write or communicate in English.

The study was designed as a prospective, observational study with no interference with routine clinical management. Patients were invited and consented on the day of UGIE and completed the survey at baseline and two and six mo after the initial assessment. Data collection was by self-completion of questionnaires and from hospital records. In order to avoid any interference with the study objectives, patients were only contacted once at the defined follow-up time points, no measures to increase compliance with medication or behavioural interventions outside routine clinical care were provided.

The study was approved by the human research ethics committees of both hospitals, and each patient gave informed consent. The study was registered at the Australian New Zealand Clinical Trials Registry as "Multivariate analysis of predictors for severity of mucosal lesions in patients with gastro-oesophageal reflux symptoms: a clinical, epidemiological and endoscopic survey" (ACTRN12609000045213).

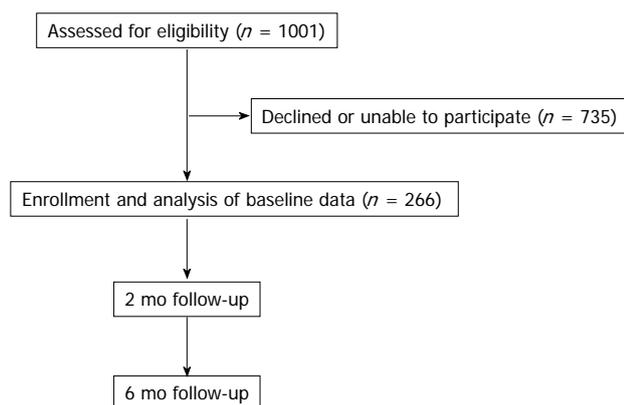


Figure 1 Consort diagram.

Treatment, lifestyle and sociodemographic data collection

In addition to the questionnaires (see below), hospital records and UGIE reports were reviewed to collect relevant data. Endoscopic findings at baseline were recorded using the Los Angeles (LA) classification^[13]. The SES of the patients was established according to patients' residential postcodes using the Social Health Atlas of South Australia^[12] and patients categorised into one of 3 groups: advantaged, average and disadvantaged.

Survey instruments

The postal survey included the patient assessment of upper gastrointestinal disorders symptom severity^[14,15]. A symptom severity score (SSS) was calculated from it using 4 items covering reflux symptoms during the past week. The items used were: (1) heartburn (burning rising in your chest or throat) during the day; (2) regurgitation or reflux (fluid or liquid from your stomach coming up into your throat) during the day; (3) heartburn (burning rising in your chest or throat) at night (when recumbent); and (4) regurgitation or reflux (fluid or liquid from your stomach coming up into your throat) at night (when recumbent).

Severity of the symptoms was rated on a 6-point Likert scale, ranging from "none" = 0 to "very severe" = 5, therefore the SSS could range from 0-20. This score was used as the outcome variable to assess patients' reflux symptom severity over time. For this instrument we defined a clinically relevant improvement as change of at least 25% of symptom severity.

Statistical analysis

Means, standard deviations and percentages were calculated. *t*-tests or when appropriate non-parametric test were used to compare characteristics of patient groups. To assess which factors may affect symptom severity over time, bivariate correlations and non-parametric tests (Mann-Whitney) were used between individual factors and the change in SSS from baseline to 2 and 6 mo. Variables which were significant in initial analyses, or thought to be biologically relevant, were then included in a multivariable logistic regression model. Two-sided *P* values

less than 0.05 were regarded as statistically significant. SPSS 15 was used for all analyses (2006, SPSS Inc., Chicago, IL, United States). A sample size of > 100 subjects was considered sufficient to identify relevant effect at an alpha level of 0.05 and a beta > 0.75.

RESULTS

Patient numbers and flow

Across the 2 sites, 1001 patients were eligible to participate (598 at the RAH; 403 at the LHM), (52.2% female overall). In total, 266 participated, 173 from the RAH [response rate (RR) = 29%] and 93 from LMH (RR = 23%) (*P* = 0.35), 145 participants were female (54.5%). Patient flow and follow-up are shown in Figure 1.

Baseline demographic details are shown in Table 1. Of note, at baseline 63% of participants were on treatment with a PPI (while all patients had PPI for at least 4 wk in the past), almost one third were regular smokers and 15.6% had high alcohol intake (daily or greater than 5 standard drinks/d).

As shown in Table 2 participants from the community hospital were significantly older, more socioeconomically disadvantaged, had more NERD, included fewer Barrett's surveillance cases, were more often smokers and had a trend for a greater proportion to be obese than those at the city hospital. However, there were no significant differences in gender mix or the proportion with high alcohol consumption between the hospitals.

Baseline endoscopic findings and symptoms

Higher grades of esophagitis (LA grade C + D) were associated with male gender (*M* = 38.8% *vs* *F* = 18.6%, *P* < 0.001), older age (*P* = 0.014) and with heavy alcohol consumption (15/37 with heavy alcohol consumption *vs* 15/125 with nil or moderate alcohol consumption, *P* = 0.002). Interestingly, there was an inverse association between higher grades of esophagitis and current smoking (15.4% in smokers *vs* 29.9% in non-smokers, *P* = 0.017).

The cohort had a mean SSS of 7 (SD 5.5) out of a possible maximum of 20. Reflux symptoms were rated as moderate to severe by 22.1% of the patients. In patients with NERD, the symptom score was significantly higher than in those with erosive or complicated GERD (*e.g.*, Barretts, Figure 2). Unemployed participants had a significantly higher mean symptom score at baseline than those who were employed (*P* = 0.007). Similarly, subjects with a lower SES had significantly more severe symptoms at baseline (8.1 ± 0.66) as compared to other patients (5.8 ± 0.52 , *P* < 0.05); this difference was not explained by variation in the use of antisecretory drugs at baseline (*P* > 0.4) and overall there was no significant difference in symptom score at baseline for patients with and without PPI therapy (without PPI 6.8 ± 0.75 *vs* with PPI 7.1 ± 0.47).

Follow-up

At 2 mo, 128 patients (48.1% of initial responders) agreed

Table 1 Baseline clinical and demographic data

| LA-classification | NERD | Grade A | Grade B | Grade C | Grade D |
|--|-------------------------|-----------------------|------------------------|------------------------|------------------------|
| n (F) | 138 (90) | 21 (13) | 33 (15) | 19 (5) | 55 (22) |
| (%, 95%CI) | (51.9, 45.9-57.8) | (7.9, 5.2-11.8) | (12.4, 9.0-16.0) | (7.1, 4.6-10.9) | (20.7, 16.2-25.9) |
| Age, yr, mean \pm SD (range) | 49 \pm 12.2 (19-65) | 42 \pm 12.9 (19-62) | 50 \pm 11.9 (22-65) | 46 \pm 12.8 (29-65) | 53 \pm 8.1 (37-65) |
| BMI, kg/m ² , mean \pm SD (range) | 28.8 \pm -6.4 (19-49) | 30 \pm 8.5 (21-53) | 29.7 \pm 6.1 (21-44) | 26.3 \pm 5.5 (15-35) | 28.7 \pm 7.6 (16-53) |
| Percentage BMI > 30 kg/m ² | 39.7% | 38.9% | 43.3% | 27.8% | 24.5% |
| PPI at enrolment | 61.5% | 50% | 71% | 57.9% | 70.4% |
| Tobacco | 25.2% | 30% | 8.7% | 21.4% | 7.9% |
| Alcohol (daily or > 50 g/d) | 7.7% | 13.3% | 23.8% | 14.3% | 34.2% |
| Employed | 54.4% | 66.7% | 55% | 50% | 44.7% |
| Married | 48.6% | 40% | 52.2% | 42.9% | 51.3% |
| Socioeconomically disadvantaged | 54.9% | 56.3% | 50% | 43.8% | 51.2% |

LA: Los-Angeles; NERD: Non erosive gastroesophageal reflux disease; BMI: Body mass index; PPI: Proton pump inhibitor.

Table 2 Baseline comparisons of patients from the different study sites

| Variable | RAH-tertiary referral centre (n = 173) | LMH-community Hospital (n = 93) | P value |
|--------------------------------------|--|---------------------------------|---------|
| Age, yr, mean \pm SD (range) | 48 \pm 12.5 (19-65) | 51.5 \pm 10.2 (23-65) | 0.027 |
| Disadvantaged SES | 56% | 74.4% | < 0.001 |
| LA-Grade NERD | 46.8% | 61.3% | 0.024 |
| LA-Grade C/D | 21.4% | 6.4% | 0.024 |
| Smoking | 14.7% | 31.3% | 0.007 |
| Alcohol (daily or > 5 Std. drinks/d) | 17.1% | 12.7% | 0.701 |
| Male gender | 48.6% | 60.2% | 0.171 |
| BMI > 30 kg/m ² | 32.1% | 44.1% | 0.064 |

SES: Socioeconomic status; LA: Los-Angeles; NERD: Non erosive gastroesophageal reflux disease; BMI: Body mass index; Std.: Standard; RAH: Royal Adelaide Hospital; LMH: Lyell McEwin Hospital.

to be reassessed and 94 complete questionnaires were returned. At 6 mo, 108 participants (40.6% of initial responders) responded, providing 77 completed questionnaires.

Descriptive and univariate comparisons: During follow-up the vast majority of patients had PPI therapy. Only 9% never had PPI, and only 6% started PPI after the baseline visit. The overall mean symptom score significantly improved from baseline at two and six months (Figure 3). However, looking at individual improvements, only 15% of patients had improvement in their symptom score at 2 mo and 19% at 6 mo. The majority of subjects had residual reflux symptoms and 19% and 17% still rated their reflux symptoms as moderate to severe, at 2 and 6 mo respectively, compared to 22.1% at baseline. At 2 mo, a higher body mass index (BMI) correlated with a greater improvement in SSS (mean \pm SD, 6.7 \pm 28.8, $P = 0.031$). Only minor gender differences were noted; with a greater change of the absolute SSS from baseline to 2-mo seen in women compared to men (mean \pm SD, 1.1 \pm 4.3, $P = 0.046$), however there was no gender difference in symptom responsiveness at the 6-mo evaluation.

Multiple linear regressions: Multiple linear regression analyses were separately performed for the 2- and 6-mo time-points to identify factors were associated with chang-

es in symptom severity over time. Factors included in the model were SES, BMI > 30 kg/m², PPI use, tobacco smoking, alcohol consumption, marital and employment status. Neither model (2 or 6 mo) reached overall significance ($P = 0.104$, adjusted $R^2 = 0.066$ at 2 mo; $P = 0.732$, adjusted $R^2 = -0.043$ at 6 mo); indicating that the chosen set of socio-demographic and life style factors did not explain a significant proportion of the variability in change in symptom severity.

Amongst individual predictors; social status and employment status were each associated with significant improvement in symptoms score at 2 mo. Patients of advantaged social status had an average 3.3 points greater improvement in symptoms score than patients of average SES and 2.5 points gain on those of disadvantaged SES ($P = 0.031$ and 0.014 respectively). Unemployed patients had an average 2.2 points greater SSS improvement compared to those employed or studying ($P = 0.02$). No individual factors were significantly associated with change in symptom score from baseline to 6 mo.

DISCUSSION

The main findings of this study are: (1) In the routine clinical setting more than 90% of patients referred for the assessment of suspected GERD are or have been on treatment with a PPI by the time of endoscopy. Nevertheless slightly more than 50% (138/266) do not have

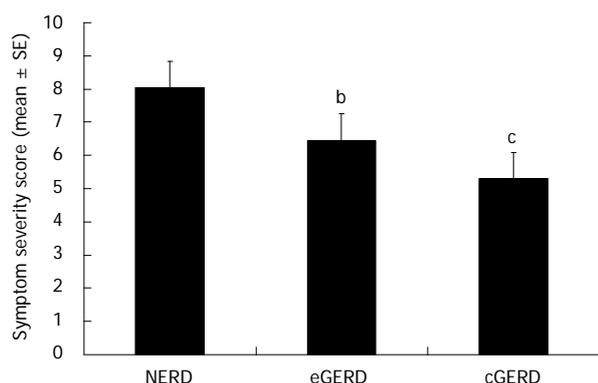


Figure 2 Mean symptom severity score for patients with non erosive reflux disease, erosive gastroesophageal reflux disease and complicated gastroesophageal reflux disease. Complicated gastroesophageal reflux disease (cGERD) includes all patients with Barretts esophagus. ^b $P < 0.01$ vs non erosive reflux disease (NERD); ^c $P < 0.05$ vs erosive gastroesophageal reflux disease (eGERD) and NERD.

any mucosal lesions; (2) Patients without mucosal lesions have significantly more severe symptoms as compared to patients with erosive or complicated GERD; and (3) There is statistically significant improvement of symptoms over 6 mo. However, while the available treatments (*e.g.*, PPI) are considered highly effective for the healing of lesions and relief of symptoms, it is remarkable that the majority of patients continue to have GORD symptoms.

It is important to note that the majority of patients referred for endoscopic assessment of GERD symptoms, received PPI therapy at the time of endoscopy or had received PPI before. In spite of that 48% of patients were found to have mucosal lesions at the time of endoscopy. However, symptoms do not appear to be “driven by lesions” as symptom severity was significantly higher in patients without mucosal lesions as compared to those with lesions. Moreover, symptoms persisted in the majority of patients although there was a modest, even though statistically significant improvement of symptoms during follow-up.

Previous clinical trials have clearly shown that GERD patients can be effectively treated with PPI^[16]. While there is no reason to question the data of these clinical trials, the typical patient now referred in the routine clinical setting for an endoscopy is already treated with PPI before an endoscopy is even considered. To our knowledge this is the first non-interventional prospective study that specifically examined this cohort of patients and aimed to define the response to therapy and possible influence of socio-demographic and lifestyle factors in a real world clinical setting. It is very striking that only a very small proportion of patients experience a substantial improvement of symptoms after endoscopy and being treated with a PPI.

Whilst medication adherence could not be verified in this observational study, it is possible that all patients have taken the medication at the appropriate time or at the individually prescribed dosage. However, it seems

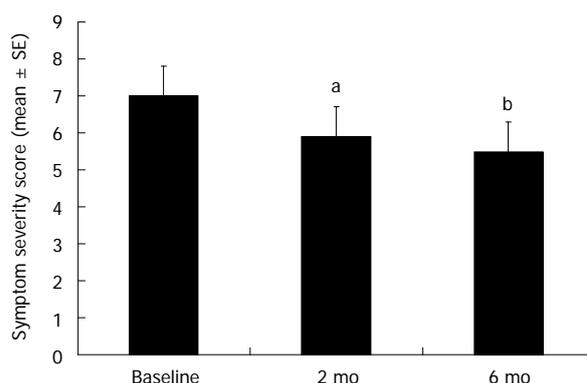


Figure 3 Mean symptom severity score at baseline and after 2 and 6 mo. ^a $P < 0.05$, ^b $P < 0.01$ vs baseline.

unlikely poor compliance is the major explanation for this apparent failure of PPI, as they give rapid symptom relief^[17]. We therefore hypothesize that in this patient group excess oesophageal acidification is not likely to be the major driver for the symptoms. It is likely that some of the symptoms are manifestation(s) of functional gastrointestinal disorders. These are known to commonly co-exist in subjects with reflux^[18], and would substantially account for the lack of response to PPI therapy. On the other side it is interesting that 9% of the group reported not being offered PPI therapy despite having the cardinal symptoms of reflux (heartburn/regurgitation).

The marginal improvement in symptoms over time (SSS 7 at baseline and 5.5 at 6 mo) shows that the usual care approach falls short with regard to improvement of symptoms when compared with trial data^[16]. Altogether, less than one quarter of patients improved over 6 mo and the percentage of patients with moderate to severe symptom severity only decreased from 22% to 19% after 2 mo and was still 17% at 6 mo. This may reflect the fact that now predominantly non-responders to PPI therapy are referred for endoscopy. Our data may raise the question if other diagnostic and therapeutic approaches might be needed for these patients. Our results showed at baseline that higher LA grades were more likely found in men, older subjects, and participants with higher alcohol consumption, consistent with several studies^[1,19,20]. Interestingly, whilst these factors are associated with more severe baseline reflux symptoms, they did not modify the symptomatic response to therapy, again suggesting that symptoms in our cohort may not be entirely attributable to GERD. This is likely to be due to our patient selection process due to biases inherent in their referral, which appears to have resulted in a group with “reflux symptoms” not due to clear-cut GERD (high proportion referred with ongoing symptoms despite PPI therapy).

Tobacco smoking is listed as a major risk factor for many diseases, but there are few studies in regard to reflux symptoms. Due to lowering the pressure of the lower esophageal sphincter it is proposed as a possible risk factor for erosive esophagitis or Barrett’s esophagus^[9,21]. Our data however, revealed greater tobacco use

in patients with non-erosive or low grades esophagitis. This observation is based upon the *post-hoc* data analysis. Thus it needs to be independently confirmed before firm conclusions can be drawn with regard to this point. Of note, however, tobacco use did not influence the response to therapy over time in our cohort.

Whilst in the multivariable models, the set of socioeconomic and life style factors did not influence symptom severity over time, BMI > 30 kg/m², SES and employment status, as individual factors, did influence symptom severity in our population. Patients with a BMI > 30 kg/m² had greater improvement in SSS at both 2 and 6 mo, and advantaged social status and unemployment were both associated with a greater improvement in symptom severity over time. While this finding is based upon a *post-hoc* analysis and also requires independent prospective validation, it is reasonable to assume that a BMI > 30 kg/m² increases reflux of acidic content into the esophagus. Thus inhibiting acid secretion would reduce esophageal acid exposure and improve symptoms.

Our study has the strength that it reflects the routine clinical setting. Patients were studied with minimal interference. Thus our “real-life” setting did not reflect the setting of clinical trials with regular contacts that facilitate compliance with medication. While all patients had been informed and consent obtained at baseline and symptoms assessed during the follow-up, there were no other interferences with routine care that potentially could affect the outcome. While this provides insights into the real world, this must be balanced against some limitations including a possible participation bias by including only those choosing to complete the questionnaires, and the appreciable dropout rate after 2 mo. However, consistent with most clinical trials a considerable proportion of patients declined to participate or could not participate for various reasons. However, characteristics of patients included into the study were not different from patients not included. Thus our finding and conclusions appear to be relevant for the whole population. These are also part of the strength of the study as this is far more representative of what happens in real world clinical medicine.

In summary, contrasting general beliefs, the majority of patients with reflux symptoms referred for endoscopy continue to have symptoms in spite of the use of highly potent PPI's. Patients without endoscopic lesions appear to have more severe symptoms. The obvious persistence of symptoms suggests that there is the need to better monitor the response to therapy in these patients and to develop and properly use diagnostic and therapeutic strategies for patients with reflux symptoms who do not respond to the routine therapy with PPIs.

COMMENTS

Background

Authors aimed to study at 2 and 6 mo after endoscopy, the outcome of patients with symptoms of gastroesophageal reflux disease (GERD) referred for endoscopy under real life conditions. Little naturalistic data on reflux symptoms under usual care conditions exist. The majority of patients with GERD symptoms referred for endoscopy have already been or are currently treated with proton

pump inhibitors (PPIs). In this setting symptoms are more severe in patients without erosive lesions. After endoscopy with continued PPI therapy, there is a significant but modest improvement of symptoms however, the majority of patients continue to have symptoms. Thus long-term PPI therapy appears to be insufficient in providing relief of symptoms in a considerable proportion of patients. Patients unresponsive to PPI therapy for reflux symptoms may benefit from investigations other than upper gastrointestinal endoscopy. There is a need to develop and test new approaches for these patients.

Research frontiers

Numerous trials suggest that in patients with gastro-oesophageal reflux disease the currently available treatments (*i.e.*, PPI) provide rapid control of symptoms and healing of lesions. Since these treatments are now widely available, they are frequently used prior to endoscopy and patients non-responsive to PPI are more likely to be referred for endoscopy. This study clearly demonstrates that there is a considerable unmet need with regard to symptom control in patients with gastro-oesophageal reflux disease.

Innovations and breakthroughs

This is the first study that prospectively assessed the symptoms of GERD patients' referred for endoscopy. The fact that the majority of patients continued to have symptoms contrasts general beliefs. Endoscopy alone might not be appropriate to target therapy in patients with GERD symptoms.

Applications

This research has considerable implications in the clinical setting. While highly potent treatments (*e.g.*, PPI) are widely available and used, patients referred for endoscopy are more likely to have symptoms that do not respond to PPI. Thus the study suggests that other treatment modalities might be required.

Peer review

In this manuscript, the authors investigated the outcome of patients with symptoms of GERD referred for endoscopy at 2 and 6 mo post endoscopy. The study was uniquely performed and the results were well discussed with no serious methodological issues.

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Trans-arterial chemo-embolization is safe and effective for very elderly patients with hepatocellular carcinoma

Matan J Cohen, Allan I Bloom, Orly Barak, Alexander Klimov, Tova Neshet, Daniel Shouval, Izhar Levi, Oren Shibolet

Matan J Cohen, Orly Barak, Tova Neshet, Daniel Shouval, Izhar Levi, Oren Shibolet, Division of Internal Medicine, Hadassah-Hebrew University Medical Center, Jerusalem 91120, Israel

Allan I Bloom, Alexander Klimov, Interventional Radiology Unit, Department of Radiology, Hadassah-Hebrew University Medical Center, Jerusalem 91120, Israel

Oren Shibolet, Liver Unit, Department of Gastroenterology, Tel Aviv Sourasky Medical Center and The Sackler Faculty of Medicine Tel-Aviv University, Tel-Aviv 64239, Israel

Author contributions: Cohen MJ, Levi I, Shouval D and Shibolet O designed the research; Cohen MJ, Barak O, Bloom AI, Klimov A and Neshet T performed the research; Cohen MJ, Levi I and Shibolet O analyzed the data; and Cohen MJ, Levi I, Bloom AI, Shouval D and Shibolet O wrote the paper.

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Correspondence to: Oren Shibolet, MD, Liver Unit, Department of Gastroenterology, Tel Aviv Sourasky Medical Center and The Sackler Faculty of Medicine Tel-Aviv University, Tel-Aviv 64239, Israel. orensh@tasmc.health.gov.il

Telephone: +972-3-6973984 Fax: +972-3-6966286

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Abstract

AIM: To assess the safety and efficacy of trans-arterial chemo-embolization (TACE) in very elderly patients.

METHODS: A prospective cohort study, from 2001 to 2010, compared clinical outcomes following TACE between patients ≥ 75 years old and younger patients (aged between 65 and 75 years and younger than 65 years) with hepatocellular carcinoma (HCC), diagnosed according to the European Association for the Study of the Liver and the American Association for the Study of Liver Diseases criteria. The decision that patients were not candidates for curative therapy was made by a

multidisciplinary HCC team. Data collected included demographics, co-morbidities, liver disease etiology, liver disease severity and the number of procedures. The primary outcome was mortality; secondary outcomes included post-embolization syndrome (nausea, fever, abdominal right upper quadrant pain, increase in liver enzymes with no evidence of sepsis and with a clinical course limited to 3-4 d post procedure) and 30-d complications. Additionally, changes in liver enzyme measurements were assessed [alanine and aspartate aminotransferase (ALT and AST), gamma-glutamyl transpeptidase and alkaline phosphatase] in the week following TACE. Analysis employed both univariate and multivariate methods (Cox regression models).

RESULTS: Of 102 patients who underwent TACE as sole treatment, 10 patients (9.8%) were > 80 years old at diagnosis; 13 (12.7%) were between 75 and 80 years, 45 (44.1%) were between 65 and 75 years and 34 (33.3%) were younger than 65 years. Survival analysis demonstrated similar survival patterns between the elderly patients and younger patients. Age was also not associated with the adverse event rate. Survival rates at 1, 2 and 3 years from diagnosis were 74%, 37% and 31% among patients < 65 years; 83%, 66% and 48% among patients aged 65 to 75 years; and 86%, 41% and 23% among patients ≥ 75 years. There were no differences between the age groups in the pre-procedural care, including preventive treatment for contrast nephropathy and prophylactic antibiotics. Multivariate survival analysis, controlling for disease stage at diagnosis with the Barcelona Clinic Liver Cancer score, number of TACE procedures, sex and alpha-fetoprotein level at the time of diagnosis, found no significant difference in the mortality hazard for elderly vs younger patients, and there were no differences in post-procedural complications. Serum creatinine levels did not change after 55% of the procedures, in all age groups. In 42% of all procedures, serum creatinine levels increased by no more than 25% above

the baseline levels prior to TACE. Overall, there were 69 post-embolization events (23%). Hepatocellular enzymes often increased following TACE, with no association with prognosis. In 40% of the procedures, ALT and AST levels rose by at least 100%. The increases in hepatocellular enzymes occurred similarly in all age groups.

CONCLUSION: TACE is safe and effective in very elderly patients with HCC, and is not associated with decreased survival or increased complication rates.

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Key words: Hepatocellular carcinoma; Chemoembolization; Therapeutic; Elderly; Prognosis; Complications

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INTRODUCTION

Hepatocellular carcinoma (HCC) is the fifth most common cancer worldwide and the second cause of cancer-related deaths in men. It is the seventh most common cancer and sixth cause of cancer-related deaths among women^[1]. The mean age of HCC diagnosis is increasing, and likewise the proportion of older patients seeking treatment for this deadly malignancy^[2]. Latest estimates indicate that HCC incidence peaks above the age of 70 years^[2,3].

Treatment modalities of HCC are limited. Curative treatment includes: liver resection, liver transplantation and, in small tumors, radiofrequency ablation (RFA). Only a small fraction of patients are eligible for these treatments. Most remaining patients receive palliative treatments including trans-arterial chemo-embolization (TACE), palliative RFA, sorafenib, or supportive care.

Compared to young patients with HCC, elderly patients have more co-morbidities and are considered poorer surgical candidates. Furthermore, patients older than 65-70 years of age are usually not considered to be potential candidates for liver transplantation^[4]. Recently, sorafenib, the only proven chemotherapy to show efficacy against HCC, was evaluated in elderly patients (> 70 years), and was shown to have similar survival benefits as in patients younger than 70 years^[5].

TACE has been previously shown to prolong survival among patients diagnosed with HCC who are not candidates for curative treatment. These data were recently challenged when a meta-analysis, using stringent inclusion and exclusion criteria, did not demonstrate a survival benefit for TACE^[6]. One limitation of that study was that most of the data were derived from case series and

clinical trials which included patients who were younger than 65 years^[7,8].

There are only a few studies which have assessed the role of TACE among elderly patients. Most of these studies defined elderly as patients over 70 years of age, including a Chinese cohort of 196 patients older than 70 years^[9] and an Italian cohort of 158 patients^[10]. In both studies, the majority of elderly patients were between 70 and 75 years of age.

Since 2000, we have prospectively enrolled patients in our HCC database. We noticed that our population is aging and includes patients in their late seventies, eighties and even nineties, seeking treatment. Our aim was to assess efficacy, survival and safety of TACE among very elderly patients defined as ≥ 75 years old, compared to younger patients in our cohort.

MATERIALS AND METHODS

Local institutional board approval was received for this study, with waiver of the need for informed consent as identifying data were omitted after data collection and there was no intervention performed (HMO 0604-10). All patients diagnosed with HCC between 2000 and 2010 who underwent TACE were included and prospectively followed until January 2012. HCC diagnosis was determined in accordance with established guidelines published by both the European Association for the Study of the Liver and the American Association for the Study of Liver Diseases^[11,12]. In cases where the radiological findings were inconclusive in establishing the diagnosis, percutaneous image-guided liver biopsy was performed. The decision that patients were not candidates for curative therapy was made by a multidisciplinary HCC team including the treating physician.

Demographic and clinical features were collected from patients' medical records. Patients were designated into three cohorts, stratified by age at diagnosis (< 65 years of age, 65-75 years of age, ≥ 75 years of age). All patient data were collected using a national identification number.

TACE technique

Using a standard angiographic approach, transfemoral visceral arteriography was performed. Whenever possible, super selective TACE was attempted using a co-axial microcatheter and a mixture of 50 mg doxorubicin with lipiodol oil and gelfoam slurry or powder. If dictated by tumor burden and feasibility (hepatic reserve, Child Pugh status), segmental or lobar TACE was performed. Percutaneous vascular closure devices were routinely employed from 2007 onward.

Statistical analysis

All TACE procedures were recorded in the patient electronic records and all procedures were confirmed through the computerized hospital billing database. Inpatient notes and computer records were reviewed in order to extract clinical events, laboratory test results and medica-

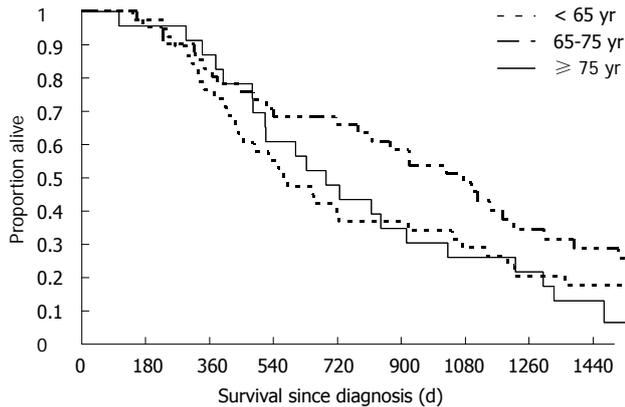


Figure 1 Survival curves comparing the age groups. Kaplan-Meier survival curves presenting the proportion of patients alive since hepatocellular diagnosis, stratified by age groups: < 65, 65-75 and \geq 75 years. The survival curves are not statistically different (log-rank $P = 0.19$).

tion provisions. The primary outcome of this analysis was overall mortality. Mortality rates and dates were determined *via* the national population registry. Secondary outcomes included post-embolization syndrome (nausea, fever, abdominal right upper quadrant pain, increase in liver enzymes with no evidence of sepsis and with a clinical course limited to 3-4 d post procedure); 30-d complications (sepsis, acute kidney injury, vascular events), as recorded in the medical charts; 30-d all-cause-readmission or all-cause-return to the emergency department. We also recorded pre-procedure (one week prior) and post-procedure (one week after) levels of creatinine, alanine and aspartate aminotransferase (ALT and AST), and gamma-glutamyl transpeptidase (GGT) and alkaline phosphatase (ALKP). We cross-referenced clinical impressions documented in the medical files by examining all medical documents available to reduce bias resulting from human error and ascertainment bias.

Descriptive statistics were employed to presented age group characteristics. Categorical variables are depicted with percentages and distributions, and continuous variables are presented with mean \pm SD. Associations between categorical variables were assessed with the Fisher exact test, and comparison of continuous variables was performed with the Student's *t*-test, or with the Mann-Whitney *U* test. Survival charts were generated employing the Kaplan-Meier method, and survival curves were compared with the log-rank test. After confirming that the proportional hazard assumptions were met, we examined the association of age group with mortality using Cox proportional hazard regression analysis. Co-variables found on univariate analyses to have a seemingly probable association with mortality ($P < 0.1$) were included in the model. There were two types of missing data - treatment data and patient laboratory data. Treatments not specifically documented in the patient chart orders were considered not to have been provided (for example, preparation for contrast injection, antibiotic prophylaxis). In cases where laboratory tests were not taken, they were treated as missing and not included in the relevant analy-

ses. In all analyses, two-tailed $P < 0.05$ was considered statistically significant. We examined whether absolute and relative differences between pre-procedure and post-procedure laboratory tests were different between the age groups using one-way analysis of variance and plotted the relationship between these measures.

RESULTS

Between 2000 and 2010, 235 patients were diagnosed with HCC. Of these, 102 patients were treated with TACE alone. Thirty-day follow-up was complete (all living patients were evaluated in the liver clinic outpatient service) and survival follow-up was complete (none of the patients left the country and the population registry is updated regularly). Data collection was complete for laboratory data, and all patient files were available for clinical assessment.

We divided our population into 3 cohorts: age < 65 years (group 1), age between 65 and 75 years (group 2) and age \geq 75 years (group 3). There were 38 patients in group 1, 41 patients in group 2 and 23 patients in group 3. Patient characteristics are presented in Table 1. There were 27 males and 75 females, with only three males in the younger age group. Younger patients had more advanced disease, as assessed by the Cancer of the Liver Italian Program (CLIP), Okuda or Barcelona Clinic Liver Cancer (BCLC) staging systems. The differences were less pronounced with the BCLC system, which accounts for functional status parameters, and we used this staging system to assess the primary outcome with multivariate analysis. Elderly patients had a mean alpha-fetoprotein (AFP) level at diagnosis that was higher than the two other age groups. The distribution of the number of TACE procedures per patient was similar between the age groups.

Survival analysis demonstrated similar survival patterns among the elderly patients and younger patients (Figure 1, $P = 0.19$). Overall, the cumulative follow-up time was 258 patient years. Median survival from diagnosis was 574 d (range: 143 d to 6 years), 1032 d (range: 154 d to 10 years) and 688 d (range: 104 d to 4.2 years) among patients in groups 1, 2 and 3, respectively. Respective survival rates at 1, 2 and 3 years from diagnosis were 74%, 37% and 31% among group 1 patients; 83%, 66% and 48% among group 2 patients; and 86%, 41% and 23% among group 3 patients. Multivariate survival analysis using the Cox proportional hazard regression model with variables of age (according to group), disease stage at diagnosis, number of TACE procedures, sex and AFP level at diagnosis found no significant difference in the mortality hazard of very elderly *vs* younger patients. The analysis was repeated with both the CLIP and Okuda staging systems, and the results were stable and consistent (Table 1).

Next, we assessed whether the number of TACE procedures was different among the groups. We hypothesized that elderly patients may have received different TACE regimens. The cohort patients described above under-

Table 1 Patient characteristics *n* (%)

| | < 65 yr (<i>n</i> = 38) | 65-75 yr (<i>n</i> = 41) | ≥ 75 yr (<i>n</i> = 23) | <i>P</i> value | HR (95%CI) |
|--|--------------------------|---------------------------|--------------------------|----------------|-------------------------------|
| Age group multivariate HR (95%CI) ¹ | 1 | 1.03 (0.58-1.83) | 1.04 (0.56-1.9) | | - |
| Female | 35 (92.2) | 24 (58.6) | 16 (69.6) | 0.003 | 0.55 (0.31-0.98) ¹ |
| Cirrhosis at diagnosis | 35 (92.1) | 41 (1) | 22 (95.6) | 0.338 | |
| Ascites at diagnosis | 4 (10.5) | 4 (9.7) | 4 (17.3) | 0.632 | |
| Hepatitis B virus | 12 (31.5) | 7 (17.0) | 2 (8.6) | 0.06 | 0.85 (0.49-1.47) ¹ |
| Hepatitis C virus | 21 (55.2) | 29 (70.7) | 18 (78.2) | 0.14 | |
| Cancer of the Liver Italian Program ² | | | | 0.008 | |
| 0 | 8 (21.0) | 20 (48.7) | 7 (30.4) | | 1.9 (1.5-2.4) ² |
| 1 | 6 (15.7) | 13 (31.7) | 9 (39.1) | | |
| 2 | 17 (44.7) | 8 (19.5) | 5 (21.7) | | |
| 3 | 6 (15.7) | 0 (0.0) | 2 (8.6) | | |
| 4 | 1 (2.6) | 0 (0.0) | 0 (0.0) | | |
| Okuda ² | | | | 0.001 | |
| 1 | 21 (55.2) | 39 (95.1) | 19 (82.6) | | 5 (2.7-9.1) ² |
| 2 | 16 (42.1) | 2 (4.8) | 4 (17.3) | | |
| 3 | 1 (2.6) | 0 (0.0) | 0 (0.0) | | |
| Child-Pugh-Turcot ² | | | | 0.98 | |
| 1 | 31 (81.5) | 35 (85.3) | 19 (82.6) | | 2.1 (1.2-3.6) ² |
| 2 | 6 (15.7) | 5 (12.1) | 3 (13.0) | | |
| 3 | 1 (2.6) | 1 (2.4) | 1 (4.3) | | |
| Barcelona Clinic Liver Cancer | | | | 0.027 | |
| 1 | 3 (7.8) | 15 (36.5) | 3 (13.0) | | 2.3 (1.65-3.2) ² |
| 2 | 7 (18.4) | 6 (14.6) | 5 (21.7) | | |
| 3 | 26 (68.4) | 19 (46.3) | 15 (65.2) | | |
| Procedures | | | | 0.16 | |
| 1 | 12 (31.5) | 4 (9.7) | 7 (30.4) | | |
| 2 | 12 (31.5) | 12 (29.2) | 8 (34.7) | | |
| 3 | 6 (15.7) | 11 (26.8) | 2 (8.6) | | |
| > 3 | 8 (20.9) | 14 (31.4) | 6 (26.0) | | |
| Number of procedures | 2.4 ± 1.6 | 3.4 ± 2.0 | 2.7 ± 2 | 0.07 | |
| Albumin at diagnosis | 36.37 ± 4.4 | 36.3 ± 4.7 | 36.0 ± 5.2 | 0.96 | 0.97 (0.84-1.11) ¹ |
| Alpha-fetoprotein at diagnosis | 944 ± 2162 | 337 ± 729 | 9232 ± 31376 | 0.05 | |
| International normalized ratio at diagnosis | 1.14 ± 0.23 | 1.19 ± 0.25 | 1.12 ± 0.18 | 0.46 | 1 ¹ |
| Bilirubin at diagnosis | 2.9 ± 7.8 | 1.02 ± 0.66 | 1.17 ± 0.56 | 0.19 | |

¹Hazard ratio (HR) (95%CI) when controlling for Barcelona Clinic Liver Cancer (BCLC) in multivariate model; ²Multivariate HRs used instead of BCLC.

Table 2 Trans-arterial chemo-embolization procedure characteristics *n* (%)

| | < 65 yr | 65-75 yr | ≥ 75 yr | <i>P</i> value |
|--|------------|------------|-----------|----------------|
| Number of procedures | 93 | 129 | 61 | |
| Preparation for contrast material exposure | 18 (19) | 38 (29) | 14 (22) | 0.37 |
| Iodine allergy and preparation | 1 (1) | 21 (16) | 0 (0) | < 0.001 |
| Antibiotic prophylaxis | 68 (73) | 95 (73) | 44 (72) | 0.7 |
| Cefamezine | 63 (67) | 87 (67) | 44 (71) | |
| Ceftazidime | 1 (1) | 1 (0.7) | 0 (0) | |
| Clindamycin | 2 (2) | 1 (0.7) | 0 (0) | |
| Clindamycin and Ciprofloxacin | 0 (0) | 1 (0.7) | 0 (0) | |
| Vancomycin | 2 (2) | 1 (0.7) | 0 (0) | |
| Right upper quadrant abdominal pain | 16 (17) | 24 (18) | 5 (8) | 0.22 |
| Nausea | 9 (9.6) | 17 (13.1) | 1 (1.6) | 0.057 |
| Fatigue | 6 (6) | 6 (4) | 1 (1) | 0.36 |
| Fever | 21 (22) | 26 (2) | 5 (8) | 0.07 |
| Post-embolization syndrome | 23 (24) | 36 (27) | 10 (16) | 0.3 |
| Readmission | 3 (3.2) | 8 (6.2) | 1 (1.6) | 0.34 |
| Total length of stay | 3.6 (1.14) | 3.55 (1.1) | 3.3 (0.9) | 0.19 |

went a total of 299 TACE procedures (Table 2). There were no differences between the age groups in pre-procedural care, including preventive treatment for contrast

nephropathy and prophylactic antibiotics.

There were also no differences in post-procedural complications. There was a trend towards fewer post-embolization syndrome events among the elderly patients. Overall, there were 69 post-embolization syndrome events (23%). Some complications were very rare, including two cases of acute kidney injury (0.6%), two cases of sepsis (0.6%), two cases of hemorrhage (0.6%) and two cases of hemodynamic instability (0.6%). There was one event of vascular dissection and one event of pseudo-aneurysm.

There were 10 patients aged 80 years or older at diagnosis who were included in the analysis. Eight were women, two had BCLC stage I, three had BCLC stage II and five had BCLC stage III. Eight were HCV carriers. These patients underwent 34 procedures; there were five post-embolization events (15%) and one readmission within 30 d of the procedure. Nine patients survived one year; of these, three survived two years, while two survived three years.

We hypothesized that older patients may be more prone to contrast-induced renal injury following TACE. Serum creatinine levels did not change after 55% (group 1), 58% (group 2) and 55% (group 3) of the procedures (*P* = 0.98). In 42% of all procedures, serum creatinine

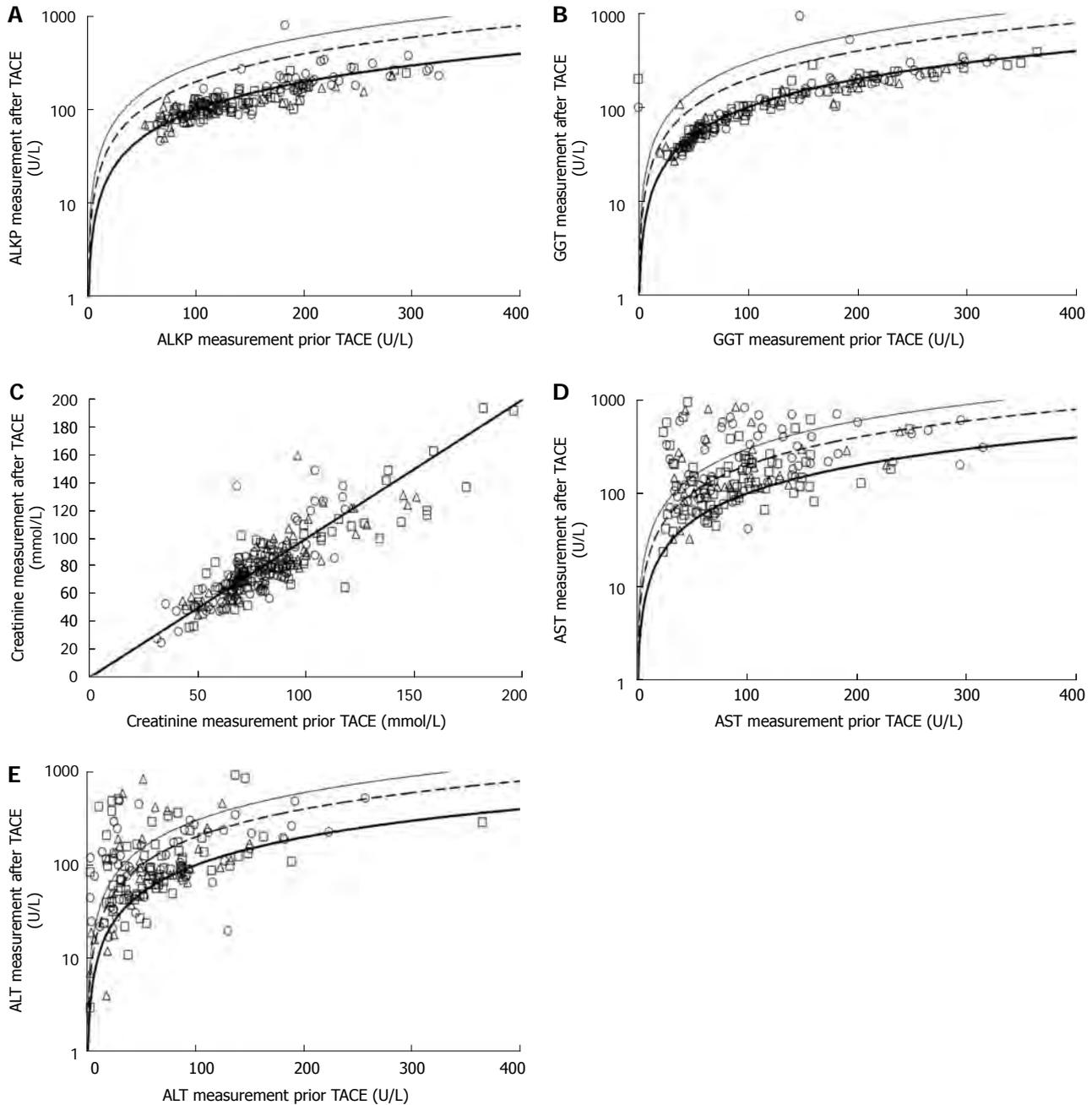


Figure 2 Laboratory results before and after trans-arterial chemoembolization procedures. A: Alkaline phosphatase (ALKP); B: Gamma-glutamyl transferase (GGT); C: Creatinine; D: Aspartate aminotransferase (AST); E: Alanine aminotransferase (ALT). Values before trans-arterial chemo-embolization (TACE) are in the x-axis, values after TACE are in the y-axis. In panels A, B, D and E, the vertical axis is in a logarithmic scale. In all panels the thick line represents $y = x$, the dashed line represents $y = 2 \times x$ and the thin line represents $y = 3 \times x$. In all panels patients aged < 65 years are represented in circles, patients aged 65-75 years are presented with squares and patients aged ≥ 75 years are represented with triangles.

levels increased by no more than 25% above the baseline measurement taken prior to TACE. There were only two cases in which creatinine more than doubled (Figure 2). Increases in creatinine levels were not associated with increased mortality.

Both ALT and AST levels frequently rose following TACE. These increases were evenly distributed among age groups ($P = 0.17$ for ALT, and $P = 0.69$ for AST). ALT and AST levels were not increased after 25% and 17% of TACE procedures, respectively. In contrast, ALT and AST more than doubled in 40% and 43% of the

procedures, respectively (Figure 2). GGT and ALKP did not increase following TACE procedures in any of the age groups (Figure 2). There was no association between post-TACE rise in hepatocellular enzymes and mortality.

DISCUSSION

In this study, we show that TACE is safe and effective in very elderly patients (≥ 75 years of age) who were diagnosed with HCC. Our results support previous findings which show that both survival and post-TACE

complications do not differ between older and younger patients.

Current guidelines for the management of HCC do not stratify strategies according to age^[11]. Still, the treating clinician may be concerned that TACE will be more hazardous for elderly patients, due to the perceived increased chance of renal or vascular complications, or the potential for liver function deterioration. Furthermore, given that elderly patients have a shorter life expectancy, the treating physician may assume that elderly patients might not survive long enough to benefit from any potential gains that the TACE confers on younger patients.

Previous studies used different cutoffs to define elderly. Some even defined elderly patients as those above 60 or 65 years^[13-17]. In a meta-analysis of randomized controlled trials, published in 2002, increased age was not associated with decreased prognosis. However, mean patient age in the included studies ranged from 41 to 66 years of age. In most of the included trials, more than 50% of the participating patients had a Child-Pugh score below 7^[7]. In these cohorts there was a significant misrepresentation of the older patients, far less than their proportion among patients with HCC, suggesting marked selection bias^[18].

Reports of treatment outcomes in patients older than 70 years have shown inconsistent results with regard to prognosis, although most have shown that advanced age is not associated with worse prognosis. Poon *et al.*^[19] have shown that among patients older than 70 years, those offered resection and those offered TACE had comparable prognosis with treatment-matched younger controls. In a recent report describing the role of TACE among patients with HCC excluded from transplantation or surgical resection, the mean age of the patients was 70 years, survival was not stratified by age and was estimated at 91%, 86% and 80% at 1, 2 and 3 years, respectively^[20]. A similar study, with 95 elderly patients (defined as above 70 years old, though most were younger than 75 years) demonstrated that after excluding patients referred for transplantation, age was not associated with poorer survival. Survival rates among patients above 70 years of age were 51%, 36% and 23% at 1, 2 and 3 years, respectively^[21].

The Italian Liver Cancer group compared treatments for HCC over a twenty-year period. They included a comparison of elderly and younger patients who underwent TACE with an age cutoff of 70 years and a mean age in the elderly group of 74.9 years. They report no difference in prognosis between elderly and younger patients who underwent TACE^[10]. Another study from China presented similar results^[9].

Only a few studies have included a larger proportion of older patients. Dohmen *et al.*^[22] analyzed a cohort of 36 patients with HCC who were older than 80 years, and showed that disease stage rather than age was the major determinant of survival. The patients in the study received various interventions including TACE, chemotherapy and surgical resection. Two other studies

showed that patients older than 75 years with HCC had a worse prognosis than younger patients, but attributed these findings to poorer treatment rather than age effect^[23,24]. An analysis that focused on all treatment modalities in 40 patients older than 75 years compared to younger patients showed no difference in survival rates. In that study, 43% of the younger controls underwent liver transplantation. Among the elderly, TACE was the most frequent treatment modality^[25]. In 2010, a study of patients who underwent TACE, which included 131 patients aged 70 to 79 years and 69 patients older than 80 years, showed that age was a predictor of increased mortality^[26].

We found a 23% (69/299) post-embolization syndrome rate and a 2.4% (12/299) hospital readmission rate in patients undergoing TACE. Both complications were not increased in elderly patients. A recent review of adverse events associated with TACE reported the incidence of hepatic insufficiency as ranging between 1% and 50%, and that of cholecystitis was between 0% and 10%^[16]. Post-embolization syndrome rates have been reported between 2% and 80%^[6,8]. Previous assessment of acute kidney injury among patients undergoing TACE found no association between this adverse event and age^[26].

Because our study was conducted in a "real-life" setting, it suffers from selection bias. This limitation is shared by all previously published studies. The strengths of our study are the prospective design, with complete patient follow-up, record analysis and data acquisition. We use a clear age cut-off of 75 years to define elderly patients. An important strength of our study is the systematic documentation of post-embolization complications, segregated to the age groups, and also the presentation of biochemistry result dynamics.

Given the body of literature and the natural history of non-curable HCC, our study provides data to support the use of TACE in selected, very elderly patients (older than 75 years old). These patients should be offered similar treatment regimens, including TACE and palliative care, as younger patients. TACE should not be withheld from elderly patients based on age criteria alone.

COMMENTS

Background

Hepatocellular carcinoma (HCC) incidence is highest among patients over 70 years old. Though many are not candidates for curative therapy, they can benefit from life-prolonging interventions. Most existing literature provides information about younger patients. Trans-arterial chemo-embolization (TACE) has been shown to prolong survival among HCC patients, though there is little evidence of the procedure's safety and efficacy among very elderly HCC patients (above 75 years of age).

Research frontiers

HCC is one of the most common neoplastic malignancies worldwide. Therapeutic options aiming for cure include liver transplantation, liver resection and radiofrequency ablation (RFA). TACE, sorafenib, palliative RFA and supportive care are palliative options, which in some cases also offer life prolongation. Current clinical research challenges include: (1) Innovative discovery of novel therapeutic modalities; (2) Improving the use of known treatment options by identifying patient characteristics which can predict better outcomes, which therapeutic option will maximize outcome and to broaden the population eligible to receive

treatment. These challenges are met with an aging patient population, as the proportion of newly diagnosed elderly patients is consistently increasing.

Innovations and breakthroughs

In this study the authors included very elderly patients (older than 75 years), who have not been sufficiently represented in most published research so far. The authors compared survival time since diagnosis between the very elderly and younger patients. Additionally, the authors included critical information regarding TACE-associated complications and changes in common biochemistry tests. The authors show that the very elderly HCC patients enjoy the same survival benefits conferred by TACE on younger patients and that they do not experience more complications following this procedure.

Applications

There are several very practical implications which can be taken from this study: (1) TACE can be offered to patients above 75 years of age using the same clinical considerations which apply to younger patients; (2) Increases in hepatocellular enzymes alanine and aspartate aminotransferase are common and, by themselves, do not indicate poor or better response to treatment. In contrast, increases in the cholestatic enzymes gamma-glutamyl transpeptidase and alkaline phosphatase and in creatinine are not common and should warrant clinical investigation; (3) Post-embolization syndrome is common and has no prognostic implications.

Terminology

HCC is a malignant disease of the liver, often presenting as a complication of long-standing liver disease and cirrhosis due to viral, alcoholic and metabolic etiologies. TACE is a minimally invasive procedure in which an artery (most often the femoral artery) is punctured; through it a catheter is introduced and advanced towards the blood vessels which provide arterial blood to the liver. After identifying which specific vessels provide blood supply to the liver tumor, toxic chemotherapy is injected in order to cause cancer cell death, and additionally, the arteries are blocked (embolized) in order to stop the blood supply to the tumor.

Peer review

The study results are interesting and important with regard to epidemiological data. The theme is interesting and the study evaluations, as well as the statistical analysis, are well done. It is of great significance in providing evidence for clinicians to expand the age limit for TACE, and helps a lot for treatment decision making in elderly HCC patients.

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Effects of *Nigella sativa* on outcome of hepatitis C in Egypt

Eman Mahmoud Fathy Barakat, Lamia Mohamed El Wakeel, Radwa Samir Hagag

Eman Mahmoud Fathy Barakat, Department of Tropical Medicine, Faculty of Medicine, Ain Shams University, Cairo 11566, Egypt

Lamia Mohamed El Wakeel, Department of Clinical Pharmacy, Faculty of Pharmacy, Ain Shams University, Cairo 11566, Egypt

Radwa Samir Hagag, Department of Clinical Pharmacy, Faculty of Pharmacy, Egyptian Russian University, Cairo 16686, Egypt

Author contributions: Barakat EMF contributed to study concept and design, acquisition and interpretation of data, and critical revision of the manuscript for important intellectual content; El Wakeel LM contributed to acquisition, analysis and interpretation of data, and critical revision of the manuscript for important intellectual content; Hagag RS contributed to data collection and material support; all authors contributed to drafting of the manuscript and final approval of the version to be published.

Correspondence to: Lamia Mohamed El Wakeel, PhD, Assistant professor of Clinical Pharmacy, Faculty of Pharmacy, Ain Shams University, 4 Street 292, New Maadi, Cairo 11566, Egypt. lamywak@yahoo.com

Telephone: +20-100-5201099 Fax: +20-100-5201099

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Abstract

AIM: To evaluate the safety, efficacy and tolerability of *Nigella sativa* (*N. sativa*) in patients with hepatitis C not eligible for interferon (IFN)- α .

METHODS: Thirty patients with hepatitis C virus (HCV) infection, who were not eligible for IFN/ribavirin therapy, were included in the present study. Inclusion criteria included: patients with HCV with or without cirrhosis, who had a contraindication to IFN- α therapy, or had refused or had a financial constraint to IFN- α therapy. Exclusion criteria included: patients on IFN- α therapy, infection with hepatitis B or hepatitis I virus, hepatocellular carcinoma, other malignancies, major severe illness, or treatment non-compliance. Various parameters, including clinical parameters, complete blood

count, liver function, renal function, plasma glucose, total antioxidant capacity (TAC), and polymerase chain reaction, were all assessed at baseline and at the end of the study. Clinical assessment included: hepato and/or splenomegaly, jaundice, palmar erythema, flapping tremors, spider naevi, lower-limb edema, and ascites. *N. sativa* was administered for three successive months at a dose of (450 mg three times daily). Clinical response and incidence of adverse drug reactions were assessed initially, periodically, and at the end of the study.

RESULTS: *N. sativa* administration significantly improved HCV viral load (380808.7 ± 610937 vs 147028.2 ± 475225.6 , $P = 0.001$) and TAC (1.35 ± 0.5 vs 1.612 ± 0.56 , $P = 0.001$). After *N. sativa* administration, the following laboratory parameters improved: total protein (7.1 ± 0.7 vs 7.5 ± 0.8 , $P = 0.001$), albumin (3.5 ± 0.87 vs 3.69 ± 0.91 , $P = 0.008$), red blood cell count (4.13 ± 0.9 vs 4.3 ± 0.9 , $P = 0.001$), and platelet count (167.7 ± 91.2 vs 198.5 ± 103 , $P = 0.004$). Fasting blood glucose (104.03 ± 43.42 vs 92.1 ± 31.34 , $P = 0.001$) and postprandial blood glucose (143.67 ± 72.56 vs 112.1 ± 42.9 , $P = 0.001$) were significantly decreased in both diabetic and non-diabetic HCV patients. Patients with lower-limb edema decreased significantly from baseline compared with after treatment [16 (53.30%) vs 7 (23.30%), $P = 0.004$]. Adverse drug reactions were unremarkable except for a few cases of epigastric pain and hypoglycemia that did not affect patient compliance.

CONCLUSION: *N. sativa* administration in patients with HCV was tolerable, safe, decreased viral load, and improved oxidative stress, clinical condition and glycaemic control in diabetic patients.

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Key words: Hepatitis C virus; *Nigella sativa*; Oxidative stress; Viral load

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INTRODUCTION

Egypt has the highest prevalence of hepatitis C virus (HCV) worldwide (15%) and the highest prevalence of HCV-4 (67%) with a predominance of subtype 4a (55%)^[1-4].

The natural history of HCV infection and disease progression are influenced by several factors such as age at infection onset, sex, duration of infection, co-infection with hepatitis B virus (HBV), level of HCV viremia and its genotype^[5].

HCV is an important etiological factor for the development of hepatocellular carcinoma (HCC) and 23% of HCV patients develop HCC^[6]. It has been shown that there is an alarming increase in the incidence of HCC in HCV patients in Egypt^[7].

Presently, the only approved therapy for HCV is pegylated interferon- α (PEG-IFN- α) and ribavirin treatment, and their success is heavily influenced by patient adherence, which correlates directly with tolerance to their side effects^[8]. Moreover, financial constraints for the combined therapy in many patients often contribute to therapy non-adherence, potentially lowering its success rates^[9].

Oxidative-stress-related molecules may act as mediators modulating cellular events responsible for progression to liver fibrosis^[10,11]. It has been shown that increased production of reactive oxygen species, in part catalyzed by iron overload, is involved in HCV-related liver damage through a pathway that involves DNA oxidative injury^[12].

Silymarin is one of the alternative therapies that has been previously tested for the management of HCV patients who are not candidates for PEG-IFN; however, it has not shown any appreciable effects on viral load^[13].

Nigella sativa (*N. sativa*) is used as a food condiment in the Middle East, and its seeds/oil have been shown to possess anti-inflammatory, antiviral and antineoplastic activity in various *in vitro* and *in vivo* studies^[14]. The antioxidant effects of *N. sativa* have been shown in the essential oil obtained from six different extracts of its seeds, as well as from a commercial fixed oil^[15]. The crude *N. sativa* oil and its fractions have shown potent *in vitro* radical scavenging activity^[16].

The effect of *N. sativa* has been evaluated in animal studies. There are many reports of its biological activities including: immunopotential, antitumor, anti-inflammatory, analgesic, antihypertensive, antidiabetic, respiratory stimulation, antibacterial, antifungal, anticestode and antinematode effects^[17-19].

A striking reduction of murine cytomegalovirus (CMV) virus titer in both spleen and liver was found in mice treated with *N. sativa* seed oil compared with control mice^[20]. Moreover, oral feeding with *N. sativa* extract suppressed

chemically induced hepatic tumors in rats^[21]. *N. sativa* treatment has been shown to ameliorate disturbed hematological parameters in diabetic rabbits through modulation of lipid peroxide red blood cell (RBC) membrane content, leading to an increase in RBC count^[22].

To date, no studies have addressed the use of *N. sativa* in HCV patients and its potential benefits; hence, we sought to evaluate the efficacy, safety, and tolerability of *N. sativa* supplementation as an alternative therapy in the management of HCV patients who are non-candidates for IFN- α therapy.

MATERIALS AND METHODS

This was a prospective, single-armed, self-controlled pilot study, conducted at the Tropical Medicine Department, El-Demerdash Hospital, Ain Shams University, Cairo, Egypt.

Patients

All HCV patients presenting to the department were assessed for eligibility. Inclusion criteria included all patients diagnosed with HCV with or without cirrhosis who either had a contraindication to IFN- α therapy^[23], or had refused or had a financial constraint to IFN- α therapy. Exclusion criteria included: patients on IFN- α therapy; infection with HBV or hepatitis I virus; HCC or other malignancies; major severe illness such as renal failure, congestive heart failure, respiratory failure or autoimmune disease; or non-compliance to treatment. Informed consent was obtained from all patients, and the institutional ethical committee approved the study protocol, which conformed with the ethical guidelines of the 1975 Declaration of Helsinki.

Methods

Hepatitis markers were assessed for all patients at enrollment, including: hepatitis B core immunoglobulin G, hepatitis B surface antigen, and HCV antibody. All eligible patients were subjected to the following at enrollment and after 3 mo therapy: (1) Full clinical assessment with an emphasis on hepato- and/or splenomegaly, jaundice, palmar erythema, flapping tremors, spider naevi, lower-limb edema, and ascites; (2) Abdominal ultrasonography; (3) Laboratory investigations including complete blood count, liver functions [aspartate aminotransferase (AST), alanine aminotransferase (ALT), total proteins, albumin, total and direct bilirubin, prothrombin time and international standard ratio (INR)], renal function (serum creatinine, blood urea nitrogen), serum α -fetoprotein, polymerase chain reaction (PCR) for HCV (lower detection limit, < 50 copies) and total antioxidant capacity (TAC); (4) The antioxidants assessed in the estimation of TAC included enzymes such as superoxide dismutase, catalase, glutathione peroxidase; macromolecules such as albumin, ceruloplasmin, ferritin; small molecules, including ascorbic acid, α -tocopherol, β -carotene, reduced glutathione, uric acid, and bilirubin; (5) The assay principle depended

Table 1 Clinical assessment data at baseline and after treatment *n* (%)

| Characteristic | Baseline | After treatment | <i>P</i> value |
|-----------------------------|------------|-----------------|----------------|
| Hepato and/or splenomegaly | 19 (63.30) | 19 (63.30) | |
| Jaundice | 8 (26.70) | 5 (16.70) | 0.25 |
| Palmar erythema | 10 (33.30) | 8 (26.70) | 0.5 |
| Spider naevi | 8 (26.70) | 4 (13.30) | 0.125 |
| Lower limb edema | 16 (53.30) | 7 (23.30) | 0.004 |
| Clinically detected ascites | 13 (43.30) | 8 (26.70) | 0.063 |

After treatment: 3 mo *Nigella sativa* treatment. McNemar's test was used to compare categorical data overtime.

on the determination of the antioxidative capacity by the reaction of antioxidants in the sample with a defined amount of exogenously provide H₂O₂. The antioxidants in the sample eliminated a certain amount of the provided H₂O₂. The residual H₂O₂ was determined colorimetrically by an enzymatic reaction that involved the conversion of 3,5-dichloro-2-hydroxy benzenesulfonate to a colored product; (6) TAC was analyzed using a TAC kit from Bio-diagnostic and measured spectrophotometrically using KENZA (Biolabo) analyzer; and (7) Real-time PCR was performed on COBAS TaqMan 48 PCR analyzer, using Roche COBAS Ampliprep Taqman Kit.

Drug administration

After performing the baseline evaluation, all patients received one capsule of *N. sativa* seed oil (450 mg) available as soft gelatin capsules (Baraka; Pharco Pharmaceuticals) three times daily after meals continuously for 3 mo. Patients were followed up every 2 wk throughout the study period for assessing treatment adherence, tolerability and incidence of adverse reactions.

Statistical analysis

Statistical analysis was performed using SPSS version 17 software. Numerical data were summarized using means and standard deviations or medians and ranges. Categorical data were summarized as percentages. Differences between numerical variables over two time measurements were tested using paired *t* test or medians test for non-normally distributed data. Repeated measures analysis of variance was used to test differences between three-time numerically normally distributed variables and Friedman test was used for non-normally distributed variables. McNemar's test was used to compare categorical data overtime. All *P* values were two-sided, and *P* < 0.05 was considered significant. All authors had access to the study data and reviewed and approved the final manuscript.

RESULTS

Thirty patients (16 male, 14 female) with a mean age of 47 ± 10.2 years fulfilled the inclusion criteria and were enrolled in the study. Four of those patients (13.33%) had diabetes and 26 (86.67%) did not. Fifteen patients (30%) had chronic liver disease, five (16.7%) had com-

Table 2 Laboratory data assessment at baseline and after treatment

| Parameter | Base line | After 3 mo treatment | <i>P</i> value |
|-----------------------------------|-------------------|----------------------|----------------|
| Hemoglobin (g%) | 11.8 ± 2.1 | 12.2 ± 2.2 | 0.1 |
| RBCs (× 10 ⁶ /μL) | 4.13 ± 0.9 | 4.3 ± 0.9 | 0.001 |
| WBCs (× 10 ³ /μL) | 6.4 ± 2.1 | 5.6 ± 2.2 | 0.013 |
| Platelets (× 10 ³ /μL) | 167.7 ± 91.2 | 198.5 ± 103 | 0.004 |
| Hematocrit (%) | 35.5 ± 6.3 | 37.3 ± 6.3 | 0.056 |
| ALT (IU/L) | 35.0 ± 15.7 | 41 ± 24.4 | 0.255 |
| AST (IU/L) | 40.9 ± 30.4 | 46.8 ± 32.2 | 0.307 |
| Total protein (g/dL) | 7.1 ± 0.7 | 7.5 ± 0.8 | 0.001 |
| Albumin (g/dL) | 3.5 ± 0.9 | 3.69 ± 0.9 | 0.008 |
| Direct bilirubin (mg/dL) | 0.5 ± 0.8 | 0.57 ± 1.5 | 0.745 |
| Total bilirubin (mg/dL) | 1.46 ± 1.5 | 1.36 ± 1.3 | 0.428 |
| Prothrombin time (s) | 14.1 ± 2.7 | 13.8 ± 2.2 | 0.562 |
| INR | 1.18 ± 0.2 | 1.2 ± 0.2 | 0.974 |
| BUN (mg/dL) | 13.5 ± 6.2 | 14.1 ± 5 | 0.540 |
| Creatinin (mg/dL) | 0.99 ± 0.4 | 0.88 ± 0.2 | 0.102 |
| Serum AFP (IU/mL) | 5.07 ± 1.8 | 4.67 ± 2.3 | 0.194 |
| Sodium (mmole/L) | 135.5 ± 6.1 | 133.5 ± 6 | 0.064 |
| Potassium (mmole/L) | 4.1 ± 0.5 | 4 ± 0.5 | 0.350 |
| TAC (mmol/L) | 1.35 ± 0.5 | 1.61 ± 0.6 | 0.001 |
| Fasting blood sugar (mg/dL) | 104.03 ± 43.4 | 92.1 ± 31.3 | 0.001 |
| Post prandial blood sugar (mg/dL) | 143.67 ± 72.6 | 112.1 ± 42.9 | 0.001 |
| PCR (copies) | 380808.7 ± 610937 | 147028.2 ± 475225.6 | 0.001 |

Paired *t*-test for all parameters, median test (equivalent to Wilcoxon matched pairs test) for polymerase chain reaction (PCR) levels. RBCs: Red blood cells; WBCs: White blood cells; ALT: Alanine aminotransferase; AST: Aspartate aminotransferase; INR: International normalized ratio; BUN: Blood urea nitrogen; AFP: α-fetoprotein; TAC: Total antioxidant capacity.

pensated cirrhosis, and 10 (33.3%) had decompensated cirrhosis.

Patients' clinical assessment data before and after treatment are presented in Table 1. After treatment, there was a significant decrease in the percentage of patients with lower-limb edema, while there was no change in the percentage of patients with jaundice, palmar erythema, spider naevi or ascites. Laboratory parameters before and after treatment are presented in Table 2.

Liver functions tests

After 3 mo of *N. sativa* treatment, the mean HCV RNA levels (PCR) (147028.2 ± 475225.6) significantly decreased relative to their baseline levels (380808.7 ± 610937, *P* = 0.001) (Figure 1A). Table 3 presents the PCR responses after 3 mo treatment in patients with chronic liver disease and compensated and decompensated cirrhosis. Figure 2 presents individual patients' HCV RNA (PCR) values before and after treatment. Table 4 presents the Child-Pugh score and PCR response at baseline and after 3 mo in patients with compensated and decompensated cirrhosis. All cirrhotic patients (compensated and decompensated) showed no change or an improvement in their Child-Pugh score, patients presented with variable Child-Pugh score, yet the proportions' numbers were small for a valid statistical test. There was a significant increase in total

Table 3 Polymerase chain reaction response after treatment *n* (%)

| | |
|--------------------------|----------------|
| Total responders | 5 (16.67) |
| Chronic liver disease | 3 |
| Compensated cirrhosis | 1 |
| Decompensated cirrhosis | 1 |
| Total partial responders | 15 (50) |
| Chronic liver disease | 5 |
| Compensated cirrhosis | 4 |
| Decreased 1 log | 1 |
| Decreased 2 log | 3 |
| Decompensated cirrhosis | 6 ¹ |
| Total non-responders | 10 (33.33) |
| Chronic liver disease | 7 |
| Compensated cirrhosis | |
| Decompensated cirrhosis | 3 |

¹Patients decreased polymerase chain reaction (PCR) but in same log. Non-responders: Patients did not show a decrease or showed an increase in PCR after 3 mo treatment with *Nigella sativa* (*N. sativa*); Responders: Patients became seronegative after 3 mo treatment with *N. sativa*; Partial responders: Patients showed a decrease in PCR but were still seropositive after 3 mo treatment with *N. sativa*.

protein and albumin levels after treatment. However, there was no significant change in liver enzymes (AST and ALT), bilirubin, or INR. Renal function did not show a significant change from baseline. TAC showed a significant increase after treatment (1.612 ± 0.56) relative to the baseline values (1.35 ± 0.05 , $P = 0.001$, Figure 1B). Hematological functions varied significantly after 3 mo of *N. sativa* treatment. There was a significant increase in RBCs ($P = 0.001$) and platelets ($P = 0.004$) and a significant decrease ($P = 0.013$) in white blood cells.

Blood glucose

There was a significant decrease in both fasting and post-prandial blood glucose after treatment ($P = 0.001$).

Incidence of side effects and drug interactions

The reported side effects throughout the study period were gastritis in one patient (3.33%) and hypoglycemia in five (16.76 %); of whom two had insulin-dependent diabetes, and the other three had advanced liver cirrhosis with possible glycogen depletion. Both side effects were treated and did not hinder completion of therapy. The only reported drug interaction was hypoglycemia due to concurrent use of insulin and *N. sativa*, which aggravated its hypoglycemic effects.

DISCUSSION

The main findings of our study were that administration of *N. sativa* significantly decreased HCV viral load, increased total antioxidant activity and total protein and albumin levels, lowered blood glucose levels, and improved lower-limb edema.

The anti-inflammatory, antiviral and antineoplastic activities of *N. sativa* have been previously documented in various *in vitro* and *in vivo* studies^[14]. In the current

Table 4 Child-Pugh score at baseline and after 3 mo in patients with compensated and decompensated cirrhosis

| Patients | Child-Pugh score at baseline | Child-Pugh score after 3 mo of treatment | HCV RNA (PCR) response |
|----------|------------------------------|--|------------------------|
| 1 | B | B | Partial responder |
| 2 | C | B | Partial responder |
| 3 | A | A | Partial responder |
| 4 | B | B | Partial responder |
| 5 | A | A | Partial responder |
| 6 | C | C | Partial responder |
| 7 | C | B | Non-responder |
| 8 | C | B | Partial responder |
| 9 | A | A | Responder |
| 10 | B | B | Non-responder |
| 11 | C | B | Partial responder |
| 12 | B | A | Responder |
| 13 | C | B | Non-responder |
| 14 | A | A | Partial responder |
| 15 | A | A | Partial responder |

HCV: Hepatitis C virus; PCR: Polymerase chain reaction.

study, *N. sativa* administration resulted in a significant decrease in viral load, with 16.67% of patients becoming seronegative, and 50% showing a significant decrease in the quantitative viral count. Among these, 66.7% had cirrhosis and 33.3% had chronic liver disease, implying antiviral activity. Patients with compensated and decompensated cirrhosis, either improved or maintained their baseline clinical condition and viral load, and none of them deteriorated, which signified the potential beneficial effects of *N. sativa* administration, as reflected by improvement in HCV RNA responses and clinical condition reflected in Child-Pugh class. Although the subcategory of cirrhosis patients was not large enough to detect significance, we recommend that larger studies should be conducted in patients with cirrhosis to confirm the potential beneficial effects offered by *N. sativa*, which might improve patients' overall outcome. To the best of our knowledge, this is the first human study to evaluate the effects of *N. sativa* on viral load in patients with HCV infection. Our findings of improved viral load could be explained by the results of a previous study of murine CMV^[20], which showed a significant increase in macrophages and CD4⁺ T cells, with a significant decrease in viral titer and increased serum IFN- γ levels in animals treated with *N. sativa*^[24].

Oxidative-stress-related molecules have been shown to modulate cellular events responsible for the progression of liver fibrosis^[10,11]. Moreover, HCV-related fibrosis, cirrhosis and liver failure have been found to be the result of an adaptive immune response to HCV-infected cells^[25], which is mediated by induction of endoplasmic reticulum and oxidative stress and downregulation of antiapoptotic proteins nuclear factor- κ B and Bcl-xl in infected hepatocytes^[26].

In our study, *N. sativa* administration significantly increased TAC in HCV patients, implying the potential protective effect of *N. sativa* by halting the oxidative stress that contributes to disease progression. Further-

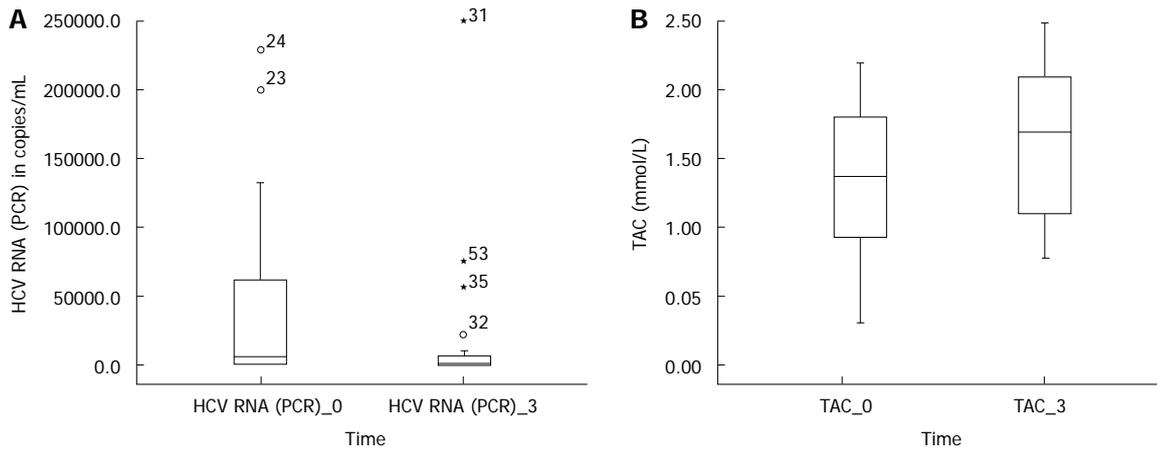


Figure 1 Box plot for hepatitis C virus RNA (polymerase chain reaction) levels (A), total antioxidant capacity (B) before and after treatment. A: Median test (equivalent to Wilcoxon matched pairs test), $P < 0.001$. Hepatitis C virus (HCV) RNA [(polymerase chain reaction (PCR))_0: PCR values of patients before treatment; HCV RNA (PCR)_3: PCR values of patients after 3 mo treatment; B: Paired t test, $P < 0.001$. Total antioxidant capacity (TAC)_0: TAC levels of patients before treatment; TAC_3: TAC levels of patients after 3 mo treatment.

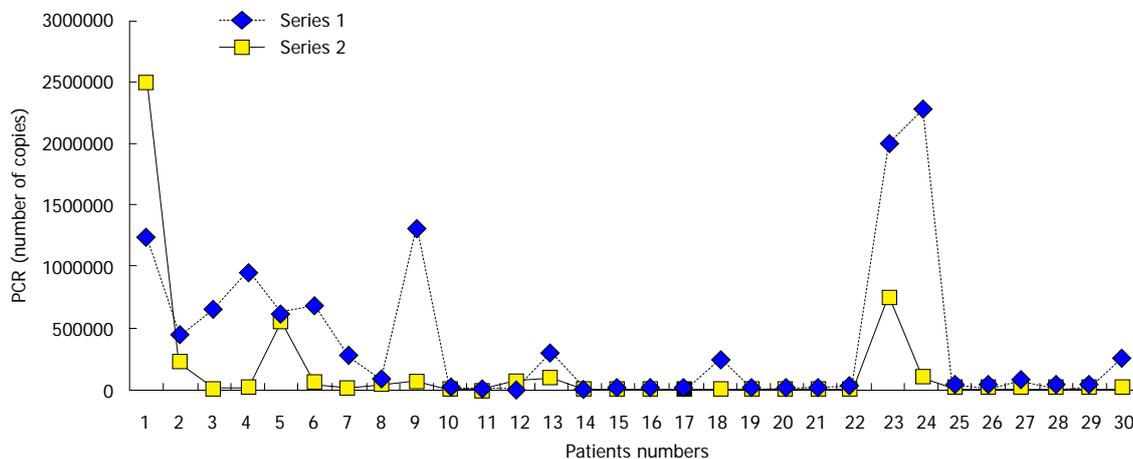


Figure 2 Line plot for polymerase chain reaction levels in individual patients at baseline and after 3 mo of treatment. Series 1: Polymerase chain reaction (PCR) values in all patients at baseline; Series 2: PCR values in all patients after 3 mo of treatment.

more, it is tempting to propose that increasing antioxidant capacity, with its cytoprotective role, contributed to decreasing the viral load.

The antioxidant effects of *N. sativa* have been previously elaborated in animal models of liver ischemia, in which it improved the antioxidant capacity and reduced oxidative stress^[27]. Moreover, *N. sativa* increased hepatic glutathione and reduced elevated hepatic serum enzymes in carbon-tetrachloride-treated mice, ameliorating its hepatotoxic potential^[15,28].

Some patients with acute and chronic liver disease develop diabetes mellitus^[29,30]. HCV infection may also contribute to the development of diabetes, which has been observed in 21% of HCV-infected patients^[31], and glucose intolerance has been seen in patients with HCV infection, compared with controls with liver diseases^[32-35].

Insulin resistance is one of the pathological features in patients with HCV infection that may be associated with life-threatening complications, making HCV-associated insulin resistance a therapeutic target at any stage of

HCV infection^[36].

Our study showed that *N. sativa* treatment significantly decreased blood glucose levels in HCV patients, implying that it might offer a potential modulatory effect on HCV-induced glucose intolerance. This effect was beneficial in the control of diabetes in HCV patients because it allowed us to lower the insulin requirement. Similar results have been previously shown in a study of patients with diabetes, in whom administration of *N. sativa* (2 g/d) caused significant reductions in fasting blood glucose and 2-h postprandial blood glucose and hemoglobin A1c, and improved insulin resistance^[37].

HCV infection itself can induce autoimmune hemolytic anemia, leukopenia, and thrombocytopenia, even in the absence of IFN- α treatment^[38-42]. Hematopoietic growth factors modulating these complications have shown a beneficial role in HCV patients^[43].

N. sativa therapy in our study significantly improved RBC and platelet counts in HCV patients, indicating a potential amelioration/prevention of HCV-induced

hematological disorders. Hence, *N. sativa* may positively affect clinical outcome in HCV patients.

The ability of *N. sativa* to improve hematological indices has also been reported in animal studies in which it increased both the packed cell volume and hemoglobin in treated rats^[18], as well as increased RBC count in diabetic rabbits^[44]. The increased RBC count was attributed to lowering of the membrane lipid peroxide level, leading to decreased susceptibility to hemolysis.

Serum albumin is the most abundant plasma protein^[45] and is essential for maintaining oncotic pressure of the vascular system^[46]. Chronic HCV patients may suffer a decrease in serum albumin level^[47], and improvement in hypoalbuminemia has been shown to improve prognosis^[48] and quality of life^[49]. Concentrations of < 30 g/L were associated with an 85% chance of liver-related complications at 5 years and a 3-year mortality of 70%^[50], and was predictive of morbidity and mortality in patients with liver cirrhosis^[51,52].

In the current study, *N. sativa* administration significantly increased serum albumin levels and significantly reduced lower-limb edema, indicating an improvement in clinical condition. Prior animal studies have shown similar effects in rats^[53] and broiler chickens^[54] in a dose-dependent manner^[55].

N. sativa is used in Arab folk medicine as a diuretic plant^[56], the mechanism that can also contribute to its efficacy in decreasing lower limb edema, and its resolution in many patients.

In our study, the number of patients with ascites decreased after treatment with *N. sativa*, although the change was not significant; nevertheless, the change in ascites severity could not be totally denied, because the degree of ascites was not assessed sonographically. We hence recommend assessment of ascites incidence and severity in future studies to confirm these results.

The safety and tolerability of *N. sativa* have been previously documented in various clinical trials^[57-60]. However, to date, clinical studies addressing *N. sativa* efficacy, safety and tolerability in HCV patients are lacking. Our study has shown that *N. sativa* was tolerable in all patients, and the only side effects reported were one patient with epigastric pain that was controlled with antacids, and five patients with hypoglycemia, two of whom had diabetes and were receiving concomitant insulin and the hypoglycemia did not recur after decreasing the insulin dose. Of note, the dose of *N. sativa* used in the current study was (1.35 g/d), which was slightly lower than in the other studies - 2 g/d used by Bamosa *et al.*^[37] - because this dose was available in the Egyptian market and was close to the doses previously used. Although *N. sativa* in such patients had significantly positive effects on many parameters, perhaps higher doses or longer durations of therapy may accentuate such appreciable effects. Further studies are needed to confirm such findings.

It can therefore be concluded that *N. sativa* administration can have a potential beneficial effect on HCV disease progression and outcome through its prominent antiviral, antioxidant and immunomodulatory effects and

can minimize HCV-related hematological complications.

Our study had some limitations. This was the first clinical study to be performed in HCV patients and larger studies are required to confirm the results of the current study. We did not assess all patients for the amount of ascites after therapy sonographically, because such a favorable effect of *N. sativa* was not anticipated. Hence, in view of significant improvement of serum albumin, this effect of *N. sativa* on the amount of ascites needs further study. Liver biopsy was not performed, because the patients were either not eligible or refused the procedure.

In conclusion, *N. sativa* administration in HCV patients is safe and tolerable and results in a significant improvement in viral load, oxidative stress and laboratory markers. Moreover, the clinical improvement and better glycemic control in patients with diabetes indicate a potential role for *N. sativa* in improving the clinical outcome of HCV patients. We recommend larger controlled multicenter randomized studies for longer periods for evaluation of the potential beneficial role of *N. sativa* in HCV patients with and without concurrent IFN therapy.

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COMMENTS

Background

Hepatitis C virus (HCV) is an important etiological factor for the development of hepatocellular carcinoma. Pegylated interferon- α (PEG-IFN- α) and ribavirin treatment are the only currently approved therapy for HCV with variable response rate, and a success that is heavily influenced by patients' response rate, adherence to treatment, and tolerance to side effects. Moreover, the financial constraints for the combined therapy in many patients often contribute to their non-adherence to therapy, potentially lowering its success rates. *Nigella sativa* (*N. sativa*), a food condiment used in the Middle East, has shown anti-inflammatory, antiviral, antioxidant and anticancer activities in various *in vitro* and *in vivo* studies. To date, no studies have addressed the use of *N. sativa* in HCV patients and its potential benefits.

Research frontiers

N. sativa is a natural food supplement, and has shown beneficial antioxidant, antiviral, anticancer and immunopotentiating properties in various *in vitro* and *in vivo* studies, but HCV studies are lacking. In exploring the potential role of *N. sativa* in improving HCV patients' clinical outcome, the research hot spot is its beneficial effects on reducing viral load, improving antioxidant capacity, alleviating hematological parameters, and improving blood glucose control, especially in diabetes. All of which could have a potential beneficial effect on HCV patients' responses and amelioration of HCV-related complications.

Innovations and breakthroughs

No prior clinical trials in HCV patients have evaluated the use of *N. sativa* and its potential beneficial effects. No studies have addressed any alternative treatments for IFN non-eligible patients or those who refuse or cannot tolerate IFN therapy. *N. sativa* offers hope for a safe tolerable alternative to those patients who cannot tolerate IFN or have a contraindication to its use. Moreover, *N. sativa* has a potential benefit in improving clinical outcome. It showed a preliminary improvement in viral load and antioxidant levels that could provide a potential cure for HCV infection. *N. sativa* also improved the hematological profile and to-

tal protein and albumin levels, which contribute to HCV-induced complications. Moreover, *N. sativa* decreased blood glucose levels, and hence decreased insulin requirement in patients with diabetes.

Applications

The study results suggest that *N. sativa* is a potentially beneficial, safe and tolerable alternative in IFN non-eligible HCV patients. It can improve clinical outcome, ameliorate HCV-induced hematological and diabetic complications, and improve lower-limb edema.

Terminology

Viral load, also known as viral burden or viral titer, is a measure of the severity of a viral infection, and can be calculated by estimating the amount of virus in an involved body fluid, for example, RNA copies/mL blood plasma. Human serum albumin is the most abundant protein in human blood plasma. It is produced in the liver and constitutes about half of the blood serum protein. It transports hormones, fatty acids, and other compounds, buffers pH, and maintains osmotic pressure, among other functions. Total antioxidant capacity measures collectively the amount of antioxidant components of the body that reflects the body's capacity to combat oxidative stress.

Peer review

This was an interesting study in which the authors treated HCV patients with *N. sativa*, a food condiment used in the Middle East.

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ABO blood type, long-standing diabetes, and the risk of pancreatic cancer

Naoto Egawa, Yingsong Lin, Taku Tabata, Sawako Kuruma, Seiichi Hara, Ken Kubota, Terumi Kamisawa

Naoto Egawa, Taku Tabata, Sawako Kuruma, Seiichi Hara, Ken Kubota, Terumi Kamisawa, Department of Internal Medicine, Tokyo Metropolitan Komagome Hospital, Tokyo 113-8677, Japan

Naoto Egawa, Department of Internal Medicine, Tokyo Metropolitan Matsuzawa Hospital, Tokyo 156-0057, Japan

Yingsong Lin, Department of Public Health, Aichi Medical University School of Medicine, Aichi 480-1195, Japan

Author contributions: Egawa N designed the research; Egawa N and Lin Y analyzed the data and wrote the paper; Tabata T, Kuruma S, Hara S, Kubota K and Kamisawa T collected the data. Correspondence to: Dr. Naoto Egawa, Department of Internal Medicine, Tokyo Metropolitan Matsuzawa Hospital, 2-1-1 Kamikitazawa, Setagaya-ku, Tokyo 156-0057, Japan. naoto_egawa@tmhp.jp

Telephone: +81-3-33037211 Fax: +81-3-33045331

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blood type O. Compared with the non-DM group, the DM group had a higher frequency of blood type B [odds ratio (OR) = 2.61, 95%CI: 1.24-5.47; reference group: blood type A]. Moreover, male (OR = 3.17, 95%CI: 1.67-6.06), older than 70 years of age (OR = 2.19, 95%CI: 1.20-3.98) and presence of a family history of diabetes (OR = 6.21, 95%CI: 3.38-11.36) were associated with long-standing type 2 diabetes. The mean ages were 64.8 ± 9.2 years, 67.1 ± 9.8 years, and 71.7 ± 7.0 years in the subgroups with the duration of diabetes, 3-5 years, 5.1-14.9 years, and 15 years or more, respectively ($P = 0.007$). A comparison of ABO blood type distribution among the subgroups also showed a significant difference ($P = 0.03$).

CONCLUSION: The association of pancreatic cancer with blood type and duration of diabetes needs to be further examined in prospective studies.

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Key words: Pancreatic cancer; ABO blood type; Diabetes mellitus; Risk factor; Screening

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Abstract

AIM: To retrospectively study pancreatic cancer patients with respect to their ABO blood type and diabetes.

METHODS: Our analysis included a cohort of 1017 patients with pancreatic ductal cancer diagnosed at our hospital in Tokyo. They were divided into two groups: 114 patients with long-standing type 2 diabetes (DM group, defined as diabetes lasting for at least three years before the diagnosis of pancreatic cancer) and 903 patients without diabetes (non-DM group). Multivariate analysis was performed to identify factors that are associated with long-standing diabetes. The DM group was further divided into three subgroups according to the duration of diabetes (3-5 years, 5.1-14.9 years, and 15 years or more) and univariate analyses were performed.

RESULTS: Of the 883 pancreatic cancer patients with serologically assessed ABO blood type, 217 (24.6%) had

INTRODUCTION

Pancreatic cancer is the fifth leading cause of cancer deaths in Japan, accounting for approximately 26000 deaths each year^[1]. Because of the poor prognosis, identifying high-risk individuals and the modifying risk factors are important strategies for preventing pancreatic cancer. Despite intensive research efforts, the etiology of spo-

radic pancreatic cancer remains largely unknown. Epidemiologic studies have consistently shown that smoking and long-standing type 2 diabetes are two modifiable risk factors for pancreatic cancer^[2,3]. The association between diabetes and pancreatic cancer is complex because diabetes and pancreatic cancer development may involve a similar pathogenesis and share common risk factors, such as obesity, smoking and insulin resistance. Moreover, although long-standing type 2 diabetes is a risk factor for pancreatic cancer, new-onset diabetes may also result from pancreatic cancer^[4,5]. There is a lack of data on the proportion of pancreatic cancer cases that can be attributed to long-standing diabetes and the prevalence of pancreatic cancer-induced new-onset diabetes.

In addition, although an association between the ABO blood type and various diseases was proposed 50 years ago^[6,7], the ABO blood type has recently been confirmed to be associated with malignant tumors, including skin cancer^[8], esophageal cancer^[9], hepatocellular carcinoma^[10] and pancreatic cancer^[11-16]. Regarding pancreatic cancer risk, both epidemiologic^[11-15] and genome-wide association studies (GWAS)^[16] showed that individuals carrying the O blood type had the lowest risk compared with those with non-O blood types.

Although diabetic patients may represent a high-risk group for pancreatic cancer, the increasing prevalence of type 2 diabetes in the general population and the lack of specific biomarkers do not justify screening all diabetic patients for the early detection of pancreatic cancer. It is possible that among diabetic patients, a subset of diabetics who are at high risk of developing pancreatic cancer may show different characteristics from other diabetics, including the duration of diabetes and blood type distribution. In this study, we retrospectively examined 1017 patients with pancreatic cancer, focusing on the duration of type 2 diabetes and the ABO blood type.

MATERIALS AND METHODS

Patients

We reviewed the medical records of patients with pancreatic ductal cancer diagnosed between 1975 and 2009 at Tokyo Metropolitan Komagome Hospital. A total of 1022 patients were included in the present analysis. Overall, 66.3% had histological confirmation, and the remaining patients were diagnosed based on either endoscopic retrograde cholangiopancreatography or at least two imaging modalities. To exclude the possibility that new-onset diabetes was caused by pancreatic cancer, we defined individuals with long-standing diabetes as those who had diabetes for at least 3 years before the diagnosis of pancreatic cancer. Among the 1022 patients, we excluded 5 patients with long-standing diabetes due to diagnoses other than type 2 diabetes.

The subjects were divided into two groups: 114 patients with long-standing type 2 diabetes (DM group) and 903 patients without long-standing type 2 diabetes (non-DM group). Furthermore, we classified the DM group into 3 subgroups according to the duration of

preexisting diabetes: a relatively short period of 3-5 years (DM-S group: 31 patients), a medium range of 5.1-14.9 years (DM-M group: 48 patients), and a relatively long period of 15 years or more (DM-L group: 35 patients). Information on gender, age, smoking status, ABO blood type, diabetes, a family history of diabetes and tumor location was recorded from medical charts. The ABO blood type was assessed serologically, and the information of DM was primarily based on self-report. For 92 patients in the DM group, their medical history revealed the type of medical treatment that they had received for diabetes.

This study was approved by the Institutional Review Board of Tokyo Metropolitan Komagome Hospital.

Statistical analysis

First, age, gender, smoking status (never *vs* former or current), a family history of diabetes (present *vs* absent in a first-degree relative), the location of the cancer (head *vs* body or tail) and the ABO blood type were compared using univariate analysis. A two-sample *t*-test was conducted with age as a continuous variable. A χ^2 test was used for categorical variables. The unconditional logistic regression method was used to compare the DM group with the non-DM group using variables that showed a *P* value of less than 0.15 in the univariate analyses. Variables for which the *P* value exceeded 0.05 were eliminated in a stepwise fashion such that only those that had a statistically significant association with long-standing type 2 diabetes were included in the final regression model. In this analysis, blood type A was used as a reference group. The final models were evaluated for goodness-of-fit with the Hosmer-Lemeshow test.

Similar to the analyses mentioned above, we performed univariate analyses among 3 subgroups of the DM group. A one-way analysis of variance was conducted for continuous variables, and a χ^2 test was used for categorical variables.

We used the χ^2 test to compare the ABO blood type distribution in our pancreatic cancer patients with the distribution reported from a nationally representative sample of the Japanese population^[17].

All of the *P* values were two-sided, with statistical significance set at *P* < 0.05. All of the statistical analyses were performed using the SPSS (Statistical Package for the Social Sciences) 19 statistical package software (IBM Japan, Tokyo).

RESULTS

Table 1 shows the characteristics of the DM group and the non-DM group and the results of univariate analyses and multivariate analysis. The sex ratio (male/female) was significantly higher in the DM group than in the non-DM group (*P* = 0.002). The mean age of the DM group was 1.8 years older at the diagnosis of pancreatic cancer than the non-DM group (67.9 ± 9.2 years *vs* 66.1 ± 10.6 years, *P* = 0.08). In accordance with this result, there were more patients older than 70 years of age

Table 1 Characteristics of the diabetes mellitus group and the non-diabetes mellitus group *n* (%)

| Variables | DM group (<i>n</i> = 114) | Non-DM group (<i>n</i> = 903) | <i>P</i> value | OR (95%CI) |
|-------------------------------|-------------------------------|-----------------------------------|-------------------|-------------------|
| Sex | | | 0.002 | |
| Female | 33 (28.9) | 398 (44.1) | | Reference |
| Male | 81 (71.1) | 505 (55.9) | | 3.17 (1.67-6.06) |
| Age (yr) | | | 0.009 | |
| < 70 | 53 (46.5) | 541 (59.9) | | Reference |
| ≥ 70 | 61 (53.5) | 362 (40.1) | | 2.19 (1.20-3.98) |
| Smoking status | <i>n</i> = 99 ¹ | <i>n</i> = 715 | 0.28 | |
| Former and current smokers | 60 (60.6) | 392 (54.8) | | |
| Non-smokers | 39 (39.4) | 323 (45.2) | | |
| Tumor location | | | 0.37 | |
| Head | 58 (50.9) | 501 (55.5) | | |
| Body/tail | 56 (49.1) | 402 (44.5) | | |
| Family history of DM | <i>n</i> = 76 | <i>n</i> = 393 | < 0.001 | |
| No | 41 (53.9) | 335 (85.2) | | Reference |
| Yes | 35 (46.1) | 58 (14.8) | | 6.21 (3.38-11.36) |
| ABO blood type | <i>n</i> = 104 | <i>n</i> = 779 | 0.06 | |
| A | 35 (33.7) | 338 (43.4) | | Reference |
| B | 28 (26.9) | 175 (22.5) | | 2.61 (1.24-5.47) |
| O | 34 (32.7) | 183 (23.5) | | 1.92 (0.95-3.87) |
| AB | 7 (6.7) | 83 (10.7) | | 0.94 (0.28-3.14) |

¹Because of missing data, the numbers of subjects are presented for smoking status, family history of diabetes, and ABO blood type in each group. Diabetes mellitus (DM) group represents pancreatic cancer patients with long-standing type 2 diabetes, defined as diabetes lasting at 3 years prior to the diagnosis of pancreas cancer. Non-DM group represents pancreatic cancer patients without long-standing type 2 diabetes. OR: Odds ratio determined by the logistic regression method.

in the DM group than the non-DM group ($P = 0.009$). Subjects in the DM group were more likely to have a family history of diabetes than those in the non-DM group ($P < 0.001$). The distribution of the ABO blood type seemed to differ between the DM group and non-DM group ($P = 0.06$). There were no significant differences in the smoking status ($P = 0.28$) or tumor locations ($P = 0.37$) between the two groups.

The logistic regression method using candidate variables resulting from the univariate analyses revealed that sex, age, a family history of diabetes and ABO blood type were associated with long-standing type 2 diabetes. Interestingly, the frequency of blood type B was significantly higher in the DM group than in the non-DM group (Table 1). The Hosmer-Lemeshow test showed that the regression model had an acceptable goodness-of-fit ($P > 0.05$).

There were no significant differences in gender, smoking status, a family history of diabetes or the location of cancer among the 3 subgroups defined by the duration of diabetes. However, significant differences in age ($P = 0.007$) and the ABO blood type ($P = 0.03$) were observed among the 3 subgroups (Table 2). We found a significant difference in the ABO blood type distribution between our pancreatic cancer patients and the general Japanese population^[17] ($P = 0.02$). As shown in Table 3, our patients had a lower frequency of blood type O and a higher frequency of blood type A.

Table 2 Comparison of characteristics among 3 diabetes mellitus subgroups according to duration of diabetes

| Variables | DM-S (<i>n</i> = 31) | DM-M (<i>n</i> = 48) | DM-L (<i>n</i> = 35) | <i>P</i> value |
|-------------------------------|----------------------------|--------------------------|--------------------------|-------------------|
| Sex ratio (M/F) | 2.44 | 2.69 | 2.18 | 0.91 |
| Age, yr (mean ± SD) | 64.8 ± 9.2 | 67.1 ± 9.8 | 71.7 ± 7.0 | 0.007 |
| ≥ 70 | 41.9% | 47.9% | 71.4% | 0.03 |
| Smoking status | <i>n</i> = 26 ¹ | <i>n</i> = 43 | <i>n</i> = 30 | 0.12 |
| Former and current smokers | 50.0% | 72.1% | 53.3% | |
| Tumor location: Head | 54.8% | 47.9% | 51.4% | 0.83 |
| Family history of DM | <i>n</i> = 16 | <i>n</i> = 36 | <i>n</i> = 24 | 0.64 |
| Positive family history of DM | 56.3% | 44.4% | 41.7% | |
| ABO blood type | <i>n</i> = 26 | <i>n</i> = 44 | <i>n</i> = 34 | 0.03 |
| A | 19.2% | 52.3% | 20.6% | |
| B | 26.9% | 18.2% | 38.2% | |
| O | 42.3% | 27.3% | 32.4% | |
| AB | 11.5% | 2.3% | 8.8% | |

¹Because of missing data, the numbers of subjects are presented for smoking status, family history of diabetes and ABO blood type in each group. F: Female; M: Male; DM: Diabetes mellitus; DM-S: Patients with diabetes of 3-5 years; DM-M: Patients with diabetes of 5.1-14.9 years; DM-L: Patients with diabetes of 15 years or more.

Table 3 Comparison of the distribution of ABO blood type between our cases and the general Japanese population *n* (%)

| ABO blood type | Our pancreatic cancer patients (<i>n</i> = 883) | General Japanese population (<i>n</i> = 4465349) ¹ |
|----------------|---|---|
| A | 373 (42.2) | 1725950 (38.7) |
| B | 203 (23.0) | 988996 (22.2) |
| O | 217 (24.6) | 1305924 (29.3) |
| AB | 90 (10.2) | 444479 (10.0) |

¹The data was referred to Fujitas' article^[17].

DISCUSSION

In our retrospective examination of 1017 pancreatic cancer patients, we found that the distribution of the ABO blood type in our cases is different from that of the general Japanese population. Furthermore, the distribution of the blood type also seemed to differ between the DM group and the non-DM group, with the DM group having a higher frequency of blood type B. This finding suggests that long-standing type 2 diabetes and other underlying factors that are associated with diabetes, such as blood type, might play a role in predisposing diabetic patients to pancreatic cancer.

Because of the increasing prevalence of type 2 diabetes in the general population and the absence of specific biomarkers, it is not cost-effective to screen for pancreatic cancer in asymptomatic diabetics. Therefore, it is important to identify a subset of diabetics with a higher susceptibility to pancreatic cancer than other diabetics. We addressed this issue by focusing on the duration of diabetes and the ABO blood type.

It remains unclear whether the duration of diabetes significantly predicts pancreatic cancer risk. Previous studies have noted an inverse association between the duration of diabetes and the pancreatic cancer risk; the

association appeared to be strongest among individuals with a duration of diabetes less than 4 years, with a relative risk of 2.1 (95%CI: 1.9-2.3)^[3]. However, in a large Korean cohort study, the pancreatic cancer risk was significantly increased with an increasing duration of diabetes in men: the hazard ratios were 2.0, 2.4 and 3.0 for individuals with a duration of diabetes less than 4.9 years, 5.0-9.9 years, and 10 years or more, respectively^[18]. Despite the inverse association observed in a meta-analysis published in 2005, individuals with long-standing diabetes (> 5 years) were still at a 50% increased risk of pancreatic cancer^[3]. Interestingly, when we divided the DM group into 3 subgroups according to the duration of diabetes, we found that among long-standing diabetes-related pancreatic cancer cases, there may be several subgroups that are associated with a specific blood type and characterized by the period from the onset of diabetes to the occurrence of pancreatic cancer. This finding suggests that patients with long-standing type 2 diabetes might not be considered a single uniform group.

Regarding the ABO blood type, several lines of evidence in recent years have shown that the ABO blood type is associated with a risk of pancreatic cancer. A prospective cohort study noted an elevated risk of incidental pancreatic cancer among subjects with blood type A, AB or B compared with blood type O, and those with blood type B had the highest risk^[11]. In addition, they also reported increased risk with the addition of each non-O allele^[12]. A recent GWAS, which mainly involved Caucasian populations, identified an association between a single-nucleotide polymorphism (SNP) in the ABO gene locus (rs505922) and pancreatic cancer^[16]. Accordingly, an article by Nakao and co-workers, which is the only study on pancreatic cancer and the ABO blood type alleles in Japanese subjects, showed that the risk of pancreatic cancer was higher among those with the non-O blood type than those with the O blood type^[14]. The distribution of the ABO blood type in our overall pancreatic cancer patients was similar to that reported in the Nakao's article. In fact, when the ABO blood type distribution in our pancreatic cancer patients was compared with their cases^[14], univariate analysis with the chi-square test showed no difference between them ($P = 0.56$). Moreover, considering that the frequency of the O blood type in our patients was lower than that observed in the general Japanese population, our study provided indirect evidence that the O blood type may be associated with a lower risk of pancreatic cancer in Japanese people.

Another interesting finding is that the B blood type is more common in pancreatic cancer patients with long-standing type 2 diabetes than in those without diabetes. The association between the ABO blood type and diabetes is controversial. Advances in genome-wide sequencing have provided novel insights into the pathogenesis of diabetes mellitus. A recent GWAS showed that genetic variants in the ABO locus were associated with not only diabetes risk, with blood group B showing a decreased risk compared with blood group O^[19], but also the plasma levels of soluble intercellular adhesion molecule 1 and

soluble E selectin^[19-22], both of which are markers of inflammation and are thought to be related to the risk of type 2 diabetes mellitus^[23,24]. In addition, a SNP at the ABO locus was reported to be strongly associated with serum tumor necrosis factor alpha^[25], which is a pro-inflammatory cytokine that modulates rates of pancreatic ductal cell apoptosis^[26], and an adipocytokine that has been implicated in the development of insulin resistance^[27]. Although the mechanism underlying the association between ABO blood type, diabetes and pancreatic cancer has not been clarified, these findings suggest interactions among ABO blood types, inflammatory markers, type 2 diabetes and pancreatic cancer.

A major strength of this study is a large cohort of pancreatic cancer patients. Our study has several limitations. First, the major limitation is the lack of an appropriate control group comprising long-standing type 2 diabetes patients without pancreatic cancer. Although our finding showed that the B blood type is more common among pancreatic cancer patients with long-standing type 2 diabetes, a prospective cohort study of diabetics is warranted to confirm whether long-term diabetics with the B blood type have an increased risk of pancreatic cancer. Second, because the study subjects were selected from one hospital, the generalization of our results to other populations is unclear. As mentioned above, with regard to the distribution of the ABO blood type, pancreatic cancer cases in Nakao's study were comparable to ours. Thus, our subjects are not particularly unique. Third, the history of diabetes was mainly based on self-reporting, and the accuracy of the self-reported information is unknown. However, because it is unlikely that a patient would be forgetful regarding the minimum duration of 3 years, a self-report that the duration was 3 years or more than 3 years was likely to be reliable. Fourth, we cannot exclude the possibility that the significant differences observed in ABO blood types among the 3 subgroups were due to chance because of the small number of subjects in each subgroup. This issue warrants further examination in a larger population.

In summary, the retrospective examination of a large cohort of pancreatic cancer patients showed that the B blood type is more common in pancreatic cancer patients with long-standing type 2 diabetes than in those without diabetes. Further studies are needed to better define the set of factors associated with an increased susceptibility to pancreatic cancer in diabetic patients.

COMMENTS

Background

Pancreatic cancer is a dismal disease and refractory to almost all current therapies. Because of the poor prognosis, identifying high-risk individuals and modifying risk factors are important strategies for preventing pancreatic cancer. Currently, smoking habits and type 2 diabetes are well-known modifiable risk factors for pancreatic cancer. However, the prevalence of smoking and the increasing incidence of type 2 diabetes in the general population do not justify screening all subjects for the early detection of pancreatic cancer.

Research frontiers

Recently, there has been emerging evidence that the ABO blood type is associ-

ated with pancreatic cancer risk. A prospective cohort study noted an elevated risk of incidental pancreatic cancer among subjects with blood type A, AB or B compared with blood type O, and those with blood type B had the highest risk.

Innovations and breakthroughs

In their retrospective examination of a large cohort of pancreatic cancer patients, authors found that the distribution of the ABO blood type seemed to differ between patients with long-standing type 2 diabetes and those without, with the former showing a higher frequency of blood type B. In addition, when they divided the former group into 3 subgroups according to the duration of diabetes, they found that there may be several subgroups associated with a specific blood type and characterized by the duration of diabetes. These findings suggest that long-standing type 2 diabetes and other underlying factors, such as blood type and period of diabetes, may play a role in predisposing diabetic patients to pancreatic cancer.

Applications

Although their results should be replicated in prospective studies, they may be useful to define a subset of diabetics that is associated with increased susceptibility to pancreatic cancer.

Terminology

Long-standing type 2 diabetes: Type 2 diabetes represents a complex interaction between hereditary conditions and environmental factors and is essentially different from diabetes secondary to pancreatic cancer. Long-standing diabetes here is defined as diabetes for at least 3 years prior to the diagnosis of pancreatic cancer. This duration should be sufficient to rule out diabetes secondary to the tumor due to the rapid fatal course of pancreatic cancer.

Peer review

Nice retrospective study that is well supported by advanced statistical methodology. Extremely well written in idiomatic English, and limitations are appropriately described. All in all, a good study with marginal clinical relevance.

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Computed tomography findings for predicting severe acute hepatitis with prolonged cholestasis

Sang Jung Park, Jin Dong Kim, Yeon Seok Seo, Beom Jin Park, Min Ju Kim, Soon Ho Um, Chang Ha Kim, Hyung Joon Yim, Soon Koo Baik, Jin Yong Jung, Bora Keum, Yoon Tae Jeen, Hong Sik Lee, Hoon Jai Chun, Chang Duck Kim, Ho Sang Ryu

Sang Jung Park, Yeon Seok Seo, Soon Ho Um, Chang Ha Kim, Hyung Joon Yim, Jin Yong Jung, Bora Keum, Yoon Tae Jeen, Hong Sik Lee, Hoon Jai Chun, Chang Duck Kim, Ho Sang Ryu, Department of Internal Medicine, College of Medicine, Korea University, Seoul 136-705, South Korea

Jin Dong Kim, Department of Internal Medicine, Cheju Halla General Hospital, Jeju 690-766, South Korea

Beom Jin Park, Min Ju Kim, Department of Radiology, College of Medicine, Korea University, Seoul 136-705, South Korea

Soon Koo Baik, Department of Internal Medicine, Yonsei University Wonju College of Medicine, Wonju Christian Hospital, Wonju 220-701, South Korea

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Correspondence to: Soon Ho Um, MD, PhD, Department of Internal Medicine, College of Medicine, Korea University, No. 126-1 Anam-dong 5-ga, Seongbuk-gu, Seoul 136-705, South Korea. umsh@korea.ac.kr

Telephone: +82-2-9206608 Fax: +82-2-9531943

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Abstract

AIM: To evaluate the significance of computed tomography (CT) findings in relation to liver chemistry and the clinical course of acute hepatitis.

METHODS: Four hundred and twelve patients with

acute hepatitis who underwent enhanced CT scanning were enrolled retrospectively. Imaging findings were analyzed for the following variables: gallbladder wall thickness (GWT), arterial heterogeneity, periportal tracking, number and maximum size of lymph nodes, presence of ascites, and size of spleen. The serum levels of alanine aminotransferase, alkaline phosphatase, bilirubin, albumin, and prothrombin time were measured on the day of admission and CT scan, and laboratory data were evaluated every 2-4 d for all subjects during hospitalization.

RESULTS: The mean age of patients was 34.4 years, and the most common cause of hepatitis was hepatitis A virus (77.4%). The mean GWT was 5.2 mm. The number of patients who had findings of arterial heterogeneity, periportal tracking, lymph node enlargement > 7 mm, and ascites was 294 (80.1%), 348 (84.7%), 346 (84.5%), and 56 (13.6%), respectively. On multivariate logistic regression, male gender [odds ratio (OR) = 2.569, 95%CI: 1.477-4.469, $P = 0.001$], toxic hepatitis (OR = 3.531, 95%CI: 1.444-8.635, $P = 0.006$), level of albumin (OR = 2.154, 95%CI: 1.279-3.629, $P = 0.004$), and GWT (OR = 1.061, 95%CI: 1.015-1.110, $P = 0.009$) were independent predictive factors for severe hepatitis. The level of bilirubin (OR = 1.628, 95%CI: 1.331-1.991, $P < 0.001$) and GWT (OR = 1.172, 95%CI: 1.024-1.342, $P = 0.021$) were independent factors for prolonged cholestasis in multivariate analysis.

CONCLUSION: In patients with acute hepatitis, GWT on CT scan was an independent predictor of severe hepatitis and prolonged cholestasis.

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Key words: Acute hepatitis; Cholestasis; Computed tomography; Prognosis; Gallbladder

Core tip: Previous studies on the correlation between imaging and laboratory findings in acute hepatitis have shown conflicting results. This study revealed a correlation between abdominal computed tomography findings and liver biochemical parameters. In particular, gallbladder wall thickness (GWT) was the only independent imaging finding that predicts severe hepatitis and prolonged cholestasis. The results of this study suggest that GWT measurement, which is a relatively easy and objective procedure, is helpful to predict severe acute hepatitis or prolonged cholestasis.

Park SJ, Kim JD, Seo YS, Park BJ, Kim MJ, Um SH, Kim CH, Yim HJ, Baik SK, Jung JY, Keum B, Jeon YT, Lee HS, Chun HJ, Kim CD, Ryu HS. Computed tomography findings for predicting severe acute hepatitis with prolonged cholestasis. *World J Gastroenterol* 2013; 19(16): 2543-2549 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v19/i16/2543.htm> DOI: <http://dx.doi.org/10.3748/wjg.v19.i16.2543>

INTRODUCTION

The principal causes of acute hepatitis include viral infection, use of certain drugs, heavy alcohol consumption, chemicals and autoimmunity. In laboratory testing, an increase in serum aminotransferase provides a basis for diagnosis; however, other tests may be conducted to determine the cause of disease and assess severity and prognosis. Abdominal ultrasonography and computed tomography (CT) may help to exclude other conditions that resemble acute hepatitis, such as biliary obstruction, cirrhosis, malignant metastasis to the liver, and diseases that alter liver biochemistry^[1].

The general findings of abdominal ultrasonography in acute hepatitis include hepatomegaly, increased periportal echogenicity, decreased echogenicity in liver parenchyma and thickening of the gallbladder wall^[2]. Computed tomography may also reveal lymphadenopathy around the hepatoduodenal ligament, fatty deposits around the liver, gallbladder changes, periportal tracking, hepatomegaly, splenomegaly and fluid retention within the pelvis in patients with acute hepatitis A^[3].

The purpose of this study was to evaluate the significance of CT findings in relation to liver chemistry and the clinical course in patients with acute hepatitis.

MATERIALS AND METHODS

Patients

We conducted this study through a retrospective review of the medical records of 435 patients who had been hospitalized with acute hepatitis and examined with CT from January 2006 to May 2010. Patients who had previous chronic diseases, such as congestive heart failure, pulmonary diseases, chronic renal insufficiency, or uncontrolled diabetes mellitus, were not included. Twenty-three patients were also excluded because of a lack of relevant

clinical chemistry findings or CT imaging obtained without contrast medium. The patients were treated with general symptomatic care with proper hydration and hepatotonic. The clinical and CT data of the 412 eligible patients were analyzed for this study. The cause of acute hepatitis was determined through obtaining a thorough medical history of alcohol consumption, drug use, and coexisting diseases, performing various serological and polymerase chain reaction (PCR) assays to diagnose a variety of viral, bacterial and protozoal infections of the liver caused by hepatitis viruses, cytomegalovirus, Epstein-Barr virus, human immunodeficiency virus, *Toxoplasma*, *Leptospira*, *Candida*, *Mycobacteria*, *Brucella*, pneumocystis, and if necessary, special biochemical tests for metabolic or hereditary hepatic diseases, including serum ceruloplasmin and 24-h urine copper quantification for Wilson's disease. The institutional review board approved this study and waived the written informed consent requirement.

Laboratory examinations

The serum levels of alanine aminotransferase (ALT), alkaline phosphatase (ALP), bilirubin, albumin and prothrombin time (PT) were determined with a conventional autoanalyzer using commercial reagents on the day of admission and CT scan. These same examinations were repeated every 2-4 d for all subjects during hospitalization.

Diagnosis and definitions

The diagnosis of viral hepatitis was based on the positivity of hepatitis A virus, hepatitis B virus, or hepatitis C virus (HCV) markers. The laboratory criteria for confirming each type of acute hepatitis were as follows: acute hepatitis A, positive immunoglobulin M (IgM) antibody to hepatitis A virus; and acute hepatitis B, positive IgM antibody to hepatitis B core antigen and positive hepatitis B surface antigen with seroconversion at least 6 mo after initial presentation. Acute hepatitis C was considered to be present if the following criteria were met: an elevated serum ALT level with a documented normal level during the year before admission, no previous medical history of chronic hepatitis C, positive HCV RNA by PCR with known or suspected exposure to HCV within the preceding four months, and seroconversion of anti-HCV antibody^[4,5]. Autoimmune hepatitis was diagnosed based on the recommendations of the International Autoimmune Hepatitis Group^[6]. Toxic hepatitis (drug-induced liver injury) was confirmed in patients who had taken relevant various causative medications, herbs, or other xenobiotics within two months before admission and the aforementioned viral markers were all negative.

To identify a relationship between the severity of acute hepatitis and CT findings, we divided patients into two groups, one with and one without severe hepatitis (defined as serum bilirubin ≥ 10 mg/dL or PT $\leq 40\%$ despite the administration of vitamin K in the most severe phase)^[7,8]. To determine the factors associated with prolonged cholestasis, we arbitrarily divided patients into those with serum bilirubin ≥ 10 mg/dL for longer than

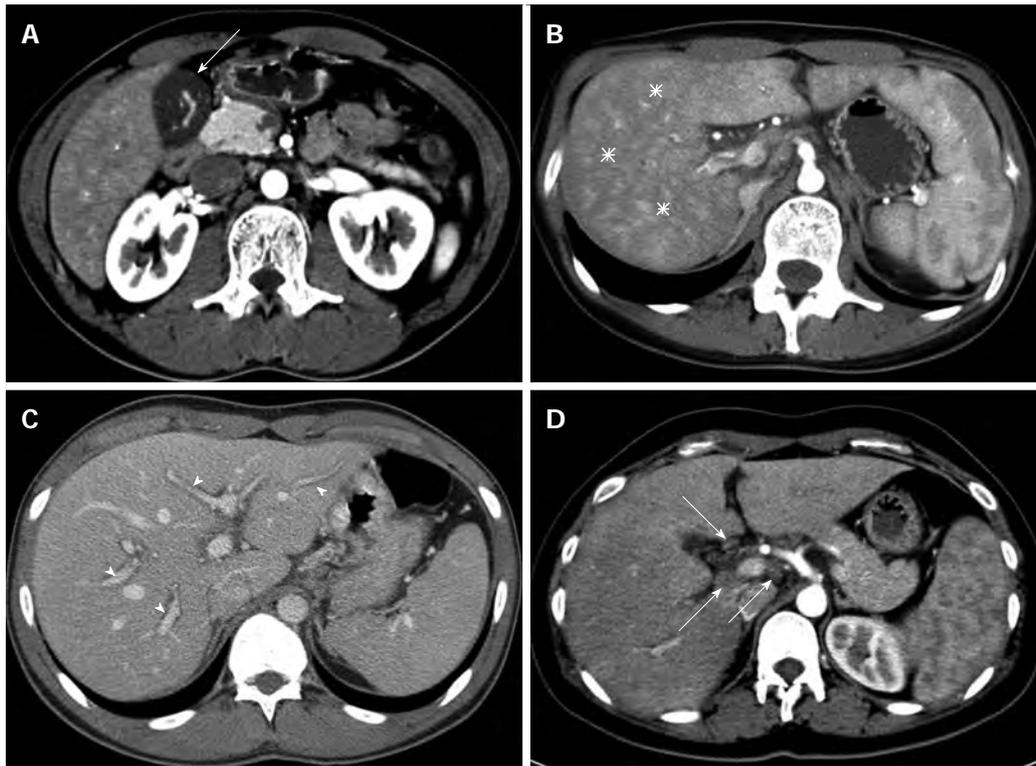


Figure 1 Typical multi-channel computed tomography findings in patients with acute hepatitis. A: Gallbladder wall thickening (arrow); B: Arterial heterogeneity (asterisks), diffuse heterogeneous attenuation of liver parenchyma in the arterial phase; C: Periportal tracking (arrowheads), decreased attenuation, which highlights the portal vein; D: Lymphadenopathy (arrows) in portal hepatitis. Other usual findings, *e.g.*, ascites and splenomegaly, are not shown in this figure.

14 d and those having lower bilirubin levels or higher levels for less than 14 d.

Imaging

All examinations were performed with a 64-channel CT scanner (Brilliance 64, Phillips Medical Systems, Cleveland, OH, United States). The scanning parameters used were as follows: tube voltage, 120 kV; effective tube current, 200 mAs with dose modulation (D-Dom, Phillips Medical System); rotation time, 0.5 s; and collimation, 64 mm × 0.625 mm. In all patients, the abdominal CT was conducted with contrast enhancement. The delay between contrast medium administration and the commencement of scanning was determined individually for each patient using standard bolus-tracking software (Automatic Bolus Tracking, Phillips Medical Systems). Scanning began 7 s after a threshold attenuation of 300 HU was reached in the suprarenal aorta. For each patient, 100 mL of iomeprol (400 mg iodine per mL, Iomeron 400; Bracco, Milan, Italy) was injected into an antecubital vein. Contrast medium was injected monophasically at a rate of 3 mL/s. The portal venous and delayed phases started 75 and 120 s later, respectively.

Abdominal CT analysis

Two radiologists (Park BJ and Kim MJ, each with 10 years of experience in abdominal imaging) interpreted the CT images and reached an opinion by consensus. Both radiologists were blinded to the patient's condition and blood test results. Six features of the CT image were in-

terpreted (Figure 1): (1) gallbladder wall thickness (GWT) where the gallbladder is vertical to the surface of the liver in the part adjacent to the liver; (2) non-homogeneity of liver parenchyma in the arterial phase (arterial heterogeneity); (3) decreased attenuation along the portal vein, which is usually expressed as periportal tracking; (4) the maximum size and number of lymph nodes around the liver; (5) presence of ascites; and (6) size of the spleen.

Statistical analysis

The results were presented as the mean ± SD or number of patients (%) as appropriate. The Mann-Whitney *U* test was used to compare groups of continuous data. Categorical data were evaluated using Fisher's exact test or the χ^2 test. Correlations between two continuous variables were assessed using linear regression analysis with the Spearman correlation coefficient. Multivariate analysis was conducted on variables with $P < 0.1$ in univariate analysis using logistic regression analysis to identify factors related to the severity of acute hepatitis or prolonged cholestasis. P values less than 0.05 were considered statistically significant. SPSS version 12.0 for Windows (SPSS Inc., Chicago, IL, United States) was used for statistical analysis.

RESULTS

Baseline characteristics of the patients

The mean age (± SD) of 412 patients at the time of diagnosis was 34.4 (± 11.4) years (range, 11-92 years),

Table 1 Baseline characteristics of the patients *n* (%)

| Characteristics | Total (<i>n</i> = 412) | Severe hepatitis (<i>n</i> = 92) | Non-severe hepatitis (<i>n</i> = 320) | <i>P</i> value | Prolonged cholestasis (<i>n</i> = 21) | Non-prolonged cholestasis (<i>n</i> = 391) | <i>P</i> value |
|--------------------------------|----------------------------|--------------------------------------|---|-------------------|---|--|-------------------|
| Age ¹ (yr) | 34.4 ± 11.4 | 36.5 ± 11.5 | 33.8 ± 11.4 | 0.035 | 42.6 ± 15.0 | 34.0 ± 11.1 | 0.013 |
| Gender, male | 237 (57.5) | 62 (67.4) | 175 (54.7) | 0.032 | 11 (52.4) | 226 (57.8) | 0.655 |
| Etiology | | | | | | | |
| Hepatitis A | 319 (77.4) | 67 (72.8) | 252 (78.8) | | 8 (38.1) | 311 (79.5) | |
| Toxic | 44 (10.7) | 17 (18.5) | 27 (8.4) | | 10 (47.6) | 34 (8.7) | |
| Unknown | 25 (6.1) | 1 (1.1) | 24 (7.5) | | 0 (0) | 25 (6.4) | |
| Hepatitis B | 16 (3.9) | 4 (4.3) | 12 (3.8) | | 2 (9.5) | 14 (3.6) | |
| Alcohol | 4 (1.0) | 1 (1.1) | 3 (0.9) | | 0 (0) | 4 (1.0) | |
| Hepatitis C | 2 (0.5) | 1 (1.1) | 1 (0.3) | | 1 (4.8) | 1 (0.3) | |
| Autoimmune | 2 (0.5) | 1 (1.1) | 1 (0.3) | | 0 (0) | 2 (0.5) | |
| Etiology, toxic | 44 (10.7) | 17 (18.5) | 27 (8.4) | 0.001 | 10 (47.6) | 34 (8.7) | < 0.001 |
| ALT ¹ (IU/L) | 2053 ± 1759 | 2145 ± 2207 | 2027 ± 1611 | 0.392 | 998 ± 1090 | 2110 ± 1772 | 0.001 |
| ALP ¹ (IU/mL) | 155 ± 75 | 152 ± 72 | 156 ± 76 | 0.983 | 153 ± 110 | 156 ± 73 | 0.265 |
| Bilirubin ¹ (mg/dL) | 5.6 ± 4.9 | 11.2 ± 6.7 | 3.9 ± 2.6 | < 0.001 | 18.6 ± 6.9 | 4.9 ± 3.7 | < 0.001 |
| Albumin ¹ (g/dL) | 3.9 ± 0.4 | 3.8 ± 0.4 | 4.0 ± 0.4 | < 0.001 | 3.8 ± 0.4 | 3.9 ± 0.4 | 0.174 |
| PT ¹ (%) | 81.0 ± 18.0 | 75.2 ± 22.7 | 83.7 ± 16.6 | 0.003 | 84.6 ± 15.0 | 81.7 ± 18.6 | 0.721 |
| GWT ¹ , mm | 5.2 ± 5.5 | 7.2 ± 6.0 | 5.5 ± 5.3 | 0.009 | 9.6 ± 6.7 | 5.7 ± 5.4 | 0.003 |
| > 3 | 233 (56.5) | 70 (70.7) | 168 (51.7) | 0.004 | 17 (81.0) | 216 (55.2) | 0.025 |
| > 7 | 142 (34.5) | 36 (39.1) | 106 (33.1) | 0.040 | 11 (52.4) | 131 (33.5) | 0.005 |
| Arterial heterogeneity (+) | 294 (80.1) | 68 (73.9) | 226 (70.6) | 0.755 | 15 (71.5) | 279 (71.3) | 1.000 |
| Periportal tracking (+) | 348 (84.7) | 78 (84.8) | 270 (84.4) | 0.869 | 18 (85.7) | 330 (84.4) | 1.000 |
| LN number ¹ | 4.8 ± 3.0 | 5.1 ± 3.3 | 4.8 ± 2.9 | 0.581 | 5.3 ± 3.7 | 4.8 ± 3.0 | 0.701 |
| LN size ¹ (cm) | 0.97 ± 0.26 | 0.99 ± 0.28 | 0.96 ± 0.26 | 0.465 | 0.97 ± 0.30 | 0.97 ± 0.26 | 0.743 |
| ≥ 0.8 | 346 (84.5) | 79 (85.9) | 267 (83.4) | 0.632 | 17 (81.0) | 329 (84.1) | 0.759 |
| > 1 | 162 (39.3) | 42 (45.7) | 120 (37.5) | 0.183 | 10 (47.6) | 152 (38.9) | 0.494 |
| Ascites (+) | 56 (13.6) | 17 (18.5) | 39 (12.2) | 0.123 | 7 (33.3) | 49 (12.5) | 0.015 |
| Spleen size ¹ (cm) | 10.9 ± 1.6 | 11.04 ± 1.68 | 10.84 ± 1.60 | 0.419 | 10.44 ± 1.7 | 10.91 ± 1.6 | 0.094 |
| ≥ 12 | 95 (23.1) | 24 (26.1) | 71 (22.2) | 0.483 | 3 (14.3) | 92 (23.5) | 0.431 |

¹Data presented as mean ± SD. ALT: Alanine aminotransferase; ALP: Alkaline phosphatase; PT: Prothrombin time; GWT: Gallbladder wall thickness; LN: Lymph node.

and 237 (57.5%) were male. The most common cause of acute hepatitis was hepatitis A virus (*n* = 319, 77.4%). The other causes in order of frequency were toxicity, indeterminate, hepatitis B virus, alcoholism, HCV, and autoimmunity. Contrast-enhanced abdominal CT was performed at 1.5 d, on average, after admission (the second hospitalization day). The mean GWT on CT was 5.2 (± 5.5) mm, and 233 patients (56.5%) showed thickening of over 3 mm. The numbers of patients who showed arterial heterogeneity, periportal tracking, enlarged lymph nodes greater than 7 mm, and presence of ascites were 294 (80.1%), 348 (84.7%), 346 (84.5%), and 56 (13.6%), respectively. Spleen enlargement by greater than 12 cm was found in 95 patients (23.1%) (Table 1).

Correlation between CT findings and liver chemistry

GWT was related to serum ALT ($r = 0.234$, $P < 0.001$), ALP ($r = 0.180$, $P < 0.001$), bilirubin ($r = 0.319$, $P < 0.001$), albumin ($r = -0.282$, $P < 0.001$), and PT ($r = -0.118$, $P = 0.017$). Lymph node size was correlated with serum ALT ($r = 0.164$, $P = 0.001$), ALP ($r = 0.135$, $P = 0.006$), and bilirubin ($r = 0.138$, $P = 0.005$). The number of lymph nodes was correlated with serum ALP ($r = 0.150$, $P = 0.003$) and bilirubin ($r = 0.202$, $P < 0.001$). The spleen size was correlated with serum ALT ($r = 0.119$, $P = 0.015$) and bilirubin ($r = 0.190$, $P < 0.001$). Serum ALT levels ($P < 0.001$) and PT ($P = 0.032$) varied depending on the

presence of arterial heterogeneity. The presence of periportal tracking was related to serum ALP ($P = 0.007$), bilirubin ($P < 0.001$), and albumin ($P = 0.002$). The presence of ascites was related to serum ALT ($P = 0.005$), bilirubin ($P = 0.012$), and albumin ($P < 0.001$) levels and PT ($P = 0.006$) (Table 2).

Factors associated with acute hepatitis severity

On univariate analysis, age, gender, frequency of toxic hepatitis, serum bilirubin and albumin, PT at the time of CT scanning and GWT showed significant differences ($P < 0.05$) between the group with severe hepatitis (defined as bilirubin ≥ 10 mg/dL or PT $\leq 40\%$ in the most severe phase) and the group with non-severe hepatitis. On multivariate analysis, male gender [odds ratio (OR) = 2.569, 95%CI: 1.477-4.469, $P = 0.001$], toxic hepatitis (OR = 3.531, 95%CI: 1.444-8.635, $P = 0.006$), low serum albumin (OR = 2.154, 95%CI: 1.279-3.629, $P = 0.004$), and high GWT (OR = 1.061, 95%CI: 1.015-1.110, $P = 0.009$) independently predicted severe hepatitis (Tables 1 and 3).

Factors associated with prolonged cholestasis

Patient age, frequency of toxic hepatitis, serum ALT and bilirubin levels at the time of CT scan, GWT, and the frequency of ascites differed significantly between patients with prolonged cholestasis and those with non-prolonged

Table 2 Relationship between computed tomography findings and liver chemistry (mean \pm SD)

| | GWT | LN maximum size | LN number | Spleen size | Arterial heterogeneity | | | Periportal tracking | | | Ascites | | |
|-------------------|------------------|-----------------|-----------------|-----------------|------------------------|--------------------|---------|---------------------|--------------------|---------|-------------------|-------------------|---------|
| | | | | | Absence (n = 73) | Presence (n = 294) | P value | Absence (n = 63) | Presence (n = 348) | P value | Absence (n = 356) | Presence (n = 56) | P value |
| ALT (IU/L) | 0.234 (< 0.001) | 0.164 (0.001) | -0.013 (0.798) | 0.119 (0.015) | 1504 \pm 1478 | 2216 \pm 1794 | < 0.001 | 1994 \pm 2139 | 2064 \pm 1688 | 0.074 | 1981 \pm 1769 | 2509 \pm 1641 | 0.005 |
| ALP (IU/mL) | 0.180 (< 0.001) | 0.135 (0.006) | 0.150 (0.003) | 0.086 (0.082) | 145 \pm 76 | 159 \pm 75 | 0.108 | 133 \pm 63 | 159 \pm 76 | 0.007 | 154 \pm 76 | 164 \pm 66 | 0.152 |
| Bilirubin (mg/dL) | 0.319 (< 0.001) | 0.138 (0.005) | 0.202 (< 0.001) | 0.190 (< 0.001) | 5.6 \pm 5.2 | 5.6 \pm 4.9 | 0.451 | 4.3 \pm 5.1 | 5.8 \pm 4.9 | < 0.001 | 5.3 \pm 4.7 | 7.0 \pm 5.9 | 0.012 |
| PT (s) | -0.118 (0.017) | -0.038 (0.445) | 0.072 (0.149) | -0.092 (0.061) | 86 \pm 16 | 81 \pm 18 | 0.032 | 81 \pm 20 | 82 \pm 18 | 0.795 | 82 \pm 18 | 76 \pm 17 | 0.006 |
| Albumin (g/dL) | -0.282 (< 0.001) | -0.018 (0.717) | -0.017 (0.739) | -0.062 (0.206) | 4.0 \pm 0.4 | 3.9 \pm 0.4 | 0.404 | 4.1 \pm 0.4 | 3.9 \pm 0.4 | 0.002 | 4.0 \pm 0.4 | 3.7 \pm 0.4 | < 0.001 |

ALT: Alanine aminotransferase; ALP: Alkaline phosphatase; PT: Prothrombin time; GWT: Gallbladder wall thickness; LN: Lymph node.

Table 3 Multivariate logistic regression analysis for the factors associated with severe acute hepatitis and prolonged cholestasis in acute hepatitis

| Variables | OR | 95%CI for OR | | P value |
|--|-------|--------------|--------|---------|
| | | Lower | Upper | |
| Severe acute hepatitis | | | | |
| Age (yr) | 1.007 | 0.928 | 1.033 | 0.579 |
| Gender, male | 2.569 | 1.477 | 4.469 | 0.001 |
| Etiology, toxic | 3.531 | 1.444 | 8.635 | 0.006 |
| Albumin (g/dL) | 2.154 | 1.279 | 3.629 | 0.004 |
| GWT (mm) | 1.061 | 1.015 | 1.110 | 0.009 |
| Prolonged cholestasis in acute hepatitis | | | | |
| Age (yr) | 0.393 | 0.059 | 2.599 | 0.333 |
| Etiology, toxic | 6.848 | 0.960 | 48.859 | 0.055 |
| ALT (IU/L) | 1.000 | 1.000 | 1.001 | 0.607 |
| Bilirubin (mg/dL) | 1.628 | 1.331 | 1.991 | < 0.001 |
| GWT (mm) | 1.172 | 1.024 | 1.342 | 0.021 |
| Ascites | 0.484 | 0.119 | 6.039 | 0.869 |

GWT: Gallbladder wall thickness; ALT: Alanine aminotransferase; OR: Odds ratio.

cholestasis (Table 1). In this study, prolonged cholestasis was defined as serum bilirubin levels of 10 mg/dL or higher sustained for longer than 14 d. On multivariate analysis, elevated serum bilirubin (OR = 1.628, 95%CI: 1.331-1.991, P < 0.001) and increased GWT (OR = 1.172, 95%CI: 1.024-1.342, P = 0.021) were independently predictive for prolonged cholestasis. Three hundred and fifty-six of 357 patients with bilirubin levels less than 10 mg/dL at the time of CT scan were assigned to the non-prolonged cholestasis group. Among patients with serum bilirubin levels \geq 10 mg/dL (n = 55), 79% (22/28) of patients with GWT less than 5 mm were classified in the non-prolonged cholestasis group, and 71% (5/7) of those with GWT of 13 mm or higher were classified in the prolonged cholestasis group (Tables 1 and 3).

DISCUSSION

Hepatomegaly, increased periportal echogenicity, decreased parenchymal echogenicity, lymph node enlargement,

and a thickened gallbladder wall are frequently observed during the ultrasonographic examination of patients with acute hepatitis^[2,9-12]. Similar findings also appear on CT scan^[13-16], and CT easily detects lymphadenopathy and gallbladder wall thickening. The “periportal tracking” observed on CT may correspond to the same pathology as “increased periportal echogenicity” on ultrasonography. The non-homogeneity of liver parenchyma observed in the arterial phase of dynamic CT (arterial heterogeneity) is a CT-specific image generated by contrast media in patients with acute hepatitis^[17]. These image findings are most commonly observed at the icteric phase of acute hepatitis^[3].

Thickening of the gallbladder wall has attracted attention because it may be detected by ultrasonography in 51%-91% of patients with acute hepatitis^[18-22]. In a recent study, ultrasonography within 7 d after symptomatic onset of acute hepatitis showed morphological changes in the gallbladder in more than 80% of patients, and these findings normalized as the hepatitis improved clinically^[20]. In the present study, 56.6% of patients with acute hepatitis demonstrated an abnormal GWT when GWT > 3 mm was defined as abnormal. In addition, we observed other CT findings in patients with acute hepatitis, including lymph node enlargement, periportal tracking, and arterial heterogeneity at frequencies of 84.5%, 84.7% and 80.1%, respectively. These values were all higher than the frequency of GWT.

Correlations between imaging and laboratory findings in acute hepatitis have not been closely investigated. Studies on the correlation between GWT and the liver chemistry index show conflicting results. Some studies show that GWT increases at elevated serum aminotransferase levels^[22,23], while other studies do not show this correlation^[18,19,24]. Suk *et al.*^[24] showed a significant correlation between GWT and increases in serum bilirubin. In the present study, GWT clearly correlated with elevated serum bilirubin and liver enzymes, which supports the hypothesis that inflammation and necrosis in the liver parenchyma induce an inflammatory reaction and hyperemia in muscular and serosal layers of the gallbladder

wall^[23]. We also found a negative correlation between GWT and serum albumin levels, which suggests that hypoalbuminemia caused by acute hepatitis contributes to gallbladder wall edema. Patients with chronic renal insufficiency or cirrhosis who also have hypoalbuminemia frequently present gallbladder wall thickening^[14].

In the present study, imaging findings other than GWT showed significant correlations with the serum levels of ALT, ALP, bilirubin, albumin and PT. These imaging findings included lymph node enlargement around the hepatoduodenal ligament, arterial heterogeneity, and periportal tracking. These correlations suggested that image findings may reflect liver damage and hepatic dysfunction, at least to some degree (Table 2). We therefore tested the prognostic value of imaging data in patients with acute hepatitis. First, we designated patients with serum bilirubin levels ≥ 10 mg/dL or PT index $\leq 40\%$ despite the administration of vitamin K as having severe hepatitis, and we then sought to identify factors that may predict this outcome. In our analysis, male gender, toxic hepatitis, low serum albumin, and increased GWT independently predicted severe hepatitis. Furthermore, increased GWT and high serum bilirubin levels at the time of CT scanning independently predicted prolonged cholestasis. Because only approximately 20% of patients with hyperbilirubinemia (serum bilirubin > 10 mg/dL) at the time of CT scan showed prolonged cholestasis if their GWT was < 5 mm, the detection of GWT may be of value in predicting prolonged cholestasis in patients with acute hepatitis. Of course, other imaging modalities could substitute for CT scan unless CT is absolutely necessary, especially in young patients, to avoid unnecessary radiation exposure if they could also depict changes in the gallbladder, considering arguments on the safety of radiation exposure caused by CT scan in young patients^[25,26].

This study is limited by its retrospective design. Therefore, we could not investigate the correlation between imaging findings and the duration of hospitalization because the criteria for patient discharge were not set in advance. Similarly, we could not determine the time of symptomatic onset of hepatitis in many patients. However, this study is strengthened by its size, which allowed us to confirm significant correlations between imaging findings and liver chemistry data in patients with acute hepatitis. In addition, this study revealed the value of GWT for predicting the outcome of acute hepatitis.

In conclusion, abdominal CT findings and liver biochemical parameters in patients with acute hepatitis showed some correlations, and GWT was the only imaging variable that independently predicted the severity of acute hepatitis or prolonged cholestasis.

COMMENTS

Background

In patients with suspected acute hepatitis, imaging studies, such as ultrasonography or computed tomography (CT), are usually performed to rule out other diseases presenting with similar clinical and biochemical abnormalities (e.g., extrahepatic cholestasis, diffuse metastatic disease or cirrhosis).

Research frontiers

Studies on the correlation between imaging and laboratory findings in acute hepatitis have shown conflicting results.

Innovations and breakthroughs

This large-scale study has revealed significant correlations between abdominal CT findings and liver biochemical parameters. Furthermore, gallbladder wall thickness (GWT), which can be easily measured by other imaging techniques as well, was found to be valuable for predicting the outcome of patients with acute hepatitis.

Applications

The results of this study suggest that GWT measurement by imaging modalities is helpful to classify patients with severe acute hepatitis or prolonged cholestasis.

Terminology

GWT refers to the thickness of the gallbladder wall vertical to the surface of the liver in the part adjacent to the liver.

Peer review

The authors describe the CT findings of severe acute hepatitis in 412 hospitalized patients. They found that GWT was an independent predictive factor for severe hepatitis. My main criticism is the relevance of such a paper. This study involved exposing young people to unnecessary radiation.

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New-style laparoscopic and endoscopic cooperative surgery for gastric stromal tumors

Hai-Yan Dong, Yu-Long Wang, Jie Li, Qiu-Ping Pang, Guo-Dong Li, Xin-Yong Jia

Hai-Yan Dong, Qiu-Ping Pang, Guo-Dong Li, Xin-Yong Jia, Department of Endoscopy, Qianfoshan Hospital Affiliated to Shandong University, Jinan 250014, Shandong Province, China
Yu-Long Wang, Jie Li, Department of General Surgery, Qianfoshan Hospital Affiliated to Shandong University, Jinan 250014, Shandong Province, China

Author contributions: Dong HY, Pang QP, Li GD and Jia XY performed the surgery and experiment; Dong HY and Wang YL wrote the paper; Jia XY, Li J and Dong HY designed the study.
Correspondence to: Xin-Yong Jia, Professor, Department of Endoscopy, Qianfoshan Hospital Affiliated to Shandong University, No. 16766, Jingshi Road, Jinan 250014, Shandong Province, China. jjaxinyong19620723@163.com

Telephone: +86-531-82968900 Fax: +86-531-82967114
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Abstract

AIM: To evaluate the feasibility and safety of a new style of laparoscopic and endoscopic cooperative surgery (LECS), an improved method of laparoscopic intragastric surgery (LIGS) for the treatment of gastric stromal tumors (GSTs).

METHODS: Six patients were treated with the new-style LECS. Surgery was performed according to the following procedures: (1) Exposing and confirming the location of the tumor with gastroscopy; (2) A laparoscopy light was placed in the cavity using the trocar at the navel, and the other two trocars penetrated both the abdominal and stomach walls; (3) With gastroscopy monitoring, the operation was carried out in the gastric lumen using laparoscopic instruments and the tumor was resected; and (4) The tumor tissue was removed orally using a gastroscopy basket, and puncture holes and perforations were sutured using titanium clips.

RESULTS: Tumor size ranged from 2.0 to 4.5 cm (av-

erage 3.50 ± 0.84 cm). The operative time ranged from 60 to 130 min (average 83.33 ± 26.58 min). Blood loss was less than 20 mL and hospital stay ranged from 6 to 8 d (average 6.67 ± 0.82 d). The patients were allowed out of bed 12 h later. A stomach tube was inserted for 72 h after surgery, and a liquid diet was then taken. All cases had single tumors which were completely resected using the new-style LECS. No postoperative complications occurred. Pathology of all resected specimens showed GST: no cases of implantation or metastasis were found.

CONCLUSION: New-style LECS for GSTs is a quick, optimized, fast recovery, safe and effective therapy.

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Key words: Laparoscopic and endoscopic cooperative surgery; Gastric stromal tumor

Core tip: A new style of laparoscopic and endoscopic cooperative surgery (LECS) was used to treat gastric stromal tumors (GSTs) originating from the muscularis propria in this study. The operation was carried out in the gastric cavity, and the GST was completely removed using a gastroscopic light source and laparoscopic instruments. This method is minimally invasive and avoids many complications such as bleeding and intra-abdominal infection. Furthermore, the integrity of stomach structure and function is preserved. New-style LECS is a safe and effective method for the treatment of GSTs.

Dong HY, Wang YL, Li J, Pang QP, Li GD, Jia XY. New-style laparoscopic and endoscopic cooperative surgery for gastric stromal tumors. *World J Gastroenterol* 2013; 19(16): 2550-2554 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v19/i16/2550.htm> DOI: <http://dx.doi.org/10.3748/wjg.v19.i16.2550>

INTRODUCTION

Gastric stromal tumors (GSTs) are potentially malignant tumors, accounting for approximately 60%-70% of gastrointestinal stromal tumors (GISTs)^[1]. GSTs diffuse mainly by hematogenous metastasis and direct violation, are less likely to occur in lymph metastasis, and are not sensitive to chemotherapy or radiotherapy. The main treatment option is complete tumor resection^[2-5]. Laparoscopic and endoscopic cooperative surgery (LECS) is an important therapy in the treatment of GSTs^[6-8]. In this study, we used this new improved method of laparoscopic intragastric surgery (LIGS) to completely resect GSTs, and achieved good results.

MATERIALS AND METHODS

General information

From January 2011 to May 2012, 6 cases of GSTs originating from the muscularis propria were confirmed by endoscopic ultrasound (EUS) and gastroscopy. The patients were 2 males and 4 females, aged 42-63 years, with a mean age of 53.83 ± 7.94 years. All cases had a single tumor with an average size of 3.50 ± 0.84 cm. The tumors were located in the fundus fornix in 3 cases, in the posterior wall of the gastric body in 1 case and in the gastric cardia in 2 cases (Table 1).

Surgical methods

Using tracheal intubation with general anesthesia, the patients were placed in the supine position with their heads slightly to the left. Both the surgeon and the endoscopist stood on the patient's left side. The location of the tumor was determined by the endoscopist (Figure 1A). The surgeon made a curved incision of approximately 0.5 cm at the superior border of the umbilicus, a CO₂ pneumoperitoneum with pressure of 12 mmHg was established, and a 0.5 cm laparoscope was inserted for observation. Two punctures of about 0.5 cm at the left upper quadrant of the abdomen were then established. Two sutures were placed in the avascular area of the anterior wall of the stomach and exported from one of the puncture holes, in order to pull the anterior stomach wall close to the abdominal wall. The stomach was inflated during gastroscopy. Under laparoscopic monitoring, the surgeon inserted 2 ordinary 0.5 cm puncture cannulas into the stomach from the anterior wall, at a distance of more than 3 cm (Figure 1B and C). Under gastroscopic guidance, the surgeon used an ultrasound knife to completely resect the tumors (Figure 1D). Specimens were removed *via* the mouth using grasping forceps (Figure 1E and F). If there was no perforation, the two puncture cannulas in the gastric lumen were pulled out one by one, and the puncture holes were clipped using titanium clips *via* the endoscope. Otherwise, the perforation was clipped from the edge to the center using clips, the puncture cannulas were then pulled out, and the puncture holes closed by clips (Figure 1H and I). If the stomach was repeatedly inflated without leak, the gas was exhausted and a gastric

Table 1 Patient demographics and gastric stromal tumor characteristics

| Patient | Sex | Age (yr) | Tumor location | Tumor size (cm) | Operation time (min) | Hospital stay (d) | Complications |
|---------|-----|----------|------------------------|-----------------|----------------------|-------------------|---------------|
| 1 | M | 48 | Fundus fornix | 3.5 | 80 | 7 | None |
| 2 | F | 42 | Posterior wall of body | 4.5 | 50 | 7 | None |
| 3 | F | 56 | Cardia | 2.5 | 130 | 7 | None |
| 4 | F | 63 | Fundus fornix | 2.0 | 70 | 5 | None |
| 5 | F | 53 | Cardia | 3.0 | 90 | 7 | None |
| 6 | M | 61 | Fundus fornix | 3.5 | 80 | 7 | None |

M: Male; F: Female.

tube was inserted. The pneumoperitoneum was reestablished and the hanging line in the stomach wall was removed. Intra-abdominal exudate and bleeding was fully suctioned. The pneumoperitoneum was removed when the surgeon pulled the puncture cannula out of the abdominal wall and sutured the puncture subcutaneously.

RESULTS

The average operative time was 83.33 ± 26.58 min and blood loss was less than 20 mL. A stomach tube was inserted for 72 h after surgery, and a liquid diet was then taken. After surgery, there was no significant abdominal pain or pneumoperitoneum. Patients were allowed out of bed 12 h later. The average length of hospital stay was 6.67 ± 0.82 d. Pathological results were GSTs in all patients, and the structure of the basal and cutting edge was normal. On the 3rd and 6th mo after surgery, the patients were reassessed by endoscopy and EUS, and all patients had healed well without metastasis.

DISCUSSION

With the development of endoscopic technology, LECS and endoscopic submucosal dissection (ESD) have been used more and more widely in the treatment of GSTs^[6-13]. Conventional LECS, according to the different locations of GSTs, is mainly divided into 2 types: the laparoscope-assisted endoscopic technique (LAET) and endoscope-assisted laparoscopic technique (EALT). In addition, EALT can be divided into endoscope-assisted wedge resection (EAWR) and LIGS^[6,8,14,15].

LAET mainly refers to the procedure where laparoscopy is used to closely monitor the endoscopic resection of tumors throughout the surgical process, and the timely treatment of perforation, bleeding and other complications^[16]. However, when a tumor is too large or located in the posterior wall or gastric fundus, gastroscopy is very difficult to achieve. In EAWR, the tumor is resected by laparoscopy, while endoscopy plays an important role in the location of tumors, and is usually used for GSTs at the lesser curvature and the anterior wall of the stomach^[17]. When the lesions are near the pylorus or cardia, a wedge resection may lead to stenosis^[18].

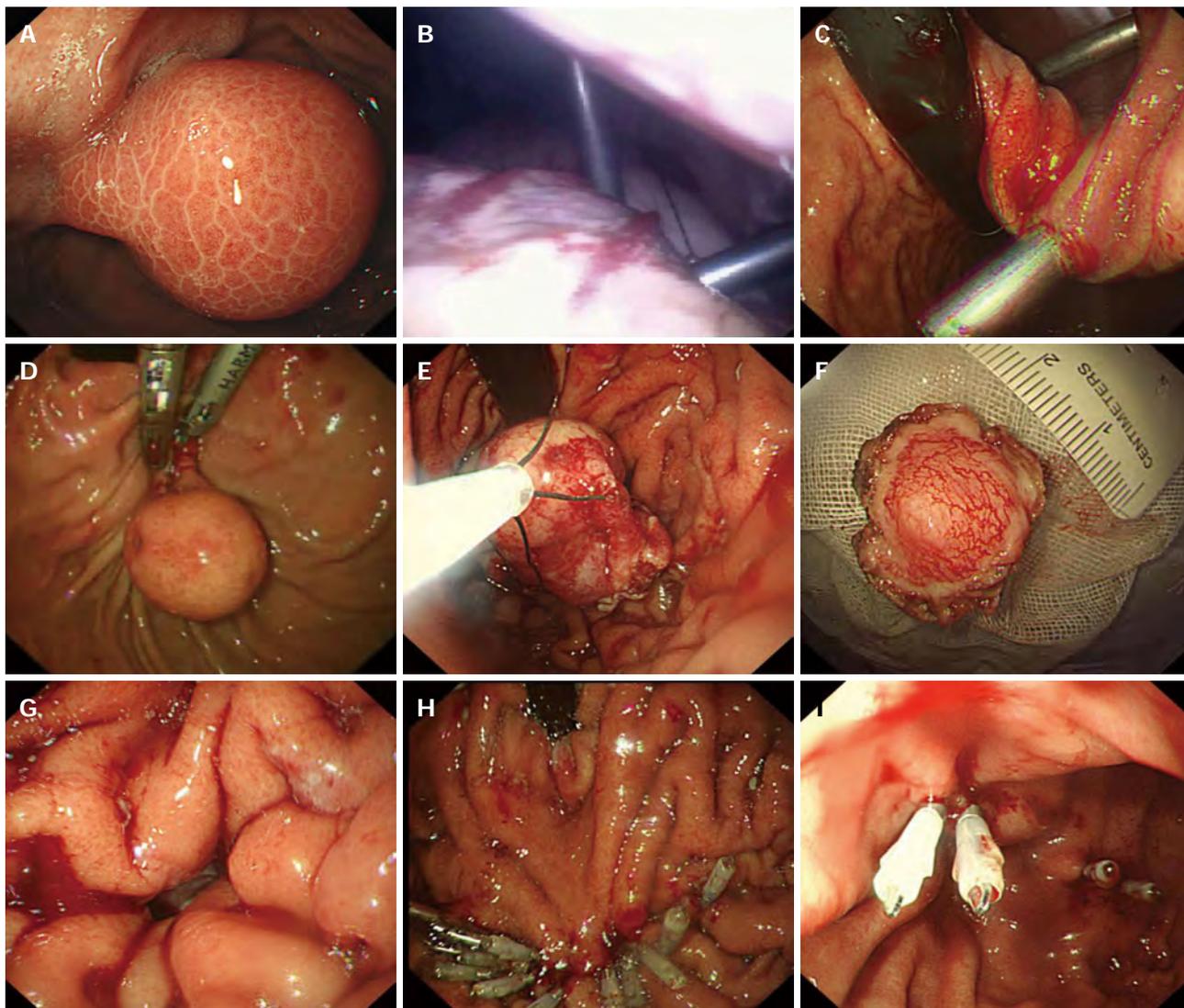


Figure 1 New-style laparoscopic and endoscopic cooperative surgery for gastric stromal tumors. A: Tumor in fundus fornix; B, C: Two puncture cannulas were inserted into the stomach; D: The tumor was resected by ultrasound knife under gastroscopic guidance; E: Specimen was taken out via the mouth using endoscopic basket; F: The tumor; G: Perforation in fundus. H: Perforation was closed by clips via gastroscopy; I: The 2 puncture holes in stomach were clipped by clips.

Laparoscopic instruments are used in LIGS, including a laparoscopic light source, to puncture the gastric cavity directly and to carry out the surgery within it. The tumor is then removed from the abdominal wall, and the gastric wall and puncture holes are sutured using laparoscopic instruments^[19-21].

Compared with the traditional LIGS, the advantages of the new-type LECS are as follows: (1) Using gastroscopic light, doctors can reduce the laparoscopic light holes in the gastric wall, and close laparoscopic operational holes with clips *via* the gastroscopy. Compared with normal laparoscopic suturing, this technique is minimally invasive; (2) Tumors less than 4.5 cm in size can be removed *via* gastroscopy from the mouth to reduce trauma to the abdominal wall; and (3) Both the exterior and interior of the gastric cavity can be observed by laparoscopy and gastroscopy, respectively, in order to reduce the incidence of postoperative complications.

ESD is a technique which uses gastroscopy alone to

resect tumors^[22-24]. However, for tumors greater than 2 cm or exogenic or mixed type tumors, or if the tumor is located in an area where it is difficult to operate, ESD can result in problems, such as the risk of bleeding or perforation. For large perforations, it is more difficult to suture using clips during gastroscopy. Furthermore, longer operative time, and no peritoneal lavage and suction, can lead to pneumothorax, pneumoperitoneum, abdominal cavity cysts, peritoneal abscesses and other complications^[25,26]. Compared to ESD, the advantages of new-type LECS are as follows: (1) Shortens the operative time; (2) Reduces the risk of bleeding; (3) In the event of perforation, the use of laparoscopy to suction exudate and bleeding and to execute peritoneal washing, will reduce the chance of intra-abdominal infection and other complications; and (4) Avoids the situation where observation and the operative field are unclear after perforation.

In conclusion, new-type LECS can be used to resect

GSTs greater than 2 cm in size which originate from the muscularis propria, mixed tumors or where difficulty in resection is encountered by laparoscopy or gastroscopy.

Although this new type of LECS has unique advantages in the treatment of certain GSTs, this surgery has been used for less than one year. It is still at the stage of exploration and practice, and requires more cases to verify its usefulness. In addition, it requires good laparoscopic physicians, endoscopists and endoscopy nurses to help each other to accomplish the safe and effective use of this technique.

COMMENTS

Background

Gastric stromal tumors (GSTs) are potentially malignant tumors, and the main outcome of treatment is complete tumor resection. Laparoscopic and endoscopic cooperative surgery (LECS) is now being used more widely in the treatment of GSTs. In order to decrease complications and minimize trauma, a new type of LECS was used for GSTs in the present study.

Research frontiers

Conventional LECS is used for the treatment of GSTs originating from the muscularis propria. However, use of the new type of LECS has rarely been reported. The authors investigated the clinical safety and efficacy of the new type of LECS for GSTs.

Innovations and breakthroughs

This is the first report on the new type of LECS for GSTs to date. This new surgery is similar to laparoscopic intragastric surgery. The operation is carried out in the stomach and the GST is completely removed using a gastroscopic light source and laparoscopic instruments. The integrity of the stomach structure and function is preserved. It is an innovative and effective operation for GSTs, and has unique advantages in the treatment of certain GSTs.

Applications

A new type of LECS was used for GSTs greater than 2 cm in size which originated from the muscularis propria, in mixed tumors and in tumors which were difficult to resect using only laparoscopy or gastroscopy.

Terminology

LECS is an operation for resecting gastrointestinal tumors using laparoscopy and endoscopy. This method includes a laparoscope-assisted endoscopic technique and an endoscope-assisted laparoscopic technique.

Peer review

The new-type of LECS described in this study is relatively new, and the procedures are feasible and may have some localizing advantages in selected patients.

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Endoscopic mucosal resection for rectal carcinoids under micro-probe ultrasound guidance

Fu-Run Zhou, Liu-Ye Huang, Cheng-Rong Wu

Fu-Run Zhou, Liu-Ye Huang, Cheng-Rong Wu, Department of Gastroenterology, Yu Huang Ding Hospital affiliated to Qingdao University School of Medicine, Yantai 264000, Shandong Province, China

Author contributions: Zhou FR designed the study, performed the endoscopic mucosal resection and wrote the manuscript; Huang LY and Wu CR performed the endoscopic procedures; all authors have read and approved the final version for publication. Correspondence to: Fu-Run Zhou, Associate Professor, Department of Gastroenterology, Yu Huang Ding Hospital affiliated to Qingdao University School of Medicine, No. 20, Yudong Street, Zhifu District, Yantai 264000, Shandong Province, China. frz_1205@126.com

Telephone: +86-535-6691999 Fax: +86-535-6691999
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Abstract

AIM: To assess the therapeutic value of endoscopic mucosal resection (EMR) under micro-probe ultrasound guidance for rectal carcinoids less than 1 cm in diameter.

METHODS: Twenty-one patients pathologically diagnosed with rectal carcinoids following colonoscopy in our hospital from January 2007 to November 2012 were included in this study. The patients consisted of 14 men and 7 women, with a mean age of 52.3 ± 12.2 years (range: 36-72 years). The patients with submucosal tumors less than 1 cm in diameter arising from the rectal and muscularis mucosa detected by micro-probe ultrasound were treated with EMR and followed up with conventional endoscopy and micro-probe ultrasound.

RESULTS: All of the 21 tumors were confirmed by micro-probe ultrasound as uniform hypoechoic masses originating from the rectal and muscularis mucosa, without invasion of muscularis propria and vessels, and less than 1 cm in diameter. EMR was successfully com-

pleted without bleeding, perforation or other complications. The resected specimens were immunohistochemically confirmed to be carcinoids. Patients were followed up for one to two years, and no tumor recurrence was reported.

CONCLUSION: EMR is a safe and effective treatment for rectal carcinoids less than 1 cm in diameter.

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Key words: Micro-probe ultrasound; Endoscopic mucosal resection; Rectal carcinoid; Endoscopic submucosal dissection; Submucosal tumors

Core tip: Rectal carcinoids are rare neuroendocrine tumors that are often missed or misdiagnosed in clinical settings due to the absence of specific symptoms in the early stage. This study reports on a hospital-based series of 21 patients who were successfully treated and followed up for 1-2 years for small rectal carcinoids less than 1 cm by means of endoscopic mucosal resection (EMR) under micro-probe ultrasound guidance. EMR is found to be a safer option with fewer complications, shorter operation time, and comparable rate of complete removal to endoscopic submucosal dissection, therefore, EMR is more suitable for treating rectal carcinoids less than 1 cm in diameter.

Zhou FR, Huang LY, Wu CR. Endoscopic mucosal resection for rectal carcinoids under micro-probe ultrasound guidance. *World J Gastroenterol* 2013; 19(16): 2555-2559 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v19/i16/2555.htm> DOI: <http://dx.doi.org/10.3748/wjg.v19.i16.2555>

INTRODUCTION

Rectal carcinoids are rare neuroendocrine tumors that

are often missed or misdiagnosed in clinical settings due to the absence of specific symptoms in the early stage^[1]. With the widespread popularity of colonoscopy, however, the detection rate of rectal carcinoids has been increasing in recent years, enabling us to achieve better outcomes for patients with submucosal tumors (SMTs) derived from the muscular layer of rectal mucosa using endoscopic mucosal resection (EMR) combined with micro-probe ultrasound examination. The following is a summary of a retrospective analysis of 21 patients with pathologically confirmed rectal carcinoid tumors treated in our hospital from January 2007 to November 2012.

MATERIALS AND METHODS

Subjects

Twenty-one patients pathologically diagnosed with rectal carcinoids following colonoscopy in our hospital from January 2007 to November 2012 were included in this study. The patients consisted of 14 men and 7 women, with a mean age of 52.3 ± 12.2 years (range: 36-72 years).

Methods

Equipment: Olympus GIF-260 Endoscope, Fujinon-Sp702 micro-probe system, with a micro-probe frequency of 20 MHz and probe diameter of 2.5 mm.

Procedures: Lesions were assessed by endoscopic ultrasonography (Figures 1-7), and endoscopic submucosal resection was performed for lesions less than 1 cm in diameter and not deeper than the submucosa. Normal saline was injected into the root of each tumor under colonoscopy to form a bulge before EMR. Metal hemoclipping was applied to the excision wounds to prevent postoperative perforation, bleeding and other complications. The removed tissues were sent for pathological identification. All specimens were fixed with 10% formalin, and stained with hematoxylin-eosin for immunohistochemistry in the Department of Pathology.

RESULTS

Colonoscopy was performed for subjects with unexplained changes in bowel habit (including stool frequency and consistency) and those who underwent routine physical examinations, and 21 patients were diagnosed as having rectal carcinoids. Among the 21 cases of rectal carcinoids, 16 (76.2%) cases were located within 8 cm and 5 (23.8%) cases were located within 8-10 cm from the anal margin. The typical submucosal lesions with smooth mucosal surface were the predominant endoscopic manifestations (85.7%, 18/21). Three (14.31%, 3/21) lesions were depressed and erosive at the top region, and 17 (80.9%, 17/21) appeared tough or hard, and only 1 lesion was soft (0.5%). Seventeen (80.9%) cases appeared yellow, and 4 (19.1%) cases appeared red or normal.

All of the 21 tumors were confirmed by micro-probe



Figure 1 Endoscopic findings of rectal carcinoids. Endoscopy shows yellow or gray submucosal nodules, which are hard and covered by normal-appearing mucosa.



Figure 2 Rectal carcinoids under micro-probe ultrasound. The hypoechoic lesions are mainly derived from submucosa or muscularis mucosa.



Figure 3 Endoscopic loop ligation after injection.

ultrasound as uniform hypoechoic masses originating from the rectal and muscularis mucosa, without invasion of muscularis propria and vessels, and less than 1 cm in diameter. EMR was successfully completed without bleeding, perforation or other complications. The resected specimens were immunohistochemically diagnosed as carcinoids. The post-operative follow-up lasted 1-2 years, during which colonoscopy was performed in the first month, and then in months 3, 6, 12 and 24. No tumor relapse was noted.



Figure 4 Endoscopic resection of the lesions. The whole rectal carcinoid mass is visible in the endoscopically resected lesion.



Figure 5 Wounds after endoscopic resection. After the endoscopic resection of the rectal carcinoid, the wound is complete and no residual mass is present.



Figure 6 Wounds after endoscopic clipping.

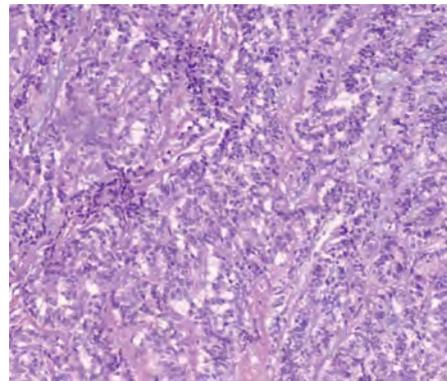


Figure 7 Pathological manifestations of rectal carcinoids. The carcinoid cells are small oval or polygonal, nested or glandular-like, and with a regular and small nucleus under microscope (magnification $\times 400$).

DISCUSSION

Carcinoid tumors are derived from argyrophilic cells in the intestinal glandular base (Kulchitsky cells) and are known for their affinity for silver staining, thus, these tumors are also known as argyrophil cell carcinomas. Rare as they are, carcinoids can occur in multiple body systems, with the gastrointestinal tract being the most commonly involved system. Rectal carcinoids are the third leading gastrointestinal carcinoids, accounting for about 14% of all conditions of this type^[1], with an increasing incidence in recent years potentially attributable to extensive endoscopic application and increased awareness of carcinoid tumors. Epidemiological surveys have revealed that the incidence rate of gastrointestinal carcinoids has increased year by year with the popularity of endoscopy, and ranges from 0.27 to 1.33 per 100000 people. The highest incidence was found in the appendix (38%), followed by the small intestine (29%), colon (13%), and stomach (12%). Based on the epidemiological statistics in Europe and North America conducted by Shields *et al*^[2], the median age for rectal carcinoids is 55 years, the average tumor size is 10 mm, and the average age of onset for the surveyed patients is 52 years, similar to the findings in this study.

Accounting for about 1.33% of rectal tumors, rectal carcinoids can give rise to non-specific symptoms such as general discomfort, constipation, blood in stool, ab-

dominal pain, weight loss, and change in bowel habits^[3]. Based on their origins, carcinoids can be divided into foregut, midgut and hindgut tumors. Rectal carcinoids, originating in the hindgut, generally secrete no or few active substances, such as 5-hydroxytryptamine (5-HT), resulting in normal 5-HT and 24 h 5-hydroxyindoleacetic acid levels and a very low risk of carcinoid syndromes in patients^[4].

Colonoscopy is an important tool in the detection of rectal carcinoids. Typical manifestations include single or multiple yellow or gray submucosal nodular masses covered with normal mucosa. Atypical carcinoids are often associated with irregular, hard ulcers. Endoscopic ultrasonography has become a necessary part of the diagnosis of rectal carcinoids and helps to determine the size, origin, contour, borders and depth of submucosal invasion, which are important for the differential diagnosis. Carcinoid lesions mostly originate in the submucosa or muscularis mucosa, and have a hypoechoic appearance. In addition, pathological examination is an important means of diagnosing rectal carcinoids. Typical histology includes solid nesting, nodular, insular, trabecular, palisading and rosette-like patterns, with small oval or polygonal, nested or glandular-like carcinoid cells, a regular and small nucleus, and little or no mitotic figures under the microscope. Chromogranin A, neuron-specific

enolase and synaptophysin are the most sensitive and specific markers of neuroendocrine cells^[5].

Studies have shown that lymph node metastasis and liver metastasis of a given rectal carcinoid are significantly correlated with the size of the tumor. The incidence rates of lymph node metastasis are 3%, 7% and 64% for rectal carcinoids < 1 cm, 1-2 cm and > 2 cm in diameter, respectively. Therefore, it is generally believed that metastasis is rare in rectal carcinoids less than 1 cm, but more often seen in those > 2 cm^[6]. Carcinoid metastasis occurs by direct invasion, including invasion of the surrounding tissue by serosal penetration, while lymph node metastasis or hematogenous metastasis can also be observed.

It is generally accepted that lymphatic invasion, muscularis propria invasion and lymph node metastasis are unlikely to occur in rectal carcinoid tumors < 1 cm in diameter and such small, well differentiated tumors can be removed endoscopically or by local excision. Endoscopic ultrasound must be employed to determine the exact size and depth of invasion of the tumor before surgery. The absence of preoperative staging will result in a suspicious or even positive pathological resection margin in 31.8%-83% of patients^[7,8]. In contrast, appropriate endoscopic ultrasound staging before endoscopic submucosal dissection (ESD) reduces the postoperative positive margin rate to between 4.8% and 17%^[9]. Therefore, this technique should be routinely performed before local excision of rectal carcinoids. Traditionally, most rectal carcinoid tumors of 1 cm or smaller are treated with EMR.

With the development of endoscopic techniques, many clinicians have been using ESD for the local resection of these tumors. Compared with traditional EMR, *en bloc* removal can be achieved using ESD regardless of tumor size, but it is also associated with a higher risk of complications (such as intraoperative or postoperative bleeding and perforation) and longer duration of operation. Yamaguchi *et al.*^[10] reported complete removal in 90% of their 20 patients with rectal carcinoids of < 1 cm using ESD, with an average operation time of 45 min, and one case of perforation. As reported by Lee *et al.*^[11], complete removal was achieved in 89.3% and 100% of patients undergoing EMR and ESD, without a significant difference in the size of tumors, respectively. EMR was successfully completed in all 21 patients with a rectal carcinoid of less than 1 cm in diameter without bleeding, perforation or other complications. The mean operation time was 15 min. The operation duration in the ESD group was significantly longer compared with the EMR group. It can be seen that, for lesions less than 1 cm in diameter, there is no considerable difference in the rate of complete removal between the EMR and ESD techniques, although a higher risk of complications and longer operation are associated with the latter technique.

For regional lymph node-negative patients with rectal carcinoids < 1 cm without muscular and vascular invasion, the 5-year survival rate can be 98.9% to 100% after treatment. Those who have positive lymph nodes but no distant metastases have a 5-year survival rate of 54% to

73%, and those with metastases have a 5-year survival rate of approximately 15%-30%^[12]. Wang *et al.*^[13] found that the presence or absence of muscularis propria infiltration is the only determining factor in the 5-year survival rate, which is closely related to tumor size. Yoon *et al.*^[14] reported that, the possibility of distant metastasis from rectal carcinoids increases with tumor volume and T staging, as well as the presence of lymphatic, vascular or neural invasion. EMR was performed for the 21 patients with a rectal carcinoid of less than 1 cm in diameter. Pre-operative micro-probe ultrasound showed homogeneous hypoechoic masses derived from rectal mucosa and/or muscularis mucosa, but without infiltration in the muscularis propria or blood vessels. The tumor was completely removed. The resected specimens were immunohistochemically diagnosed as carcinoids. No relapse was noted during the follow-up of 1-2 years. The resection rate was high in our cohort and no relapse was noted, which may be explained by the small sample size and short follow-up. Therefore, studies with larger sample size and longer follow-up are warranted.

Although rectal carcinoid tumors are potentially malignant, they often grow slowly and present with a fairly long disease course, with favorable outcomes. To sum up, since lymphatic invasion, muscularis propria invasion and lymph node metastasis are unlikely to occur in rectal carcinoid tumors < 1 cm in diameter, these small, well differentiated tumors can be treated by endoscopic resection. A growing number of rectal carcinoids can be detected early with endoscopy, and endoscopic ultrasonography is a necessary part of the diagnosis of rectal carcinoids and can help to determine the size, origin, contour, borders and depth of submucosal invasion, which are important for the differential diagnosis and choice of treatment. EMR and ESD are the primary endoscopic treatments for these conditions, and EMR is a safer option with fewer complications, shorter operating time, and comparable rate of complete removal to ESD. We believe that EMR is more suitable for treating rectal carcinoids less than 1 cm in diameter.

COMMENTS

Background

Rectal carcinoids are rare neuroendocrine tumors that are often missed or misdiagnosed in clinical settings due to the absence of specific symptoms in the early stage. Rare as they are, carcinoids can occur in multiple body systems, with the gastrointestinal tract being the most commonly involved system. Rectal carcinoids are the third leading gastrointestinal carcinoids, accounting for about 14% of all conditions of this type, with an increasing incidence in recent years potentially attributable to extensive endoscopic application and increased awareness of carcinoid tumors.

Research frontiers

With the development of endoscopic techniques, many clinicians have been using endoscopic submucosal dissection (ESD) for the local resection of these tumors. Compared with traditional endoscopic mucosal resection (EMR), *en bloc* removal can be achieved using ESD regardless of tumor size, but it is also associated with a higher risk of complications (such as intraoperative or postoperative bleeding and perforation) and longer duration of operation.

Innovations and breakthroughs

A growing number of rectal carcinoids can be detected early with endoscopy,

and endoscopic ultrasonography is a necessary part of the diagnosis of rectal carcinoids and can help to determine the size, origin, contour, borders and depth of submucosal invasion, which are important for the differential diagnosis and choice of treatment. EMR and ESD are the primary endoscopic treatments for these conditions, and EMR is a safer option with fewer complications, shorter operation time, and comparable rate of complete removal to ESD. The authors believed that EMR is more suitable for treating rectal carcinoids less than 1 cm in diameter.

Applications

The authors evaluated the role of EMR under micro-probe ultrasound guidance in treatment of rectal carcinoids, and concluded that EMR is a safe and effective treatment for rectal carcinoids less than 1 cm in diameter.

Terminology

ESD is an advanced technique of therapeutic endoscopy for superficial gastrointestinal neoplasms. EMR represents a major advance in minimally invasive surgery in the gastrointestinal tract. EMR is based on the concept that endoscopy provides visualization and access to the mucosa, the innermost lining of the gastrointestinal tract.

Peer review

This is a retrospective case series of endoscopic removal of rectal carcinoid smaller than 1 cm. The authors evaluated the role of EMR under micro-probe ultrasound guidance in treatment of rectal carcinoids, and concluded that EMR is a safe and effective treatment for rectal carcinoids less than 1 cm in diameter. The paper is well organized and correctly reported. Some issues are needed to be concerned.

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Acid suppressive drugs and gastric cancer: A meta-analysis of observational studies

Jeong Soo Ahn, Chun-Sick Eom, Christie Y Jeon, Sang Min Park

Jeong Soo Ahn, Sang Min Park, Department of Family Medicine, Seoul National University Hospital, Seoul National University College of Medicine, Seoul 110-744, South Korea
Chun-Sick Eom, Department of Family Medicine, Hallym University Chuncheon Sacred Heart Hospital, Chuncheon 200-702, South Korea

Christie Y Jeon, Center for Cancer Prevention and Control Research, UCLA Fielding School of Public Health, Los Angeles, CA 90095, United States

Author contributions: Ahn JS and Park SM had full access to all the data in the study and take responsibility for the integrity of the data and the accuracy of the data analysis; Ahn JS and Park SM designed the study; Ahn JS, Park SM and Eom CS performed the data acquisition; Ahn JS and Jeon CY analyzed the data; Ahn JS, Park SM and Jeon CY wrote the paper; all the authors have approved the final submitted manuscript.

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Correspondence to: Dr. Sang Min Park, Department of Family Medicine, Seoul National University Hospital, Seoul National University College of Medicine, 101 Daehangno, 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 evaluate the association between acid suppressive drug use and the development of gastric cancer.

METHODS: A systematic search of relevant studies that were published through June 2012 was conducted using the MEDLINE (PubMed), EMBASE, and Cochrane Library databases. The search included observational studies on the use of histamine 2-receptor antagonists (H₂RAs) or proton pump inhibitors and the associated risk of gastric cancer, which was measured using the adjusted odds ratio (OR) or the relative risk and 95%CI. An independent extraction was performed by

two of the authors, and a consensus was reached.

RESULTS: Of 4595 screened articles, 11 observational studies ($n = 94558$) with 5980 gastric cancer patients were included in the final analyses. When all the studies were pooled, acid suppressive drug use was associated with an increased risk of gastric cancer risk (adjusted OR = 1.42; 95%CI: 1.29-1.56, $I^2 = 48.9%$, $P = 0.034$). The overall risk of gastric cancer increased among H₂RA users (adjusted OR = 1.40; 95%CI: 1.24-1.59, $I^2 = 59.5%$, $P = 0.008$) and PPI users (adjusted OR = 1.39; 95%CI: 1.19-1.64, $I^2 = 0.0%$, $P = 0.377$).

CONCLUSION: Acid suppressive drugs are associated with an increased risk of gastric cancer. Further studies are needed to test the effect of acid suppressive drugs on gastric cancer.

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Key words: H₂-receptor antagonists; Proton pump inhibitors; Gastric cancer; Meta-analysis

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INTRODUCTION

Acid suppressive drugs were the second most prescribed medication worldwide in 2005. Histamine 2-receptor antagonists (H₂RAs) and proton pump inhibitors (PPIs) were widely used for the treatment of peptic ulcers, gastroesophageal reflux disease (GERD), and other benign conditions of the stomach, esophagus, and duodenum^[1-4]. PPIs were one of the most commonly prescribed medications by primary physicians and are frequently used

over the long term.

The safety of these drugs and their potential adverse effects is of great importance to public health. Several case reports suggested that acid suppressive drugs may increase the occurrence of gastric polyps or cancer^[5-13], and several epidemiological studies have evaluated the association between long-term gastric acid suppression and the risk of gastrointestinal neoplasms. Several researchers have proposed that acid suppressive drugs may suppress gastric acid secretion and interfere with bacterial growth and nitrosamine formation^[14-16]. In addition, the reduction of gastric acid secretion with acid suppressive drugs can lead to hypergastrinemia^[17,18], which has been identified as a possible risk factor for gastric polyps and gastric and colonic carcinomas^[19-22]. However, those findings are contradictory: several studies have found an increased risk of gastric cancer among acid suppressive drug users^[23-25], whereas other studies found no evidence of an increased risk^[26-28]. To date, no systematic meta-analysis has been published on this topic.

Therefore, in this study, we sought to investigate the association between the use of acid suppressive drugs and the risk of gastric cancer *via* a meta-analysis of cohort studies and case-control studies.

MATERIALS AND METHODS

Data sources and searches

Our review followed the Meta-analysis of Observational Studies in Epidemiology guidelines^[29]. We performed our search in MEDLINE (PubMed) (inception to June 2012), EMBASE (inception to June 2012), and the Cochrane Library (inception to June 2012) using common key words regarding acid suppression and gastric cancer in case-control studies, cohort studies, and randomized controlled trials (RCTs). However, there were no RCTs among the search results that satisfied our inclusion criteria.

In addition, we searched the bibliographies of relevant articles to identify additional studies of interest. For the studies that did not directly report the association between the use of acid suppressive drugs and gastric cancer incidence, we contacted the authors in the field for any unpublished data. However, the authors did not have any available data to use in our meta-analysis. We used the following keywords in the literature search: “histamine receptor antagonist”, “H₂ receptor antagonist”, “cimetidine”, “ranitidine”, “famotidine”, “nizatidine”, “proton pump inhibitor”, “proton pumps”, “omeprazole”, “nexium”, “lansoprazole”, “rabeprazole”, “pantoprazole”, or “esomeprazole” for the exposure factors and “stomach cancer”, “stomach neoplasia”, “gastric cancer”, “gastric neoplasia”, “stomach neoplasm” or “gastric neoplasm” for the outcome factors.

Study selection and data extraction

We included case-control studies and cohort studies that investigated the association between acid suppressive

drug use and gastric cancer risk, which reported an adjusted odds ratio (OR) or relative risk (RR) and the corresponding 95%CI. We only selected articles that were written in English and excluded studies with no available data for outcome measures.

All the studies that were retrieved from the databases and bibliographies were independently evaluated by two authors of this paper (Ahn JS and Eom CS). Of the articles that were found in the three databases, duplicate articles and articles that did not meet the selection criteria were excluded. We extracted the following data from the remaining studies: the study names (first author), the year of publication, the country of publication, the study design, the study period, the population characteristics, the type of drugs, the adjusted OR or RR with a 95%CI: the study quality, and the adjustment. The data abstraction and the study selection were performed in duplicate.

Quality assessment

We assessed the methodological quality of the included studies using the Newcastle-Ottawa Scale (NOS) for the case-control and cohort studies in the meta-analysis^[30]. The NOS is comprehensive and has been partially validated for assessing the quality of non-randomized studies in meta-analyses. The NOS is based on the following three broad subscales: the selection of the study groups (4 items), the comparability of the groups (1 item), and the ascertainment of the exposure and the outcome of interest for case-control studies and cohort studies, respectively (3 items). A “star system” (a score range from 0-9) has been developed for quality assessment. Each study can be awarded a maximum of one star for each numbered item within the selection and exposure categories, whereas a maximum of two stars can be assigned for comparability. In this study, we considered a study that was awarded 7 or more stars as a high-quality study because standard criteria have not been established.

Statistical analysis

The outcome of the meta-analysis was the risk of gastric cancer. We used the adjusted data (adjusted OR or RR with a 95%CI) for the meta-analysis. In addition, we conducted subgroup analyses by type of study design (case-control studies *vs* cohort studies), type of acid suppressive drugs (H₂RAs *vs* PPIs), duration of acid suppressive drug use (within 5 years *vs* more than 5 years), location of gastric cancer (gastroesophageal junction, cardia, or non-cardia), and study quality (high-quality *vs* low-quality).

We pooled the adjusted ORs, RRs and 95%CIs based on both fixed-effects and random-effects models. Because the incidence of gastric cancer was low (< 5%), as evidenced in the cohort studies, we concluded that the outcome was sufficiently rare to assume that the OR could be used to approximate the RR. Heterogeneity was assessed using the Higgins *I*² value, which measures the percentage of total variance across studies, which is attributable to heterogeneity rather than chance. Negative

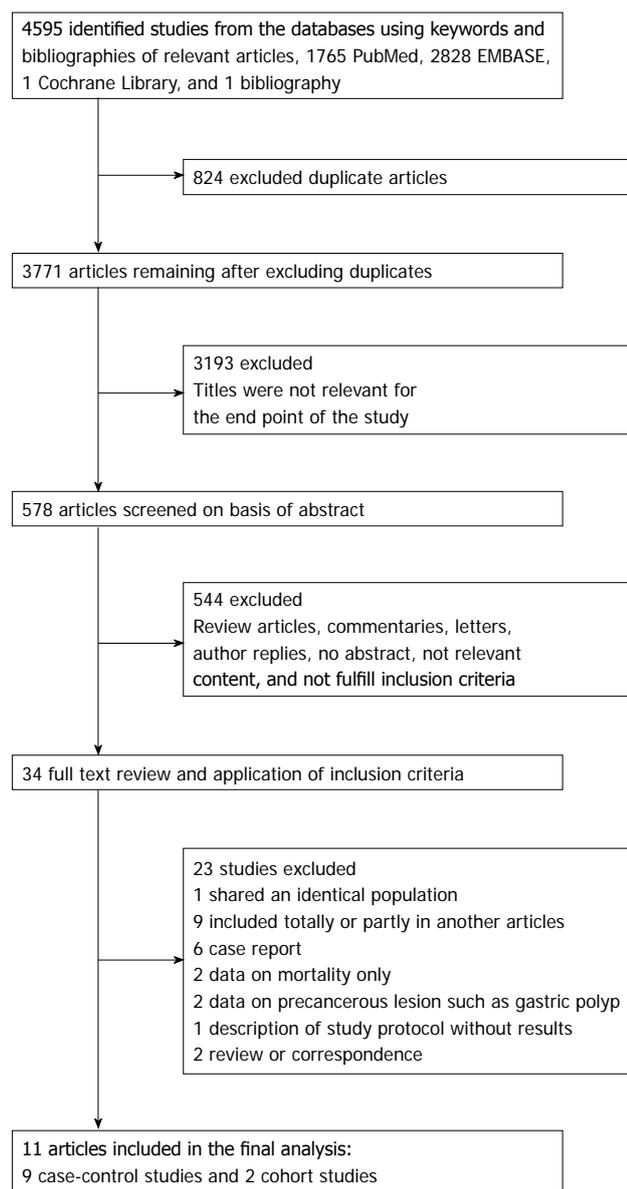


Figure 1 Flow diagram for identification of relevant studies.

I^2 values were set at zero to ensure that I^2 fell between 0% (no observed heterogeneity) and 100% (maximal heterogeneity)^[31,32]. We considered an I^2 value greater than 50% to represent substantial heterogeneity. When the heterogeneity was substantial, we conducted sensitivity analyses: the changes in the I^2 values were examined by removing each study from the analysis to determine which studies contributed most significantly to the heterogeneity. We assessed between-group heterogeneity using analysis of variance. When heterogeneity was found, we performed a meta-regression using the subgroup categories.

We used the Woolf method (inverse variance method) for a fixed-effects analysis^[33,34], and we used the DerSimonian and Laird method for a random-effects analysis^[35]. Begg's funnel plot and Egger's test were used to identify publication bias. Publication bias was detected in studies when the funnel plot was asymmetrical or the P -value was less than 0.05 using Egger's test. We used the

Stata/SE version 10.1 software program (StataCorp, College Station, TX, United States) for the statistical analysis.

RESULTS

Identification of relevant studies

Figure 1 shows a flow diagram of the study selection. A total of 4595 articles were identified by searching the three databases and relevant bibliographies. We excluded 824 duplicate articles and 3737 articles that did not satisfy the selection criteria. After the full texts of the remaining 34 articles were reviewed, the following 23 articles were excluded: one article from a study that shared an identical population^[36]; 6 articles that were case reports^[5-10]; 2 articles that included data on precancerous lesions, such as gastric polyps^[37,38]; one article that described a study protocol without results^[39]; 2 articles that only included mortality data^[40,41]; 2 articles that were reviews or correspondence^[25,42]; and 9 articles that were included totally or partially in another article^[43-51]. As a result, we included 11 observational studies (9 case-control studies and 2 cohort studies), which ultimately met our inclusion criteria.

Characteristics of the studies included in the final analysis

Table 1 shows the main characteristics of the 11 reviewed studies. Five studies were published in the 1990s^[23-27], and 6 studies were published in the 2000s^[52-57]. The countries where the studies had been conducted were as follows: the United States ($n = 5$), the United Kingdom ($n = 2$), Denmark ($n = 2$), Canada ($n = 1$), and Italy ($n = 1$). We identified a total of 94558 participants, which included 5980 cases and 88578 controls from the following articles: 1 article on a medical record-based case-control study, 3 articles on nested case-control studies, 2 articles on population-based case-control studies, and 2 articles on prospective cohort studies. Seven studies evaluated the association between H₂RA use and the risk of gastric cancer, and 4 studies assessed the association between the use of H₂RAs or PPIs and the risk of gastric cancer. The mean value for the methodological quality of the 11 studies was 6.64 stars according to the NOS (Table 1).

Overall use of acid suppressive drugs and the risk of gastric cancer

Figure 2 shows the association between the use of acid suppressive drugs and gastric cancer risk. The overall use of H₂RAs and PPIs was associated with an increased risk of gastric cancer in 9 case-control studies [adjusted OR = 1.36; 95%CI: 1.23-1.50, $n = 9$, $I^2 = 24.5%$ (range, 0.0%-64.0%)] and in 2 cohort studies [adjusted RR, 2.01; 95%CI: 1.51-2.68, $n = 2$, $I^2 = 63.0%$ (not available)] using a fixed-effects model, and this association was observed in a combined study [adjusted OR and RR, 1.42; 95%CI: 1.29-1.56, $n = 11$, $I^2 = 48.9%$ (range, 0.0%-74.0%)]. As shown in Table 2, an increased risk of gastric cancer was found with H₂RA use [adjusted OR = 1.40; 95%CI: 1.24-1.59, $n = 10$, $I^2 = 59.5%$ (range, 19.0%-80.0%)] and with PPI use [adjusted OR = 1.39; 95%CI: 1.19-1.64,

Table 1 Characteristics of observational studies on acid suppressive drugs and gastric cancer

| Ref. | Country | Design | Study period | Range of age (yr) | Population | Drugs | Adjusted OR or RR (95%CI) | Study quality ¹ | Adjustment for covariates |
|---|----------------|--------|--------------|-------------------|---|---|---------------------------|----------------------------|--|
| La Vecchia <i>et al</i> ^[23] | Italy | CC | 1985-1989 | 27-74 | 563 cases and 1501 controls | Cimetidine, Ranitidine | 1.80 (1.20-2.70) | 5 | Age, sex, education, smoking, alcohol, coffee |
| Johnson <i>et al</i> ^[24] | United States | CC | 1988-1992 | NA | 113 cases and 452 matched controls | Cimetidine, Ranitidine | 2.00 (1.00-3.90) | 5 | Age, sex, first pharmacy use |
| Møller <i>et al</i> ^[25] | Denmark | PCC | 1977-1990 | NA | 134 cases among 16739 | Cimetidine | 2.30 (1.60-3.10) | 6 | Age, sex, diagnosis, method of diagnosis |
| Schumacher <i>et al</i> ^[26] | United States | CC | 1981-1987 | 17-94 | 99 cases and 365 controls | Cimetidine | 2.30 (0.80-6.90) | 6 | Age, sex, date of first pharmacy use |
| Chow <i>et al</i> ^[27] | United States | MCC | 1986-1992 | NA | 196 cases and 200 controls | Cimetidine, Ranitidine, Famotidine, Nizatidine | 0.60 (0.30-1.30) | 6 | Race, smoking, BMI, number of composite conditions ² |
| Farrow <i>et al</i> ^[52] | United States | PBCC | 1993-1995 | 30-79 | 293 esophageal adenoca, 221 esophageal SCC, 261 gastric cardia adenoca, 368 non-cardia gastric adenoca and 695 controls | Cimetidine, Ranitidine, Famotidine, Nizatidine | 1.00 (0.60-1.90) | 7 | Age, sex, study center, smoking, BMI, history of gastric or duodenal ulcer, GERD symptom frequency, alcohol |
| Suleiman <i>et al</i> ^[53] | United Kingdom | NCC | 1990-1992 | NA | 231 cases among 9876 controls | Cimetidine, Ranitidine | 2.56 (0.17-38.09) | 7 | Age, sex, MI, antacid, steroid, smoking, alcohol, social class, height, weight |
| García Rodríguez <i>et al</i> ^[54] | United Kingdom | NCC | 1994-2001 | 40-84 | 287 esophageal adenoca, 195 gastric cardiac adenoca, 327 gastric non-cardia adenoca and 10000 controls | H ₂ RAs, PPIs | 1.24 (0.88-1.75) | 8 | Age, sex, calendar year, smoking, alcohol, BMI, UGI disorder, hiatal hernia, GU, DU, dyspepsia |
| Tamim <i>et al</i> ^[55] | Canada | NCC | 1995-2003 | NA | 1589 cases and 12991 controls | H ₂ RAs, PPIs | 1.37 (1.22-1.53) | 8 | Age, sex number of prescriptions to any drug, total length of hospitalizations and number of visit to GPs, specialist, emergency rooms |
| Duan <i>et al</i> ^[56] | United States | PBCC | 1992-1997 | NA | 220 esophageal adenoca, 277 gastric cardiac adenoca, 441 distal gastric adenoca and 1356 controls | H ₂ RAs, PPIs | 1.15 (0.58-2.29) | 7 | Age, sex, race, birthplace, education, smoking, BMI, history of UGI symptom |
| Poulsen <i>et al</i> ^[57] | Denmark | PCC | 1990-2003 | NA | 161 cases among users of 18790 PPIs or 17478 H ₂ RAs | Omeprazole, Lansoprazole, Esomeprazole, Pantoprazole, Rabeprazole, Cimetidine, Ranitidine, Nizatidine, Famotidine | 1.30 (0.70-2.30) | 8 | Age, sex, calendar period, history of <i>H. pylori</i> , gastroscopy year, COPD, alcohol, NSAID |

¹Study quality was judged based on the Newcastle-Ottawa Scale (range, 1-9 stars). The mean value for the methodological quality of 11 studies was 6.64 stars; ²Number of composite conditions were created to include gastroesophageal reflux, hiatal hernia, esophagitis/esophageal ulcer, or difficulty swallowing. OR: Odds ratio; RR: Relative risk; CC: Case-control; MCC: Medical record based case-control; PBCC: Population-based case-control study; NCC: Nested case-control study; PCC: Prospective cohort study; NA: Not available; Adenoca: Adenocarcinoma; SCC: Squamous cell carcinoma; H₂RAs: Histamine 2-receptor antagonists; PPIs: Proton pump inhibitors; BMI: Body mass index; GERD: Gastroesophageal reflux disease; MI: Myocardial infarction; UGI: Upper gastrointestinal; GU: Gastric ulcer; DU: Duodenal ulcer; GPs: General practitioners; COPD: Chronic obstructive pulmonary disease; NSAID: Nonsteroidal anti-inflammatory drug.

$n = 3$, $I^2 = 0.0\%$ (range, 0.0%-90.0%). In a sensitivity analysis, when the study by Møller *et al*^[25] was removed, the I^2 values of the H₂RA studies decreased from 59.5% to 34.3%, but the effect remained significant. In addition, when stratified by the study design, H₂RA use was positively associated with gastric cancer in both case-control [adjusted OR = 1.30; 95%CI: 1.13-1.49, $n = 8$, $I^2 = 42.0\%$ (range, 0.0%-74.0%)] and cohort [adjusted RR, 1.84; 95%CI: 1.41-2.41, $n = 2$, $I^2 = 80.4\%$ (not available)] studies. The use of PPIs was associated with an increased risk

of gastric cancer in case-control studies [adjusted OR = 1.44; 95%CI: 1.21-1.71, $n = 2$, $I^2 = 23.5\%$ (not available)], whereas only one cohort study was conducted to evaluate the use of PPIs (adjusted RR, 1.20; 95%CI: 0.80-1.80) (data were not shown).

Subgroup meta-analysis

In a subgroup meta-analysis, acid suppressive drugs were associated with an increased gastric cancer risk within 5 years of use [adjusted OR = 1.58; 95%CI: 1.35-1.81,

Table 2 Association between acid suppressive drugs use and gastric cancer risk in subgroup meta-analysis

| Category | No. of studies | Adjusted OR/RR (95%CI) | Heterogeneity, I ² % (95%CI) | Model used | Heterogeneity between groups |
|----------------------------|-------------------------------------|------------------------|---|---------------|------------------------------|
| Type of drugs | | | | | P = 0.01 |
| H ₂ RAs | 10 ^[23-27,52-55,57] | 1.40 (1.24-1.59) | 59.5 (19.0-80.0) ¹ | Fixed-effects | |
| PPIs | 3 ^[54,55,57] | 1.39 (1.19-1.64) | 0.0 (0.0-90.0) | Fixed-effects | |
| Location of gastric cancer | | | | | P = 0.03 |
| GE junction | 2 ^[24,26] | 2.28 (0.97-5.35) | 0.0 (NA) | Fixed-effects | |
| Gastric cardia | 4 ^[52,54-56] | 0.88 (0.63-1.24) | 9.2 (0.0-86.0) | Fixed-effects | |
| Non-cardia | 6 ^[24,26,52,54-56] | 1.42 (1.12-1.79) | 0.0 (0.0-75.0) | Fixed-effects | |
| Duration of drugs use | | | | | P = 0.27 |
| Within 5 yr | 7 ^[23,24,26,27,52,55,57] | 1.58 (1.35-1.81) | 60.2 (9.0-83.0) ¹ | Fixed-effects | |
| Over 5 yr | 6 ^[23,24,26,27,52,57] | 1.24 (0.84-1.84) | 25.4 (0.0-69.0) | Fixed-effects | |
| Study quality | | | | | P = 0.01 |
| High-quality ² | 6 ^[52,53,54,55-57] | 1.34 (1.21-1.48) | 0.0 (0.0-75.0) | Fixed-effects | |
| Low-quality | 5 ^[23-27] | 1.86 (1.49-2.32) | 63.5 (4.0-86.0) ¹ | Fixed-effects | |

¹I² value greater than 50% represents substantial heterogeneity. We conducted sensitivity analyses by removing each study to determine the studies that contributed most to the heterogeneity; ²High quality study was considered a study awarded 7 or more stars, as standard criteria have not been established. OR: Odds ratio; RR: Relative risk; H₂RAs: Histamine 2-receptor antagonists; PPIs: Proton pump inhibitors; GE junction: Gastroesophageal junction; NA: Not available.

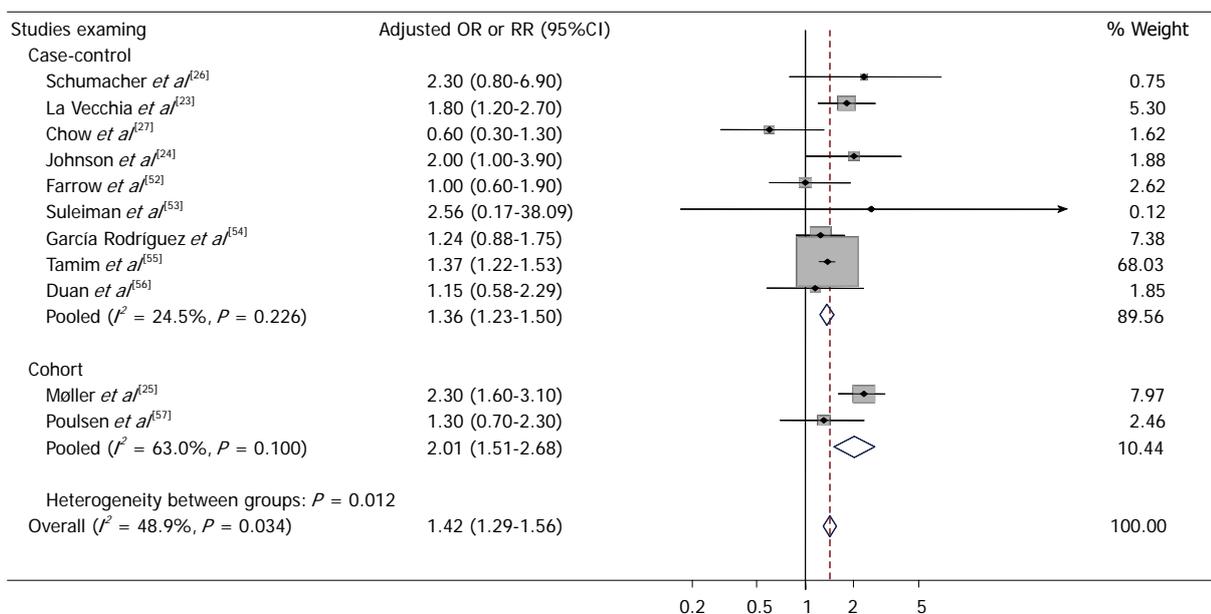


Figure 2 Meta-analysis results of individual and pooled adjusted odds ratio or relative risk of gastric cancer. The size of each square is proportional to the study's weight. Diamonds are the summary estimate from the pooled studies with 95%CI. OR: Odds ratio; RR: Relative risk.

n = 7, I² = 60.2% (not available)]. In a sensitivity analysis, when the study by La Vecchia *et al*^[23] was removed, the I² values decreased from 60.2% to 41.4%; however, the summary estimate indicated an elevated risk of gastric cancer. Additionally, a positive association was observed in patients with more than 5 years of acid suppressive drug use. However, there was no statistical significance [adjusted OR = 1.24; 95%CI: 0.84-1.84, n = 6, I² = 25.4% (not available)] (Table 2). The between-group differences in the effect estimates for within 5 years of exposure *vs* more than 5 years of exposure were not significant (P = 0.27).

Regarding the location of gastric cancer, a significant positive association was observed between the use of acid suppressive drugs and non-cardia cancer risk [ad-

justed OR = 1.42; 95%CI: 1.12-1.79, n = 6, I² = 0.0% (not available)], whereas a marginal significance was observed in gastroesophageal junction cancer and the use of acid suppressive drugs [adjusted OR = 2.28; 95%CI: 0.97-5.35, n = 2, I² = 0.0% (not available)]. However, there was no significant association between the use of acid suppressive drugs and gastric cardia cancer [adjusted OR = 0.88; 95%CI: 0.63-1.24, n = 4, I² = 9.2% (0.0%-86.0%)]. The between-group differences in the effect estimates for non-cardia cancer *vs* gastroesophageal junction cancer *vs* gastric cardia cancer were significant (P = 0.03). The meta-regression according to the site of cancer indicated that the effect estimates for non-cardia cancers were significantly higher than those for cardia cancers (P = 0.02), and the effect estimates for gastroesophageal junction

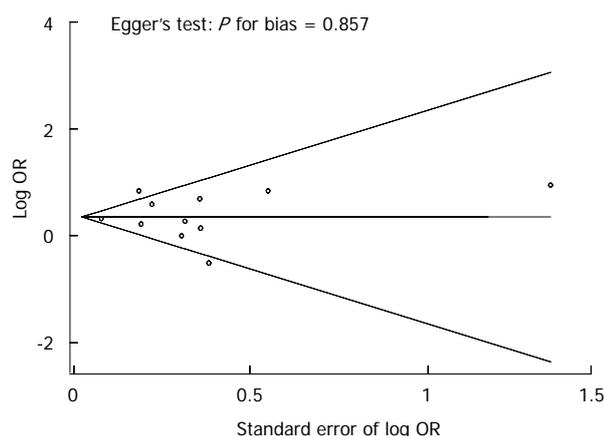


Figure 3 Begg's funnel plots and Egger's test for identifying publication bias ($P = 0.857$) in a meta-analysis of observational studies ($n = 11$). OR: Odds ratio.

cancers were significantly different from those of cardia cancers ($P = 0.04$).

There was an increased risk of gastric cancer that was associated with the use of acid suppressive drugs in both high-quality [adjusted OR = 1.34; 95%CI: 1.21-1.48, $n = 6$, $I^2 = 0.0\%$ (range, 0.0%-75.0%)] and low-quality [adjusted OR = 1.86; 95%CI: 1.49-2.32, $n = 5$, $I^2 = 63.5\%$ (range, 4.0%-86.0%)] studies. No publication bias was observed in the selected studies (Figure 3, Begg's funnel plot was symmetrical; Egger's test, P for bias = 0.857).

DISCUSSION

In this meta-analysis of 11 observational studies, we found that both H₂-receptor antagonist use and proton pump inhibitor use were associated with an increased risk of gastric cancer. In site-specific analyses, an increased risk of non-cardia gastric cancer was observed in patients who used acid suppressive drugs, whereas acid suppressive drug use was not associated with the risk of gastric cardia cancer.

Our meta-analysis has several strengths. This systematic review was the most comprehensive meta-analysis to date of observational studies that addressed the association between the use of acid suppressive drugs and the risk of gastric cancer, which included a large number of studies and participants. We performed a detailed analysis by stratifying the type of drugs (H₂RAs or PPIs), the location of gastric cancer (gastroesophageal junction, cardia, or non-cardia), the duration of acid suppressive drug use, and the study quality.

Previous studies suggested that long-term H₂RA use may increase gastric cancer^[11-13], and long-term PPI treatment induced gastric fundic polyps, which led to the development of precancerous lesions^[5-8]. There is biological evidence of the effect of acid suppressive drug use on the risk of gastric cancer. First, PPIs and H₂RAs can reduce gastric acidity by modulating H(+)-K(+)-ATPase or competitive inhibitors of histamine binding sites in gastric parietal cells. Decreased gastric acidity,

whether caused by gastric atrophy or acid suppressive drug-induced hypochlorhydria, may result in increased bacterial colonization and a greater number of bacteria that can produce nitrosamines^[16,58], which are compounds that are associated with an increased risk of gastric adenocarcinoma^[59]. Second, the reduction of gastric acid secretion by acid-suppressive drugs switches on the positive feedback of a gastric acid-producing cascade, which leads to hypergastrinemia^[19]. This condition is a possible cause of carcinoids, gastric polyps, and gastric and colonic carcinomas because elevated serum gastrin could have a trophic effect on neoplastic growth in the gastrointestinal tract^[60]. The use of long-term PPIs can cause hyperplasia in enterochromaffin-like cells and increase the incidence of atrophic gastritis and gastric polyps, which are a precursor to gastric cancer^[37,38,43-46].

We found that acid suppressive drugs increased the risk of non-cardia gastric cancer, whereas acid suppressive drug use was not associated with a risk of gastric cardia cancer. One possible mechanism may be associated with the augmented effects of acid suppressive drugs and other risk factors of non-cardia gastric cancer, such as gastric atrophy and *Helicobacter pylori* (*H. pylori*) infection^[61,62]. Several authors suggested that omeprazole treatment was associated with an elevated incidence of gastric corpus mucosal atrophy^[63], and long-term PPI treatment in *H. pylori* infected patients could accelerate the development of corpus atrophic gastritis^[64,65]. In addition, recent studies suggested that a history of *H. pylori* eradication prior to long-term PPI therapy could prevent the development of atrophic gastritis^[57,66,67]. Considering this knowledge, further studies are needed to clarify the association between acid suppressive drug use and the risk of non-cardia gastric cancer, especially for patients with gastric atrophy and *H. pylori* infection.

We found that within 5 years of use, acid suppressive drugs increased the risk of gastric cancer, whereas there was a non-significant increased risk of gastric cancer among users of acid suppressive drugs for more than 5 years. Our meta-analysis was underpowered to detect statistically significant differences between the studies according to the duration of exposure; however, the qualitative differences were noteworthy. Early gastric symptoms of stomach cancer are typically similar to those of benign conditions, such as peptic ulcers, GERD, or functional gastrointestinal disease^[23], which could lead to the use of acid suppressive drugs. Therefore, we could not exclude the possibility of misspecification and the protopathic bias of gastric cancer, especially among 5-year acid suppressive drug users. We found that two of the earliest studies (Møller *et al.*^[25] and La Vecchia *et al.*^[23]) contributed most significantly to the heterogeneity in the studies of H₂RAs and in the studies that examined exposure within 5 years of the incidence of cancer. These studies may have been influenced by misspecification because endoscopic tools that would have detected early stage cancer were not readily available when the studies were conducted. There could be bias due to existing gastric conditions; however, we recommend that future

studies carefully consider the appropriate control of previous gastric conditions.

Our meta-analysis has several limitations. First, most of the studies in our meta-analysis were observational studies. Observational studies, even when well-controlled, are susceptible to various biases, which may reduce the quality of the analysis. Second, most of the studies in our meta-analysis came from Western countries. The occurrence of gastric cancer is rapidly increasing in the United States and in Western Europe^[68]; however, this disease is a more significant public health problem in Asia. Third, we did not have access to individual data on dose-response relationships that may have affected gastric acid production. Finally, we could not evaluate the effect of underlying gastric conditions, *e.g.*, *H. pylori* infection, because these data were not presented in each study.

Our meta-analysis demonstrated that the use of acid suppressive drugs was associated with an increased risk of gastric cancer. Our findings should be confirmed by more prospective cohort studies that are designed with larger sample sizes and longer follow-up durations to test the effect of acid suppressive drugs on the risk of gastric cancer. These studies should focus on previous underlying gastric conditions for which acid suppressive drugs are prescribed and the dose response of acid suppressive drugs use.

COMMENTS

Background

The widespread use of acid suppressive drugs has led to concern about the development of adverse effects owing to prolonged gastric acid suppression, particularly the development of gastric polyps or gastric neoplasms. Authors performed a meta-analysis of cohort studies and case-control studies to determine whether the use of acid suppressive drugs, such as histamine 2-receptor antagonists (H₂RAs) and proton pump inhibitors (PPIs), can increase the risk for gastric cancer.

Research frontiers

Several case reports suggested that antacids may increase the risk of gastric polyps or cancer. Several epidemiological studies have evaluated the association between long-term gastric acid suppression and the risk of gastrointestinal neoplasms. However, epidemiological studies have reported inconsistent findings regarding the association between the use of acid suppressive drugs and gastric cancer risk. To date, no systematic meta-analysis has been published on the use of acid suppressive drugs and the risk of gastric cancer.

Innovations and breakthroughs

In this meta-analysis of case-control and cohort studies, the authors found that both H₂RA and PPI use were associated with an increased risk of gastric cancer. In site-specific analyses, an increased risk of non-cardia gastric cancer was observed in patients who used acid suppressive drugs, whereas acid suppressive drug use was not associated with a risk of gastric cardia cancer.

Applications

The use of acid suppressive drugs is associated with an increased risk of gastric cancer. However, authors could not exclude the possibility of misspecification and the protopathic bias of gastric cancer. The early gastric symptoms of stomach cancer are typically similar to those of benign conditions, such as peptic ulcers, gastroesophageal reflux disease, or functional gastrointestinal disease, which could lead to the use of acid suppressive drugs. There could be bias due to existing gastric conditions; therefore, they recommend that future studies carefully consider the appropriate control of previous gastric conditions.

Terminology

Acid suppressive drugs can reduce gastric acidity by modulating H(+)-K(+)-

ATPase or competitive inhibitors of histamine binding sites in gastric parietal cells, which leads to hypergastrinemia. This condition is a possible cause of carcinoids, gastric polyps, and gastric and colonic carcinomas because elevated serum gastrin could have a trophic effect on neoplastic growth in the gastrointestinal tract.

Peer review

This study may lead conclusion that acid suppressive drugs such as H₂R blocker or PPI may act as a stimulator for gastric cancer. It is difficult to obtain a substantial conclusion from 11 studies adopted by this paper because all 11 papers were observational study and no information on *Helicobacter pylori* infection. This paper is good for publication.

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Portal vein stenosis after pancreatectomy following neoadjuvant chemoradiation therapy for pancreatic cancer

Yosuke Tsuruga, Hirofumi Kamachi, Kenji Wakayama, Tatsuhiko Kakisaka, Hideki Yokoo, Toshiya Kamiyama, Akinobu Taketomi

Yosuke Tsuruga, Hirofumi Kamachi, Kenji Wakayama, Tatsuhiko Kakisaka, Hideki Yokoo, Toshiya Kamiyama, Akinobu Taketomi, Department of Gastroenterological Surgery I, Hokkaido University Graduate School of Medicine, Sapporo 060-8638, Japan

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Correspondence to: Yosuke Tsuruga, MD, PhD, Department of Gastroenterological Surgery I, Hokkaido University Graduate School of Medicine, North 15, West 7, Kita-ku, Sapporo 060-8638, Japan. ytsuruga@med.hokudai.ac.jp

Telephone: +81-11-7065927 Fax: +81-11-7177515

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Abstract

Extrahepatic portal vein (PV) stenosis has various causes, such as tumor encasement, pancreatitis and as a post-surgical complication. With regard to post-pancreaticoduodenectomy, intraoperative radiation therapy with/without PV resection is reported to be associated with PV stenosis. However, there has been no report of PV stenosis after pancreatectomy following neoadjuvant chemoradiation therapy (NACRT). Here we report the cases of three patients with PV stenosis after pancreatectomy and PV resection following gemcitabine-based NACRT for pancreatic cancer and their successful treatment with stent placement. We have performed NACRT in 18 patients with borderline resectable pancreatic cancer since 2005. Of the 15 patients who completed NACRT, nine had undergone pancreatectomy. Combined portal resection was performed in eight of the nine patients. We report here three patients with PV stenosis, and thus the ratio of post-operative PV stenosis in patients with PV resection following NACRT is 37.5% in this series. We encountered no case of PV stenosis

among 22 patients operated with PV resection for pancreaticobiliary cancer without NACRT during the same period. A relationship between PV stenosis and NACRT is suspected, but further investigation is required to determine whether NACRT has relevance to PV stenosis.

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Key words: Pancreatic cancer; Portal vein stenosis; Neoadjuvant chemoradiation therapy; Pancreatectomy; Expandable metallic stent

Core tip: Intraoperative radiation therapy for pancreatic cancer with/without portal vein (PV) resection is reported to be associated with PV stenosis. However, there has been no report of PV stenosis after pancreatectomy following neoadjuvant chemoradiation therapy (NACRT). Here we report the cases of three patients with PV stenosis after pancreatectomy and PV resection following gemcitabine-based NACRT for pancreatic cancer and their successful treatment with stent placement. We have performed pancreatectomy with PV resection following NACRT in 8 patients with borderline resectable pancreatic cancer since 2005. The ratio of post-operative PV stenosis is 37.5% in this series.

Tsuruga Y, Kamachi H, Wakayama K, Kakisaka T, Yokoo H, Kamiyama T, Taketomi A. Portal vein stenosis after pancreatectomy following neoadjuvant chemoradiation therapy for pancreatic cancer. *World J Gastroenterol* 2013; 19(16): 2569-2573 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v19/i16/2569.htm> DOI: <http://dx.doi.org/10.3748/wjg.v19.i16.2569>

INTRODUCTION

Extrahepatic portal vein (PV) stenosis can occur due to

tumor encasement^[1], pancreatitis^[2] and as a post-surgical complication-especially post-liver transplantation^[3]. With regard to post-pancreaticoduodenectomy (PD), intraoperative radiation therapy (IORT) with/without PV resection is reported to be associated with PV stenosis^[4-6]. The incidence rate for PV stenosis is reported to be 11% to 23%^[5,6].

Portal hypertension secondary to PV stenosis causes gastrointestinal bleeding from gastroesophageal or jejunal varices, and refractory ascites^[1]. Gastrointestinal bleeding is the most serious life-threatening complication. Refractory ascites is not fatal but affects the patient's quality of life.

Here we provide the case reports of three patients with PV stenosis after pancreatectomy and PV resection following neoadjuvant chemoradiation therapy (NACRT) for pancreatic cancer, and we discuss the relationship between PV stenosis and NACRT and the indications for stent placement. To the best of our knowledge, PV stenosis after pancreatectomy following NACRT has not been described in the literature.

CASE REPORT

Case 1

A 54-year-old man underwent PD and PV resection for pancreatic cancer following NACRT. The protocol consisted of external-beam radiotherapy to the pancreatic bed and regional lymphatics for a total dose of 50.4 Gy in 28 fractions. Concomitant chemotherapy consisted of gemcitabine at a dose of 150 mg/m² once weekly. The reconstruction between the PV and the superior mesenteric vein (SMV) was end-to-end anastomosis using a continuous running suture of 6-0 prolene. The splenic vein was not reconstructed.

Refractory ascites and malnutrition were recognized at 9 postoperative months (POMs). Computed tomography (CT) showed short segmental stenosis of the PV in the region of the anastomosis, severe ascites, and liver atrophy (Figure 1). Intraoperative portography through the catheter via the ileocolic vein and balloon dilation were performed but were not sufficiently effective because the region had elastic stenosis. Therefore, an expandable metallic stent (EMS; 1 cm diameter, 3 cm length) was inserted into the stenotic region. The portal venous pressure decreased from 14.5 to 8.6 cm H₂O, and the pressure gradient of 6.5 cm H₂O across the PV stenosis disappeared. Anticoagulant therapy was initiated immediately after stent placement. Heparin was administered at a dose of 10000 IU/d by intravenous infusion for 3 d initially, and then oral warfarin was administered. The warfarin was switched to aspirin 3 mo later. After the stent placement, a follow-up CT showed that the patient's ascites decreased and his liver atrophy improved (Figure 2). Stent patency is maintained at present, 5 years after the placement.

Case 2

A 44-year-old man underwent distal pancreatectomy and

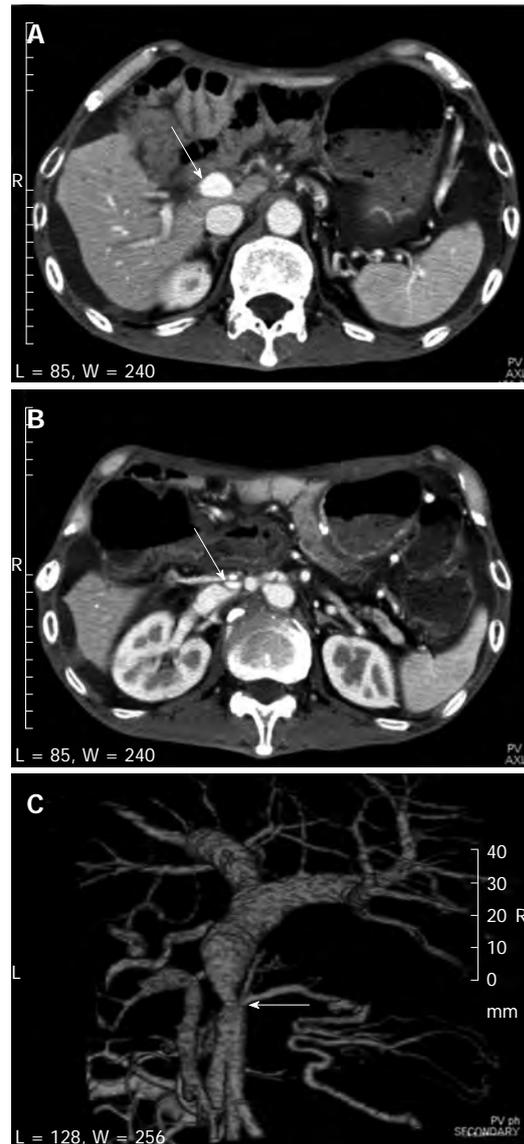


Figure 1 Computed tomography showed short segmental stenosis of the portal vein in the region of the anastomosis, severe ascites, and liver atrophy. A, B: Computed tomography (CT) scan shows severe ascites and liver atrophy (arrow) (A), and stenosis of the portal vein (arrow) behind the superior mesenteric artery (B); C: The image of the 3D reconstruction of the portal vein shows short segmental stenosis in the region of the anastomosis (arrow).

PV resection simultaneously with splenectomy and total gastrectomy for pancreatic cancer following NACRT using the same protocol as that described for Case 1. The PV was preoperatively occluded by tumor thrombus, and a cavernous transformation was identified. The reconstruction between the PV and the SMV was end-to-end anastomosis using the same procedure as that described for Case 1. Refractory diarrhea, ascites and malnutrition were recognized at 5 POMs. Initially, malabsorption was suspected, and thus total parenteral nutrition support was initiated. There was no improvement in symptoms after the improvement in nutrition status. CT showed short segmental stenosis of the PV in the region of the anastomosis, collateral circulation through the cavernous transformation of the pancreatic head, severe ascites, and thickness of the intestinal wall (Figure 3).

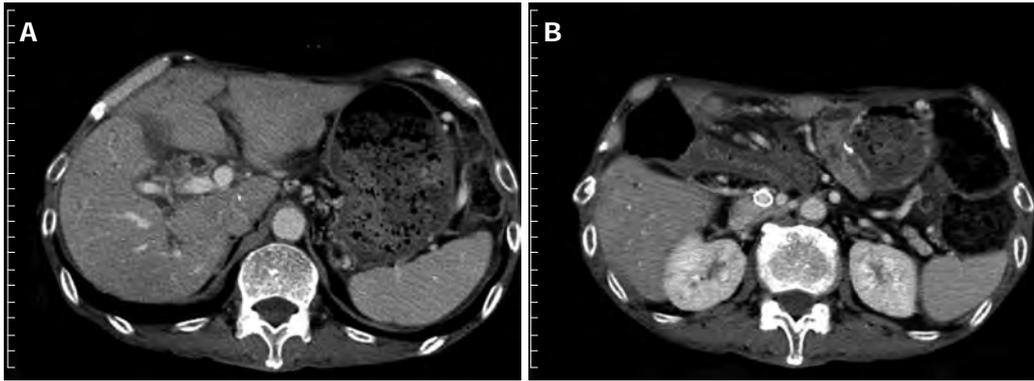


Figure 2 Computed tomography scan 3 mo after the expandable metallic stent placement. A: Shows that the ascites decreased and the liver atrophy improved; B: The stent placed in the portal vein remained patent.

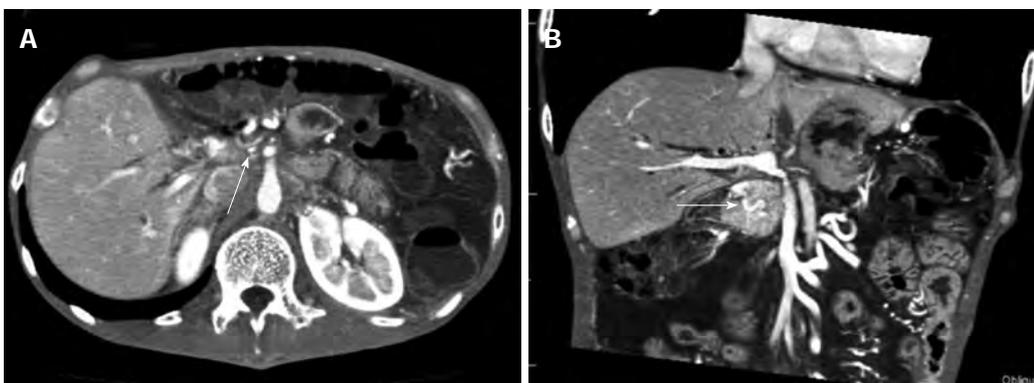


Figure 3 Computed tomography showed short segmental stenosis of the portal vein in the region of the anastomosis, collateral circulation through the cavernous transformation of the pancreatic head, severe ascites, and thickness of the intestinal wall. A: Computed tomography scan showing severe portal vein stenosis (arrow) in the region of the anastomosis; B: Multiplanar reconstruction revealed the collateral circulation through the cavernous transformation of the pancreatic head (arrow), severe ascites and thickness of the intestinal wall.

We suspected portal hypertension secondary to PV stenosis, even though the portal venous flow seemed to be sustained by the collateral circulation. Percutaneous transhepatic direct portography was performed (Figure 4A). The portal venous pressure was 31.0 cm H₂O, and the pressure gradient across the PV stenosis was 21.0 cm H₂O. An EMS (1 cm diameter, 4 cm length) was inserted into the stenotic region (Figure 4B). The portal venous pressure decreased to 17.0 cm H₂O, and the pressure gradient decreased to 2.0 cm H₂O. Anticoagulant therapy was performed as that described for Case 1. After a stent placement, the patient's diarrhea and ascites improved. Stent patency is maintained at present, 1.5 years after the placement.

Case 3

A 68-year-old woman underwent PD and PV resection for pancreatic cancer following NACRT using the same protocol as that described for Case 1. The reconstruction between the PV and the SMV was end-to-end anastomosis using the same procedure as that described for Case 1. Malnutrition and ascites were recognized at 5 POMs. Total parenteral nutrition support and repeated drainage of ascites were performed, but there was no improvement of the ascites. Cytology of the ascites showed no

evidence of malignancy. CT showed short segmental stenosis of the PV in the region of the anastomosis. Percutaneous transhepatic direct portography was performed (Figure 4C). The portal venous pressure was 24.5 cm H₂O, and the pressure gradient across the PV stenosis was 12.0 cm H₂O. An EMS (1 cm diameter, 3 cm length) was inserted into the stenotic region (Figure 4D). The portal venous pressure decreased to 11.5 cm H₂O, and the pressure gradient disappeared. Anticoagulant therapy was performed as that described for Case 1. After the stent placement, the patient's ascites improved. Stent patency is maintained at present, 4 mo after the placement.

DISCUSSION

Despite considerable research, the prognosis for pancreatic cancer remains poor. For all stages combined, the 1- and 5-year relative survival rates are 25% and 6%, respectively^[7]. Complete surgical resection is the only therapy to afford a chance of cure^[8], but patients with borderline resectable pancreatic cancer are at high risk of having positive surgical margins due to vascular involvement^[9]. NACRT for borderline resectable pancreatic cancer is expected to increase the margin-negative resection rate and improve survival^[10].

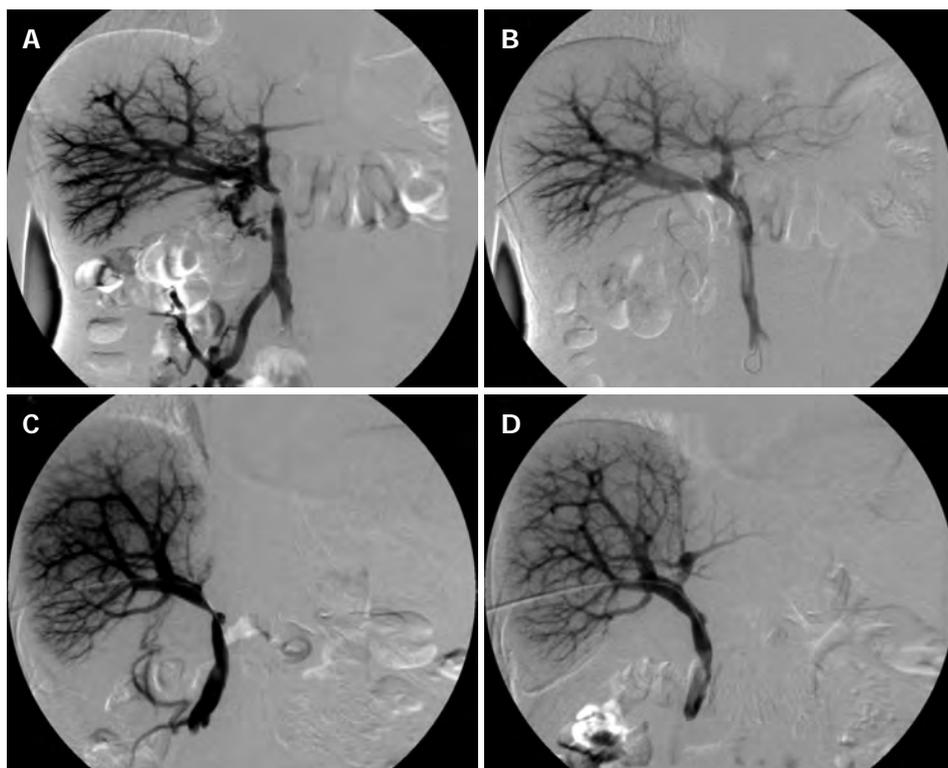


Figure 4 Percutaneous transhepatic direct portography showing short segmental stenosis of the portal vein in the region of the anastomosis. A: Collateral circulation of the pancreatic head; B: After the expandable metallic stent (EMS) placement, the stenosis was improved, and the collateral circulation disappeared; C: The blood flow of the umbilical portion of the left portal vein was unclear; D: The stenosis was improved, and the blood flow of the umbilical portion became clear after the EMS placement.

Table 1 The clinical characteristics of three patients

| Pt. No. | Age (yr) | Sex | Operative procedure | Symptoms | Months before onset | Procedure of stent placement | Pressure gradient (cmH ₂ O) | | Improvement in symptoms |
|---------|----------|-----|---------------------|---------------------------------|---------------------|------------------------------------|--|-------|-------------------------|
| | | | | | | | Before | After | |
| 1 | 54 | M | PD | Ascites, malnutrition | 9 | Intraoperative, via ileocolic vein | 6.5 | 0 | Yes |
| 2 | 44 | M | DP | Ascites, malnutrition, diarrhea | 5 | Percutaneous transhepatic | 21.0 | 2.0 | Yes |
| 3 | 68 | F | PD | Ascites, malnutrition | 5 | Percutaneous transhepatic | 12.0 | 0 | Yes |

PD: Pancreaticoduodenectomy; DP: Distal pancreatectomy; M: Male; F: Female.

We have performed gemcitabine-based NACRT in 18 patients with borderline resectable pancreatic cancer since 2005. Of 15 patients who completed the NACRT, nine had pancreatectomy. Combined portal resection was performed in 8 of the 9 patients. We report here the cases of three patients with PV stenosis (Table 1), and thus the ratio of post-operative PV stenosis in patients with PV resection following NACRT increases to 37.5% in our patient series.

There was no case of PV stenosis among 22 patients who underwent PV resection for pancreatobiliary cancer without NACRT in the same period in our department. The incidence of PV obstruction after PD with PV resection has been reported as 1.5%^[11] and 25%^[12]. The incidence of PV stenosis after PD with PV resection is rarely reported. Leach *et al.*^[12] reported the incidence 18% (5 of 29 patients), but the 18% includes 14 patients

who received IORT. Mitsunaga *et al.*^[5] suggested that the mechanism of the development of extrahepatic PV occlusion after IORT is associated with the periportal changes induced by IORT and periportal fibrosis. We did not find any reports of PV stenosis after pancreatectomy following NACRT for pancreatic cancer because most papers about NACRT for pancreatic cancer report only perioperative complications^[13,14]. However, it seems possible that the periportal changes are induced by neoadjuvant radiation, similar to those induced by IORT, and they increase the risk of the development of PV stenosis.

The first choice of treatment for PV stenosis after liver transplantation is balloon dilation^[15]. The indication of stent placement is limited to elastic stenosis and recurrent stenosis. Case 1 had an elastic stenosis, and thus the EMS placement was done after the venoplasty. However, we placed the stents in Cases 2 and 3 before the

venoplasty. Placing a stent for benign stenosis before a venoplasty is controversial. Takaki *et al.*^[16] speculated that stent placement is essential for the treatment of early anastomotic stenosis because such stenosis is caused by reactive edema or technical problems, and balloon angioplasty fails to dilate the vessel due to recoil. The onset of the stenosis in the three cases presented here was also early (5-9 POMs). Considering the poor prognosis of pancreatic cancer, early improvement of a patient's symptoms and quality of life is important.

In Case 1, we placed the stent *via* ileocolic vein because massive ascites interfered with transhepatic puncture. However, in Cases 2 and 3, we could safely performed percutaneous transhepatic stent placement after draining the ascites.

In conclusion, we have reported the cases of three patients with PV stenosis after pancreatectomy and PV resection following NACRT for pancreatic cancer. A relationship between PV stenosis and NACRT is suspected, but further investigation is required to determine whether NACRT has relevance to PV stenosis.

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Henoch-Schonlein purpura with intestinal perforation and cerebral hemorrhage: A case report

Hong-Liang Wang, Hai-Tao Liu, Qi Chen, Yang Gao, Kai-Jiang Yu

Hong-Liang Wang, Hai-Tao Liu, Qi Chen, Yang Gao, Kai-Jiang Yu, Intensive Care Unit, the Second Affiliated Hospital of Harbin Medical University, Harbin 150086, Heilongjiang Province, China

Author contributions: Wang HL and Liu HT contributed equally to this work; Gao Y collected materials and prepared figures; Yu KJ and Chen Q revised the manuscript.

Correspondence to: Kai-Jiang Yu, MM, Intensive Care Unit, the Second Affiliated Hospital of Harbin Medical University, Harbin 150086, Heilongjiang Province, China. drkaijiang@sohu.com

Telephone: +86-451-86296376 Fax: +86-451-86296376

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INTRODUCTION

Henoch-Schonlein purpura (HSP), also known as anaphylactoid purpura, is the most common form of systemic vasculitis in children^[1]. It occurs most commonly in children between 3 and 10 years old^[2] and affects small vessels. Typical symptoms of HSP include palpable purpura, arthralgia, stomach ache and hematochezia. Pathologically, HSP is characterized by the development of leukocytoclastic vasculitis in small vessels with deposition of immune complexes mainly consisting of immunoglobulin A. Although the etiology is still unclear, HSP is known to develop after upper respiratory infection or drug allergy^[3] and is therefore considered an autoimmune disease. It is reported that the incidence of HSP with intestinal perforation is 0.38%, mainly affecting the ileum and occasionally the jejunum, where intestinal perforation appears about 10 d after the onset of initial symptoms^[4]. The incidence of HSP with central nervous system symptoms is between 0.9% and 6.9%, and HSP with intracranial hemorrhage has an even lower incidence^[5]. The most common sign of HSP with intracranial hemorrhage is seizure, followed by headache, changes in mental status, and focal neurologic deficits^[6]. To date, there have been only 10 reported cases of HSP with cerebral hemorrhage. Herein we report a case of HSP with intestinal perforation and cerebral hemorrhage.

Abstract

Henoch-Schonlein purpura (HSP) with intestinal perforation and cerebral hemorrhage is a very rare clinical condition. There has been no report of HSP complicated with both intestinal perforation and cerebral hemorrhage until October 2012. Here we describe a case of HSP with intestinal perforation and cerebral hemorrhage in a 5-year-old girl. Plain abdominal radiograph in the erect position showed heavy gas in the right subphrenic space with an elevated diaphragm. Partial resection of the small intestine was performed, and pathological analysis suggested chronic suppurative inflammation in all layers of the ileal wall and mesentery. Seventeen days after surgery, cerebral hemorrhage developed and the patient died.

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Key words: Henoch-Schonlein purpura; Anaphylactoid purpura; Small intestine; Cerebral hemorrhage; Child

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CASE REPORT

A 5-year-old Chinese girl was admitted to our intensive care unit with complaints of abdominal pain for 22 d, rash for 21 d, and hematuria for 2 wk. She was previously healthy and had a history of allergy to aminopyrine.



Figure 1 Abdominal bulge was found on examination at admission.

Upon physical examination, her general condition was poor. Her blood pressure was 112/69 mmHg, heart rate 153 beats/min, body temperature 37.2 °C, and arterial oxygen saturation 87%. She was unconscious and presented with difficulty breathing, cyanotic lips, rales, abdominal bulge (Figure 1), marked abdominal tenderness, rebound tenderness and muscle tension, and marked edema of the bilateral lower extremities up to the level of her knees. She had purpuric papules, 1-2 cm in diameter, over her ankles, buttocks and lower legs, as well as raised flesh-colored papules over her upper medial legs, but her upper trunk and perineum were not involved. Neurologic exam was within normal limits. Laboratory data on admission were as follows: white blood cell count 45900/ μ L with an increase in the percentage of neutrophils (93.5%); hemoglobin 69 g/L; hematocrit 20.9%; platelet count 40000/L; prothrombin time 10.6 [international normalized ratio (INR) 0.99]; serum total protein 33 g/L; albumin 12 g/L; cholinesterase 223 U/L; alkaline phosphatase 128 U/L; urea 15.3 mmol/L; serum creatinine 84.0 μ mol/L; urine protein 2+; and urine occult blood 5+. Cerebral and chest computed tomography (CT) demonstrated slight widening of cerebral sulci and cisterns, inflammation of the lungs, and bilateral pleural effusions. Plain abdominal radiograph in the erect position showed heavy gas in the right subphrenic space with an elevated diaphragm (Figure 2). Abdominal ultrasonography showed remarkable intestinal expansion in the lower abdomen and increased bilateral renal artery resistance index. To improve the patient's condition, symptomatic treatments were given, including immediate intubation, mechanical ventilation, improvement of circulation, correction of disturbances of the internal environment, and application of broad-spectrum antibiotics. On January 13, the girl underwent emergency laparotomy. Intraoperative findings included necrosis of the intestine (10-30 cm long), with multiple perforations visible from the terminal ileum to the ileocecal valve, severe intestinal adhesion, and the presence of a large number of pus masses on the surface of the liver, intestine and peritoneum. Surgical treatment strategies were enterectomy, enterostomy and peritoneal lavage. Postoperative pathological analysis suggested chronic suppurative inflammation in all layers of the ileal wall and



Figure 2 Plain abdominal radiograph in the erect position showed heavy gas in the right subphrenic space with an elevated diaphragm.

mesentery (Figure 3). Thus, the patient was diagnosed with Henoch-Schönlein purpura complicated by intestinal necrosis and perforation, nephritis, respiratory failure, and severe sepsis.

Postoperatively, comprehensive treatments were given, including broad-spectrum antibiotics, gamma-globulin, hormones, nutritional support, and mechanical ventilation. Following these treatments, the girl's condition showed significant improvement: her inspired oxygen concentration was 40% and arterial oxygen saturation was 97%; the tension of the abdominal wall was reduced; and the surgical incision healed well. Apart from blood pressure (maintained at 130/90 mmHg) that was not controlled effectively by antihypertensive therapy, all other vital signs were satisfactory. Laboratory data after surgery were as follows: white blood cell count 24700/ μ L with an increased percentage of neutrophils (93.3%); hemoglobin 69 g/L; hematocrit 20.9%; platelet count 157000/L; prothrombin time 12 (INR 1.29); serum total protein 38 g/L; albumin 19 g/L; urea 13.2 mmol/L; serum creatinine 100 μ mol/L; urine protein 2+; and urine occult blood 3+.

On January 28, the patient became unconscious suddenly and developed ptosis and mydriasis. Cerebral CT showed subarachnoid hemorrhage, right thalamic and intraventricular hemorrhage, and multiple ischemic changes in the left cerebellar hemisphere and bilateral cerebral hemispheres (Figure 4). Despite active rescue, the girl died on January 28.

DISCUSSION

HSP is a systemic vasculitis which affects small vessels, with typical symptoms including palpable purpura, arthralgia, stomach ache and bloody stools. Pathologically, HSP is characterized by the development of leukocytoclastic vasculitis in small vessels with deposition of immune complexes consisting mainly of immunoglobulin A^[3]. Although all HSP patients invariably develop cutaneous purpura, other symptoms, such as arthritis, abdominal pain, and renal involvement, do not occur in all patients, which make the diagnosis of HSP more challenging^[7]. In a few cases, HSP can affect other systems or organs and

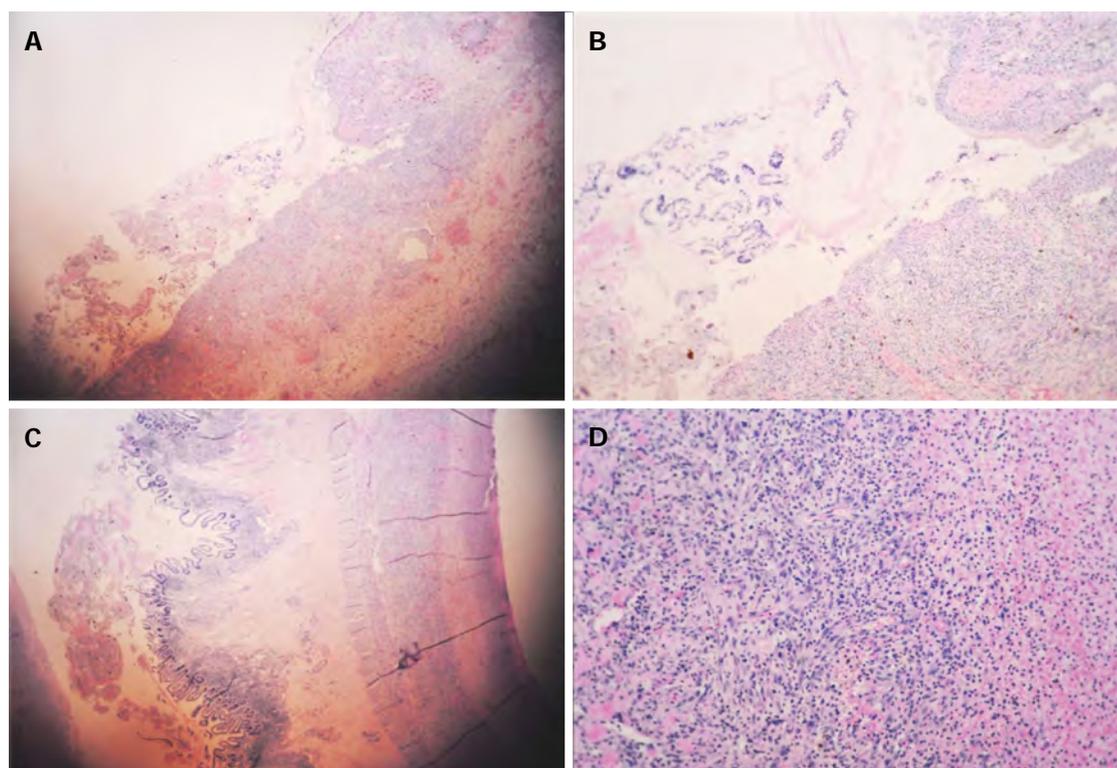


Figure 3 Histopathological analyses of the patient's ileal biopsy specimens. A: Hematoxylin and eosin-stained sections of the biopsy specimens revealed mucosal discontinuity (magnification $\times 10$); B: Multiple site bleeding (magnification $\times 20$); C: Inflammatory cell infiltration in all layers of the ileal wall (magnification $\times 20$); D: Infiltration of a large number of neutrophils and cell deformation in the area of edema (magnification $\times 40$).

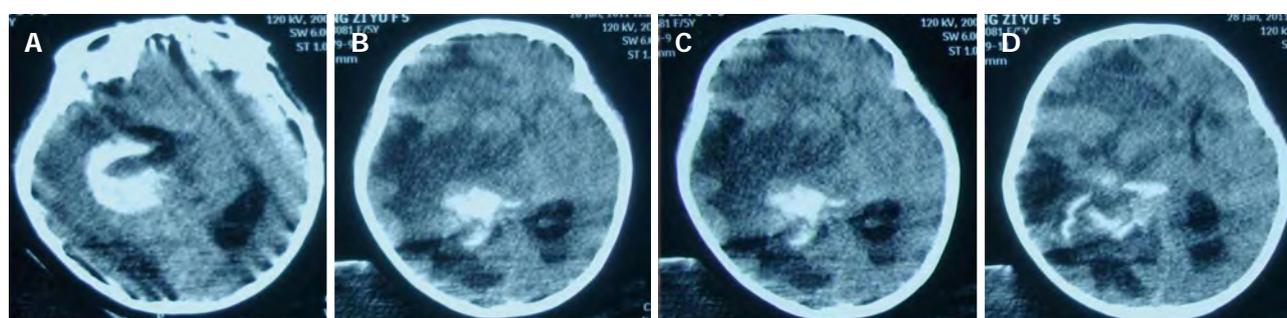


Figure 4 Cerebral computed tomography demonstrated subarachnoid hemorrhage, right thalamic and intraventricular hemorrhage, and multiple ischemic changes in the left cerebellar hemisphere and bilateral cerebral hemispheres. A: Right thalamic hemorrhage ruptured into the ventricle; B-D: Thalamic hemorrhage complicated with subarachnoid hemorrhage, cerebral hemispheres multiple focal ischemia.

results in the occurrence of transient paralysis, convulsions, myocarditis, hypertension, and other symptoms^[4]. Although palpable purpura is often considered the hallmark of HSP, skin findings were not described as the presenting feature in nearly 25% of patients^[8].

Gastrointestinal manifestations are present in 45% to 75% of patients with HSP, with 14% to 36% of patients developing these symptoms prior to the appearance of purpura^[9], as occurred in our case. After being treated with corticosteroids, gastrointestinal lesions in HSP are usually reversible and can heal, although a few patients (2%-6%) develop conditions requiring surgery^[10]. It is reported that approximately 52% of HSP patients have varying degrees of gastrointestinal bleeding, mostly self-limiting; however, cases with serious intestinal bleeding

(0%-8.2%) call for intervention, and misdiagnosis can be life-threatening^[11]. Patients most commonly develop abdominal pain without complications. The most commonly reported complication is intussusception^[12,13], while bowel ischemia and infarction, perforation, stricture, fistula, hemorrhage, pancreatitis, and appendicitis are infrequent. In our case, heavy gas in the right subphrenic space with an elevated diaphragm was detected radiographically, and surgical treatment strategies were enterectomy, enterostomy and peritoneal lavage. This observation may be of great clinical significance, as these gastrointestinal manifestations are usually serious and can result in invasive diagnostic techniques, including laparotomy. The incidence of HSP with central nervous system symptoms ranges from 0.9% to 6.9%, and HSP with intracranial hemor-

rhage has an even lower incidence^[5]. The most common sign of HSP with intracranial hemorrhage is seizures, followed by headache, changes in mental status, and focal neurologic deficits. HSP as a form of necrotizing vasculitis results in a wide range of pathologic manifestations. According to the severity and site of the vascular lesions, diffuse encephalopathy, focal ischemia, or intracranial hemorrhages may develop^[6,14]. With respect to the causes of cerebral hemorrhage in our case, the primary disease may play a major role because it can affect small vessels and lead to systemic vasculitis. Interestingly, we found the coexistence of cerebral ischemia and cerebral hemorrhage. It may be assumed that immunoglobulin A immune complex deposition initiates arteriolar inflammation in the cerebral vasculature as well as in the systemic vessels^[12,14]. Cerebral hemorrhage was possibly secondary to resistant hypertension and cerebral vasculitis. Both purpura nephritis and increased bilateral renal artery resistance index could lead to resistant hypertension. In addition, cerebral hemorrhage might be associated with cerebral vascular malformation^[5]; however, this possibility could not be ruled out in our case because of postoperative mechanical ventilation.

In conclusion, we herein describe a case of intestinal perforation and cerebral hemorrhage secondary to HSP. Treatment of the primary disease is very important in preventing the occurrence of serious complications, such as intestinal perforation and cerebral hemorrhage. HSP patients should be closely monitored for early serious complications such as intestinal perforation and given timely surgery. Surgery is the most effective treatment. Despite its rarity, HSP should be considered when gastrointestinal perforation is observed. In addition, blood pressure should be controlled effectively in HSP patients to prevent the occurrence of serious neurological complications such as cerebral hemorrhage.

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Surgical treatment of a patient with peliosis hepatis: A case report

Wei Pan, Hai-Jie Hong, Yan-Ling Chen, Sheng-Hua Han, Chang-Yue Zheng

Wei Pan, Hai-Jie Hong, Yan-Ling Chen, Sheng-Hua Han, Department of Hepatobiliary Surgery, Fujian Medical University Union Hospital, Fuzhou 350001, Fujian Province, China
Chang-Yue Zheng, Department of General Surgery, Affiliated Hospital of Putian University, Putian 351100, Fujian Province, China

Author contributions: Pan W and Hong HJ contributed equally to this work; Chen YL designed the report; Chen YL, Han SH, Hong HJ and Pan W are attending doctors for patient treatment; Chen YL and Han SH performed operation; Zheng CY conducted literature search; Chen YL organized the report; Pan W wrote the paper.

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Correspondence to: Yan-Ling Chen, MD, Department of Hepatobiliary Surgery, Fujian Medical University Union Hospital, 29 Xinquan Road, Gulou District, Fuzhou 350001, Fujian Province, China. ylchen@medmail.com.cn

Telephone: +86-591-87665700 Fax: +86-591-87665700

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Abstract

This report describes a case of a space-occupying lesion in the right liver in a 38-year-old man who was found to have peliosis hepatis. Clinical data of this patient were presented, including medical history, laboratory test and imaging results, and postoperative pathological findings (hematoxylin and eosin staining). Review of his medical history showed that the patient had been bitten by a dog three years earlier. B-mode ultrasonography revealed an uneven echo mass in the right hemiliver, and magnetic resonance imaging scans also showed a mass in the anterior segment of the right liver. Upon surgical removal, the mass was found to be 4.0 cm × 3.8 cm × 3.8 cm in size and located in segment VI. The mass had a dark and soft appearance, with an irregular edge on intraoperative ultrasonography. Postoperative pathological findings revealed

many small capsules filled with blood cells. The patient was diagnosed with peliosis hepatis based on his medical history of having been bitten by a dog, presence of mild anemia, and lack of characteristic symptoms, including fever of unknown origin, abdominal pain, and hepatosplenomegaly, combined with intraoperative and postoperative pathologic findings. The operation was successful, and after being treated with anti-infection agents, the patient had a good recovery.

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Key words: Peliosis hepatis; Surgical treatment; Ultrasonography; Space-occupying lesion

Core tip: This report describes a case of a space-occupying lesion in the right liver in a 38-year-old man who was diagnosed with peliosis hepatis based on the medical history of having been bitten by a dog, presence of mild anemia, and lack of characteristic symptoms, including fever of unknown origin, abdominal pain, and hepatosplenomegaly, combined with intraoperative and postoperative pathologic findings. The operation was successful, and after being treated with anti-infection agents, the patient had a good recovery.

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INTRODUCTION

Peliosis hepatis (PH) is a rare clinical disease, appearing mostly as diffuse hepatic lesions. PH is considered a benign vasogenic lesion and is characterized by the presence of cystic blood-filled cavities distributed randomly throughout the liver parenchyma. This disease is more

common in adults than in children or adolescents. We herein report a case of PH in a 38-year-old man.

CASE REPORT

A 38-year-old man presented with a space-occupying lesion in his right liver. The patient had been bitten by a dog three years earlier, but his health was generally good. The patient did not have hepatosplenomegaly or symptoms of tuberculosis intoxication, nor did he have a history of blood transfusions or long-term use of glucocorticoids or anabolic hormones. Physical examination revealed no abnormalities. Blood tests showed that his hemoglobin level was 90 g/L, whereas his blood biochemistry, liver and kidney function tests, and alpha fetal protein, cancer embryo antigen, carbohydrate antigen 19-9 concentrations were all within normal ranges. He was positive for hepatitis B surface antibody (323.21 IU/L) and core antibody (4.25 S/C.0), but negative for other hepatitis B antigens and antibodies and negative for human immunodeficiency virus. Hepatobiliary magnetic resonance imaging (MRI) revealed a 3.8 cm × 3.1 cm mass with irregular boundaries in the anterior segment of his right liver.

Enhanced scanning showed inhomogeneous enhancement, peaking at the arterial phase (Figure 1). Color ultrasonography showed that his liver had a normal appearance, but there was a 3.9 cm × 3.9 cm mass with inhomogeneous echo in his right hemiliver with unclear boundaries and weak blood flow signal within the mass (Figure 2). He underwent surgery, which revealed the mass located in segment VI. Intraoperative ultrasonography showed that the mass was 4.0 cm × 3.8 cm × 3.8 cm in size, and had a dark and soft appearance, with irregular edges (Figure 3). No abnormality was observed in any other abdominal organs. Rapid pathological examination on frozen sections suggested the possibility of a hepatic hemangioma, although pathological assessment confirmed PH (Figure 4). The operation was successful, and after being treated with anti-infection agents, the patient had a good recovery.

DISCUSSION

At gross inspection, the peliotic lesions in a PH liver look like “Swiss cheese slices”. Microscopically, there are two types of peliosis: “parenchymal peliosis” and “phlebotatic peliosis”^[1], the main difference being whether the cavities are lined by endothelium or fibrotic tissue. The patient described here had parenchymal peliosis.

The pathogenesis of PH remains unknown, and pathogenic factors vary. Roughly they can be divided into three categories: drug-related, autoimmune and infectious. PH has been associated with the use of hormones and immunosuppressive medications, especially α -alkyl steroid hormones and thiopurine^[2]. Autoimmune factors are those associated with secondary immunodeficiency caused by certain potential consumptive diseases, such

as tuberculosis, hematological malignancies^[3,4], acquired immunodeficiency syndrome^[5], immune deficiency after transplantation^[6], and hepatocellular carcinoma (HCC)^[7]. Infectious factors include Bartonella infection, which leads to cat-scratch disease^[8,9]. PH has also been observed in dogs infected with Bartonella^[10]. Despite the absence of symptoms of cat-scratch disease, the patient’s dog-bite experience suggests the possibility of Bartonella infection.

PH is usually caused by autoimmune disorders triggered by endogenous and exogenous risk factors. These autoimmune disorders can induce primary dysfunction in the endothelial cells of the liver sinus, as well as hepatocyte necrosis. This causes angiectasis and hyperemia, producing multiple blood-filled cystic spaces in the liver parenchyma.

Clinical development of PH is not apparent, and it is difficult to detect at its onset. The diagnosis of PH is based on pathological examination and liver imaging. Imaging can disclose either intrahepatic space-occupying or diffuse lesions. MRI scans of the patient reported here showed a mass in the anterior segment of the right liver, approximately 3.8 cm × 3.1 cm in size, with irregular boundaries, that showed a weak T1 and a strong T2 signal, resulting in inhomogeneous enhancement. In addition, the diffusion weighted imaging phase showed a strong signal with increasing B values. This result is consistent with those observed in other patients with PH^[11].

PH shows a broad spectrum of appearances on radiography, primarily because the demonstrations of MRI scans largely depend on the blood supply to the lesions^[12]. This lack of specificity can easily result in a misdiagnosis of other hypervascular tumors^[13]. In addition, PH and HCC may occur together^[7]. Therefore, only pathologic findings are considered the gold standard for the diagnosis of PH.

PH is a benign vasogenic lesion, which usually can be cured by removal of the mass or part of the liver. No patient has shown recurrence or metastasis. Its special pathological structure nonetheless makes patients prone to liver rupture and hemorrhage^[14] or death due to hepatic failure. The incidence of PH is relatively low, but it has high risks. Thus, it should be aggressively treated once diagnosed, in order to prevent complications. Treatment should be tailored based on the location and extent of the lesion, the damage to liver function, and whether or not serious amalgamative complications can occur.

Because of its insidious onset and insufficient specific clinical manifestations, the diagnosis of PH is often delayed, making treatment more difficult. Most patients are not diagnosed until PH develops into diffuse lesions accompanied by severe liver failure, at which point liver transplantation is life-saving^[15]. The treatments for diffuse lesions without severe damage to liver function typically include finding and removing the cause (*i.e.*, treating the primary disease, controlling infection, or stopping any associated drugs), assisted by medicines that protect liver function. Liver transplantation is indicated, how-

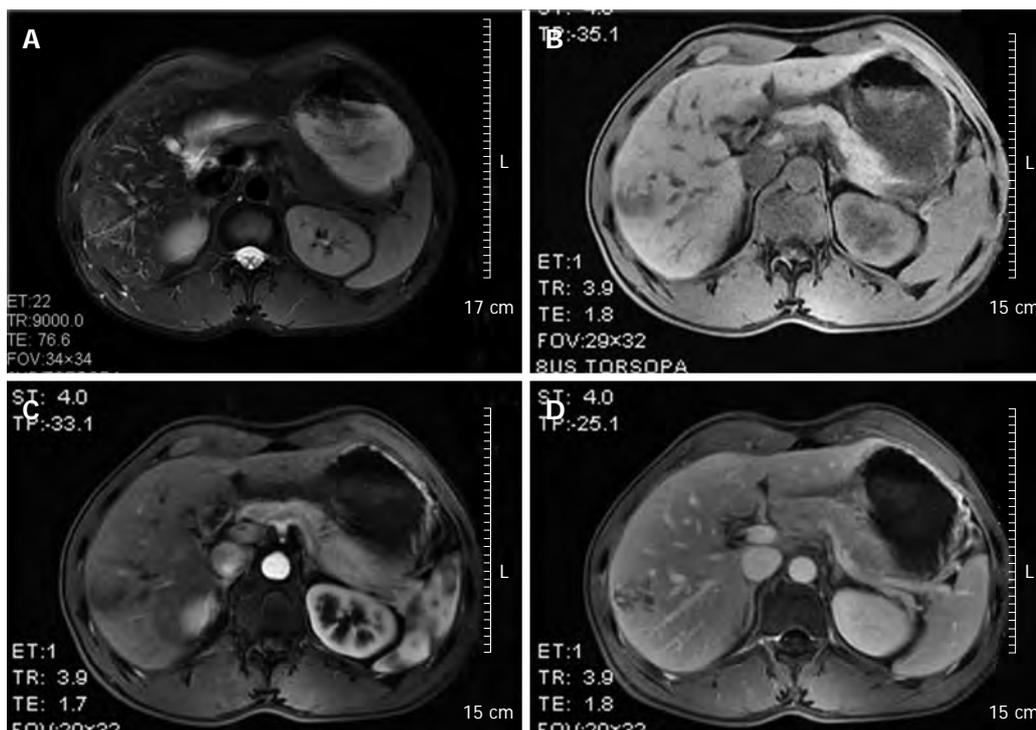


Figure 1 Magnetic resonance imaging scans. A: Hepatobiliary magnetic resonance imaging scans revealed a 3.8 cm × 3.1 cm mass in the anterior segment of the right liver with irregular boundaries, producing low-intensity T1 signals and high-intensity T2 signals; B-D: Gadolinium diethylenetriamine-pentaacid enhanced scanning showed inhomogeneous enhancement, peaking during the arterial phase and declining during the portal vein and parenchymal phases. The lumen and collateral vessels of the portal vein were well defined without filling defects.



Figure 2 B-mode ultrasonography. A: B-mode ultrasonography showing a 3.9 cm × 3.3 cm mass with inhomogeneous echo in the right hemiliver; B: The boundary was unclear and some blood-flow signal was detected within the mass.

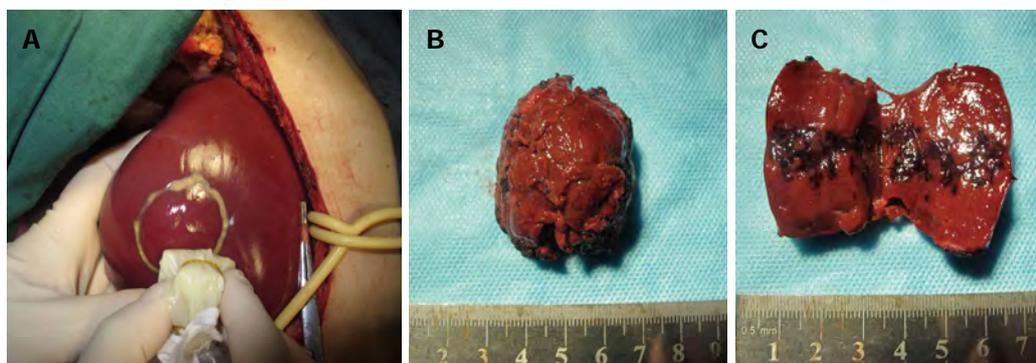


Figure 3 Gross appearance of the mass after surgical removal. A: There were no clear boundaries or lining membrane between the mass and the liver; B: The specimen had an irregular shape; C: Several blood-filled cystic spaces were observed after opening the mass.

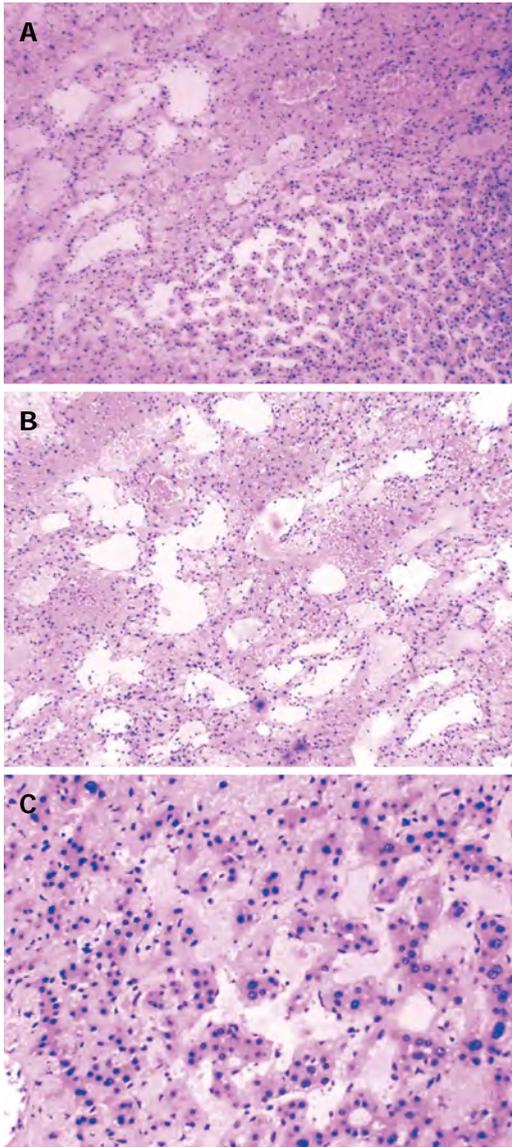


Figure 4 Pathologic examination, showing irregular and dilated lacunae, some of which were filled with blood and without endothelial linings. Hematoxylin and eosin staining, original magnification A: $\times 100$; B: $\times 200$; C: $\times 400$.

ever, for patients with severely diffuse lesions accompanied by hepatic failure. If focal lesions are diagnosed by laparoscopy, biopsy or fine-needle aspiration and the cause has been clearly determined, the treatment usually consists of removing the cause and closely monitoring the patient. Patients with ruptured lesions and bleeding should be promptly treated by transcatheter superselective embolization or even surgical hemostasis (if necessary)^[16,17]. If the cause is unknown or conservative treatment is ineffective, surgery should be performed. Although the lesion was focal in the patient described in this report, its space-occupying nature could not be clearly estimated. The patient had no liver function abnormality (Child-Pugh grade A) and no relevant basic disease or medical history. However, the patient had mild anemia that might be caused by hemorrhage of the lesion. Since surgical removal can effectively prevent com-

plications such as abdominal bleeding, and can result in a clear diagnosis to guide further treatments, we decided to perform a precise hepatectomy. The long-term therapeutic effects remain to be determined.

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Role of molecular analysis in the adjuvant treatment of gastrointestinal stromal tumours: It is time to define it

Margherita Nannini, Maria A Pantaleo, Guido Biasco

Margherita Nannini, Maria A Pantaleo, Guido Biasco, Department of Hematology and Oncological Sciences "LA Seragnoli", Sant'Orsola-Malpighi Hospital, University of Bologna, 40138 Bologna, Italy

Maria A Pantaleo, Guido Biasco, "Giorgio Prodi" Cancer Research Center, University of Bologna, 40138 Bologna, Italy

Author contributions: All the authors contributed to this letter. Correspondence to: Dr. Margherita Nannini, Department of Hematology and Oncological Sciences "LA Seragnoli", Sant'Orsola-Malpighi Hospital, University of Bologna, Via Mas-sarenti 9, 40138 Bologna, Italy. maggie.nannini@gmail.com

Telephone: +39-51-6364078 Fax: +39-51-6364037

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Abstract

Sendur *et al* pointed out the attention on the importance of mutational analysis for adjuvant treatment of gastrointestinal stromal tumor (GIST) in an article published in *World Journal of Gastroenterology*. In particular, they suggested that the optimal dose and duration of adjuvant therapy could be defined by the mutational status of the primary disease. This comment would underline the importance of centralised laboratories, given the increasingly important role of molecular analysis in the work-flow of all GIST, and the need of retrospective analyses for subgroups population stratified for the mutational status from the available studies in the adjuvant setting, in order to define the role of mutational analysis in choosing the optimal dose and duration of adjuvant therapy.

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Key words: Gastrointestinal stromal tumours; Platelet-derived growth factor receptor alpha; KIT; Wild-type; Molecular analysis; Imatinib; Adjuvant treatment

Core tip: Sendur *et al* pointed out the attention on the

importance of mutational analysis for adjuvant treatment of gastrointestinal stromal tumor (GIST). In particular, they suggested that the optimal dose and duration of adjuvant therapy could be defined by the mutational status of the primary disease. This topic represents a big challenge in GIST's management by now, because even if the molecular analysis is strictly recommended in the work-flow of all GIST, its role in the adjuvant setting remains still unsettled due to the lack of prospective large clinical trials. In particular we pointed out the attention on the KIT/platelet-derived growth factor receptor alpha wild type GIST, that are extremely heterogeneous both in clinical and molecular background, making difficult their management also in the adjuvant setting. Our comment would underline the importance of centralised laboratories, given the increasingly important role of molecular analysis in the work-flow of all GIST, and the need of retrospective analyses for subgroups population stratified for the mutational status from the available studies in the adjuvant setting, in order to define the role of mutational analysis in choosing the optimal dose and duration of adjuvant therapy.

Nannini M, Pantaleo MA, Biasco G. Role of molecular analysis in the adjuvant treatment of gastrointestinal stromal tumours: It is time to define it. *World J Gastroenterol* 2013; 19(16): 2583-2586 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v19/i16/2583.htm> DOI: <http://dx.doi.org/10.3748/wjg.v19.i16.2583>

TO THE EDITOR

We read with great interest the article by Sendur *et al*^[1] entitled "Is exon mutation analysis needed for adjuvant treatment of gastrointestinal stromal tumor?", that has been recently published in the January issue (2013) of *World Journal of Gastroenterology*. The authors pointed out the likely importance of mutational analysis for guiding

the clinicians in the treatment's choice of gastrointestinal stromal tumor (GIST) patients also in the adjuvant setting^[1]. In particular they suggest that the optimal dose and duration of adjuvant treatment could be defined by the mutational status of the primary disease. Their account comes from the consolidated evidence that the mutation status is predictive of response to imatinib treatment in the advanced disease^[2-5]. In fact it is well known that *KIT* exon 11 mutations are associated to the highest response rate to imatinib in comparison to patients exon 9 mutations^[2,3]. On the contrary exon 18 *PDGFRA* D842V point mutation confers primary resistance to imatinib^[4,5]. Furthermore it has been widely shown that the subgroup of patients with *KIT* exon 9 mutation treated with imatinib 800 mg had an higher progression-free survival in comparison with those treated with the standard dose of 400 mg^[6]. Translating these evidences from the advanced disease to the limited disease the authors suggested that it may be reasonable to assume that patients with a substantial risk of GIST relapse harbouring *KIT* exon 9 mutation should be considered for higher dose of adjuvant imatinib.

Moreover, in the last years the potential role of mutational status as a prognostic factor has been progressively appeared^[7]. In particular, it has been shown that deletions of *KIT* exon 11, especially those involving codon 557 and/or codon 558 are associated with a shorter progression-free and overall survival whereas most platelet-derived growth factor receptor alpha (*PDGFRA*)-mutant GISTs generally have a lower potential for malignancy^[8-15].

By now even if the optimal duration of adjuvant imatinib therapy is still unclear, adjuvant imatinib for three years of duration as a standard of care in high-risk operable GISTs is recommended^[16,17].

Whether the role of mutational status as a prognostic factor should be confirmed in large series, the authors suggest that the optimal duration of adjuvant treatment could be different in relation to the kind of mutation found and not only to the standard prognostic factors.

Based on the above considerations, the mutational analysis is strictly recommended in the work-flow of all cases of primary GIST, because it provides information about tumour sensitivity to imatinib and, although not yet included in any risk stratification system, it may provide prognostic information^[17-19]. However, the decision-making on adjuvant therapy treatment based on mutational analysis alone is not still supported by consistent data, with the exception of *PDGFRA* D842V mutation^[17].

In particular, the adjuvant therapy in the *KIT/PDGFRA* wild type (WT) GIST, which notably may be less sensitive to imatinib than most mutated GIST, remains controversial. In fact, in recent years is emerging more and more the idea that *KIT/PDGFRA* WT GIST should be considered as a heterogeneous group of disorders rather than a single molecular subtype of GIST, both in clinical behaviour and molecular background^[20-27]. For example, it has been recently identified a subgroup of *KIT/PDG-*

FRA WT GIST, characterized by germline and somatic mutations in succinate dehydrogenase (*SDH*) subunits B, C and A and defined in different ways as *SDH*-deficient GIST, or type-2 or pediatric-type GIST^[24-27]. These patients have in common several pathological and clinical features, such as the epithelioid pattern, the multifocal presentation, the female prevalence, the gastric primary tumor localization, and the indolent course of disease despite the presence of lymph nodes and liver metastases up-front and independently to standard prognostic parameters. Moreover it seems that they have also a questionable sensitivity to imatinib. Given their indolent behaviour when metastatic, *KIT/PDGFRA* WT GIST *SDH*-deficient may not benefit from adjuvant treatment irrespective to the standard risk stratification, whereas more aggressive *KIT/PDGFRA* WT GIST without *SDH*-impairment, may be probably considered as all mutated GIST.

Therefore also the effect of adjuvant imatinib on *KIT/PDGFRA* WT GIST may be variable and clinical decision-making should be individualised case by case taking into account various molecular data and shared with the patient^[17].

In conclusion, given the increasingly important role of molecular analysis in the work-flow of all GIST, centralised laboratories should be widely warranted. Furthermore, the special attention pointed up by the authors on the "optimal dose" and the "duration" of adjuvant treatment defined by the mutational status of the primary disease should be used at first for the decision to suggest or not the imatinib treatment in this setting. Finally, since prospective clinical trials with large series for definitely defining the role of mutational analysis for patients stratification, dose selection and treatment duration in the adjuvant setting, are difficult because the rarity of disease, retrospective analyses for subgroups population stratified for the mutational status from the available studies in the adjuvant setting are necessary.

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