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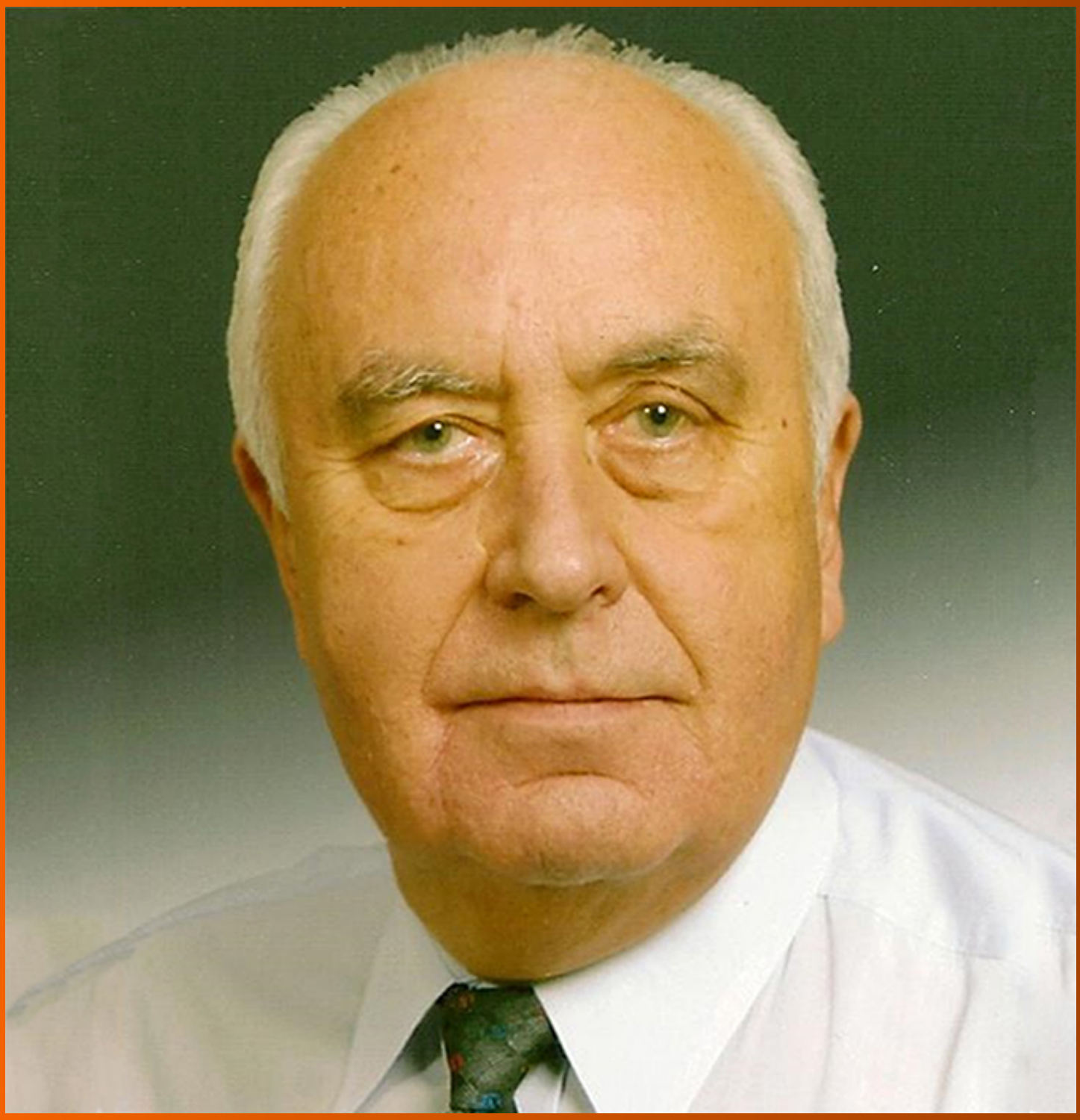

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OPINION REVIEW

Diagnosing coeliac disease: Out with the old and in with the new?

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Abstract

Coeliac disease (CD) is a complex condition resulting from an interplay between genetic and environmental factors. When diagnosing the condition, serological testing and genotyping are useful in excluding CD, although the gold standard of testing is currently histopathological examination of the small intestine. There are drawbacks associated with this form of testing however and because of this, novel forms of testing are currently under investigation. Before we develop completely novel tests though, it is important to ask whether or not we can simply use the data we gather from coeliac patients more effectively and build a more accurate snapshot of CD through statistical analysis of combined metrics. It is clear that not one single test can accurately diagnose CD and it is also clear that CD patients can no longer be defined by discrete classifications, the continuum of patient presentation needs to be recognised and correctly captured to improve diagnostic accuracy. This review will discuss the current diagnostics for CD and then outline novel diagnostics under investigation for the condition. Finally, improvements to current protocols will be discussed with the need for a holistic "snapshot" of CD using a number of metrics simultaneously.

Key words: Coeliac disease; Diagnostics; Histology; Serology; Microbiome; Metabolome

Core tip: Due to the complexity of the condition, the diagnosis of coeliac disease can pose unique challenges. This review will discuss the current diagnostics for the condition and then outline novel diagnostics currently under investigation. Finally improvements to the current testing protocols will be discussed with the need for a holistic "snapshot" of the condition, using a number of metrics simultaneously.

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INTRODUCTION

Coeliac disease (CD) is a chronic autoimmune enteropathy which results from a complex interplay between genetic and environmental factors[1,2]. Inheritance of altered forms of the human leukocyte antigen receptor (HLA-DQ2 or HLA-DQ8) in patients with CD bind epitopes of a dietary protein, gluten, with high affinity. Once cells within the small intestine are sensitised to these epitopes, a destructive autoimmune reaction is triggered which ultimately results in the destruction of the small intestinal wall[3-5]. The prevalence of CD is increasing, with around 1% of the general population now affected by the condition and presentation of the disease in populations not classically affected[1,6,7]. The symptoms of CD are varied; but can include diarrhoea, weight loss, abdominal pain and failure to thrive in children. If left untreated, CD can lead to complications associated with nutrient deficiency; such as anemia, alopecia, fertility issues or increased bone fracture risk[2,8-11]. Long-term untreated CD has been associated with increased risk of enteropathy-associated T-cell lymphoma (EATL) and adenocarcinoma of the small intestine[12,13]. The only current form of treatment is a strict, lifelong gluten-free diet[2,14]. This review will focus on the diagnosis of CD, outlining the current diagnostic guidelines and highlighting the benefits and shortcomings associated with these. Novel diagnostics currently under investigation or improvements to the current diagnostics will then be discussed.

CURRENT DIAGNOSTICS - SEROLOGY

The frontline testing for the presence of CD is serological examination. Currently, serological testing for CD is recommended for patients presenting with chronic/intermittent diarrhoea, unexpected weight loss, recurrent abdominal pain or persistent gastrointestinal symptoms. Serological screening for CD is also offered to patients with associated conditions such as autoimmune thyroid disease, irritable bowel syndrome, or type 1 diabetes[15]. Titres of three main types of antibody are assessed in CD screening; IgA-based antibodies against the enzyme tissue transglutaminase (tTG), IgA and IgG-based antibodies against deamidated gliadin peptides (DGP) and IgA-based antibodies against the endomysium (EMA). Of these tests, high titres of IgA-tTG and IgA-EMA accounts for nearly 95% reliability in serological screening[10,15]. IgG/IgA-anti-deamidated gliadin peptide (IgG/IgA-DGP) is used as a companion test for improved accuracy by very specifically detecting antibodies directed against immunogenic peptides of gluten in patients with suspected CD[10,16,17]. Serological testing can also be used as a less invasive method of monitoring treatment progression and adherence to a gluten-free diet after diagnosis[18].

There are shortcomings associated with serological screening however. Firstly, the patient must be on a diet containing gluten for results to be meaningful[10,19]. Secondly, many of the tests rely on IgA-based antibodies and IgA deficiency is far more common in CD patients than in the general population, with a prevalence rate of around 2%-3%[10,19,20]. This can be overcome however by measuring total IgA at the start of testing to ensure sufficient levels and incorporating IgG-based tests into the panel[19]. Furthermore, it has also been shown that the sensitivity of serological tests for CD is far lower than reported when patients with milder pathology are tested[21]. Due to these limitations, patients with negative serology but who are still highly suspected of having CD are often referred for further testing, either by examining biopsy material or genotyping (Figure 1).

CURRENT DIAGNOSTICS - GENOTYPING

Currently, patients who have negative serology (but are suspected of having CD), patients with a family history of CD or patients who are following a gluten-free diet at the time of diagnosis (and unwilling to undergo a gluten challenge) are offered genetic testing for CD in the form of HLA genotyping (Figure 1)[2,19]. Ninety-nine percent of patients with CD express either of the MHC Class II antigens HLA-DQ2 or HLA-DQ8, variants of the Human Leukocyte Antigen class II receptors[3,5]. As MHC II molecules are heterodimers, on a genetic level these variants result from the inheritance of several key alleles. HLA-DQ2 results from the expression of two alleles, HLA-DQA1*0501 and HLA-DQB1*02 whose gene products combine to form the altered MHC II receptor. HLA-DQ8 results from the expression of the variant HLA-DQB1*0302 and HLA-DQB1*03 alleles[22]. These variants are MHC II molecules that favour the binding of negatively charged residues in a 3-anchor point configuration,

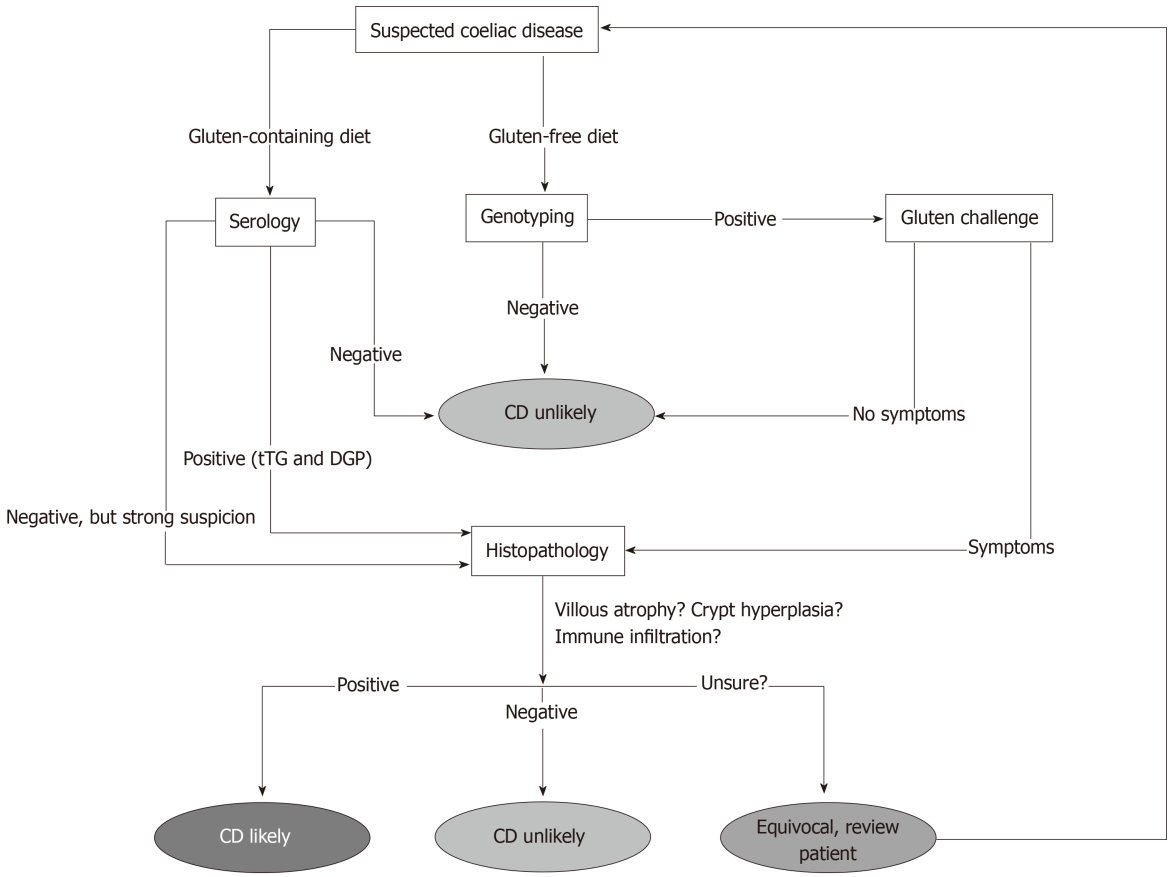

Figure 1 Current diagnostic pathway for suspected coeliac disease. Currently, serology forms the front line of testing for patients currently on a diet containing gluten whilst genotyping can be used for patients who aren't consuming gluten. Histopathology is currently the most conclusive test for the presence of the condition, even though diagnosis can be difficult for patients with mild or equivocal pathology. CD: Coeliac disease; tTG: Tissue transglutaminase; DGP: Deamidated gliadin peptides.

usually at positions 4, 6 and 7 on the gliadin epitope. HLA-DQ8 is a very similar molecule, although with anchor points usually at positions 1, 4 and 9 on the gliadin epitope[23,24].

HLA-genotyping is therefore useful in excluding a diagnosis of CD in cases where serological or histological results are difficult to interpret or to determine the prevalence of CD amongst relatives[22,25]. The test can be performed either with blood or buccal samples and a negative result effectively rules out the presence of CD entirely. It is also not dependent on gluten intake, so can be administered without the need for the patient to commence a gluten-containing diet[15,19].

Unfortunately, the frequency of these genes has been reported to vary geographically, with the DQ2.5 allele being reported at higher frequency in north-western Europeans, such as those from Ireland[26] and the DQ8 allele showing a higher frequency in Amerindian populations[27], thus creating differences in expression independent of the presence of CD. In Australia specifically, approximately 20% of the population have been estimated to have the DQ2 allele, whilst less than 5% of the population have been estimated to have the DQ8 allele[28-30]. At the same time, the cost of the test excludes its use as a front line diagnostic for CD and it also cannot diagnose CD effectively in its own right, as only around 1 in 30 people with the DQ2 or DQ8 variants will eventually develop the condition[22]. Thus HLA-genotyping only provides a risk profile for developing CD. For this reason, even if the gene test returns a positive result, clinical guidelines state that patients still need to undergo small bowel biopsy to confirm the diagnosis[2,15,19].

CURRENT DIAGNOSTICS - HISTOLOGY

Histopathological examination of duodenal biopsy material is currently the most conclusive test for the presence of CD. Using image enhancement on modern endoscopes, it is currently recommended that if villous atrophy is suspected during upper-gastrointestinal assessment that 2-3 biopsies are taken from the duodenal bulb

and 4-6 biopsies are taken from along the distal duodenum[10,15,18,19,31]. Biopsies from these regions are used as they are the first point of contact with the digesta[10]. The histopathological findings associated with active CD are well documented and include three main findings (Figure 1); blunted or atrophic villi (including complete destruction of the epithelial surface), crypt hyperplasia and mononuclear/lymphocytic infiltration into the lamina propria[15,18,32].

Villous atrophy and crypt hyperplasia are usually assessed by calculating the crypt-villous ratio, a measure of the height of the villous when compared to the depth of the adjacent intestinal crypt. Using this method, a normal ratio of villous:crypt height in adults is around 3:1 to 5:1, whilst this figure in children is around 2:1. Values significantly less than these give an indication of the degree of villous atrophy present[33,34]. Lymphocytic infiltration can be assessed by directly examining the numbers of lymphocytes present within the lamina propria in the late stages of the condition (usually T and B lymphocytes) and by assessing the numbers of intraepithelial lymphocytes (IEL)[15,34]. The IELs are a specialised, critical part of the gut-associated lymphoid tissue and do not need priming by other immune cells to release cytokines[34-36]. As the population of these cells is expanded in CD, the current diagnostic cut-off is 25 IELs per 100 enterocytes to demonstrate intraepithelial lymphocytosis in the condition[19,34,37,38].

Morphological changes in CD mucosa can then graded according to the Marsh Score[39,40], with "0" indicating no detectable changes and "3a/3b/3c" indicating severely inflamed tissue affected by autoimmune destruction. It should be noted however that some pathologists will prefer to use descriptive terms instead of the Marsh score in routine assessment of CD rather than the Marsh score[10]. There has been considerable debate as to the accuracy of the Marsh score system however, as it is based on subjective observations of intestinal histological sections which must be made by an experienced pathologist[10,39,40,41]. It has been suggested that subjective interpretation of biopsy material may potentially lead to significant inter-observer disagreement and therefore negative or delayed patient outcomes[41]. Further confounding the histological diagnosis of CD is the patchy presentation of the condition and the fact that the lesions that appear during active CD may not be entirely specific and can often be seen in other enteropathies such as giardiasis or gastroenteritis[37,42-44]. However, at present the Marsh score system in conjunction with serology is currently the gold standard for the assessment of CD and a vast majority of pathologists are able to readily recognise active lesions (Marsh type 3). The difficulty arises when assessment of milder lesions is required. Thus, equivocal patients with subtle changes may be missed by the current histological criteria, leading to ambiguity in diagnosis.

DO WE NEED NOVEL DIAGNOSTICS, MORE DATA OR JUST IMPROVEMENTS TO THE CURRENT PROTOCOLS?

It is clear that the current testing regimen for CD is complicated, as shown in Figure 1, and it is also clear that each diagnostic has significant drawbacks associated with its use. Therefore, it is at this point that we need to ask a critical question. Do we need to develop completely novel tests for coeliac disease or can we use the data we currently generate from patients more effectively? A number of studies have investigated whether or not the application of statistical analysis to existing measurements can increase the diagnostic sensitivity of CD screening. One such technique is linear discriminant analysis (LDA). This technique, when applied to biological data, aims to assign patients to one or more groups on the basis of a series of measurements from which a linear function has been defined[45,46]. Discriminant analysis has been shown to be able to predict patient groupings in conditions such as rheumatoid arthritis[47], Parkinson's disease[48], diabetes[49], Alzheimer's disease[50] and coronary disease[51]. In CD, improving diagnosis with discriminant function analysis has been previously investigated with IgA/IgG and absorptive serology[52,53], IEL counts with crypt/villous ratios[54] and immunohistochemistry data[55]. More recently, studies in CD have used this technique with capsule endoscopy images[56], histology data[57] and gene expression data[58]. The use of this technique has further highlighted the inherit difficulties in classifying CD patients using the discrete divisions of the Marsh subclassifications[59]. Clearly, the full spectrum of CD presentation needs to be captured with continuous categories along a scale to be able to accurately diagnose all who present with CD-like symptomology. Improved and more accurate diagnostics could then also be used to separate other inflammatory conditions, such as Crohn's disease.

As the current technique of sampling from the small intestinal mucosa relies on the

patient being on a gluten-containing diet and actively having CD damage to evaluate, previous research has also focused on determining other biopsy-based tests which could be implemented after a patient has commenced treatment. It has been shown that a rectal challenge with gluten can induce CD-like pathology in the rectal mucosa that is specific enough to attain a diagnosis of CD as both a screening and confirmatory test, with reported sensitivities of 90%-100% and specificities of 91%-100%[60,61]. As sensitised gluten-specific T lymphocytes circulate within the gastrointestinal mucosa, they can be rapidly deployed to sites of localised antigen presentation to initiate a localised inflammatory reaction[60,62]. Although experimental, this test involves the introduction of a slurry of gluten to the rectum that the patient is instructed to retain for as long as possible, preferably for at least 2-4 hours. Biopsies are then taken from the rectal mucosa and the intraepithelial lymphocyte numbers are assessed within the tissue[60-63].

More recently, a novel serological test for CD[64] has also been demonstrated to show high sensitivity and specificity for CD patients who possess the DQ2.5 allele and does not require the patient to be on a gluten-containing diet. Using a whole blood cytokine release assay (primarily used for infectious diseases) focused on IFN-γ, these authors took whole blood from treated CD patients after an oral challenge of gluten and cultured it in the presence of gliadin epitopes. They found that there was no test which could detect changes between the treated CD patients and controls before the gluten challenge; but after gluten was consumed by the treated CD patients, significant elevations in IFN-γ and IFN-γ inducible protein 10 (IP-10; CXCL10) resulted in 85% IFN-γ and 94% IP-10 sensitivity and 100% specificity for DQ2.5+ CD patients. These authors concluded that further clinical studies investigating the utility of these tests were required[64]. Similar testing using tetramers of gluten and HLA has recently been shown to be able to specifically detect gluten-reactive T cells in coeliac patients with a high degree of accuracy and regardless of current gluten intake[65].

Due to the complexity of the coeliac reaction, it is clear that not one single test can fully capture the coeliac continuum, data from many different parameters would need to be combined for the most accuracy. So where should this data come from and how could it be used as a single diagnostic? Histology of the duodenal mucosa should always a play a part in CD diagnosis, however we must first define what a normal duodenal mucosa is before we can begin to compare pathological specimens. The upper and lower borders of the mucosal surface need to be defined, in particular where the exact border of the crypt and the villus meet, perhaps through the use of mRNA expression[59]. At the same time, the surface of the duodenum is a complex and 3-dimensional environment which is poorly represented on a 2-dimensinal microscope slide. Computerised analysis is needed to fully understand the 3-dimensional structure of the duodenal mucosa and how this relates to our 2-dimensional representations[59,66]. Once we can overcome these shortfalls, we need to take numerical values of histological parameters from slides instead of making subjective assessments or attempting to put patients into discrete categories. These numbers can then be used to improve diagnostics as previously shown[57].

There is a wealth of data which could potentially be collected from CD patients, with the most recent being insights into the microbiome of these patients. In the mouth of CD patients, it has been shown that differences occur in the microbial population and that these organisms display proteolytic activity against gliadin, possibly generating immunogenic peptides in the process[67]. In the small intestine, although it was demonstrated that the microbiome did not differ in children with CD when compared to healthy controls, this same effect has not been well investigated in adults to date[68]. It is hypothesised however that changes in the small intestinal microbiome may be involved in the pathogenesis of CD through immune reactions generated against translocated bacterial proteins, resulting from decreased intestinal barrier integrity[69-71]. Within the large intestine, shifts in microbial populations have been shown in CD with a number of genera, including *Lactobacillus, Streptococcus* and *Clostridium* demonstrating proteolytic activity against gluten proteins. Members of these strains may possibly be used in the treatment of the condition by digesting immunogenic fragments of gliadin[72]. Significant changes in the colonic microbiome, including increases in the *Veillonellaceae* family and other taxa involved in starch metabolism, have also been observed in patients who have started treatment with a gluten-free diet in CD[73]. If it is possible to numerically categorise these changes in CD patients when compared to non-CD patients, this data could then be used in a diagnostic sense. At the same time, the collection of faeces and saliva is more efficient and less invasive than intestinal tissue sampling.

Along a similar vein is the categorisation of the CD metabolome; that is, the complete set of metabolites present in a patient sample at a given time point[74,75]. This holistic assessment of end-products can therefore indirectly take into account a variety of changes which may occur from genotype to phenotype[74]. In CD, the

metabolites studied are most commonly from pathways associated with malabsorption, energy metabolism and alterations in intestinal permeability and these can be assessed in a diverse range of fluids, including saliva, CSF, amniotic fluid, breath condensate and faecal extract[74,75]. As there is currently no one particular metabolite which has been shown to have a high predictive value for CD, assessing panels of these potential biomarkers currently holds the most promise for novel diagnostic tests[74-77]. Most recently, this approach has identified a phospholipid signature in HLA at-risk infants which has diagnostic capacity for CD well before antibodies or clinical symptoms appear[78].

"Fingerprinting" of microbial and metabolomic signatures in CD therefore has potential to generate a large amount of data from individuals from a relatively non-invasive test. With the use of LDA, the more variables which can be added to build diagnostic equations, the more accurate the outcome[45]. Combining these new definitions of CD with current diagnostics would allow for a snapshot of CD presentation from all angles. Measuring these parameters (histology, mRNA expression, microbiome change, metabolome change) simultaneously would allow for the most accurate diagnosis and secure the best outcome for patients, as shown in Figure 2.

CONCLUSION

Having accurate diagnostics for CD is critical moving forward, with increasing prevalence of the condition and the risk of serious effects if treatment is not commenced early enough. Current diagnostics have significant drawbacks however and the accuracy of these tests needs to be improved to successfully detect CD in all patients who present with the condition. This is particularly true for those who lack classical symptomology or those who have very mild histopathology. This is also true for tracking treatment progression and healing in patients once a gluten-free diet has been commenced. We need to move away from the discrete definition of CD and towards a continuous scale to fully capture the complete spectrum of patient presentation. To do this, we need diagnostic tests which are holistic; that is, they can take a range of measures from a patient at once and can then be combined to improve diagnostic accuracy. This is where new diagnostic tests need to be defined which can assess CD less invasively. Of most interest is the changes which appear in the microbiome of CD patients and if these changes can be numerically defined, this could lead to a range of novel tests for the condition, either alone or in combination with the traditional CD diagnostics.

With our original question in mind then, novel diagnostic advances in CD are welcomed, particularly if they can assess the condition less invasively and increase the accuracy and speed of screening. The current diagnostics for the condition need to be revisited for the next generation of CD patients and their accuracy needs to be improved, particularly for equivocal presentation. It is hoped then that a balance can be found between novel tests and traditional methods to provide an accurate snapshot of the condition and improve the outcomes of CD patients.

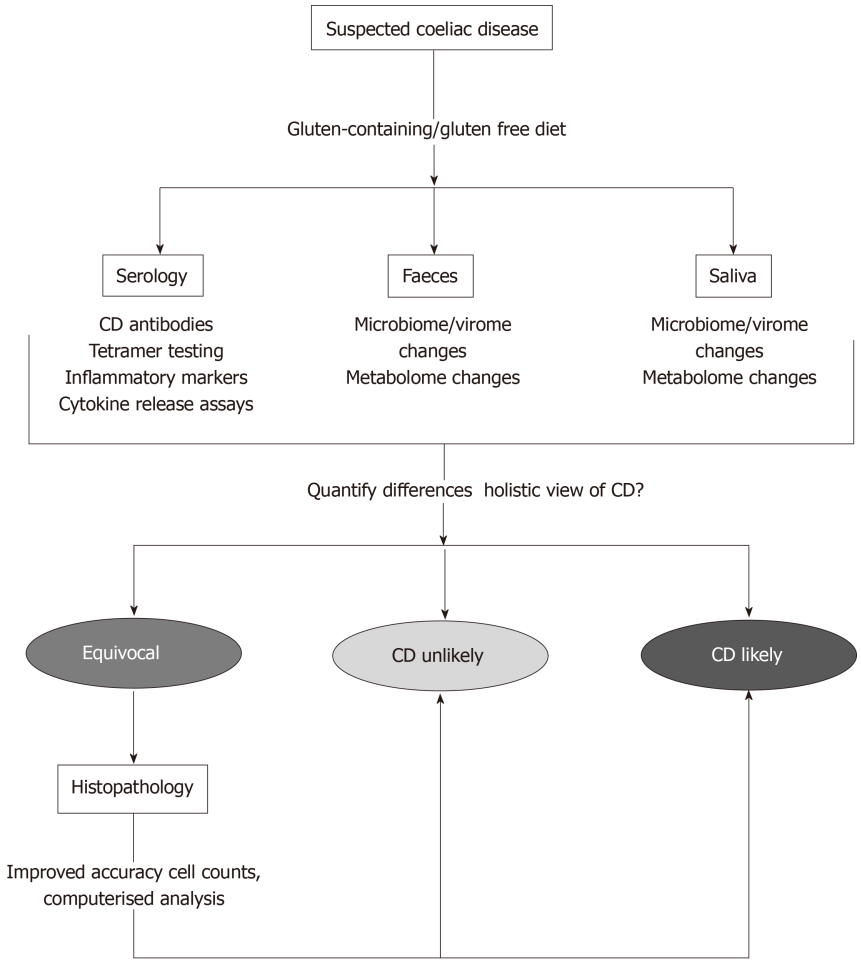

Figure 2 Proposed diagnostic pathway for suspected coeliac disease. Several less invasive measures could be investigated at once using markers in serum, faeces and saliva. If reliable differences could be quantified in coeliac disease, then patients could be diagnosed rapidly and with increased accuracy. For equivocal patients, histopathology of the small intestine would still be used, although increasing the accuracy of these measures through cell counts and computerised analysis would need to be considered. CD: Coeliac disease.

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MINIREVIEWS

Hepatic hemangioma: What internists need to know

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Abstract

Hepatic hemangioma (HH) is the most common benign liver tumor and it is usually found incidentally during radiological studies. This tumor arises from a vascular malformation; however, the pathophysiology has not been clearly elucidated. Symptoms usually correlate with the size and location of the tumor. Less commonly the presence of a large HH may cause life-threatening conditions. The diagnosis can be established by the identification of HH hallmarks in several imaging studies. In patients that present with abdominal symptoms other etiologies should be excluded first before attributing HH as the cause. In asymptomatic patient's treatment is not required and follow up is usually reserved for HH of more than 5 cm. Symptomatic patients can be managed surgically or with other non-surgical modalities such as transcatheter arterial embolization or radiofrequency ablation. Enucleation surgery has shown to have fewer complications as compared to hepatectomy or other surgical techniques. Progression of the tumor is seen in less than 40%. Hormone stimulation may play a role in HH growth; however, there are no contraindications for hormonal therapy in patients with HH due to the lack of concrete evidence. When clinicians encounter this condition, they should discern between observation and surgical or non-surgical management based on the clinical presentation.

Key words: Hepatic hemangioma; Liver masses; Liver; Vascular lesion

Core tip: Hepatic hemangioma is the most common benign liver tumor and it is usually found incidentally during radiological studies. This tumor arises from a vascular malformation. Symptoms usually correlate with the size and location of the tumor. Symptomatic patients can be managed surgically or with other non-surgical modalities.

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INTRODUCTION

Hepatic hemangioma (HH) is a mesoderm-derived tumor consisting of a blood-filled space, fed by hepatic arterial circulation and lined by a single layer of flat endothelial cells[1]. It is the most common benign liver tumor, presenting as a well- circumscribed hypervascular lesion, more commonly found in women with a prevalence that ranges from 0.4% to 7.3% (based on autopsy findings) and an incidence of 0.4%-20% in the general population[1-5].

HH presents commonly as an incidental finding during radiological imaging and are describe as solitary or multiple lesions. They may be confined to one lobe (more in the right hepatic lobe) or extend throughout the entire liver. According to their dimension they can be small or giant (> 5 cm) and may range from 1 mm up to 50 cm[2,6]. HH are classified by their nature as cavernous, capillary and sclerosing hemangioma; the latter is characterized by degeneration and fibrous replacement and can be misdiagnosed as a malignant tumor[7,8].

PATHOGENESIS

The pathophysiology of HH is not completely understood, and in some cases, a genetic predisposition has been described[9]. HH arises from a vascular malformation with a growing pattern secondary to dilation rather than hypertrophy or hyperplasia.

One hypothesis suggest HH results from abnormal angiogenesis and an increase in pro-angiogenic factors[10].

Vascular endothelial growth factor (VEGF) is an important pro-angiogenic factor for endothelial cells. Mammalian target of rapamycin (mTOR) stimulates an autocrine loop of VEGF signaling and increase cell proliferation in vascular endotelial cells. TOR proteins are a group of serine/threanine kinases involved in ribosomal biogenesis, mRNA translation and cell mass growth and proliferation[11]. Zhang *et al*[12] found an increased expression of VEGF-A, pro-matrix metalloproteinase 2, and activated metalloproteinase 2 in HH cells compared to normal human liver endothelial cells.

Rapamycin inhibits mTOR and has been studied in mouse models and mouse cells as a possible treatment for vascular cell growths (mainly malignancies)[11].

Rapamycin is currently used as an antifungal, antineoplastic and antibacterial macrolide drug, but no human studies aimed to HH have been done.

Hormones such as estrogens play a role in HH growth, as they are seen more frequently among women and their size increase after hormone replacement therapy (HRT), oral contraceptive pills (OCPs), and pregnancy[13,14]. The direct mechanisms of hormone effects are unknown, as HH are negative for estrogen and progesterone receptors and current evidence does not support a contraindication of OCPs/HRT/anabolic steroids in patients with HH[15-18].

SYMPTOMS

HH are usually asymptomatic, however symptoms may present when a HH is larger than > 5 cm[19]. Symptoms are nonspecific, patients usually describe abdominal pain, discomfort and fullness in the right upper quadrant, secondary to stretching and inflammation of the Glisson's capsule. Tumors > 10 cm present with abdominal distention[19,20]. The location of the liver mass may cause pressure and compression of adjacent structures causing other symptoms such as nausea, early satiety, and postprandial bloating. Less commonly associated symptoms include fever, jaundice, dyspnea, high-output cardiac failure, and haemobilia[21-24].

Giant HH may cause a life-threatening coagulation disorder known as Kasabach-Merrit syndrome (thrombocytopenia, disseminated intravascular coagulation, and systemic bleeding) presenting with coagulopathy secondary to thrombocytopenia, anemia, hypofibrinogenimia, a decrease in prothrombin time, and increase in D-dimer. This syndrome has been reported with an incidence ranging from 0.3% of all

HH to 26% in tumors > 15 cm[19,25].

Another serious complication is bleeding from spontaneous or traumatic rupture (in peripherally located and exophytic giant lesions), however the risk is extremely low (0.47%)[26].

GROWTH PATTERN

The natural progression of HH varies, previously these lesions were considered to remain stable. However, multiple studies have shown progression and increase in size when followed throughout the years[27,28]. In a study of 236 patients with a median follow up of 48 mo (3-26), 61% experienced HH size increase with a peak growth rate when HH was 8-10 cm (0.80 ± 0.62 cm/year) and in patients less than 30 years of age[29].

In another study with 123 patients (163 HH) a 50.9% grew by any amount in absolute mean linear dimension with an annual growth rate of 0.03 cm for all lesions and 0.19 cm for those that grew > 5%. This study also found a correlation with increased annual growth in HH of 5 cm or more at initial size. They predicted an annual growth rate for all HH of 0.34 mm[5].

DIAGNOSIS

HH unique features by imaging are the presence of peripheral nodular enhancement and a progressive centripetal fill-in. Ultrasound (US), computed tomography (CT), and magnetic resonance imaging (MRI) are the most common imaging tests. Atypical lesions may require more than one imaging test.

US is usually the first diagnostic imaging test due to its availability. HH appears as a well-defined, homogeneously hyper echoic mass with posterior acoustic enhancement (Figure 1). Color-Doppler US does not improve accuracy in diagnosis as it only shows blood flow in HH with an intra arterio-portal shunt[30,31].

US has a sensitivity of 96.9% and a specificity of 60.3%[32]. Some malignant hepatic lesions (Hepatocellular carcinoma and hepatic metastases) may produce similar acoustic patterns and other imaging modality must be used to confirm diagnosis.

Contrast-enhanced US (CEUS) uses gas-filled micro bubbles that delineate the signal produced by blood flow. HH shows a peripheral nodular contrast enhancement in the early phase (arterial) with centripetal filling in later phases.

Some studies have proven CEUS improves characterization and specificity for HH diagnosis[33,34].

CT has a sensitivity of 98.3% and a specificity of 55%[32]. HH are described as well-demarcated hypodense masses (Figure 2). When contrast is used, a peripheral nodular enhancement with centripetal homogeneous filling is expected, however small lesions and HH with cystic areas, fibrosis or thrombosis may show an atypical pattern[35].

MRI shows a well-defined, smooth, homogenous lesion, hypointense on T1 and hyperintense on T2 weighted images (Figure 3). Some malignant lesions may show a similar hyperintensity on T2, to differentiate HH from solid neoplastic liver lesions the echo time is increased which causes signal from malignant lesions to decrease and signal from HH to increase. Gadolinium administration shows a peripheral enhancement on arterial phase and contrast retention on delayed phases, which allows differentiating from hypervascular tumors that usually have a contrast washout on delayed phase. MRI has been considered the best imaging method for HH with a sensitivity of 90%-100% and a specificity of 91%-99%[32,36].

Angiography is the best option for atypical HH that are difficult to diagnose with other imaging test. HH appears as a "snowy-tree" or "cotton wool" with a large feeding vessel and diffuse pooling of contrast that continues during delayed phase. Technetium-99m pertechnetate-labeled red blood cell pool scintigraphy, single photon emission computed tomography and positron emission tomography/CT are other imaging modalities available to diagnose HH in patients with atypical tumors, history of chronic liver disease or malignancy[37,38].

Needle aspiration biopsy is not recommended because of the high risk of hemorrhage and a low diagnostic yield[39-41].

MANAGEMENT

Small, asymptomatic HH do not require treatment or follow up. Some authors suggest to follow-up in HH > 5 cm at 6-12 mo to asses for rapid growth with the same

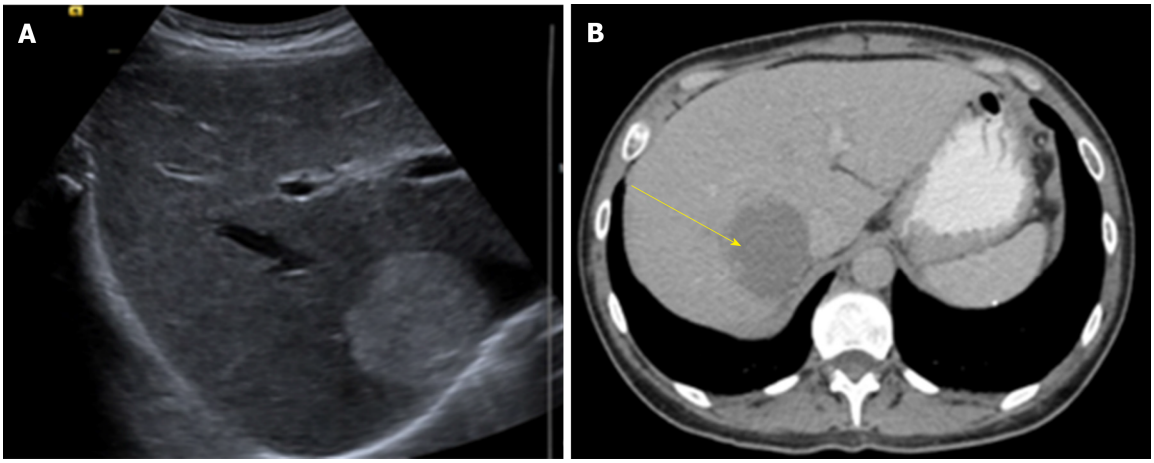

Figure 1 Liver ultrasound and computed tomography abdomen with contrast of a patient with hepatic hemangioma (55 mm × 46 mm). A: Image of ultrasound; B: Image of computed tomography.

imaging test used at diagnosis[42].

Treatment should be restricted to symptomatic patients, with continuous mass growth, compression of adjacent organs (gastric outlet obstruction, Budd-Chiari syndrome) or complications such as rupture with intraperitoneal bleeding or Kasabach-Merrit syndrome.

Abdominal pain should be carefully evaluated in patients with HH and other possible causes should be kept in mind before definitive treatment is decided. Farges *et al*[43] diagnosed 87 patients with abdominal pain and HH, from these, 54% were found to have other condition responsible from the abdominal pain. Specific treatment for abdominal pain and HH was required in 14 patients and half of them remained symptomatic after treatment, suggesting another etiology causing the pain. In another study, the majority of patients with abdominal pain and HH were found to have symptoms attributable to different gastrointestinal diseases (Irritable bowel syndrome, gastroesophageal reflux disease, hepatitis, peptic ulcer, gallbladder disease) and in only 21.7% of symptomatic patients, abdominal pain was attributable to HH[44].

Surgery

Surgery continues to be the most common treatment for HH. Surgical management includes liver resection, enucleation, hepatic artery ligation and liver transplantation. The most common procedures worldwide are liver resection and enucleation (open surgery, laparoscopy or robot)[45-48].

The first hepatic resection for HH was done in 1987 by Schwartz *et al*[49] and in 1988 Alper *et al*[50] reported the first nine patients treated with enucleation.

The choice of procedure depends on the size, number of lesions, location, surgeon experience, and institutional resources. Both techniques carry minimal postoperative morbidity.

In the last years several studies have evaluated enucleation vs hepatectomy and most have concluded that enucleation is associated with lower morbidity, shorter operation time, less blood loss and fewer complications[47,48,51]. However, when HH is larger than 10 cm, Zhang *et al*[52] found no difference in operation time, blood loss, complications or hospital stay between enucleation and resection.

Enucleation is technically easier in peripherally located HH, when done in centrally located HH the procedure causes a longer vascular inflow occlusion time, longer operating time and more blood loss[53]. Centrally located HH (Segments I, IV, V and VIII) are treated with extended right and left hepatectomy. This therapy may remove 60% to 80% of liver parenchyma, which convey a higher risk of postsurgical liver failure. Some lesions are suitable for a wedge resection[53].

Improvement in laparoscopic surgery has increased the cases treated with minimally invasive surgery for either resection or enucleation. Laparoscopic liver surgery is preferred in small, left lateral lesions with minor resections[32,54].

A recent retrospective study compared open versus laparoscopic liver surgery for HH; results favored laparoscopic therapy with less blood loss, lower complication rates, and a shorter postoperative hospital stay. However, baseline patient characteristics between the two groups were not equal as surgeons decided open or laparoscopic surgery based on tumor characteristics[54].

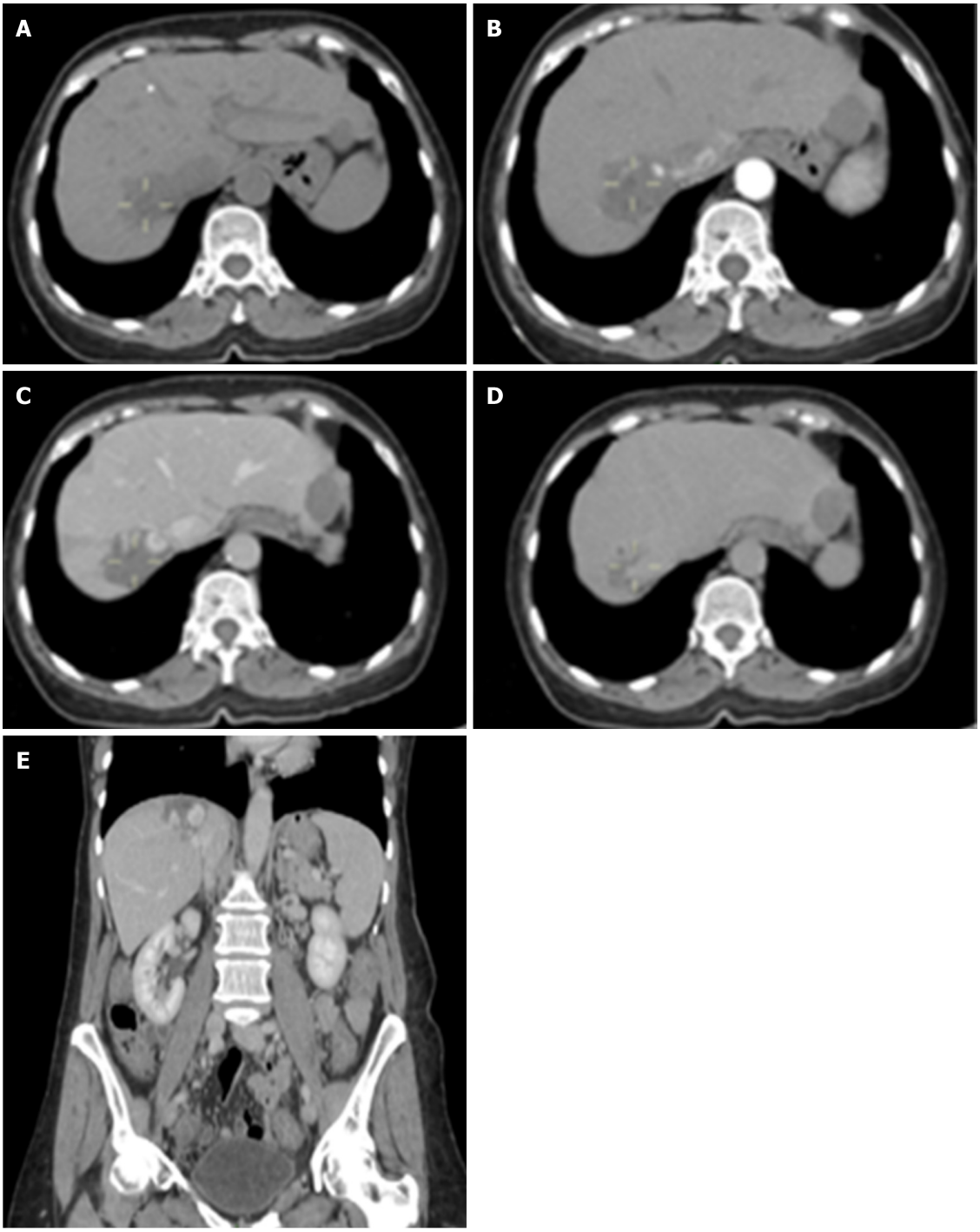

Figure 2 Hepatic hemangioma of 49 mm × 30 mm. A: Non-contrast phase; B: Arterial phase; C: Venous phase; D: Delayed phase; E: Coronal view of computed tomography scan (venous phase).

Liver transplantation for benign solid tumors is not considered a first line treatment due to morbidity and organ shortage. A study published in 2015 analyzed data from the United Network of Organ Sharing from 1988 to 2013 and found 147 (0.17%) liver transplants in US patients were performed for benign tumors of the liver, including 25 for HH[55].

Liver transplantation is reserved for unresectable giants HH causing severe symptoms (respiratory distress, abdominal pain), failure of previous interventions or life-threating complications such as Kasabach Merrit syndrome[56,57].

Non-surgical management

Transcatheter arterial embolization (TAE) is used to control acute bleeding or shrink HH prior to surgery with metallic coils, gelform particles, polyvinyl alcohol and liquid agents such as N-butyl-2-cyanoacrylate, bleomycin-lipiodol[58-61]. However, TAE

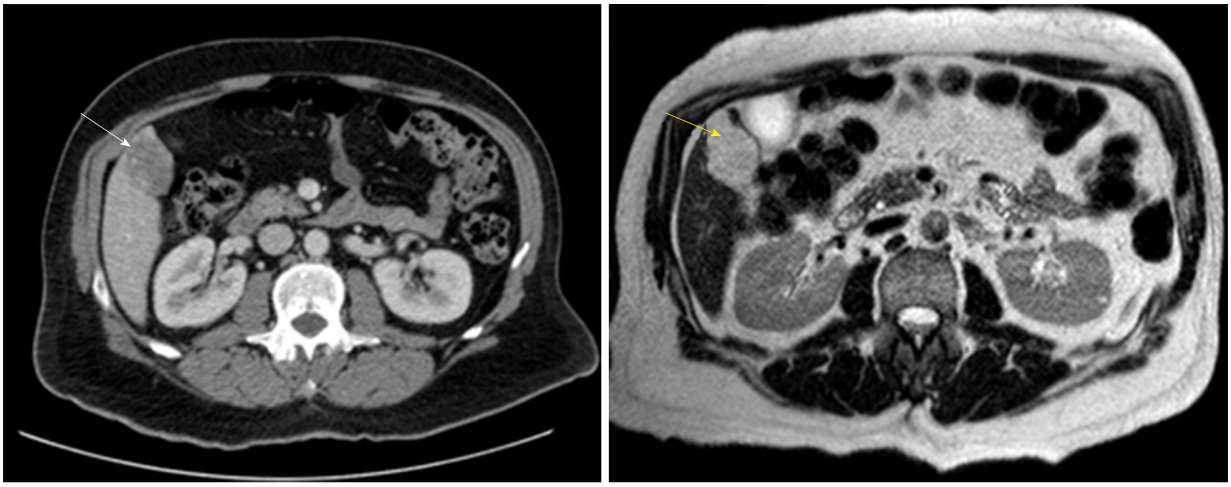

Figure 3 Computed tomography abdomen with contrast (white arrow) and magnetic resonance imaging T2-weighted scan (yellow arrow) with hepatic hemangioma 45 mm × 30 mm.

as also been used as single treatment with acceptable results[62,63].

A mix of pingyangmycin/lipiodol was first studied as a single treatment for HH. Two studies reported good results with significant reduction of HH volume and relief of symptoms[64,65]. Pingyagmycin is only available in China, similar studies have been carried in other places with bleomycin as substitute for pingyagmycin[62,63].

A study with 23 patients (29 HH) managed with TAE with bleomycin-lipiodol concluded 73.9% of patients had > 50% volume regression of HH[62]. Bleomycin administration results in micro-thrombi formation, which leads to atrophy and fibrosis of the tumor. It also induces a non-specific inflammatory process around the HH and in the portal area. Acute liver failure, liver infarction, abscess, intrahepatic biloma, cholecystitis, splenic infarction, hepatic artery perforation, and sclerosing cholangitis have been reported as associated complications of TAE with Bleomycin[66].

Radiofrequency ablation (RFA) can be used percutaneously, laparoscopically or by open surgery. RFA induces a thermal damage to endothelial vascular structures and promotes thrombosis. RFA is usually performed under US guidance; CT guidance for percutaneous RFA is suitable for HH located deeply in liver parenchyma[67].

Laparoscopic RFA with US guidance is preferred for subcapsular HH[68]. Laparoscopic RFA compared with open resection is associated with shorter operative time, less pain, shorter hospital stay and the lower hospital cost[69,70].

Lengthy RFA is prone to cause hemolysis, hemoglobinuria and acute kidney injury, thus is not suitable for large HH[71]. Other complications of RFA include bleeding at the electrode entry site, rupture of HH and injury to adjacent organs by puncture or thermal injury.

The established indications for RFA in this population are maximum diameter of HH > 5 cm, tumor gaining enlargement > 1 cm within 2 years, persistent HH related abdominal pain with exclusion of other GI diseases. Contraindications include patients with severe bleeding tendency, malignant tumors, Kasabach-Merrit syndrome, infection (biliary system inflammation), low immune function, and severe organ failure[67].

The use of anti-VEGF such as sorafenib and bevacizumab have been reported in case reports to incidentally reduce HH size[72,73]. A retrospective study aimed to study HH size reduction with anti VEGF (bevacizumab or sunitinib) showed no significant volume reduction[10]. Metformin has also been reported in a case report to incidentally reduce HH size[74].

Liver transplant with liver resection graft of HH

In the last years, the donor's criteria for liver transplant has expanded to overcome organ shortage. Liver donors with the discarded partial liver resection from HH have proved to be a viable source for liver transplant with acceptable receptor outcomes and no growth of HH[75-77].

CONCLUSION

Most HH are diagnosed incidentally on imaging tests since most patients remain

asymptomatic throughout their life. Patients who present with symptoms are usually due to larger lesions.

Since the natural history of HH is benign and an increase in size progression occurs in less than 40%, most patients can be reassured and only observed. When a patient is symptomatic, the first step is to exclude other causes of their symptoms. Once excluding other etiologies and HH is considered the cause of symptoms, treatment modalities are decided based on size, anatomy and comorbidities of the patient.

Over the last years, non-surgical minimal invasive procedures for tumor reduction and laparoscopic surgery have proven good results in selected patients.

Rarely HH present with life-threatening conditions such as an acute traumatic rupture or coagulation disorders. Only in these instances, emergent surgical management is warranted.

Clinicians should discern between observation and the best optimal management based on the clinical presentation. If treatment is needed, a minimal invasive approach should be pursued. Future research will help clinicians understand HH pathogenesis and guide management.

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ORIGINAL ARTICLE

Basic Study

Feedback regulation between phosphatidylinositol-3,4,5-trisphosphate dependent Rac exchange factor 1 and transforming growth factor β1 and prognostic value in gastric cancer

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Abstract

BACKGROUND

Phosphatidylinositol-3,4,5-trisphosphate dependent Rac exchange factor 1 (PREX1) was reported to be overexpressed in some cancers and involved in cancer development, but its expression and significance in gastric cancer remain unclear.

AIM

To evaluate the expression of PREX1 in gastric cancer and its significance in the development of gastric cancer, especially to evaluate the potential mechanism of PREX1 in gastric cancer.

METHODS

Bioinformatic analysis was performed in order to examine the expression of PREX1 in gastric cancer. The relationship between the survival rate of gastric cancer patients and PREX1 expression was assessed by Kaplan Meier portal. The Gene Set Enrichment Analysis and the correlation between PREX1 and transforming growth factor (TGF) β1 pathway-related mediators were evaluated by cBioPortal for Cancer Genomics. Western blotting and reverse transcriptase polymerase chain reaction assay were used to test the role of TGFβ1 on the expression of PREX1. Western blotting and dual-luciferase reporter system was used to evaluate the effect of PREX1 on the activation of TGFβ1 pathway. Wound healing and Transwell assay were used to assess the effect of PREX1 on the metastasis activity of gastric cancer cells.

RESULTS

PREX1 was overexpressed in the gastric tumors, and the expression levels were positively associated with the development of gastric cancer. Also, the high expression of PREX1 revealed poor prognosis, especially for those advanced and specific intestinal gastric cancer patients. PREX1 was closely involved in the positive regulation of cell adhesion and positively correlated with TGFβ1-related

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mediators. Furthermore, TGFβ1 could induce the expression of PREX1 at both the protein and mRNA level. Also, PREX1 could activate the TGFβ1 pathway. The induced PREX1 could increase the migration and invasion activity of gastric cancer cells.

CONCLUSION
PREX1 is overexpressed in gastric cancer, and the high level of PREX1 predicts poor prognosis. PREX1 is closely associated with TGFβ signaling and promotes the metastasis of gastric cancer cells.

Key words: Phosphatidylinositol-3,4,5-trisphosphate dependent Rac exchange factor 1; Gastric cancer; High expression; Poor prognosis; Metastasis; Transforming growth factor β1 pathway

Core tip: In this study, we fully identified the overexpression of phosphatidylinositol-3,4,5-trisphosphate dependent Rac exchange factor 1 (PREX1) in gastric cancer and its positive correlation with the gastric cancer progression. High PREX1 expression predicts poor prognosis in the specific intestinal-type gastric cancer patients. PREX1 is closely involved with cell adhesion, and PREX1 has a positive feedback loop regulation with transforming growth factor β1 pathway to promote the metastasis of gastric cancer cells. PREX1 may be a novel target for the drug development of gastric cancer, and PREX1 acts as a predictor for prognosis of intestinal-specific gastric cancer.

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INTRODUCTION

Gastric cancer is one of the most common digestive tract tumors. It has the characteristics of high malignancy and high metastasis[1]. In recent years, the incidence of gastric cancer continues to increase, which is closely related to food safety, poison exposure, and bad living habits[2], such as environmental toxins, malnutrition, *etc*. At present clinical practice, the curative treatment of gastric cancer is relatively rare. Furthermore, due to the poor early diagnosis of gastric cancer, most patients with gastric cancer are diagnosed at the advanced stage, so the efficiency of treatment is not satisfactory[3-5]. The clinical summary represents that the 5-year survival rate is relatively low, and due to the tumor type, the patient's quality of life is significantly reduced[6]. Therefore, exploring novel mediators of gastric cancer is of great significance in the therapy of gastric cancer.

Phosphatidylinositol-3,4,5-trisphosphate dependent Rac exchange factor 1 (PREX1) was identified as an activator of Rac[7,8]. A previous publication showed that PREX1 interacts with a G protein-coupled receptor and activates a downstream signaling pathway[9,10]. PREX1 is an indispensable factor in the regulation of neutrophil function[11,12]. Welch *et al*[13] reported that PREX1 was an important regulator in neutrophil functions such as the induction of reactive oxygen species and the inflammation response. Moreover, PREX1 has the ability to regulate neutrophil chemotaxis. A detailed molecular mechanism study showed that PREX1 can activate Rac1/2, thus regulating neutrophil migration[14,15]. In addition, PREX1 mediates the signaling regulation between the T-cell antigen receptor and CXCR4[16,17]. Therefore, PREX1 may be a prospective target for immune disease.

Many reports of PREX1 also focus on the study of cancer. PREX1 was shown to be overexpressed in breast tumor tissues and to be positively correlated with the clinical-pathological feature, which could predict the prognosis for breast cancer patients[18-20]. Other studies also reported the overexpression of PREX1 in some cancers, including prostate cancer, kidney cancer, ovarian cancer, glioblastoma and thyroid cancer, and that this overexpression was involved in the proliferation and migration of cancer

cells[21-24]. Notably, the latest study found abnormal methylation of PREX1 in gastric cancer. Liu *et al*[25] reported that hypermethylation of PREX1 represented a higher survival rate. However, the expression of PREX1 and its significance in gastric cancer remain unclear. In this study, we identified the expression of PREX1 in different types of gastric cancer. This study also revealed the association between PREX1 expression with the development of gastric cancer. Furthermore, the clinical significance of PREX1 was assessed using survival analysis. We firstly report that PREX1 expression is a specific prognosis biomarker for intestinal-type gastric cancer. Notably, we also evaluated the potential mechanism regulation in gastric cancer. Interestingly, we report for the first time that PREX1 has a positive feedback loop with the transforming growth factor (TGF) β1 signaling pathway and participates in the process of gastric cancer metastasis. This study further demonstrates the expression of PREX1 in gastric cancer and evaluates its significance in the clinical finding. Furthermore, the feedback loop regulation between TGFβ1 and PREX1 has been firstly addressed, and PREX1 might be a novel target for the treatment of gastric cancer.

MATERIALS AND METHODS

Reagents

SGC-7901, BGC-823 cells, and HEK293T cells were obtained from the American Type Culture Collection (ATCC, Rockville, MD, United States). The recombinant human TGFβ1 protein (240-B-002) was purchased from R&D Systems (Minneapolis, MN, United States). Anti-TGF-β 1 antibody (ab92486) and anti-PREX1 antibody (ab124231) were purchased from Abcam (Cambridge, MA, United States). Anti-β actin was provided from Santa Cruz Biotechnology (Dallas, TX, United States). Lipofectamine 2000 transfection reagent, Dulbecco's modified eagle's medium, fetal bovine serum (FBS), antibiotic reagent, and RMPI 1640 medium were purchased from Thermo Fisher (Waltham, MA, United States). Dual-luciferase reporter kit and crystal violet staining were obtained from Beyotime (Wuhan, China). Protease inhibitor cocktail was purchased from APExBio (Houston, TX, United States). Lentivirus concentration solution, radioimmunoprecipitation assay lysis buffer, bicinchoninic acid protein concentration kit, total RNA isolation reagent, first-strand cDNA synthesis kit, enhanced chemiluminescence system, and SYBR quantitative polymerase chain reaction (PCR) kit were obtained from Yeasen Biotechnology (Shanghai, China).

Oncomine *analysis*

The Cancer Genome Atlas (TCGA) analysis of gastric cancer was achieved by *Oncomine* portal. The relative gene expression of PREX1 was expressed as copy number units. The different histology of gastric cancer included diffuse gastric adenocarcinoma, gastric tubular adenocarcinoma, gastric intestinal-type adenocarcinoma, mucinous gastric adenocarcinoma, and gastric mixed adenocarcinoma. The 236 cases of blood sample from gastric cancer patients were also included in this study. The detailed analysis condition was as follows: *P* value < 1E-4, fold change > 2, the gene ranks was top 10%. The patient's information was obtained from sample filters with clinical outcome, and the expression of PREX1 in gastric cancer patients with different stage and tumor grade and with lymph node metastasis or not were assessed in this study. Datasets included in the survival analysis are shown in Table 1[26-31].

Survival analysis with Kaplan Meier plotter

The overall survival (OS) and post-progression survival (PPS) rate were conducted in Kaplan Meier plotter. The classification of high expression patients and low expression patients was split by median expression value. The biased arrays were excluded in the survival analysis. In total, 631 patients were included to compare OS between PREX1 high expression and low expression patients, and 384 patients were included in the PPS analysis. The restrict analysis to the subtype was the Lauren classification method. In the OS analysis, 269 samples with intestinal gastric cancer and 240 samples with diffuse gastric cancer were included. The statistical analysis was performed using the logrank test and Cox regression analysis.

Co-expression network analysis of PREX1 in gastric cancer

The co-expression analysis of PREX1 in gastric cancer was performed in the cBioPortal for Cancer Genomics. *P* value less than 0.01 was set as a filter for significant co-expression network analysis. The co-expression gene list was subjected to the Gene Set Enrichment Analysis (GSEA) with a biological process. The enrichment score and ranked list metric were included in the GSEA analysis. The correlation analysis between PREX1 and TGFβ1, TGFβ2, intercellular adhesion molecule-1, integrin alpha

Table 1 Datasets included in the survival analysis

GEO accession	Sample number	Platform	Ref.
GSE14210	146	GPL571	[26]
GSE15459	200	GPL570	[27]
GSE22377	43	GPL570	[28]
GSE29272	268	GPL96	[29]
GSE51105	94	GPL570	[30]
GSE62254	300	GPL570	[31]

GEO: Gene Expression Omnibus.

(ITGA) 4, and ITGA5 of TCGA gastric cancer were conducted with cBioPortal for Cancer Genomics. The correlation level analysis was assessed by the Pearson method.

Cell culture and transfection

Human embryonic kidney HEK293T cells were cultured in the Dulbecco's modified eagle's medium with 10% FBS. SGC-7901 and BGC-823 cells were cultured in RMPI1640 medium and supplemented with 10% FBS, 200 mg/mL streptomycin, and 200 U/mL penicillin. Lipofectamine 2000 was applied in the cell transfection. HEK293T cells were plated in the 10 cm cell dish, and when the cell density was about 80%, the cells were subjected to the transfection. The full length of human PREX1 or control vector plasmid was transfected combined with pCMV-dR8.91 and pCMV-VSVG plasmids into HEK293T cells. The detailed procedure was as follows: the overexpression or vector plasmid, pCMV-dR8.91 plasmid, and pCMV-VSVG plasmid were diluted in the Opti-MEM medium, the ratio between the three plasmids was 5:3:2. Lipofectamine 2000 reagent was diluted in the Opti-MEM medium, and the concentration was confirmed according to the manufacturer's protocol. After further culture for 48 h, the supernatant was collected for lentivirus concentration with solution kit. The viral particle of PREX1 overexpression and vector control were supplemented in the culture medium with 5 μg/mL polybrene. With the incorporation of polybrene, the effect of overexpression transfection could be enhanced.

PREX1 transcripts' construction

The human full length of PREX1 is 4980 bp. The full length of PREX1 was cloned into pCDH-CMV-MCS-EF1-Puro lentivirus system. The primer for PREX1 transcript was as follows: forward primer, 5'-GCTCTAGAATGGAGGCGCCCAGCGGCAG-3'; reverse primer, 5'-CGGAATTCTCAGAGGTCCCCATCCAC-3'. The PREX1 transcript amplification was constructed with PrimeStar DNA polymerase. Due to the high content of GC in the forward primer, the PCR reaction solution contained 10% dimethylsulfoxide. Furthermore, considering the big Tm value difference between forward and reverse primer, the two-step PCR method was applied. The restriction enzymes *Xba*I and *EcoR*I were used to insert PREX1 full length into a pCDH-CMV-MCS-EF1-Puro vector.

Western blotting assay

The cells were washed with cold phosphate-buffered saline (PBS) buffer twice, and the cells were lysed with radioimmunoprecipitation assay solution on ice for 30 min. The lysis buffer was supplemented with protease inhibitor cocktail at the work concentration. The cell lysis buffer was collected and centrifuged at 12000 g/min for 15 min, and the supernatant was collected and subjected to the bicinchoninic acid protein concentration assay. The equivalent protein was subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis and transferred to polyvinylidene difluoride membrane. The membrane was blocked with non-fatty milk at room temperature for 1 h, and then the membrane was washed with Tris buffered saline with tween buffer. The membranes were incubated with indicated primary antibody (1:1000) at 4 °C overnight. The membranes were washed with Tris buffered saline with tween three times and incubated with the indicated second antibody at room temperature for 1 h. Enhanced chemiluminescence system was used to detect the protein expression.

Real-time quantitative polymerase chain reaction

The cells were collected and washed with cold PBS buffer twice, and the cells were lysed with total RNA isolation reagent at room temperature for 10 min.

Trichloromethane was added, and the sample was vortexed for 1 min and centrifuged at 12000 *g* for 10 min. The supernatant was collected in a new tube and supplemented with cold isopropanol. The solution was mixed and centrifuged at 12000 *g* for 10 min. The white sediment was the total RNA and washed with 75% ethanol twice for RNA purification. The first-strand cDNA synthesis was conducted according to the manufacturer's protocol. Relative mRNA levels of the indicated target gene was evaluated with SYBR green assay, and the 2^{-CT} method was used to display the mRNA levels. The primer for real-time quantitative polymerase chain reaction as followed: PREX1 (forward primer: 5′-GGCATTCCTGCATCGCATC-3′, reverse primer: 5′-CGGGTGTAAACAATACTCCAAGG-3′, amplicon size: 151 bp), β-actin (forward primer: 5′-CATGTACGTTGCTATCCAGGC-3′, reverse primer: 5′-CTCCTT AATGTCACGCACGAT-3′, amplicon size: 250 bp).

Dual-luciferase reporter assay

HEK293T cells were seeded in a 24-well plate, and the cell density was approximately 80%. The cells were transfected with Smad3 reporter plasmid pGL3.PT3 and housekeeping Rellina plasmid. Simultaneously, the culture medium was supplemented with lentivirus particle of PREX1 overexpression and vector control. Twenty-four h later, the cells were treated with recombinant human TGFβ1 protein at the density of 20 ng/mL. The cells were washed with cold PBS buffer and lysed with reporter lysis on ice for 15 min, and the lysis was collected and centrifuged at 12000 g at 4 °C. The supernatant was collected into a white 96-well plate, and the firefly luciferin substrate was added into the supernatant and immediately mixed for 10 s, and the fluorescence value was measured using Rellina luciferase substrate.

Wound healing assay

The cells were seeded into 6-well plates and cultured for 24 h. When the cell confluence was about 100%, a yellow tip was used to develop a scratch, and the cells were washed with PBS three times. The lentivirus for PREX1 overexpression and vector control were supplemented, and the cells were cultured for another 48 h. Cell images were acquired by Zeiss microscope.

Transwell invasion assay

The Transwell unit was pretreated with matrigel and stored at 37 °C for 6 h, and the cells were seeded into the Transwell unit for culture. After 48 h culture, the matrigel was removed from the Transwell and stained with crystal violet. The invasive cells were numbered by Zeiss microscope.

Statistical analysis

The gene levels are expressed as log2 (copy number units) in *Oncomine* analysis, and the difference between two groups was analyzed by Student's *t*-test. The survival rate analysis was conducted with the Kaplan Meier method, and logrank *P* value was conducted to evaluate the statistical significance. The correlation analysis was achieved by the Pearson method.

RESULTS

Overexpression of PREX1 on gastric cancer

Previous studies demonstrated that PREX1 was overexpressed in some cancers[15,18,23], but its expression in gastric cancer remains unclear. To understand fully the value of PREX1 in gastric cancer, we firstly examined the expression of PREX1 in gastric cancer. As shown in Figure 1A, the analysis of TCGA gastric cancer revealed that PREX1 was highly expressed in different histology types of gastric tumor tissues. To confirm the overexpression of PREX1 in gastric tumor tissues, Deng gastric RNA-sequencing analysis was conducted and demonstrated that PREX1 was highly expressed in different histology of gastric cancer (Figure 1B), suggesting that PREX1 was a general mediator in gastric cancer[32].

PREX1 expression is positively correlated with the development of gastric cancer

In this study, we identified that PREX1 was overexpressed in the gastric tumor tissues. Considering the significance of PREX1 in the function regulation of neutrophils and macrophage[10,12], we examined the expression of PREX1 in the blood of 236 gastric cancer patients. The results showed that the level of PREX1 in gastric cancer patient's blood was not significantly that than in normal gastric cancer. To understand the expression of PREX1 in the development of gastric cancer, the level of PREX1 in the different stages of gastric cancer patients was assessed. As shown in Figure 2B, the level of PREX1 in patients with stage I was not remarkably different

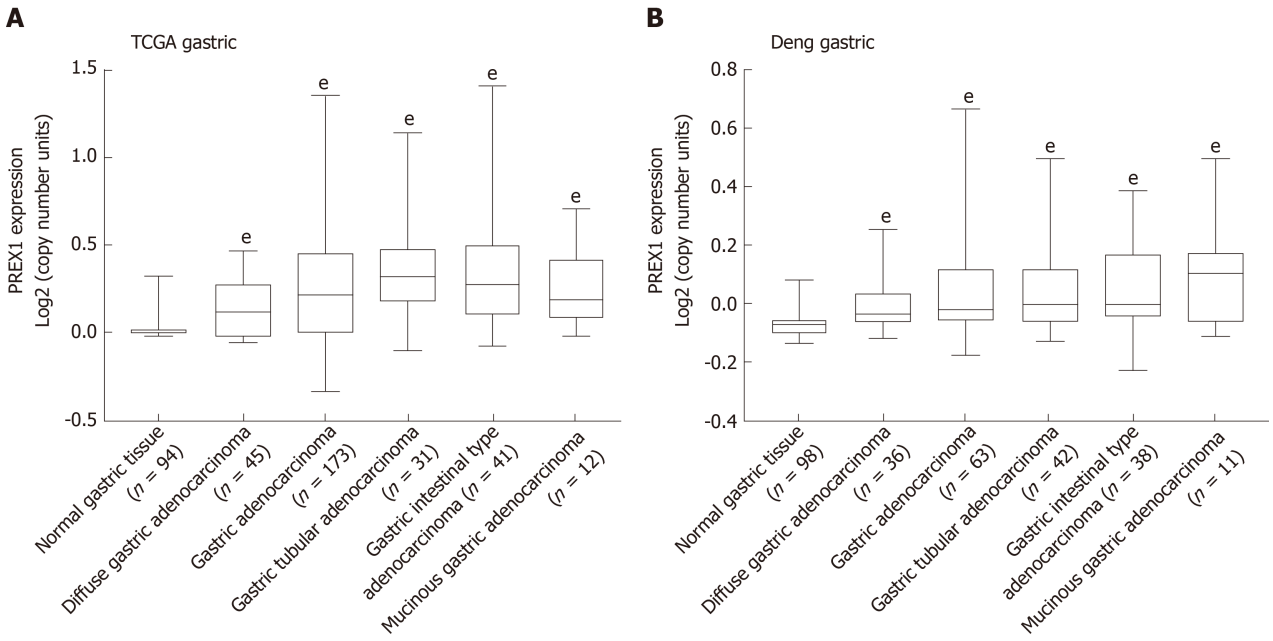

Figure 1 The expression of phosphatidylinositol-3,4,5-trisphosphate dependent Rac exchange factor 1 was evaluated in the *Oncomine* portal. A: The expression of phosphatidylinositol-3,4,5-trisphosphate dependent Rac exchange factor 1 in the cancer genome atlas gastric cancer database was conducted; B: Deng's gastric tissues from *Oncomine* portal[32] were selected to evaluate the phosphatidylinositol-3,4,5-trisphosphate dependent Rac exchange factor 1 mRNA levels in different histology types of gastric cancer, the data was evaluated by high-resolution single nucleotide polymorphism arrays. Statistical significance is expressed as $^{e}P < 0.001$ *vs* normal gastric tissue. PREX1: Phosphatidylinositol-3,4,5-trisphosphate dependent Rac exchange factor 1; TCGA: The Cancer Genome Atlas.

than in normal gastric tissues. However, the expression of PREX1 was significantly enhanced in the advanced stage. A similar result was also observed in the PREX1 expression analysis of different tumor grades. There was no significant difference between patients with tumor grade I and normal gastric tissues, and PREX1 level was remarkably elevated in the higher tumor grade (Figure 2C). Furthermore, the expression of PREX1 in gastric cancer patients with lymph node metastasis or not was evaluated, and the result showed that the level of PREX1 was significantly increased in patients with gastric cancer tumor with lymph node metastasis compared to those without lymph node metastasis (Figure 2D). Based on these findings, we hypothesized that PREX1 expression might be a marker for advanced-stage gastric cancer.

Overexpression of PREX1 predicts poor prognosis for specific intestinal gastric cancer patients

As mentioned previously, PREX1 expression was varied in gastric cancers with different histology, PREX1 expression was elevated in the advanced stage of gastric cancer patients, and PREX1 might serve as a marker for the diagnosis of advanced gastric cancer patients. Thus, we hypothesized that PREX1 might be a prognosis marker for gastric cancer patients. To confirm the hypothesis, we evaluated the relationship between PREX1 expression and the OS and PPS rates of gastric cancer patients. As shown in Figure 3A, the gastric cancer patients with high expression of PREX1 represented a lower OS rate [logrank $P = 0.006$, hazard ratio = 1.46 (1.17-1.81)]. In detail, the median survival of the high expression cohort was 35.9 mo, but the median survival of the low expression cohort was 78.6 mo. PREX1 expression was elevated in the advanced gastric cancer patients compared with the early-stage patients. Regarding PPS, patients with high PREX1 expression displayed a lower survival rate [Figure 3B, logrank $P = 6.6E-07$, hazard ratio = 1.986 (1.5-2.6)]. Combined with higher expression in the advanced gastric cancer patients, PREX1 might be a significant biomarker as a prognosis predictor of gastric cancer patients, especially for those patients with advanced stage. In clinical practice, gastric cancer has two main histology types, diffuse gastric cancer and intestinal gastric cancer[33]. In further evaluation, we examined if PREX1 expression affected the survival rate in different histology types of gastric cancer patients. Interestingly, as shown in Figure 3C and D, high PREX1 expression patients display a lower survival rate in specific intestinal gastric cancer patients. However, the survival rate of high PREX1 expression and low expression patients was not significantly different in diffuse gastric cancer patients.

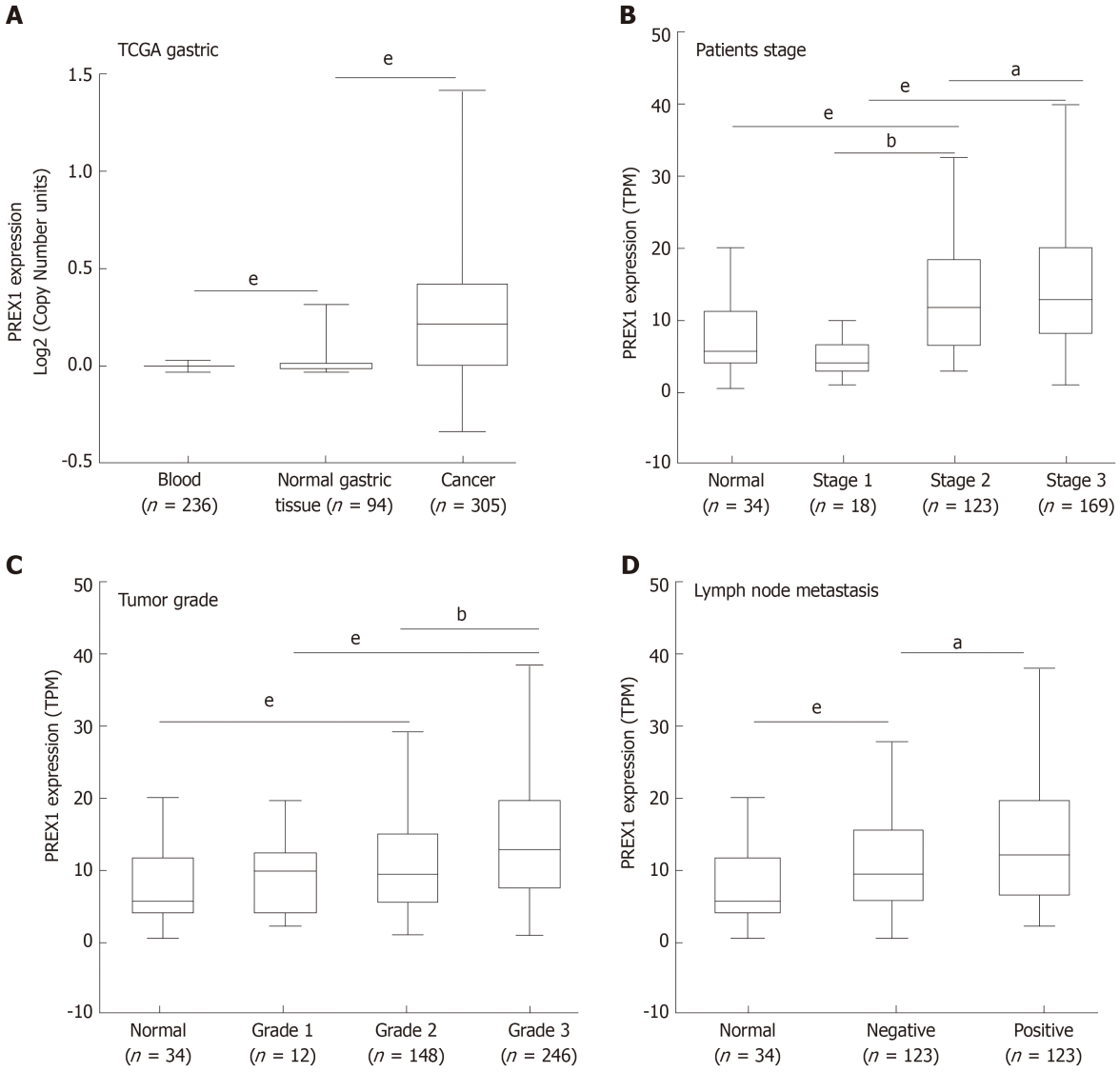

Figure 2 The association between phosphatidylinositol-3,4,5-trisphosphate dependent Rac exchange factor 1 expression and clinical features. A: The expression of phosphatidylinositol-3,4,5-trisphosphate dependent Rac exchange factor 1 (PREX1) in blood samples of gastric cancer patients, normal gastric tissues, and gastric tumor tissues; B: The expression of PREX1 was examined in gastric cancer patients with the different patient stage; C: The level of PREX1 was assessed in gastric cancer patients with different tumor grade; D: The level of PREX1 was examined in those gastric cancer patients with lymph node metastasis or not. Statistical significance is expressed as $^{a}P < 0.05$, $^{b}P < 0.01$, $^{e}P < 0.001$. TPM: Transcript per million; PREX1: Phosphatidylinositol-3,4,5-trisphosphate dependent Rac exchange factor 1; TCGA: The Cancer Genome Atlas.

So, PREX1 expression was a novel prognosis biomarker for gastric cancer patients, especially for advanced patients and intestinal gastric cancer patients.

PREX1 expression is involved in the positive regulation of cell adhesion

PREX1 was overexpressed in the gastric tumor tissues, and the expression of PREX1 could serve as a prognosis predictor for gastric cancer patients, especially for those patients with advanced-stage and intestinal histology. These results demonstrated that PREX1 might be an important mediator in the development of gastric cancer. Thus, we evaluated the potential mechanism regulation involved in the function of PREX1 in gastric cancer. Firstly, the co-expression network of PREX1 was conducted, and the GSEA was applied to study the potential effect of PREX1 on the biological process. As shown in Figure 4A, PREX1 expression was closely associated with the positive regulation of cell adhesion, with the higher enrichment score (normalized enrichment score = 1.56, false discovery rate = 0.003). To confirm further this result, the correlation between PREX1 and other important mediators were conducted. TGFβ signaling pathway is an important mediator in cancer development and is was closely involved in the migration and invasion of cancer cells[34]. As PREX1 expression was positively associated with cell adhesion, we evaluated the correlation between PREX1 and TGFβ signaling pathway. PREX1 was positively correlated with TGFβ1 (Figure

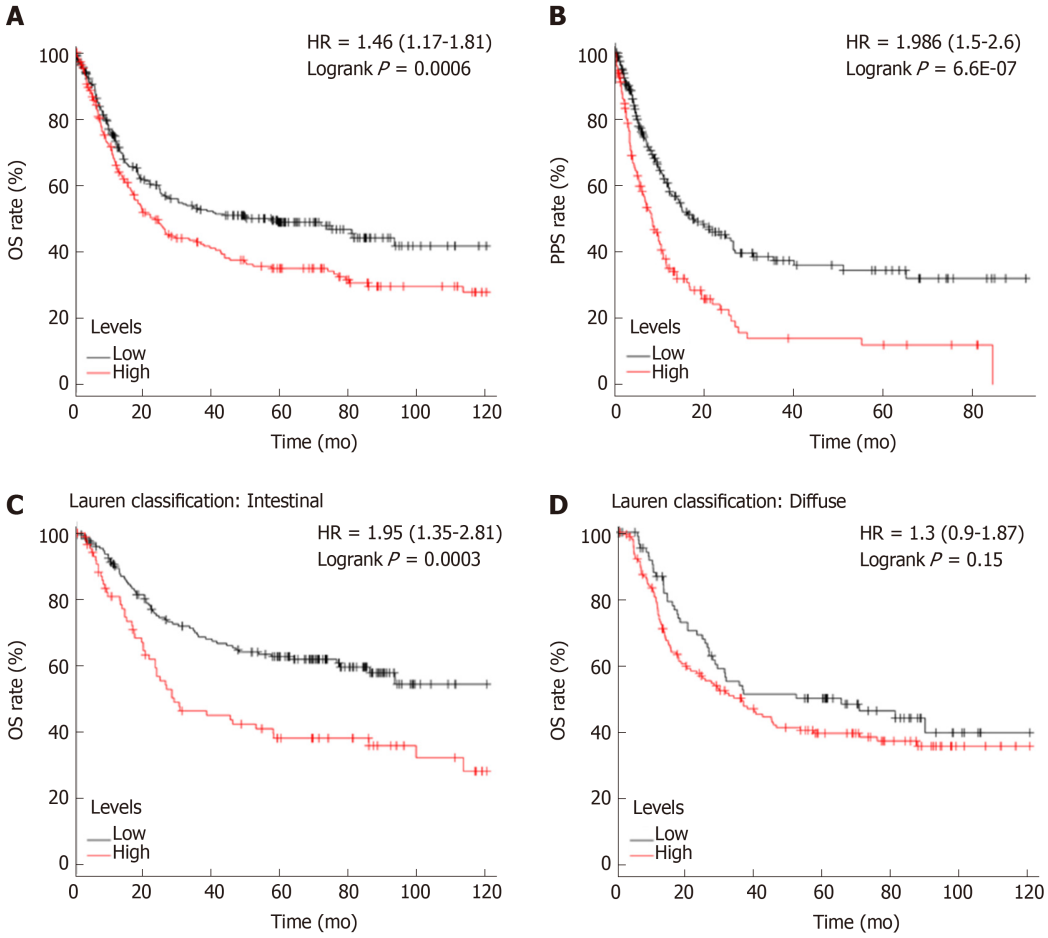

Figure 3 The survival analysis between high and low phosphatidylinositol-3,4,5-trisphosphate dependent Rac exchange factor 1 expression cohorts. A: The overall survival rate was conducted in the high and low phosphatidylinositol-3,4,5-trisphosphate dependent Rac exchange factor 1 (PREX1) expression gastric cancer patients; B: The post-progression survival analysis was conducted in the high and low PREX1 expression gastric cancer patients; C: In the intestinal gastric cancer patients; D: diffuse gastric cancer patients, the overall survival was assessed in the high and low PREX1 expression patients. OS: Overall survival; PPS: Post-progression survival; HR: Hazard ratio.

4B), and there was no significant correlation between PREX1 and TGFβ2 (Figure 4C). In further analysis downstream of TGFβ pathway, a similar phenomenon was observed in the correlation analysis of intercellular adhesion molecule-1 (intercellular adhesion molecule-1, R = 0.44, Figure 4D), ITGA4 (ITGA4, R = 0.76, Figure 4E), and ITGA5 (ITGA5, R = 0.18, Figure 4F). These data revealed that PREX1 was positively associated with the cell adhesion and migration process, which are important stimulus factors in the development of some cancers.

Positive feedback loop regulation of PREX1 and TGFβ1 in gastric cancer

PREX1 was closely involved in the TGFβ1 signaling pathway, as described above. In this study, we further confirm the interaction between PREX1 and TGFβ1. As shown in Figure 5A and B, recombinant human TGFβ1 protein treatment could induce PREX1 mRNA levels in SGC-7901 and BGC-823 cell lines. The protein expression was evaluated by western blotting, and the result showed recombinant human TGFβ1 protein could remarkably increase PREX1 protein expression (Figure 5C). Feedback regulation is an essential pattern in the signaling transduction. As TGFβ1 can induce PREX1 expression, we hypothesized that PREX1 might affect the TGFβ1 pathway. To confirm the hypothesis, SGC-7901 and BGC-823 cells were overexpressed with PREX1, and we found that overexpression of PREX1 could activate the TGFβ1 pathway (Figure 5D and E). Furthermore, a Smad3-luciferase reporter (Luc-PT3) was utilized to study the effect of PREX1 on the TGFβ downstream signaling pathway. As shown in Figure 5F, overexpression of PREX1 could enhance TGFβ1-induced Smad3 luciferase activity. Hence, PREX1 has a feedback loop with TGFβ1 pathway.

Overexpression of PREX1 could promote migration and invasion of gastric cancer cells

PREX1 was overexpressed in gastric cancer, PREX1 was closely involved in the

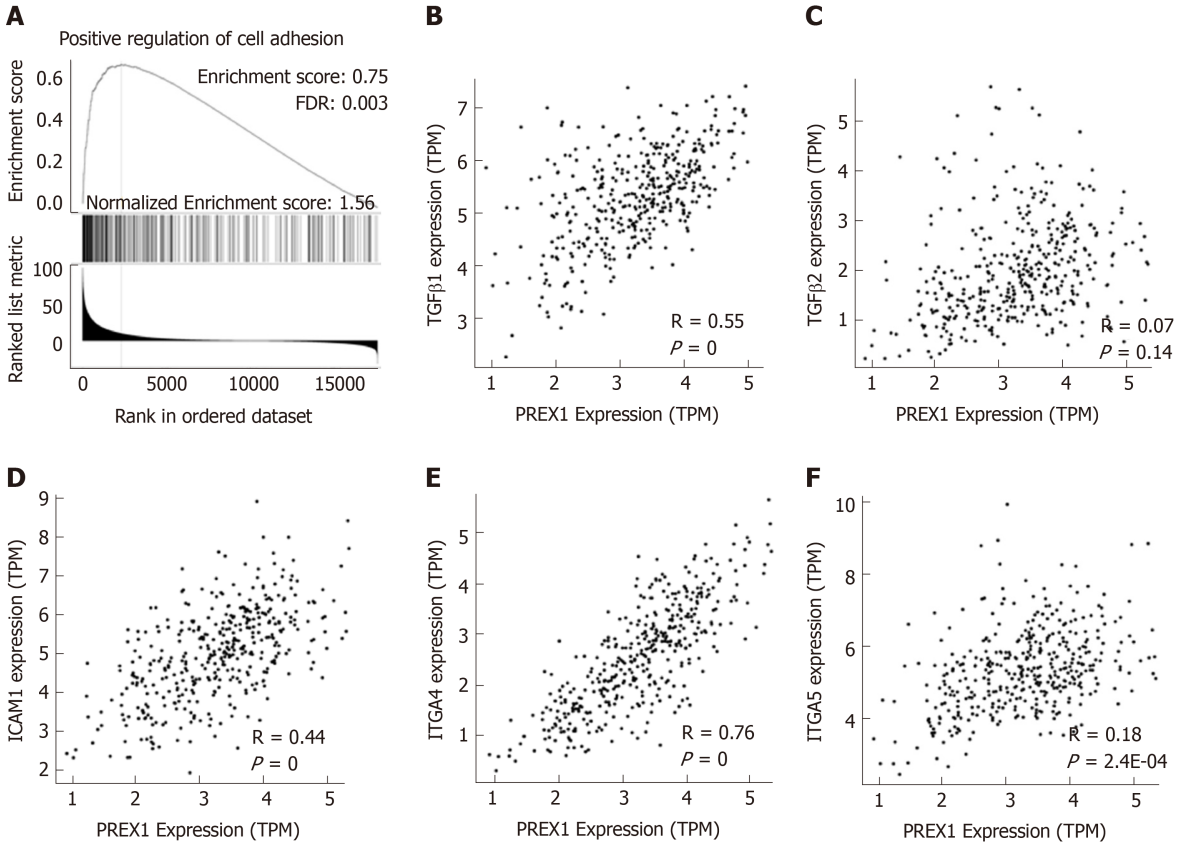

Figure 4 Phosphatidylinositol-3,4,5-trisphosphate dependent Rac exchange factor 1 was closely associated with the regulation of cell adhesion. A: The Gene Set Enrichment Analysis analysis of phosphatidylinositol-3,4,5-trisphosphate dependent Rac exchange factor 1 (PREX1) and co-expression network in gastric cancer; B: The correlation between PREX1 and transforming growth factor β1 were conducted in gastric cancer; C: The correlation between PREX1 and transforming growth factor β2 were conducted in gastric cancer; D: The correlation between PREX1 and intercellular adhesion molecule-1 were conducted in gastric cancer; E: The correlation between PREX1 and integrin alpha 4 were conducted in gastric cancer; F: The correlation between PREX1 and integrin alpha 5 were conducted in gastric cancer. TPM: Transcript per million; FDR: False discovery rate; NES: Normalized enrichment score; TGFβ1: Transforming growth factor β1; TGFβ2: Transforming growth factor β2; ICAM1: Intercellular adhesion molecule-1; ITGA4: Integrin alpha 4; ITGA5: Integrin alpha 5; PREX1: Phosphatidylinositol-3,4,5-trisphosphate dependent Rac exchange factor 1.

biological process of cell adhesion, and a mechanism study revealed that PREX1 has a feedback loop with the TGFβ1 pathway, which has been widely reported as a promoter in cancer metastasis[34]. Therefore, we examined the effect of PREX1 in the migration and invasion of gastric cancer cells. As Figure 6A and 6B show, when PREX1 is overexpressed with lentivirus particle in SGC-7901 cells, the migration rate was increased compared with lentivirus vector control. Furthermore, the Transwell assay assessed that overexpression of PREX1 enhances invasion in SGC-7901 cells. Taken together, these results revealed that PREX1 was closely associated with TGFβ1 and regulated the metastasis process of gastric cancer cells.

DISCUSSION

The incidence and mortality of gastric cancer continue to rise in recent years, and the current clinical treatment of gastric cancer mainly depends on surgery, chemotherapy, and targeted therapy[35]. As targeted therapy can be directly applied to tumors, compared with surgery, radiotherapy, and chemotherapy, its side effects are relatively small[35,36]. Targeted therapeutic therapies for gastric cancer are mainly targeted to epidermal growth factor receptor and vascular endothelial growth factor receptor (VEGFR)[37]. However, more and more studies have confirmed the presence of epidermal growth factor receptor and vascular endothelial growth factor receptor mutations in gastric cancer patients, resulting in the failure of targeted therapy[38]. Therefore, the development of new targets is of great significance for the treatment of gastric cancer.

PREX1 has been reported to be overexpressed in some cancers, including breast, prostate, ovarian, and thyroid cancer, and PREX1 expression is closely associated with cancer development[18,39]. Furthermore, some studies about the mechanism regulation

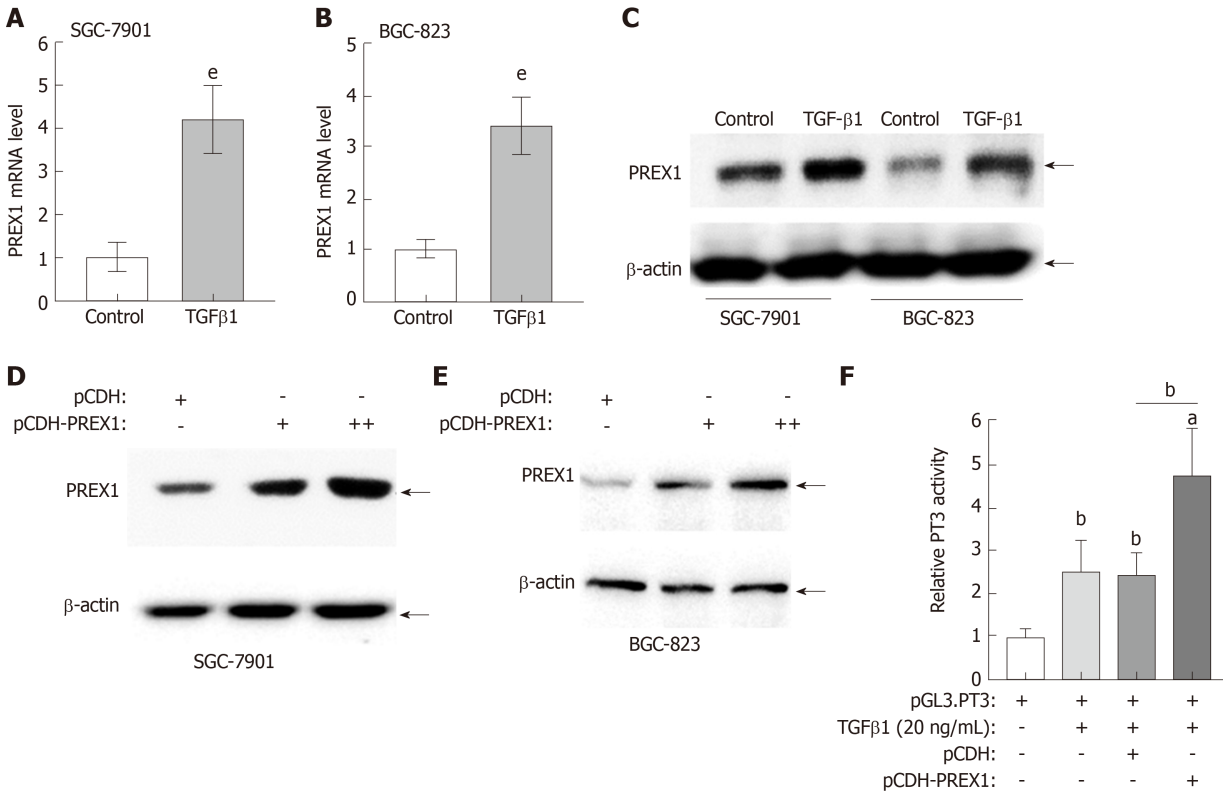

Figure 5 Feedback regulation between phosphatidylinositol-3,4,5-trisphosphate dependent Rac exchange factor 1 and transforming growth factor β1 in gastric cancer cells. A and B: SGC-7901 cells and BGC-823 cells were treated with recombinant human transforming growth factor (TGF) β1 protein at the concentration of 20 ng/mL for 12 h, the mRNA level was assessed by reverse transcriptase quantitative polymerase chain reaction; C: SGC-7901 and BGC-823 cells were treated with recombinant human TGFβ1 protein at the concentration of 20 ng/mL for 12 h, the phosphatidylinositol-3,4,5-trisphosphate dependent Rac exchange factor 1 (PREX1) protein was examined by western blotting; D and E: SGC-7901 and BGC-823 cells were cultured with lentivirus of PREX1 overexpressing particle in dose concentration and control for 24 h, the cells were subjected to western blotting to evaluate the activation of TGFβ1; F: HEK293T cells were transfected with Luc-PT3 and Rellina plasmids, the culture medium was supplemented with lentivirus particle of PREX1 overexpression and vector control. For another 24 h culture, the cells were treated with recombinant TGFβ1 (20 ng/mL) for 6 h, then the cells were subjected to dual-luciferase reporter assay. Statistical significance is expressed as $^{a}P < 0.05$, $^{b}P < 0.01$, $^{e}P < 0.001$. PREX1: Phosphatidylinositol-3,4,5-trisphosphate dependent Rac exchange factor 1; TGFβ1: Transforming growth factor β1.

of PREX1 revealed that PREX1 could activate phosphatidylinositol 3-kinase signaling pathway, a cancer facilitating factor[18]. However, the study of PREX1 in gastric cancer remains unclear and needs further elaboration. In this study, we firstly identified that PREX1 is highly expressed in different types of gastric cancer (Figure 1) and that PREX1 expression is positively correlated with progression of gastric cancer (Figure 2), suggesting that PREX1 is a potential target for gastric cancer. To understand fully the significance of PREX1 in the development of gastric cancer, the effect of PREX1 on the survival analysis was conducted, and high PREX1 expression in patients predicted poor prognosis (Figure 3A and B). Interestingly, the OS analysis in intestinal-type gastric cancer revealed PREX1 is a significant biomarker for the prediction of poor prognosis, but it is not applicable to diffuse-type gastric cancer (Figure 3C and D). As PREX1 was overexpressed in both diffuse-type and intestinal-type gastric cancer (Figure 1), PREX1 might be a specific molecule in the heterogeneity of gastric cancer. Based on these data, we believe that PREX1 may be a novel target for gastric cancer therapy, especially in the intestinal-type gastric cancer.

PREX1 was reportedly involved in the regulation of some signaling pathways[39]. For example, PREX1 could activate phosphatidylinositol 3-kinase/AKT and MEK/ERK pathways to promote the development of breast cancer[18], and PREX1 could also interact with protein kinase A and cooperate with platelet-derived growth factor receptor β in the regulation of cell migration[14,40]. As PREX1 is an important regulator in the development of gastric cancer, the mechanism study of PREX1 in gastric cancer showed that PREX1 is closely involved with regulation of cell adhesion (Figure 4). Further evaluation represents that PREX1 has a positive feedback loop with TGFβ pathway (Figure 5), which is an essential factor in the development of some cancers, especially in the promotion of metastasis. Indeed, the metastasis promoting activity of PREX1 was also confirmed in the wound healing and Transwell assay (Figure 6). In summary, this study fully evaluated the overexpression of PREX1 in gastric cancer and demonstrated that PREX1 expression could act as a poor

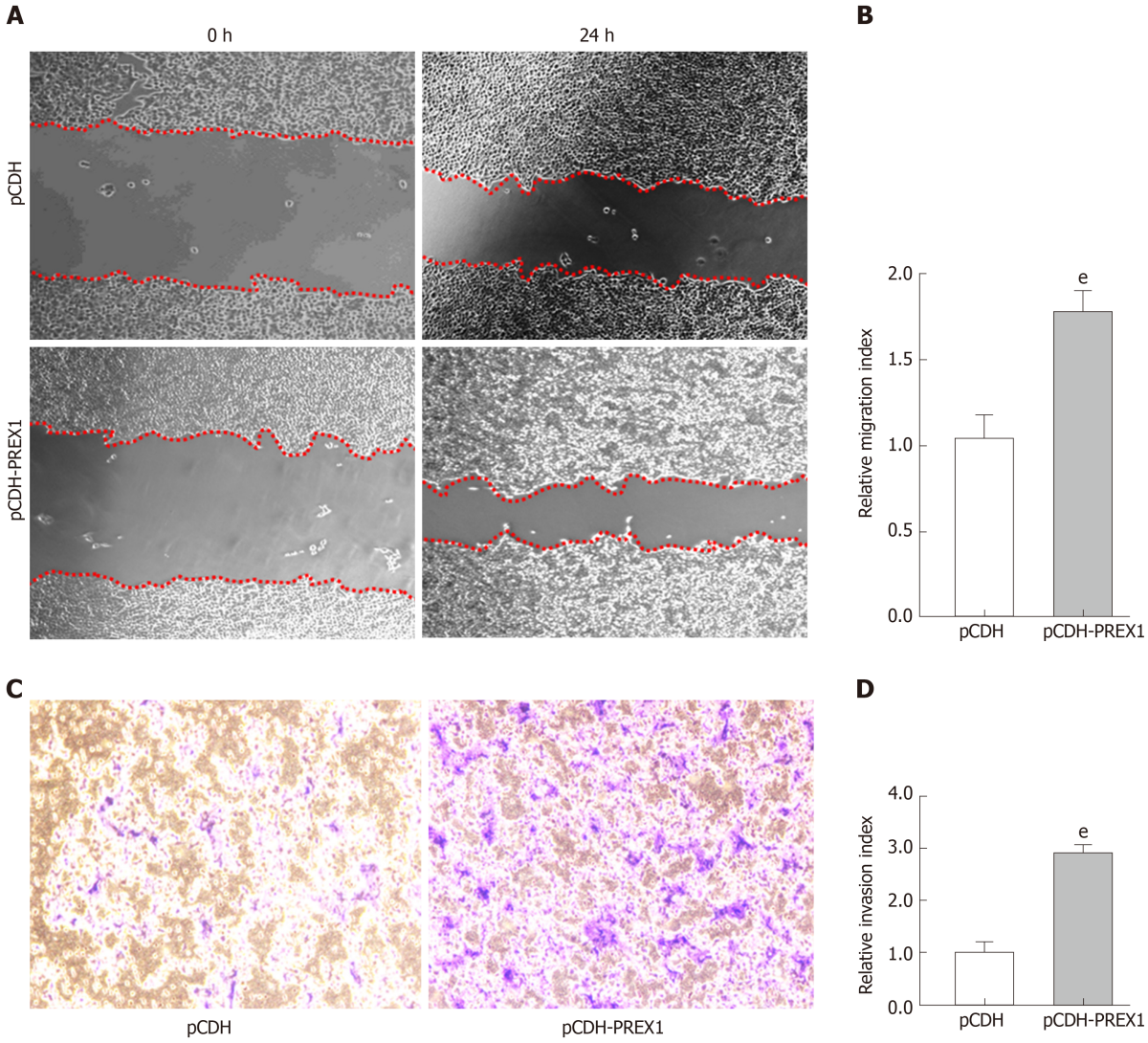

Figure 6 Overexpression of phosphatidylinositol-3,4,5-trisphosphate dependent Rac exchange factor 1 could promote the metastasis of gastric cancer cells. A: SGC-7901 cells were subjected to wound healing assay, and the cells were treated with phosphatidylinositol-3,4,5-trisphosphate dependent Rac exchange factor 1 overexpressing lentivirus and vector control lentivirus particles for 24 h; B: The relative migration index was calculated by the width of the wound scratch of panel A; C: Transwell assay was used to examine the effect of phosphatidylinositol-3,4,5-trisphosphate dependent Rac exchange factor 1 in the invasion of SGC-7901 cells; D: The relative invasion index was calculated by the number of invasive cells in three different fields. $^{e}P < 0.001$ *vs* pCDH. PREX1: Phosphatidylinositol-3,4,5-trisphosphate dependent Rac exchange factor 1.

prognosis predictor for the specific intestinal-type gastric cancer. PREX1 is a prompting factor in the metastasis of gastric cancer cells, and PREX1 might be a novel drug target and a valuable prognostic biomarker for gastric cancer.

ARTICLE HIGHLIGHTS

Research background

Phosphatidylinositol-3,4,5-trisphosphate dependent Rac exchange factor 1 (PREX1) is considered a pro-oncogene is some cancer types and is involved in some common cancer-promoting pathways, such as phosphatidylinositol 3-kinase/AKT and MEK/ERK. However, its expression and clinical value in gastric cancer has not been reported. Gastric cancer is a common high mortality disease, and identifying a novel therapeutic target could be valuable.

Research motivation

The goal was to confirm the expression of PREX1 in gastric cancer tissues and to correlate PREX1 level and disease development. The potential mechanism of PREX1 in the regulation of transforming growth factor (TGF) β pathway was also examined.

Research objectives

To evaluate the level and clinical value of PREX1 in the gastric cancer and to determine the potential mechanism in the regulation of gastric cancer metastasis.

Research methods
The Cancer Genome Atlas and *Oncomine* portal were used to test the level of PREX1 in gastric cancer tissues. The Kaplan Meier was utilized to evaluate the survival rate in high and low PREX1 expressing patient groups, including the examination of overall survival (OS) and post-progression survival (PPS). The Gene Set Enrichment Analysis was applied to screen the potential PREX1-mediated pathways. The correlation analysis was achieved in the cBioPortal for Cancer Genomics. The mRNA level of PREX1 was examined by reverse transcription quantitative polymerase chain reaction, and the protein level was evaluated by western blotting assay. The dual luciferase reporter assay was applied to test the activation of the TGFβ pathway. The effect of PREX1 in the regulation of metastasis in gastric cancer was assessed by wound healing and Transwell assays.

Research results
PREX1 was highly expressed in gastric cancer tissues, and the expression level of PREX1 was positively correlated with patient stage, tumor grade, and lymph node metastasis. Furthermore, the high PREX1 level patients showed lower survival rate in OS and PPS, and the difference in OS was only discovered in the intestinal-specific gastric cancer patients. PREX1 expression was also closely linked with the cell adhesion and TGF-β related regulators. TGF-β1 could induce PREX1 expression, and PREX1 could activate TGF-β pathway. Overexpression of PREX1 could enhance the migration and invasion activity *in vitro*.

Research conclusions
PREX1 is overexpressed in gastric cancer tissues and is involved in the development of gastric cancer. PREX1 could serve as a value prognostic biomarker in the prediction of OS and PPS. The mechanism study showed PREX1 is closely involved with the regulation of the cell adhesion process and TGF-β pathway in gastric cancer. PREX1 has a feedback regulation relationship with TGF-β and acts an enhancer in the regulation of metastasis in gastric cancer.

Research perspectives
In this study, we firstly identified that PREX1 was overexpressed in gastric cancer and involved in the development of disease. PREX1 could act as a valuable prognostic marker, and the feedback regulation between PREX1 and TGF-β signaling pathway might contribute to the metastasis of gastric cancer cells. In future work, the detailed regulation between TGF-β and PREX1 should be studied as well as whether PREX1 could directly interact with TGF-β.

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ORIGINAL ARTICLE

Basic Study

Abdominal paracentesis drainage ameliorates myocardial injury in severe experimental pancreatitis rats through suppressing oxidative stress

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Author contributions: Wen Y, Sun HY and Tan Z contributed equally to this work; Tang LJ designed the experiments; Wen Y, Tan Z, Liu RH and Huang SQ performed the experiments; Wen Y, Sun HY and Tan Z analyzed the data; Chen GY and Hao Q prepared and finished all the figures; Wen Y and Tan Z drafted the manuscript; Tang LJ and Sun HY refined and edited the manuscript; Tang LJ supervised the study.

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Abstract

BACKGROUND

Abdominal paracentesis drainage (APD) is a safe and effective strategy for severe acute pancreatitis (SAP) patients. However, the effects of APD treatment on SAP-associated cardiac injury remain unknown.

AIM

To investigate the protective effects of APD on SAP-associated cardiac injury and the underlying mechanisms.

METHODS

SAP was induced by 5% sodium taurocholate retrograde injection in Sprague-Dawley rats. APD was performed by inserting a drainage tube with a vacuum ball into the lower right abdomen of the rats immediately after SAP induction. Morphological staining, serum amylase and inflammatory mediators, serum and ascites high mobility group box (HMGB) 1, cardiac-related enzymes indexes and cardiac function, oxidative stress markers and apoptosis and associated proteins were assessed in the myocardium in SAP rats. Nicotinamide adenine dinucleotide phosphate oxidase activity and mRNA and protein expression were also examined.

RESULTS

APD treatment improved cardiac morphological changes, inhibited cardiac dysfunction, decreased cardiac enzymes and reduced cardiomyocyte apoptosis, proapoptotic Bax and cleaved caspase-3 protein levels. APD significantly

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Conflict-of-interest statement: The authors declare that there is no conflict of interest related to this study.

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decreased serum levels of HMGB1, inhibited nicotinamide adenine dinucleotide phosphate oxidase expression and ultimately alleviated cardiac oxidative injury. Furthermore, the activation of cardiac nicotinamide adenine dinucleotide phosphate oxidase by pancreatitis-associated ascitic fluid intraperitoneal injection was effectively inhibited by adding anti-HMGB1 neutralizing antibody in rats with mild acute pancreatitis.

CONCLUSION
APD treatment could exert cardioprotective effects on SAP-associated cardiac injury through suppressing HMGB1-mediated oxidative stress, which may be a novel mechanism behind the effectiveness of APD on SAP.

Key words: Abdominal paracentesis drainage; Severe acute pancreatitis; Myocardial injury; High mobility group box 1; Nicotinamide adenine dinucleotide phosphate oxidase; Oxidative stress

Core tip: In the present study, we provided the first evidence that abdominal paracentesis drainage (APD) treatment exerts beneficial effects on severe acute pancreatitis-associated cardiac injury. Our key findings are that (1) APD treatment decreases the serum levels of cardiac enzymes and improves cardiac function; (2) APD treatment alleviates cardiac oxidative stress and accompanied cardiomyocyte apoptosis; and (3) The beneficial effects of APD treatment in ameliorating severe acute pancreatitis-associated cardiac injury are due to the inhibition of oxidative damage *via* downregulating high mobility group box 1-mediated nicotinamide adenine dinucleotide phosphate oxidase expression. Our data manifest that APD is a promising treatment in severe acute pancreatitis-associated cardiac injury.

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INTRODUCTION

Severe acute pancreatitis (SAP) is a fatal abdominal disease and usually complicated with multiple organ dysfunction syndrome[1]. SAP-associated cardiac injury (SACI) occurs in some patients, and cardiac decompensation even causes death[2-4]. SACI frequently exhibits cardiomyocyte hypoxia, apoptosis and hypertrophy and can even lead to death[4,5]. Recent studies have shown that the elevated levels of myocardial enzymes are associated with the severity and outcome of SAP[6]. Despite constant understanding of the pathogenesis of SAP and significant improvement in clinical management, the mortality rate remains high, and the incidence rate of related complications is still unacceptable. Therefore, it is necessary to develop novel treatment strategies for improving cardiac injury and outcomes in SAP patients.

In our previous clinical study, early abdominal paracentesis drainage (APD) effectively relieved or controlled the severity of SAP, and this treatment strategy was an important development and supplement for the minimally invasive step-up approach[7]. Through removal of pancreatitis-associated ascitic fluid (PAAF), APD exerts beneficial effects supported by a delay or avoidance of multiple organ failure, decreased mortality rate and no increase in infection in patients with SAP[8,9]. Experimental evidence also indicates the effectiveness of APD treatment on SAP-associated lung and intestinal mucosa injury in rats[10,11]. However, the effect of APD treatment on SACI and the potential underlying mechanisms are yet to be elucidated.

Recently, high mobility group box 1 (HMGB1), a DNA-binding intranuclear protein, has attracted increasing attention because of its vital role as a late mediator in lethal systemic inflammation[12,13]. HMGB1 is derived from active secretion by activated macrophages and passive secretion by necrotic and apoptotic cells. Extracellular HMGB1 can trigger a lethal inflammatory process and participate in the

development of multiple organ injury in SAP[14,15]. It has been reported that serum HMGB1 level is significantly elevated in patients with SAP on admission and is positively related to severity of SAP as well as organ dysfunction and infection during the clinical course[16]. In particular, the level of HMGB1 in PAAF is higher compared with that in serum in experimental SAP, indicating that HMGB1 is first produced and released by the pancreas and peritoneal macrophages/monocytes during SAP[17]. In addition, HMGB1 plays an important role in the pathogenesis of cardiac dysfunction in many diseases. Active neutralization with anti-HMGB1 antibodies or HMGB1-specific blockage *via* box A could prevent cardiac dysfunction in mice with ischemia-reperfusion injury[18], sepsis[19] and diabetic cardiomyopathy[20]. Therefore, we propose that APD treatment by draining PAAF may decrease the level of HMGB1 in serum, thereby exerting a protective role in SACI.

The role of oxidative injury in the development of SACI has been demonstrated[4,21]. It is suggested that HMGB1 causes increased production of reactive oxygen species (ROS) through activation of nicotinamide adenine dinucleotide phosphate oxidase oxidases (NOXs)[22]. NOX acts as a major source of ROS in the heart in pathological conditions[23]. Our recent study has confirmed that NOX hyperactivity contributes to cardiac dysfunction and apoptosis in rats with SAP[24]. Despite these advances, whether APD influences oxidative stress *via* HMGB1-mediated cardiac NOX is not known.

Based on these findings, we hypothesized that APD treatment could protect rats from cardiac injury induced by SAP *via* antioxidative stress, through inhibiting HMGB1/NOX signaling. To test this hypothesis, we systematically investigated the role of APD treatment in SACI and determined whether HMGB1 plays a pivotal role during this treatment process.

MATERIALS AND METHODS

Reagents

The sodium taurocholate was purchased from Sigma-Aldrich (Merck KGaA, Darmstadt, Germany). The dihydroethidium (DHE) was purchased from Beyotime Institute of Biotechnology (Haimen, China). The antibody for HMGB1 (BM3965) was purchased from Boster Biological Technology, Inc. (Wuhan, China). The antibody specific for apoptosis regulator Bax (cat. No. ab32503), NOX2/gp91phox (cat. No. ab31092) and NOX4 (cat. No. ab133303) were purchased from Abcam (Cambridge, United Kingdom). The antibody for caspase-3 (cat. No. 9662S) was purchased from Cell Signaling Technology, Inc. (Danvers, MA, United States). The antibody for apoptosis regulator protein Bcl-2 (cat. No. GTX100064) was purchased from GeneTex Inc. (Irvine, CA, United States). All other chemicals used in this study were of analytical grade and were commercially available.

Animals and experimental design

Adult male Sprague-Dawley rats (weight, 200-220 g) were used in this study. The animals were purchased from Chengdu Dossy Animal Science and Technology Co. Ltd. (Chengdu, China). They were kept separately in an individually ventilated cage system maintained at 23 °C with a 12-h light/dark cycle and fed with standard laboratory food and water *ad libitum* for 3 d prior to the experiments. The animals were fasted overnight prior to the experiment but had free access to water. The experimental procedures were approved by the Institutional Animal Care and Use Committee at the Chengdu Military General Hospital and were conducted in accordance with the established International Guiding Principles for Animal Research.

In the first series of experiments, 45 rats were randomly divided into three groups of 15: sham operation, SAP and SAP + APD. The rats were anesthetized with 5% isoflurane (*via* an induction box) prior to surgery. The SAP model was induced by a standardized pressure-controlled retrograde infusion of 5% sodium taurocholate into the biliopancreatic duct at a rate of 12 mL/h using a micro-infusion pump (0.13 mL/100 g body weight); the microvascular clamp and puncture needle were removed after 5 min. The abdomen was closed according to the classical method of Chen *et al*[25] with modifications. In the SAP + APD group, a drainage tube connected to a negative-pressure ball device was inserted into the lower right abdomen immediately following SAP induction (Figure 1). Following the operation, all rats received 10 mL/200 g body weight of sterile saline by subcutaneous injection in the back to compensate for anticipated fluid loss. For the sham operation group, an incision was made in the abdomen and was subsequently closed. The animals were denied access to food or water for 24 h after the procedure.

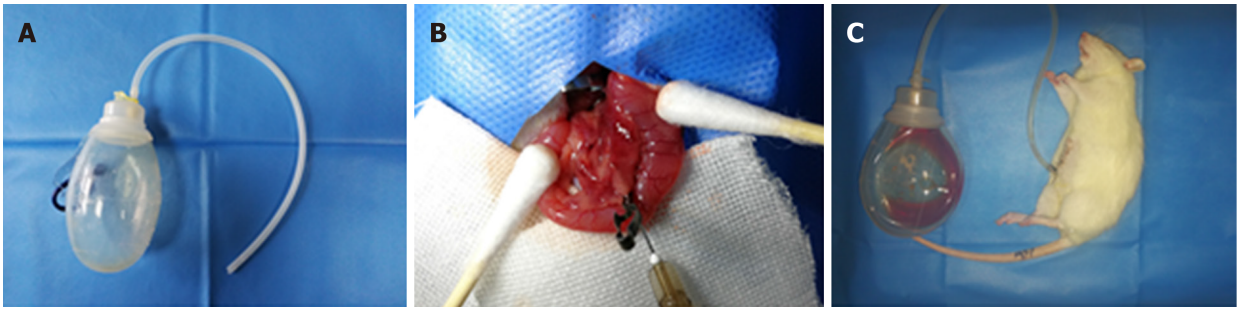


Figure 1 Abdominal paracentesis drainage in rats with severe acute pancreatitis. A: Abdominal paracentesis drainage device; B: Sodium taurocholate (5%) was injected into the biliopancreatic duct using a micro infusion pump; C: Ascitic fluid drained by abdominal paracentesis drainage.

The second experiment was designed to determine whether cardiac NOX activated by HMGB1 was involved in the beneficial effects of APD treatment. Another 36 rats with mild acute pancreatitis (MAP) were used. MAP was induced by six consecutive intraperitoneal injections of cerulein (20 μg/kg at 1-h intervals) as previously described[21]. Fresh PAAF was aseptically obtained from the abdominal cavity of rats with SAP in the first experiment, and the sterile supernatant was collected after centrifuged at 4000 x *g* for 15 min at 4 °C. The rats with MAP were randomly divided into the following six groups of six: (1) Controls: rats were intraperitoneally injected with 8 mL saline solution; (2) PAAF injection (PI): rats were intraperitoneally injected with 8 ml of PAAF; (3) PAAF + anti-HMGB1 neutralizing antibody 50 μg (PIH 50): rats were intraperitoneally injected with 8 mL PAAF and 50 μg anti-HMGB1 neutralizing antibody; (4) PAAF + anti-HMGB1 neutralizing antibody 100 μg (PIH 100): rats were intraperitoneally injected with 8 mL PAAF and 100 μg anti-HMGB1 neutralizing antibody; (5) PAAF + anti-HMGB1 neutralizing antibody 200 μg (PIH 200): rats were intraperitoneally injected with 8 mL PAAF and 200 μg anti-HMGB1 neutralizing antibody; and (6) PAAF + control IgY 200 μg: rats were intraperitoneally injected with 8 mL PAAF and 200 μg control IgY. Nonimmune chicken IgY (Boster Biological Technology, Wuhan, China) acted as a control antibody for HMGB1 neutralization. Anti-HMGB1 neutralizing antibody (rabbit anti-HMGB1 monoclonal antibody) recognized rat HMGB1. The specificity and neutralizing activity of this antibody was confirmed by western blotting. PAAF and anti-HMGB1 neutralizing antibody were previously incubated at 36 °C for 30 min. Eight hours after the injections, rats were anesthetized, and blood and heart tissues were collected, immediately frozen and stored at -80 °C until use.

Echocardiography and blood pressure analysis

At 24 h after induction of SAP, all rats were anesthetized by 5% isoflurane, and echocardiography was performed using LOGIQ E9 ultrasound apparatus (GE, Boston, MA, United States) equipped with a 12-MHz transducer. Heart rate, left ventricular end-diastolic dimension and left ventricular end-systolic dimension were measured, and left ventricular ejection fraction and fractional shortening were calculated from M-mode recordings. Measurements were analyzed by a blinded observer, and all the results were averaged from five consecutive cardiac cycles measuring from the M-mode images. Following the measurements, blood pressure was assessed using the AD Instruments PowerLab system (Bella Vista, NSW, Australia). In the process of this measurement, a microtip catheter transducer (22G IV cannula; ShiFeng, Inc., Chengdu, China) was gradually inserted 2 cm into the right carotid artery. The signals were continuously recorded, and systolic and diastolic pressure were processed using LabChart 7 (version 7.3.7) analytical software. After completion of all the measurements, under strict aseptic conditions, PAAF was obtained from the abdominal cavity of rats with SAP, blood samples were collected from the ventral aorta, serum was obtained by centrifugation at 3000 rpm for 15 min at 4 °C and an appropriate number of aliquots were separated and stored at -80 °C until use. The pancreas and heart were quickly removed, and part of the pancreas and left ventricle were fixed in 4% paraformaldehyde or flash-frozen in liquid nitrogen until use.

Histological assessment

Twenty-four hours after SAP induction, the terminal pancreas and heart tissue samples were paraformaldehyde-fixed, paraffin-embedded and sectioned at 4 μm. The sections were then stained with hematoxylin and eosin. The slides were read by a consultant histopathologist blinded to the groups under Leica DM 3000 light microscope (Leica Microsystems GmbH, Wetzlar, Germany) with a digital

photography system (Leica application suite, version 4.4.0). For the heart, we assessed interstitial edema, hemorrhage, neutrophil infiltration and contraction band necrosis[26]. For the pancreas, the severity of pancreatic injury was evaluated based on a 0-4 scoring method, the scores of several parameters including edema, fat necrosis, hemorrhage, inflammatory cell infiltrate and acinar necrosis[27].

Tissue edema index

The heart was placed in a preweighed plastic bag, weighed again (wet), then samples were oven-dried at 57 °C for 48 h and reweighed (dry). The edema index was calculated as: Edema Index = Weight (wet)/Weight (dry).

Biochemical analysis

Serum HMGB1, TNF-α, endotoxin, IL-1β, creatine kinase-MB, cardiac troponin-I and HMGB1 in PAAF were detected using an enzyme linked immunosorbent assay kit (Nanjing Jiancheng Bioengineering Institute, China) according to the manufacturer's protocol. The serum levels of amylase and lactate dehydrogenase were measured according to the manufacturer's instructions using an automatic biochemistry analyzer (TC6010L; Tecom Science Corporation, Jiangxi, China).

Measurement of oxidative stress and NOX activity

To determine the degree of oxidative stress in the heart, lipid peroxidation (LPO), superoxide dismutase (SOD) and reduced glutathione (GSH) were measured in heart homogenates. Heart tissue (0.1 g) was collected and homogenized in ice-cold normal saline (weight:volume = 1:9). The homogenates used for the determination of SOD activities and LPO and GSH levels were centrifuged at 3000 rpm for 10 min at 4 °C, and the supernatants were collected. SOD activity and LPO and GSH levels were measured with test kits (Nanjing Jiancheng Bioengineering Institute, China). To detect myocardial NOX activity, 0.5 g heart tissue was collected, washed with reagent buffer and then placed in cryogenic vials to stay overnight in liquid nitrogen. The next day, we ground the tissue into powder and added lysis buffer for 30 min under ice-cold conditions. After centrifugation at 10000 *g* for 10 min at 4 °C, the supernatants were collected. NOX activity in the samples was measured with test kits (Nanjing Jiancheng Bioengineering Institute). Protein quantification was performed using the Bradford method.

Detection of ROS generation by DHE fluorescence staining

ROS can oxidate DHE, which forms ethidium bromide. This will intercalate DNA, which will emit red fluorescence. Frozen rat heart tissues were analyzed using DHE. Myocardium cross-sections (10 μm) were incubated with DHE (5 μmol/L) in PBS in a light-protected incubator at 37 °C for 30 min. The sections were washed three times with PBS to remove excess DHE. Red fluorescence was assessed by fluorescence microscope (IX81; Olympus, Tokyo, Japan) with green light. The ROS content increased in proportion to the intensity of red fluorescence. Quantitative analysis of fluorescent images was performed with Image J (NIH, United States) software and expressed as arbitrary units of fluorescence.

Transferase-mediated dUTP nick end labeling assay

Myocardium frozen sections (10 μm) were used to detect the myocardial cells apoptosis with a terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling assay kit (Fluorescein in situ cell death detection kit; Boster biological Technology Co., Ltd., Wuhan, China). According to the manufacturer's instructions, all of the cells showed blue nuclear DAPI staining, but the terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling positive cells display green nuclear staining. The stained slices were analyzed by laser-scanning confocal microscopy (Eclipse ti2; Nikon, Tokyo, Japan).

Fluorescent quantitative real-time polymerase chain reaction

Total RNA was extracted from heart tissue using TRIzol reagent (Beyotime Biotechnology, Shanghai, China), which is an RNA extraction kit. RNA purity and concentration were determined using the Thermo NanoDrop-2000 spectrophotometer (Thermo Fisher Scientific Inc., Waltham, MA, United States) at A260/A280 nm. The purity of RNA obtained was 1.8-2.0. The primers were synthesized by Sangon Biotech Co. Ltd. (Shanghai, China) (Table 1). Reverse transcription of RNA and PCR amplification were performed with One Step SYBR PrimeScript TM RT-PCR Kit II (Takara Biotechnology, Dalian, China) by C1000 TM Thermal Cycler (Bio-Rad, Hercules, CA, United States). The cycling program was as follows: 5 min at 42 °C, 10 s at 95 °C, followed by 40 cycles of 5 s at 95 °C and 30 s at 60 °C and then dissociation. The reactions were quantified according to the amplification cycles when the PCR

products of interest were first detected (threshold cycle, Ct). Each reaction was performed in triplicate. The expression of the transcripts was normalized to the levels of *β-actin* in the samples. Data were analyzed using CFX Manager TM Software 1.6 (Bio-Rad).

Western blotting

The proteins from the rat left-ventricular tissue or cells were extracted using a protein extraction kit (Nanjing Jiancheng Bioengineering Institute). The protein concentrations were measured using an enhanced BCA Protein Assay kit (Nanjing Jiancheng Bioengineering Institute). Equal amounts of protein for each sample were separated by SDS-PAGE in a minigel apparatus (MiniPROTEAN II; Bio-Rad). Then they were transferred to a 0.22- or 0.40-μm polyvinylidene difluoride membrane. Membranes were blocked with 5% nonfat milk in Tris-buffered saline/Tween 20 and were incubated overnight at 4 °C with anti-NOX2 (1:2000), anti-NOX4 (1: 5000), anti-Bax (1:2000), anti-Bcl-2 (1:1000), anti-caspase-3 (1:1000) and anti-GADPH (1:5000; loading control) antibodies. After incubation with horseradish-peroxidase-conjugated secondary antibody, Chemiluminescence Detection Reagent (Millipore, Billerica, MA, United States) was added drop-wise onto the membranes. Then the membranes were examined with the BioSpectrum4 apparatus (UVP, Upland, CA, United States).

Statistical analysis

All data are presented as mean ± SD and analyzed using SPSS version 18.0 (Chicago, IL, United States). Normally distributed data were compared using a one-way analysis of variance followed by the SNK test for multiple comparisons, and nonparametrically distributed variables were compared by the Mann-Whitney test with Bonferroni corrections. Values of $P < 0.05$ was recognized as statistically significant.

RESULTS

APD treatment alleviated cardiac injury induced by SAP in rats

To study the effect of APD treatment on cardiac injury in a sodium-taurocholate-induced SAP rat model, we examined cardiac histopathology, tissue edema index and cardiac-related enzymes. The sham group showed normal myocardial architecture, whereas SAP rats exhibited obvious structural and cellular changes, including myocardial degeneration, cellular edema and mononuclear infiltration (Figure 2A). This result was consistent with early changes in myocarditis demonstrated by the histology severity score and tissue edema index (Figure 2B and 2C). Compared with the SAP group, the cardiac lesion in the SAP + APD group was mild with normal structure, and the score and tissue edema index were lower than those in the SAP group. The serum levels of cardiac-related enzymes were sensitive and specific indexes to reflect cardiac lesions. Compared with the sham group, the serum levels of cardiac enzymes in the SAP groups increased significantly ($P < 0.01$) (Figure 2D-2F). However, the levels of cardiac enzymes in the SAP + APD group were significantly lower compared with those in the SAP group ($P < 0.01$), especially creatine kinase-MB and cardiac troponin-I, which are specific indexes of cardiac injury. These results suggested that APD treatment preserved histopathology and decreased the levels of cardiac-related enzymes.

APD treatment improved cardiac function in SAP rats

To evaluate the effect of APD treatment on cardiac functional abnormality caused by SAP, echocardiographic and hemodynamic changes were recorded 24 h after SAP challenge. Echocardiography was a sensitive indicator of cardiac function during SAP. Figure 3A-3F shows representative M-mode images and parameters from echocardiographic analysis. No significant differences were evidenced among the three groups in terms of left ventricular end-diastolic dimension. Compared with the sham group, increasing left ventricular end-systolic dimension was observed in SAP, but the increased level was attenuated by APD treatment. Heart rate, fractional shortening and ejection fraction decreased significantly after SAP challenge, but this was reversed with APD treatment. In the hemodynamic change evaluation, blood pressure was measured. Systolic and diastolic blood pressure were significantly reduced in the SAP group compared with the sham group (Figure 3G and 3H). APD treatment improved systolic and diastolic blood pressure at 24 h after SAP challenge. The results indicated that APD treatment attenuated cardiac function in SAP rats.

APD treatment alleviated oxidative stress in myocardium induced by SAP in rats

Given that NOX hyperactivity plays an important role in cardiac dysfunction in rats

Table 1 Sequences and conditions of primers used in reverse transcription-polymerase chain reaction

Gene		Primer sequences
NOX2	Forward	5′-GACCATTGCAAGTGAACACCC-3′
	Reverse	5′-AAATGAAGTGGACTCCACGCG-3′
NOX4	Forward	5′-TTCGCGGATCACAGAAGGTC-3′
	Reverse	5′-AAGTTCAGGGCGTTCACCAA-3′
β-actin	Forward	5′-ACGGTCAGGTCATCACTATCG-3′
	Reverse	5′-GGCATAGAGGTCTTTACGGATG-3′

NOX2: Nicotinamide adenine dinucleotide phosphate oxidase-2; NOX4: Nicotinamide adenine dinucleotide phosphate oxidase-4.

with SAP, we measured the effect of APD treatment on protein expression of NOX2 and NOX4 in myocardial tissue in SAP rats. Western blotting manifested that NOX2 and NOX4 in the myocardium were significantly increased in SAP compared with the sham group, in accordance with enhanced activity of NOX (Figure 4). APD treatment markedly decreased expression of NOX2 and NOX4. These results were further confirmed by assessing DHE oxidation in which a remarkable increase in ROS production was noted in myocardial tissue in SAP group compared with the sham group. APD treatment resulted in a decrease in ROS level in the sham group compared to the SAP group. Given that accumulated ROS can cause oxidative damage, we found an increase in LPO level and a decrease in SOD activity and GSH level in line with the observed changes in ROS content in the SAP group. After treatment with APD, the SOD activity and GSH level in the SAP + APD group were higher and the LPO level was lower than in the SAP group. These data suggest that APD treatment reduced ROS production and oxidative damage in the heart induced by SAP.

APD treatment reduced myocardial cell apoptosis and expression of apoptosis-related proteins

Given that the degree of myocardial apoptosis secondary to oxidative stress reflects the extent of myocardial injury, we used terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling to measure myocardial cell apoptosis. The apoptotic index was significantly increased in the SAP group (26.36 ± 4.54 *vs* 0.0 ± 0.0, $P < 0.01$) compared with the sham group (Figure 5A). APD treatment induced a significant decrease in apoptosis (6.35 ± 2.19 *vs* 26.36 ± 4.54, $P < 0.01$) in the sham group compared with the SAP group. Furthermore, apoptosis-associated protein expression was analyzed by western blotting. APD treatment downregulated the levels of proapoptotic Bax and cleaved caspase-3, and upregulated antiapoptotic Bcl-2 levels (Figure 5B-5E). These results confirmed that APD treatment relieved myocardial injury in SAP rats.

APD treatment attenuated pancreatic injury and decreased inflammatory indexes in SAP rats

Considering that the key feature of acute pancreatitis is damage to the pancreas, we evaluated the effect of APD treatment on pancreatic injury and inflammatory indexes in SAP and measured changes in pancreatic histopathology, amylase activity and levels of proinflammatory cytokines. For pancreatic histopathology, hematoxylin and eosin-stained sections of rat pancreas tissue were analyzed. The sham group exhibited normal pancreatic structures while the SAP group displayed notable morphological changes with large areas of tissue necrosis and inflammatory infiltration, supported by the histological severity scores (Figure 6A and 6B). Compared with the features of the SAP group, the pancreatic damage in the SAP + APD group was significantly alleviated with a lower severity score. Furthermore, as shown in Figure 6C-6F, the serum amylase activity and levels of IL-1β, TNF-α and endotoxin in the SAP group were significantly increased compared with those in the sham group ($P < 0.05$). APD treatment after SAP induction resulted in a marked decrease in the levels of these indexes ($P < 0.05$).

APD treatment decreased the levels of serum HMGB1 by removing PAAF

Given that HMGB1 acts as a key inflammatory mediator and plays an important role in the course of lethal systemic inflammatory response and distant organ injury, we

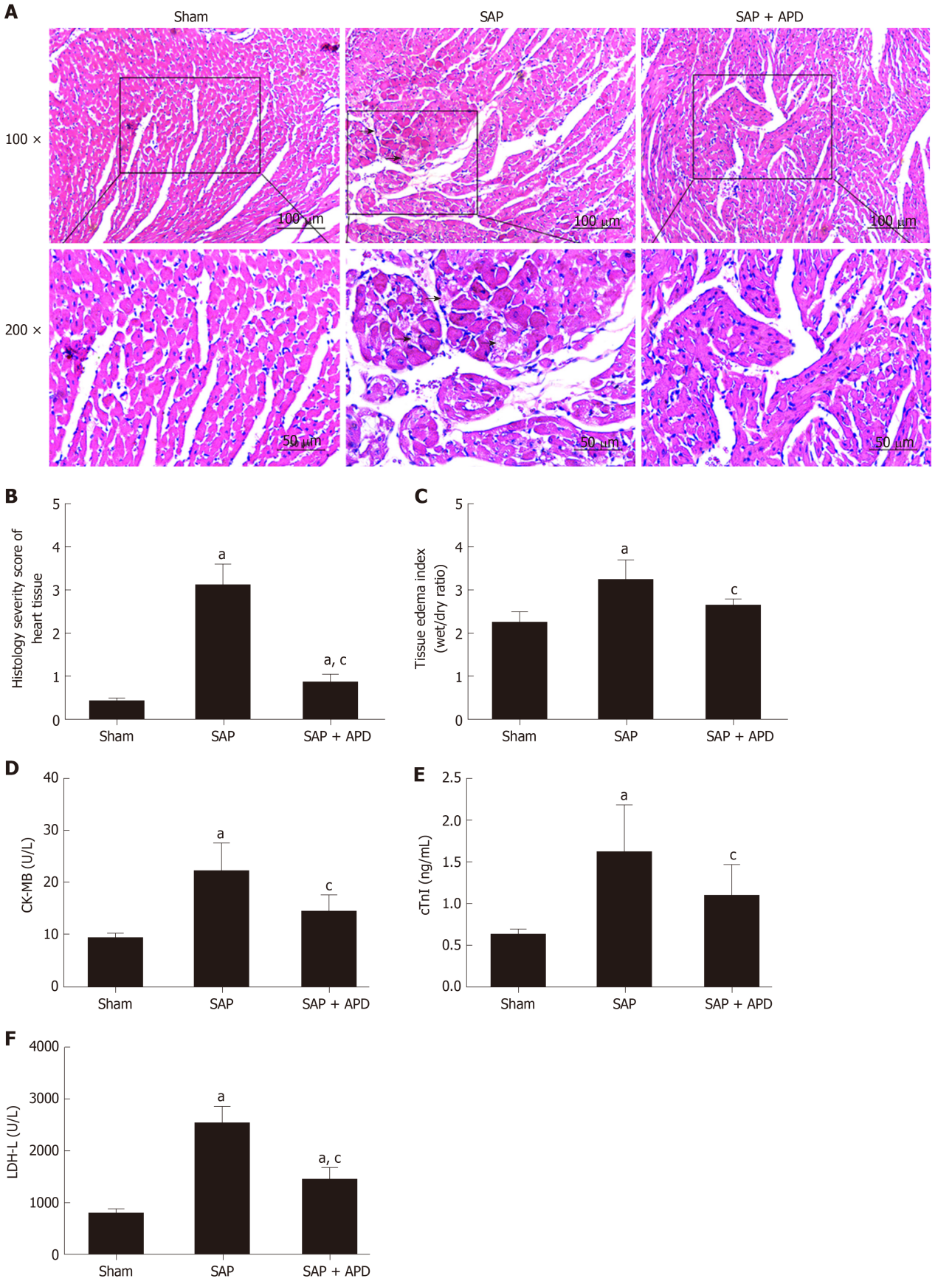
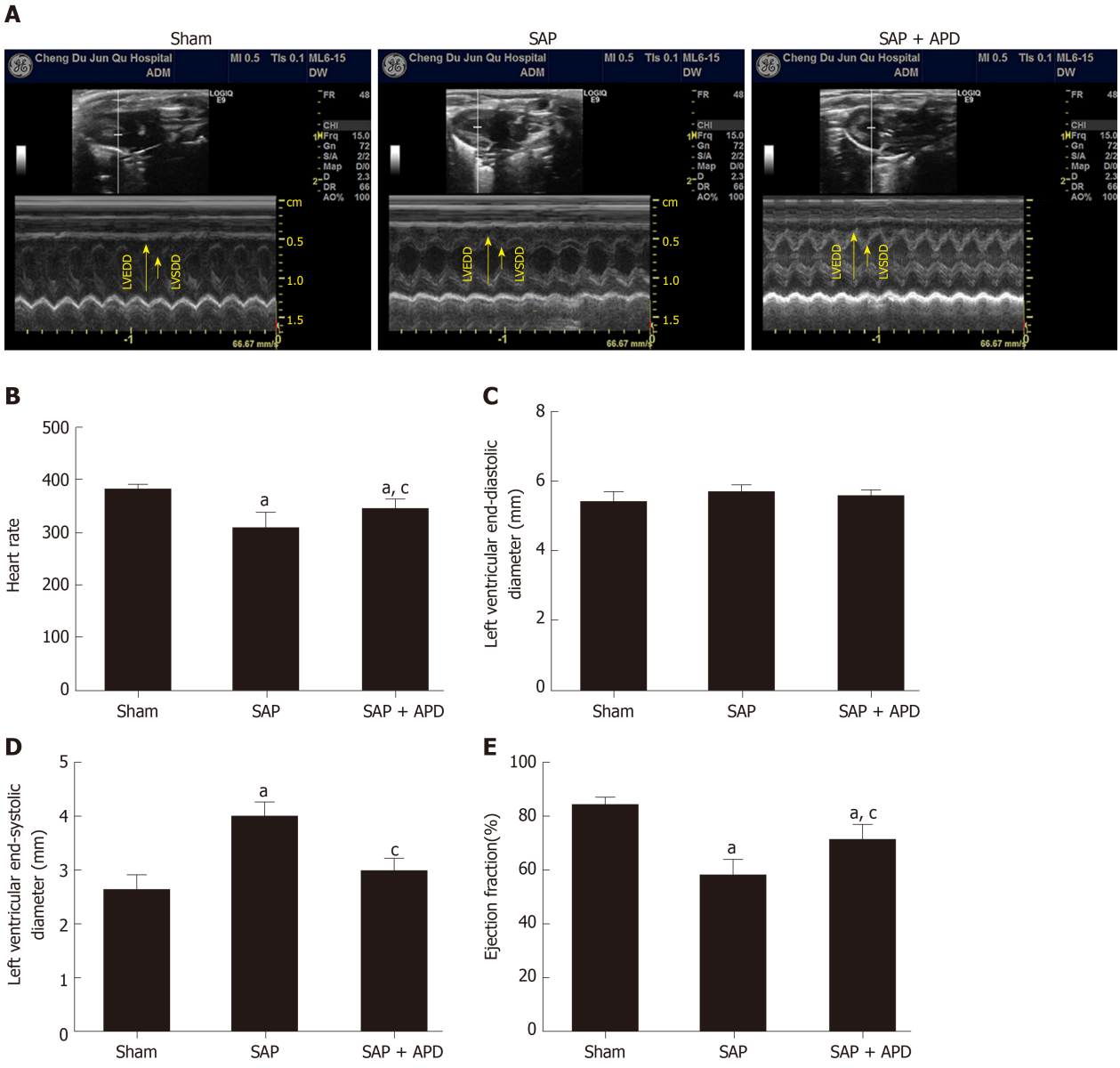


Figure 2 Effects of abdominal paracentesis drainage on cardiac histopathology, tissue edema index and cardiac-related enzymes 24 h after severe acute pancreatitis induction. A: Representative micrographs of hematoxylin-eosin stained sections of rat heart tissue from different groups. The arrow shows disruptive myocardial fibers. Images were taken under 100 × and 200 × magnification; B: Histology severity score of the heart; C: Tissue edema index of the heart; D-F: The serum levels of Creatine Kinase Isoenzyme MB, Cardiac troponin-I and Lactic Dehydrogenase-L, respectively. Data indicate the mean ± standard deviation obtained from six animals in each group (C-E). $^{a}P < 0.05$ *vs* sham group; $^{c}P < 0.05$ *vs* severe acute pancreatitis group. SAP: Severe acute pancreatitis; APD: Abdominal paracentesis drainage.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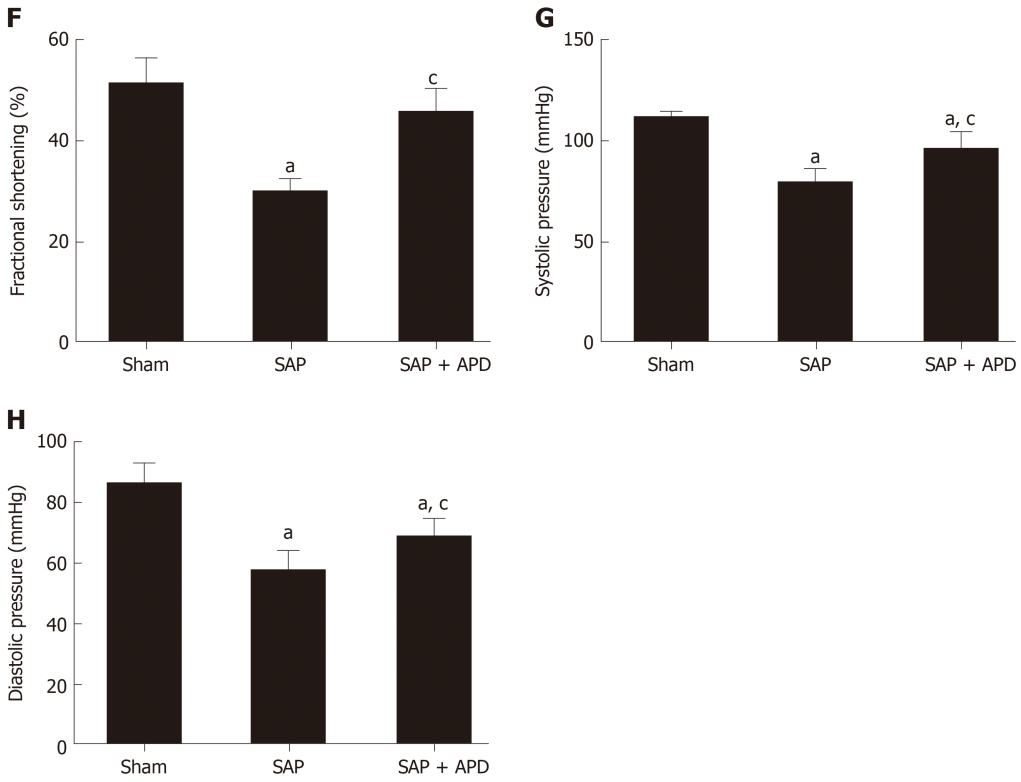


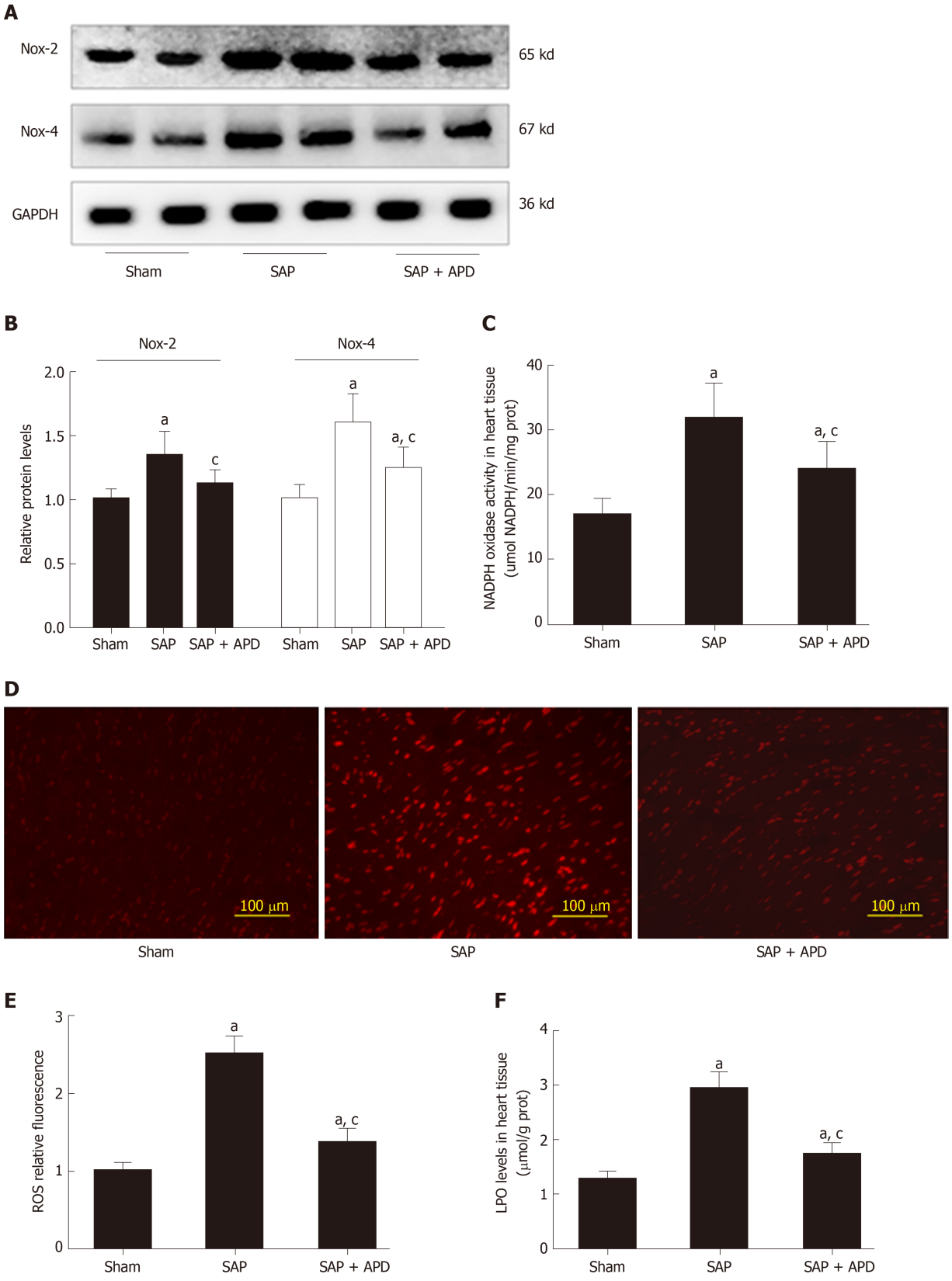
Figure 3 Effect of abdominal paracentesis drainage on echocardiographic and hemodynamic properties. A: Representative M-mode images in groups; B: Heart rate; C: Left ventricular end-diastolic diameter (mm); D: Left ventricular end-systolic diameter (mm); E: Ejection fraction (%); F: Fractional shortening (%); G: Systolic pressure (mmHg); H: Diastolic pressure (mmHg). Data indicate the mean ± standard deviation obtained from six animals in each group. $^{a}P < 0.05$ *vs* sham group; $^{c}P < 0.05$ *vs* severe acute pancreatitis group. SAP: Severe acute pancreatitis; APD: Abdominal paracentesis drainage.

compared the levels of HMGB1 in PAAF and serum samples with or without APD treatment. Twenty-four hours after SAP induction, we found that serum HMGB1 level was significantly higher than that in sham rats. HMGB1 level in PAAF was higher than that in serum (Figure 6G). The results were consistent with previous studies, suggesting that HMGB1 is produced and released by the pancreas and peritoneal macrophages/monocytes in response to inflammatory mediators during SAP. Because APD treatment is a strategy for removing PAAF directly, we predicted that it would reduce the circulating concentration of HMGB1. As expected, serum HMGB1 level in SAP + APD rats was markedly reduced compared with that in the SAP group (Figure 6G). These data demonstrated that APD treatment, by removing PAAF, significantly decreased the serum level of HMGB1 in SAP rats, suggesting that HMGB1 signaling is responsible for the cardioprotective effects of APD.

Decreased HMGB1 level by APD treatment resulted in reduced NOX expression

HMGB1 plays an important role in the pathogenesis of cardiac injury in many diseases; therefore, we determined whether HMGB1 in PAAF affected cardiac NOX expression. We intraperitoneally injected PAAF or PAAF + anti-HMGB1 neutralizing antibody (PI and PIH groups, respectively) and investigated the expression of NOX in heart samples from rats with MAP. We measured the serum level of HMGB1 after intraperitoneal injection of PAAF, with or without HMGB1 neutralizing antibody. The circulating level of HMGB1 in the PI group was significantly higher than that in the control group, suggesting that HMGB1 in PAAF could enter the bloodstream (Figure 7A). With the injection of HMGB1 neutralizing antibody into PAAF, we observed that when the dose was increased to 200 μg, the serum level of HMGB1 was significantly decreased compared with that in the PI group.

Next, we examined the influence of HMGB1 in PAAF on mRNA and protein expression of NOX2 and NOX4 from the heart (Figure 7B). Eight hours after injection, the mRNA and protein expression of NOX2 was significantly increased in the PI group compared with the control group, while only *NOX4* mRNA expression was affected. The effects on expression in the PAAF + control IgY 200 μg group were similar to those in the PI group. In the 200 μg HMGB1 neutralizing antibody group, mRNA expression of *NOX2* and *NOX4* and protein expression of NOX2 was reduced compared with that in the PI group. These results demonstrated that high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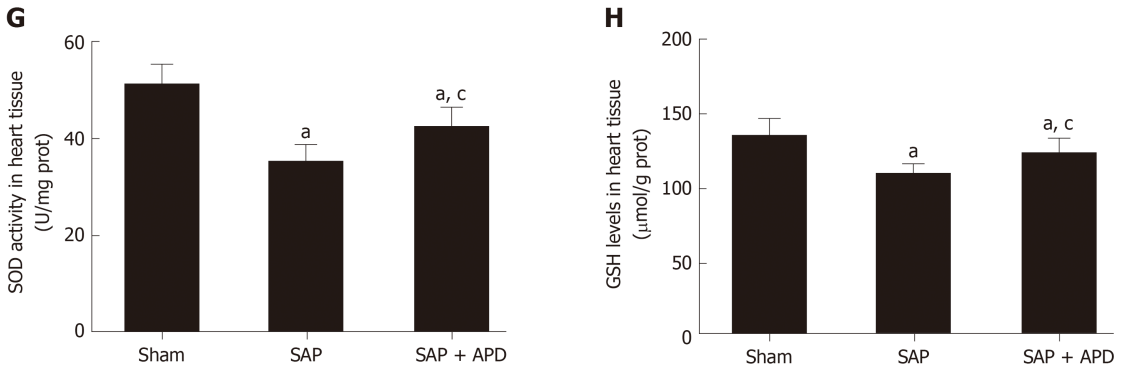


Figure 4 Effect of abdominal paracentesis drainage on the protein expression and activity of nicotinamide adenine dinucleotide phosphate oxidase, reactive oxygen species production and oxidative indexes. A: Immunoblot of nicotinamide adenine dinucleotide phosphate oxidase-2 (NOX2) and NOX4 protein expression from heart samples; B: Densitometry analysis of NOX2 and NOX4; C: NOX activity was measured by the colorimetric method in heart tissues; D: Dihydroethidium staining in frozen sections by fluorescent microscopy (100 × magnification); E: The fluorescence values are expressed as the ratio to the levels in the sham group; F-H: The levels of lipid peroxidation, the activity of superoxide dismutase and reduced glutathione in heart tissues, respectively. Data are representative of at least three independent experiments (A-B). Data indicate the mean ± standard deviation obtained from six animals in each group (C-H). $^{a}P < 0.05$ *vs* sham group; $^{c}P < 0.05$ *vs* severe acute pancreatitis group. NOX2: Nicotinamide adenine dinucleotide phosphate oxidase-2; NOX4: Nicotinamide adenine dinucleotide phosphate oxidase-4; GAPDH: Glyceraldehyde-3-phosphate dehydrogenase; SAP: Severe acute pancreatitis; APD: Abdominal paracentesis drainage; ROS: Reactive oxygen species; LPO: Lipid peroxidation; SOD: Superoxide dismutase; GSH: Reduced glutathione.

concentration of HMGB1 in PAAF could lead to NOX overexpression, and the decreased HMGB1 level caused by APD treatment results in reduced NOX expression.

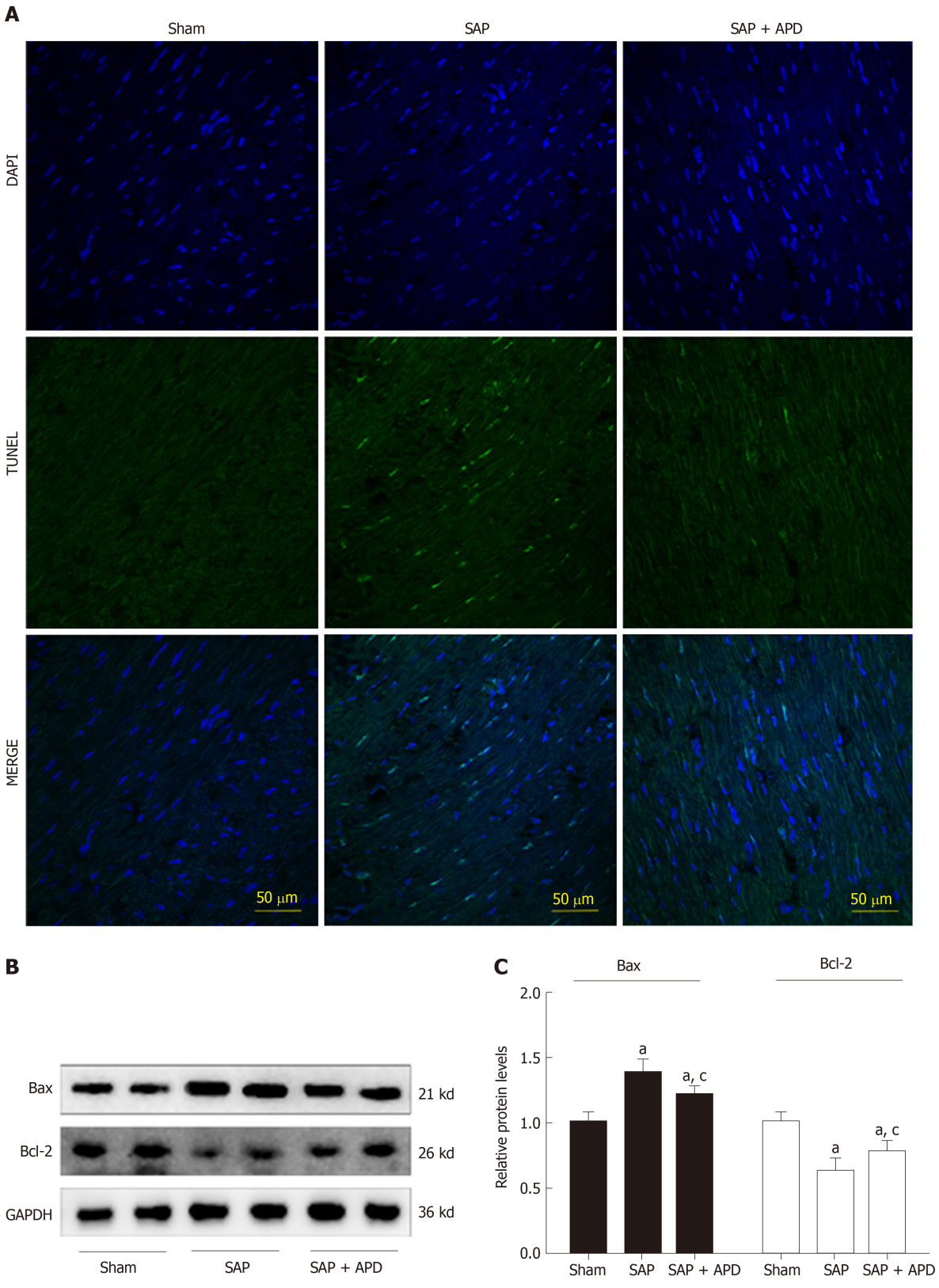
Based on the above findings, we propose a schematic diagram for the possible mechanism behind the effect of APD on SACI (Figure 8). Once SAP occurs, HMGB1 is mainly released from injured pancreas and peritoneal macrophages/monocytes. High levels of HMGB1 in the bloodstream can trigger a lethal inflammatory process and participate in the development of remote organ injury in SAP. Following APD treatment, the levels of HMGB1 in the circulation decreased significantly, resulting in inhibition of expression and activity of cardiac NOX, which can effectively alleviate apoptosis *via* downregulating ROS production and thereby protecting against SACI.

DISCUSSION

In the present study, we provided the first evidence that APD treatment exerts a beneficial effect on SACI. Our key findings were that: (1) APD treatment decreases the serum levels of cardiac enzymes and improves cardiac function; (2) APD treatment alleviates cardiac oxidative stress and accompanied cardiomyocyte apoptosis; and (3) the beneficial effects of APD treatment in ameliorating SACI are due to the inhibition of oxidative damage *via* downregulating HMGB1-mediated expression of NOX. Our data show that APD is a promising treatment in SACI.

SAP is a dangerous and lethal acute abdominal disease. PAAF is a common local complication and is important in the progression of systemic inflammatory response during SAP[28]. PAAF causes elevated intra-abdominal pressure and aggravates abdominal viscus injuries. Moreover, the toxic substances in PAAF can be reabsorbed into the circulation to amplify the systemic inflammatory response and induce distant organ injury[28]. Therefore, any strategies or methods to remove PAAF may be effective in treating SAP and its related complications. In our previous studies, we discovered that APD treatment exerts beneficial effects on patients or animals with SAP[7,10]. Our results demonstrated early APD could effectively relieve or control the severity of SAP without increase in infection rate, improve tolerance of enteral nutrition and reduce intra-abdominal pressure[9,29,30]. It was an important development and supplement for the minimally invasive step-up approach with important clinical implications.

SAP is usually complicated with multiple organ injury[31]. Cardiac injury is an important cardiovascular complication, and cardiac decompensation even causes death. In this study, we produced a well-characterized SAP-induced cardiac injury model by retrograde injection of 5% sodium taurocholate, as described previously with some modifications[25]. SAP was demonstrated by morphological changes, hemodynamic and echocardiographic abnormalities, elevated cardiac enzymes, increased LPO production and myocardial cell apoptosis. Unlike other system failures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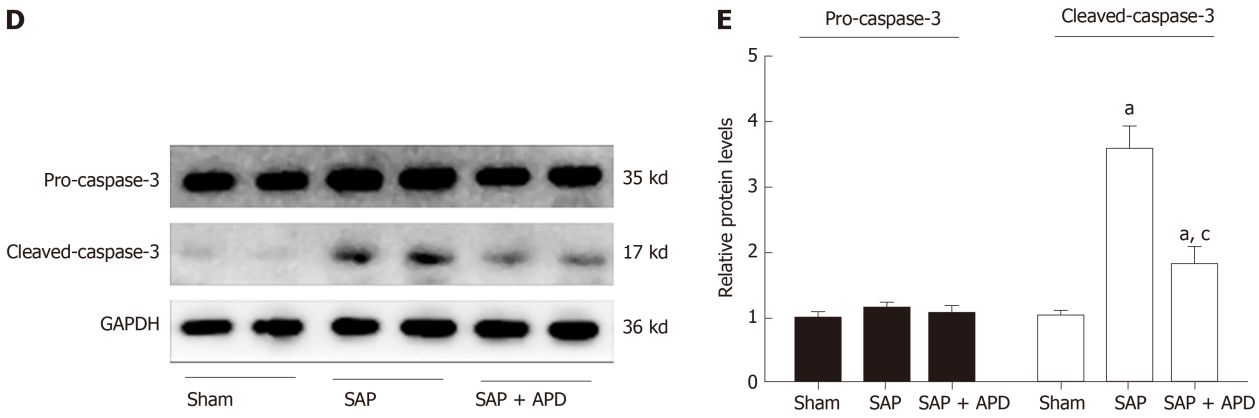

Figure 5 Abdominal paracentesis drainage attenuates myocardial cell apoptosis and expression of apoptosis associated protein in myocardium. A: Representative images (400 × magnification) of terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling assay. Cells with green staining in the nuclei were determined to be apoptotic cells; B: Immunoblot of Bax and Bcl-2 protein expression from heart samples; C: Densitometry analysis of Bax and Bcl-2. Data are representative of at least three independent experiments; D: Immunoblot of pro-caspase-3 and cleaved-caspase-3 protein expression from heart samples; E: Densitometry analysis of pro-caspase-3 and cleaved-caspase-3. Data are representative of at least three independent experiments. $^{a}P < 0.05$ *vs* sham group; $^{c}P < 0.05$ *vs* severe acute pancreatitis group. TUNEL: Terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling; Bax: Bcl-2 related X protein; Bcl-2: B cell lymphoma/leukemia-2 gene; GAPDH: Glyceraldehyde-3-phosphate dehydrogenase; SAP: Severe acute pancreatitis; APD: Abdominal paracentesis drainage.

during SAP that have been extensively studied, cardiac injury and intervention for preservation of heart function have been less emphasized in the management of SAP. Thus, we assessed the effect of APD treatment on SAP-evoked myocardial injury and explored the potential mechanisms.

Through a series of animal experiments, we found that APD treatment markedly improved histopathological changes in the heart tissues and reversed the alterations in serum cardiac enzymes and cardiac dysfunction. The serum levels of lactate dehydrogenase, creatine kinase-MB and cardiac troponin-I in SAP rats were significantly decreased following APD treatment, indicating the cardioprotective effect of APD induced by SAP. In line with the above findings, morphological changes indicative of cardiac injury that occurs in SAP, including disruption of myocardial fibers, cellular edema and intensive infiltration, were markedly alleviated following APD treatment. Preserving near normal heart tissue architecture will yield beneficial effects to maintain near-normal heart function, which was reflected by near-normal hemodynamics and echocardiography in SAP rats receiving APD treatment. These results indicate the important contribution of APD treatment to myocardial injury in SAP models.

Oxidative stress is a key contributor to the initiation and progression of SAP-induced remote organ injury[32]. Oxidative stress is inseparable from abnormal activation of oxidases[33]. Recent studies have indicated that NOX makes a major contribution to ROS generation, mediates ROS in the heart and then increases in response to various stimuli[34]. The present study was prompted by our previous finding that NOX hyperactivity was present in the heart of SAP rats and could cause oxidative injury and increase myocardial cell apoptosis and that inhibition of NOX had a protective effect against cardiac injury induced by SAP[24]. We hypothesized that the cardioprotective effect of APD treatment was achieved through ameliorating oxidative injury *via* the modulation of NOX. When measurements were implemented at 24 h after SAP induction, treatment with APD significantly decreased protein expression and activity of NOX in the heart. This was supported by the decreased ROS production and LPO level and increase in SOD activity and GSH level in heart tissues in the APD-treated group. Furthermore, myocardial cell apoptosis secondary to oxidative stress that accounts for contractility declines was also observed in SAP rats at 24 h[35]. APD reduced the number of myocardial cell apoptosis and modulated the expression of apoptosis-related proteins. As shown in this study, the expression levels of proapoptotic markers, *i.e*. cleaved caspase-3 and Bax, were markedly decreased, whereas the expression of antiapoptotic marker Bcl-2 was increased in the heart tissues of SAP rats following APD treatment. These data demonstrated that APD treatment alleviated oxidative stress damage and myocardial cell apoptosis induced by SAP. However, it is unclear how the elimination of PAAF *via* APD treatment reduces cardiac NOX. A possible explanation is that APD decreases the levels of some lethal factors that can act on cardiac NOX.

Oxidative stress has a direct relationship with systemic inflammatory response during SAP[36], which may involve some proinflammatory cytokines. Recently,

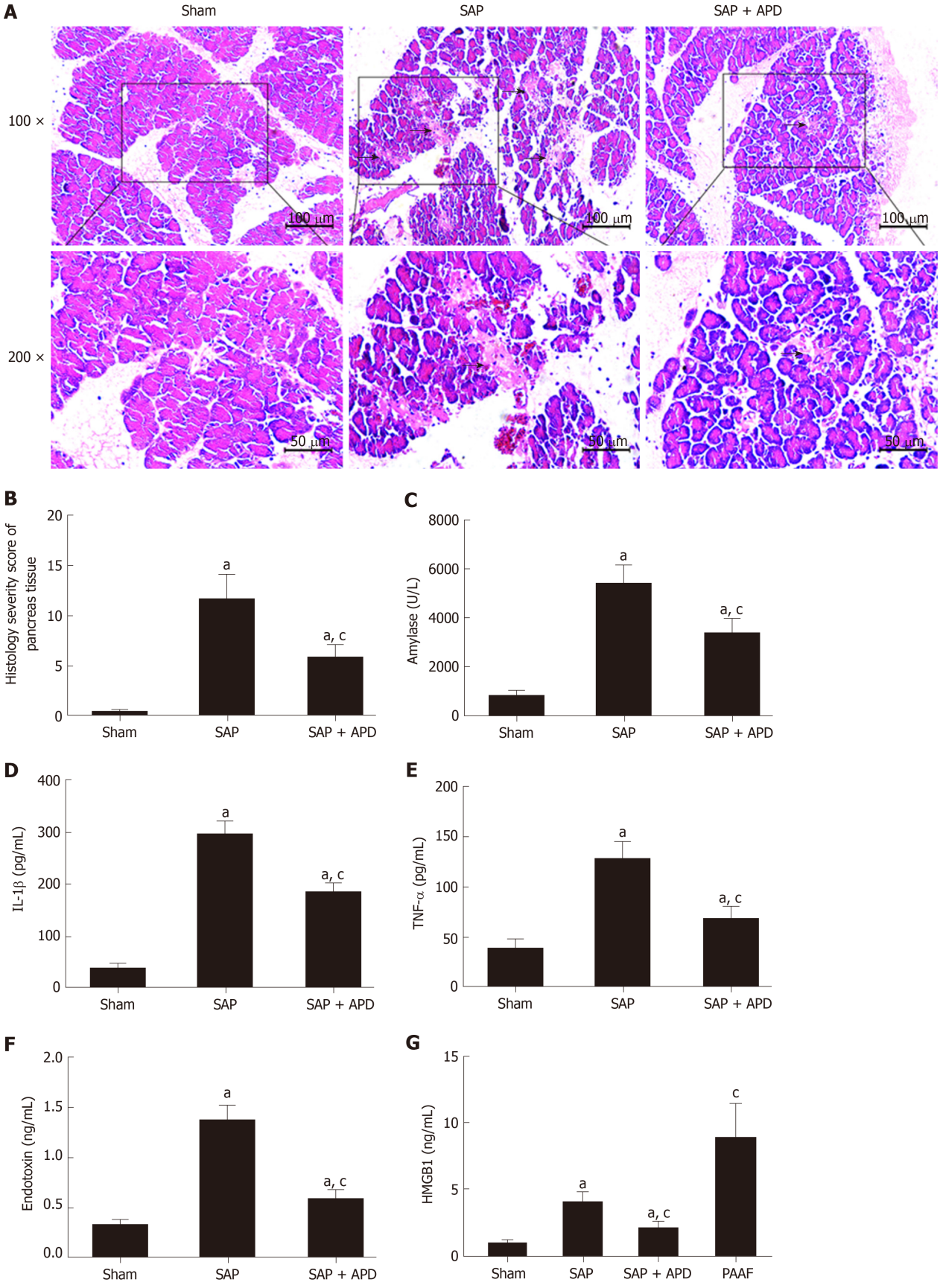

Figure 6 Effects of abdominal paracentesis drainage on pancreatic histopathology and proinflammatory cytokines. A: Representative micrographs of hematoxylin-eosin stained sections of rat pancreatic tissue from different groups. Images were taken under 100 × and 200 × magnification. The arrow indicates necrotic pancreatic tissue; B: Histology severity score of pancreas; C: Amylase; D: Interleukine-1 beta; E: Tumor necrosis factor alpha; F: Endotoxin; G: High mobility group box 1. Data indicate the mean ± standard deviation obtained from six animals in each group (C-G). [a]$P < 0.05$ *vs* sham group; [c]$P < 0.05$ *vs* severe acute pancreatitis group. IL-1β: Interleukine-1 beta; TNF-α: Tumor necrosis factor alpha; HMGB1: High mobility group box 1; PAAF: Pancreatitis associated ascitic fluids; SAP: Severe acute pancreatitis; APD: Abdominal paracentesis drainage.

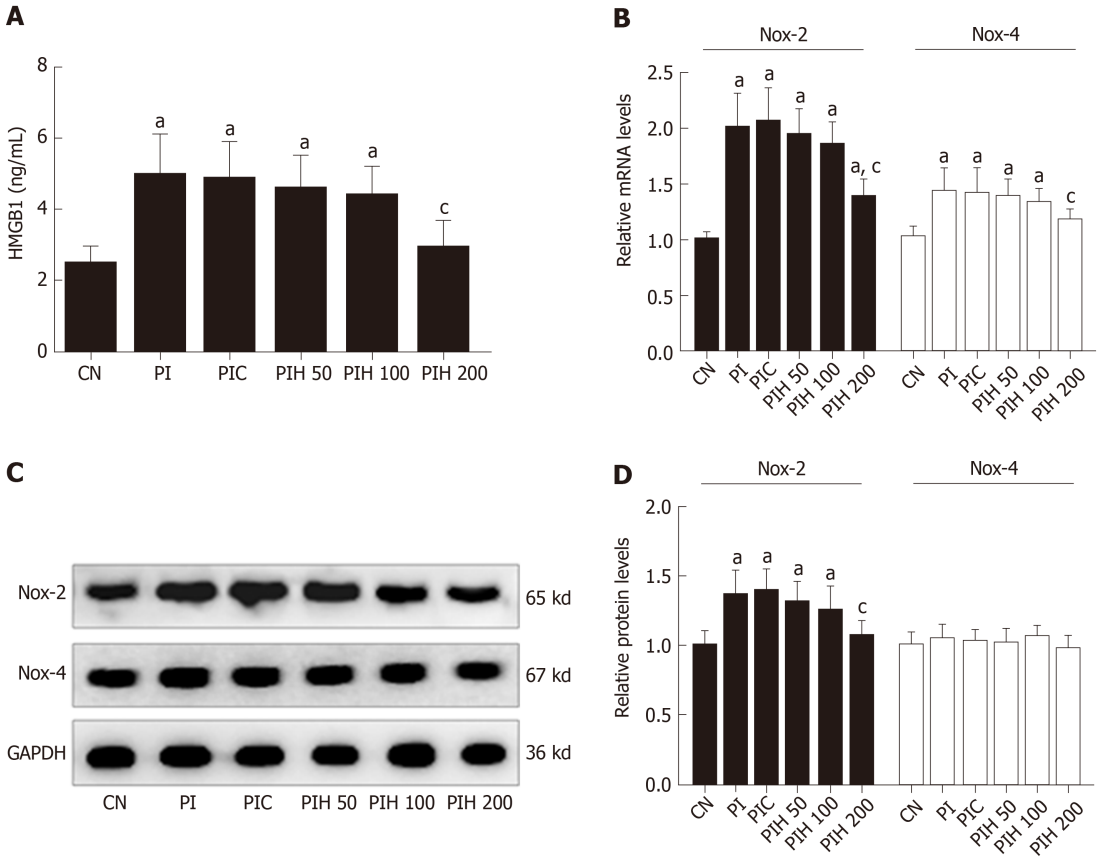

Figure 7 Effects of pancreatitis associated ascitic fluids intraperitoneal injection with or without anti-high mobility group box 1 neutralizing antibody on cardiac NOX expression in cerulein rats. A: High mobility group box 1 in the serum; B: *Nicotinamide adenine dinucleotide phosphate oxidase-2* (*NOX2*) and *NOX4* mRNA measurement by real-time polymerase chain reaction; C: Immunoblot of NOX2 and NOX4 protein expression from heart samples; D: Densitometry analysis of NOX2 and NOX4. Data indicate the mean ± standard deviation obtained from six animals in each group (A-B). Data are representative of at least three independent experiments (C-D). $^{a}P < 0.05$ *vs* controls group; $^{c}P < 0.05$ *vs* pancreatitis-associated ascitic fluid injection group. HMGB1: High mobility group box 1; NOX2: Nicotinamide adenine dinucleotide phosphate oxidase-2; NOX4: Nicotinamide adenine dinucleotide phosphate oxidase-4; GAPDH: Glyceraldehyde-3-phosphate dehydrogenase; SAP: Severe acute pancreatitis; APD: Abdominal paracentesis drainage.

extracellular HMGB1 was identified as a novel proinflammatory cytokine, which could trigger a lethal inflammatory process and participate in the development of pancreatic and remote organ injury in SAP[12,14]. Accumulated evidence indicates that the level of serum HMGB1 is significantly elevated in patients with SAP and SAP-induced animal models and is positively related to the outcome of SAP as well as organ dysfunction[16,17]. Meanwhile, HMGB1 plays an important role in the pathogenesis of cardiac dysfunction in many diseases, such as ischemia-reperfusion injury, sepsis and diabetic cardiomyopathy[12,37,38]. Thus, increased HMGB1 may have a detrimental effect on the heart in SAP. In our present study, we found that serum levels of HMGB1 increased in SAP rats, and it was noteworthy that the level of HMGB1 in PAAF was higher than that in serum of the sham group. In light of the fact that the key feature of acute pancreatitis is damage to the pancreas, our results regarding the beneficial effects of APD on pancreatic injury were consistent with previous studies, suggesting that HMGB1 was first produced and released by the pancreas and peritoneal macrophages/monocytes in response to inflammatory mediators during SAP. As a treatment strategy to eliminate PAAF, APD not only improves pancreatic histopathology in SAP but also regulates the polarization of peritoneal macrophages[39]. Therefore, it is reasonable to conceive that APD could decrease HMGB1 level in the circulation. As our results showed, there was a significant decline in serum HMGB1 level following APD treatment in the sham group compared with the SAP group. In addition, active neutralization with anti-HMGB1 antibodies or HMGB1-specific blockage *via* box A could prevent cardiac dysfunction in mice with ischemia-reperfusion injury, sepsis and diabetic cardiomyopathy[18-20]. Taken together, these results imply that downregulated HMGB1 is involved in the beneficial effects of APD in SACI.

To confirm that the decreased level of serum HMGB1 induced by APD can alleviate the hyperactivity of cardiac NOX, we intraperitoneally injected PAAF, with or

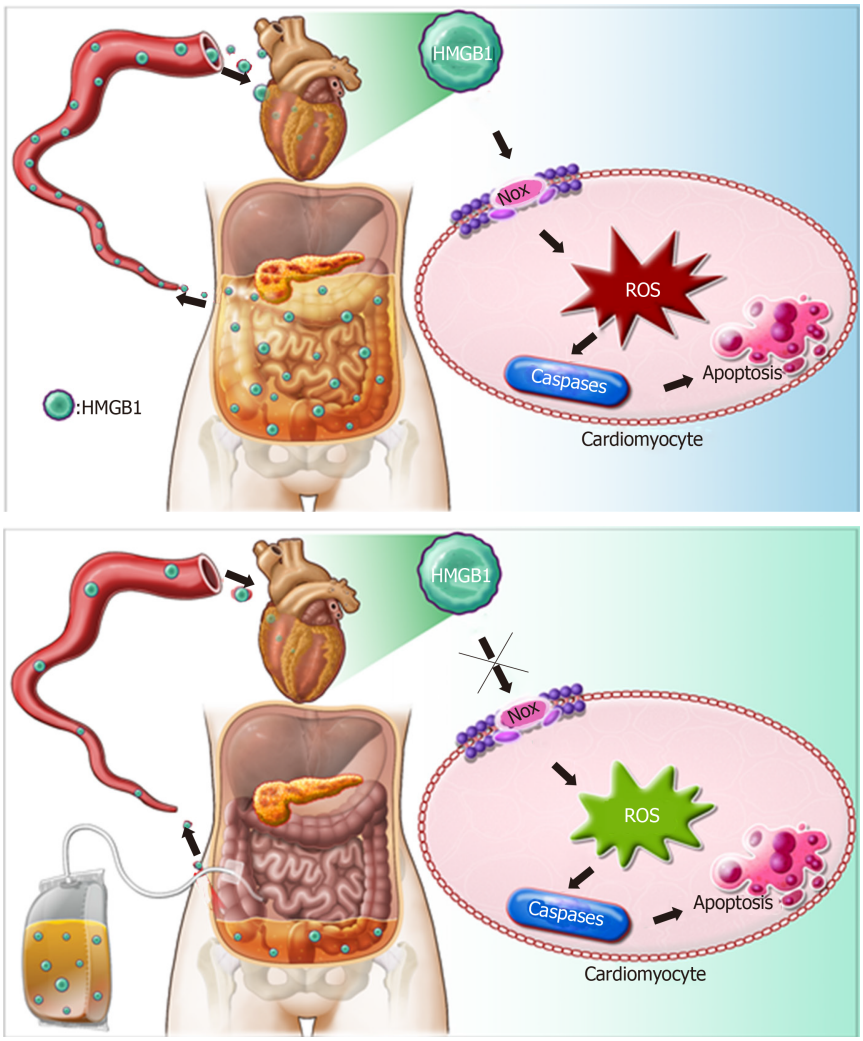

Figure 8 Possible mechanisms responsible for the protective effects of abdominal paracentesis drainage on severe acute pancreatitis associated cardiac injury. During pancreatitis, high levels of high mobility group box 1 (HMGB1) in the bloodstream can trigger a lethal inflammatory process and participate in the development of remote organ injury. Following abdominal paracentesis drainage treatment, the levels of high mobility group box 1 in the circulation decrease significantly, resulting in inhibition of expression and activity of cardiac nicotinamide adenine dinucleotide phosphate oxidase, thus reactive oxygen species production markedly decreases. These events downregulate expression of caspase-associated proteins and alleviate apoptosis, thereby yielding beneficial effects. HMGB1: High mobility group box 1; APD: Abdominal paracentesis drainage; NOX: Nicotinamide adenine dinucleotide phosphate oxidase; ROS: Reactive oxygen species.

without anti-HMGB1 neutralizing antibody, into rats with cerulein-induced pancreatitis. Eight hours after PAAF injection, the circulating level of HMGB1 in the PI group was significantly increased compared with that in the control group, suggesting that HMGB1 in PAAF can enter the bloodstream in MAP. Correspondingly, mRNA expression of *NOX2* and *NOX4* and protein expression of NOX2 were significantly increased. When the dose of anti-HMGB1 neutralizing antibody increased up to 200 μg, we observed that *NOX* mRNA and NOX protein expression decreased more than in the PI group. The important findings were consistent with previous studies that HMGB1 increases ROS production through activation of NOX. However, HMGB1 acting on cells must pass through some membrane receptors, such as Toll-like receptor (TLR) 4, which is the first target activated by extracellular HMGB1 to induce deleterious effects and is highly expressed in the heart[40]. In addition, TLR4-NOX interaction is suggested to participate in the pathogenesis of myocardial injury by activating multiple signaling pathways. For example, LPS-induced cardiac dysfunction is regulated by autophagy and ROS production in cardiomyocytes *via* the TLR4-NOX4 pathway[41]. As for SAP, whether TLR4 is also involved in HMGB1-induced cardiac NOX hyperactivity is still unclear, and future studies should be carried out.

Our results reveal that APD treatment ameliorated myocardial injury and improved cardiac function in a rat model of SAP. The cardioprotective effects of APD

treatment are potentially due to the inhibition of oxidative stress by downregulation of HMGB1-mediated expression of NOX. This study suggests a novel mechanism underlying the effect of APD on SAP and related complications.

ARTICLE HIGHLIGHTS

Research background

Severe acute pancreatitis (SAP) is a fatal systemic disease usually complicated with multiple distant organ injury. Among the distant organ injury, SAP-associated cardiac injury (SACI) occurs in a variable proportion of patients, and cardiac decompensation even causes death. Despite constant understanding in the pathogenesis of SAP and significant improvement in clinical management, the mortality rate remains high and unacceptable. In our previous study, early abdominal paracentesis drainage (APD) was found to effectively relieve or control the severity of SAP and maintenance of organ function through removing pancreatitis associated ascitic fluids (PAAF). However, the effect of APD treatment on SAP-associated cardiac injury and the possible mechanism are yet to be elucidated.

Research motivation

Inflammatory mediators exert a vital role in the initiation and progression of SAP in the early stage. High concentration of high mobility group box 1 (HMGB1) in the pancreatitis associated ascitic fluids has been confirmed. Thus, we want to further study whether HMGB1 in ascites is related to SAP-associated cardiac injury, which may be a novel mechanism behind the effectiveness of APD on SAP.

Research objectives

The aim of this study was to determine the protective effects of APD treatment on SAP-associated cardiac injury and explore the potential mechanism.

Research methods

In the present study, SAP was induced by 5% sodium taurocholate retrograde injection in Sprague-Dawley rats. Mild acute pancreatitis was induced by six consecutively intraperitoneal injections of cerulein (20 μg/kg rat weight). APD was performed by inserting a drainage tube with a vacuum ball into the lower right abdomen of the rats immediately after SAP induction. Morphological staining, serum amylase and inflammatory mediators, serum and ascites HMGB1, cardiac-related enzymes indexes and cardiac function and oxidative stress markers were performed. Cardiomyocyte apoptosis was detected by terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling. *Nicotinamide adenine dinucleotide phosphate oxidase* (*NOX*) mRNA was identified by real-time polymerase chain reaction. Apoptosis-associated proteins and protein expression of NOX were measured by western blot.

Research results

APD notably improved pancreatic and cardiac morphological changes, attenuated the alterations in serum amylase, inflammatory mediators, cardiac enzymes and function, reactive oxygen species production and oxidative markers, alleviated myocardial cell apoptosis, reversed the expression of apoptosis-associated proteins, downregulated HMGB1 level in serum and inhibited NOX hyperactivity. Furthermore, the activation of cardiac NOX by pancreatitis associated ascitic fluids intraperitoneal injection was effectively inhibited by adding anti-HMGB1 neutralizing antibody in rats with mild acute pancreatitis.

Research conclusions

APD treatment could exert cardioprotective effects on SAP-associated cardiac injury through suppressing HMGB1-mediated oxidative stress.

Research perspectives

Our study provided new evidence of the efficacy and safety of APD treatment on SAP and revealed a novel mechanism behind the effectiveness of APD on SAP. However, in this study we still do not know how HMGB1 modulates NOX under SAP conditions. In the next experiments, we should detect the expression profile of HMGB1 receptor protein in the heart and utilize a special receptor protein knockout model to clarify the precise mechanisms.

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ORIGINAL ARTICLE

Basic Study

Synergistic protection of astragalus polysaccharides and matrine against ulcerative colitis and associated lung injury in rats

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Abstract

BACKGROUND

Ulcerative colitis (UC) is a main form of inflammatory bowel disease. Due to complicated etiology and a high rate of recurrence, it is quite essential to elucidate the underlying mechanism of and search for effective therapeutic methods for UC.

AIM

To investigate the effects of astragalus polysaccharides (APS) combined with matrine on UC and associated lung injury.

METHODS

UC was induced in rats by colon mucosal tissue sensitization combined with trinitro-benzene-sulfonic acid-ethanol. Then, the effects of the treatments of salazopyrine, APS, matrine, and APS combined with matrine on histopathological changes of lung and colon tissues, disease activity index (DAI), colon mucosal damage index (CMDI), serum endotoxin (ET) level, serum diamine oxidase (DAO) activity, the contents of tumor necrosis factor-α and interleukin-1β, and the activities of myeloperoxidase, superoxide dismutase, and malondialdehyde in lung tissues, as well as the protein expression of zonula occludens (ZO)-1, Occludin, and trefoil factor 3 (TFF3) were detected in UC rats.

RESULTS

The treatments of salazopyrine, APS, matrine, and APS combined with matrine reduced DAI scores and improved histopathological changes of colon and lung tissues, as well as decreased CMDI scores, ET levels, and DAO activities in UC rats. Moreover, in lung tissues, inflammatory response and oxidative stress injury were relieved after the treatments of salazopyrine, APS, matrine, and APS combined with matrine in UC rats. Furthermore, the expression of ZO-1,

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Occludin, and TFF3 in lung and colon tissues was increased after different treatments in UC rats. Notably, APS combined with matrine exerted a better protective effect against UC and lung injury compared with other treatments.

CONCLUSION
APS combined with matrine exert a synergistic protective effect against UC and lung injury, which might be associated with regulating TFF3 expression.

Key words: Astragalus polysaccharides; Matrine; Ulcerative colitis; Lung injury; Trinitro-benzene-sulfonic acid-ethanol; Trefoil factor 3

Core tip: The results of the present study show that astragalus polysaccharides combined with matrine exert a synergistic protective effect against ulcerative colitis and lung injury, which might be associated with regulating trefoil factor 3 expression.

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INTRODUCTION

As a main form of inflammatory bowel disease (IBD), ulcerative colitis (UC) is characterized by chronic inflammation and ulcerative lesions in the intestinal mucosa[1,2]. Clinical manifestations of UC mainly include persistent or recurrent diarrhea, stools with mucus, blood, and pus, abdominal pain, and various systemic symptoms[1,2]. Recently, due to the changes in diet structure, living habits, and environments, the incidence and diagnosis rates of UC in China have increased year by year[3]. Despite the substantial progress in the diagnosis and treatment of UC, unsatisfactory therapeutic effects remain an issue due to complicated etiology and a high rate of recurrence[4]. Therefore, elucidating the underlying mechanism of and searching for effective therapeutic methods for UC are quite essential.

Previous studies have demonstrated that lung injury is closely involved in IBD, including UC, thus, the treatment for UC should be focused on large intestine and lung injury simultaneously[5-7]. In recent years, several traditional Chinese medicines (TCMs) have been evidenced to exert protective effects against UC[8] and lung diseases[9]. *Astragalus membranaceus* is a popular TCM that has been widely used for its anti-fatigue, anti-sepsis, anti-inflammation, anti-hypertension, and anti-tumor properties[10]. Astragalus polysaccharides (APS) are one of the primary bioactive ingredients extracted from *Astragalus membranaceus*, and play important roles in treating oxidative stress, immunological diseases, and cancers based on their pharmacological and biological effects[11,12]. It has been shown that APS can attenuate experimental colitis by regulating the immune response[13,14]. Also, APS are reported to protect against several lung diseases, such as pulmonary arterial hypertension[15] and chronic obstructive pulmonary disease[16]. Matrine, the extracts from another common TCM *Sophora flavescens Ait*., is also proved to be effective in treating colitis[17] and lung injury[18] due to its diverse pharmacological properties, including anti-virus, anti-inflammation, antioxidant, and anti-tumor activities[19]. However, few studies have investigated the effect of APS combined with matrine on UC and associated lung injury.

In the current study, we established a UC rat model by colon mucosal tissue sensitization combined with trinitro-benzene-sulfonic acid (TNBS)-ethanol, and then explored the effects and mechanisms of the treatments of salazopyrine, APS, matrine, and APS combined with matrine on histopathological changes of colon and lung tissues, intestinal mucosa injury, and lung injury in UC rats.

MATERIALS AND METHODS

Ethical approval

Approval from the Animal Ethics Committee of North China University of Science and Technology was obtained prior to experiments.

Preparation of antigen emulsion

Totally, ten healthy male New Zealand white rabbits weighing 2.5 kg were obtained from Jinmuyang Laboratory Animal Breeding Co., Ltd (Beijing, China). The rabbits were sacrificed by air embolization, and then colon tissues were dissected and collected. The colonic mucosa tissue was scraped using a disinfecting blade, and added into the same amount of pre-cooled physiological saline to make the tissue homogenate. After centrifugation at 3000 rpm for 30 min at 4 °C, the supernatant was obtained and mixed with the same volume of complete Freund's adjuvant to prepare an antigen emulsion.

UC rat model

UC was induced in rats by colon mucosal tissue sensitization combined with TNBS-ethanol. Briefly, the antigen emulsion (8 mg/each rat) was injected into the toes and groin of the rats on the 1st, 15th, and 22th days, respectively. On the 29th day, the rats were fasted with free access to water for 24 h, and then anesthetized with 10% chloral hydrate (0.35 mL/100g) by intraperitoneal injection. TNBS-50% ethanol was prepared by mixing TNBS solution (120 mg/mL) with 50% ethanol at a ratio of 1:1 (v/v). The rats were inverted, and then TNBS-50% ethanol was slowly injected into the intestine at 100 mg/kg body weight using a silicone tube inserted 8 cm proximal to the anus. After pinching the anus and keeping the anus elevated for 1 min, the rats were placed in the cage, with the buttock raised to prevent the drug outflow. The rats naturally awakened after the anesthetic failed.

Animal grouping, drug administration, and sample collection

A total of 150 healthy male Wistar rats (weighing 200 ± 10 g, purchased from Charles River, Beijing, China) were used for the following experiments after one week of acclimation. The grouping is shown as Figure 1. Briefly, 30 Wistar rats were randomly selected as a normal group, and the remaining 120 Wistar rats were induced as UC models. On the 3rd day after modeling, 10 rats in the normal group and 10 UC model rats were randomly selected and used for model validation by histopathological observation (0 wk). The remaining 105 UC model rats (five rats sacrificed after modeling) were randomly assigned to five groups: Model group (n = 25), salazopyrine control group (n = 25), APS treatment group (n = 15), matrine treatment group (n = 15), and monomer mixture group (n = 25). The rats in the salazopyrine group received 0.125 g/mL of salazopyrine (SASP, Sunve, Shanghai, China); rats in the APS treatment group were given 0.6 g/mL of APS (Fuzhou Rimian Technology Development Co. LTD, China); rats in the matrine treatment group were given 12 mg/mL of matrine (Xi'an Linhe Biotechnology Co. LTD, China); and rats in the monomer mixture group were administered with the mixture of APS and matrine at a ratio of 1:1. All drugs in these groups were intragastrically administered at eight times of the dose for an adult human (60 kg body weight); and rats in the normal group and model group were intragastrically administered with equal volume of drinking water once a day. Among all the experimental animals, dynamic observation at 2 wk and 4 wk after administration was performed in the normal group, model group, salazopyrine control group, and monomer mixture group. Various indicators at 4 wk before and after the treatment were observed in the APS treatment group and matrine treatment group. Rats were anesthetized with 10% chloral hydrate (3.5 μL/g) by intraperitoneal injection, and lung and colon tissues were removed aseptically and stored in liquid nitrogen for the following experiments. Meanwhile, blood samples were collected from the abdominal aorta, and then serum was separated by centrifugation at 4 °C for 15 min and stored at 80 °C.

General indicators

The general conditions, including coating gloss, mental state, activity, diet, respiration, and feces, of rats at 0 wk, 2 wk, and 4 wk after administration in each group were observed. Meanwhile, body weight was measured, and disease activity index (DAI)[20] and colon mucosal damage index (CMDI)[21] were evaluated.

Histopathological assessment

Lung and colon tissues were fixed at 4 °C for 24 h with 4% paraformaldehyde. Following paraffin embedding, the tissues were sliced into sections. After dehydration with gradient ethanol, the sections underwent hematoxylin-eosin (HE)

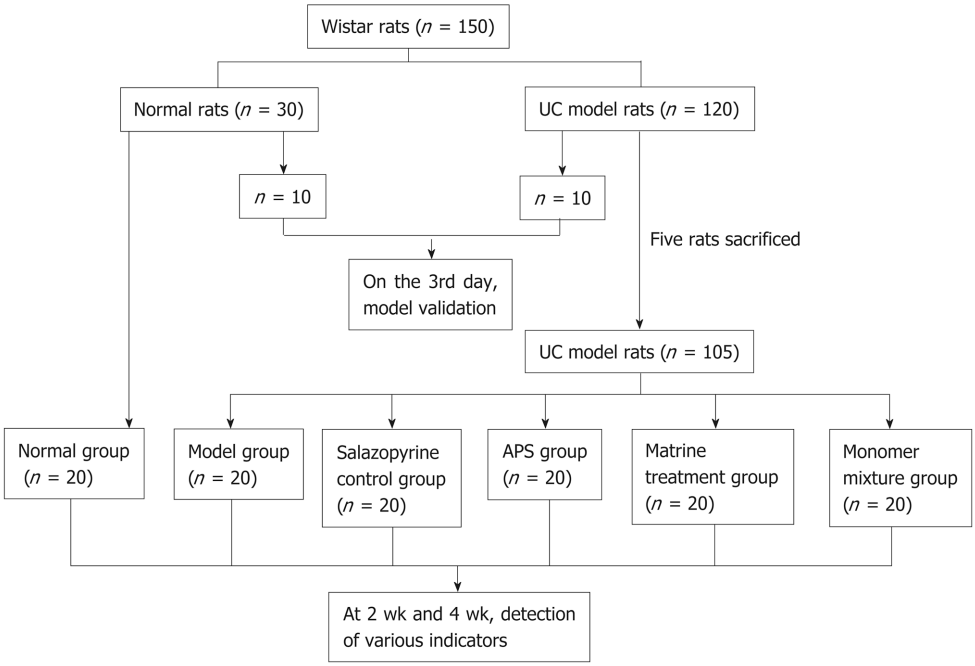

Figure 1 Flow chart of animal grouping. APS: Astragalus polysaccharides.

staining and mounting with neutral resin. Lastly, the sections were observed by light microscopy (Olympus, Japan).

Enzyme-linked immunosorbent assay

Serum samples were thawed on ice, and then determined with a commercial enzyme-linked immunosorbent assay (ELISA) kit (Boster, Wuhan, China) for the content of endotoxin (ET). In addition, lung tissues in each group were cut into pieces, and then lung tissue homogenate was obtained with an ultrasonic cell disruptor (VCX130PB, Sonics, United States). After centrifugation at 4 °C for 15 min, the supernatant was determined with commercial ELISA kits (Boster) for the contents of tumor necrosis factor (TNF)-α, and interleukins (IL)-1β according to the manufacturer's instructions.

Diamine oxidase measurement

Diamine oxidase (DAO) activity in serum samples was detected by spectrophotometry. Briefly, the standard curve of DAO was made. Then, 100 μL of serum samples were incubated with 1 mL of phosphate buffer, 50 μL of HPRO (200 μg), 50 μL of o-dianisidine (50 μg), and 50 μL of pentanediamine (1750 μg) for 1.5 h in a water bath at 37 °C. Lastly, 200 μL of mixture was added into 96-well plates, and the absorbance at 436 nm was read with a microplate reader (Molecular Devices, United States).

Myeloperoxidase, superoxide dismutase, and malondialdehyde activity detection

Lung tissues were cut into pieces, and then lung tissue homogenate was obtained with an ultrasonic cell disruptor (VCX130PB, Sonics, United States). The activities of myeloperoxidase (MPO), superoxide dismutase (SOD), and malondialdehyde (MDA) were determined with MPO, SOD, and MDA detection kits (Nanjing Jiancheng Bioengineering Institute, China), respectively, according to the manufacturer's instructions.

Western blot analysis

Lung and colon tissues were lysed with RIPA lysis buffer (Applygen, Beijing, United States), and protein was extracted by centrifugation and detected with the BCA kit (CW Biotech Co., Beijing, China). Then, protein samples were separated on an SDS-PAGE gel, and transferred to polyvinylidene fluoride membranes, followed by blockage with 5% nonfat milk for 1 h. Next, the membrane was incubated with anti-rat zonula occludens (ZO)-1 antibody (1:200, Santa Cruz, Santa Cruz, United States) or GAPDH antibody (Zhongshan Biotech, Beijing, China) overnight at 4 °C, and then washed with phosphate buffer saline, followed by the incubation with a secondary antibody (1:1000, Applygen, Beijing, United States) for 2 h at room temperature, respectively. Lastly, enhanced chemiluminescence (ECL, Millipore, United States) was

used to detect the protein levels.

Immunohistochemistry

Lung and colon tissues were fixed at 4 °C for 24 h with 4% paraformaldehyde. Following paraffin embedding, the tissues were sliced into sections. After dehydration with gradient ethanol, the sections were immunostained with Occludin (Abcam, Cambridge, MA, United States) or trefoil factor 3 (TFF3, ProSci, Poway, CA, United States) antibody, followed by staining with a secondary antibody (Zhongshan Biotech, Beijing, China), and incubation with diaminobenzidine (Zhongshan Biotech, Beijing, China). Ultimately, the sections were observed by light microscopy (Olympus, Japan).

Statistical analysis

SPSS Statistics 20.0 software (IBM, Armonk, NY, United States) was used for data statistical analyses. Data are expressed as the mean ± SD. The differences between groups were analyzed by one-way ANOVA followed by multiple comparisons by the LSD test. P values < 0.05 were considered statistically significant.

RESULTS

Effect of APS combined with matrine on general conditions and histopathological changes in UC rats

The rats in the normal group showed smooth hair, sensitive response, high activity, normal diet, normal feces, and stable breathing, while the rats in the model group presented pale yellow color hair, lack of energy, sleepiness, decreased appetite, softened feces, and dyspnea. At 2 wk after administration, compared with the model group, rats in the salazopyrine control group had increased coating glossiness, less autonomic activity, and improved feces. Meanwhile, the mental state, activity, food intake, defecation, and respiratory symptoms of rats in the matrine treatment group, APS treatment group, and the monomer mixture group were also improved compared with the model group. In addition, these general conditions of rats were obviously improved at 4 wk than those at 2 wk. Moreover, the weight of rats in the model group was significantly lower than that in the normal group (P < 0.01, Table 1) at 0 wk. At 2 wk and 4 wk, compared with the control group, the weight of rats was also lower in the other groups, and the weight of rats was increased in comparison with rats at 0 wk, especially in the APS treatment group and the monomer mixture group (Table 1). Compared with the normal group, DAI score was significantly increased in the model group (P < 0.01, Table 2) at 0 wk. Similarly, at 2 wk and 4 wk, DAI scores were also increased in the other groups compared with the normal group (Table 2). However, rats in the other groups had lower DAI scores than the model group, especially in the APS treatment group and the monomer mixture group (Table 2). Furthermore, HE staining revealed that the colonic mucosa of rats in the model group showed congestion, edema, ulceration, and a large amount of lymphocyte and neutrophil infiltration, and lung tissue of rats in the model group showed obvious congestion, edema, and a large amount of inflammatory cell infiltration (Figure 2). Nonetheless, compared with the model group, histopathological observation was improved in the other treatment groups at 2 wk and 4 wk after administration (Figure 2).

Effect of APS combined with matrine on intestinal mucosa injury in UC rats

CMDI score was used to evaluate intestinal mucosa injury. Compared with the normal group, CMDI score was significantly elevated in the model group (P < 0.01, Table 3) at 0 wk. Consistently, CMDI scores were also increased in other groups compared with those in the normal group at 2 wk and 4 wk (Table 3). However, rats in other treatment groups had lower CMDI scores than that in the model group, especially in the matrine treatment group and the monomer mixture group (Table 3). In addition, compared with the normal group, serum ET levels were obviously increased in the model group at 0 wk, 2 wk, and 4 wk (P < 0.05, Figure 3A), while lower serum ET levels were found in the other treatment groups than in the model group (Figure 3A). Similarly, the trend of DAO activity was consistent with ET level (Figure 3B).

Effect of APS combined with matrine on the expression of ZO-1 and Occludin in lung and colon tissues of UC rats

Western blot analysis showed that ZO-1 protein expression in colon tissues was significantly decreased in the model group at 2 wk and 4 wk compared with the

Table 1 Body weight of rats in various groups

Group	*n*	0 wk	*n*	2 wk	*n*	4 wk
Normal	10	414.00 ± 29.28	10	467.50 ± 21.44	10	507.60 ± 49.21
Model	10	355.90 ± 25.60[b]	10	413.10 ± 27.91[b]	10	461.30 ± 37.54
Salazopyrine control			10	420.70 ± 35.29[b]	11	451.91 ± 38.31[b]
Matrine treatment			13	429.23 ± 38.04[a]	12	458.83 ± 45.36[a]
APS treatment			13	443.62 ± 45.06	13	486.92 ± 55.67
Monomer mixture			13	426.92 ± 30.74[b]	12	489.75 ± 32.06

[a]$P < 0.05$ and
[b]$P < 0.01$ *vs* normal group. APS: Astragalus polysaccharides.

normal group ($P < 0.05$, Figure 4A), while ZO-1 protein expression in colon tissues was increased in the other treatment groups than in the model group, especially in the monomer mixture group at 4 wk ($P < 0.05$, Figure 4A). Similarly, in lung tissues, ZO-1 protein expression was obviously decreased in the model group at 2 wk and 4 wk compared with the normal group ($P < 0.01$, Figure 4B); however, the other treatment groups showed increased protein expression of ZO-1 compared with the model group, especially in the monomer mixture group at 2 wk ($P < 0.05$, Figure 4B). Moreover, immunohistochemistry analysis revealed that Occludin was usually expressed in intestinal mucosal epithelial cells and glandular cells in colon tissues in normal rats. Occludin protein expression in colon tissues was remarkably decreased in the model group at 2 wk and 4 wk compared with the normal group ($P < 0.05$, Figure 4C), while Occludin protein expression in colon tissues was increased in the other treatment groups than in the model group, especially in the APS treatment group and monomer mixture group at 4 wk ($P < 0.05$, Figure 4C). In addition, Occludin was usually expressed in alveolar epithelial cell membrane in lung tissues of normal rats. Consistently, in lung tissues, Occludin protein expression at 2 wk and 4 wk was prominently decreased in the model group compared with the normal group ($P < 0.05$, Figure 4D); however, the other treatment groups showed increased protein expression of Occludin compared with the model group, especially in the APS treatment group and monomer mixture group at 4 wk ($P < 0.05$, Figure 4D).

Effect of APS combined with matrine on inflammatory response and oxidative stress injury in ling tissues of UC rats

ELISA showed that the expression of inflammatory factors such as TNF-α and IL-1β in lung tissues was significantly increased in the model group at 2 wk and 4 wk compared with the normal group ($P < 0.05$, Figure 5A and B), while their expression was decreased in the other treatment groups compared with the model group ($P < 0.05$, Figure 4A and B). In addition, in lung tissues, SOD activity was obviously decreased in the model group at 2 wk and 4 wk compared with the normal group ($P < 0.01$, Figure 5C); however, the other treatment groups showed an increased activity of SOD compared with the model group, especially at 4 wk ($P < 0.05$, Figure 5C). Furthermore, the activities of MDA and MPO were conspicuously enhanced in the model group at 2 wk and 4 wk compared with the normal group ($P < 0.05$, Figure 5D and E); however, the activities of MDA and MPO were weakened in the other treatment groups compared with the model group (Figure 5D and E).

Effect of APS combined with matrine on TFF3 expression in lung and colon tissues of UC rats

Immunohistochemistry analysis revealed that TFF3 was usually expressed in intestinal mucosa goblet cells and intestinal gland acini in colon tissues of normal rats. TFF3 protein expression in colon tissues was remarkably decreased in the model group at 2 wk and 4 wk compared with the normal group ($P < 0.05$, Figure 6A), while TFF3 protein expression in colon tissues was increased in the other treatment groups compared with the model group, especially in the monomer mixture group at 2 wk and 4 wk ($P < 0.05$, Figure 6A). In addition, compared with the salazopyrine control group, TFF3 protein expression in colon tissues was higher in the monomer mixture group at 4 wk ($P < 0.05$, Figure 6A). Similarly, in lung tissues, TFF3 protein expression was decreased in the model group at 2 wk and 4 wk compared with the normal group, but without a significant difference (Figure 6B). Meanwhile, increased protein expression of TFF3 was found in the APS treatment group and monomer mixture group at 4 wk compared with the model group without a significant difference

Table 2 Disease activity indexes of rats in various groups

Group	*n*	0 wk	*n*	2 wk	*n*	4 wk
Normal	10	0.00 ± 0.00	10	0.00 ± 0.00	10	0.00 ± 0.00
Model	10	3.00 ± 0.59[b]	10	1.67 ± 0.90[b]	10	1.13 ± 0.63[b]
Salazopyrine control			10	0.80 ± 0.53[a]	11	0.36 ± 0.35
Matrine treatment			13	0.51 ± 0.48[ac]	12	0.33 ± 0.45
APS treatment			13	0.67 ± 0.47[b]	13	0.41 ± 0.34[a]
Monomer mixture			13	0.56 ± 0.48[ac]	12	0.56 ± 0.48[ac]

[a]$P < 0.05$ and
[b]$P < 0.01$ *vs* normal group;
[c]$P < 0.05$ *vs* model group. APS: Astragalus polysaccharides.

(Figure 6B).

DISCUSSION

In the present study, a UC rat model was successfully established by colon mucosal tissue sensitization combined with TNBS-ethanol. The treatments of salazopyrine, APS, matrine, or APS combined with matrine inhibited DAI scores, increased body weight, and improved histopathological changes of colon and lung tissues in UC rats. In addition, the treatments of salazopyrine, APS, matrine, or APS combined with matrine alleviated intestinal mucosa injury and inhibited ET levels and DAO activity in UC rats. Moreover, in lung tissues, the inflammatory response and oxidative stress injury were relieved after the treatments of salazopyrine, APS, matrine, or APS combined with matrine in UC rats. Furthermore, the results revealed that the expression of ZO-1, Occludin, and TFF3 in lung and colon tissues was increased after different treatments in UC rats. Notably, APS combined with matrine exerted a better protective effect against UC and lung injury compared with other treatments.

The roles of APS and matrine have been widely investigated in various diseases. Previous studies have demonstrated that APS can significantly improve histological and DAI scores, and increase body weight by regulating the inflammatory response in dextran sulfate sodium-induced colitis[22,23]. It is also reported that matrine can ameliorate colitis by regulating the inflammatory response[17,24]. These results are consistent with our findings. Meanwhile, CMDI, ET levels, and DAO activity were detected in this study. CMDI is a common indicator to evaluate intestinal mucosa injury in colitis, and a high CMDI score represents a serious degree of intestinal mucosa injury[25,26]. In addition, ET is a lipopolysaccharide that is present in the cell wall of Gram-negative bacteria, and is particularly accumulated in the intestine[27]. The healthy human intestinal barrier can protect ET from entering the blood circulation, while the dysfunctional intestinal barrier causes ET to pass through the intestinal mucosa and enter the blood circulation, thereby resulting in endotoxemia[28,29]. DAO is present in the mucosa or villi of mammals, and is normally abundant in intestinal mucosa, kidney, and placental tissues, but rarely in serum[30]. After intestinal mucosal epithelial damage, the cytoplasm DAO can be released into the blood circulation[30,31]. Therefore, serum ET level and DAO activity are ideal indicators reflecting the structure and function of the intestinal mucosa. This study revealed that the treatments of APS or/and matrine inhibited CMDI scores, ET levels, and DAO activity in UC rats, indicating that they can alleviative intestinal mucosa injury. Moreover, the dysfunctional intestinal barrier is closely related to the tight junctions between cells[32]; thus, the expression of tight junction-associated proteins ZO-1 and Occludin in colon tissues was determined in this study. The tight junction mainly consists of the ZO protein family, Occludin protein, Claudin protein, and connective adhesion molecules, which is an important structure for maintaining mucosal permeability[33]. Occludin can interact with the intracellular protein ZO-1, and then bind to the backbone protein[33]; thus, ZO-1 and Occludin play important roles in performing tight junction barrier functions. Several studies have suggested that both ZO-1 and occludin were lowly expressed during colitis[34,35], which is consistent with our study. Taken together, these results suggest that APS combined with matrine might exert a synergistic protective effect against UC.

It is well known that the inflammatory response and oxidative stress are involved in the pathophysiology of lung injury[36]. This study detected the expression of

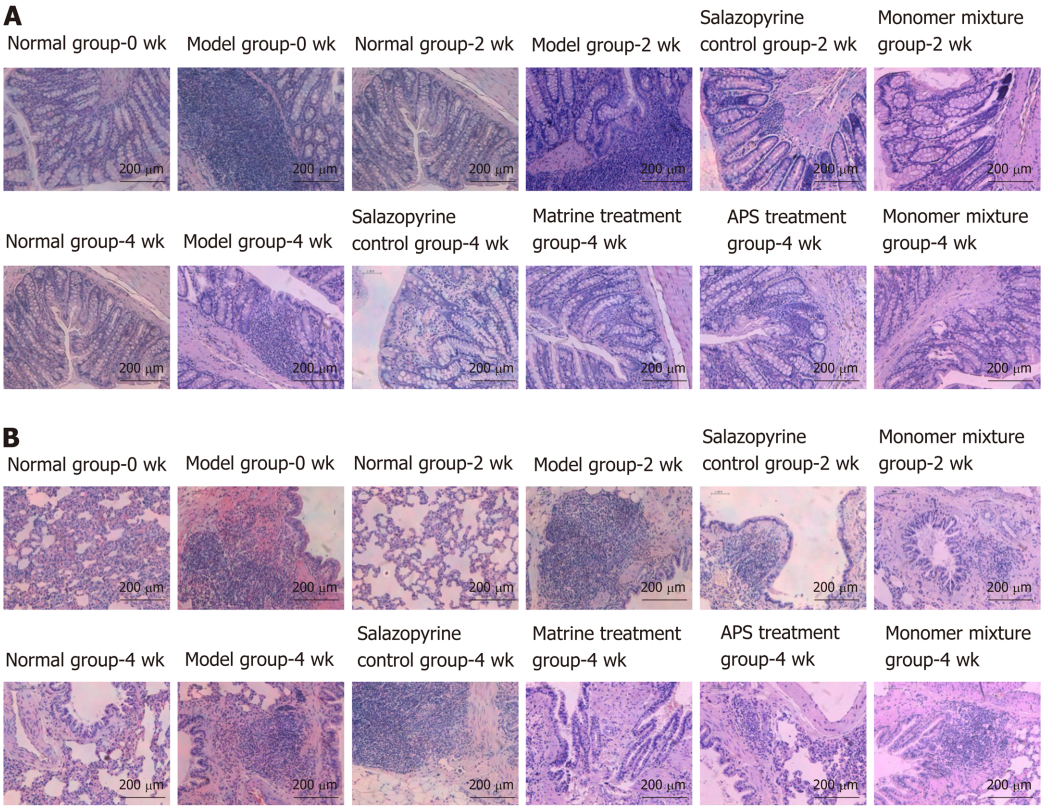

Figure 2 Astragalus polysaccharides combined with matrine improve histopathological changes in rats with ulcerative colitis. A and B: Histopathological changes of colon (A) and lung tissues (B) in various groups analyzed by hematoxylin-eosin staining. APS: Astragalus polysaccharides.

inflammatory factors such as TNF-α and IL-1β in lung tissues. Accumulating evidence has indicated that TNF-α and IL-1β levels were significantly elevated during lung injury[37,38], which is consistent with our results. In addition, the activities of SOD, MDA, and MPO in lung tissues were measured in this study, which are most representative and important indicators during the oxidation-antioxidant balance systems[39]. A previous study has confirmed the widespread abnormality of oxygen free radical metabolism in UC[40]. UC contributes to increased permeability of the intestinal mucosa and elevated oxygen consumption, then a large number of O_2^-, OH, and lipid peroxides are produced, which damage the intestinal mucosa[40]. In addition, the oxygen free radical produced in UC can destroy lung tissue, thereby leading to inflammatory injury of the lungs[40]. This study revealed that SOD activity in lung tissues was obviously decreased, and the activities of MDA and MPO in lung tissues were conspicuously enhanced during UC, which indicated that UC was accompanied with lung injury. The anti-inflammatory role of APS and matrine has also been reported. Consistently, this study showed that the treatments of APS or/and matrine inhibited the levels of TNF-α and IL-1β, reduced the activities of MDA and MPO, and increased SOD activity. All these results indicated that APS combined with matrine might exert a synergistic protective effect against lung injury by regulating the inflammatory response and oxidative stress.

Furthermore, this study detected TFF3 expression in lung and colon tissues after different treatments in UC rats. The TFF family are a kind of small molecule polypeptides, and play roles in mucosal protection, inhibition of inflammatory mediators, regulation of cellular immunity, and apoptosis[41]. TFF3 is specifically distributed in the surface of the intestinal mucosa, and is confirmed to be closely related with the onset of UC[42]. It is generally believed that the expression of TFF3 is down-regulated during the acute onset of IBD, while the up-regulated expression of TFF3 is found during the recovery phase[43,44]. Thus, TFF3 not only has a protective effect on the intestinal mucosal barrier, but its reduction is also related to the progression of UC. This study found decreased expression of TFF3 in UC rats at 2 wk and 4 wk, indicating that the down-regulation of TFF3 reduced the protective and repairing effects on the mucosa, promoted the formation of ulcers, and slowed the repair of damaged mucosa. After 2 wk and 4 wk of treatment with APS and matrine, the expression of TFF3 increased, suggesting that APS combined with matrine might increase the expression of TFF3, and then promote intestinal mucosal injury repair

Table 3 Colon mucosal damage indexes of rats in various groups

Group	*n*	0 wk	*n*	2 wk	*n*	4 wk
Normal	10	0.00 ± 0.00	10	0.00 ± 0.00	10	0.00 ± 0.00
Model	10	6.70 ± 1.89[b]	10	6.40 ± 1.78[b]	10	4.70 ± 2.06[b]
Salazopyrine control			10	3.90 ± 2.23[b]	11	4.00 ± 2.24[b]
Matrine treatment					12	1.83 ± 1.59[ac]
APS treatment					13	2.33 ± 1.97[a]
Monomer mixture			11	0.56 ± 0.48[ac]	12	1.92 ± 1.73[ac]

[a]$P < 0.05$ and
[b]$P < 0.01$ *vs* normal group;
[c]$P < 0.05$ *vs* model group. APS: Astragalus polysaccharides.

and protect intestinal mucosal barrier function. Therefore, we speculated that the synergistic protective effect of APS and matrine against UC and lung injury might be associated with regulating TFF3 expression.

In conclusion, APS combined with matrine exert a synergistic protective effect against UC and lung injury, which might be associated with regulating TFF3 expression.

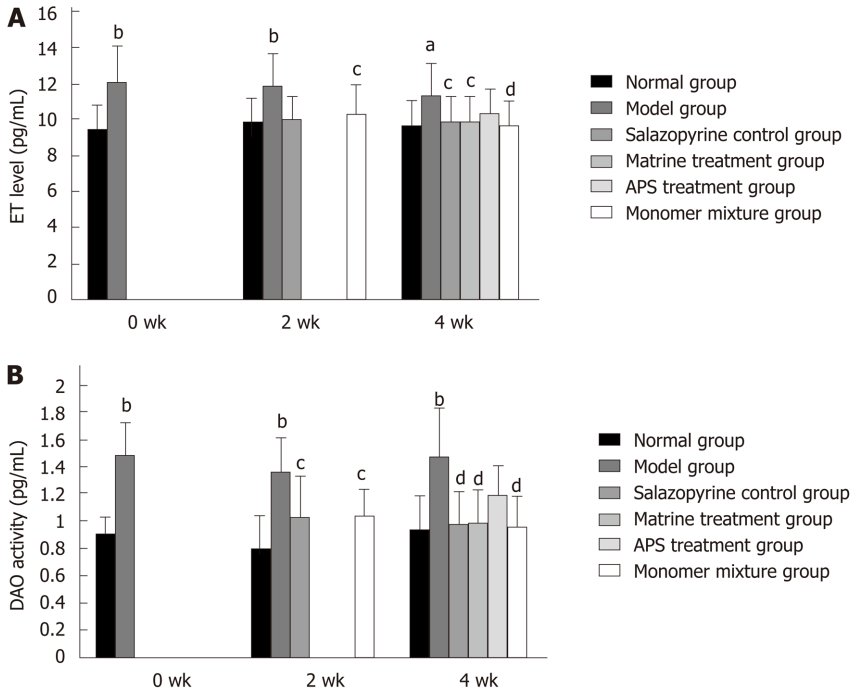

Figure 3 Astragalus polysaccharides combined with matrine inhibit diamine oxidase activity in rats with ulcerative colitis. A: Serum endotoxin levels in various groups detected by enzyme-linked immunosorbent assay; B: Serum diamine oxidase activity in various groups detected by spectrophotometry. $^{a}P < 0.05$ and $^{b}P < 0.01$ *vs* normal group; $^{c}P < 0.05$ and $^{d}P < 0.01$ *vs* model group. APS: Astragalus polysaccharides; ET: Endotoxin; DAO: Diamine oxidase.

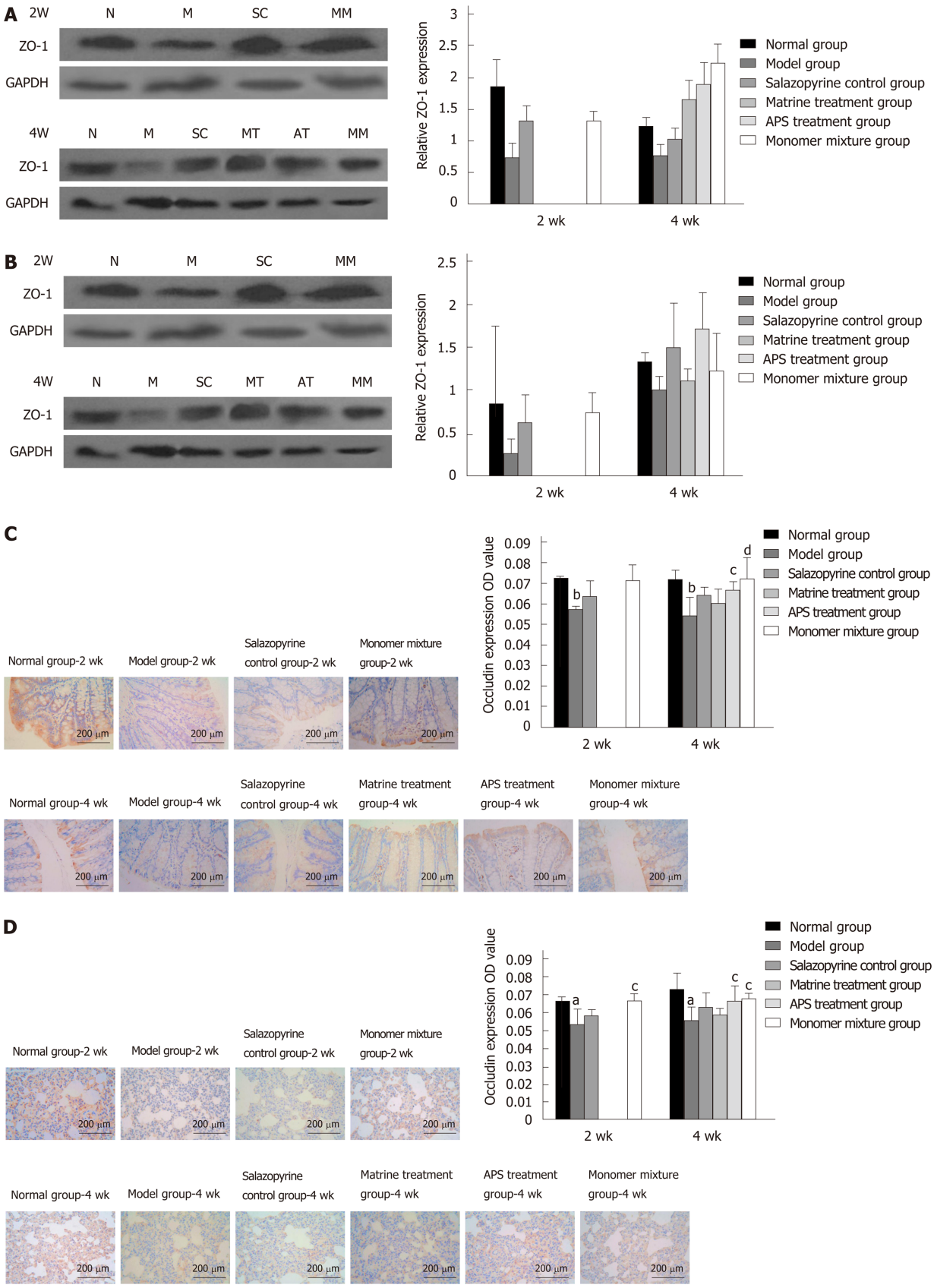

Figure 4 Astragalus polysaccharides combined with matrine inhibit the expression of zonula occludens-1 and Occludin in lung and colon tissues of rats with ulcerative colitis. A and B: Zonula occludens-1 (ZO-1) expression in colon (A) and lung tissues (B) in various groups detected by Western blot analysis; C and D: Occludin expression in colon (C) and lung tissues (D) in various groups detected by immunohistochemistry analysis. $^{a}P < 0.05$ and $^{b}P < 0.01$ *vs* normal group; $^{c}P < 0.05$ and $^{d}P < 0.01$ *vs* model group. APS: Astragalus polysaccharides; ZO-1: Zonula occludens-1; N: Normal group; M: Model group; SC: Salazopyrine control group; MT: Matrine treatment group; AT: Astragalus polysaccharides treatment group; MM: Monomer mixture group.

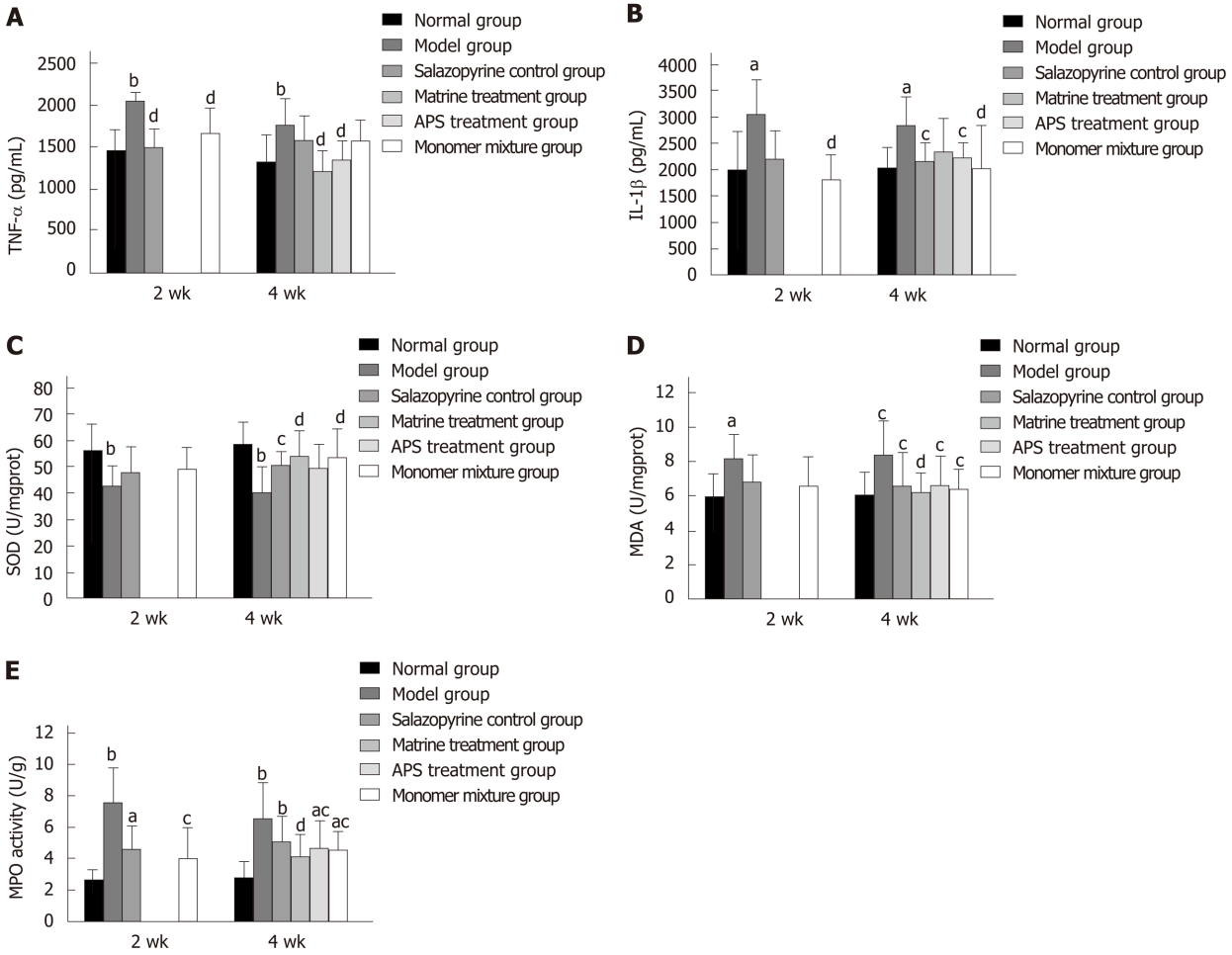

Figure 5 Astragalus polysaccharides combined with matrine relieve inflammatory response and oxidative stress injury in lung tissues of rats with ulcerative colitis. A and B: Tumor necrosis factor-α (A) and interleukin-1β levels (B) in lung tissues in various groups detected by enzyme-linked immunosorbent assay (ELISA); C-E: Activities of superoxide dismutase (C), malondialdehyde (D), and myeloperoxidase (E) in lung tissues in various groups determined with commercial detection kits. $^{a}P < 0.05$ and $^{b}P < 0.01$ *vs* normal group; $^{c}P < 0.05$ and $^{d}P < 0.01$ *vs* model group. APS: Astragalus polysaccharides; TNF-α: Tumor necrosis factor-α; IL-1β: Interleukins-1β; MPO: Myeloperoxidase.

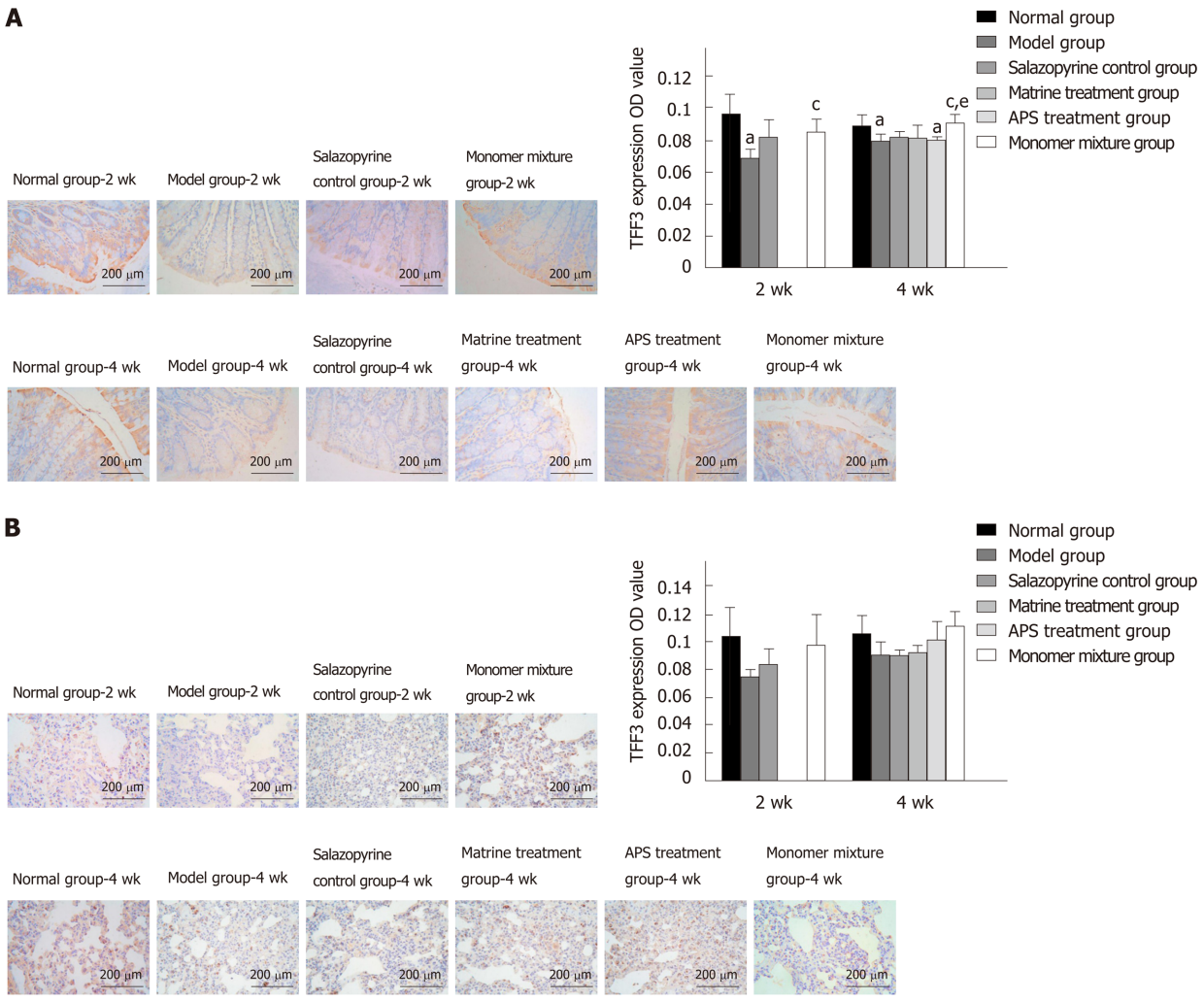

Figure 6 Astragalus polysaccharides combined with matrine increase trefoil factor 3 expression in lung and colon tissues of rats with ulcerative colitis. A and B: Trefoil factor 3 (TFF3) expression in colon (A) and lung tissues (B) in various groups detected by immunohistochemistry analysis. [a]*P* < 0.05 *vs* normal group; [c]*P* < 0.05 *vs* model group; [e]*P* < 0.05 *vs* salazopyrine control group. APS: Astragalus polysaccharides; TFF3: Trefoil factor 3.

ARTICLE HIGHLIGHTS

Research background

Astragalus polysaccharides (APS) are bioactive components extracted from the radix of *Astragalus membranaceus*, a commonly used herbal compound in traditional Chinese medicine.

Research motivation

APS was reported to have anti-inflammatory, anti-oxidative, anti-tumor, and anti-diabetic properties.

Research objectives

To evaluate the therapeutic effect of APS and its potential mechanisms in a ulcerative colitis (UC) rat model induced by colon mucosal tissue sensitization combined with trinitro-benzene-sulfonic acid-ethanol.

Research methods

First, we used two groups of Wistar rats: UC models and controls. Then, 105 UC model rats were randomly divided to five groups: Model group (n = 25), salazopyrine control group (n = 25), APS treatment group (n = 15), matrine treatment group (n = 15), and monomer mixture group (n = 25).

Research results

The inflammatory response and oxidative stress injury was relieved in colitis observed in APS combined with matrine-treated mice.

Research conclusions

APS combined with matrine may represent a potential therapeutic approach for treating inflammatory bowel disease.

Research perspectives

Drug research can provide a valuable resource to help clinicians make strategic treatment choices that will ultimately benefit patients at many levels.

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ORIGINAL ARTICLE

Retrospective Cohort Study

Risk of gastrointestinal cancer in a symptomatic cohort after a complete colonoscopy: Role of faecal immunochemical test

Noel Pin-Vieito, María J Iglesias, David Remedios, Lorena Rodríguez-Alonso, Francisco Rodriguez-Moranta, Victoria Álvarez-Sánchez, Fernando Fernández-Bañares, Jaume Boadas, Eva Martínez-Bauer, Rafael Campo, Luis Bujanda, Ángel Ferrandez, Virginia Piñol, Daniel Rodríguez-Alcalde, Jordi Guardiola, Joaquín Cubiella, on behalf of the COLONPREDICT study investigators

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the data and accuracy of the data analysis; Cubiella J and Pin-Vieito N acted as full guarantors of the research.

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Institutional review board statement: This is a post hoc cohort analysis performed within two prospective diagnostic test studies. The protocols of both studies conform to the ethical guidelines of the 1975 Declaration of Helsinki. COLONPREDICT study was approved by the Clinical Research Ethics Committee of Galicia (Code 2011/038) under resolution dated 11 April, 2012. The study of Rodríguez-Alonso *et al*. was approved by Bellvitge Hospital Clinical Research Ethics Committee (Code 21/11) on 1 December, 2011.

Informed consent statement: Patients of both studies provided written informed consent before inclusion.

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Data sharing statement: The original anonymous dataset is available on request from the corresponding author at noel.pin.vieito@sergas.es.

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Abstract

BACKGROUND

Faecal immunochemical test (FIT) has been recommended to assess symptomatic patients for colorectal cancer (CRC) detection. Nevertheless, some conditions could theoretically favour blood originating in proximal areas of the gastrointestinal tract passing through the colon unmetabolized. A positive FIT result could be related to other gastrointestinal cancers (GIC).

AIM

To assess the risk of GIC detection and related death in FIT-positive symptomatic patients (threshold 10 μg Hb/g faeces) without CRC.

METHODS

Post hoc cohort analysis performed within two prospective diagnostic test studies evaluating the diagnostic accuracy of different FIT analytical systems for CRC and significant colonic lesion detection. Ambulatory patients with gastrointestinal symptoms referred consecutively for colonoscopy from primary and secondary healthcare, underwent a quantitative FIT before undergoing a complete colonoscopy. Patients without CRC were divided into two groups (positive and negative FIT) using the threshold of 10 μg Hb/g of faeces and data from follow-up were retrieved from electronic medical records of the public hospitals involved in the research. We determined the cumulative risk of GIC, CRC and upper GIC. Hazard rate (HR) was calculated adjusted by age, sex and presence of significant colonic lesion.

RESULTS

We included 2709 patients without CRC and a complete baseline colonoscopy, 730 (26.9%) with FIT ≥ 10 μgr Hb/gr. During a mean time of 45.5 ± 20.0 mo, a GIC was detected in 57 (2.1%) patients: An upper GIC in 35 (1.3%) and a CRC in 14 (0.5%). Thirty-six patients (1.3%) died due to GIC: 22 (0.8%) due to an upper GIC and 9 (0.3%) due to CRC. FIT-positive subjects showed a higher CRC risk (HR 3.8, 95%CI: 1.2-11.9) with no differences in GIC (HR 1.5, 95%CI: 0.8-2.7) or upper GIC risk (HR 1.0, 95%CI: 0.5-2.2). Patients with a positive FIT had only an increased risk of CRC-related death (HR 10.8, 95%CI: 2.1-57.1) and GIC-related death (HR 2.2, 95%CI: 1.1-4.3), with no differences in upper GIC-related death (HR 1.4, 95%CI: 0.6-3.3). An upper GIC was detected in 22 (0.8%) patients during the first year. Two variables were independently associated: anaemia (OR 5.6, 95%CI: 2.2-13.9) and age ≥ 70 years (OR 2.7, 95%CI: 1.1-7.0).

CONCLUSION

Symptomatic patients without CRC have a moderate risk increase in upper GIC, regardless of the FIT result. Patients with a positive FIT have an increased risk of post-colonoscopy CRC.

Key words: Colonoscopy; Colorectal cancer; Faecal immunochemical test; Gastric cancer; Gastroesophageal cancer; Gastrointestinal cancer; Symptoms

Core tip: Our study, evaluates for the first time whether symptomatic patients with a positive faecal immunochemical test (FIT) result, no colorectal cancer (CRC) and a complete exploration of the colon have increased risk of related gastrointestinal cancer (GIC) detection or death. We found that this cohort of patients only have an increased risk of related CRC and death when compared with the cohort with a negative FIT result. Although the risk of upper GIC is higher than expected, the probability of detecting an upper GIC is unrelated to the FIT result and only associated with anaemia and advanced age.

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INTRODUCTION

The use of quantitative faecal immunochemical test (FIT) is increasing outside the screening setting. FIT has proved its ability to identify which symptomatic patients are more likely to have an underlying colorectal cancer (CRC) or even other significant colonic lesions (SCL). Therefore, it is useful to improve the suitability of referrals for investigation of abdominal symptoms[1].

In this sense, the National Institute for Health and Care Excellence (NICE) has recently recommended adoption of FIT in primary care to guide referral for suspected CRC in people without rectal bleeding who have unexplained symptoms but do not meet the criteria for a suspected cancer pathway referral, and results should be reported using a threshold of 10 μg Hb/g faeces[2,3].

This has been possible due to the progressive replacement of the guaiac-based faecal occult blood test by the immunochemical-based test. FIT reacts with human globin, a protein digested by enzymes in the upper gastrointestinal tract (GIT), so it should have greater specificity to detect lower GIT lesions than guaiac-based tests and is not modified by diet[4,5]. Nevertheless, some conditions (*e.g.*, altered bowel habit, prior gastrectomy) could theoretically favour blood originating in proximal areas of the GIT passing through the colon unmetabolized. A previous systematic review led to the conclusion that there is insufficient evidence to recommend for or against routine esophagogastroduodenoscopy (EGD) in patients with a positive faecal occult blood test followed by negative colonoscopy[6].

However, all the studies included were mainly based on faecal occult blood test that used the guaiac method or had been performed in a screening setting. Thus, conclusions drawn from these data cannot be extrapolated to the application of FIT in symptomatic patients. These patients may require additional diagnostic workup as long as complaints could be related to bleeding lesions located in the GIT proximal to the colon[7]. Thus, we aim to assess the risk of gastrointestinal cancers (GIC) detection and related death in symptomatic patients with a positive determination of FIT (≥ 10 μg Hb/g faeces) without CRC at baseline quality colonoscopy and to evaluate whether it might be worthwhile to perform additional evaluations to detect an upper GIC.

MATERIALS AND METHODS

Study design

This is a post hoc cohort analysis performed within two prospective diagnostic test studies evaluating the diagnostic accuracy of different FIT analytical systems for CRC and SCL detection[8,9].

We followed the Strengthening the Reporting of Observational studies in Epidemiology statement to conduct and report our study[10]. The main characteristics of the different cohorts have been detailed elsewhere[8,9].

Inclusion and exclusion criteria

The study population consisted of ambulatory patients with gastrointestinal symptoms referred consecutively for colonoscopy from primary and secondary healthcare in ten out of the thirteen hospitals that took part in the primary studies. Patients included in the analysis underwent a quantitative FIT before undergoing a complete colonoscopy. Patients were excluded from this analysis if a CRC was detected on baseline exploration or the colonoscopy was incomplete. A colonoscopy was considered complete if more than 90% of the mucosa could be evaluated according to the Aronchick scale and caecal intubation was achieved[11]. In addition, patients were excluded from this analysis if follow-up after colonoscopy was insufficient (< 2 years) or a GIC was diagnosed before basal colonoscopy.

Definition of cohorts

Patients were divided into two groups (positive and negative FIT) using the threshold of 10 μg Hb/g of faeces. All individuals collected a stool sample from one bowel movement without specific diet or medication restrictions before colonoscopy. Characteristics of the different FIT system used are shown in Table 1. Estimates of faecal haemoglobin (f-Hb) were quantitated as μg Hb/g of faeces so that results could be compared across analytical systems[12].

Colonoscopy and pathology

The colonoscopist was blinded to the FIT results. The bowel was cleansed and sedated as previously reported and all colonoscopies were performed by experienced endoscopists who reported any colorectal lesion and obtained biopsies if appropriate[13]. All polyps were removed either upon baseline exploration or afterwards.

SCL was defined as advanced adenoma (any adenoma ≥ 10 mm, with high-grade dysplasia or villous histology), histologically confirmed colitis (any aetiology), polyps ≥ 10 mm, polyposis (> 10 polyps of any histology), complicated diverticular disease (bleeding, diverticulitis), bleeding angiodysplasia and colonic ulcer. Any other colonic lesion was considered non-significant.

Follow up and main outcome

The main outcomes of this analysis are GIC detection and GIC-related death. Data from follow-up were retrieved from electronic medical records of the public hospitals involved in the research. For all patients, cancer diagnoses of any aetiology were recorded. We classified all cancers that could justify the presence of blood in the GIT as a GIC: Oral, throat, oesophageal, gastric, intestinal and CRC. We defined an upper GIC as a cancer that can be detected in an EGD exploration: Oesophageal, gastric, duodenal or ampullary cancer. The cause and date of death were recorded. We pooled the different causes of death into five categories: related to (1) GIC, (2) Upper GIC, (3) CRC, (4) Global cancer or (5) Global death.

Data analysis

We first performed a descriptive analysis of the cohorts included in the analysis. We determined whether there were differences using the Chi-square and student *t* test in the qualitative and quantitative variables, respectively. We calculated cumulative risk and number of cases per 1000 patient-years and its 95% confidence interval (CI). Differences in cumulative risk were analysed with the Chi-square test and Cochran–Mantel–Haenszel statistics and expressed as the risk ratio (RR) and its 95%CI. In order to control confounding variables, age, sex and SCL, we performed a Cox regression analysis to determine the hazard ratio (HR) of detecting a new cancer and cancer-related death respectively.

In order to determine whether there was an association between the baseline faecal haemoglobin concentration and length of time to GIC detection, we performed a descriptive analysis and a correlation analysis. We determined the Spearman correlation coefficient (r).

Finally, we evaluated which variables were associated with detection of any upper GIC during the first year after baseline colonoscopy. In this respect, we determined which variables had a statistically significant association with detection of an upper GIC using the Chi-square and the Cochran–Mantel–Haenszel statistics and expressed the differences as RR and its 95%CI. We included variables with a statistically significant association ($P < 0.05$) in a multivariate logistic regression analysis and expressed the association as the odds ratio (OR) and 95%CI. Statistical analysis was performed using SPSS statistical software, version 15.0 (SPSS Inc., Chicago, IL, USA)

The statistical methods of this study were reviewed by Noel Pin Vieito from Complexo Hospitalario Universitario de Ourense.

RESULTS

Participants and descriptive data

We excluded 1347 patients out of the 4056 symptomatic patients initially included in both studies, yielding a final sample of 2709 (Figure 1).

Of these participants, 1979 (73.1%) and 730 (26.9%) had a negative and positive FIT, respectively. The cohorts included were different in terms of age, sex, healthcare referring to colonoscopy, colonoscopy indication, findings in baseline exploration and length of follow-up as shown in Table 2.

Table 1 Characteristics of the different faecal immunochemical tests evaluated

Ref.	Country	Analytical system for estimation of faecal haemoglobin concentration
Cubiella *et al*[9], 2016 (DC)	Spain	OC-Sensor: 100%
Rodríguez-Alonso *et al*[8], 2015	Spain	OC-Sensor: 100%
Cubiella *et al*[9], 2016 (VC)	Spain	OC-Sensor: 49.7%; OC-Auto 3 Latex 13.8%; FOB Gold 2.4%; Linear i-FOB 34.1%
Overall	Spain	OC-Sensor: 81.8%; OC-Auto 3 Latex 5.0%; FOB Gold 0.9%; Linear i-FOB 12.3%

DC: Derivation cohort; VC: Validation cohort.

Cancer incidence and death

During a mean time of 45.5 ± 20.0 mo, a GIC was detected in 57 (2.1%) patients: An upper GIC (six oesophageal carcinomas, 25 gastric carcinomas, one duodenal adenocarcinoma, two ampullary carcinomas and one duodenal GIST) in 35 (1.3%), a CRC in 14 (0.5%) and other GIC (three cholangiocarcinomas, two small bowel adenocarcinomas, one small bowel lymphoma, one lingual carcinoma and one piriform sinus carcinoma) in 8 (0.3%). The distribution of the GIC according to the FIT result is shown in Figure 1. Thirty-six patients (1.3%) died due to GIC: 22 (0.8%) due to an upper GIC and 9 (0.3%) due to CRC. Finally, 205 (7.6%) patients developed a cancer and 197 (7.3%) died, 98 (3.5%) due to cancer. Cumulative risk and number of cancers and death per 1000 patient-years is shown in Table 3.

Patients with positive FIT showed greater GIC risk (≥ 10 μg/g of faeces = 3.2%, < 10 μg/g of faeces = 1.7%; RR 1.9, 95%CI: 1.1-3.2) and GIC-related mortality (≥ 10 μg/g of faeces = 2.3%, < 10 μg/g of faeces = 1.0%; OR 2.5, 95%CI: 1.3-4.6). In the subgroup analysis, patients in the positive FIT cohort had an increased risk of CRC (≥ 10 μg/g of faeces = 1.1%, < 10 μg/g of faeces = 0.3%; RR 3.6, 95%CI: 1.3-10.5) and CRC-related mortality (≥ 10 μg/g of faeces = 1.0%, < 10 μg/g of faeces = 0.1%; RR 9.5, 95%CI: 2.0-46.2) but no differences in upper GIC or upper GIC-related mortality as shown in Table 3. However, in the Cox's proportional multivariate regression analysis, patients with a positive FIT had only an increased risk of CRC (HR 3.8, 95% CI 1.2-11.9), CRC-related death (HR 10.8, 95%CI: 2.1-57.1) and GIC-related death (HR 2.2, 95%CI: 1.1-4.3), after adjusting for confounding variables. The cumulative risk of cancer and related death calculated in the Cox's multivariate regression analysis is shown in Figure 2.

Faecal haemoglobin concentration and time of cancer diagnosis

Figure 3 links time elapsed until diagnosis of each GIC throughout follow-up with the FIT result. We did not detect a correlation between time to GIC diagnosis (r = -0.1; P = 0.4) or related death (r = -0.2; P = 0.3) and FIT result as shown in Figure 4.

Detection of upper GIC during the first year of follow-up

During the first year after baseline colonoscopy, 22 (0.8%) upper GIC were detected: 17 cases of gastric carcinomas, 4 oesophageal carcinomas and one ampullary carcinoma. Only two variables were independently associated with detection of an upper GIC during the first year: anaemia (OR 5.6, 95%CI: 2.2-13.9), defined as < 11 g/100 mL in men and < 10 g/100 mL in non-menstruating women, and age ≥ 70 years (OR 2.7, 95%CI: 1.1-7.0), as shown in Table 4.

Diagnosis of gastrointestinal cancer during follow up based on SCL detection at baseline colonoscopy

The distribution of GIC according to FIT result and presence of SCL at baseline colonoscopy is shown in Figure 5. For each subgroup, the minimum diagnostic yield of an upper endoscopy performed at the time of FIT determination, has been calculated assuming a theoretical 100% sensitivity for any esophageal or gastric bleeding tumor developed over the first year since performing baseline colonoscopy.

There were no significant differences in gastroesophageal cancer (GEC) diagnoses irrespective of FIT result, both in the subgroup of patients with SCL as well as in the subgroup with normal baseline colonoscopy.

Those results were similar when the analysis was limited to people aged 50 and older (Figure 6).

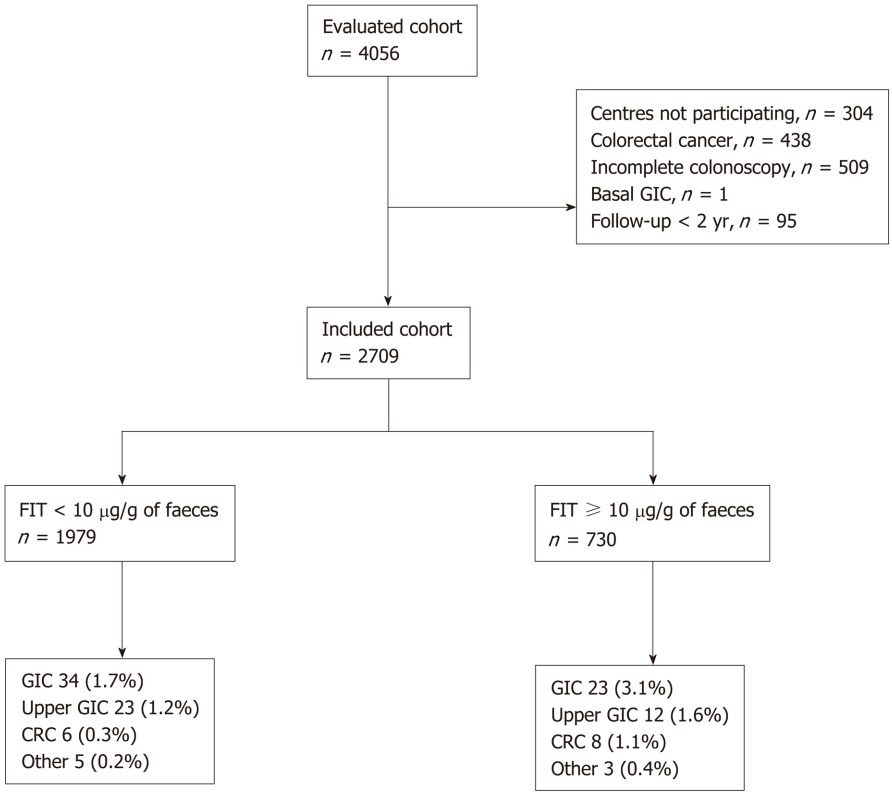

Figure 1 Study population flowchart. CRC: Colorectal cancer; FIT: Faecal immunochemical test; GIC: Gastrointestinal cancer.

DISCUSSION

Statement of principal findings

Our study, for the first time, evaluates whether symptomatic patients with a positive FIT result, no CRC and a complete exploration of the colon have increased risk of related GIC detection or death. We found that this cohort of patients only have an increased risk of related CRC and death when compared with the cohort with a negative FIT result. There are no differences in the risk of upper GIC between both cohorts. In addition, we have identified two variables independently associated with detection of an upper GIC during the first year: Anaemia and advanced age.

Strengths and weaknesses of our study

Our analysis has several strengths. The main one is that we have included a wide number of symptomatic patients who underwent FIT and colonoscopy in several public hospitals in Spain. In this sense, we have limited our analysis to subjects with complete baseline colonoscopy and resection of pre-neoplastic lesions. On the other hand, we performed follow-up analysis by means of search in the electronic medical records of our centres linked to the National Health System's Hospital Discharge Records Database (CMBD in Spanish), which receives notifications from around 98% of Spanish public hospitals that have seen to more than 99% of the Spanish population[14]. Since 2005, the CMBD also has partial coverage from private hospitals[15].

However, the main weakness of our analysis arises from differences between the cohorts in terms of demographics, basal symptoms, endoscopic findings or follow-up. Moreover, the risk of GIC during follow-up, as expected, is low. To solve this limitation, we performed a Cox multivariate regression analysis controlling by confounding variables and final results are consistent.

Strengths and weaknesses in relation to other studies with discussion of important differences in results

Our study detected a higher than expected risk of GIC in the patients evaluated, mainly related to upper GIC and CRC. Estimated 30-year risk of developing an upper GIC in the United States is 0.98%[16], which is lower than the risk detected in our symptomatic cohort. Moreover, the incidence of GEC is also notable even in patients

Table 2 Characteristics of the individuals included in the analysis, *n* (%)

Characteristics	Overall (*n* = 2709)	FIT < 10 μg/g (*n* = 1979)	FIT ≥ 10 μg/g (*n* = 730)	*P* value[8]
Demographic				
Age (yr)	62.9 ± 13.5	62.0 ± 13.5	65.5 ± 13.0	< 0.001
Female sex	1432 (52.9)	1084 (54.8)	348 (47.7)	0.001
Primary healthcare referral[1]	617 (24.2)	397 (21.2)	220 (32.2)	< 0.001
Previous colonoscopy[2]	444 (25.9)	287 (25.8)	157 (26.0)	0.9
Daily using ASA[2]	330 (19.2)	193 (17.3)	137 (22.7)	0.01
Indications				
Rectal bleeding[1]	1234 (48.3)	843 (45.1)	391 (57.2)	< 0.001
Change of bowel habit[1]	1271 (49.8)	913 (48.8)	358 (52.4)	0.1
Anaemia[3,4]	368 (16.2)	236 (13.9)	132 (23.1)	< 0.001
Abdominal pain[5]	766 (41.3)	587 (41.8)	179 (40.0)	0.4
Weight loss[5]	391 (21.1)	301 (21.4)	90 (20.1)	0.6
Basal colonoscopy findings				
Benign anorectal lesion[2]	756 (44.0)	495 (44.4)	261 (43.3)	0.6
Significant colonic lesions[6]	480 (17.7)	204 (10.3)	276 (37.8)	< 0.001
Advanced adenoma[1,7]	337 (13.2)	139 (7.4)	198 (29.0)	< 0.001
Follow-up (mo)	45.5 ± 20.0	47.9 ± 21.2	39.2 ± 14.1	< 0.001

Missing data in
[1]156,
[2]992,
[3]441 and
[4]Defined as < 11 g/100 mL in men and < 10 g/100 mL in non-menstruating women.
[5]856 patients.
[6]Advanced adenoma (≥ 10 mm, villous histology, high-grade dysplasia), polyposis (> 10 polyps of any histology), colitis (any aetiology), polyps ≥ 10 mm, complicated diverticular disease, colonic ulcer and/or bleeding angiodysplasia.
[7]Adenoma ≥ 10 mm, villous histology or high-grade dysplasia.
[8]Differences between both groups in the Chi-square test in the qualitative variables and in the student *t* test in the quantitative variables. Differences with *P* < 0.05 are considered statistically significant. Qualitative variables are expressed as absolute numbers and percentages. Quantitative variables are expressed as mean and standard deviation. ASA: Acetyl salicylic acid; FIT: Faecal immunochemical test.

with a positive FIT result who were diagnosed with a SCL in the baseline colonoscopy, which could theoretically justify the presence of haemoglobin in faeces. This is related to the lack of specificity of symptoms related to diagnosis of cancer. In this sense, we believe that most GIC detected are prevalent. As an example, anaemia, although mainly related to CRC, is related to any GIC with positive predictive values ranging between 1% and 5% of the population seen in primary healthcare[2].

FIT has been recommended for adoption in primary care to guide referral for suspected colorectal cancer in people without rectal bleeding who have unexplained symptoms but do not meet the criteria for a suspected cancer pathway referral. Furthermore, NICE has recommended 10 μg Hb/g of faeces as the threshold for further evaluation referral[3]. This recommendation is based on the high accuracy of the test for CRC detection in symptomatic patients[1,17]. However, one practical doubt when using FIT in symptomatic patients is what to do with "false positive" results. Most evidence available comes from asymptomatic patients and suggests that a positive FIT is not predictive of prevalent GIC[7,18,19]. A recently published study revealed that only 0.14% of all persons with a positive FIT result were diagnosed with gastric or oesophageal cancer within 3 years and the risk was similar to the group with negative FIT[20]. Our study evaluates, for the first time, the risk of GIC after a false positive FIT result. In this sense, the probability of detecting an upper GIC is not modified by the FIT result.

It is noteworthy that our study did not exclude patients with high risk symptoms as rectal bleeding which are outside of NICE recommendation. However, most of the studies included in the meta-analysis that supports NICE recommendation[1], were not only concerned with patients with low risk symptoms (*i.e.*, rectal bleeding is described in several patients in those studies). That clinical concern was highlighted by Fraser[21] and led to the development of an additional review and meta-analysis to obtain more information about the accuracy of FIT through the broad spectrum of symptomatic patients[17]. In our cohort, the risk of GIC cancer tends to be lower in patients with rectal bleeding. Probably, this is due to this symptom's being less subjective than others like abdominal pain and more specific to the colon. Thus, unlike other

Table 3 Risk of cancer and death according to faecal immunochemical test result

Event	Risk	Overall (*n* = 2709)	FIT < 10 μg/g (*n* = 1979)	FIT ≥10 μg/g (*n* = 730)	RR[1] (95%CI)	HR[2] (95%CI)
GIC	Cumulative[3]	2.1% (1.6-2.6)	1.7% (1.1-2.3)	3.2% (1.9-4.4)	1.9 (1.1-3.2)	1.5 (0.8-2.7)
GIC	Density[4]	5.6 (4.1-7.0)	4.3 (2.9-5.8)	9.7 (5.8-13.7)		
GIC	Cumulative death[3]	1.3% (0.9-1.8)	1.0% (0.5-1.4)	2.3% (1.2-3.4)	2.5 (1.3-4.7)	2.2 (1.1-4.3)
GIC	Death density[4]	3.5 (2.4-4.6)	2.4 (1.3-3.5)	7.1 (3.7-10.5)		
Up GIC[5]	Cumulative[3]	1.3% (0.9-1.7)	1.2% (0.7-1.6)	1.6% (0.7-2.6)	1.4 (0.7-2.8)	1.0 (0.5-2.2)
Up GIC[5]	Density[4]	3.4 (2.3-4.6)	2.9 (1.7-4.1)	5.1 (2.2-8.0)		
Up GIC[5]	Cumulative death[3]	0.8% (0.5-1.2)	0.7% (0.3-1.0)	1.2% (0.4-2.0)	1.6 (0.7-3.7)	1.4 (0.6-3.3)
Up GIC[5]	Death density[4]	2.1 (1.2-3.0)	1.6 (0.8-2.5)	3.8 (1.3-6.2)		
CRC	Cumulative[3]	0.5% (0.2-0.8)	0.3% (0.1-0.5)	1.1% (0.3-1.9)	3.6 (1.3-10.5)	3.8 (1.2-11.9)
CRC	Density[4]	1.4 (0.7-2.1)	0.8 (0.2-1.4)	3.4 (1.0-5.7)		
CRC	Cumulative death[3]	0.3% (0.1-0.5)	0.1% (0.0-0.2)	1.0% (0.3-1.7)	9.5 (2.0-46.2)	10.8 (2.1-57.1)
CRC	Death density[4]	0.9 (0.3-1.4)	0.3 (-0.1-0.6)	2.9 (0.8-5.1)		
Cancer	Cumulative[3]	7.6% (6.6-8.6)	7.3% (6.2-8.5)	8.2% (6.2-10.2)	1.1 (0.8-1.5)	1.1 (0.8-1.5)
Cancer	Density[4]	20.5 (17.7-23.3)	18.8 (15.8-21.9)	25.9 (19.4-32.5)		
Cancer	Cumulative death[3]	3.6% (2.9-4.3)	3.2% (2.4-4.0)	4.8% (3.2-6.3)	1.5 (1.0-2.3)	1.4 (0.9-2.2)
Cancer	Death density[4]	9.5 (7.6-11.4)	8.0 (6.0-9.9)	14.7 (9.8-19.6)		
Death	Cumulative[3]	7.3% (6.3-8.2)	7.0% (5.9-8.1)	7.9% (6.0-9.9)	1.1 (0.8-1.5)	1.1 (0.8-1.5)
Death	Density[4]	19.2 (16.5-21.8)	17.6 (14.7-20.5)	24.3 (18.1-30.6)		

[1]Differences in cumulative incidence were analysed with the Chi-square and the Cochran–Mantel–Haenszel statistics and expressed as the RR and its 95%CI in the qualitative variables.
[2]Differences in the risk of cancer and death adjusted by age, sex and presence of significant colonic lesion were analysed with a Cox multivariate regression and expressed as HR and its 95%CI.
[3]Cumulative risk is expressed as percentage and its 95%CI.
[4]Risk density rate is expressed per 1000 patient-years and its 95%CI.
[5]Defined as a cancer located in the oesophagus, stomach, duodenum or ampulla. CI: Confidence interval; CRC: Colorectal cancer; FIT: Faecal immunochemical test; GIC: Gastrointestinal cancer RR: Risk ratio; Up GIC: Upper gastrointestinal cancer.

indications, patients with overt bleeding who underwent a quality colonoscopy that ruled out CRC were less likely to be diagnosed with an upper GIC.

Although the risk is low, CRC risk is increased in symptomatic subjects with positive FIT even after a high-quality colonoscopy when compared to patients with a negative test. This finding is worthy of several comments. CRC detected fall into the definition of a post-colonoscopy colorectal cancer (PCCRC)[22]. In fact, the rate of PCCRC detected, approximately 3%, is located in the expected segment between 2.5% and 7.7%. However, we must highlight that the risk of PCCRC is higher after a positive FIT, probably due to the higher prevalence in this group of patients. This finding should be taken into account by physicians if symptoms persist after a normal colonoscopy. Finally, the risk of PCCRC calculated per 1000 colonoscopies is higher than the risk previously documented ranging between 0.8 and 2.4[23]. Our population consists of symptomatic patients with a CRC prevalence in the original studies ranging between 3.0% and 13.7%. We therefore suggest that the risk of PCCRC should be evaluated on the basis of the colonoscopy indication. However, the sample size of our analysis and the low number of PCCRC detected did not enable us to analyse additional factors that could predict the risk of PCCRC, such as age, comorbidity and diverticular disease, or the relationship with baseline symptoms[24].

A recent study conducted in patients taking part in CRC screening has associated the presence of detectable f-Hb with increased risk of death from a wide range of causes unrelated to CRC or even GIC[25]. In that study, Libby *et al*[26] consider the possibility of detectable f-Hb originating from subclinical colonic inflammation due to a generalised inflammatory state. We did not find such an association. However, the threshold used in our study (10 μg Hb/g faeces) is much lower than the concentration of approximately 80 μg Hb/g faeces required to attain a positive result by means of the qualitative method used by Libby *et al*[26].

Meaning of the study: Possible explanations and implications for clinicians and policymakers

Early diagnosis of GIC is challenging as long as abdominal symptoms are common, mostly related to benign diseases and non-specific to a particular cancer. In fact, abdominal symptoms are very common among patients with cancer (23%), mainly

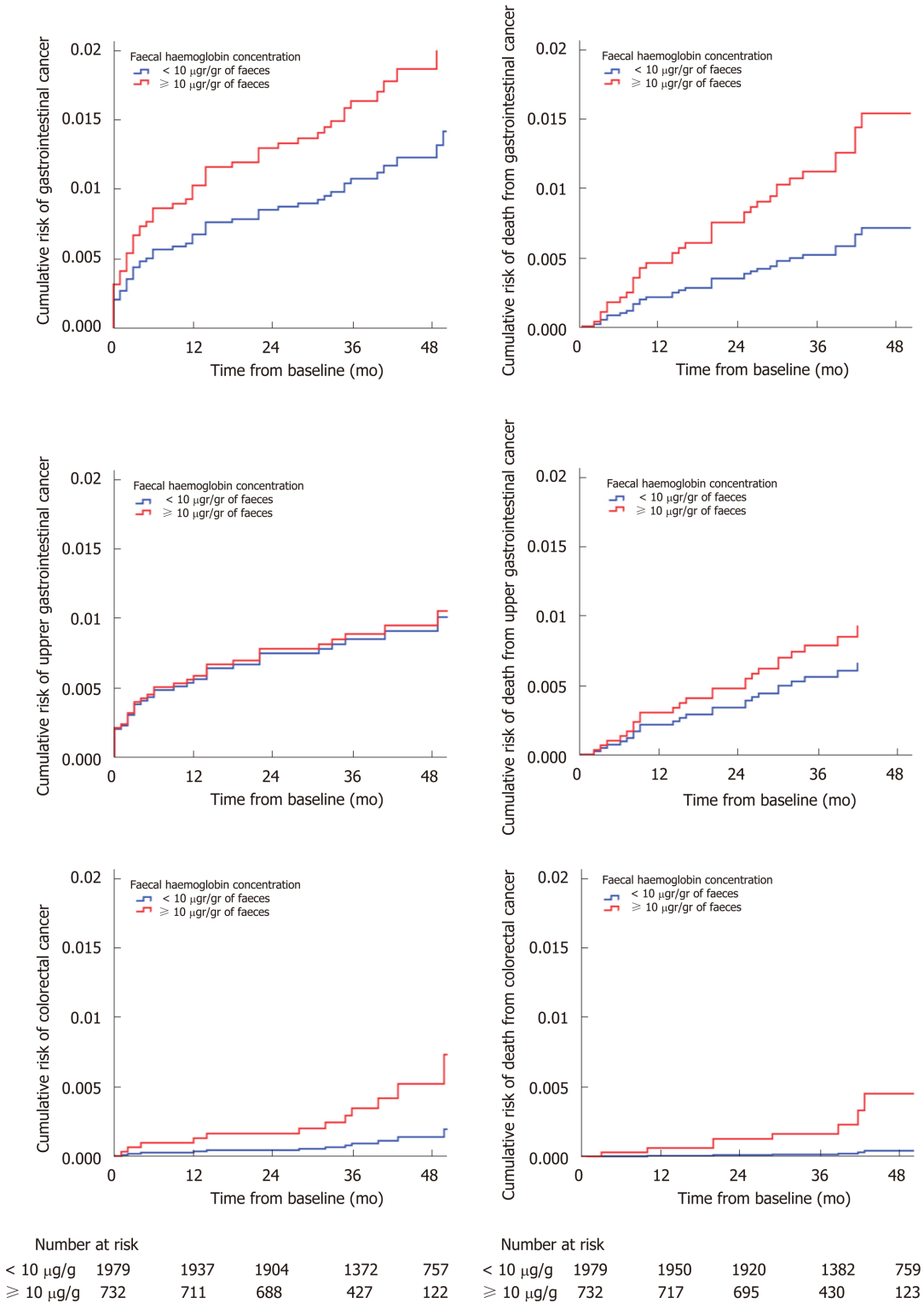

Figure 2 Cumulative risk of gastrointestinal cancer and related death. Cumulative risk of gastrointestinal cancer and related death during the first four years after baseline evaluation according to faecal haemoglobin concentration and adjusted by sex, age and presence of significant colonic lesion. The figure is calculated with a Cox's multivariate regression.

related to GIC and CRC in particular[27]. In contrast with breast cancer or melanoma, GIC have a broad symptom signature with varying predictive value[28]. In order to reduce delays in patients with lower abdominal symptoms with a low positive predictive value for CRC, FITs are recommended for adoption in primary care to guide referral for suspected CRC[3]. Our analysis aims to resolve a frequent issue that will take place when patients with lower abdominal symptoms are evaluated with a FIT. Hypothetically, 179-229 out of 1000 symptomatic patients will have a positive FIT

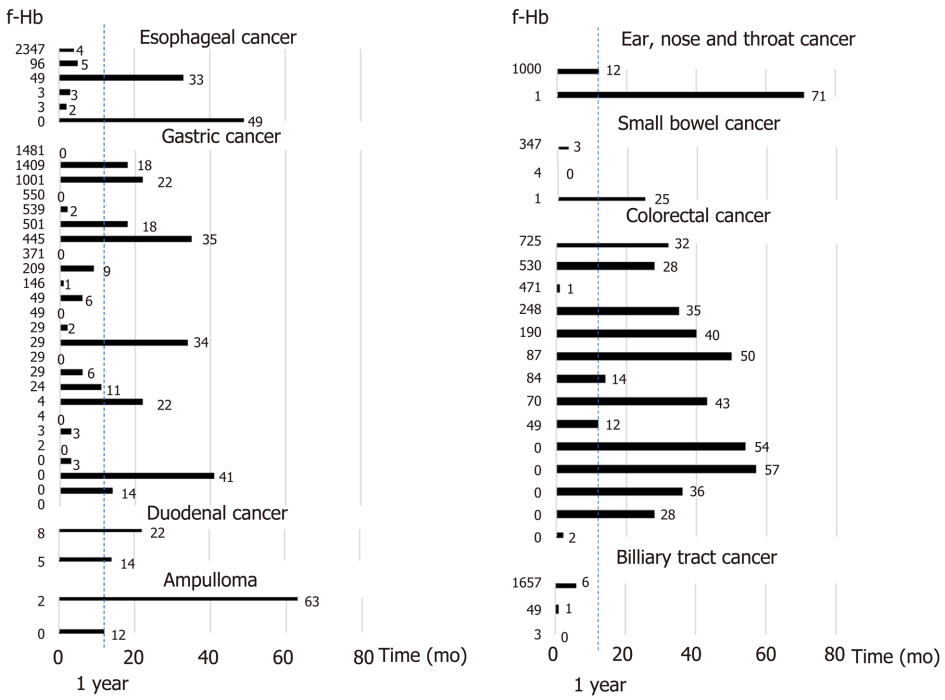

Figure 3 Time (mo) until gastrointestinal cancer diagnosis according to location, diagnostic test and faecal immunochemical test result. f-Hb: Faecal haemoglobin (μg Hb/g of faeces).

and colonoscopy without CRC[2]. As our results show, this patient cohort has a similar risk of GIC as the cohort with a negative FIT. In this situation, an EGD should be recommended in patients with anaemia especially if they are elderly. However, special caution should be taken with the risk of PCCRC after positive FIT and normal colonoscopy if abdominal symptoms persist or reappear.

Unanswered questions and future research

Our results are the basis to design a large prospective follow-up study including patients treated in primary healthcare with abdominal symptoms. In these patients, diagnostic evaluation should not be restricted to GIC. Other abdominal cancers in addition to benign gastrointestinal diseases should be evaluated to determine the positive predictive value and the best diagnostic strategy for each group of symptoms.

Additionally, a recent study concluded that endoscopic gastric cancer screening could be cost-effective if combined with a screening colonoscopy in countries with a gastric cancer risk ≥ 10 per 100000[29]. Given the gastroesophageal cancer incidences shown during the first year since FIT determination in our cohort irrespective of SCL finding in the basal colonoscopy, the cost-utility of combining upper and lower endoscopies should be investigated also in this setting.

To summarise, the risk of GIC is higher than expected in patients with low gastrointestinal symptoms and no CRC detected in a complete colonoscopy. The probability of detecting an upper GIC is unrelated to the FIT result and only associated with the presence of anaemia and advanced age. Finally, the risk of PCCRC in our study is within the ranges expected and clearly associated with the FIT result.

Table 4 Factors associated with upper gastrointestinal cancer detection the first year after baseline colonoscopy, *n* (%)

	Upper gastrointestinal cancer	Odds ratio (95 %CI)[1]	Odds ratio (95 %CI)[2]
Sex			
Female (*n* = 1432)	10 (0.7)	1	
Male (*n* = 1277)	12 (0.9)	1.3 (0.6-3.1)	
Age			
< 70 yr (*n* = 1757)	8 (0.5)	1	1
≥ 70 yr (*n* = 952)	14 (1.5)	3.3 (1.4-7.8)	2.7 (1.1-7.0)
Primary healthcare referral			
No (*n* = 1936)	19 (1.0)	1	
Yes (*n* = 617)	3 (0.5)	0.5 (0.1-1.7)	
Rectal bleeding			
No (*n* = 1319)	16 (1.2)	1	
Yes (*n* = 1234)	6 (0.5)	0.4 (0.1-1.0)	
Change of bowel habit			
No (*n* = 1282)	12 (0.9)	1	
Adequate (*n* = 1271)	10 (0.8)	0.8 (0.4-1.9)	
Anaemia[3]			
No (*n* = 2077)	13 (0.6)	1	1
Yes (*n* = 191)	8 (4.2)	6.9 (2.8-17.0)	5.6 (2.2-13.9)
Abdominal pain			
No (*n* = 1319)	12 (1.1)	1	
Yes (*n* = 1234)	5 (0.7)	0.6 (0.2-1.7)	
Weight loss			
No (*n* = 1462)	12 (0.8)	1	
Yes (*n* = 391)	5 (1.3)	1.5 (0.5-4.4)	
Faecal immunochemical test			
< 10 μg/g (*n* = 1979)	14 (0.7)	1	
≥ 10 μg/g (n = 730)	8 (1.1%)	1.5 (0.6-3.7)	
Benign anorectal lesion			
No (*n* = 961)	7 (0.7)	1	
Yes (*n* = 756)	6 (0.8)	1.1 (0.4-3.2)	
Significant colonic lesion[4]			
No (*n* = 2216)	16 (0.7)	1	
Yes (*n* = 480)	6 (1.3)	(0.7-4.5)	
Advanced adenoma[5]			
No (*n* = 2968)	16 (0.7)	1	
Yes (*n* = 337)	6 (1.8)	2.5 (1.0-6.4)	

[1]Differences were analysed with the Chi-square and Cochran–Mantel–Haenszel statistics and expressed as the odds ratio and its 95%CI.
[2]Variables with statistically significant differences were introduced in a multivariate logistic regression analysis. The association is expressed as odds ratio and its 95 %CI.
[3]Defined as < 11 g/100 mL in men and < 10 g/100 mL in non-menstruating women.
[4]Advanced adenoma (≥ 10 mm, villous histology, high-grade dysplasia), polyposis (> 10 polyps of any histology), colitis (any aetiology), polyps ≥ 10 mm, complicated diverticular disease, colonic ulcer and/or bleeding angiodysplasia.
[5]Adenoma ≥ 10 mm with villous histology or high-grade dysplasia.

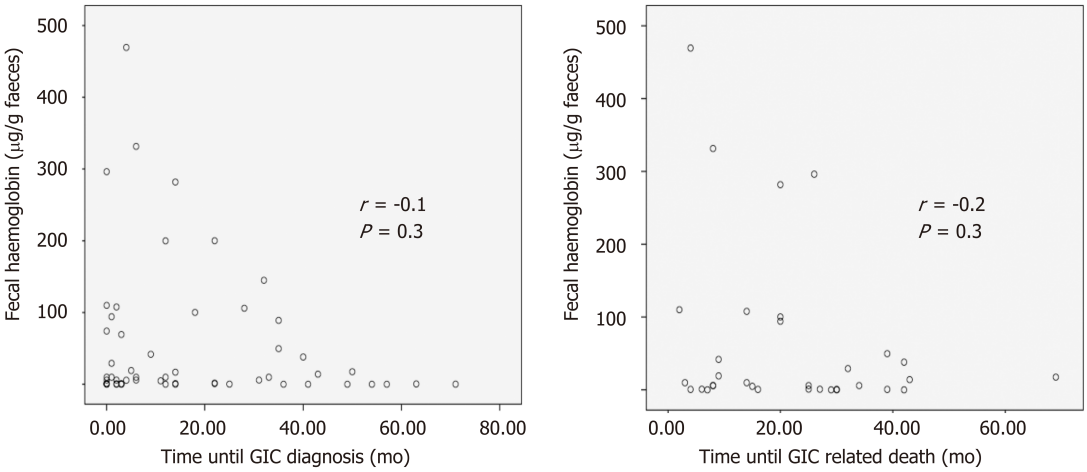

Figure 4 Correlation between faecal haemoglobin concentration and time to gastrointestinal cancer detection and gastrointestinal cancer-related death. GIC: Gastrointestinal cancer; *r*: Spearman correlation coefficient.

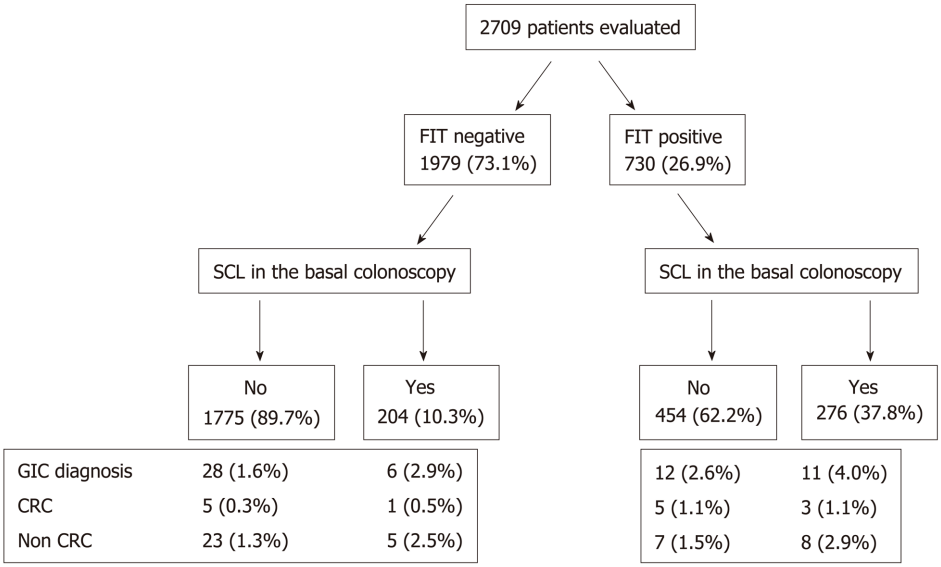

Figure 5 Gastrointestinal cancer diagnosis during follow up based on faecal immunochemical test result and significant colonic lesion in the basal colonoscopy. CRC: Colorectal cancer; FIT: Faecal immunochemical test; GITN: Gastrointestinal tract neoplasm; SCL: Significant colonic lesion.

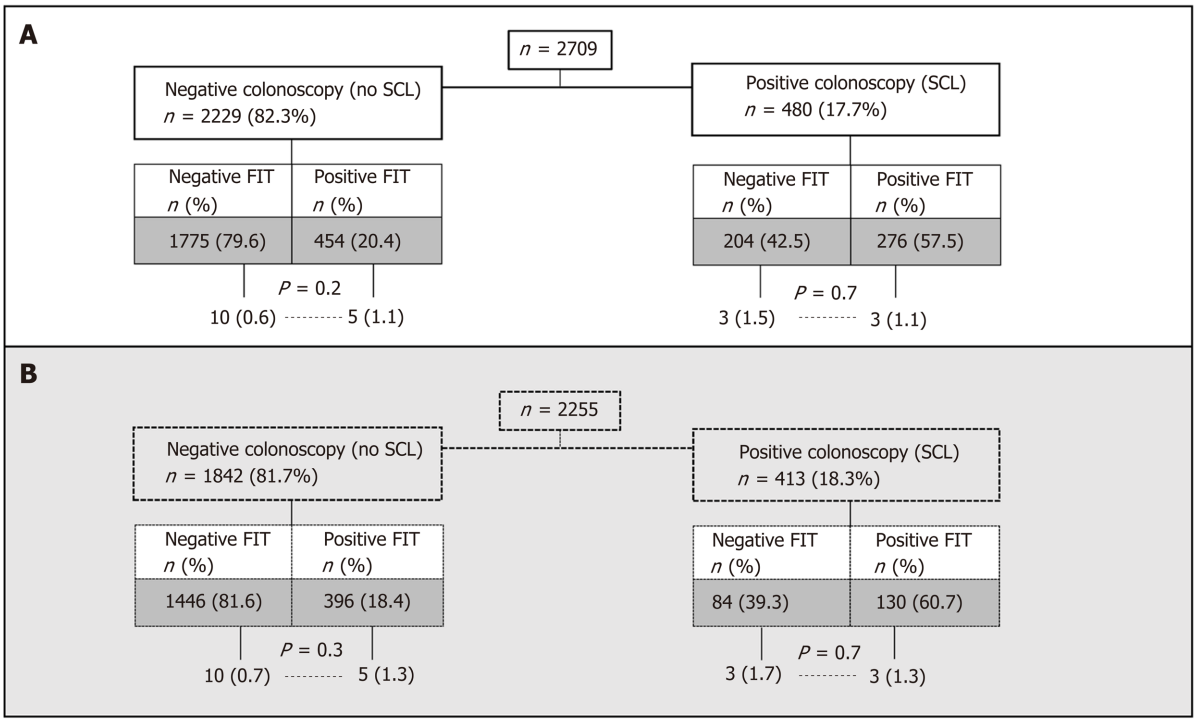

Figure 6 Minimum diagnostic yield of an upper endoscopy performed at the time of faecal immunochemical test for haemoglobin determination. A: All subjects; B: subjects aged 50 years old and older. FIT: Faecal immunochemical test for haemoglobin; SCL: Significant colonic lesion; *P*: Differences were analysed with the Chi-square statistics.

ARTICLE HIGHLIGHTS

Research background

Faecal immunochemical test for haemoglobin (FIT) is more specific and appears to be equal to or more sensitive than guaiac-based tests when used for colorectal cancer (CRC) screening. FIT reacts with human globin, so it should have greater specificity to detect lower gastrointestinal tract (GIT) lesions than guaiac-based tests. However, a previous systematic review led to the conclusion that there is insufficient evidence to recommend for or against routine esophagogastroduodenoscopy in asymptomatic patients with a positive faecal occult blood test followed by negative colonoscopy.

Research motivation

Out of a screening setting, several approaches have been developed to improve the suitability of referrals for investigation of symptoms suggestive of CRC and reduce delays in diagnosis and some include using FIT. Therefore, it will be increasingly common for clinicians to face the uncertainty of a patient with non-specific digestive symptoms, a positive FIT result and normal colonoscopy.

Research objectives

We aim to assess the risk of gastrointestinal cancer (GIC) detection and related death in symptomatic patients with a positive determination of FIT (threshold 10 μg Hb/g faeces) without CRC at baseline quality colonoscopy.

Research methods

We performed a post hoc cohort analysis within two prospective diagnostic test studies evaluating the diagnostic accuracy of FIT for CRC detection. Outpatients with gastrointestinal symptoms referred consecutively for colonoscopy from primary and secondary healthcare were divided into two groups (positive and negative FIT) using the threshold of 10 μg Hb/g of faeces and data from follow-up were retrieved from their electronic medical records. We determined the cumulative risk of GIC, CRC and upper GIC. Hazard rate was calculated adjusted by age, sex and presence of significant colonic lesion on basal colonoscopy.

Research results

This study revealed high neoplasia and death rates in our cohort (n = 2709) of people consulting with a physician for non-acute symptoms suggestive of lower gastrointestinal tract disorders. FIT-positive patients have higher incidence of GIC during follow-up. However, this did not result in a statistically significant increase in the risk of upper GIC development after multivariate adjustment. Moreover, we found that this cohort of patients only has an increased

risk of related CRC and death when compared to the cohort with a negative FIT result.

Research conclusions

This study suggests that FIT positivity using the threshold of 10 μg Hb/g of faeces is not enough to differentiate which patients would benefit from continuing workup to rule out a GIC out of screening setting. Nevertheless, small amounts of f-Hb may originate in the upper GI tract or the small bowel and this possibility must be considered along with other false-positive risk factors when interpreting FIT requested to rule out CRC or another significant colonic lesion.

Research perspectives

We hypothesize that benign lesions (*i.e.* due to non-steroid anti-inflammatory drugs) are much more prevalent than GIC in the upper tract regardless of symptoms. Thus, it is much more likely that a small amount of detectable (unmetabolized) haemoglobin, originally from any kind of lesion located in the upper tract or the small bowel will be unrelated to a GIC. However, the study design is not suitable to prove this hypothesis. A large prospective follow-up study which takes competitive FIT positive causes and other risk factors into consideration would provide a predictive model to guide decision-making.

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The contents of this manuscript have not been published previously in any journal nor deposited on a pre-print server. Furthermore, this paper has not been presented at any scientific meeting in abstract form.

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ORIGINAL ARTICLE

Retrospective Study

Prognostic value of serum microRNA-122 in hepatocellular carcinoma is dependent on coexisting clinical and laboratory factors

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Author contributions: Franck M performed the experiments; Schütte K and Malfertheiner P provided clinical material; Franck M, Malfertheiner P and Link A did the analysis and interpretation of the data and drafting of the manuscript; Link A created the study concept and design and is the guarantor of the study; all authors edited and approved the final version of the manuscript.

Institutional review board statement: The study was reviewed and approved by the ethical board of the Otto-von-Guericke University.

Informed consent statement: In this study we used retrospectively collected anonymized samples from a previous study. No additional informed consent other than from the primary study was obtained. Ethical committee approved the study protocol.

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Abstract

BACKGROUND

There is ongoing search for new noninvasive biomarkers to improve management of patients with hepatocellular carcinoma (HCC). Studies, mostly from the Asian-Pacific region, demonstrated differential expression of liver-specific microRNA-122 (miR-122) in tissue as well as in sera of patients with hepatitis B virus- and hepatitis C virus-induced HCC.

AIM

To evaluate prognostic value of miR-122 in patients with HCC in a European population and determine potential factors related to alteration of miR-122 in sera.

METHODS

Patients with confirmed HCC (n = 91) were included in the study over a two-year period. Patients were characterized according to Child-Pugh score, Barcelona clinic liver cancer (BCLC) staging system, etiology of liver disease, laboratory parameters and overall survival. MiR-122 was measured in sera using TaqMan assay normalized to spiked-in cel-miR-39.

RESULTS

Serum miR-122 quantity was independent of the Child-Pugh score, the BCLC stage or the underlying etiology. Significant positive correlation was found between miR-122 and alanine aminotransferase ($P < 0.0001$), aspartate aminotransferase ($P = 0.0001$), alpha-fetoprotein (AFP) ($P = 0.0034$) and hemoglobin concentration ($P = 0.076$). Negative correlation was observed

Data sharing statement: Technical appendix and dataset available from the corresponding author at alexander.link@med.ovgu.de. Participants gave informed consent for data analysis and publication. Since no patients consent to data sharing was obtained, no additional data will be made available. The data that support the findings of this study are available from the corresponding author upon reasonable request.

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between miR-122 level and creatinine concentration (P = 0.0028). AFP, Child-Pugh score and BCLC staging system were associated with survival differences. In overall cohort low miR-122 in sera was only associated with a trend for a better overall survival without reaching statistical significance. Subgroup analysis revealed that low miR-122 was significantly associated with better prognosis in patients with advanced cirrhosis (Child-Pugh class B/C), advanced tumor stage (BCLC B/C/D) and normal AFP (< 7 ng/mL).

CONCLUSION
Our results strongly support the value of miR-122 as potential biomarker of liver injury and probably prognosis. Nevertheless, the value of miR-122 in prediction of prognosis of HCC patients was limited to certain patients' subgroups. Since circulating miR-122 may be influenced by impaired renal function, AFP and hemoglobin concentration, those factors need to be considered while interpreting miR-122 level.

Key words: Hepatocellular carcinoma; MicroRNA; Prognosis; MicroRNA-122; Influencing factors

Core tip: Small non-coding RNAs are in focus of liver biomarker research. Here we confirm that the most abundant liver-specific microRNA-122 (miR-122) is a potential biomarker for liver injury and has potential value to predict the outcome of patients with hepatocellular carcinoma, but several influencing factors need to be taken into account while interpreting the miR-122 level. Besides clinical aspects, several coexisting factors like impairment of renal function, hemoglobin concentration, alpha-fetoprotein level and liver injury may strongly influence circulating miR-122 level and potential clinical translational application of miR-122.

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INTRODUCTION

Hepatocellular carcinoma (HCC) is among the most common cancers with high mortality risk. The incidence is rising because of an increasing prevalence of chronic liver injury related to dietary and environmental factors[1,2]. Majority of HCC is developed in patients with liver cirrhosis. Prognosis of patients with HCC is strongly dependent on liver function as well as related complications of liver disease. Several different scores have been developed to estimate prognosis of HCC patients [*e.g.*, Barcelona Clinic Liver Cancer (BCLC) staging system[3], Okuda staging system[4], CLIP score[5]]. Most widely used is the BCLC staging system[3], which estimates prognosis based on morphology of the tumor and the clinical presentation (liver function, portal vein thrombosis among others) without taking molecular biology into account. The BCLC staging system has been validated in multiple studies, but has limitations in the prognostic assessment of patients with intermediate or advanced HCC stages[6,7].

There is a need for biomarkers to optimize the prognostic assessment in HCC patients which would contribute to personalized management. So far, the only biomarker of HCC with world-wide clinical application is alpha-fetoprotein (AFP). AFP is broadly implemented for surveillance of patients at high-risk for developing HCC. Clear recommendations to applicability of AFP for prognostic assessment are still lacking[8]. Different molecules such as AFP, des-γ-carboxyprothrombin, Lens culinaris agglutinin-reactive AFP, Insulin-like growth factor-1, vascular endothelial growth factor, and Angiopoetin 2 were also evaluated regarding their prognostic value but have not made it into routine clinical management as individual parameters[9], although have been included into prognostic staging systems (*e.g.*, CLIP score[5], ALBI grade[10], BALAD-2[11]).

MicroRNAs (miRNA) are still a relatively new class of molecules that show exceptional stability against degradation[12]. Alterations in miRNA expression pattern in liver tissue have been shown in various liver diseases and HCC[13-15]. Equally, variation of miRNA in serum and plasma were shown in different liver diseases[16-18]. MiR-122 is a liver-specific miRNA and with an average expression of 52% it is the most common miRNA in human liver tissue[19,20]. MiR-122 has been shown to play a crucial role in hepatitis C virus infection[21]. Chronic inflammation, for instance chronic hepatitis B virus, alcohol damage or non-alcoholic steatohepatitis, is associated with reduced miR-122 expression in hepatocytes[13,22,23]. Several studies suggested that the deregulation of miR-122 is associated with an aggressive type of HCC[24-26]. Overall, a reduced level of tissue miR-122 was shown in HCC compared to non-tumorous tissue[27-29]. However, opposite miR-122 behavior was described in plasma or sera of HCC patients compared to healthy people[17,30,31].

Several attempts to integrate miR-122 into various algorithms for HCC diagnosis have been made based on tissue[14,15,32] or blood analyses[16,17,30]. But it is clear that miRNA biogenesis follows its own cascade and it is crucial to characterize and identify potential influencing factors in order to implement miR-122 in clinical settings. Furthermore, systematic review of the literature revealed that there is a high heterogeneity of miRNA-biomarker studies related to technical, methodological aspects and quality reporting, which may affect the applicability and reproducibility of generated data[33].

Aim of our study was to evaluate the prognostic value of serum miR-122 in patients with HCC in a European cohort. In addition, we aimed to identify potential liver disease-, tumor-related or other factors that may influence circulating miR-122 level in HCC patients.

MATERIALS AND METHODS

Study design

We analyzed miRNA level in retrospectively collected serum samples (January 2009-April 2011, n = 91) from well characterized patients with histologically or clinically confirmed HCC. The study was performed according to the World Medical Association "Declaration of Helsinki – Ethical Principles for medical research involving human subjects" and approved by the local Institutional Review Board of Otto-von-Guericke University Magdeburg (Number: 99/10). All patients provided written informed consent prior inclusion in the primary study.

Description of the patients

Patient characteristics are presented in Table 1. In comparison to existing data, this cohort consisted of HCC patients with mostly alcohol-related liver damage (45.1%). After blood sampling, all patients were characterized with respect to clinical and laboratory parameters and Child-Pugh score and the BCLC stage were documented. We used survival data to evaluate the prognosis of HCC patients. The overall survival time was defined as the time between inclusion into our study (blood withdrawal) and death or the last documented contact to the patient.

Extraction of total RNA

After centrifugation and taking the supernatant serum samples were stored by 80°C. Extraction of total RNA (including miRNA) was performed using miRNeasy Mini Kit (QIAGEN, Hilden, Germany) as previously described[34]. One hundred microliter of serum were added to 700 μL QIAzol Lysis Reagent and were homogenized in vortex mixer. Five μL of a 5 nmol/L cel-miR-39 (miR-39) were added for internal normalization. Following precipitation and washing steps, RNA was finally eluted in 30 μL RNase free water. UV-Spectrophotometry was used for analysis of RNA quality.

Reverse transcription and polymerase chain reaction

Reverse transcription was performed using TaqMan® MicroRNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA, United States). Quantitative real time PCR (qPCR) (TaqMan® Universal Master Mix II, no UNG, Applied Biosystems, Foster City, CA, United States) was performed for miR-122 (Assay ID: 002245) and cel-miR-39 (Assay ID: 000200) according to the manufacturer's instructions. The analyses were performed on the BioRad CFX Cycler System (BioRad, Hercules, CA, United States). Cel-miR-39 was used for normalization of miR-122 with method. All analysis were performed in duplicates and samples with known quantity were used for interplate normalization.

Table 1 Clinical and laboratory characteristics of the patients with hepatocellular carcinoma, *n* (%)

Characteristics	Value
Patient number	91
Gender	
Women	17 (18.7)
Men	74 (81.3)
Age in yr, mean ± SD	67.91 ± 8.98
Etiology	
Alcohol abuse	41 (45.1)
Viral hepatitis	12 (13.2)
NASH	13 (14.3)
Hemochromatosis	6 (6.6)
Rare or other cause	19 (20.8)
BCLC stage	
0	0 (0.0)
A	16 (17.6)
B	37 (40.6)
C	32 (35.2)
D	6 (6.6)
Child-Pugh score	
No liver cirrhosis	16 (17.6)
A	45 (49.4)
B	27 (29.7)
C	3 (3.3)
Treatment	
Therapy naive	26 (28.6)
Pretreated	65 (71.4)

NASH: Non-alcoholic steatohepatitis; BCLC: Barcelona clinic liver cancer.

Statistical methods

GraphPad Prism® Version 6.0 (GraphPad Software, San Diego, CA, United States) was used for statistical analysis. Two-sided P value ≤ 0.05 was considered as significant. Based on the data distribution, we used nonparametric tests (Spearman correlation, Mann-Whitney test, Kruskal-Wallis test, Post-hoc Dunn's test). The data are shown as boxplots with whiskers for the minimum and maximum, a lower and upper quartile and the median. Overall survival was analyzed using Kaplan-Meier survival curves and comparison was performed using nonparametric log-rank test.

RESULTS

Serum miR-122 is independent of Child-Pugh score, etiology and BCLC stage

Liver function, etiology of liver disease and accordingly tumor stage may be important factors that could impact miR-122 quantity in serum. Analysis of sera samples from patients with different Child-Pugh scores revealed no significant differences of miR-122 between different stages (P = 0.3060) (Figure 1A). In similar manner, we observed no significant differences of the serum miR-122 level with regard to BCLC staging system (P = 0.5289) or underlying etiology of liver disease (P = 0.2456) (Figure 1B and C).

Correlation between serum miR-122 and AFP, hepatocellular damage, renal function and hemoglobin level

To estimate possible influencing factors that may impact miR-122 concentration in sera, we analyzed various laboratory parameters in correlation to miR-122. Our analysis revealed positive association between miR-122 and alanine aminotransferase (ALAT) (r = 0.4731, P < 0.0001) and aspartate aminotransferase (ASAT) (r = 0.3937, P = 0.0001). In both cases miR-122 level was higher in the group with pathologically

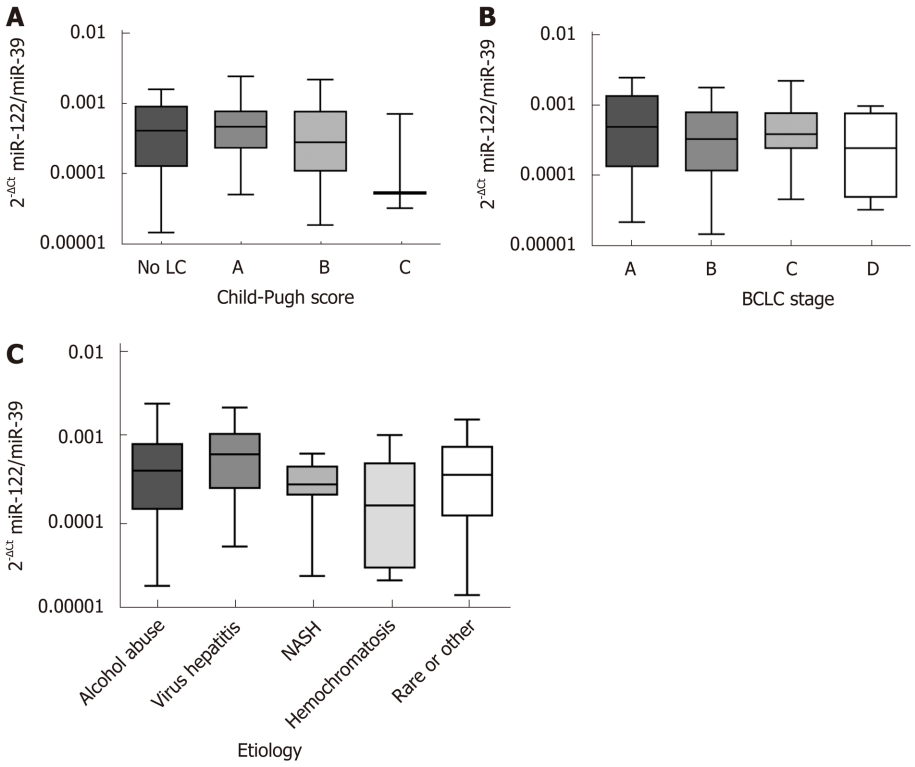

Figure 1 Serum microRNA-122 level in correlation to Child-Pugh score, Barcelona clinic liver cancer stage and underlying etiology. A: Serum microRNA-122 (miR-122) level in relation to Child-Pugh score: No liver cirrhosis (n = 16), class A (n = 45), class B (n = 27), and class C (n = 3); B: Serum miR-122 level in relation to Barcelona clinic liver cancer staging system: Stage A (n = 16), stage B (n = 37), stage C (n = 32), and stage D (n = 6); C: Serum miR-122 level in relation to underlying etiology of the hepatocellular carcinoma: Alcohol abuse (n = 41), viral hepatitis (n = 12), non-alcoholic steatohepatitis (n = 13), hemochromatosis (n = 6), rare or other (n = 19). Kruskal-Wallis test and post-hoc Dunn's test were used for statistical analysis. LC: Liver cirrhosis; NASH: Non-alcoholic steatohepatitis; BCLC: Barcelona clinic liver cancer.

elevated transaminases (ALAT: P = 0.0050, ASAT: P = 0.0214) (Figure 2A and B). Next, in patients with HCC elevated AFP was associated with elevated miR-122 concentration (r = 0.3043, P = 0.0034). After subdividing patients into a group with normal (< 7 ng/mL), with slightly increased (7 ng/mL ≤ AFP ≤ 400 ng/mL) and with strongly increased AFP (> 400 ng/mL), we observed significant differences between the group with normal AFP compared to both groups with increased AFP values (P = 0.0071 and P = 0.0144), while no difference was observed between both AFP-elevated groups (Figure 2C). Impairment of the renal function is a frequent consequence of the chronic advanced liver disease. Negative association was observed between miR-122 and creatinine levels (r = 0.3100, P =0.0028), where patients with pathological creatinine value had lower miR-122 levels (P = 0.0027) (Figure 2D). Also, an anemia is a common event in patients with cancer. Despite positive association between miR-122 and hemoglobin levels (r = 0.2783, P = 0.0076), there was only a non-significant trend for lower miR-122 in patients with anemia compared to subjects with normal hemoglobin values (P = 0.0618) (Figure 2E). Supplemental Tables 1 and 2 show analysis of additional parameters that did not show significant differences.

Survival analysis and prognostic value of serum miR-122

First, to confirm the suitability of survival data in our cohort, we evaluated the impact of known prognostic parameters on survival of HCC patients. As expected, higher AFP level (P = 0.0038), higher Child-Pugh score (P = 0.0129) and higher BCLC stage (P = 0.0001) were all associated with worse overall survival of patients with HCC (Figure 3). To evaluate the prognostic potential of miR-122 in sera, we subdivided our study cohort into three groups by taking the 25th and the 75th percentile which would allow better subdivision. As shown in Figure 3, only a non-significant trend was observed between the groups (P = 0.1019). Based on this observation, we hypothesized that low miR-122, but not intermediate or high, may be of the greatest prognostic value, and therefore, we used 25th percentile as cut-off value for the subsequent analysis. By applying this subgrouping of patients the statistical trend was improved but with P = 0.0610 remain non-significant suggesting that additional factors may influence the

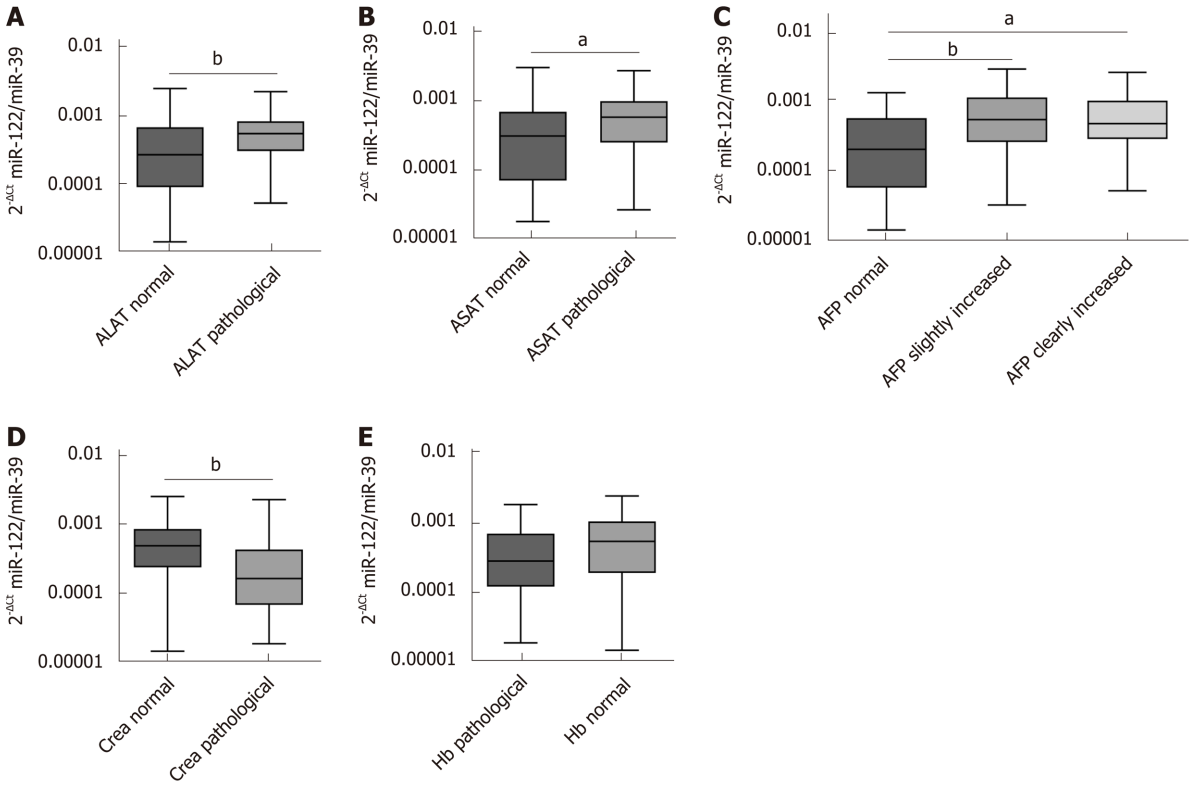

Figure 2 Relation between serum microRNA-122 level and other laboratory parameters. A: Differences of serum microRNA-122 (miR-122) in relation to alanine aminotransferase (ALAT) [ALAT normal: ≤ 0.58 (w) / 0.83 (m) μmol/Ls, ALAT pathological: > 0.58 (w) / 0.83 (m) μmol/Ls]; B: Differences of serum miR-122 in relation to aspartate aminotransferase (ASAT) [ASAT normal: ≤ 0.58 (w) / 0.83 (m) μmol/Ls, ASAT pathological: > 0.58 (w) / 0.83 (m) μmol/Ls]; C: Differences of serum miR-122 in relation to alpha-fetoprotein (AFP) (AFP normal: AFP < 7 ng/mL, AFP slightly increased: 7 ng/mL ≤ AFP ≤ 400 ng/mL, AFP clearly increased: AFP > 400 ng/mL); D: Differences of serum miR-122 in relation to creatinine [Crea normal: ≤ 84 (w) / 104 (m) μmol/L, Crea pathological: > 84 (w) / 104 (m) μmol/L]; E: Differences of serum miR-122 in relation to hemoglobin [Hb normal: ≥ 7.4 (w) / 8.6 (m) mmol/L, Hb pathological: < 7.4 (w) / 8.6 (m) mmol/L]. Mann-Whitney test, Kruskal-Wallis test and post-hoc Dunn's test were used for statistical analysis. [a]$P < 0.05$ and [b]$P < 0.01$ *vs* normal group, not significant- not shown. ASAT: Aspartate aminotransferase; ALAT: Alanine aminotransferase; AFP: Alpha-fetoprotein; Crea: creatinine.

performance of miR-122 as prognostic biomarkers (Figure 4A). To address this issue, we performed subgroup analyses based on the Child-Pugh score, BCLC staging and AFP. Interestingly, low miR-122 level was associated with better overall survival in patients with advanced cirrhosis (Child-Pugh B/C) ($P = 0.0129$) (Figure 4B). In similar fashion, low miR-122 level in patients with BCLC B-D was also associated with better overall survival ($P = 0.0157$) (Figure 4C). In subgroup of patients with normal AFP, low miR-122 level was also associated with better prognosis ($P = 0.0353$) (Figure 4D). The results of the subgroup analysis support the potential of miR-122 as potential prognostic biomarker, however, critical attention and consideration of confounding factors need to be considered.

DISCUSSION

Deregulation of miR-122 has been reported in several studies for patients with HCC; however, translational and clinicopathological value of serum miR-122 in real-life setting is still unknown. In this study, we systematically characterized the prognostic value of serum miR-122 in HCC patients in a European cohort. Although, we observed only a trend for a better prognosis in patients with low miR-122 level in total cohort, we identified several valuable tumor- and liver disease-related factors that may influence miR-122 biogenesis or its biomarker performance. In particular, our data demonstrate strong positive correlation between miR-122 level and biomarkers of liver injury (transaminases ALAT and ASAT, but not liver function), AFP and hemoglobin and negative correlation with renal function.

An association between miR-122 and unspecific liver injury has been previously suggested but exact mechanism remains poorly understood[18,35,36]. A release of miR-122 during hepatocellular damage into blood because of the extraordinary high expression in liver tissue may be the best possible explanation[19,20]. Our results support the assumption showing positive correlation between miR-122 levels and

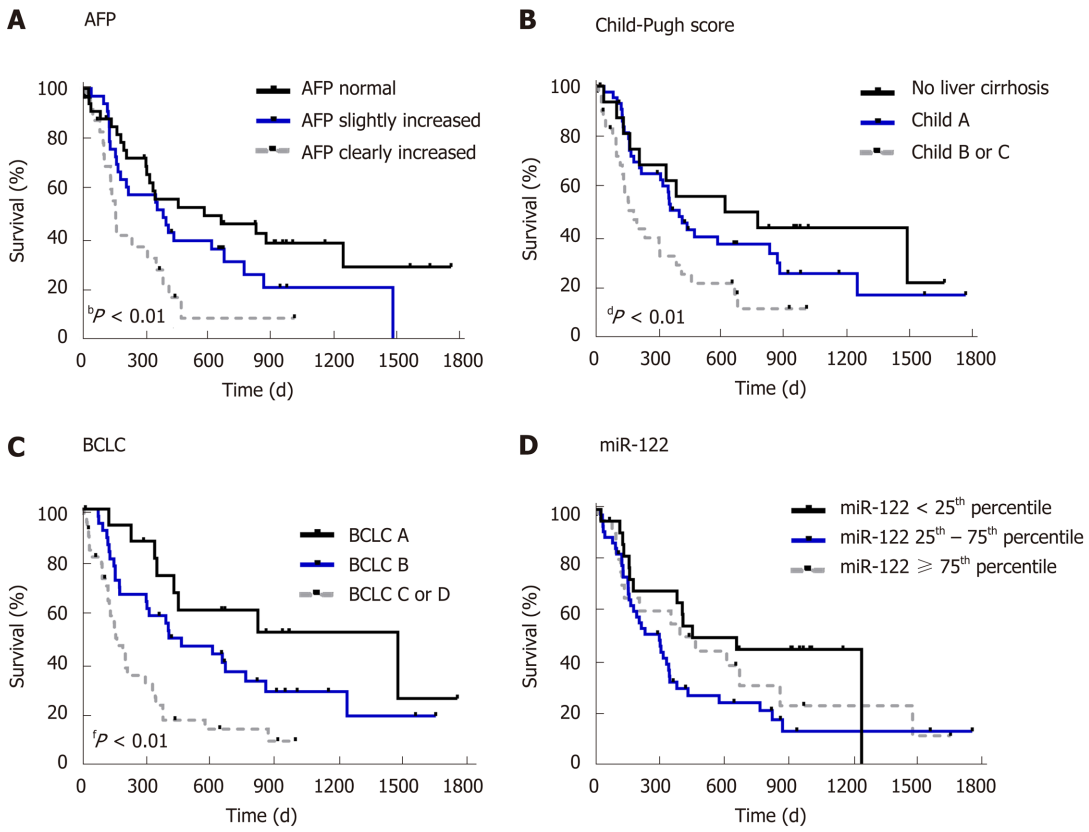

Figure 3 Survival analysis in relation to alpha-fetoprotein, Child-Pugh score, Barcelona clinic liver cancer stage and serum microRNA-122 level. A: Survival analysis in hepatocellular carcinoma (HCC) patients in relation to alpha-fetoprotein (AFP) [divided into three groups: patients with regular AFP (< 7 ng/mL, *n* = 33), patients with slightly (7 ng/mL ≤ AFP ≤ 400 ng/mL, *n* = 33) and patients with clearly increased AFP (> 400 ng/mL, *n* = 25)]; B: Survival analysis in HCC patients in relation to Child-Pugh score [divided into three groups: patients without cirrhosis (*n* = 16), patients with Child-Pugh class A (*n* = 45), and patients with Child-Pugh class B or C (*n* = 30), class B and class C were summarized, because only a few patients were in the more severe class (*n* = 3)]; C: Survival analysis in HCC patients in relation to Barcelona clinic liver cancer (BCLC) staging system [divided into three groups: patients with BCLC A (*n* = 16), with BCLC B (*n* = 37), with BCLC C or D (*n* = 38), BCLC C and BCLC D were summarized, because only a few patients were in the more severe stage (*n* = 6)]; D: Survival analysis in HCC patients in relation to serum miR-122 level [divided into three groups: < 25th percentile (*n* = 22), 25th-75th percentile (*n* = 46), ≥ 75th percentile (*n* = 23)]. Nonparametric log-rank test was used for statistical analysis. $^{b}P < 0.01$: AFP normal *vs* AFP slightly increased *vs* AFP clearly increased; $^{d}P < 0.01$ No liver cirrhosis *vs* Child A *vs* Child B or C; $^{f}P < 0.01$ BCLC A *vs* BCLC B *vs* BCLC C or D. BCLC: Barcelona clinic liver cancer; AFP: Alpha-fetoprotein.

elevated transaminases and potential value of miR-122 as biomarker of hepatocellular damage is also supported by others[35,36]. From another point of view, two previous publications suggested miR-122 as a biomarker of residual liver function in patients with cirrhosis and HCC[37,38] even though we observed no correlation to liver function.

AFP is among the most recognized diagnostic and prognostic biomarkers for HCC[9]. The potential link between AFP and miR-122 has been suggested in a mouse model[23]. Our data also strongly support this positive interaction between serum miR-122 and serum AFP levels. Since negative correlation between AFP and miR-122 has been described in HCC tissue[26], we conclude that the link between AFP and circulating miR-122 may be rather indirect and reflect general liver injury and not HCC-specific alterations.

Among various studied influential factors, kidney function may deserve a key attention potentially affecting miRNA biogenesis. Negative association between total small RNA level and creatinine has been previously described in patients with severe kidney injury[39]. Here we showed that HCC patients with renal impairment have significant lower miR-122 values in serum. Similar result has been shown for patients with liver cirrhosis[38] and in a cohort of critically ill patients[35]. From one side, alterations in liver and renal function may lead to relative dilution of miR-122. From another side, either excretion of miRNAs or stability and degradation of miRNAs in exosomes or protein-bond miRNAs may be affected. In similar fashion, we showed that miR-122 correlates positively with hemoglobin. It is important to mention that secretion of miR-122 has been recently linked to development of inflammation-induced anemia[40]. Simply, isotonic hyperhydration, frequently observed in liver cirrhosis patients, could be another possible explanation for our result.

Having shown the impact of various factors on miR-122 in serum one may question

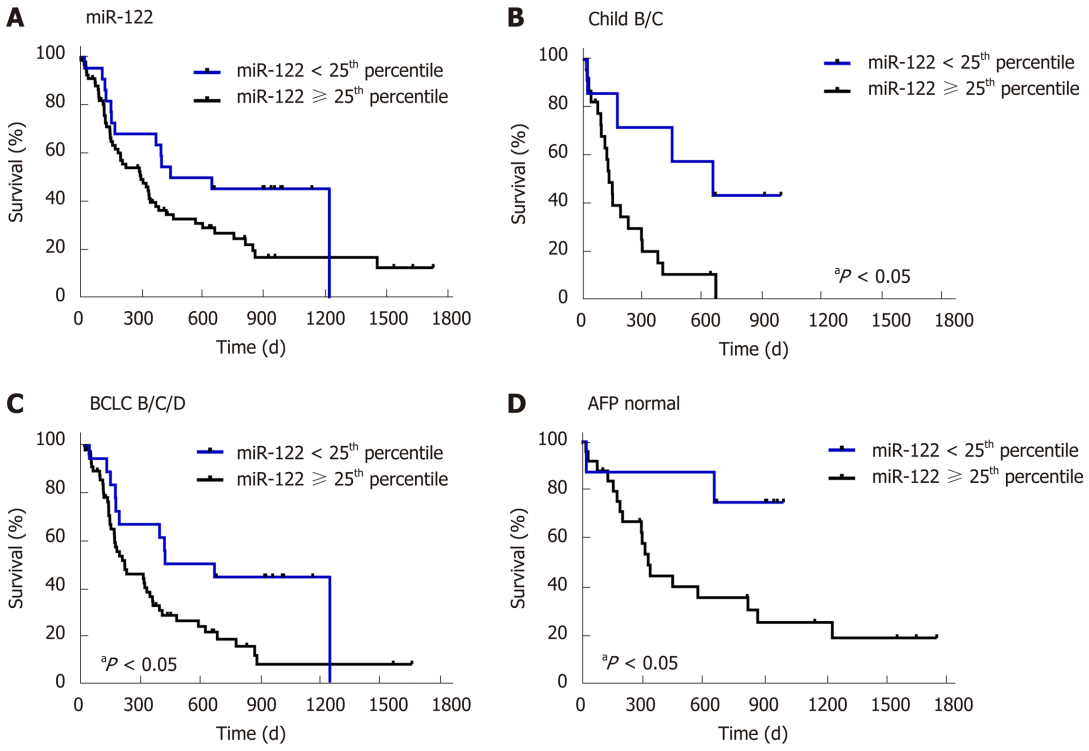

Figure 4 Survival analysis of subgroups of patients depending on serum microRNA-122 level. A: Survival analysis in relation to serum miR-122 in all patients [divided into two groups: < 25th percentile (n = 22), ≥ 25th percentile (n = 69)]; B: Survival analysis in relation to serum miR-122 in patients with Child-Pugh class B or C [divided into two groups: < 25th percentile (n = 7), ≥ 25% percentile (n = 23)]; C: Survival analysis in relation to serum miR-122 in patients with Barcelona clinic liver cancer stage B/C/D [divided into two groups: < 25th percentile (n = 18), ≥ 25th percentile (n = 57)]; D: Survival analysis in relation to serum miR-122 in patients with normal alpha-fetoprotein (AFP) (AFP < 7 ng/mL) [divided into two groups: < 25th percentile (n = 8), ≥ 25th percentile (n = 25)]. Nonparametric log-rank test was used for statistical analysis. P < 0.05 miR-122 < 25th percentile *vs* miR-122 ≥ 25th percentile. BCLC: Barcelona clinic liver cancer; AFP: Alpha-fetoprotein.

the validity of miR-122 for prognostic assessment. Our data do not support the use of miR-122 as general biomarker for HCC prognosis. However, the subgroup analyses still highlight the potential value in predefined groups, but the knowledge of confounding factors and understanding of related mechanism is crucial. This may explain why several studies have reported the prognostic role of serum miR-122 in HCC patients with contrary results[37,41]. Supporting our results, Liu *et al*[41] showed that low miR-122 was associated with better prognosis in a cohort with large portion of HCC patients with BCLC B and BCLC C stage.

There is an increasing evidence for the decoupling of miR-122 in tissue and blood[23,35]. Reduced miR-122 expression is described for HCC tissue in human samples[20,27,29]. Higher miR-122 blood values are measured in patients with hepatitis or HCC compared to healthy people[30,31,36]. Following these results, miR-122 level in serum does not allow any statement related to miR-122 expression in liver tissue, where lower expression is associated with worse prognosis[24,42]. Interestingly, preoperative and postoperative miR-122 revealed significant reduction of miR-122 in patients with HCC[31]. This may be related to the reduced liver volume, although the HCC may still be a potential source of circulating miRNAs.

With an increasing number of studies related to miRNAs as biomarkers, our work highlight additional aspects to be considered in the future. There are strong patient-independent factors that may impact comparability between studies[33]. Some of those factors may be related to miRNA extraction and qPCR methods. Also, appropriate normalization is still a great limitation in miRNA-based research. In particular, miR-16 has been used in various studies[31,37,43,44], but it is prone to strong bias in HCC[45] and from our point of view it needs to be abandoned. Nevertheless, our work has several limitations to be mentioned as well. The number of samples may be still too low and larger studies with prospective study design would be needed. Even though our data are close to the real-life setting, the role of miR-122 in HCC patients' needs to be evaluated in larger cohort of patients with at different diseases stages if possible in prospective manner.

In summary, out data support the unique biomarker value of miR-122 in liver-related diseases and specifically in HCC. Although low miR-122 was associated only with a trend for better prognosis in total cohort of patients with HCC, the prognostic

output could be improved by consideration of various factors in subgroup analyses. This work clearly emphasizes the need for accurate assessment of potential systemic cofactors in miRNA-based biomarker research. Serum miR-122 level may be strongly affected by various patient-related conditions including AFP level, liver injury, renal function and anemia. Therefore, future studies with careful and systematic characterization of those potential cofactors are urgently needed.

ARTICLE HIGHLIGHTS

Research background

Hepatocellular carcinoma (HCC) is among the deadliest conditions worldwide. One of the challenges related to HCC is the identification of specific and sensitive diagnostic and prognostic biomarkers. MicroRNAs have been shown to be deregulate in HCC and microRNA-122 (miR-122) is among the most promising liver-specific molecules.

Research motivation

miR-122 has been studied in Asian-Pacific regions but only limited knowledge is available from European population. At present the role of miR-122 as prognostic is not independently validated. Most importantly, it is still less known about potential factors that may influence miR-122 level in blood samples.

Research objectives

Circulating miR-122 may be influenced by impaired renal function, alpha-fetoprotein (AFP) and hemoglobin concentration. Those factors may strongly influence the performance of miR-122 a potential biomarker in HCC.

Research methods

A cohort of well characterized patients with HCC were included in this study. Quantitative TaqMan assay was used to analyze miR-122 in serum and the results were normalized to spiked-in cel-miR-39. The data were stratified based on individual characteristics including liver disease, liver biochemistry, tumor staging and overall survival.

Research results

Overall miR-122 was shown to be independent to Child-Pugh score, Barcelona clinic liver cancer tumor staging classification or etiology of liver disease. Among the studied factors, we identified alanine aminotransferase, aspartate aminotransferase, AFP and renal function (creatinine) as factors that may influence miR-122 level in serum. According to our results, low miR-122 may be associated with lower overall survival, however, only if certain conditions including cirrhosis score, tumor stage or APF are considered.

Research conclusions

The results from this study strongly suggest that renal function and liver inflammation may impact microRNA biomarkers. In particular, a liver-specific miR-122 may be strongly influenced by intrahepatic inflammation which may create potential bias. Our results support the use of miR-122 as a marker for liver inflammation. The value of miR-122 in prediction of overall survival is, however, limited due to the co-existing factors. Further studies will need to determine the mechanism responsible for the influence, but also find a way of controlling the influencing factors in biomarker studies.

Research perspectives

This study clearly highlights the need for better understanding of microRNAs biogenesis in circulation. Since large number of studies focus on microRNAs as potential biomarkers, we urge for better characterization of co-existing factors to identify potential individual influencing factors. Future studies related to miR-122 need to consider also renal function and liver biochemistry.

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ORIGINAL ARTICLE

Retrospective Study

Usefulness of autotaxin for the complications of liver cirrhosis

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Abstract

BACKGROUND

Autotaxin (ATX) has been reported as a direct biomarker for estimating the evaluation of liver fibrosis. But available data on ATX as a useful biomarker for the complications of liver cirrhosis (LC) are scant.

AIM

To assess the clinical usefulness of ATX for assessing the complications of LC.

METHODS

This multicenter, retrospective study was conducted at six locations in Japan. We include patients with LC, n = 400. The ATX level was evaluated separately in men and women because of its high level in female patients. To assess the clinical

Review Board Ethics Committee of the Tokushukai Medical Group, and the protocol for this study conforms to the provisions of the Declaration of Helsinki.

Informed consent statement: This study is a retrospective observational study. Informed consent was obtained from all individual participants included in the study by the opt-out method of our hospital website.

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usefulness of ATX for the complications of LC, the area under the curve (AUC) of ATX assessing for the severe complications was analyzed in comparison with the model for end-stage liver disease score, albumin-bilirubin (ALBI) score, fibrosis-4 index, and aspartate aminotransferase-to-platelet ratio index.

RESULTS
The mean age was 68.4 ± 11.4 years, 240 patients (60.0%) were male. A total of 213 (53.3%) and 187 (46.8%) patients were compensated and decompensated, respectively. The numbers of patients with varix rupture, hepatic ascites, and hepatic encephalopathy were 35 (8.8%), 131 (32.8%), and 103 (25.8%), respectively. The AUCs of ATX in men for hepatic encephalopathy, hepatic ascites, and varix ruptures were 0.853, 0.816, and 0.706, respectively. The AUCs of ATX in women for hepatic encephalopathy, hepatic ascites, and varix rupture were 0.759, 0.717, and 0.697, respectively. The AUCs of ATX in men were higher than those in women, as were all the other biomarkers used to detect encephalopathy and varix ruptures. However, for detecting ascites, the AUC of ALBI in men was more effective than using ATX.

CONCLUSION
ATX in men was more effective than any other biomarkers for detecting hepatic encephalopathy and varix ruptures.

Key words: Autotaxin; Hepatic encephalopathy; Hepatic ascites; Liver fibrosis; Biomarker

Core tip: Autotaxin (ATX) has been reported as a novel biomarker for estimating liver fibrosis stages, and researchers have reported that this fibrosis marker correlated closely with more advanced fibrosis. Especially, the assessment for the complications of liver cirrhosis (LC) is valuable in helping to make treatment decisions. However, data on the clinical usefulness of novel fibrosis markers in assessing the complications of LC are scant. This study showed that ATX is a useful biomarker for assessing the complications of LC. Especially, the use of ATX in men was more effective than any other biomarker for detecting hepatic encephalopathy and varix ruptures.

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INTRODUCTION

Progression of liver disease to cirrhosis, liver failure, hepatic cellular cancer, varix, or ascites is correlated with the histological change in liver fibrosis[1]. Liver biopsy is the standard procedure to assess the stages of liver fibrosis and is well known to be diagnostically accurate. However, liver biopsy is not an ideal method for the diagnosis of liver fibrosis because the small sample volume with each biopsy leads to sampling errors[2]. Furthermore, liver biopsy is an invasive procedure, with a significantly high risk of life-threatening complications for patients with advanced liver cirrhosis (LC)[3,4]. These limitations have led to the development of serum biomarkers for the assessment of liver fibrosis in recent years. Serum biomarkers are categorized into direct and indirect biomarkers whether or not they reflect extracellular matrix (ECM) turnover. Direct biomarkers have clinical values involving both the evaluation of liver fibrosis and monitoring the behavior of fibrogenesis and ECM metabolism[5]. Autotaxin (ATX) has been reported as a direct biomarker for estimating the evaluation of liver fibrosis. This biomarker, ATX, also known as ectonucleotide pyrophosphatase/phosphodiesterase 2, is a secreted lysophospholipase D that hydrolyzes lysophosphatidylcholine to generate lysophosphatidic acid, a lipid mediator that activates G-protein-coupled receptors to evoke various cellular responses[6,7].

Because ATX is subsequently cleared from the circulation in liver sinusoidal endothelial cells, liver fibrosis reduces the capacity to metabolize ATX, resulting in deterioration of liver function, which in turn leads to an increase in the serum ATX level[7-9]. According to the progression of fibrosis, the degree of impairment of the uptake of various substances in the liver sinusoidal endothelium was exacerbated. Especially, for decompensated LC with ascites, hepatic encephalopathy, and varix rupture, impairment of the uptake of the phenotypic changes in liver sinusoidal endothelial cells leads to an extreme reduction[10].

Developments of serum biomarkers have focused on the diagnosis of cirrhosis, but more recent researches have emphasized the availability of these markers to assess patients with more advanced fibrosis[5]. The ATX level may be a useful biomarker to select treatment therapy for ascites, hepatic encephalopathy, and varix ruptures. And the assessment for the complications of LC is especially valuable in helping to make treatment decisions[11]. However, available data on ATX as a useful biomarker for the complications of LC are scant. The aim of this study was to assess the clinical usefulness of ATX for assessing the complications of LC.

MATERIALS AND METHODS

Ethics

This study was approved by the Institutional Review Board Ethics Committee of the Tokushukai Medical Group (No. TGE01178-024), and the protocol for this study conforms to the provisions of the Declaration of Helsinki. This study is registered in the UMIN Clinical Trials Registry as UMIN000036221. This study is a retrospective observational study. Informed consent was obtained from all individual participants included in the study by the opt-out method of our hospital website.

Patients

This multicenter, retrospective study was comprised of 400 patients with LC conducted at six locations in Japan. Cirrhosis in these patients was caused by hepatitis B virus (HBV), hepatitis C virus (HCV), alcoholic liver disease, nonalcoholic steatohepatitis, autoimmune hepatitis, primary biliary cholangitis, primary sclerosing cholangitis, the Budd-Chiari syndrome, and chronic right-sided heart failure. Patients with poorly controlled heart failure, severe renal dysfunction, and malignancies other than HCC were excluded.

Diagnosis of LC

Diagnosis of LC was based on noninvasive imaging using computed tomography and/or magnetic resonance imaging revealing a hepatic cirrhotic appearance, portosystemic collaterals, an enlarged spleen, esophageal varices, and/or nonmalignant ascites. Ultrasound-guided liver biopsy was not conducted for histological assessment.

Measurement of serum ATX

Serum concentrations of ATX were measured using a two-site enzyme immunoassay and an automated immunoassay analyzer (Tosoh Corp., Tokyo, Japan). The assay reagent was compatible with a commercial automated immunoassay analyzer AIA System (Tosho Corp., Tokyo Japan). This system included automated 10 μL of specimen dispensation, incubation of the reaction cup, bound-free washing, 4-methylumbelliferyl phosphate substrate dispensation and fluorometric detection[8-10].

Measurement of indirect biomarkers for liver fibrosis

Indirect biomarkers for the aspartate aminotransferase (AST)-to-platelet ratio index (APRI), the fibrosis-4 (Fib-4) index, the albumin-bilirubin (ALBI) score, and the model for end-stage liver disease (MELD) score were calculated as: APRI = [AST (35 IU/L)/platelets (10^3/μL)] × 100; Fib-4 index = [AST (IU/L) × age (years)]/[alanine aminotransferase (ALT; IU/L)1/2 × platelets (10^3/μL)]; ALBI score = −0.085 × [albumin(g/L)] + 0.66 × $\log_{10}$ [serum bilirubin (μmol/L)][12]; and MELD score = 3.78 × $\log_e$ [serum bilirubin (mg/dL)] + 11.2 × $\log_e$ [PT-international normalized ratio] + 9.57 × $\log_e$ [serum creatinine (mg/dL)] + 6.43[13].

Evaluation of the usefulness of ATX for the complications of LC

To assess the clinical usefulness of ATX for the complications of LC, we examined ATX levels in cirrhotic patients with severe complications and compared the area under the curve (AUC) of serum biomarkers assessing for the complications of LC. Severe complications were defined as those of a decompensated phase, such as hepatic ascites, hepatic encephalopathy, and varix ruptures according to the

guidelines by the American Association for the Study of Liver Diseases[14]. We also determined if ATX was associated with other serum biomarkers or other laboratory data. Correlations between the ATX score in LC and the characteristics were determined using Pearson's r coefficient. Laboratory tests were conducted to measure the white blood cells, hemoglobin, platelets, prothrombin time, serum albumin, blood urea nitrogen, serum creatinine, AST, ALT, total bilirubin, serum sodium, serum potassium, hemoglobin A1c, total cholesterol, the branched-chain amino acid and tyrosine ratio, ammonia, and alpha-fetoprotein levels. All laboratory data were obtained on the same day as the ATX measurements.

Statistical evaluation

Data were analyzed using SPSS version 24.0 (IBM Corp., Armonk, NY, United States). All data were expressed as mean ± SD. Continuous variables in Child Pugh class (CPC) were compared using Tukey's honestly significant difference (HSD) test, and unpaired groups were compared using the unpaired *t*-test. Receiver operating characteristic (ROC) plots were constructed to establish sensitivity-specificity relationships. All differences with a P value of < 0.05 were considered significant.

RESULTS

Patient and baseline characteristics

A total of 400 patients with LC were enrolled. The baseline clinical characteristics of the 400 patients are summarized in Table 1. The mean age was 68.4 ± 11.4 years (range 22–93 years), 240 patients (60.0%) were male, and 84 patients (21.0%) had liver cancer. The mean body weight was 62.8 ± 13.3 kg (range 31.5–97.0 kg), and the mean body mass index was 24.0 ± 4.11 (range 13.7–41.4). LC was caused by hepatic ($n = 180$) and nonhepatic viruses ($n = 220$). A total of 213 (53.3%) and 187 (46.8%) patients were compensated and decompensated, respectively. The numbers (and proportions) of decompensated LC in varix rupture, hepatic ascites, and hepatic encephalopathy were 35 (8.8%), 131 (32.8%), and 103 (25.8%), respectively.

Comparison of ATX between sexes

The average ATX levels (mg/L) were 1.58 ± 0.68 in men and 1.99 ± 0.73 in women. A significantly higher ATX level was observed in women ($P < 0.001$).

ATX for the evaluation of the etiology

The average ATX levels in men and women were 1.62 ± 0.67 and 2.09 ± 0.71 mg/L for the HCV group, 1.36 ± 0.62 and 1.82 ± 0.54 mg/L for the HBV group, and 1.49 ± 0.71 and 1.96 ± 0.79 mg/L for the nonviral group, respectively. Tukey's HSD test confirmed that the ATX levels in men were significantly different in the HCV and HBV groups ($P = 0.044$). However, the ATX levels were not significantly different in the other groups (Figure 1).

ATX for the progression fibrosis

We analyzed the proportion of patients with different ATX levels stratified by CPC. The numbers (and proportions) of cirrhosis in CPCs A, B, and C were 232 (58.0%), 127 (31.7%), and 41 (10.3%), respectively. The average ATX levels in men and women were 1.23 ± 0.39 and 1.76 ± 0.67 mg/L, respectively, for CPC A, 1.88 ± 0.59 and 2.21 ± 0.66 mg/L, respectively, for CPC B, and 2.68 ± 0.81 and 2.74 ± 0.62 mg/L, respectively, for CPC C. Tukey's HSD test confirmed that the ATX levels in both men and women increased significantly with increasing CPCs ($P < 0.001$).

ATX for the complications of LC

The average ATX levels (mg/L) in men and women were 1.21 ± 0.40 and 1.73 ± 0.57, respectively, for the compensated group and 1.98 ± 0.71 and 2.33 ± 0.80, respectively, for the decompensated group. A significantly higher ATX level in men and women was observed in the decompensated group ($P < 0.001$). The average ATX levels in men with varix rupture, hepatic ascites, and hepatic encephalopathy were 1.88 ± 0.89, 2.07 ± 0.72, and 2.18 ± 0.71, respectively. By contrast, the average ATX levels (mg/L) in men without varix rupture, hepatic ascites, and hepatic encephalopathy were 1.55 ± 0.64, 1.36 ± 0.53, and 1.38 ± 0.53, respectively (Figure 2). A significantly higher ATX levels in men were observed in patients with varix rupture, hepatic ascites, and hepatic encephalopathy ($P = 0.028$; $P < 0.001$; $P < 0.001$, respectively). The average ATX levels (mg/L) in women with varix ruptures, hepatic ascites, and hepatic encephalopathy were 2.62 ± 1.51, 2.26 ± 0.67, and 2.37 ± 0.61, respectively. By contrast, the average ATX levels in women without varix rupture, hepatic ascites, and hepatic encephalopathy were 1.94 ± 0.63, 1.85 ± 0.74, and 1.85 ± 0.72, respectively.

Table 1 Base clinical characteristics

Characteristics	Value
Number of patients, *n*	400
Age, yr	68.4 ± 11.4
Gender: Male, *n* (%)	240 (60.0)
Weight, kg	62.8 ± 13.3
Body mass index, kg/m^2	24.0 ± 4.11
Aetiology: HCV/HBV/Alcohol/NASH/AIH/PBC/*etc*., *n*	130/50/108/60/18/9/25
HCV: SVR/non-SVR, *n*	84/46
Liver cancer, *n* (%)	84 (21)
Diabetes mellitus, *n* (%)	124 (31.0)
Hypertension, *n* (%)	120 (30.0)
Compensated/Decompensated, *n*	213/187
Hepatic ascites, *n* (%)	131 (32.8)
Hepatic encephalopathy, *n* (%)	103 (25.8)
Varicose vein rupture, *n* (%)	35 (8.8)
Child-Pugh score	6.62 ± 1.88
Child-Pugh class A/B/C, *n*	232/127/41
MELD-score	7.16 ± 4.48
ALBI score	-2.29 ± 0.68
White blood cells, /μL	4845 ± 3609
Hemoglobin, g/dL	12.5 ± 2.29
Platelets, × 10^4/μL	12.1 ± 6.53
Prothrombin time, %	75.9 ± 20.2
Serum albumin, g/dL	3.66 ± 0.72
Blood urea nitrogen, mg/dL	17.4 ± 9.9
Serum creatinine, mg/dL	0.97 ± 0.68
Aspartate aminotransferase, IU/L	39.4 ± 23.5
Alanine aminotransferase, IU/L	28.1 ± 18.7
Total bilirubin, mg/dL	1.25 ± 0.97
Serum sodium, mEq/L	139 ± 2.75
Hemoglobin A1c, %	5.94 ± 1.18
Total cholesterol, mg/dL	176 ± 40.8
Branched chain amino acid	4.97 ± 2.24
Ammonia, μg/dL	52.3 ± 40.9
Alpha-fetoprotein, ng/mL	54.4 ± 564

Data are expressed as mean, number (%), or mean ± SD. A total of 400 patients with liver cirrhosis were enrolled. The mean age was 68.4 ± 11.4 years, 240 patients (60.0%) were male, and 84 patients (21.0%) had liver cancer. The mean body weight was 62.8 ± 13.3 kg, and the mean body mass index was 24.0 ± 4.11. A total of 213 (53.3%) and 187 (46.8%) patients were compensated and decompensated, respectively. The numbers (and proportions) of decompensated liver cirrhosis in varix rupture, hepatic ascites, and hepatic encephalopathy were 35 (8.8%), 131 (32.8%), and 103 (25.8%), respectively. HBV: Hepatitis B virus; HCV: Hepatitis C virus; NASH: Non-alcoholic steatohepatitis; AIH: Autoimmune hepatitis; PBC: Primary biliary cholangitis; MELD: Model for end-stage liver disease; SVR: Sustained virologic response.

Significantly higher ATX levels in women were observed in patients with varix rupture, hepatic ascites, and hepatic encephalopathy (P = 0.003; P < 0.001; P < 0.001, respectively).

ATX for patients with and those without HCC

The average ATX levels (mg/L) in men and women were 1.65 ± 0.40 and 2.02 ± 0.68, respectively, for patients with HCC and 1.56 ± 0.54 and 1.97 ± 0.76, respectively, for patients without HCC. However, the ATX levels were not significantly different among patients with and those without HCC in men and women (Men: P = 0.178, women P = 0.215).

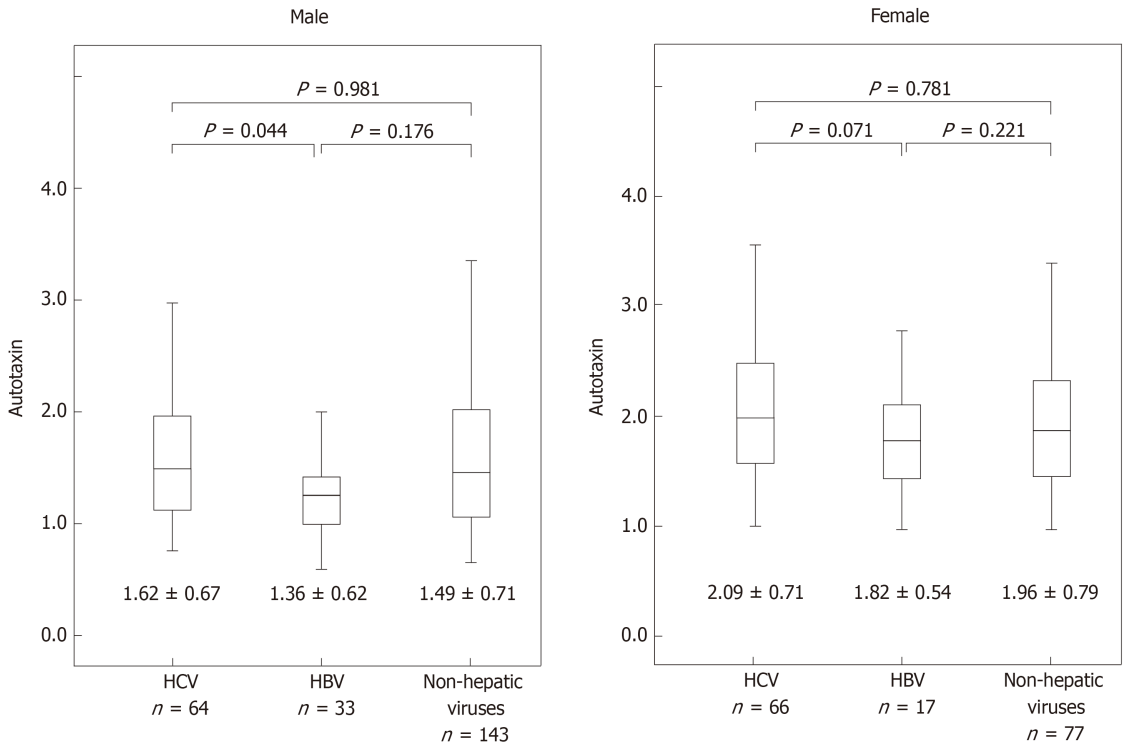

Figure 1 Comparison of autotaxin levels and the etiology. Data are expressed as mean ± SD. Separate analyses of the etiology are compared using Tukey's honestly significant difference test. HCV: Hepatitis C virus; HBV: Hepatitis B virus.

Comparison of ATX and serum biomarkers for assessment of complications of LC by ROC analysis

ATX for the assessment of complications of LC was analyzed in comparison with the MELD score, ALBI score, Fib-4 index, and APRI (Table 2). The AUCs of ATX for hepatic encephalopathy were 0.853 [95% confidence interval (CI): 0.795–0.911] in men and 0.759 (95%CI: 0.658–0.860) in women (Figure 3). The AUCs of ATX in men were higher than those in women and higher than those using other biomarkers for detecting encephalopathy. The AUCs of ATX for hepatic ascites were 0.816 (95%CI: 0.756–0.877) in men and 0.717 (95%CI: 0.616–0.819) in women. The AUCs of ATX in men were higher than those in women and higher than those using other serum biomarkers for detecting hepatic ascites. The AUCs of ALBI in men were higher than those using other biomarkers for detecting ascites. The AUCs of ATX for varix rupture were 0.706 (95%CI: 0.558–0.855) in men and 0.697 (95%CI: 0.512–0.882) in women. The AUCs of ATX in men were higher than those in women and higher than those using other serum biomarkers for detecting varix rupture (Figure 4).

Correlation between ATX and clinical characteristics using Pearson's r coefficient

Table 3 shows the correlation between ATX and clinical characteristics. The MELD score, Fib-4 index, APRI, and ALBI score were correlated with the ATX level in both men and women. The correlation coefficient between ALBI and ATX in men was strong. We also evaluated the correlation between ATX and clinical laboratory data. Platelets, prothrombin time, serum albumin, AST, ALT, total bilirubin, total cholesterol, branched-chain amino acid (BCAA), tyrosine (TYR), the BCAA-to-TYR ratio and ammonia were correlated with ATX levels in both men and women. The correlation coefficient between TYR and ATX in men was strong.

DISCUSSION

To the best of our knowledge, this is the first report that directly compared the levels of serum liver fibrosis markers and ATX in patients with the complications of LC. Direct biomarkers reflect not only hepatic fibrosis but also hepatic function[5]. We observed that ATX is a useful biomarker for assessing the complications of LC. Especially, the performance of ATX in men was better than that in women and of the ALBI score, Fib-4 index, and APRI in detecting hepatic encephalopathy and varix ruptures. These results demonstrated that ATX levels may be helpful for detecting

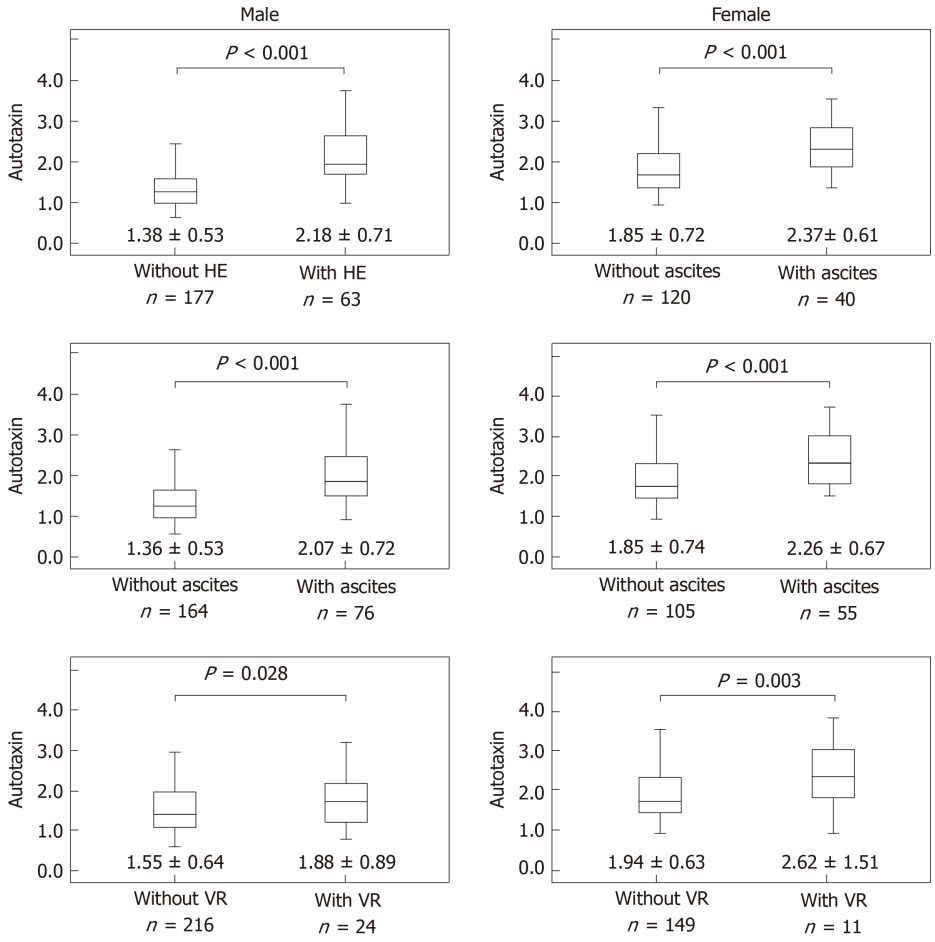

Figure 2 Comparison of autotaxin levels and severe complications of liver cirrosis. Data are expressed as mean ± SD. Separate analyses of complications were compared using the unpaired *t*-test. HE: Hepatic encephalopathy; VR: Varix ruptu.

clinically evident decompensating events due to portal hypertension.

Portal hypertension is caused by increased portal venous flow and/or enhanced intrahepatic vascular resistance resulting from the activation of hepatic stellate cells and the dysfunction of endothelial cells[10]. Because endothelial dysfunction lead to decrease ATX clearance, ATX may indicate portal hypertension. Pleli *et al*[10] reported a possible causative link between the extent of portal hypertension and ATX levels. Especially, patients suffering from portal hypertensive gastropathy and hepatic encephalopathy showed significantly higher ATX serum concentrations. Increased portal venous flow or enhanced intrahepatic vascular resistance lead to diversion of blood away from the liver toward low-resistance portosystemic vessels[10,15]. Portal hypertensive collateral formation leads to "varices" that are dilated end-organ veins with a high risk of rupture, as well as "shunts" that may lead to recurrent and refractory hepatic encephalopathy[16]. By contrast, for detecting ascites, the AUC of ATX was lower than that using ALBI. The significance of albumin in ascites has been established since the 1940s[17]. Cirrhotic patients with hypoalbuminemia developed ascites while patients without hypoalbuminemia did not[18,19]. The ALBI grades, which are calculated from albumin and serum bilirubin, may be indicated better than those of any other biomarkers for detecting hepatic ascites.

Pleli *et al*[10] reported serum levels of ATX levels from subjects with LC were elevated compared to healthy control subjects, and serum ATX levels correlated with the Child-Pugh score in predicting the severity of the disease. That is, dysfunction of endothelial cells from the progression of fibrosis lead to reduced ATX clearance and increased serum ATX. However, the mechanism for the high levels of ATX could be complicated. A unique aspect of ATX is its high level in female patients, but the exact mechanism is unknown[20-22]. Sex-dependent differences in serum ATX levels need to be considered when using the ATX level as a marker of liver fibrosis. In the present study, ATX was more accurate in male patients than in female patients as a liver fibrosis marker for detecting liver disease severity. ATX levels in women may overestimate liver disease severity compared with any other serum biomarkers.

Furthermore, previous reports indicated that elevated ATX levels were associated

Table 2 Area under the receiver operator characteristic curves

Item	Parameter	Male	Female
		AUC (95%CI)	AUC (95%CI)
Hepatic encephalopathy	ATX	0.853 (0.795-0.911)	0.759 (0.658-0.860)
	MELD	0.644 (0.563-0.725)	0.727 (0.640-0.814)
	Fib 4 index	0.651 (0.568-0.734)	0.674 (0.538-0.789)
	APRI	0.670 (0.590-0.750)	0.640 (0.516-0.778)
	ALBI	0.825 (0.757-0.893)	0.815 (0.685-0.811)
Hepatic ascites	ATX	0.816 (0.756-0.877)	0.717 (0.616-0.819)
	MELD	0.640 (0.560-0.720)	0.744 (0.665-0.823)
	Fib 4 index	0.654 (0.569-0.738)	0.653 (0.540-0.765)
	APRI	0.663 (0.581-0.744)	0.633 (0.520-0.746)
	ALBI	0.863 (0.807-0.918)	0.787 (0.690-0.878)
Varix rupture	ATX	0.706 (0.558-0.855)	0.697 (0.512-0.882)
	MELD	0.514 (0.386-0.643)	0.612 (0.413-0.810)
	Fib 4 index	0.619 (0.462-0.775)	0.566 (0.299-0.833)
	APRI	0.653 (0.474-0.833)	0.579 (0.333-0.826)
	ALBI	0.608 (0.449-0.768)	0.614 (0.524-0.803)

The area under the receiver operating characteristic curves of autotaxin in men were higher than those in women and higher than those using other biomarkers for detecting encephalopathy and varix rupture. ATX: Autotaxin; Fib-4: Fibrosis-4; ALBI: Albumin-bilirubin index; MELD: Model for end-stage liver disease; APRI: Aspartate aminotransferase-to-platelet ratio index. AUC: Area under the receiver operating characteristic curve; CI: Confidence interval.

with inflammatory liver damage[23-25]. In the present study, the ATX levels for men in the HBV and HCV groups differed slightly (P = 0.044). The reason for this may correlate with the different mechanisms for liver inflammation from HBV and HCV. Patients with chronic HCV infection exhibited persistent inflammatory responses and fibrogenesis throughout the clinical course even after progression to LC[26]. Meanwhile, chronic HBV infection was quiescent inflammation because of seroconversion from HBe antigen to HBe antibody in most cases[27]. Therefore, suppression of liver inflammation after the eradication of HCV may lead to a better clinical outcome in patients with HCV who have an elevated ATX fibrosis marker. In point of fact, the ATX levels of patients with eradicated HCV through direct-acting antiviral therapy were comparable to those of uninfected patients[28].

This study had some limitations. First, this was a retrospective study. Second, this study included 400 patients with chronic liver diseases whose etiologies were not uniform. We made ATX comparisons among HBV, HCV, and nonviral groups. However, the proportion of patients with complications of LC was different among these three etiologies. Third, the gold standard for assessment of the severity of portal hypertension is the hepatic venous pressure gradient (HVPG). Regrettably, however, the correlation between ATX and HVPG was unknown because these data were not evaluated for all the enrolled patients due to it being a retrospective study. Future studies on the HVPG may make it the first-choice biomarker for the assessment of portal hypertension. Fourth, this study was a multiple center study. Therefore, there may have been inconsistencies between the experimental equipment and standardization.

In conclusion, our study revealed that ATX is a useful clinical biomarker for assessing the complications of LC because it could reflect not only hepatic fibrosis but also hepatic function. Using ATX as a biomarker in men was more efficacious than that of any other biomarkers for hepatic encephalopathy and varix ruptures. To make treatment decisions, it is necessary to consider that patients with high ATX levels may have complications of LC.

Table 3 Correlation between autotaxin and clinical characteristics

Characteristics	Male		Female	
	r	*P* value	*r*	*P* value
Serum biomarker				
MELD-score	0.293	0.001	0.211	0.040
Fibrosis 4 index	0.470	< 0.001	0.335	< 0.001
APRI	0.438	0.001	0.414	< 0.001
ALBI	0.614	< 0.001	0.452	< 0.001
Clinical laboratory data				
White blood cells	-0.123	0.057	-0.130	0.102
Hemoglobin	-0.260	0.001	-0.268	0.001
Platelets	-0.289	0.001	-0.308	0.001
Prothrombin time	-0.443	< 0.001	-0.279	0.001
Serum albumin	-0.539	< 0.001	-0.451	< 0.001
Blood urea nitrogen	-0.093	0.152	-0.123	0.716
Serum creatinine	-0.036	0.580	-0.139	0.387
Aspartate aminotransferase	0.443	0.001	0.329	0.001
Alanine aminotransferase	0.225	0.022	0.249	0.001
Total bilirubin	0.538	< 0.001	0.300	0.001
Serum sodium	-0.282	0.001	-0.192	0.015
Hemoglobin A1c	-0.151	0.109	-0.094	0.282
Total cholesterol	-0.244	0.001	-0.231	0.001
BTR	-0.596	0.004	-0.298	0.003
Branched chain amino acid	-0.338	0.001	-0.260	0.374
Tyrosine	0.585	< 0.001	0.455	< 0.001
Ammonia	0.369	< 0.001	0.407	< 0.001
Alpha-fetoprotein	0.074	0.269	-0.051	0.549

The model for end-stage liver disease score, Fibrosis-4 index, aspartate aminotransferase-to-platelet ratio index, and albumin-bilirubin index (ALBI) score were correlated with the autotoxin level in both men and women. The correlation coefficient between ALBI and autotaxin in men was strong. Fib-4: Fibrosis-4; ALBI: Albumin-bilirubin index; MELD: Model for end-stage liver disease; APRI: Aspartate aminotransferase-to-platelet ratio index; BTR: Branched-chain amino acid and tyrosine ratio.

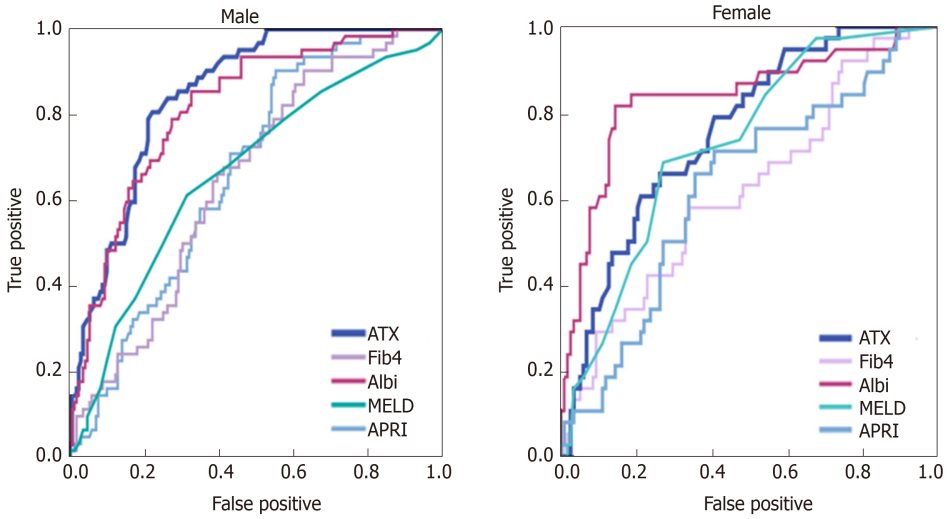

Figure 3 Comparison of autotaxin and serum biomarkers for assessment of hepatic encephalopathy by the receiver operating characteristic curve analysis. The Area under the curves of autotaxin in men were higher than those in women and all the other biomarkers for detecting encephalopathy. ATX: Autotaxin; Fib-4: Fibrosis-4; ALBI: Albumin-bilirubin index; MELD: Model for end-stage liver disease; APRI: Aspartate aminotransferase-to-platelet ratio index.

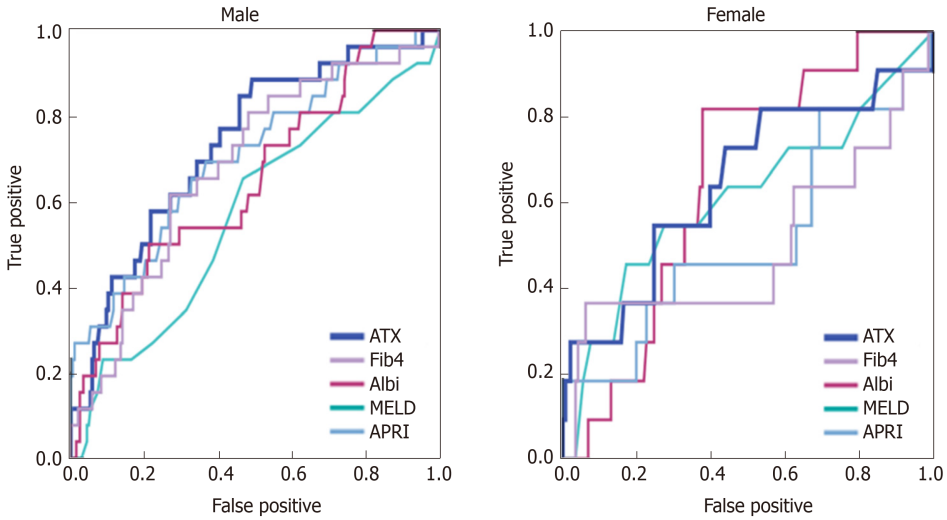

Figure 4 Comparison of autotoxin and serum biomarkers for assessment of varix ruptures by the receiver operating characteristic curve analysis. The area under the curves of autotaxin in men were higher than those in women and all the other biomarkers for detecting varix ruptures. ATX: Autotaxin; Fib-4: Fibrosis-4; ALBI: Albumin-bilirubin index; MELD: Model for end-stage liver disease; APRI: Aspartate aminotransferase-to-platelet ratio index.

ARTICLE HIGHLIGHTS

Research background

Developments of serum biomarkers have focused on the diagnosis of cirrhosis, but more recent researches have emphasized the availability of these markers to assess patients with more advanced fibrosis.

Research motivation

The autotaxin (ATX) level may be a useful biomarker to select treatment therapy for ascites, hepatic encephalopathy, and varix ruptures. And the assessment for the complications of liver cirrhosis (LC) is especially valuable in helping to make treatment decisions.

Research objectives

The aim of this study was to assess the clinical usefulness of ATX for assessing the complications of LC.

Research methods

This multicenter, retrospective study was conducted at six locations in Japan. We include patients with LC, n = 400. The ATX level was evaluated separately in men and women because of its high level in female patients. To assess the clinical usefulness of ATX for the complications of LC, the area under the curve (AUC) of ATX assessing for the severe complications was analyzed in comparison with the model for end-stage liver disease score, albumin-bilirubin (ALBI) score, fibrosis-4 index, and aspartate aminotransferase-to-platelet ratio index.

Research results

The AUCs of ATX in men for hepatic encephalopathy, hepatic ascites, and varix ruptures were 0.853, 0.816, and 0.706, respectively. The AUCs of ATX in women for hepatic encephalopathy, hepatic ascites, and varix rupture were 0.759, 0.717, and 0.697, respectively. The AUCs of ATX in men were higher than those in women, as were all the other biomarkers used to detect encephalopathy and varix ruptures. However, for detecting ascites, the AUC of ALBI in men was more effective than using ATX.

Research conclusions

ATX is a useful biomarker for assessing the complications of LC. Especially, the use of ATX in men was more effective than any other biomarker for detecting hepatic encephalopathy and varix ruptures. The ATX level is especially valuable in helping to make treatment decisions for hepatic encephalopathy and varix ruptures. ATX in men was more effective than any other biomarkers for detecting hepatic encephalopathy and varix ruptures. Developments of serum biomarkers have focused on the diagnosis of cirrhosis and the assessment of advanced fibrosis. Using ATX as a biomarker in men was more efficacious than that of any other biomarkers for hepatic encephalopathy and varix ruptures. To make treatment decisions, it is necessary to consider that patients with high ATX levels may have complications of LC. ATX is a useful clinical biomarker for assessing the complications of LC because it could reflect not only hepatic fibrosis but also hepatic function. Direct biomarkers reflect not only hepatic fibrosis but also hepatic function. The gold standard for assessment of the severity of portal hypertension is the hepatic venous pressure gradient (HVPG). Future studies on the HVPG may make it the first-choice biomarker for the assessment of portal hypertension.

Research perspectives

Direct biomarkers reflect not only hepatic fibrosis but also hepatic function. The gold standard for assessment of the severity of portal hypertension is the HVPG. Future studies on the HVPG may make it the first-choice biomarker for the assessment of portal hypertension. The best method is a direct comparison of the ATX and HVPG for assessing the complications of LC.

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