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Baishideng Publishing Group Inc
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Portal vein thrombosis in cirrhotic patients - it is always the small pieces that make the big picture

Irina Gîrleanu, Anca Trifan, Carol Stanciu, Cătălin Sfarti

Irina Gîrleanu, Anca Trifan, Carol Stanciu, Cătălin Sfarti, Department of Gastroenterology, "Grigore T Popa" University of Medicine and Pharmacy, Iași 700115, Romania

Irina Gîrleanu, Anca Trifan, Carol Stanciu, Cătălin Sfarti, Institute of Gastroenterology and Hepatology, "St. Spiridon" University Hospital, Iași 700115, Romania

ORCID number: Irina Gîrleanu (0000-0001-5925-1232); Anca Trifan (0000-0001-9144-5520); Carol Stanciu (0000-0002-6427-4049); Cătălin Sfarti (0000-0001-7074-5938).

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Correspondence to: Anca Trifan, MD, Doctor, Professor, Department of Gastroenterology, "Grigore T Popa" University of Medicine and Pharmacy, 16 Universitatii St, Iași 700115, Romania. ancatrifan@yahoo.com
Telephone: +40-72-6108428
Fax: +40-23-2211820

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Abstract

Portal vein thrombosis (PVT) is a frequent and serious complication in patients with liver cirrhosis (LC). Recently, a new classification of PVT was proposed, although the functional component was not completely included. The status of liver disease (compensated/decompensated) should be added to this classification. Reduced portal flow velocity and the acquired hypercoagulable status associated with LC are the main risk factors for PVT development, although endothelial dysfunction may play an important role that needs to be further evaluated. The European Association for the Study of the Liver and the American Association for the Study of Liver Disease recommend that the anticoagulant treatment should be considered in cirrhotic patients with PVT. Low molecular weight heparin and vitamin K antagonists proved their efficacy and relatively safety in PVT treatment, although in addition to recanalization rates, more complex endpoints such as mortality and decompensation rate should be evaluated. The new oral anticoagulant therapies offers the advantage of oral administration in the absence of laboratory monitoring, however, there are a few reports regarding their use in cirrhotic patients, most of them referring to compensated isolated cases. Transjugular intrahepatic portosystemic shunt could be an alternative if thrombosis progresses despite anticoagulant therapy and/or when PVT is associated with portal hypertension complications. The aim of this editorial is to discuss the different aspects of pathophysiology, clinical relevance, diagnosis and management of PVT in patients with LC.

Key words: Portal vein thrombosis; Liver cirrhosis; Classification; Risk factors; Anticoagulation

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Core tip: Portal vein thrombosis is a frequent and serious complication in patients with liver cirrhosis. The new classification needs to be validated and should contain the pattern of thrombus evolution and the status of liver cirrhosis- compensated or decompensated. The two main risk factors - reduced portal flow velocity and the hypercoagulable state should be addressed more extensively in large studies, considering the stage of liver disease. The anticoagulant treatment could be considered in cirrhotics with portal vein thrombosis. The end-points of the anticoagulant treatment should consider the recanalization, decompensation, and the mortality rates.

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INTRODUCTION

Portal vein thrombosis (PVT) is a frequent and serious complication in patients with liver cirrhosis (LC). The prevalence of PVT in patients with LC ranges from 0.6% to 26%^[1] compared to 0.7 -1/100000 in the general population^[2]. PVT has been increasingly diagnosed in LC using noninvasive imaging techniques such as ultrasound (US), computed tomography (CT) or magnetic resonance imaging (MRI), with the highest prevalence at the time of liver transplantation^[3]. PVT mechanisms in cirrhotic patients shifted from a hypocoagulable state, due to thrombocytopenia and decreased coagulation factors levels, towards an acquired hypercoagulable state characterized by decreased protein C, protein S and antithrombin III levels and increased factor VIII levels^[4].

PVT clinical presentation in cirrhotic patients is heterogenous ranging from incidental diagnosis by US during routine follow-up screening for hepatocellular carcinoma (HCC) to life-threatening complications such esophageal/gastric variceal bleeding or intestinal infarction^[5].

The diagnosis of PVT is based on imaging techniques: Doppler US the first choice method (widely available, cheap, without irradiation) or contrast enhanced US. Contrast enhanced CT and MRI are superior to Doppler US for the assessment of thrombotic extension to venous branches difficult to be assessed by US exam^[6]. The optimal treatment of nonmalignant PVT in cirrhotic patients remains an unmet need. The European Association for the Study of the Liver (EASL)^[7] guideline for vascular diseases of the liver, the American Association of the Study of the Liver Diseases (AASLD)^[6]

and BAVENO VI consensus^[8] recommend that the anticoagulant treatment should be considered, without any strong clinical evidence. The aim of this editorial is to discuss the different aspects of pathophysiology, clinical relevance, diagnosis and management of non-malignant PVT in patients with LC.

DEFINITIONS AND CLASSIFICATIONS:

OLD AND NEW

PVT refers to partial or complete occlusion of the portal vein trunk which can includes its right and/or left intrahepatic branches, with the possibility to extend either to the superior mesenteric vein or to the splenic vein.

PVT includes two different entities: the first one is acute PVT defined by the sudden formation of a thrombus within portal vein and often involving mesenteric or splenic veins, and the second is chronic PVT (also known as cavernoma). The AASLD defined acute PVT as the sudden formation of a thrombus within the portal vein lumen, and chronic PVT as the replacement of the obstructed portion is replaced network of collaterals resulting in the cavernomatous transformation of the portal vein^[6]. The EASL defined recent PVT as a recent formation of a thrombus within the portal vein and/or right or left branches, while chronic PVT is characterized by the absence of recanalization and development of porto-portal collaterals resulting in a cavernomatous transformation of the portal vein^[7].

These definitions, otherwise simple and easily interpreted, are based only on anatomic findings, lacking any clinically significant consequences of thrombotic occlusion of the portal vein such as portal hypertension and ascites. Taking into account this aspect, Sarin *et al*^[9] proposed that PVT should be defined as a clinical syndrome presenting itself either as an incidental finding or with variable signs and symptoms such as: abdominal pain, new onset of ascites, variceal bleeding or intestinal infarction.

During the past two decades at least eight classifications of PVT have been proposed; the first one by Stieber *et al*^[10] in 1991, another by Yerdel *et al*^[11] in 2000, and the last by de Franchis (Baveno VI classification) in 2015 (Table 1). All these classifications have several limitations: they are purely anatomic, with no functional relevance, or clear delineation between acute and chronic forms, no etiology assessment and, no clinical therapeutic decisiveness.

Sarin *et al*^[9] have proposed a new classification of PVT in cirrhosis which is an anatomico-functional classification including the site of PVT, degree of portal venous system occlusion, extend of PVT, duration and presentation (recent, chronic asymptomatic, symptomatic), type and presence of underlying liver disease (cirrhotic, non-cirrhotic, post-transplant, HCC) (Table 2).

The prognostic value and the influence of this new

Table 1 Portal vein thrombosis - Baveno VI - classification^[8]

Site of PVT	Type 1: Only trunk Type 2: Only branch: 2a - one branch, 2b - both branches Type 3: Trunk and branches
Presentation	R: Recent Ch: Chronic C: Cirrhotic
Type of underlying liver disease	N: Non-cirrhotic liver disease H: HCC and other local malignancies L: Post-liver transplant A: Absence of underlying liver disease
Degree of portal venous system occlusion	I: Incomplete: Flow visible in PV lumen through imaging T: Total: No flow visible in PV lumen on imaging
Extent of PV system occlusion	Splenic vein (S) Mesenteric vein (M) Both (SM)

PVT: Portal vein thrombosis; HCC: Hepatocellular carcinoma; PV: Portal vein.

Table 2 Anatomico-functional classification of portal vein thrombosis in cirrhosis^[9]

Site of PVT	Type 1: Only trunk Type 2: Only branch: 2a - one branch, 2b - both branches Type 3: Trunk and branches
Duration and presentation	R: Recent (first time detected in previously patent PV) Asymptomatic: (As) Symptomatic: (S)- acute PVT features (with or without ABI) Ch: Chronic (previously diagnosed PVT on follow-up, portal cavernoma and clinical features of PHT) Asymptomatic Symptomatic: Features of portal hypertension Cirrhotic Non-cirrhotic liver disease HCC and other local malignancies Post-liver transplant Local malignancies Associated conditions
Type of underlying liver disease	
Degree of portal venous system occlusion	O: Occlusive: No flow visible in PV lumen on imaging/Doppler study NO: Nonocclusive: Flow visible in PV lumen through imaging/Doppler study
Extent of PV system occlusion	Splenic vein (S) Mesenteric vein (M) Both (SM)

PVT: Portal vein thrombosis; ABI: Acute bowel infarction; PHT: Portal hypertension; HCC: Hepatocellular carcinoma; PV: Portal vein.

classification in therapeutic decision remains to be confirmed by future prospective studies. In our opinion it is important to add the pattern of PVT evolution (spontaneous recanalization, extension or stable) with or without anticoagulant treatment, and the status of the liver cirrhosis (compensated or decompensated) in order to personalize the therapeutic approach.

PREVALENCE AND PREDICTORS FOR NON-MALIGNANT PVT: ETIOLOGY OF LIVER CIRRHOSIS

PVT prevalence is estimated 0.6%-26% in patients with liver cirrhosis, increasing proportionally with LC severity^[1]. In patients with compensated cirrhosis reported prevalence ranges from 1% to 8%-26% in candidates for LT^[3,4]. However, there are few studies that reported PVT incidence in LC. Thus, Nery *et al*^[12]

demonstrated a 5-year cumulative PVT incidence in LC of 10.7%, while Maruyama *et al*^[13] performed a retrospective analysis on 150 patients with LC followed up for a median period of 66 mo and reported a 12.8% cumulative overall incidence of PVT at 1 year, 18.6% at 3 years, 20% at 5 years, and 38.7% at 8-10 years.

Multiple studies evaluated the predictive factors for PVT development in cirrhotic patients. Prior PVT^[12], severe liver disease (Child-Pugh class A and B)^[14], hypercoagulable status^[15-17], recent surgical or invasive interventions of the abdomen^[18], portal flow velocity < 15 cm/s^[16], and HCC^[12], were described as having predictive value for PVT development in cirrhotic patients. In a recent Italian national multicenter study, including 753 cirrhotic patients, Violi *et al*^[19], demonstrated that previous portal vein thrombosis, Child-Pugh class B and C, HCC, prior upper gastrointestinal bleeding, and older age were independently associated with the presence of PVT.

The influence of cirrhosis etiology on development

of PVT has not been yet clearly defined. Nonami *et al*^[20] in a research on 885 candidates for LT with PVT demonstrated that alcoholic and hepatitis B virus related cirrhosis were found to be the most frequent etiologies of LC. The association between PVT and alcoholic etiology was recently confirmed by Scheiner *et al*^[21]. Cruz *et al*^[22] demonstrated that non-alcoholic steato-hepatitis was more frequently associated with PVT (40.48%), followed by hepatitis C virus (23.81%) and autoimmune hepatitis (19.05%), all of these etiologies being characterized by a significant pro-inflammatory status. By contrast, in another study with 199 candidates for LT, no relation was found between the etiology of liver disease and PVT prevalence^[23].

MECHANISMS LEADING TO PVT IN LIVER CIRRHOSIS

The physiopathological mechanisms of PVT remain controversial, although many of them have been by now demonstrated. Even if, the interest in LC associated PVT have been increasing during these last years, considering the complex tests, such as the global test for coagulation assessment -thrombin generation assays or thrombelastometry used to characterize PVT^[24-26] in cirrhotic patients, there are still a lot of missing pieces from the big picture of PVT.

PVT is a disease with multifactorial causes and, in some particular cases it is triggered by a genetic predisposition. The components of Virchow's triad (venous stasis, hypercoagulable state and endothelial dysfunction) are recognized as the main factors involved in PVT development in cirrhotic patients^[4,13,15,16].

Reduced portal flow velocity was admitted to be as the most important risk factor for PVT development in LC, although this parameter varies significantly according to the degree of liver disease severity^[16,19]. Zocco *et al*^[16] demonstrated for the first time that portal vein velocity under 15 cm/s predisposes to PVT development and, recently, Stine *et al*^[27] confirmed these results in a match case-control study. This theory started a long-debated argument that non-selective β -blockers (NSBBs) may induce PVT in liver cirrhosis. In a small study on 56 patients with liver cirrhosis, evaluated for PVT every 6 mo, Zampino *et al*^[28] demonstrated that the use of NSBBs could be an independent predictor for PVT development, although no other study has yet confirmed this hypothesis.

Liver cirrhosis is associated with profound and complex coagulation defects, involving platelets number and function, pro- and anticoagulant factors, as well as fibrinolytic system^[4].

It's a well known fact that patients diagnosed with LC present thrombocytopenia, mostly secondary to increased splenic destruction, but also due to thrombopoietin deficiency^[29]. Although the platelet number is low, their function is not impaired, moreover platelet hyperreactivity is associated with increased

levels of von Willebrand factor and factor VIII^[30].

There is growing evidence that hypercoagulability is an important part of the hematological spectrum in cirrhosis. Tripodi *et al*^[25] demonstrated, using global hemostasis assays, that in cirrhotic patients there is a normal or even increased thrombin generation. Liver cirrhosis is characterized by a decrease in procoagulant factors (fibrinogen, factor II, V, X, VII, IX, XI, XII) and anticoagulant factors (protein C, protein S and antithrombin III)^[4,31,32]. In another study Tripodi *et al*^[33] confirmed that protein C deficiency is the most important factor that contributes to the procoagulant status in LC. Rossetto *et al*^[34] performed a more complex analysis of the coagulation cascade in cirrhotics with PVT and demonstrated that the complex factor VIIa-antithrombin was significantly higher in PVT patients compared to healthy volunteers. These data were not confirmed by two recent studies conducted by Chen *et al*^[35] and Tang W *et al*^[36], the results of their case-control analysis concluded that there was no difference regarding the pro- and anticoagulant factors between patients with and without PVT matched for age, sex and Child-Pugh score.

The fibrinolytic system also is involved in PVT development in cirrhotic patients. LC is characterized by increased tissue-type plasminogen activator and plasminogen activator inhibitor-1 levels, and decreased plasminogen, alpha 2-antiplasmin and thrombin-activable fibrinolysis inhibitor levels^[37]. This abnormalities in the fibrinolytic cascade could explain the spontaneous recanalization of PVT as described in almost a quarter of the cirrhotic patients^[38].

Inherited thrombophilic disorders are reported in up to 70% of patients with cirrhosis and PVT. The most important genetic abnormalities are factor II mutation (G20210A), factor V mutation and the homozygous polymorphisms of methylenetetrahydrofolate reductase (MTHFR) C677T gene mutation^[39,40]. D'Amico *et al*^[17] confirmed the influence of MTHFR gene mutation in PVT pathogenesis along with the plasminogen activator inhibitor-type 1 4G-4G mutation. Although, it should be mentioned that the prospective longitudinal found studies no clear relationship between inherited factors and PVT development.

Other inherited prothrombotic conditions as hyperhomocysteinemia^[40] antiphospholipid syndrome^[41] or myeloproliferative disorders^[42] were evaluated, but did not proved as major risk factors for PVT development in cirrhotic patients.

There is evidence that in cirrhotic patients, markers of endothelial dysfunction, including von Willebrand factor, P-selectin and isoprostanes, are up-regulated, suggesting that endothelial cells may favor the PVT development in cirrhosis^[4]. Also, Carnevale *et al*^[43] demonstrated that the lipopolysaccharide from *Escherichia coli* stimulates factor VIII production from the endothelial cells. Endotoxemia may play an important role in activating the clotting system in portal and systemic circulation and could represent an underlying mechanism for PVT.

The bacterial translocation determines inflammation which leads to hemodynamic alterations and ultimately to an increase in portal pressure^[44]. There are studies that describe portal endotoxemia as a triggering factor of the coagulation cascade in cirrhotic patients, although a recent small study on 49 patients with cirrhosis found that endotoxemia and platelet activity were not associated to PVT^[45]. Vascular endothelial dysfunction may play a role in the pathogenesis of PVT. All these risk factors could explain the favorable role of prophylactic administration of enoxaparin in delaying the hepatic decompensation and improving survival^[46].

The two main risk factors for PVT in LC-reduced portal flow velocity and the procoagulant status should be addressed more extensively in large studies, considering two different scenarios: compensated and decompensated liver disease. This discrimination could influence not only the understanding of the physiopathological mechanism of PVT development, but also the indication for a certain anticoagulant therapy.

Obviously, data received while using the *in vitro* complex coagulation assessment should be considered carefully and not used for generalization^[25]. Furthermore, data provided by the use of only one type of coagulation parameter should be partially considered, as there are several studies with different results in regard to pro- and anticoagulant factors levels in PVT^[35,36]. As we advanced in understanding the underlying molecular mechanism of thrombosis, the coagulation investigation in cirrhotic patients becomes more complicated, time-consuming, and expensive, thus affordable only to large clinical laboratories. Unfortunately, this kind of comprehensive specific analysis of coagulation disorders in cirrhotic patients with PVT has not yet been conducted, while most of PVT and liver cirrhosis studies remain inconclusive, being based on a small sample size.

The screening for underlying thrombophilic conditions should be considered especially in patients with compensated liver disease in whom the vascular component of the Virchow's triad is not so important. A special category of cirrhotic patients with PVT is represented by those patients in whom PVT extends despite the administration of anticoagulant therapy or reappears after spontaneous recanalization. In such patients there are other risk factors which should be identified such as endothelial dysfunction, genetic thrombophilic disorders or undiagnosed neoplasia that could predispose to PVT.

WHEN AND HOW TO TREAT PVT IN LIVER CIRRHOSIS

The main goal of PVT treatment is to restore the portal blood flow and prevent the thrombus extension.

The Baveno VI Consensus^[8], published in 2015, recommends the anticoagulant treatment in cirrhotic patients with PVT who are potential candidates for LT, while no recommendation is made for non-candidates,

thus highlighting the need for individualized treatment and randomized trials on the benefit/risk ratio of anticoagulation in cirrhotic patients.

The EASL 2015^[7] and 2018^[47] guidelines for vascular diseases of the liver and for the management of patients with decompensated liver cirrhosis state that anticoagulant treatment must be considered in cirrhotic patients with PVT following the implementation of an adequate prophylaxis for gastrointestinal bleeding, while in 2009 the AASLD^[6] recommended at least three months of anticoagulant use in the treatment of PVT, irrespective of the presence of cirrhosis.

Although the guidelines accepted the anticoagulant treatment or TIPS as therapeutic option for PVT in LC not all centers accepted the idea in the daily clinical practice, so that to treat or not to treat PVT in LC it still remains an open issue.

Low-molecular-weight heparin and vitamin K antagonists

The uncertainty regarding the real efficacy of an anticoagulant treatment derives from the data reporting the natural history of PVT in LC. Studies evaluating the anticoagulant treatment have reported that spontaneous recanalization of the portal vein in the absence of anticoagulant treatment is unusual^[12,13]. In the study by Francoz *et al.*^[48] no patient achieved recanalization in the absence of anticoagulation, while 42% achieved recanalization while under anticoagulant therapy. Senzolo *et al.*^[49] reported thrombus progression in 75% of patients who did not receive anticoagulant treatment, compared to only 15% of treated patients. There are limited studies reporting on the use of anticoagulation for PVT in patients with cirrhosis. In all these studies, complete recanalization has been described in 33%-45% of cases, while partial portal vein recanalization was observed in 15%-35% of cases^[48-53]. Small sample size is one of the major problems of nearly all such investigations. The most cited side effect was the bleeding from different sites: gastrointestinal (variceal bleeding, postligation ulcer, peptic ulcer), intracerebral hemorrhages, epistaxis and hematuria^[48-53].

In order to overcome the small sample size bias and increase the efficacy and safeness of the anticoagulant treatment in patients with PVT and LC, two meta-analysis have recently been published^[54,55]. Qi *et al.*^[54] concluded in 2015 that anticoagulation could achieve a relatively high rate of portal vein recanalization in cirrhotic patients with PVT, information confirmed by another meta-analysis published by Loffredo *et al.*^[55] in 2017.

The attendant optimism is, at least in part, based on the relatively safeness of the anticoagulant treatment, including the pleiomorphic effect of reducing fibrogenesis by thrombin antagonism^[56]. For a better evaluation of the anticoagulant treatment in patients with LC other end-points should be established such as short-term and long-term mortality and decompensation or further

decompensation rate. Achieving PVT recanalization is only one of the goals of anticoagulant therapy in cirrhotics with PVT, and it is far more important to document the real impact of recanalization on LC evolution in order to confirm the benefits of this controversial treatment.

If the anticoagulant treatment is the first therapeutic option for cirrhotic patients with PVT, the ideal anticoagulant has not been developed yet. Low-molecular-weight heparin (LMWH) and vitamin K antagonists (VKAs) are the anticoagulant drugs recommended for PVT treatment, but they have some disadvantages: efficacy of LMWH may be significantly decreased (up to 40%) due to lower levels of antithrombin III synthesis by the liver, and the coagulopathy secondary to liver disease frequently results in an elevated International Normalized Ratio (INR) and thus utilizing the INR to guide dosing of VKAs is particularly challenging^[6-8].

Direct oral anticoagulants and PVT treatment

The direct oral anticoagulants (DOACs) - thrombin inhibitors (dabigatran) and activated factor X inhibitors (rivaroxaban, apixaban or edoxaban) overcame the numerous drawbacks of traditional anticoagulants and proved their efficacy and safety in stroke prophylaxis in nonvalvular atrial fibrillation, venous thromboembolism prophylaxis in orthopaedic patients, and the treatment of acute pulmonary embolism and deep vein thrombosis^[57]. DOACs offer the advantage of oral administration, the absence of laboratory monitoring, and an antithrombin III independent mechanism of action. Rivaroxaban and apixaban are 67% metabolized in the liver, with half-lives of 5-9 h and 12 h respectively^[58], their concentration depending on the plasma binding proteins. Edoxaban is 50% metabolized by the liver with a half-life of 10-15 h^[58]. Dabigatran has limited hepatic metabolism, minimal binding to plasma proteins, and longer half-life (12-14 h)^[57]. Another advantage of dabigatran is the development of an antidote - idarucizumab - monoclonal inhibitor antibody^[59].

However, there are few reports regarding their use in cirrhotic patients, most of which in compensated isolated cases. Rivaroxaban is the most studied DOACs for the treatment of PVT^[60]. There is little scientific evidence regarding the use of DOACs in cirrhosis with or without PVT, and even fewer well-designed prospective studies.

In the VALDIG study, major bleeding requiring discontinuation of DOACs was seen in two of 258 (0.71%) patients without cirrhosis and in one of 36 (2.7%) patients with cirrhosis^[61]. Intagliata *et al.*^[62] retrospectively evaluated class Child-Pugh A and B cirrhotic patients having received DOACs treatment for different conditions. Two thirds of the patients received DOACs for PVT treatment. There are no data reported on the recanalization rate. Major bleeding events occurred in 5% of the patients and a paradoxically PVT recurrence during the anticoagulant treatment was described. In a clinical trial assessing the efficacy of VKAs, Hafany

et al.^[63] compared the DOAC rivaroxaban with warfarin in 80 patients with virus C compensated cirrhosis. They reported a 85% recanalization rate, in contrast with the 45% in patients treated with warfarin, higher short-term survival rate and fewer gastrointestinal bleeding events in patients treated with DOACs. Nagaoki *et al.*^[64] compared edoxaban and warfarin in cirrhotic patients with PVT and, concluded that edoxaban is an effective anticoagulant treatment, although most of the events involving the gastrointestinal bleeding were associated with the administration of edoxaban (15% vs 7%).

The recent literature does not establish with certainty the role of DOACs in treating PVT in cirrhotic patients, and further large clinical trials are needed confirm if the DOACs can be used effectively and safely in Child-Pugh A or B liver cirrhosis.

Transjugular intrahepatic portosystemic shunt

Classically considered contraindicated in PVT, TIPS could be an alternative particularly if thrombosis progresses despite satisfactory anticoagulation and/or when PVT is associated with severe portal hypertension complications^[1,48]. However, in such cases, TIPS is expected to be technically challenging with a higher failure rate^[48,65] and should be attempted only in experienced centres. TIPS may be a treatment option in patients with acute PVT. In chronic PVT or portal cavernoma TIPS is unsuccessful if the lumen of thrombosed portal vein is not catheterizable and cavernomatous vein is not amenable to dilatation.

FUTURES PERSPECTIVES: UNMET NEEDS

Although new data on the mechanisms of PVT development in cirrhotic patients was published and a new complex classification is proposed, there are still a lot of puzzle pieces missing in the big picture of PVT.

The prognostic value of the new PVT classification remains to be confirmed by future prospective studies, without omitting the pattern of PVT evolution and the status of the liver cirrhosis (compensated or decompensated).

Controversies persist regarding the mechanism leading to PVT in LC. The influence of each previously described risk factor, in the pathogenesis of PVT needs to be demonstrated. The role of microbiota and the influence of endotoxemia in the development of PVT in compensated LC must also to be addressed. The natural history of PVT should be described in large multicenter studies in order to identify predictors for spontaneous recanalization and risk factors for rethrombosis. Updated complex and global dynamic coagulation tests should be developed and validated to assess the coagulation disorders in cirrhotic patients with PVT and even anticoagulant therapy monitoring.

The ideal anticoagulant treatment of PVT in cirrhotic patients is not yet described. DOACs are used off label for PVT treatment in LC despite the lack of randomized

control trials confirming the safety and efficacy. The end-points of these studies should also include short-term and long-term mortality rates together with decompensation outcomes.

No doubt, many advances have been made during the last decade regarding different aspects of PVT pathophysiology and treatment in cirrhotic patients, although this complication of liver cirrhosis still has more questions than answers.

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Novel targeting approaches and signaling pathways of colorectal cancer: An insight

Ankita Tiwari, Shivani Saraf, Amit Verma, Pritish Kumar Panda, Sanjay K Jain

Ankita Tiwari, Shivani Saraf, Amit Verma, Pritish Kumar Panda, Sanjay K Jain, Pharmaceuticals Research Projects Laboratory, Department of Pharmaceutical Sciences, Dr. Hari Singh Gour Central University, Sagar 470003, India

ORCID number: Ankita Tiwari (0000-0001-6433-7831); Shivani Saraf (0000-0001-7305-6054); Amit Verma (0000-0002-1376-0514); Pritish Kumar Panda (0000-0002-1422-5345); Sanjay K Jain (0000-0002-9241-3114).

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Correspondence to: Sanjay K Jain, PhD, Full Professor, Pharmaceuticals Research Projects Laboratory, Department of Pharmaceutical Sciences, Dr. Hari Singh Gour Central University, Sagar 470003, India. drskjainin@yahoo.com
Telephone: +91-7582-265457
Fax: +91-7582-264236

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Abstract

Colorectal cancer (CRC) is the third most common cancer of mortality in the world. Chemotherapy based treatment leads to innumerable side effects as it delivers the anticancer drug to both normal cells besides cancer cells. Sonic Hedgehog (SHH), Wnt wingless-type mouse mammary tumor virus/ β -catenin, transforming growth factor- β /SMAD, epidermal growth factor receptor and Notch are the main signaling pathways involved in the progression of CRC. Targeted therapies necessitate information regarding the particular aberrant pathways. Advancements in gene therapies have resulted in the recognition of novel therapeutic targets related with these signal-transduction cascades. CRC is a step-wise process where mutations occur over the time and activation of oncogenes and deactivation of tissue suppressor genes takes place. Genetic changes which are responsible for the induction of carcinogenesis include loss of heterozygosity in tumor suppressor genes such as adenomatous polyposis coli, mutation or deletion of genes like p53 and K-ras. Therefore, many gene-therapy approaches like gene correction, virus-directed enzyme-prodrug therapy, immunogenetic manipulation and virotherapy are currently being explored. Development of novel strategies for the safe and effective delivery of drugs to the cancerous site is the need of the hour. This editorial accentuates different novel strategies with emphasis on gene therapy and immunotherapy for the management of CRC.

Key words: Colorectal cancer; Immunotherapy; Gene therapy; Signaling; Targeted therapy

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Core tip: In spite of the advancements in the diagnosis and the treatment approaches for colorectal cancer (CRC), its survival rate is quite low. Therefore, there arises an urge to develop novel targeting strategies for its effective treatment. A meticulous apprehension of the signaling cascade is necessitated for better outcomes. In a nutshell, this editorial highlights various novel targeting approaches like gene therapy and immunotherapy which could usher better targeting of CRC.

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INTRODUCTION

Colorectal cancer (CRC) is the third most predominant cancer amongst the world. In 2017, 97220 and 43030 new patients of colon and rectum cancers were reported in United States, respectively. CRC is manifested by the development of adenomatous polyps and malignant cells in the colon. These abnormal cells producing tumors are characterized by uncontrolled replication and the property of metastasis. The early detection, diagnosis, and the utilization of efficient and safe delivery systems would tremendously enhance the efficacy of therapy. The novel targeting approaches (Figure 1), of raising concern as manifested by cancer drugs in the past years, block transduction pathways leading to the cell death through apoptosis and triggering of the immune system, or deliver anticancer drugs to cancer cells, reducing the side effects. The major pathways which could be targeted for CRC therapy are, Sonic Hedgehog (SHH), Wnt/ β -catenin, transforming growth factor- β (TGF- β)/SMAD, EGFR and Notch pathways^[1,2] (Figure 2).

The Hh pathway is crucial in the normal development of various organs like gut epithelium. The Hh ligands bind to the Patched protein (Ptch) receptor, which subdues the activity of Smoothened (Smoh) receptor. Binding of the ligands to PTCH1 results in the Smoh-mediated activation of GLI transcription factors, which then modulates the expression of various Hh target genes. The expression of SHH, SMO, GLI1 mRNA in colon cancer tissues is remarkably enhanced as compared to the normal cells^[3]. Vismodegib is an Hh inhibitor which acts by targeting Smoothened which is a modulator of the Hh pathway. In order to enquire novel Hh antagonists with apoptosis-triggering activity, a group of ~300 potential smoothened antagonists were screened. In colon cancer cells, Hh003 triggered caspase-dependent apoptosis whereas no apoptotic activity was depicted by vismodegib. In comparison to vismodegib, Hh003 displayed similar suppression on the Hh pathway. Hh003 depicted more

suppression of the *in vitro* tumor forming colonies and colon cancer proliferation *in vivo*^[4].

Frizzled (Fz) receptors and low-density lipoprotein receptor-related protein 5 or 6 (LRP5 or LRP6) are the targets of the Wnt family of proteins. The primary element of the Wnt/ β -catenin signaling pathway is the β -catenin destruction complex; which is comprised of a tumor suppressor protein encoded by the antigen-presenting cells (APC) gene, Axin, CKI, and GSK3. When the receptor binding does not occur, this complex undergoes binding with the β -catenin protein (encoded by *CTNNB1* gene), which then undergoes degradation through an ubiquitin-proteasome pathway. In contrary, binding of the receptor by Wnt ligands causes the deactivation of the β -catenin destruction complex and accumulation of β -catenin. It is then translocated to the nucleus for complex formation with T-cell factor/lymphoid enhancer factor, a transcription factor, causing the transcriptional actuation of the target genes. In majority of colon cancers (sporadic) mutation of both alleles of APC (a tumor suppressor gene) occurs which leads to stabilization of β -catenin and stimulation of WNT pathway genes, like TCF, which are needed for the maintenance of colon crypt. In few colon cancers identification of point mutation in β -catenin bearing wild-type alleles of APC has been done^[5]. Aquaporin5 (AQP5), a water protein channel, has an oncogenic activity in many types of malignant cancers like CRC. The effect of AQP5 silencing on 5-fluorouracil (5-FU) sensitivity was inquired in cancer cells. It was observed that the Wnt/ β -catenin pathway mediated the 5-FU chemosensitivity. AQP5 silencing suppressed the Wnt pathway. While, overexpression of the β -catenin (S33Y) mutant (which shows resistance to degradation) reversed the apoptosis process triggered by AQP5 silencing^[6]. Berberine, which is an alkaloid derived from plants and its synthetic 13-arylalkyl derivatives have been accounted to possess antitumor potential; they were investigated for their involvement in Wnt/ β -catenin signaling cascade. The cellular levels of active β -catenin were found to decrease accompanied by a rise in the expression of E-cadherin. The berberine derivatives depicted a 100-times reduced EC50 values in comparison to berberine for Wnt-repression^[7]. Esculetin, (6, 7-dihydroxycoumarin) potentially inhibits the Wnt- β -catenin pathway. It interrupted the β -catenin-Tcf complex formation by binding with the Lys312, Gly307, Lys345, and Asn387 residues of β -catenin in tumor cells. Besides, esculetin efficaciously reduced the viability and suppressed the anchorage-independent proliferation of cancer cells^[8]. Novel Wnt signaling inhibitors, isopropyl 9-ethyl-1- (naphthalen-1-yl)-9H-pyrido (3, 4-b) indole-3-carboxylate (Z86) have been recognized. Z86 suppressed the Wnt signaling functions and genes expression in mammalian cells. It suppressed the GSK3 β (Ser9) phosphorylation, causing its overactivity and elevating the phosphorylation and β -catenin degradation^[9].

TGF- β and BMP signaling pathways are often impaired in CRC. Ligand-induced oligomerization of the TGFBR1

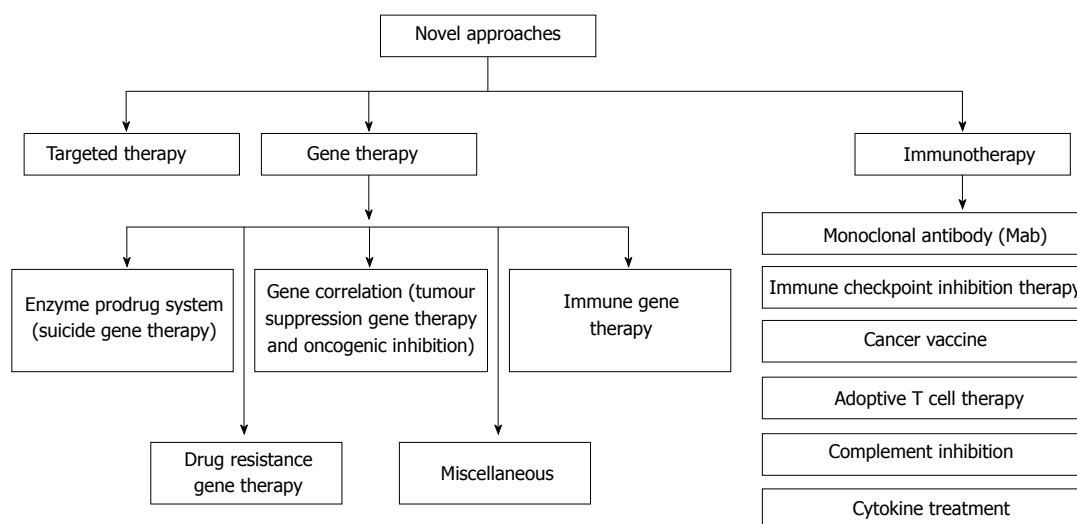


Figure 1 Various novel approaches for the treatment of colorectal cancer.

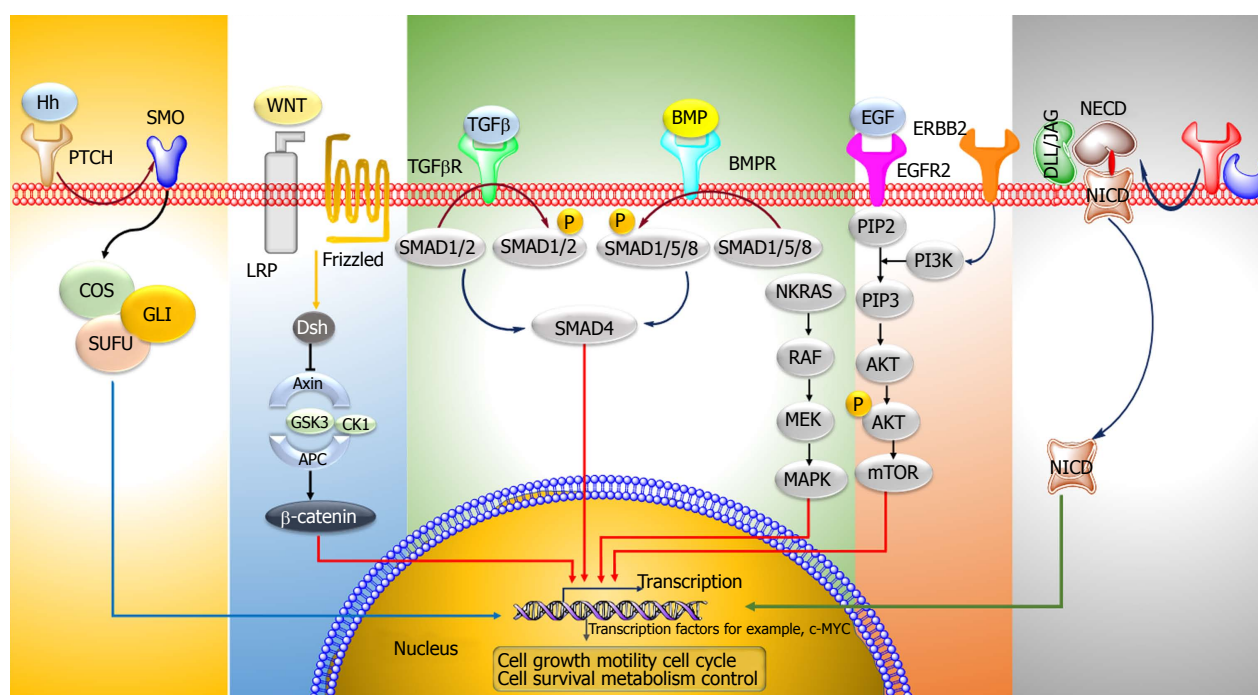


Figure 2 Signaling pathways involved in colorectal cancer. TGF- β : Transforming growth factor- β ; LRP: Lipoprotein receptor-related protein; Dsh: Phosphoprotein Dishevelled; GSK3: Glycogen synthase kinase-3; CK1: Casein kinase 1; PI3K: Phosphoinositide 3-kinase; PIP2: Phosphatidylinositol biphosphate; PIP3: Phosphatidylinositol 3,4,5-triphosphate; EGF: Epidermal growth factor; EGFR2: Epidermal growth factor receptor 2; BMPR: Bone morphogenetic proteins receptor; BMP: Bone morphogenetic proteins; RAF: Rapidly Accelerated Fibrosarcoma; MEK: Mitogen-activated protein kinase; AKT: Protein kinase B; MAPK: Mitogen-activated protein kinases; SUFU: Suppressor of fused homolog.

serine/threonine receptor kinases leads to the initiation of the signal cascade succeeded by the phosphorylation of Smad1, Smad2 and Smad3 (signaling molecules). This leads to their association with Smad4 (signaling transducer) and translocation to the nucleus. Triggered Smads modulate various biological effects by binding to transcription factors and leading to the modulation of transcription. Juvenile polyposis is observed in colon cancer due to mutated Smad4 or BMPRI. In most of sporadic colon cancers, the phosphorylation of Smad1, Smad5 and Smad8 does not occur^[10]. Genistein (obtained

from soybean) is an isoflavone possessing an anticancer potential. A dose-dependent rise in TGF- β 1 mRNA expression was found in MC-26 cells in mouse. It stimulated the generation of Smad-DNA complexes and phosphorylated Smad2 and Smad3, depicting enhanced TGF- β 1 signaling^[11].

The binding of epidermal growth factor and TGF to the EGFR, leads to the stimulation of homodimerization/heterodimerization of the receptor and phosphorylation of specific tyrosine residues (P). This in turn stimulates the downstream RAS/RAF/mitogen-activated protein

Table 1 Nanotechnology based drug delivery systems for colorectal cancer targeting

System	Chemotherapeutic agent	Significance	Ref.
Nanoparticles	Resveratrol (RSV)	Sustained release of RSV (over 72 h), and drug solubility enhancement	[17]
Micellar delivery system	Docetaxel	Enhanced the efficacy of hydrophobic chemotherapy and reduced systemic toxicity	[18]
Self-nanoemulsifying drug delivery systems (SNEDDS)	Sunitinib malate	Enhancement of <i>in vitro</i> dissolution rate and anticancer potential of drugs possessing low water solubility such as sunitinib malate	[19]
Small molecule-based theranostic system, Gal-Dox	Doxorubicin	Drug localization and site of action can be monitored	[20]
Polymeric micelles	Tanshinone IIA (TAN)	Improved efficacy of anticancer drugs and promoted the growth of beneficial commensal flora in the gut	[21]
Pressure-sensitive nanogels	5-Fluorouracil (5-FU)	Higher 5-FU intracellular accumulation and a significant cell death extension by apoptosis	[22]
Microspheres	Atorvastatin and celecoxib	Synergistic effect on colon cancer prevention and inhibition	[23]
Microbeads	Doxorubicin	Exhibited reduction-responsive character, release the DOX in reducing environments due to cleavage of the disulfide linkers	[24]
Carboxymethyl dextran (CMD) chitosan nanoparticles	Small interfering RNA	Significant changes of Epithelial mesenchymal transition genes and apoptosis	[25]
Liposomes	Apatinib	cRGD-modified liposomes displayed greater apoptosis	[26]

kinase (MAPK) and phosphoinositide 3'-kinase (PI3K) signaling pathways and expression of genes responsible for cell proliferation, angiogenesis and metastasis. KRAS2 and BRAF mutations have been seen in colon cancer. Mutations in PIK3CA which is the p110 α catalytic subunit of PI3K have also been observed in few cases of colon cancers^[12]. Everolimus (an inhibitor of mTOR) in combination with nilotinib (a platelet-derived growth factor receptors tyrosine kinase inhibitor) suppressed the growth and liver metastasis of colon cancer. The stromal reaction and cancer cell proliferation was reduced and apoptosis was stimulated in tumor cells^[13].

The Notch signaling pathway is involved in the growth of intestinal epithelium. Notch ligands *i.e.*, Delta-like (DLL) bind to their transmembrane receptors (Notch 1-4) and induce the proteolytic breakdown of the receptors by the enzymes α -secretase and γ -secretase to release the intracellular domain of the Notch receptor. The cleaved Notch receptors (NICD) are then transferred into the nucleus which forms complexes with RBP-jk (CSL or CBF-1) and lead to the stimulation of Notch-target gene Transforming growth factor- β . An overexpression of ligands namely Jagged1, Jagged2, DLL1, DLL3, DLL4, Notch receptors 1-4 and genes like hairy-enhancer-of-split (Hes-1), Deltex and Notch intracellular domain (NICS) has been observed in colorectal cancer cells^[14]. Withaferin-A is a natural compound (source *Withania somnifera*), which curbs Notch-1 signaling and downregulates various pathways like Akt/NF-kappa B/Bcl-2, in HCT-116, SW-480, and SW-620 cell lines. Besides, Withaferin-A downregulated the expression of mammalian target of rapamycin (mTOR) signaling components, pS6K and p4E-BP1, and stimulated c-Jun-NH (2)-kinase-mediated apoptosis in tumor cells^[15].

TARGETED THERAPY

Nanotechnology is a rising arena in drug delivery which furnishes many advantages over the conventional system. Colon-specific novel delivery systems would allow for the local delivery of a high concentration of drugs in the colon to improve pharmacotherapy and reduce its potential systemic toxicity and side effects. Recently, theranostic nanocarriers are introduced to simultaneously monitor and treat the disease using a single delivery system^[16]. Colon targeted nanocarriers have been described in brief in the Table 1^[17-26].

GENE THERAPY

It involves introduction of genetic components for treating various diseases including cancer. The genetic component may be the nucleic acid *i.e.*, DNA or RNA which may help to replace or correct the malfunction due to defective genes. Gene therapy can also be utilized to actuate an immune response or itself used as a therapeutic agent.

Progression of colorectal cancer is mediated by mutation and aberration of genes. Modification and correction of these defective genes and prevention of those overexpressed genes can have the capability to prevent CRC. The alteration of multiple genes is involved in the development of colon carcinogenesis. Point mutation, formation of oncogenes, de-regulation or deletion of proto-oncogenes and lack of function of suppressor-oncogenes may lead to cancer.

Till November 2017, near about 2600 clinical trials had been conducted in 38 countries and more than 50% are in phase I clinical trial^[27]. While 1309 gene therapy based trials which were performed across the

Table 2 Overview of clinical trials of colorectal cancer

Therapy	Agent	Clinical status	Ref.
Five peptides combination with oxaliplatin-based chemotherapy	Oxaliplatin	Phase II	[34]
Panitumumab with infusional fluorouracil, leucovorin, and oxaliplatin (FOLFOX4) <i>vs</i> FOLFOX4 alone	Fluorouracil, Oxaliplatin	Phase III trial	[35]
Checkpoint inhibitors	Nivolumab and pembrolizumab	Phase 2 study	[36]
Combination vaccine treatment of five therapeutic epitope-peptides	Fluorouracil, irinotecan or oxaliplatin	Phase I	[37]
Autologous dendritic cell based adoptive immunotherapy	-	Phase I-II	[38]
Autologous antigen-activated dendritic cells in the treatment of CRC	-	Phase I-II	[39]
Adjuvant chemotherapy (FOLFOX)	5-fluorouracil (FU)/leucovorin (LV)	Phase III	[40]

world, merely 45 reached the phase III. Eleven gene therapies for CRC are being subjected for trial in the United Kingdom^[28]. There are about 50000 to 100000 genes which exist in the body and a few of them take part in the cell cycle. Defective genes could be most allied factors for CRC and it has been discovered that at least 30% of colon cancers are due to defective genes. Few of them are associated with familial colon cancers. The core benefit of gene therapy is the transfer of the specific genes to the specific tumors cells so that the abnormal function of mutated gene would be suppressed and tumor progression could be inhibited^[29-32].

IMMUNOTHERAPY

Tumor immunotherapy has seized researchers in this scenario as it depicts remarkable clinical potential in CRC. Presently, there are various immunotherapies which are being subjected to clinical trials in human CRC. Various immunotherapy approaches employed in CRC are monoclonal antibody (mAb) therapy, immune checkpoint inhibitors therapy, cancer vaccines, adoptive cell therapy, complement inhibition and cytokine treatment. Majority of them are in phase I and II clinical trials and some of these trials showed promising results. So far, more than 24 immunotherapy-based clinical trials for human CRC have been completed and more than 40 clinical trials are recruiting or about to recruit patients^[33]. Table 2^[34-40] depicts various clinical studies of CRC.

Monoclonal antibody therapy

In this therapy, humanized antibodies like Cetuximab and Panitumumab which selectively recognize the epidermal growth factor receptor (EGFR) are employed for the treatment of metastatic CRC. There are some MAb's presently in various phases of clinical trials for CRC such as adecatumumab against EpCAM, labetuzumab against carcinoembryonic antigen (CEA), and pemtumomab against Mucins^[41].

Immune checkpoint inhibitors therapy

T cell activation is down-regulated by CTLA-4 which is an immune checkpoint moiety by binding to CD80/

CD86 entities on antigen-presenting cells (APC). T cell function is negatively regulated by programmed death receptor ligand 1/2 (PD-L1/L2) by binding to PD-1 receptor present on T cells usually stimulated by their various ligands which are expressed on either tumor cells (e.g., PDL1/ L2→PD-1) or APCs (e.g., CD80/86→CTLA-4; PD-L1/L2→PD-1), activated CTLA-4 and PD-1 immune checkpoint signaling pathways efficiently inhibit the tumor-reactive T cell activation and consequent tumor detection^[42]. A phase II clinical trial of individual drug Nivolumab and also a combination of dual drugs like Nivolumab plus Ipilimumab is in undergoing process for CRC (ClinicalTrials.gov Identifier: NCT02060188).

Cancer vaccines

They have been designed to induce antigen specific T-cell or B-cell activity against cancer by rendering antigens to APC like dendritic cells (DCs). Besides, vaccines likewise include constituents proposed to activate DCs pulsed with antigens and aim them to move to a local lymph node.eg DC vaccine and OncoVAX.

DC vaccine: Because majority of CRCs express carcinoembryonic antigen (CEA) which is a tumor-associated antigen DCs, can be pulsed with CEA mRNA or CEA peptides. Most of the CRC patients who were administered with DC vaccine evoked CEA-specific T cell immune activities.

Oncovax: It has been developed to use patients' own cancer cells with an immune-stimulating adjuvant to evoke antitumor immune activities to evade the relapse of colon cancer after surgery. A combination of specific immunotherapy with surgery depicts a remarkable improvement in the survival of the patients^[43].

Adoptive T cell therapy

This therapy possesses the potential to raise antitumor immunity and increase vaccine efficacy. Recent researches have riveted on endowing effector T cells with desired antigen receptors, like chimeric antigen receptor T cells. An *ex vivo* expanded human Vδ1 γδ T cells displayed a remarkable therapeutic activity in

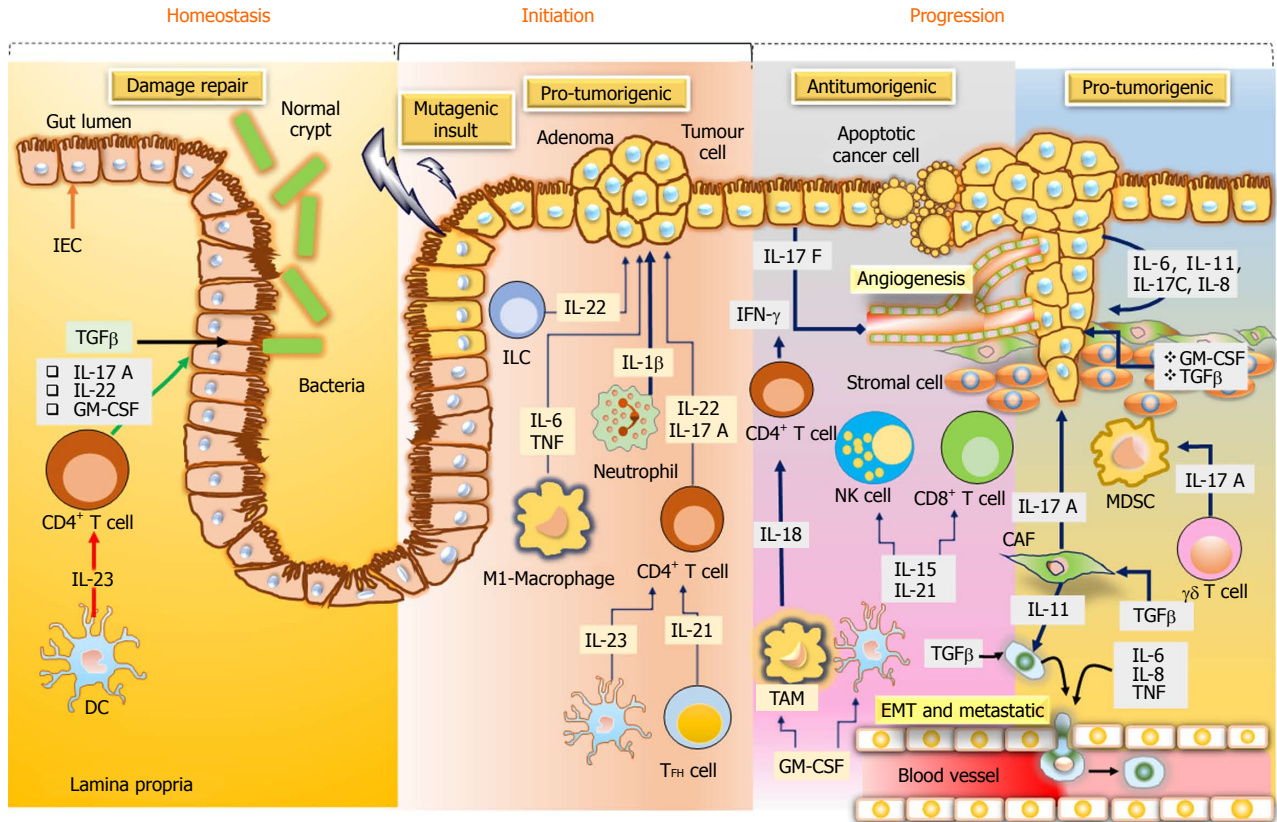


Figure 3 Cytokines involvement in the progression of colorectal cancer. IL: Interleukin; TNF: Tumour necrosis factor; TGF- β : Transforming growth factor- β ; EMT: Epithelial to mesenchymal transition; TAM: Tumour-associated macrophage; ILC: Innate lymphoid cells; GM-CSF: Granulocyte-macrophage colony-stimulating factor; MDSCs: Myeloid-derived suppressor cells; CAF: Cancer-associated fibroblast; CIC: Cancer-initiating cell; IEC: Intestinal epithelial cell; DCs: Dendritic cells; TFH: T follicular helper cells; NK: Natural killer cells.

human colon cancer xenografted mouse model^[44].

Complement inhibition

Complement is a key part of immune system and its stimulation has been taken as an essential component of the immune surveillance response against CRC. Complement comprises of more than 30 proteins and fragments, is a part of the innate and adaptive immune system. Various protein inhibitors of complement such as cobra venom factor, humanized cobra venom factor, and recombinant *staphylococcus aureus* super antigen-like protein 7 have been assessed in murine colon cancer model. Complement depletion presents an efficient type of immunotherapy in CRC by its capability to vitiate tumor progression by raising the host's immune responses to cancer and reducing the immunosuppressive effect generated by the tumor microenvironment and finally could be employed as a constituent of combination immunotherapy^[45].

Cytokine therapy

Cytokines are considered as essential aspects of tumour immunology, particularly for CRC, in which the tumor growth is determined by the inflammatory process and immunogenic responses. Cytokines like tumour necrosis factor and interleukin-6 are considered as important

factors in CRC, triggering the stimulation of the central oncogenic factors nuclear factor- κ B and inducer of transcription 3 (STAT3), respectively, in the intestinal cells to enhance the proliferation and the development of apoptosis resistance^[46] (Figure 3).

CONCLUSION

Increasing evidences show that several signaling pathways play an essential role in the development and progression of CRC. Targeting these signaling cascades using nanocarriers might be advantageous for the treatment of CRC. The identification of various genes and other biomarkers improved the conventional therapy and target the specific tumor cells. The gene therapy and various immunotherapy including cytokine therapy, cancer vaccine, adoptive cell therapy, monoclonal antibody etc. have been recently introduced which may unravel new ways for the treatment of CRC and provide its efficient management in comparison to the conventional therapy.

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Carcinogenesis on the background of liver fibrosis: Implications for the management of hepatocellular cancer

Joanne Marie O'Rourke, Vandana Mridhu Sagar, Tahir Shah, Shishir Shetty

Joanne Marie O'Rourke, Vandana Mridhu Sagar, Shishir Shetty, Centre for Liver Research, Institute of Biomedical Research, Birmingham B15 2TT, United Kingdom

Joanne Marie O'Rourke, Vandana Mridhu Sagar, Tahir Shah, Shishir Shetty, NIHR Birmingham Biomedical Research Centre, University of Birmingham and University Hospitals Birmingham NHS Foundation Trust, United Kingdom

ORCID number: Joanne Marie O'Rourke (0000-0001-7851-0703); Vandana Mridhu Sagar (0000-0003-2662-755X); Tahir Shah (0000-0002-0420-0304); Shishir Shetty (0000-0002-4729-2173).

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Correspondence to: Shishir Shetty, MBChB, MRCP, PhD, Senior Research Fellow, Centre for Liver Research, Institute of Biomedical Research, University of Birmingham, Birmingham B15 2TT, United Kingdom. s.shetty@bham.ac.uk
Telephone: +44-121-3714852

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Abstract

Hepatocellular carcinoma (HCC) is now the second leading cause of cancer-related deaths globally and many patients have incurable disease. HCC predominantly occurs in the setting of liver cirrhosis and is a paradigm for inflammation-induced cancer. The causes of chronic liver disease promote the development of transformed or premalignant hepatocytes and predisposes to the development of HCC. For HCC to grow and progress it is now clear that it requires an immunosuppressive niche within the fibrogenic microenvironment of cirrhosis. The rationale for targeting this immunosuppression is supported by responses seen in recent trials with checkpoint inhibitors. With the impact of immunotherapy, HCC progression may be delayed and long term durable responses may be seen. This makes the management of the underlying liver cirrhosis in HCC even more crucial as studies demonstrate that measures of liver function are a major prognostic factor in HCC. In this review, we discuss the development of cancer in the setting of liver inflammation and fibrosis, reviewing the microenvironment that leads to this tumourigenic climate and the implications this has for patient management.

Key words: Hepatocellular cancer; Carcinogenesis; Inflammation; Fibrosis; Immunotherapy

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Core tip: In this review, we discuss the development of hepatocellular carcinoma in the setting of liver inflammation and fibrosis, reviewing the microenviron-

ment that leads to this tumorigenic climate and the implications this has for patient management.

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INTRODUCTION

Hepatocellular carcinoma (HCC) is now the fifth most commonly diagnosed cancer in men worldwide, and in women it is ranked ninth. HCC is the second most common cause of cancer related deaths and is reported to have been responsible for nearly 745000 deaths in 2012^[1]. Incidence rates are highest in Asia and Africa with Central Europe having intermediate rates^[2]. Different risk factors predominate depending on the region of the world. In Africa and Asia infection with hepatitis B virus (HBV) and aflatoxin B1 exposure are the major risk factors. In developed countries the hepatitis C virus (HCV), alcohol and the metabolic syndrome have predominated^[3].

Despite increasing knowledge on the aetiologies of cirrhosis and progress in diagnosing and managing risk factors, the incidence rates for HCC are increasing. In England, HCC incidence increased from 0.63 per 100000 in 1990 to 2.48 in 2009^[4]. In the United States (US), HCC incidence increased by 4.5% (95%CI: 4.3-4.7) annually between 2000 and 2009 but only 0.7% annually (95%CI: 0.2-1.6) after that. The post 2009 slowing in overall rates, seen in the US, may represent a plateau created from increases in vaccination against HBV and improved chronic HBV antiviral treatment^[5].

It is uncommon to see HCC in the absence of liver fibrosis but it does occur. Table 1 lists some of the aetiologies associated with non-cirrhotic HCC^[6,7]. Chronic hepatitis B is a major risk factor for the development of HCC in the non-cirrhotic setting^[8]. In Europe and the United States the 5-year cumulative incidence of developing HCC was found to be 1% in non-cirrhotic chronic HBV hepatitis. This incidence increased to 10% in HBV with cirrhosis^[9]. Other causes of HCC in the non-cirrhotic setting include hereditary conditions for example porphyria and type 1 glycogen storage disease, metabolic syndromes and genotoxin exposure. Genotoxins are agents which damage the genetic information within a cell. For example, the aflatoxin B1, which is produced by *Aspergillus flavus*, is a pathogenic fungus and can lead to non-cirrhotic HCC induction^[10]. The global epidemic of non-alcoholic fatty liver disease (NAFLD) which is characterised by macrovesicular steatosis can lead to cirrhosis. It is however observed that a significant proportion of patients with NAFLD develop HCC in the non-cirrhotic setting^[11,12]. However

Table 1 Conditions which have been associated with hepatocellular carcinoma development in the non-cirrhotic liver^[6,7]

Viral	HBV
Metabolic	Porphyria Type 1 glycogen storage disease NAFLD A1 antitrypsin Haemochromatosis Type 1 hypercitrullinemia
Genotoxins	Aflatoxin B1
Congenital	Alagille syndrome Congenital hepatic fibrosis
Sex hormones	Anabolic steroids Hepatic adenoma transformation
Vascular	Hepatic vascular pathology, e.g., Budd Chiari

HBV: Hepatitis B virus; NAFLD: Non-alcoholic fatty liver disease.

worldwide at present the majority (70%-90%) of HCC cases occur on a background of cirrhosis^[13].

When data from the World Health Organization (WHO) mortality database was examined by Ascione *et al.*^[14], the age-standardised death rate for liver cirrhosis in European countries between 1970 and 2010 showed cause for concern for the United Kingdom (UK), Finland, Ireland and Denmark. Looking at percentage change in mortality, the UK in those four decades showed a high increase (+284.8%), Finland, Ireland and Denmark also saw increases. However these countries were the exceptions and in all other countries in Europe there was a reduction in mortality for liver cirrhosis. The same database provided comparable data, between 1980-2010 with a 85.4% increase in death from HCC over this period^[14]. The overall decrease in liver cirrhosis related deaths in Europe and the increasing mortality for HCC is confounding and concerning.

Cirrhosis mortality in the UK has been the subject of extensive discussion and patterns of alcohol consumption may account for the discrepancies between the UK and other parts of Europe. The rise in HCC cases in Europe over the last 30 years seems confounding when it is reported that in many countries mortality from cirrhosis is reducing. However, our knowledge and the management of chronic liver disease has over this timeframe improved, and it is suggested that with increased survival we are seeing increased development of HCC^[14]. This would be in keeping with our knowledge that cirrhosis creates a microenvironment for tumour development and is considered a precursor for HCC.

Over 3 decades ago the 5-year survival for HCC was 3%. Despite improvements in earlier detection 5-year survival is less than 20% for this cancer^[15].

PATHOGENESIS

Setting of inflammation and fibrosis

Liver fibrosis is a risk factor for the development of HCC with up to 90% of cases occurring on the background

of a cirrhotic liver^[16] and is a leading cause of death in this population. The major global causes of liver disease which are associated with HCC include viral hepatitis, alcoholism and non-alcoholic steatohepatitis (NASH). The effects of HBV infection have started to decline due to increased use of antivirals and immunisation programs. It is hoped that in the age of new direct acting antiviral agents with time we will see a reduction in HCV associated cirrhosis. The impact of alcohol and the development of NASH cirrhosis will prove to be more challenging to prevent and cases are predicted to continue to rise.

Fibrosis occurs when the liver is repeatedly and continuously injured. Liver volume is formed from 80% parenchymal and 20% non-parenchymal cells^[17]. Hepatocytes are the parenchymal cells and they are the target for hepatotoxic agents. Damage to hepatocytes triggers the release of reactive oxygen species (ROS) and mediators of fibrosis inducing activation of hepatic stellate cells (HSCs). HSCs with phagocytic Kupffer cells (KCs) and liver sinusoidal endothelial cells (LSECs) are central players in fibrosis development^[18]. The activation of HSCs, extracellular matrix (ECM) producing myofibroblasts, is said to be the key step in fibrosis development. Paracrine signals from injured hepatocytes and activated KCs play a prominent role in HSC activation. KCs also generate ROS in the liver and this enhances HSC activation and collagen synthesis leading to fibrosis^[19,20].

In addition to the multitude of cells involved in the development of cirrhosis there are also several cytokines that have been identified to play significant roles. They include platelet derived growth factor (PDGF), transforming growth factor- β (TGF- β), tumour necrosis factor- α (TNF- α), interferon and interleukins (ILs). A variety of hepatotoxic agents can induce KC to synthesise PDGF^[21] which binds to the HSC membrane and activates them. There are different isoforms of PDGF and two of these, PDGF B and D have been shown to have a role in activating HSCs leading to liver fibrosis^[22]. TGF- β is the most potent stimulator of fibrogenesis and is produced by a variety of cells in the liver: HSCs, KCs, LSECs, and hepatocytes. The TGF- β family has multiple members and the one that has been implicated as a notable player in hepatic fibrosis is TGF- β 1. It is reported to contribute not only to activation of HSCs but also the inhibition of ECM breakdown^[23] and the induction of apoptosis of hepatocytes^[24]. TNF- α has also been shown to activate HSCs to synthesise ECM^[25], however, results from murine studies on TNF- α are complex and it appears to also have antifibrogenic effects in some reports^[26]. ILs are expressed by many cells with the majority of ILs being produced by helper CD4 T lymphocytes. In the liver ILs have both pro-fibrogenic and antifibrogenic roles^[18]. IL-1 can activate HSCs^[27] and IL-17 has a role inducing fibrosis through the activation of HSCs and KCs^[28]. ILs with antifibrogenic roles have been identified as IL-6, IL-10

and IL-22^[29-31].

Setting for tumour development and progression in fibrosis

The inception and progression of HCC is described as being largely influenced by the microenvironment of the liver. This includes influences from chronic inflammation, liver remodelling, changes in genetics and cellular signalling. These pathways can be affected by chemical toxins, viruses, immune cells, hypoxia, ECM changes, microflora from the gastrointestinal tract and extra cellular microvesicles which carry altering signals, cytokines and oncogenic miRNAs.

Chronic inflammation and fibrosis are seen in the background of many HCCs and the most common aetiologies are viruses and ethanol. The immune mediated cell death seen in viral infections leads to increased production of ROS. This leads to increased hepatocellular oxidative stress which induces DNA mutations contributing to HCC development. Ethanol consumption is associated with increased ROS concentrations in hepatocytes resulting in hepatic DNA damage^[32]. Chronic inflammation leads to increased proliferation of hepatocytes, shortening of telomeres and therefore chromosomal instability and a predisposition to malignant transformation^[33]. Genomic alterations which have been identified in HCC and are considered to be drivers in progression include mutations affecting telomere maintenance, Wnt pathway activation, inactivation of p53, chromatin remodelling, Ras signalling, mechanistic target of rapamycin (mTOR) signalling and ROS pathway initiation^[34].

Chronic inflammation can progress to fibrosis and cirrhosis and this in turn induces several further changes in the microenvironment. Firstly, it creates altered blood flow and hypoxic hepatocytes which produce reactive nitrogen species^[35]. Areas of hypoxia in the liver parenchyma lead to changes in molecular signalling and we know that the response is to upregulate angiogenic factors including vascular endothelial growth factor (VEGF)^[36]. In a tumour this facilitates angiogenesis and tumour growth. The hepatocytes may provide the genetic mutation but it is the unique surrounding microenvironment that enables the tumour to establish.

We have explained that chronic inflammation has effects on cytokine expression within the liver, ECM production by HSC, TNF- α receptors and also the mitogenic cytokine IL6 is significantly increased in advanced cirrhosis leading to a propensity towards cancer^[37]. IL6 regulates immune cells and the growth of tumour cells^[38] and this dual role therefore is an example of the association between the tumour and the microenvironment. The effects of IL6 are controlled by nuclear factor- κ B signalling. Both pathways are altered in liver inflammation and hepatocarcinogenesis^[39]. EGFR overexpression also promotes liver cancer progression when present in macrophages^[40]. It has also been shown that hepatic stellate cells can promote the pro-

tumourigenic change in macrophages^[41]. The expansion of liver progenitor cells to replace hepatocytes, in the presence of cytokines and increases in oxidative stress promotes the accruing of mutations^[42].

Hepatocytes have great potential to regenerate but we know this predisposes cells to malignant transformation^[43]. The cell underlying the inception of HCC is also critical to understand. The human liver is not just made up of hepatocytes but also adult stem cells and progenitor cells which maybe potential cells of origin for cancer^[44].

The gut microbiota has in recent years received much attention in many disease processes including liver disease. It has been described that the microbes in our bodies encompass 100 trillion cells, with the majority residing in the gut^[45]. It is increasingly recognised that the gastrointestinal tract plays a pivotal role in liver diseases, including HCC. As we have previously described HCC usually occurs in inflamed and fibrotic livers and intensive immune cell infiltration is seen. *Via* the portal vein the liver is exposed to gut-derived bacterial products and in advanced liver diseases there is increased intestinal permeability to gut-derived bacterial products including lipopolysaccharides (LPS)^[46]. Accumulation of LPS is said to contribute to HCC development by generating inflammatory reactions in the hepatic environment^[47], activating KCs and endothelial cells to release pro-inflammatory cytokines which contributes to liver injury. Levels of LPS are increased in animal models of hepatocarcinogenesis and in patients with HCC^[47-49]. Dapito *et al*^[50] found that toll like receptor 4 activation by LPS contributed to driving inflammation and tumour progression and that gut sterilisation suppressed hepatocarcinogenesis. To date studies have been on preclinical animal models but there is potential that manipulating the microbiome may one day be an option in the prevention and perhaps treatment of HCC^[51].

Tumour antigen tolerance promotes carcinogenesis

Dysregulation of the immune system has been implicated in the pathogenesis of HCC. Changes in the innate and adaptive immune system makes the immune system tolerant to cancer and facilitates tumour progression. Understanding these processes is therefore essential to tailor therapeutic approaches. Key cells implicated include T lymphocytes, myeloid-derived suppressor cells (MDSCs), dendritic cells and natural killer (NK) cells^[52]. The innate immune system key players are dendritic cells, macrophages, MDSCs and NK cells. The adaptive immune system comprises the T lymphocyte subsets. Failure of HCC antigen presentation by dendritic cells is one defect in the immune system seen in HCC. Activated dendritic cells in HCC are not able to infiltrate cancer tissue effectively^[53] and tumour associated macrophages express cytokines that favour tumour growth, invasion and suppress the anti-tumour immune response^[54]. MDSCs possess strong immunosuppressive activities and expand in cancer and regulate T cell responses,

increased quantities of these cells are seen in the tumour environment of a HCC^[55]. NK cells are cytotoxic lymphocytes and they can modulate the activity of other immune cells, including dendritic cells and macrophages, *via* cytokine release. They are critical to the innate immune system and are capable of rapid responses and can destroy tumour cells without prior priming. In HCC a reduction in NK cell subsets has been reported with reduced cytotoxic ability^[56].

The adaptive immune system has a significant role in thwarting the development and advancement of cancer. CD8+ cytotoxic T cells play a salient part in anti-tumour mechanisms and CD4+ helper cells have a role in generating CD8+memory T cells^[57] which assist in the destruction of tumour cells. In the setting of cirrhosis there is a reduction in CD4+ cells^[58]. Tregs expressing CD4+, CD25+ and forkhead box P3 (Foxp3) have an inhibitory role and they can suppress effective anti-tumour responses^[59]. There are increased Tregs seen in patients with HCC and depletion can increase anti-tumour responses^[60] and lead to a reduction in tumour growth^[61]. In advanced HCC there are increased numbers of CD8+FoxP3+ regulatory T cells perhaps helping the tumour evade the immune system^[62]. NK T cells accumulate in the tumour environment and they appear to be able to function either as anti-tumour cells or can promote tumour tolerance depending on the subset^[63]. We can therefore conclude that a complex, partially understood, dysregulated immune environment has a key role in the development and evolution of liver tumours. An overview of the key responses to hepatic injury leading to fibrosis and HCC development together with therapeutic strategies are summarised in Figure 1.

THE SAME FIBROTIC ENVIRONMENT WHICH PROMOTES THE DEVELOPMENT OF HCC ALSO IMPACTS NEGATIVELY ON TREATMENT OPTIONS

Management of advanced HCC and the impact of cirrhosis

The fact that the majority of HCC arise in the cirrhotic liver, which affects liver function, can severely impact on therapeutics. A detailed review of the management of HCC has been covered elsewhere^[64]. There are several algorithms for the management of HCC including TNM stage, the Japanese integrated system, Cancer of the Liver Italian group and the Hong Kong Liver Cancer staging system. The most well recognised being the Barcelona Liver Cancer (BCLC) criteria which is recommended by several international guidelines^[65,66]. Considering the underlying liver disease is vital in HCC, the BCLC guidelines include both tumour stage and the severity of underlying liver cirrhosis (Child Pugh score) and helps guide treatment and to predict overall prognosis. The seminal study by Hoshida *et al*^[67]

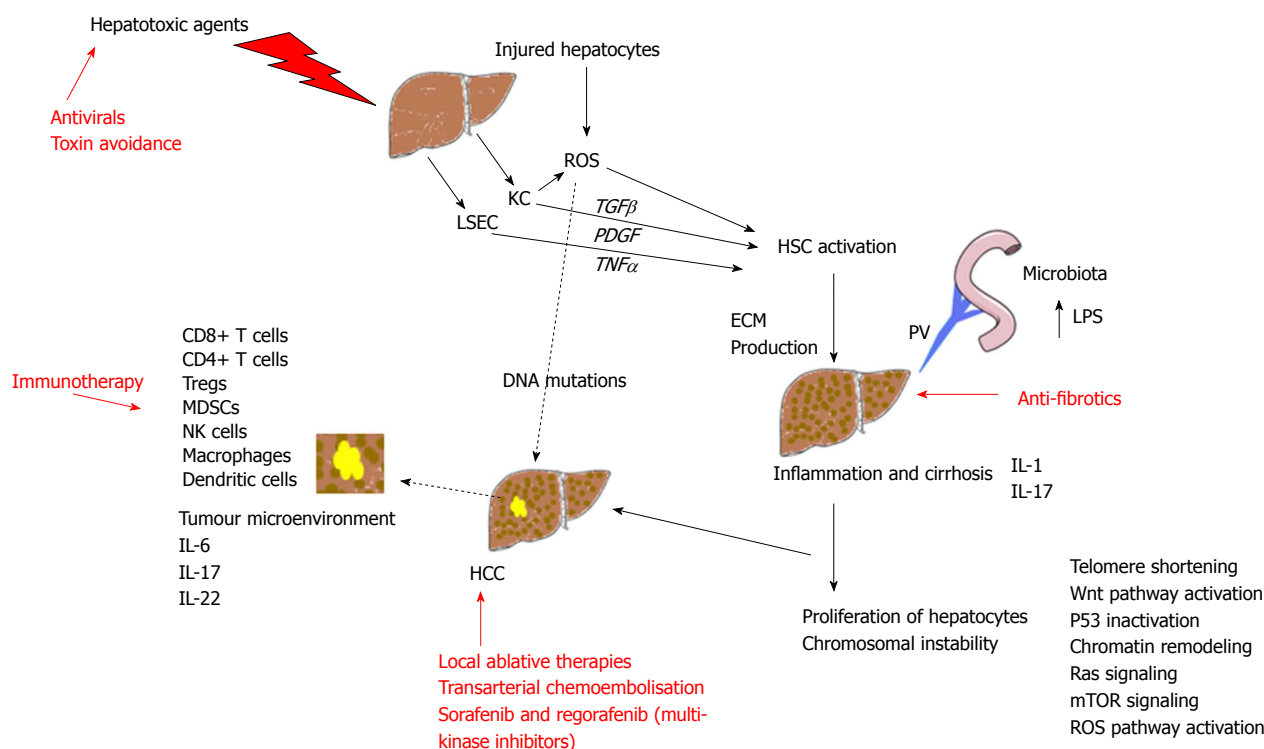


Figure 1 Overview of the key factors associated with fibrosis development and progression to hepatocellular carcinoma. Hepatotoxic agents damage key liver cells triggering reactive oxygen species and cytokine release culminating in hepatic stellate cell activation, the key step in fibrosis development. Chronic inflammation and fibrosis instigates several changes in the microenvironment predisposing to hepatocellular carcinoma (HCC) and creating distinct immune changes which promote HCC progression. Key therapeutic strategies are highlighted in red. LSEC: Liver sinusoidal endothelial cells; KC: Kupffer cells; ROS: Reactive oxygen species; HCC: Hepatocellular carcinoma; TGFβ: Transforming growth factor β; PDGF: Platelet-derived growth factor; HSC: Hepatic stellate cell; ECM: Extracellular matrix; PV: Portal vein; LPS: Lipopolysaccharides; IL: Interleukins; Treg: Regulatory T cell; MDSC: Myeloid-derived suppressor cells; NK: Natural killer cells.

highlighted the importance of underlying liver disease to overall prognosis in HCC. In this study the gene expression of the tumour was not associated with overall survival but the gene expression in adjacent non-tumour liver tissue correlated strongly with survival. More recently the severity of underlying liver disease as a prognostic marker has been highlighted with a study focusing on a scoring system based on bilirubin levels and albumin values. Johnson *et al.*^[68] developed a model incorporating just bilirubin and albumin levels called the ALBI score (Figure 2) which was an accurate discriminatory method for assessing liver function in HCC. Within the Child-Pugh class A patients, the ALBI score was able to differentiate patient groups with different prognoses. The model across a database of 3887 patients identified a median ten month difference in survival between ALBI grade 1 and ALBI grade 2 within the Child-Pugh class A group for European and US patients^[64].

Patients who present with early HCC are amenable to curative treatments including surgery (resection or transplant) and local ablative therapies. Those with more intermediate stage HCC have non curative options such as transarterial chemoembolisation which has been shown to improve survival in randomised control trials^[69]. A significant proportion of patients present with advanced incurable disease; these patients have a poor prognosis and the only licensed medical treatment has been the multi-kinase inhibitor Sorafenib^[70]. The SHARP

trial demonstrated an improved survival in patients with advanced HCC who took Sorafenib but this was by only a median of three months. Several other agents have been studied in randomised trials but none have successfully demonstrated superiority to Sorafenib but most recently a phase 3 trial demonstrated Lenvatinib, an inhibitor of VEGF receptors 1-3, FGF receptors 1-4, PDGF receptor alpha, RET and KIT, had similar efficacy to Sorafenib^[71]. Trials in which Sorafenib has been combined with other treatment such as TACE have not been successful in improving efficacy of these treatments^[72]. Until recently no second line agents in randomised trials had demonstrated clinical benefit after Sorafenib therapy, but a recent trial with Regorafenib, another multikinase inhibitor, has finally demonstrated improved survival with a second line agent^[73]. This has led to FDA approval for patients who have failed therapy with Sorafenib but overall survival for advanced HCC remains poor. These experiences have provided impetus to explore immunotherapy in HCC. We have already described that progressive HCC is associated with an immunosuppressive microenvironment. Recent early stage trials with checkpoint inhibitors which activate T cells have shown promising results. Checkpoint inhibitors currently include cytotoxic T-lymphocyte-associated protein 4 (CTLA-4) blockers and inhibitors of programmed cell death protein-1 (PD-1)/programmed cell death protein ligand-1 (PDL-1) interaction with

ALBI-score
 $[\log_{10} \text{ bilirubin } (\mu\text{mol/L}) \times 0.66] + [\text{albumin (g/L)} \times -0.085]$
 ALBI grade is defined by the resulting score:
 Grade 1 ≤ -2.60
 Grade 2 > -2.60 to ≤ -1.39
 Grade 3 > -1.39

Figure 2 Formula to calculate the ALBI-score and translate the result to ALBI grade^[64].

three studies being reported in the context of HCC. One study with 30 patients involved the administration of Tremelimumab, a CTLA-4 blocker, combined with ablative therapy, showed some patients did demonstrate immune responses and led to the accumulation of CD8+ T cells in tumours. A further study with the same drug was performed with 20 patients and demonstrated both anti-tumour and antiviral activity^[74]. Recently, a large phase II trial (CheckMate-040 trial) with the agent Nivolumab (anti-PD-1 drug) has led to significant attention because of strong anti-tumour responses, leading to improved survival and led to accelerated approval by the FDA in 2017 for the treatment of patients after failure with Sorafenib^[75].

Novel therapeutics which alter T-cell regulation are now being pursued for HCC. A study by Sia *et al*^[76] looked at over 900 HCC samples and identified that around 25% exhibited high expression levels of programmed death ligand-1 (PD-L1) and programmed cell death protein 1 (PD-1) and a subgroup expressed many genes that are regulated by TGF β 1, a cytokine which is linked to aggressive cancers and suppresses the immune response. TGF- β is involved in cell proliferation, angiogenesis, migration, immune infiltration, metastases dissemination, and drug resistance^[77]. There are ongoing trials in HCC using the TGF β 1 inhibitor Galunisertib. The phase 2 trial using Galunisertib as monotherapy has shown promise with improved overall survival in AFP responders^[78]. We are entering an era where we may be able to identify which tumours are most likely to respond to immunotherapy, tailoring treatment to the tumour biology.

With these advances in treatment of advanced HCC, clinicians will need to consider how best to manage the underlying chronic liver disease that is associated with HCC. Attempts to improve liver function will have the aim of (1) increasing the number of patients eligible for these novel therapies; (2) to minimise the potential liver related side effects of these novel agents and (3) to prolong the overall survival in patients whose tumours respond to these agents.

Management approaches of chronic liver disease for patients with HCC

Targeting the initiating factors of chronic liver disease can significantly improve liver function in patients even when they have established cirrhosis. Animal

models have demonstrated that severe fibrosis can undergo resolution and healing through cell-mediated mechanisms including reducing the number of activated HSCs and the contribution of macrophages^[79]. Patients with identifiable factors such as excess alcohol intake and metabolic syndrome benefit significantly from becoming abstinent and improving their metabolic risk factors respectively. Those with active chronic hepatitis B undergo significant improvement of liver function by suppressing viral replication. The treatment of HCV has seen dramatic improvements with the advent of direct acting antivirals^[80]. Significant improvement of fibrosis has also been demonstrated in patients with HBV and HCV antiviral medication^[81]. One assumption would be that the clearance of Hepatitis C would be beneficial in patients to improve liver function and reduction of future HCC recurrence. This is countered by recent reports suggesting that the viral clearance of HCV could alter immunological surveillance of tumour cells. Case series have described aggressive HCC in patients with cirrhosis after completing successful treatment of hepatitis C^[82]. It is not possible to draw conclusions from these findings currently and further studies are required to clarify the situation.

In addition to treating the cause of chronic liver disease, there may also be benefit in preventing the complications of cirrhosis. Variceal bleeding is a complication of cirrhosis associated with a very high mortality rate (20%)^[83]. It is well established that patients with HCC have a higher mortality rate compared to matched cirrhosis groups^[84,85]. Ripoll *et al*^[86] in their study confirmed this and furthermore demonstrated that less than half of HCC patients who were eligible for primary prophylaxis for bleeding were actually prescribed this medication. They also suggested that secondary prophylaxis improved survival in HCC patients.

Loss of muscle mass and function is provided the term sarcopenia and is frequently seen in advanced liver disease. The prevalence is estimated to be between 40%-70% in patients with cirrhosis alone^[87]. The underlying mechanism is complex and not fully understood but includes inadequate intake, malabsorption and abnormal metabolism favouring proteolysis for gluconeogenesis. The addition of anti-cancer therapies can further compound the situation. A study looking at whether sarcopenia predicts the prognosis of patients treated with Sorafenib showed that skeletal muscle depletion is an independent prognostic factor^[88]. The same has also been shown to apply to patients who have undergone transarterial chemoembolisation treatment for their HCC^[89]. Although there is no evidence that sarcopenia is impacting on HCC progression, there is an impact on outcomes and therefore it should be recognised and addressed as part of the patient management pathway.

Carcinogenesis: Prevention is better than cure

With the aim to reduce the complications of cirrhosis

Table 2 Summary of some of the antifibrotic agents being actively pursued in clinical trials, outlining mechanism and key outcomes to date

Drug	Mechanism	Comment	Ref.
BMS-986263/ND-LO2-s0201	siRNA that inhibits HSP47, reducing type 1 collagen synthesis	A lipid nanoparticle containing siRNA that inhibits HSP47. Vitamin A conjugated to the nanoparticle surface target facilitating targeted delivery to HSC and preclinical studies suggest disruption of collagen synthesis which may reverse fibrosis. A phase 1 study has demonstrated tolerability	[94]
Simtuzumab	Inhibition of Lysyl oxidases (LOX) mediated collagen cross linking reduces the breakdown of collagen by proteases such as MMPs	Simtuzumab is a humanized monoclonal antibody. It binds to LOXL2 and acts as an immunomodulator. However in a large phase 2 clinical trial in patients with NASH fibrosis the results were disappointing and focus has been diverted to LOXL1 inhibition where expression appears constant in carbon tetrachloride induced fibrosis in mouse models	[95,96]
Selonsertib	Inhibits apoptosis signal-regulating kinase 1 which in the setting of oxidative stress activates pathways which lead to fibrosis	In patients with NASH has shown promise that it may lead to a reduction in fibrosis in a phase 2 trial where it was given with and without Simtuzumab and compared to Simtuzumab alone	[97,98]
Cenicriviroc	Dual antagonist of C-C motif chemokine receptor (CCR) types 2 and 5	Demonstrated anti-fibrotic activity in animal models of liver fibrosis. In a phase 2 study improvements were seen in noninvasive markers of hepatic fibrosis. Antagonism of CCR2 reduces pro-inflammatory monocytes and macrophages. CCR5 antagonism impairs the activation of HSCs	[99-101]
Emricasan	Inhibitor of apoptotic and inflammatory caspases	Emricasan in the murine NASH model attenuated HSC activation. In phase 2 clinical trials for the regression of hepatic fibrosis caused by HCV infection after liver transplantation, the study didn't reach its primary endpoint but the results from a phase 2 trial in NASH are awaited	[102]
GR-MD-02	Targets galectin-3	Phase 3 studies are already planned for GR-MD-02. Phase 1 and 2 have been completed in the NASH cohort. Preclinical data showed some reversal of fibrosis and a reduction in portal pressures in cirrhosis	[103]
Erlotinib	Epidermal growth factor (EGF) receptor inhibitor	In preclinical models the FDA approved inhibitor regressed fibrosis in some animals and blocked the development of HCC. A pilot phase1/2 trial is underway	[104]

including decompensation there has been progress in recent years to try to prevent or reverse liver fibrosis and some evidence that this reduces HCC risk^[90]. The WHO recognise that the viral hepatitis pandemic contributes to an estimated 1.4 million deaths per year including HCC and cirrhosis^[91]. The published strategy outlines aims of reducing transmission of HCV and creating global access to treatment by 2030.

Identification and addressing of specific aetiologies, such as viral hepatitis and addressing metabolic risk factors in those with NAFLD is clearly essential but there is also a drive to develop specific anti-fibrotic agents. Whether we can reduce carcinogenesis by interrupting or reversing fibrogenic process with specific anti-fibrotic agents remains to be seen but certainly improvement in liver function is desirable to facilitate management and improve outcomes when cancer occurs.

With a better understanding of the processes that lead to fibrogenesis and fibrolysis it is now possible to target the relevant cytokines and effector cells including HSCs, myofibroblasts, profibrogenic immune

cells and other ECM targets including collagens and matrix metalloproteinases (MMP) inhibitors like tissue metalloproteinase inhibitor 1. We know that there are collagen types which are increased in liver fibrosis and are potentially targetable by small interfering RNA (siRNA) or antisense oligonucleotides. The therapeutic advantage of these nucleic acid based therapies is that they can now be delivered in vehicles which are preferentially taken up by the cell being targeted achieving knockdown effects only at the site of interest^[92,93]

The identification of novel targets in the fibrotic environment has led to the development of therapeutic agents with efficacy in preclinical models and we have seen the first clinical trials take place. We summarise in Table 2^[94-104] some of the therapeutic targets being actively pursued for their anti-fibrotic effects. Unfortunately trial design for direct anti-fibrotic agents are not at present designed to assess if they have an effect on HCC prevention.

Pre-clinical experiments have demonstrated inhibiting the pathways for pro-fibrogenic cytokines including

TGF- β and PDGF are also potential anti-fibrotic strategies to be explored further^[105]. Integrins are transmembrane receptors that promote ECM adhesion. Some integrins can facilitate MMP activation and activate fibrogenic mediators such as TGF- β 1. The identification of integrins which are important in fibrosis has led to mouse studies involving their direct and indirect inhibition^[106]. Other preclinical studies are looking at targeting transmembrane collagen receptors, proteins expressed on myofibroblasts and manipulating the inflammatory environment to deactivate matrix producing myofibroblasts and hepatic stellate cells.

CONCLUSION

Hepatocellular cancer is a deadly complication of cirrhosis and remains difficult to diagnose at an early curative stage. The prognosis in advanced cases remains extremely poor despite significant changes in epidemiology and causes of chronic liver disease, with the rising epidemic of obesity. Nevertheless, new studies with novel agents are demonstrating increasing rates of tumour response and stability and there are exciting developments with immunotherapies. The challenge in patients who do respond to anti-cancer therapies is to maintain their underlying liver function in order that intolerable liver related side effects are minimised and patients have the best chance for a prolonged overall survival. Furthermore, standard of care for cirrhosis may not be met in patients with hepatocellular cancer because of assumptions of lack of benefit, but with new treatments leading to improved survival this will need to be reconsidered. There have been significant improvements in elucidating the underlying cellular mechanisms which drive fibrosis and cancer within the liver. Hopefully, new therapies will take advantage of these findings leading to personalised therapeutic combinations for these patients which have the dual effect of promoting fibrosis regression and anti-cancer effects.

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Basic Study

Sheng-jiang powder ameliorates obesity-induced pancreatic inflammatory injury *via* stimulating activation of the AMPK signalling pathway in rats

Yi-Fan Miao, Juan Li, Yu-Mei Zhang, Lv Zhu, Huan Chen, Ling Yuan, Jing Hu, Xiao-Lin Yi, Qiu-Ting Wu, Mei-Hua Wan, Wen-Fu Tang

Yi-Fan Miao, Juan Li, Yu-Mei Zhang, Lv Zhu, Huan Chen, Ling Yuan, Jing Hu, Xiao-Lin Yi, Qiu-Ting Wu, Mei-Hua Wan, Wen-Fu Tang, Department of Integrative Medicine, West China Hospital, Sichuan University, Chengdu 610041, Sichuan Province, China

ORCID number: Yi-Fan Miao (0000-0002-3483-2345); Juan Li (0000-0002-5775-9355); Yu-Mei Zhang (0000-0001-9802-776X); Lv Zhu (0000-0002-4302-3339); Huan Chen (0000-0002-4763-6730); Ling Yuan (0000-0002-0921-713X); Jing Hu (0000-0002-8952-0357); Xiao-Lin Yi (0000-0003-0872-1155); Qiu-Ting Wu (0000-0002-6282-5791); Mei-Hua Wan (0000-0002-1237-9455); Wen-Fu Tang (0000-0001-9294-6634).

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Correspondence to: Wen-Fu Tang, PhD, Professor, Department of Integrative Medicine, West China Hospital, Sichuan University, No. 37, Guoxue Lane, Wuhou District, Chengdu 610041, Sichuan Province, China. tangwf@scu.edu.cn
Telephone: +86-28-85423546
Fax: +86-28-85423373

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Abstract

AIM

To investigate the mechanisms by which Sheng-jiang powder (SJP) ameliorates obesity-induced pancreatic inflammatory injury.

METHODS

Sprague-Dawley rats were randomized into three groups: normal group (NG), obese group (HLG), or SJP treatment group (HSG). Obesity was induced by feeding a high-fat diet in the HLG and HSG, while the NG received standard chow. Rats were euthanized after 12 wk, and blood and pancreatic tissues were collected for histopathological analyses. Nuclear factor kappa-light-chain-enhancer of activated B cells (NF- κ B) and transforming growth factor beta (TGF- β) expression, serum triglyceride and adiponectin levels, and apoptosis in pancreatic acinar cells were assessed. A high-fat AR42J acinar cell injury model was established using very low-density lipoprotein (VLDL). AR42J acinar cell culture supernatant, treated with different interventions, was applied to seven groups of pancreatic stellate cells (PSCs). The proliferation of PSCs and the expression of fibronectin and type I collagenase were assessed.

RESULTS

Compared with the NG, we found higher pathological scores for pancreatic tissues, lower serum adiponectin levels, higher expression levels of NF- κ B in pancreatic tissues and TGF- β in pancreatic inflammatory cells, and increased apoptosis among pancreatic acinar cells for the HLG ($P < 0.05$). Compared with the HLG, we found reduced body weight, Lee's index scores, serum triglyceride levels, and pathological scores for pancreatic tissues; higher serum adiponectin levels; and lower expression levels of NF- κ B, in pancreatic tissue and TGF- β in pancreatic inflammatory cells for the HSG ($P < 0.05$). The *in vitro* studies showed enhanced PSC activation and increased expression levels of fibronectin and type I collagenase after SJP treatment. An adenosine 5'-monophosphate-activated protein kinase (AMPK) inhibitor inhibited PSC activation.

CONCLUSION

SJP may ameliorate obesity-induced pancreatic inflammatory injury in rats by regulating key molecules of the adiponectin-AMPK signalling pathway.

Key words: Obesity; Sheng-jiang powder; Adiponectin; Adenosine 5'-monophosphate-activated protein kinase; Pancreatic inflammatory injury

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Core tip: Obesity is a risk factor for non-alcoholic fatty pancreas disease and induces pancreatic inflammatory injury. Sheng-jiang powder (SJP) can ameliorate obesity-induced pancreatic inflammatory injury; however, the specific mechanisms remain unclear. This study demonstrates that SJP may inhibit the inflammatory response, prevent pancreatic fibrosis, promote pancreatic acinar cell repair, and ultimately ameliorate obesity-induced pancreatic inflammatory injury in rats by regulating the key molecules of the AMPK signalling pathway.

Miao YF, Li J, Zhang YM, Zhu L, Chen H, Yuan L, Hu J, Yi XL, Wu QT, Wan MH, Tang WF. Sheng-jiang powder ameliorates obesity-induced pancreatic inflammatory injury *via* stimulating activation of the AMPK signalling pathway in rats. *World J Gastroenterol* 2018; 24(39): 4448-4461 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v24/i39/4448.htm> DOI: <http://dx.doi.org/10.3748/wjg.v24.i39.4448>

INTRODUCTION

Obesity rates have increased sharply over the past 40 years, creating a global public health crisis^[1]. According to the results of the Global Burden of Disease Study 2013, the number of overweight and obese individuals increased to 2.1 billion worldwide in 2013, which is 2.28 times more than that in 1980^[2]. Obesity or excess weight can lead to high morbidity for many noncommunicable diseases, including 75% of hypertension, 44% of the diabetes burden, 23% of ischaemic heart disease, and 7%-41% of certain cancers^[3]. Additionally, the prevalence of non-alcoholic fatty pancreas disease (NAFPD), which is characterized by pancreatic fat infiltration due to obesity, ranges from 16% to 35% in Asian populations^[4,5]. In addition, NAFPD may play an important role in the development of type 2 diabetes (T2DM), acute pancreatitis, and even pancreatic cancer^[5]. As a result, obesity- and excess weight-related complications have led to a considerable burden on patients and the society. Withrow and Alter (2011)^[6] indicated that obesity accounted for between 0.7% and 2.8% of the total healthcare costs of a country. Therefore, due to the side effects of the current treatments for obesity and the lack of specific drugs, people have gradually begun to focus on interventions using traditional Chinese medicine (TCM), such as Sheng-jiang powder (SJP)^[3,7].

The pathogenesis of obesity-induced tissue injury is complex and diverse. The most common pathogenesises are endoplasmic reticulum (ER) stress and the inflammatory response^[5,8]. According to experimental reports, maternal obesity and postnatal obesogenic diets can result in NAFPD because of an ER imbalance and an alteration in circadian metabolic patterns^[9]. Obesity-induced inflammation is a chronic and low-grade form of inflammation, which starts in adipose tissue, with abundant macrophage infiltration, followed by the increased secretion of pro-inflammatory cytokines, such as tumour necrosis factor alpha (TNF- α), interleukin 6 (IL-6), and C-reactive protein, while the production of anti-inflammatory cytokines, such as interleukin 10 (IL-10) and adiponectin, drastically decreases^[10]. Gotoh *et al.*^[11] found that obesity reduced the production of spleen-derived IL-10, which can protect against the development of NAFPD. In addition, insulin resistance (IR) and β -cell dysfunction also play important roles^[12]. IR decreases the inhibitory activity of insulin on peripheral

lipolysis, leading to an increase in circulating free fatty acids (FFAs). The chronic exposure of β -cells to elevated FFAs results in β -cell dysfunction and creates a vicious cycle resulting in the continuous deterioration of the glucometabolic state^[13]. Although obesity-induced pancreatic injury is known to be related to the inflammatory response^[7], the specific and detailed mechanisms involved remain unclear.

According to the TCM theory, obesity belongs to the category of “Turbidity”, which is primarily caused by the “ascending and descending dysfunction” of the spleen^[14]. As a classic representative formula for ascending lucidity and descending turbidity, SJP originates from a Nei-Fu-Xian-Fang decoction in *Wanbing Huichun*, which was compiled by Ting-Xian Gong during the Ming dynasty in China and is composed of Jiangchan (*Bombyx Batryticatus*), Chantui (*Periostracum cicada*), Jianghuang (*Curcuma longa* L.), and Dahuang (*Rheum palmatum* L.)^[15]. Several clinical studies have confirmed that SJP is effective in regulating lipid metabolism and improving IR, and SJP is widely used to treat obesity-related diseases, such as hyperlipidaemia, fatty liver, and diabetes^[16-18]. Our previous studies have demonstrated that SJP can ameliorate the inflammatory response and histopathological lesions in the pancreas of obese rats^[7]. However, the specific mechanisms underlying the amelioration of obesity-induced pancreatic inflammatory injury by SJP are far from being sufficiently understood. Therefore, we designed this study to further investigate the specific mechanisms of SJP on obesity-induced pancreatic inflammatory injury.

MATERIALS AND METHODS

Preparation of SJP for oral administration to rats

The spray-dried drug particles of SJP ingredients, including Dahuang (batch No. 16110150), Jianghuang (batch No. 16080008), Jiangcan (batch No. 16100147), and Chantui (batch No. 16080020), were purchased from the Affiliated Hospital of Chengdu University of TCM (Chengdu, China) and authenticated by Professor Wang WM (Department of Herbal Pharmacy, West China Hospital, Sichuan University, China), according to the Chinese Pharmacopoeia (The Pharmacopoeia Commission of People's Republic of China, 2010). Voucher specimens were deposited at our laboratory. The spray-dried drug particles were mixed in the proportions of 4:3:2:1, according to Ting-Xian Gong's *Wanbing Huichun*, a famous, classic TCM book from the Ming dynasty^[15], and they were completely reconstituted with sterile double-distilled water (concentration: 1 g/mL). This SJP solution was stored at 4 °C until ready for use, and it was administered orally to the rats at a dose of 5 mL/kg of body weight (BW).

Preparation of SJP for cell treatment

Our previous study determined that the serum peak concentration of rhein in the plasma of rats that received

orally administered SJP (Dahuang, Jianghuang, Jiangcan, and Chantui proportions: 12:9:6:3) was 4388 ± 957 μ g/L; thus, for convenience, we used 5000 μ g/L for calculations^[19]. According to the above concentration and the content of rhein in the SJP compound formula (Dahuang, Jianghuang, Jiangcan, and Chantui proportions: 12:9:6:3; the ratio of rhein to the SJP compound formula was 0.5 mg/g)^[20], we calculated the compound dosage for cell treatments as follows: 1 g of SJP compound formula was added to 100 mL of PBS (SH30256.01B, HyClone, Logan, UT, United States) to dilute to a 1 \times working concentration. In this study, 1 mL of the above 1 g/mL SJP solution for rat oral administration was diluted to 100 \times , filtered, and sterilized to prepare the highest concentration of the compound for *in vitro* use.

Preparation of adenosine 5'-monophosphate-activated protein kinase inhibitor Compound C

One gram of Compound C (171260, Merck KGaA, Darmstadt, Hessen, Germany) was dissolved in 1000 mL of phosphate buffer solution (PBS), and the mixture was diluted to a 2.5 mmol/L stock solution (100 \times), sterilized by filtration, and stored at -20 °C. Before use, the appropriate amount of the above stock solution was diluted 100 \times , for a final working concentration of 25 μ mol/L.

Induction of obesity, animal treatments, and sample collection

The protocol was reviewed and approved by the Institutional Animal Care and Use Committee of West China Hospital of Sichuan University. Twenty-four male Sprague-Dawley rats, weighing 60-80 g, were purchased from Chengdu Dashuo Experimental Animal Co., Ltd. (Chengdu, China). The protocol was designed to minimize the pain and discomfort of the rats. All rats were acclimatized to laboratory conditions (22 \pm 2 °C, 65% \pm 10% relative humidity, 12-h light/12-h dark cycle, *ad libitum* access to water and food) for one week prior to the special feeding. Special feeding meant that the rats had free access to a high-fat diet (HFD; 60% of calories derived from fat; TP23300; Trophic Animal Feed High-tech Co., Ltd., Nantong, China) to induce obesity, or to a control diet (16.7% of calories derived from fat; LAD3001G; Trophic Animal Feed High-tech Co., Ltd., Nantong, China).

All rats were randomly divided into a normal group (NG, control diet), an obese group (HLG, HFD), or an SJP treatment group (HSG, HFD plus SJP), with 8 rats in each group. The whole study lasted for 12 wk. Rats in the HSG were intragastrically administered with SJP (5 g/kg) once daily, beginning in the third week, while the rats in the other two groups were instead administered with equal volumes of normal saline. Food intake was monitored daily. After 12 wk of feeding, the rats were anesthetized (2% sodium pentobarbital, intraperitoneal injection, 40 mg/kg of BW), heart blood samples were taken to test the levels of triglyceride and adiponectin,

and the BW and naso-anal length were measured for Lee's index calculations, using the following formula^[21]:

$$\text{Lee's index} = \sqrt[3]{\text{body weight(g)} \times 10^3 / \text{naso - anal length(cm)}}.$$

Pancreatic tissue samples were obtained for histopathological analyses, immunohistochemistry tests for nuclear factor kappa-light-chain-enhancer of activated B cells (NF- κ B) and transforming growth factor- β (TGF- β), and terminal deoxynucleotidyl transferase-mediated dUTP nick-end labelling (TUNEL). Then, all rats were euthanized with a 2% sodium pentobarbital overdose (intraperitoneal injection, 200 mg/kg of BW).

Measurement of serum triglyceride and adiponectin levels

The blood samples were centrifuged at 1000 r/min for 5 min to collect supernatants for analysis. The levels of triglyceride were measured with a HITACHI automatic biochemical analyser (7170A, HITACHI, Tokyo, Japan), and the levels of adiponectin were measured with ELISA kits (EKT246253, eBio, Wuhan, China). According to the manufacturer's protocol, absorbance was measured at 450 nm with a High Throughput Universal Microplate Assay. The sample values were then read off the standard curve, and the relative concentrations were calculated.

Histopathological analysis of pancreatic tissues

Fresh pancreatic tissue samples were fixed with 40 g/L paraformaldehyde (AR1068, BOSTER, Wuhan, China), embedded in paraffin, sectioned into 5 μ m sections, and stained with haematoxylin and eosin. All histopathological sections were observed and scored in a blinded manner by two independent pathologists using the scoring system described by Kusske *et al.*^[22] (0-4 points: oedema, inflammation, haemorrhage, and necrosis). The total histopathology score is the mean of the combined scores for each parameter from both investigators.

Immunohistochemistry

Paraffin-embedded pancreatic samples were deparaffinized and then rehydrated. Endogenous peroxidase was quenched for 10 min with 30 g/L H₂O₂ and washed three times with distilled water. Sections were immersed in 0.01 mol/L citric acid buffer (pH 6.0), heated in a microwave oven until they were boiled, and then de-energized; the process was repeated 5 min later. After a wash with PBS, the sections were blocked with 5% bovine serum albumin (BSA) confining liquid (AR0004, BOSTER, Wuhan, China) for 10 min, at room temperature, and then excess liquids were removed. Sections were incubated overnight at 4 °C with primary antibody against NF- κ B p65 (sc-8008, Santa Cruz Biotechnology, Santa Cruz, CA, United States; 1:200 dilution) or TGF- β (sc-146, Santa Cruz Biotechnology, Santa Cruz, CA, United States; 1:100 dilution). After washing with PBS, the sections were incubated with biotinylated goat-anti-mouse or goat-anti-rabbit IgG (SA2010, BOSTER, Wuhan, China) at 37 °C for 30 min

and then incubated with a SABC-POD Kit (SA2010, BOSTER, Wuhan, China) for 30 min at 37 °C. Finally, the sections were stained with a DAB-kit (AR1022, BOSTER, Wuhan, China) for 20 min. The sections were rinsed in tap water and counterstained with haematoxylin. Immunohistochemistry sections were observed and scored in a blinded manner by specialists, using the scoring system described by Xu *et al.*^[23]. Briefly, the evaluation of the nuclear or cytoplasmic staining reaction was performed in accordance with the immunoreactive score (IRS): IRS = staining intensity (SI) \times percentage of positive cells (PP). SI was determined as follows: 0, colourless; 1, light yellow; 2, brownish yellow; and 3, brown. PP was defined as follows: 0, negative; 1, 10% positive cells; 2, 11%-50% positive cells; 3, 51%-75% positive cells; and 4, 75% positive cells. Ten visual fields from different areas of each pancreatic section were used for the IRS evaluation, using the average for statistical analysis.

TUNEL assay for apoptotic cells in pancreatic tissues

The levels of apoptotic cells in pancreatic tissue samples were analysed using a TUNEL detection kit (14590900, Roche, San Francisco, CA, United States), following the manufacturer's instructions. Briefly, the pancreatic tissue sections were covered with proteinase K solution (20 μ g/mL proteinase K + 0.01 mol/L Tris/HCL, pH 7-8.0) at room temperature for 15 min before the addition of 50 μ L of the TUNEL reaction mixture. After incubation in a humid chamber in the dark for 1 h, the sections were incubated with 50 μ L of converter-POD solution at 37 °C for 30 min, followed by a final PBS wash. Next, 100 μ L of DAB solution (5 μ L 20 \times DAB + 1 μ L 300 g/L H₂O₂ + 94 μ L PBS) was added for 10 min at room temperature to develop the slides, followed by three washes with PBS and haematoxylin counterstaining for 2 min. Images were captured using a fluorescence microscope (AX10 imager A2/AX10 cam HRC, Carl Zeiss Jena, Oberkochen, Germany), and the apoptotic index was calculated as the number of apoptotic cells/total number of cells \times 100%.

Cell culture

Rat pancreatic acinar AR42J cells (CRL-1492, ATCC, Manassas, VA, United States) were maintained at 37 °C in DMEM/F12 medium (SH30023.01B, HyClone, Logan, UT, United States) supplemented with 10% foetal bovine serum (FBS; 16000044, Gibco, Waltham, MA United States), 100 IU penicillin, and 100 μ g/mL streptomycin (SV30010, HyClone, Logan, UT, United States) in a 50 mL/L CO₂ atmosphere. Prior to stimulation, cells in the logarithmic growth phase were seeded at 1×10^6 cells/well in 6-well plates and incubated until completely adherent.

Rat pancreatic stellate cells (PSCs; RAT-iCell-g003, Shanghai Deyu Bio-tech Co., Ltd, Shanghai, China) were cultured under the same conditions described above, but the culture medium was changed to 90% RPMI 1640 (SH30809.01B, HyClone, Logan, UT, United

States) supplemented with 10% FBS, 100 IU penicillin, and 100 µg/mL streptomycin. PSCs in the logarithmic growth phase were seeded on polylysine-treated slides to perform cell-climbing. After the PSCs covered the slides, they were treated according to the following experimental design.

Induction of a cell model and stimulation

A high-fat AR42J acinar cell injury model was established by stimulation with 0.06 mg/mL of very low-density lipoprotein (VLDL; LP1, Merck-Millipore, Billerica, MA, United States)^[24]. AR42J cells were divided into five groups: normal group (AR42J cells + culture medium), model group (AR42J cells + VLDL), SJP group (AR42J cells + VLDL + SJP), VLDL + Compound C group (AR42J cells + VLDL + Compound C), and SJP + Compound C group (AR42J cells + VLDL + SJP + Compound C). After the AR42J cells were completely adherent, 0.5 mL of medium (80% DMEM/F12 + 20% FBS + 100 IU penicillin + 100 µg/mL streptomycin) was added to the normal group and the model group; 0.25 mL of medium and 0.25 mL of SJP were added to the SJP group; 0.25 mL of medium and 0.25 mL of Compound C were added to the VLDL + Compound C group; and 0.25 mL of SJP and 0.25 mL of Compound C were added to the SJP + Compound C group. Thirty minutes later, 30 µL of culture medium was added to the normal group, and 30 µL of VLDL (5 mg/mL) was added to the other groups for model induction. Culture supernatants were collected 24 h after treatment administration to treat the PSCs, according to the following experimental design.

PSCs that covered the slides were divided into seven groups: A, normal PSCs (VLDL-, culture supernatant-); B, PSCs stimulated directly with VLDL (VLDL+, culture supernatant-); C, PSCs stimulated with normal acinar cell culture supernatant (VLDL-, culture supernatant+); D, PSCs stimulated with acinar cell culture supernatant treated with VLDL (VLDL+, culture supernatant+); E, PSCs stimulated with acinar cell culture supernatant treated with SJP (VLDL+, culture supernatant+, SJP+); F, PSCs stimulated with acinar cell culture supernatant treated with Compound C (VLDL+, culture supernatant+, Compound C+); G, PSCs stimulated with acinar cell culture supernatant treated with Compound C and SJP (VLDL+, culture supernatant+, SJP+, Compound C+). The culture medium was added to group A, diluted VLDL (30 µL VLDL + 2.5 mL medium) was added to group B, and the appropriate acinar cell culture supernatants, as described above, were added to each of the remaining five groups for 6 h, according to a ratio of 100 µL/mL. Then, the slides were collected for immunofluorescence analysis of the expression of fibronectin and type I collagenase.

Immunofluorescence

Slides were fixed in 40 g/L paraformaldehyde for 30 min and rinsed with PBS three times (10 min each time). Slides were permeabilized with 1% Triton X-100

(Sigma, Saint Louis, MO, United States) for 30 min and rinsed with PBS three times (10 min each time). Slides were blocked with 10% goat serum (AR0009, BOSTER, Wuhan, China) at 37 °C for 2 h. Anti-collagen I antibody (ab34710, Abcam, Cambridge, MA, United States) and anti-fibronectin antibody (ab6328, Abcam, Cambridge, MA, United States) were added separately and incubated overnight at 4 °C. After washing with PBS, the secondary antibodies, goat anti-rabbit IgG H&L (Alexa Fluor® 488) (ab150077, Abcam, Cambridge, MA, United States) and goat anti-mouse IgG H&L (Alexa Fluor® 488) (ab150113, Abcam, Cambridge, MA, United States), were added separately and protected against light for 2 h at room temperature. The nucleus was stained with DAPI, and the sections were sealed with glycerine. A fluorescence microscope (AX10 imager A2/AX10 cam HRC, Carl Zeiss Jena, Oberkochen, Germany) was used for observation.

Statistical analysis

The statistical methods of this study were reviewed by Dr. Hai Niu from College of Mathematics, Sichuan University. All values are expressed as the mean ± standard deviation. GraphPad Prism 6.01 software (GraphPad Prism 6.01 software Inc., San Diego, CA, United States) was used for statistical analyses. For each test, the experimental unit was an individual animal. Normality was assessed by the Shapiro-Wilk normality test, and homogeneity of variance was assessed by the Bartlett's test. If data were normally distributed and the variances of three experimental groups were equal, one-way analysis of variance was used for multi-group comparisons, and Dunnett-t test was used for comparisons of two groups. Statistical significance is expressed as ^a*P* < 0.05 vs NG or ^b*P* < 0.05 vs HLG.

RESULTS

SJP reduces BW, Lee's index, and serum triglyceride levels of obese rats

After 12 wk of experimental diet consumption, BW, Lee's index, which is a rapid means of determining obesity, and the levels of serum triglyceride of the rats in the HLG were significantly higher than those of the rats in the NG (*P* < 0.05; Table 1). Conversely, the above three parameters of the HSG were significantly lower than those of the HLG (*P* < 0.05; Table 1). However, food intake did not differ significantly among the experimental groups.

SJP relieves the pathological damage to pancreatic tissues in obese rats

The histopathological evaluation results showed significantly higher pathological scores for pancreatic tissues from rats in the HLG than for those in the NG (*P* < 0.05; Figure 1A). Conversely, SJP treatment distinctly lowered the pathological scores of the pancreas, with reduced inflammatory cell infiltration, mild tissue oedema, and

Table 1 Body weight, Lee's index, serum triglyceride levels, and dairy food intake of rats in the three experimental groups

Parameter	NG	HLG	HSG
Initial body weight (g)	69 ± 4	70 ± 5	69 ± 8
Final body weight (g)	461 ± 56	537 ± 46 ^a	467 ± 49 ^b
Lee's index	3.13 ± 0.07	3.43 ± 0.16 ^a	3.12 ± 0.13 ^b
Triglyceride (mmol/L)	1.57 ± 0.46	3.24 ± 1.48 ^a	1.39 ± 0.41 ^b
Food intake (g/d)	19.57 ± 0.87	18.91 ± 1.12	18.01 ± 0.77

The results are presented as the mean ± SD, $n = 8$ for each group. ^a $P < 0.05$ vs NG; ^b $P < 0.05$ vs HLG. NG: Normal group; HLG: High-fat diet group; HSG: High-fat diet group treated with Sheng-jiang powder.

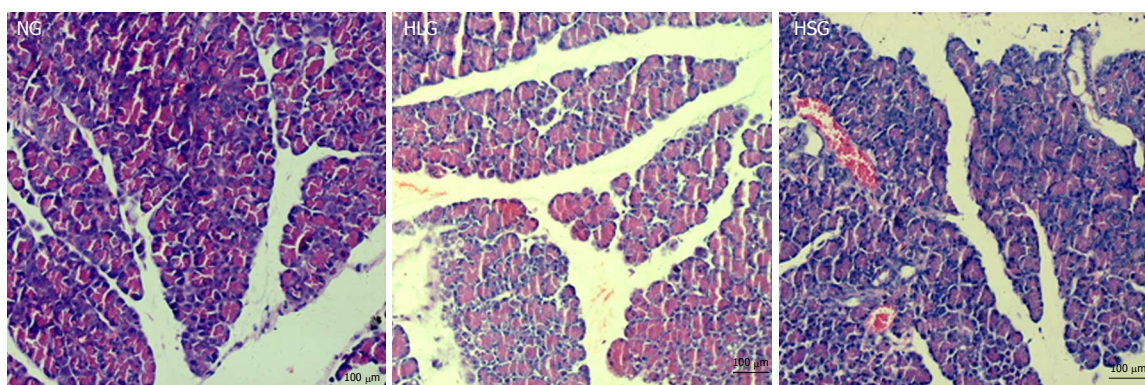
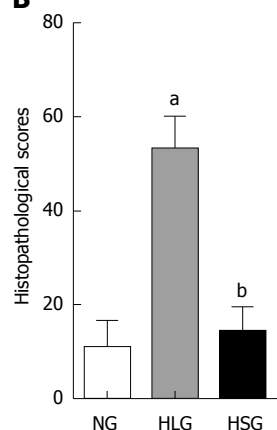
A**B**

Figure 1 Histological images and pathologic scores of pancreatic tissues from the three experimental groups. A: Pathological images of the pancreatic tissues ($\times 200$); B: Histological scores of the pancreatic tissues. The results are presented as the mean ± SD. ^a $P < 0.05$ vs NG; ^b $P < 0.05$ vs HLG. NG: Normal group; HLG: High-fat diet group; HSG: High-fat diet group treated with Sheng-jiang powder.

reduced cell necrosis (Figure 1A and B).

SJP stimulates the expression of serum adiponectin in obese rats

Adiponectin is an adipokine with anti-inflammatory, anti-oxidant, anti-atherogenic, pro-angiogenic, vasoprotective, and insulin-sensitizing properties, which is markedly decreased in obesity^[25]. Thus, we determined the levels of adiponectin in serum after SJP administration. Adiponectin levels were significantly reduced in the HLG ($P < 0.05$; Figure 2), whereas they were absent in the NG. After SJP administration, adiponectin levels were much higher in rats in the HSG than in rats in the HLG ($P < 0.05$;

Figure 2).

Effect of SJP on the expression levels of NF- κ B and TGF- β in pancreatic acinar cells and inflammatory cells from obese rats

As shown in Figure 3A, the expression levels of NF- κ B in both pancreatic acinar cells and inflammatory cells from rats were higher in the HLG than in the NG ($P < 0.05$). After SJP administration, the expression of NF- κ B in both types of cells was inhibited. Although no differences were found in TGF- β expression levels among the groups in the pancreatic acinar cells, an expression pattern similar to that of NF- κ B was observed in inflammatory cells,

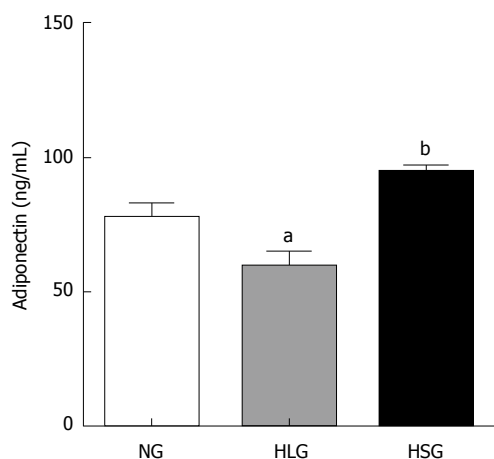


Figure 2 Levels of serum adiponectin. The results are presented as the mean \pm SD. ^a $P < 0.05$ vs NG; ^b $P < 0.05$ vs HLG. NG: Normal group; HLG: High-fat diet group; HSG: High-fat diet group treated with Sheng-jiang powder.

where TGF- β expression was stimulated by HFD and inhibited by SJP administration (Figure 3A).

Effects of SJP on apoptosis of pancreatic acinar cells in obese rats

Apoptosis of pancreatic acinar cells was significantly higher in the HLG than in the NG ($P < 0.05$; Figure 4). Although we found no significant differences in apoptosis following treatment with SJP, we found a downward trend in the HSG (Figure 4).

SJP promotes PSC activation

To explore whether the repair effects of SJP on pancreatic acinar cell damage occur through adenosine 5'-monophosphate-activated protein kinase (AMPK) signalling, we performed cellular tests of AR42J cells and PSCs. The results showed that the expression of fibronectin and type I collagenase was weak in normal PSCs (Figure 5A). After stimulation with VLDL or normal acinar cell culture supernatant, the expression of fibronectin and type I collagenase increased (Figure 5B and C). The supernatant of VLDL-treated AR42J cells stimulated PSCs to trigger increased expression levels of fibronectin and type I collagenase (Figure 5D), and the expression of these two proteins was enhanced after stimulation with acinar cell culture supernatant treated with SJP (Figure 5E). The fibronectin and type I collagenase expression levels were reduced (Figure 5F and G) in the two groups of PSCs treated with the AMPK inhibitor.

DISCUSSION

In the present study, HFD successfully induced an obese rat model, as in our previous study^[7]. Our results showed significantly higher BW, Lee's index scores, and serum triglyceride levels, lower serum adiponectin levels, higher expression levels of NF- κ B in pancreatic tissues, and increased apoptosis of pancreatic acinar

cells in obese rats, while SJP effectively reduced BW, Lee's index scores, and serum triglyceride levels, stimulated the expression of serum adiponectin, and inhibited the expression of NF- κ B in pancreatic tissues. In addition, the expression levels of TGF- β in inflammatory cells of the pancreas were significantly higher in obese rats, while SJP could reduce the expression of TGF- β in inflammatory cells but had no influence in acinar cells. The *in vitro* studies have shown that the culture supernatant from AR42J acinar cells that were incubated with VLDL stimulated the proliferation and matrix synthesis of PSCs. After SJP treatment, PSC activation was enhanced, and the expression of fibronectin and type I collagenase was further increased. Interestingly, AMPK inhibitors inhibited the PSC activation process described above.

Adipose tissue is considered to be an endocrine organ with an important role in local and systemic homeostasis. It has been demonstrated that adipose is responsible for the production and release of many potent signalling molecules, including adipokines, lipokines, and inflammatory mediators^[25]. Adiponectin is a well-known adipokine that promotes insulin sensitivity and has an anti-inflammatory effect, and its production becomes blunted as adiposity increases^[26]. Generally, obesity in humans is a symptom of energy imbalance, where energy intake exceeds energy output, while AMPK plays a key role in controlling energy homeostasis^[27]. Importantly, adiponectin can regulate energy intake and consumption by stimulating the phosphorylation of AMPK; adiponectin phosphorylates and subsequently inhibits acetyl-CoA carboxylase and inhibits malonyl-CoA synthesis, thereby decreasing the inhibitory effect of carnitine acyltransferase 1 (the key enzyme required for activated fatty acid entry into the mitochondria) and leading to increased fatty acid oxidation and glucose uptake^[28]. Therefore, with the long-term intake of HFD, the decrease in adiponectin observed in obese rats may affect the energy imbalance and promote fat infiltration or accumulation; in turn, fat accumulation may affect the expression of adiponectin, which leads to a vicious cycle.

In addition to its effect on controlling glucose and lipid metabolism, adiponectin can also inhibit lipopolysaccharide (LPS)-primed inflammasome activation in macrophages via AMPK signalling-dependent mechanisms^[29], while adiponectin-AMPK signalling can be inhibited during chronic low-grade inflammatory responses, including obesity, non-alcoholic fatty liver disease, atherosclerosis, IR, and T2DM^[30]. Therefore, the decrease in adiponectin observed in obese rats may reduce its inhibitory effect on inflammasome activation and promote the inflammatory response. In our study, SJP ameliorated the expression of adiponectin in rats with obesity induced with an HFD. Similarly, some studies showed that SJP could significantly increase serum adiponectin levels in obesity-related glomerulopathy patients and T2DM patients with dyslipidaemia^[18,31]. As a

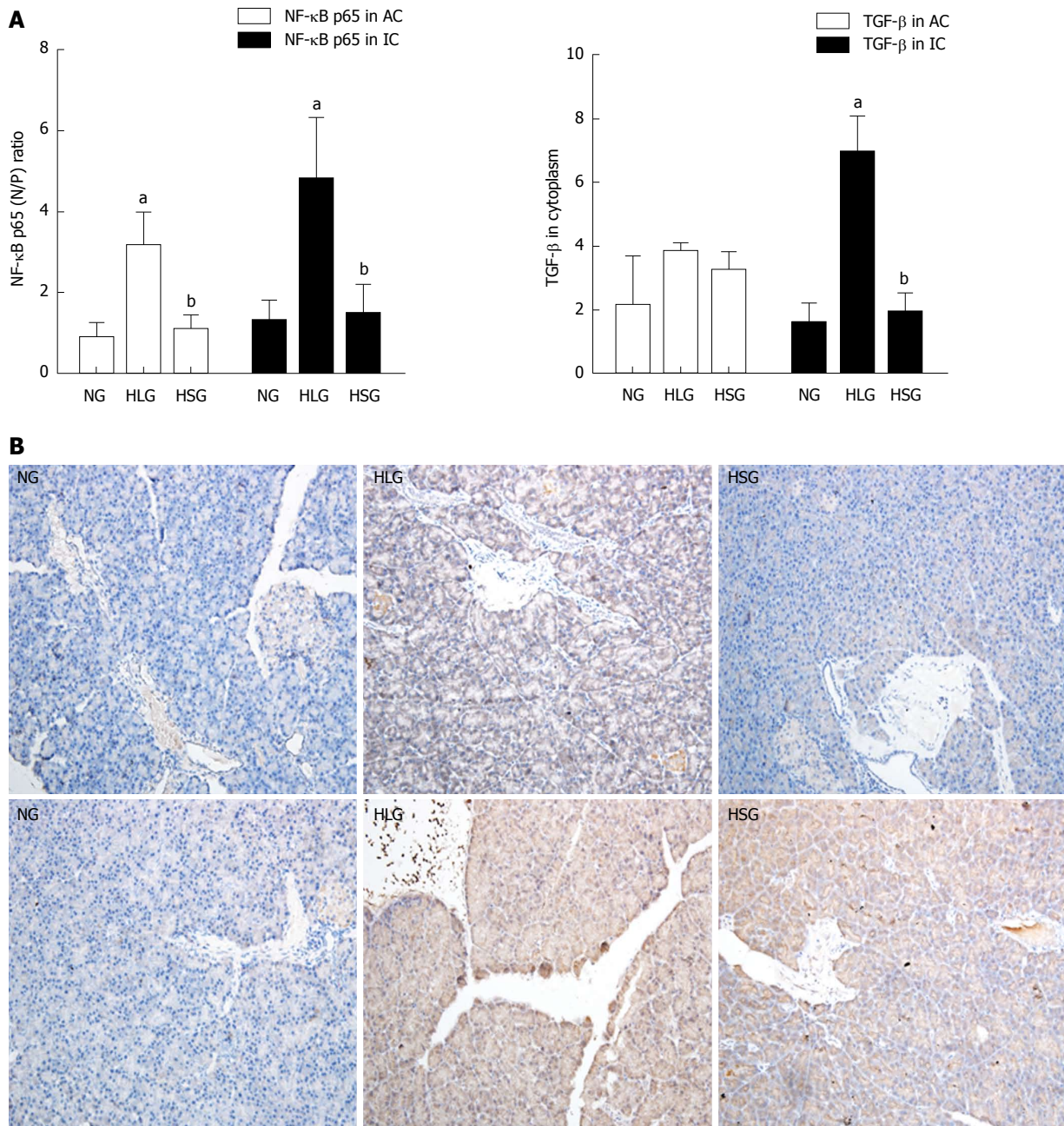


Figure 3 Nuclear factor kappa-light-chain-enhancer of activated B cells and transforming growth factor beta expression in pancreatic tissues. A: Nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) and transforming growth factor beta (TGF-β) expression in pancreatic acinar cells and inflammatory cells; B: Immunohistochemistry assay for NF-κB expression; C: Immunohistochemistry assay for TGF-β expression. Negative: Blue; Positive: Yellow-brown. The results are presented as the mean \pm SD. $^aP < 0.05$ vs NG; $^bP < 0.05$ vs HLG. NF-κB: Nuclear factor kappa-light-chain-enhancer of activated B cells; TGF-β: Transforming growth factor beta; NG: Normal group; HLG: High-fat diet group; HSG: High-fat diet group treated with Sheng-jiang powder; N/P ratio: Nuclear/cytoplasmic ratio; AC: Acinar cells; IC: Inflammatory cells.

component of *Curcuma longa*, curcumin could attenuate HFD-induced hepatic steatosis by regulating hepatic lipid metabolism via AMPK activation^[32]. Thus, combined with the anti-inflammatory effect of SJP, we speculate that SJP may reduce the suppressive effect of obesity on the adiponectin-AMPK signalling pathway, which may contribute to energy consumption and further inhibit the inflammatory response, eventually regulating lipid metabolism in obese rats.

In addition to the aforementioned decrease in adiponectin levels associated with the inflammatory

response in obese rats, the NF-κB signalling pathway and ER stress are two other important mechanisms of the obesity-induced inflammatory response. As adiposity increases, the balance between pro-inflammatory and anti-inflammatory cytokines secreted by adipocytes gradually becomes deregulated. In addition, those unbalanced inflammatory cytokines can activate macrophages via the Toll-like receptor 4 signalling pathways, whereas the binding of TNF-α released by macrophages to TNF-α receptors on adipocytes activates the NF-κB signalling pathway^[33] and promotes the

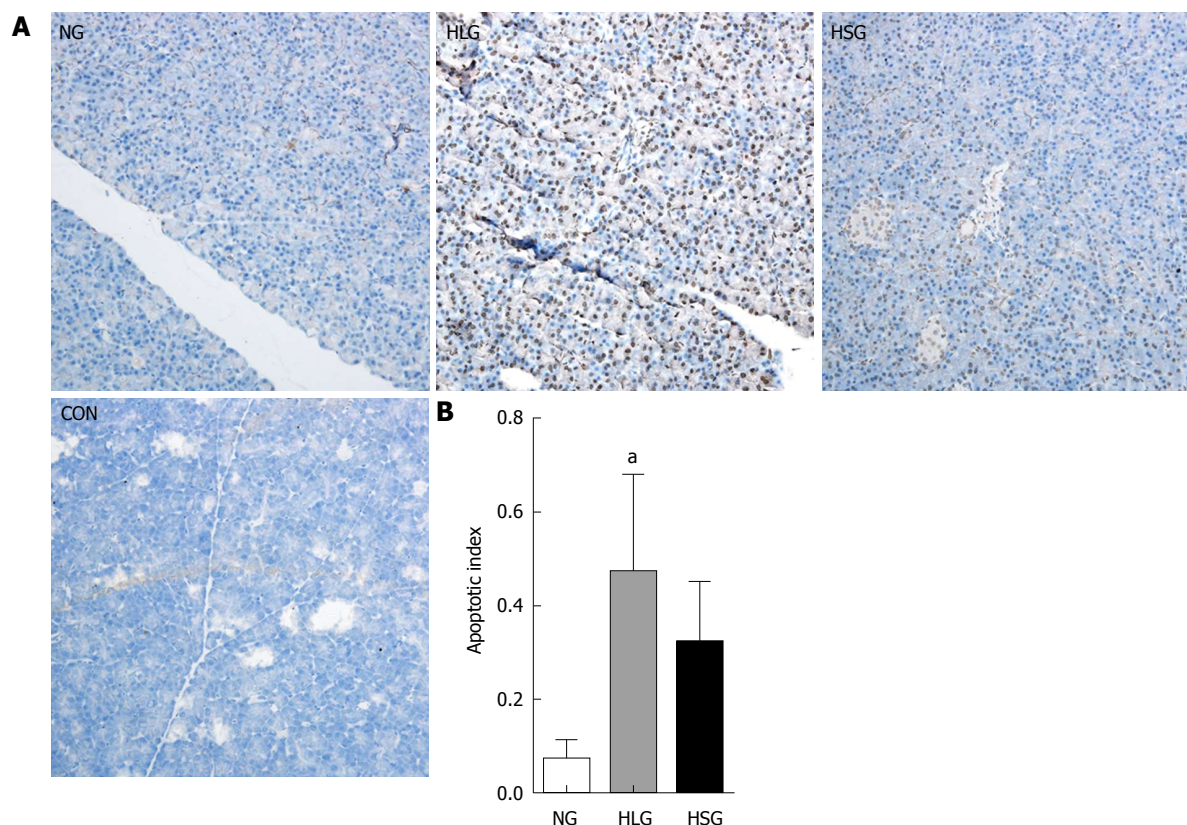


Figure 4 Results of terminal deoxynucleotidyl transferase-mediated dUTP nick-end labelling staining of pancreatic acinar cells. A: TUNEL staining images of pancreatic acinar cells; B: Apoptotic index of pancreatic acinar cells. Negative: blue; positive: yellow-brown. The results are presented as the mean \pm SD. ^a $P < 0.05$ vs NG. TUNEL: Terminal deoxynucleotidyl transferase-mediated dUTP nick-end labelling; NG: Normal group; HLG: High-fat diet group; HSG: High-fat diet group treated with Sheng-jiang powder group; CON: Negative staining control group.

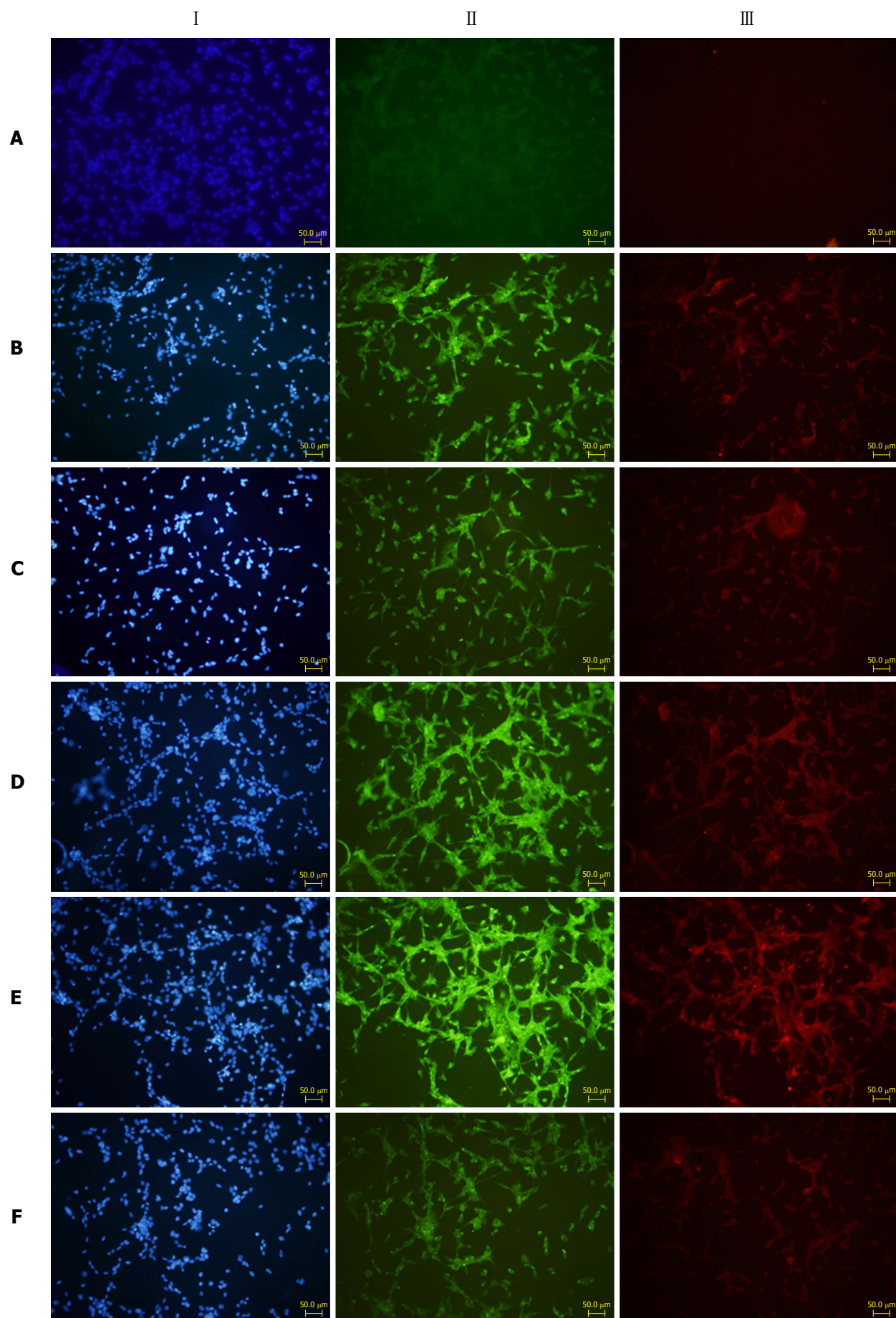
amplification of inflammatory responses. In addition, ER stress is currently recognized to be a mechanism of the obesity-induced inflammatory response. Obesity-induced ER stress primarily manifests itself in the activation of two classic signalling pathways: The nuclear factor of kappa light polypeptide gene enhancer in B cells inhibitor ($\text{I}\kappa\text{B}$)/ $\text{NF-}\kappa\text{B}$ signalling pathway and the c-Jun N-terminal kinase (JNK) signalling pathway^[34,35]. The phosphorylation of $\text{I}\kappa\text{B}$ and JNK triggers the activation of transcription factors, such as $\text{NF-}\kappa\text{B}$, that are closely related to the inflammatory response downstream, thereby promoting the development of inflammatory responses^[36]. We know that SJP was effective for anti-inflammation and could significantly downregulate the expression of $\text{NF-}\kappa\text{B}$ in inflammatory diseases, such as acute lung injury and glomerulonephritis^[37,38]. This study also confirmed that SJP reduced the expression of $\text{NF-}\kappa\text{B}$ in pancreatic tissues. Therefore, SJP may reduce the inflammatory response in the pancreas of obese rats via the $\text{NF-}\kappa\text{B}$ signalling pathway.

TGF- β is a regulatory molecule with pleiotropic effects on cell proliferation, differentiation, migration, and survival and affects multiple biological processes, including development, carcinogenesis, fibrosis, wound healing, and immune responses^[39]. Although TGF- β is an important cytokine that regulates tissue inflammation and repair, its overexpression induces fibrosis, and

the inhibition of TGF- β improves fibrotic disorder^[40,41]. Matsuda *et al.*^[42] found that, in Zucker diabetic fatty rats fed a chronic HFD, fat could accumulate in pancreatic acinar cells, which was related to subsequent pancreatic fibrosis and acinar cell injury. Similarly, Yoshikawa *et al.*^[43] demonstrated that TGF- β 1 could extend from perislets to the exocrine pancreas to become involved in pancreatic fibrosis in Otsuka Long-Evans Tokushima fatty rats, a model of naturally occurring obesity-related diabetes.

In our study, the expression of TGF- β in pancreatic acinar cells was rarely increased after 12 wk of HFD intake, but it was highly expressed in pancreatic inflammatory cells. Therefore, we speculate that obesity, a persistent chronic injury with an accompanying inflammatory response, may result in pancreatic fibrosis. Interestingly, after SJP treatment, the expression of TGF- β in pancreatic inflammatory cells was significantly decreased with a dramatic reduction in $\text{NF-}\kappa\text{B}$ and an increase in adiponectin. Thus, given the common use of anti-inflammatory drugs in the treatment of fibrosis^[40] and the anti-inflammatory effect of SJP, we speculate that SJP may prevent pancreatic fibrosis by inhibiting the inflammatory response in the pancreas of obese rats.

It has been well established that brain, liver, and heart cells undergo apoptosis under obesity conditions^[44-46]. To confirm whether obesity induces pancreatic acinar cell



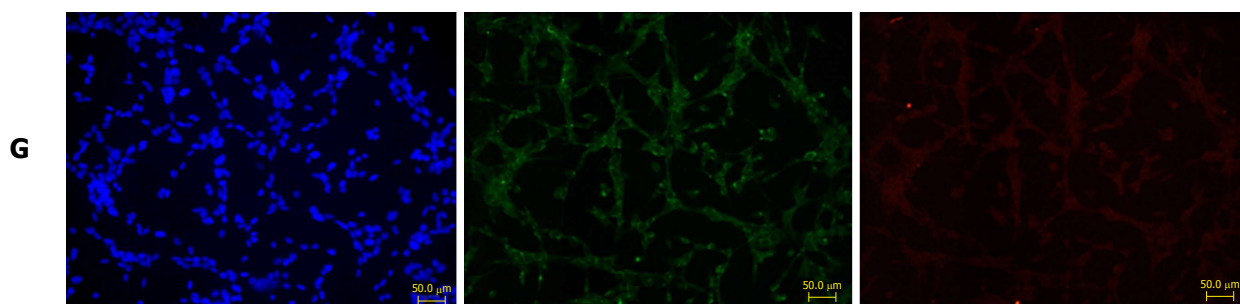


Figure 5 Immunofluorescence results of fibronectin and type I collagenase expression in cells growing on glass coverslips from each group. I: Nuclear staining of rat pancreatic stellate cells (blue fluorescence); II: Fibronectin staining (green fluorescence); III: Type I collagenase staining (red fluorescence). A: Normal PSC (VLDL-, culture supernatant-); B: PSC stimulated directly with VLDL (VLDL+, culture supernatant-); C: PSC stimulated with normal acinar cell culture supernatants (VLDL-, culture supernatant+); D: PSC stimulated with acinar cell culture supernatant treated with VLDL (VLDL+, culture supernatant+); E: PSC stimulated with acinar cell culture supernatant treated with SJP (VLDL+, culture supernatant+, SJP+); F: PSC stimulated with acinar cell culture supernatant treated with Compound C (VLDL+, culture supernatant+, Compound C+); G: PSC stimulated with acinar cell culture supernatant treated with Compound C and SJP (VLDL+, culture supernatant+, SJP+, Compound C+). PSC: Pancreatic stellate cell; VLDL: Very low-density lipoprotein; SJP: Sheng-jiang powder.

apoptosis, we performed TUNEL staining. The results showed that obesity could induce apoptosis of pancreatic acinar cells. Moreover, one study has shown that the phosphorylation of AMPK induced by adiponectin could block interleukin 8-mediated endothelial cell death and exert an anti-apoptotic effect^[47]. Considering the fact that SJP increased the levels of serum adiponectin in our study and that curcumin effectively reduced apoptosis of pancreatic acinar cells caused by the long-term intake of alcohol and different amounts of proteins^[48], we speculated that SJP might play a role in the obesity-induced apoptosis of pancreatic acinar cells. However, there was no significant difference in the amount of apoptosis in acinar cells after SJP treatment in this study. A possible explanation for this finding might be that a single dose or the dosing concentration was insufficient. To the best of our knowledge, only one study has demonstrated that SJP inhibited the apoptosis of brain cells in rats with vascular dementia, and its administration method was intravenous drip at 10 mL/kg of BW SJP^[49].

Almost immediately, from the start of injury, multiple types of cells participate in the process of exocrine pancreas repair and regeneration. These cells include not only acinar cells, which are both villains and victims in pancreatic injury, but also ductal epithelial cells, inflammatory cells of the immune system, and PSCs^[50]. Given the importance of epithelial-mesenchymal interactions during pancreas development^[51], interactions between parenchymal cells and PSCs are almost certain to be important for proper pancreatic repair. Under physiological conditions, PSCs are at rest. In the presence of profibrogenic mediators, such as inflammatory cytokines and oxidative stress, PSCs are activated^[52]. Activated PSCs produce large amounts of α -smooth muscle actin and extracellular matrix proteins, particularly fibronectin and type I collagenase, to achieve the replacement of inflammatory infiltrates and the repair or regeneration of tissue injuries^[53]. On the basis of the *in vivo* data, under obesity conditions, inflammation occurred in pancreatic tissues, and subsequently, acinar

cell injury arose. Furthermore, culture media treated with fat could stimulate acinar cells to produce profibrogenic mediators. In addition, the supernatants collected from acinar cells stimulated the proliferation of PSCs and the synthesis of extracellular matrix proteins, particularly fibronectin and type I collagenase. After treatment with the AMPK inhibitor, the expression levels of fibronectin and type I collagenase were reduced. For the first time, we found that the inhibition of the AMPK signalling pathway could impair the therapeutic effects of SJP by diminishing the expression of fibronectin and type I collagenase. Therefore, we boldly speculate that SJP may promote acinar cell injury repair through the activation of the AMPK signalling pathway.

This study expanded on the research from our previous study^[7]. However, some limitations exist. First, in the *in vivo* experiment, no critical upstream or downstream factors were detected in the adiponectin-AMPK pathway in pancreatic tissue other than serum adiponectin. Second, the relationship between dose or dose frequency and the concentration effect requires further study. Finally, the specific effective monomer components of SJP should be taken under consideration.

In conclusion, we demonstrated that obesity exacerbates pancreatic inflammatory injury in rats and promotes the apoptosis of pancreatic acinar cells. SJP can inhibit the inflammatory response, prevent pancreatic fibrosis, and promote pancreatic acinar cell repair, through the regulation of key molecules of the adiponectin-AMPK signalling pathway, and eventually ameliorate obesity-induced pancreatic inflammatory injury in rats.

ARTICLE HIGHLIGHTS

Research background

Obesity is a risk factor for non-alcoholic fatty pancreas disease and induces pancreatic inflammatory injury. Sheng-jiang powder (SJP) can ameliorate obesity-induced pancreatic inflammatory injury, but the specific mechanisms remain unclear. Therefore, the investigation of the specific mechanisms underlying the SJP amelioration of obesity-induced pancreatic inflammatory

injury is urgently required.

Research motivation

Our previous studies have demonstrated that SJP can ameliorate the inflammatory response and histopathological lesions in the pancreas of obese rats. However, the specific mechanisms underlying ameliorating effects of SJP on obesity-induced pancreatic inflammatory injury are far from sufficiently understood. Therefore, this study aimed to further explore the specific mechanisms of SJP on obesity-induced pancreatic inflammatory injury, to provide evidence for its clinical application in the future.

Research objectives

This study aimed to investigate the specific mechanisms by which SJP can ameliorate obesity-induced pancreatic inflammatory injury.

Research methods

In the *in vivo* study, an obese rat model was induced by high-fat diet feeding, which is widely accepted and used for the induction of obesity in rats. The serum adiponectin levels were measured by enzyme-linked immunosorbent assay (ELISA), which is a simple, rapid, accurate, and sensitive method. The expression levels of nuclear factor kappa-light-chain-enhancer of activated B cells (NF- κ B) and transforming growth factor beta (TGF- β) in pancreatic tissues were measured by immunohistochemistry. The levels of apoptotic cells in pancreatic tissue samples were analysed by terminal deoxynucleotidyl transferase-mediated dUTP nick-end labelling (TUNEL) assay.

In the *in vitro* study, a high-fat AR42J acinar cell injury model was established with very low-density lipoprotein (VLDL), and the AR42J acinar cell culture supernatants, treated with different interventions, were applied to pancreatic stellate cells (PSCs). The proliferation of PSCs and the expression of fibronectin and type I collagenase were measured by immunofluorescence analysis.

All statistical analyses were performed with GraphPad Prism 6.01 software. Quantitative data are expressed as the mean \pm standard deviation when normally distributed. One-way analysis of variance followed by multiple pair-wise comparisons using Dunnett-*t* test was used to detect differences among the above parameters.

Research results

In the *in vivo* study, compared to the obese group (HLG), we found reduced body weight, Lee's index scores, serum triglyceride levels, and pathological scores of pancreatic tissues; higher serum adiponectin levels; and lower expression levels of NF- κ B in pancreatic tissue and TGF- β in the inflammatory cells of the pancreas in the SJP treatment group (HSG) ($P < 0.05$). In the *in vitro* study, PSC activation was enhanced after SJP treatment, and the expression levels of fibronectin and type I collagenase were increased after SJP treatment. An adenosine 5'-monophosphate-activated protein kinase (AMPK) inhibitor inhibited the PSC activation process described above.

What remains to be determined is the relationship between dose or dose frequency and the concentration effect. Furthermore, the specific effective monomer components of SJP should be taken under consideration to provide more systematic and comprehensive evidence for the clinical application of this Chinese decoction.

Research conclusions

This study demonstrates, for the first time, that obesity exacerbates pancreatic inflammatory injury in rats and promotes apoptosis in pancreatic acinar cells. In addition, SJP can inhibit the inflammatory response, prevent pancreatic fibrosis, promote pancreatic acinar cell repair, through the regulation of key molecules of the adiponectin-AMPK signalling pathway, and eventually ameliorate obesity-induced pancreatic inflammatory injury in rats. Therefore, our study provides molecular mechanisms as evidence for the clinical application of SJP.

Research perspectives

As we have found that SJP may ameliorate obesity-induced pancreatic inflammatory injury in rats by regulating key molecules of the adiponectin-AMPK signalling pathway, further investigation regarding the potential active components of SJP and the interactions among these components is urgently

required to provide evidence for wider clinical usage and to optimize and simplify the formula.

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Case Control Study

Molecular evaluation of glutathione S transferase family genes in patients with sporadic colorectal cancer

Gabriela Helena Rodrigues-Fleming, Glaucia Maria de Mendonça Fernandes, Anelise Russo, Patrícia Matos Biselli-Chicote, João Gomes Netinho, Érika Cristina Pavarino, Eny Maria Goloni-Bertollo

Gabriela Helena Rodrigues-Fleming, Glaucia Maria de Mendonça Fernandes, Anelise Russo, Patrícia Matos Biselli-Chicote, Érika Cristina Pavarino, Eny Maria Goloni-Bertollo, Genetics and Molecular Biology Research Unit - UPGEM, São José do Rio Preto Medical School, FAMERP, São José do Rio Preto, SP 15090-000, Brazil

João Gomes Netinho, Department of Surgery and Coloproctology, São José do Rio Preto Medical School, FAMERP, São José do Rio Preto, SP 15090-000, Brazil

ORCID number: Gabriela Helena Rodrigues-Fleming (0000-0002-6714-6931); Glaucia Maria de Mendonça Fernandes (0000-0002-8113-3598); Anelise Russo (0000-0003-1963-2043); Patrícia Matos Biselli-Chicote (0000-0001-6936-4716); João Gomes Netinho (0000-0003-0264-1883); Érika Cristina Pavarino (0000-0003-0959-0695); Eny Maria Goloni-Bertollo (0000-0002-2622-4673).

Author contributions: Rodrigues-Fleming GH planned and conducted the study, collected and interpreted data, and drafted and wrote the manuscript; Fernandes GMM participated in the collection of the genetic material, performed the analytical assessments, and revised the manuscript; Russo A participated in the collection of the genetic material; Biselli-Chicote PM critically revised the analytical tools and the manuscript; Netinho JG collected data on sporadic colorectal cancer patients; Pavarino EC served as scientific advisor; Goloni-Bertollo EM was the guarantor, planned the study, and critically revised the manuscript.

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Correspondence to: Eny Maria Goloni-Bertollo, PhD, Adjunct Professor, Postdoc, Genetics and Molecular Biology Research Unit - UPGEM, Department of Molecular Biology, São José do Rio Preto Medical School (FAMERP), Av. Brigadeiro Faria Lima, - 5416 - Vila São Pedro, São José do Rio Preto, SP 15090-000, Brazil. eny.goloni@famerp.br
Telephone: +55-17-32015720
Fax: +55-17-32015708

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Abstract

AIM

To evaluate the association between polymorphisms

in glutathione S transferases (GSTs) and the risk of sporadic colorectal cancer (SCRC), tumor progression and the survival of patients.

METHODS

A case-control study of 970 individuals from the Brazilian population was conducted (232 individuals from the case group with colorectal cancer and 738 individuals from the control group without a history of cancer). PCR multiplex and PCR-RFLP techniques were used to genotype the GST polymorphisms. The tumors were categorized according to the TNM classification: tumor extension (T), affected lymph nodes (N), and presence of metastasis (M). Logistic regression, multiple logistic regression and survival analysis were used to analyze the data. The results are presented in terms of odds ratio (OR) and 95% confidence interval (CI). The level of significance was set at 5% ($P \leq 0.05$).

RESULTS

Age equal to or over 62 years (OR = 8.79; 95%CI: 5.90-13.09, $P < 0.01$) and female gender (OR = 2.91; 95%CI: 1.74-4.37; $P < 0.01$) were associated with increased risk of SCRC. Analysis of the polymorphisms revealed an association between the *GSTM1* polymorphisms and a risk of SCRC (OR = 1.45; 95%CI: 1.06-2.00; $P = 0.02$), as well as between *GSTT1* and a reduced risk of the disease (OR = 0.65; 95%CI: 0.43-0.98; $P = 0.04$). An interaction between the presence of the wild-type allele of *GSTP1* Ile105Val polymorphism and tobacco consumption on risk of SCRC (OR = 2.33; 95%CI: 1.34-4.05; $P = 0.05$) was observed. There was an association between the *GSTM1* null genotype and the presence of advanced tumors (OR = 2.33; 95%CI: 1.23-4.41; $P = 0.009$), as well as increased risk of SCRC in the presence of a combination of *GSTT1* non-null/*GSTM1* null genotypes (OR = 1.50; 95%CI: 1.03-2.19; $P = 0.03$) and *GSTT1* non-null/*GSTM1* null/*GSTP1* Val* (OR = 1.85; 95%CI: 1.01-3.36, $P = 0.04$). Combined *GSTT1* non-null/*GSTM1* null genotypes (OR = 2.40; 95%CI: 1.19-4.85; $P = 0.01$) and *GSTT1* non-null/*GSTM1* null/*GSTP1* Val* (OR = 2.92; 95%CI: 1.05-8.12; $P = 0.04$) were associated with tumor progression. Polymorphisms were not associated with the survival of patients with SCRC.

CONCLUSION

Females aged 62 years or older are more susceptible to SCRC. Polymorphisms of *GSTT1* and *GSTM1* null genotypes modulated the susceptibility to SCRC in the population studied.

Key words: Colorectal neoplasms; Smoking; Alcohol; Glutathione S transferase; Genetic polymorphisms

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Core tip: Sporadic colorectal cancer (SCRC) is the third most common cancer worldwide and includes malignancies that occur in the colon and rectum. Age

greater than 60 years, smoking, and alcohol habits are some of the risk factors for SCRC. Detoxification and elimination of carcinogens contained in tobacco and alcohol require metabolic activation mediated by enzymes that metabolize the xenobiotics (XME). Polymorphisms in genes such as *GSTP1*, *GSTT1*, and *GSTM1* that encode enzymes involved in XMEs may be related to important processes in colorectal carcinogenesis.

Rodrigues-Fleming GH, Fernandes GMM, Russo A, Biselli-Chicote PM, Netinho JG, Pavarino EC, Goloni-Bertollo EM. Molecular evaluation of glutathione S transferase family genes in patients with sporadic colorectal cancer. *World J Gastroenterol* 2018; 24(39): 4462-4471 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v24/i39/4462.htm> DOI: <http://dx.doi.org/10.3748/wjg.v24.i39.4462>

INTRODUCTION

Colorectal cancer is the third most frequent cancer worldwide^[1] and the fifth most frequent type of cancer in Brazil^[2]. Estimates for the year 2018 in Brazil are 17380 new cases for men and 18980 new cases for women^[2].

Sporadic colorectal cancer (SCRC) develops from polyps (adenomas) in the colon and rectum walls, of varying sizes, and can change to dysplasia, triggering the development of cancer^[2-4].

SCRC is a multifactorial disease, influenced by genetic factors, such as mutations or polymorphisms in genes that participate in pathways responsible for regulating cell growth, including tumor suppressor genes and proto-oncogenes^[5,6]. Other related factors are age, gender, environmental factors, and lifestyle habits such as smoking and alcohol consumption^[7]. Genetic factors may influence the effect of the environment on predisposition to the disease. Therefore, the incidence of SCRC varies among populations^[1,8,9].

There are many genes encoding enzymes responsible for the metabolism of xenobiotics, in which detoxification occurs. Some of the major genes involved in phase II are the cytosolic glutathione S transferase (GST) superfamily, including GST mi (*GSTM1*), theta (*GSTT1*), and pi (*GSTP1*)^[10,11]. These catalyze the conjugation of structurally different by-products of oxidative stress and xenobiotics to glutathione (GSH), which leads to the elimination of toxic substances from the cells and the protection of important cellular components such as nucleic acids and proteins^[12]. GST gene expression varies between different tissues and cell types^[13].

In addition to being very common in the general population, the complete absence of *GSTT1* and/or *GSTM1* may alter their expression or the activity of the protein itself^[14]. In general, *GSTP1* appears to be highly expressed in proliferating cells compared with differentiated cells. In addition, many of the GSTs are overexpressed in various neoplastic cells and higher

levels are observed in aggressive cancer cells^[15]. The change in the GSTP1 gene also significantly alters the enzymatic activity^[16,17], influencing the detoxification of carcinogens, causing DNA damage, and exerting an indirect effect on the risk of cancer development^[18].

Therefore, the objectives of this study were to evaluate the association of epidemiological risk factors and these polymorphisms with the development of SCRC, the interaction between these polymorphisms and both smoking and alcohol habits, and the association between the polymorphisms and clinical-histopathological parameters and survival among patients with SCRC.

MATERIALS AND METHODS

Approval and consent

The study was approved by the Ethics Committee-Medical School of Sao Jose do Rio Preto - FAMERP (No. 012/2012). The 970 individuals who agreed to participate in the study signed a consent form. The variables analyzed included gender, age, ethnicity, profession, smoking, alcohol consumption, and personal and familial history of cancer.

Study populations

The case group consisted of 232 (112 men and 120 women) patients from the Department of Coloproctology of the Base Hospital of Sao Jose do Rio Preto who received the clinical and/or histopathological diagnosis of SCRC between 2010 and 2016. The exclusion criterion was previous treatment with chemotherapy and/or radiotherapy. The control group included 738 (370 men and 368 women) blood donors from the Blood Center of Sao Jose do Rio Preto. The exclusion criterion for controls was personal and family history of cancer in at least three previous generations. Individuals who had smoked at least 100 cigarettes throughout their lives were considered smokers, and those who drank more than four servings of alcohol per week (one serving corresponded to 30 mL of liquor, a 102-mL glass of wine containing 12% alcohol, or a 340-mL can of beer) were considered alcohol consumers^[19,20]. SCRC was categorized according to TNM classification: tumor extension (T), affected lymph nodes (N), and presence of metastasis (M)^[21].

Molecular analysis

Analysis of the *GSTT1* and *GSTM1* polymorphisms was performed using the polymerase chain reaction (PCR) multiplex technique, with the *CYP1A1* gene as the internal positive control of amplification^[22]. PCR products were analyzed on 1.5% agarose gel stained with red gel.

Analysis of the *GSTP1* A313G polymorphism was performed using the polymerase chain reaction-polymorphism restriction fragment chain reaction (PCR-RFLP) technique with primers described by Harries *et al.*^[23]. The 176 base pair (bp) PCR products were analyzed by electrophoresis in 1.5% agarose gel stained

with red gel. The restriction enzyme digestion was performed using *Bsm*AI. The results and genotyping were performed after 2.0% agarose gel electrophoresis stained with red gel. The presence of 91 and 85 bp bands corresponded to the GG polymorphic genotype; the 176, 91, and 85 bp bands corresponded to the heterozygous genotype AG; and the 176 bp band corresponded to the wild-type AA genotype.

Statistical analysis

Descriptive statistics included mean values, standard deviation for continuous data, and percentage for categorical data. The Hardy-Weinberg equilibrium (HWE) was evaluated using the chi-square test through the BioEstat Program version 5.0. The binary logistic regression model, using the Minitab/Windows-Version 12.22 program, was used to evaluate the association of age, gender, smoking, and drinking habits with SCRC, and to evaluate the association between SCRC and clinical-histopathological parameters. Binary multiple logistic regression, adjusted for age, gender, and smoking and drinking habits, was used to evaluate the association between the genetic models of the polymorphisms and the development of SCRC using the SNPStats program (available at: http://bioinfo.iconcologia.net/SNPstats_web). The effect of the polymorphisms was evaluated in the models as (1) codominant (heterozygous vs wild-type homozygous and polymorphic homozygous vs wild-type homozygous); (2) dominant (heterozygous + polymorphic homozygous vs wild-type homozygous); (3) recessive (homozygous polymorphic vs wild-type homozygous + heterozygous); (4) overdominant (heterozygous vs wild-type homozygous + polymorphic homozygous); or (5) additive (polymorphic homozygous with 2 + heterozygous vs wild-type homozygous). The SNPStats program was used to evaluate the interaction between the polymorphisms and smoking habit, adjusted for age, gender, and alcohol consumption, and to evaluate the interaction between polymorphisms and alcohol consumption, adjusted for age, gender, and smoking, in SCRC risk. The effect of the polymorphisms on the overall survival time of SCRC patients was analyzed by the Kaplan-Meier curve and log rank test using the StatsDirect version 2.7.2 program. The results are presented in terms of odds ratio (OR) and 95% confidence interval (CI). For all statistical analyses the level of significance was set at 5% ($P < 0.05$).

RESULTS

Sociodemographic data

Table 1 presents the demographic data of SCRC patients and controls. Age equal to or above 62 years (OR = 8.79; 95%CI: 5.90-13.09; $P < 0.01$) and female gender (OR = 2.91; 95%CI: 1.74-4.37; $P < 0.01$) were associated with a risk of SCRC. The genotypic frequencies of *GSTP1* Ile105Val polymorphism were observed in the HWE in both groups (Case: $P = 1$, Control: $P = 0.29$).

Table 1 Sociodemographic characteristics, risk factors, and polymorphisms *GSTT1*, *GSTM1*, *GSTP1* A313G in patients with colorectal cancer and controls *n* (%)

Variables		Case (<i>n</i> = 232)	Control (<i>n</i> = 738)	OR ¹ (95%CI)
Gender				
Male		112 (48)	370 (50)	1.00
Female		120 (52)	368 (50)	2.91 (1.94-4.37) ^a
Age [yr (mean) ± SD]		(62) ± 12	(48) ± 12	
< 62		112 (49)	621 (84)	1.00
≥ 62		120 (51)	117 (16)	8.79 (5.90-13.09) ^a
Smoking Habit				
Non-smoker		130 (56)	465 (63)	1.00
Smoker		102 (44)	273 (37)	1.45 (0.98-2.14)
Alcohol Consumption				
Non-drinker		132 (57)	395 (54)	1.00
Drinker		100 (43)	343 (46)	1.28 (0.85-1.91)
<i>GSTP1</i>				
Codominant	A/A	227 (43.7)	107 (46.1)	1.00
	A/G	224 (43.2)	102 (44)	1.06 (0.73-1.54)
	G/G	68 (13.1)	23 (9.9)	0.88 (0.48-1.59)
Dominant	A/A	227 (43.7)	107 (46.1)	1.00
	A/G-G/G	292 (56.3)	125 (53.9)	1.02 (0.71-1.45)
Recessive	A/A-A/G	451 (86.9)	209 (90.1)	1
	G/G	68 (13.1)	23 (9.9)	0.85 (0.48-1.50)
Overdominant	A/A-G/G	295 (56.8)	130 (56)	1.00
	A/G	224 (43.2)	102 (44)	1.09 (0.76-1.55)
Additive		-	-	0.97 (0.75-1.27)
<i>GSTT1</i>				
	+/+	573 (77.6)	192 (82.8)	1.00
	0/0	165 (22.4)	40 (17.2)	0.65 (0.43-0.98) ^a
<i>GSTM1</i>				
	+/+	385 (52.2)	100 (43.1)	1.00
	0/0	353 (47.8)	132 (56.9)	1.45 (1.06-2.00) ^a

¹OR adjusted for age, gender, and alcohol and smoking habits and polymorphisms; ^a*P* < 0.05 vs control. OR: Odds ratio.

Individual polymorphism analysis

GSTM1 null genotype carriers had a higher risk of developing the disease (OR = 1.45; 95%CI: 1.06-2.00; *P* = 0.022). On the other hand, the *GSTT1* polymorphism was associated with a reduced risk of SCRC (OR = 0.65; 95%CI: 0.43-0.98; *P* = 0.037; Table 1).

In the present study, there was a significant interaction between the presence of the wild-type allele of the *GSTP1* Ile105Val polymorphism and smoking habit on the risk of SCRC (OR = 2.33; 95%CI: 1.34-4.05; *P* = 0.049). However, there was no interaction between the other polymorphisms and smoking or drinking habits on the risk of the disease (Table 2).

With regard to the clinical-histopathological parameters of the SCRC samples, the rectum was the most frequent primary site (60%), in addition to aggressive tumors (69.65%; Table 3). There was an association between the *GSTM1* null genotype and the presence of aggressive tumors (OR = 2.33, 95%CI: 1.23-4.41; *P* = 0.0087).

Analysis of the combined polymorphisms

An increased risk of SCRC was observed in the presence of the combination of the *GSTT1* non-null/*GSTM1* null genotypes (OR = 1.50; 95%CI: 1.03-2.19; *P* = 0.033) and the *GSTT1* non-null/*GSTM1* null/*GSTP1* Val* (*with the presence of at least one polymorphic

allele) (OR = 1.85; 95%CI: 1.01-3.36; *P* = 0.045). The combined *GSTT1* non-null/*GSTM1* null genotypes (OR = 2.40; 95%CI: 1.19-4.85; *P* = 0.015) and *GSTT1* non-null/*GSTM1* null/*GSTP1* Val* (OR = 2.92; 95%CI: 1.05-8.12; *P* = 0.040) were associated with tumor progression (Table 4).

Survival analysis

Kaplan-Meier curve analysis showed that the survival time of carriers of the polymorphic allele *GSTP1* Ile105Val, and the *GSTM1* and *GSTT1* null genotypes, were not significantly different from the survival time of non- carriers of these polymorphisms (Table 5).

DISCUSSION

In the present study, it was observed that individuals with advanced age (≥ 62 years) were more susceptible to SCRC, which is consistent with previous reports where old age was considered to be an etiological factor for this tumor type^[2,24]. In terms of gender, women are more susceptible to SCRC. Other studies have observed a similar trend in gender among patients with SCRC and the control group^[25-27]. An increase in the number of cases among women due to an increase in cigarette smoking and alcohol consumption has been observed^[28,29]. It is important to note that the group

Table 2 Interaction between polymorphisms in the genes *GSTP1*, *GSTT1*, and *GSTM1* and smoking or alcohol habits on the risk of sporadic colorectal cancer

	Tobacco consumption						Alcohol consumption					
	Non-smoker			Smoker			Non-smoker			Smoker		
	Case	Control	OR ¹ (95%CI)	Case	Control	OR ¹ (95%CI)	Case	Control	OR ¹ (95%CI)	Case	Control	OR ¹ (95%CI)
<i>GSTP1</i>												
A/A	50	136	1.00	57	91	2.33 (1.34-4.05) ^a	59	116	1.00	48	111	1.31 (0.74-2.31)
A/G-G/G	80	177	1.40 (0.87-2.27)	45	115	1.59 (0.91-2.77)	73	147	1.12 (0.69-1.81)	52	145	1.19 (0.70-2.04)
<i>GSTT1</i>												
+/+	110	362	1.00	82	211	1.42 (0.98-2.08)	108	300	1.00	84	273	0.76 (0.52-1.12)
0/0	20	103	0.60 (0.34-1.04)	20	62	1.03 (0.57-1.88)	24	95	0.63 (0.37-1.07)	16	70	0.53 (0.28-1.01)
<i>GSTM1</i>												
+/+	52	231	1.00	48	154	1.40 (0.86-2.28)	56	206	1.00	44	179	0.76 (0.46-1.26)
0/0	78	234	1.38 (0.90-2.10)	54	119	2.19 (1.34-3.57)	76	189	1.42 (0.93-2.18)	56	164	1.14 (0.72-1.82)

¹OR adjusted for age, gender, alcohol and smoking habits and polymorphisms; ^aP < 0.05 vs control. OR: Odds ratio.

of women with SCRC evaluated in this study had a mean age of 62 ± 13 years, which may suggest that hormonal factors might contribute to SCRC. Some studies have associated postmenopausal state with the incidence of colorectal cancer in women^[30-32]. In addition, hormone replacement therapy has been proved to be a protective factor for SCRC^[33-36]. A meta-analysis demonstrated an association between the protective effect of soy estrogen in women with SCRC who were postmenopausal^[37].

Smoking and drinking habits were not associated with SCRC in the present study. On the other hand, Koh *et al.*^[38] observed a threefold increased risk of colorectal cancer among smokers compared to those who had never smoked. Some data on the risk of SCRC due to alcohol consumption are inconsistent, which can be explained by the variation in the amount of alcohol consumption analyzed in the different studies^[39,40]. Analysis of the HWE revealed that the *GSTP1* Ile105Val polymorphism was in equilibrium in both the case and control groups. This result was similar to that observed by other studies in SCRC^[26,41]. With regard to the *GSTT1* and *GSTM1* polymorphisms, the HWE test was not possible because the molecular analysis did not distinguish wild-type homozygous and heterozygous individuals^[25].

In the present study, the *GSTP1* gene polymorphism showed no association with SCRC, corroborating other investigations in Bulgarian and Chinese populations^[3,25,27,42]. However, one study in a Tunisian population observed a significant difference in the frequency of polymorphisms between the case and control groups and was associated with the risk of SCRC^[26]. A single study observed a reduced risk of SCRC in the presence of the *GSTP1* Ile105Val polymorphism; however, there are no consistent data to explain the biological relevance of this finding^[16].

The *GSTP1* gene polymorphism results in an alteration of the amino acid sequence of the protein and a consequent reduction in enzymatic activity and inefficient detoxification^[43]. However, although the *GSTP1* Ile105Val polymorphism was not associated with SCRC in this study, the level of expression of this gene may be an important factor, which is not dependent on this genetic change. A hepatocellular carcinoma (HCC) study found that increased *GSTP1* gene expression *in vivo* and *in vitro* resulted in reduced cell proliferation in tumor cells, inhibition of Akt phosphorylation, and cell cycle disruption in G1/S by increasing p21 and p27 cell cycle inhibitors^[44]. High *GSTP1* expression was also associated with better prognosis in patients with HCC^[44]. In addition, hypermethylation of *GSTP1* has been observed in several types of cancers, such as prostate, breast, lung, and liver cancers^[45].

In relation to the *GSTT1* and *GSTM1* gene polymorphisms, the *GSTT1* null genotype was associated with a reduced risk of the development of SCRC, whereas the presence of the *GSTM1* null genotype was associated with increased risk of SCRC. The absence of some of the GST isoenzymes in normal colorectal mucosa resulting from null genotypes such as the presence of the *GSTM1* polymorphism may alter the major detoxification function of GSTs in the metabolism of xenobiotics^[4]. In Chinese and Iranian populations, an increased risk of SCRC in the presence of *GSTT1* and *GSTM1* null genotypes was determined^[2,5,46]. On the other hand, other studies did not find an association between *GSTT1* and *GSTM1* null genotypes with SCRC^[3,16,26,46-49]. In a case-control study, Vlaykova *et al.*^[4] found no association between *GSTM1* null genotype and the risk of SCRC, but the *GSTT1* null genotype was associated with an increased risk of SCRC. These different results may be related to the time of

Table 3 Distribution of the clinical-histopathological parameters in relation to the polymorphisms in the genes *GSTP1*, *GSTT1*, and *GSTM1* in patients with colorectal cancer *n* (%)

Models	Genotypes	Tumor progression (TNM) (<i>n</i> = 201)				Primary site			
		Non-advanced 61 (31)	Advanced 140 (69)	OR ¹	95%CI	Colon	Rectum	OR ¹	95%CI
<i>GSTP1</i>									
Codominant	A/A	31 (51)	62 (44)	1.00		42 (46)	65 (46)	1.00	
	A/G	23 (38)	65 (47)	1.37	(0.70-2.66)	38 (41)	64 (45)	0.96	(0.54-1.72)
	G/G	6 (10)	11 (8)	1.14	(0.37-3.50)	11 (12)	12 (8)	0.73	(0.29-1.85)
Dominant	A/A	31 (51)	62 (44)	1.00		42 (46)	65 (46)	1.00	
	A/G-G/G	29 (48)	76 (55)	1.32	(0.71-2.48)	49 (53)	76 (53)	0.91	(0.53-1.57)
Recessive	A/A-A/G	54 (90)	127 (92)	1.00		80 (87)	129 (91)	1.00	
	G/G	6 (10)	11 (8)	1.00	(0.34-2.95)	11 (12)	12 (8)	0.74	(0.31-1.81)
Overdominant	A/A-G/G	37 (61)	73 (52)	1.00		53 (58)	77 (54)	1.00	
	A/G	23 (38)	65 (47)	1.34	(0.70-2.56)	38 (41)	64 (45)	1.02	(0.59-1.77)
Aditivo	-	-	-	1.18	(0.73-1.92)	-	-	0.89	(0.59-1.34)
<i>GSTT1</i>									
	+/+	47 (78)	47 (78)	1.00		78 (85)	114 (80)	1.00	
	0/0	13 (22)	20 (14)	0.57	(0.26-1.27)	13 (14)	27 (19)	1.47	(0.71-3.06)
<i>GSTM1</i>									
	+/+	34 (56)	53 (38)	1.00		45 (49.5)	55 (39)	1.00	
	0/0	26 (43)	85 (61)	2.33	(1.23-4.41) ^a	46 (50.5)	86 (61)	1.49	(0.87-2.57)

¹OR adjusted for age, gender, alcohol and smoking habits and polymorphisms; ^a*P* < 0.05 *vs* control. OR: Odds ratio.

exposure to environmental factors and the population heterogeneity.

It has been observed that the effect of GST polymorphisms, when combined, may increase the risk of SCRC two- or threefold^[41]. The present study demonstrated that combinations of *GSTT1* non-null/*GSTM1* null genotypes and *GSTT1* non-null/*GSTM1* null/*GSTP1* Val* (presence of at least one polymorphic allele) are associated with an increased risk of SCRC and tumor progression. These findings corroborate the results of individual analyses of polymorphisms, which indicate the influence of the *GSTT1* non-null genotype on SCRC because the null genotype was associated with a reduced risk of the disease.

In the Indian population, an association between the *GSTM1* null/*GSTT1* null genotypes and the combination of *GSTM1* null/*GSTT1* null/*GSTP1* Val* and the risk of SCRC was observed^[41]. This result was also observed in a study by Vlaykova *et al.*^[41] in the Bulgarian population. A study in the Turkish population found an association between the *GSTT1* null/*GSTM1* non-null genotypes and *GSTT1* null/*GSTM1* non-null/*GSTP1* Ile (wild-type homozygote) and SCRC^[3]. Cong *et al.*^[25] observed an increased risk in the presence of *GSTT1*/*GSTM1* genotypes, whereas the combination of *GSTT1* non-null/*GSTM1* null genotypes resulted in a significant reduced risk of SCRC, differing from the findings of this and other studies. On the other hand, other studies that analyzed the effect of the combined genotypes *GSTT1*/*GSTM1* did not find an association with the risk of SCRC^[26,47,48]. Several studies have evaluated the potential association between SCRC and the combined genotypes of these polymorphisms. The observed results vary, indicating the importance of studying the

effects of the genotypic combination in SCRC.

In the present study, a significant interaction between the presence of the wild-type allele of *GSTP1* Ile105Val polymorphism and smoking habit on the risk of SCRC was demonstrated. Differing from the results of the present study, a study in the Chinese population found no interaction between the *GSTP1* Ile105Val and smoking habit or drinking habit on the risk of SCRC^[38]. The literature is sparse in terms of studies evaluating the interaction between risk factors and the *GSTP1* Ile105Val polymorphism in the development of SCRC. The biological relevance of this finding is unclear as the presence of at least one polymorphic allele of the *GSTP1* gene combined with the nullity of *GSTM1* and the presence of the *GSTT1* allele were associated with increased risk of SCRC. In addition, smoking habit was not associated with this tumor type in the present study.

With regard to the *GSTT1* and *GSTM1* polymorphisms, this study did not find an association between smoking or drinking habits and the risk of SCRC. These results are in accordance with two other studies in a Korean and Japanese population^[46,48]. The study by Piao *et al.*^[49] did not show a relationship between drinking habit and the *GSTT1* and *GSTM1* null genotypes on the risk of SCRC. However, a study in Singapore found an increased risk for smokers carrying at least two null genotypes that caused low enzyme activity^[38].

The controversial results regarding these polymorphisms may suggest that other genes involved in the metabolism of xenobiotics may be more relevant in the development of SCRC, such as polymorphisms in genes acting on phase I xenobiotic metabolism^[27,50]. Although the polymorphisms studied change in order to reduce or eliminate the enzymatic activity, other genes can also act, compensating for the detoxification of the

Table 4 Association between the double combined *GSTT1/GSTM* genotypes and triple combined *GSTT1/GSTM/GSTP1*, colorectal cancer, tumor progression, and primary site, adjusted for gender, age, smoking, and alcohol consumption

		Colorectal cancer		Tumor progression (TNM) (n = 198)				Primary site						
		Case (n = 198)	Control	OR ¹	95%CI	Non-advanced (n = 60)	Advanced (n = 138)	OR ¹	95%CI	Colon (n = 81)	Rectum (n = 117)	OR ¹	95%CI	
Double combination of genotypes														
GSTT1														
		n = 738												
(+)	(+)	68	303	1.00		26	42	1.00		34	34	1.00		
(+)	(-)	97	270	1.50	(1.03-2.19) ^a	21	76	2.40	(1.19-4.85) ^a	36	61	1.67	(0.88-3.18)	
(-)	(+)	19	82	1.00	(0.55-1.84)	8	11	0.74	(0.26-2.15)	7	12	1.70	(0.59-4.94)	
(-)	(-)	14	83	0.61	(0.32-1.19)	5	9	1.20	(0.35-4.10)	4	10	2.60	(0.72-9.46)	
Triple combination of genotypes														
GSTM1														
		n = 519												
GSTT1														
		32	96	1.00		12	20	1.00		16	10	1.00		
(+)	(+)	36	126	1.13	(0.61-2.10)	14	22	0.93	(0.34-2.56)	12	17	1.81	(0.67-4.92)	
(-)	(+)	10	22	1.45	(0.56-3.77)	5	5	0.50	(0.12-2.18)	18	22	1.41	(0.33-5.98)	
(-)	(+)	9	34	0.90	(0.36-2.25)	3	6	1.07	(0.21-5.31)	2	7	4.57	(0.80-26.24)	
(+)	(-)	42	86	1.52	(0.81-2.83)	8	22	1.78	(0.65-4.86)	11	19	2.58	(0.99-6.75)	
(+)	(-)	55	98	1.85	(1.01-3.36) ^a	10	42	2.92	(1.05-8.12) ^a	19	33	2.06	(0.83-5.15)	
(-)	(-)	9	23	1.27	(0.48-3.40)	3	5	1.25	(0.25-6.19)	1	7	5.47	(0.92-32.60)	
(-)	(-)	5	34	1.27	(0.11-1.08)	1	3	1.01	(0.14-7.42)	2	2	1.88	(0.27-13.33)	

¹OR adjusted for age, gender, alcohol and smoking habits and polymorphisms; ^ap < 0.05 *vs* control; *Ile/Val ou Val / Ile. OR: Odds Ratio.

substances present in tobacco and alcohol.

With regard to the clinical-histopathological parameters of SCRC, some studies have shown that low activity GST genotypes can be associated with more aggressive tumors and survival in colorectal cancer patients^[51,52]. An association between the *GSTM1* null genotype and the presence of advanced tumors has been observed. One study demonstrated an association between aggressive tumors and the presence of the *GSTT1* null genotype^[47]. However, other studies that evaluated the same polymorphisms did not find an association between the polymorphic genotypes and the clinical- histopathological parameters of SCRC^[3,27,42,49].

This biological relationship between GST and progression is still not well described. However, a possible explanation for this is that GSTs play important roles in the regulation of genes related to activation of cellular maintenance, proliferation and apoptosis evasion. Thus, GSTs interact with tumor necrosis factor (TNF) receptor associated factor 2 (TRAF2) and decrease signal transduction from receptors in the TNF alpha-like (TNF-α) and c-Jun NH2-terminal kinase (JNK kinase) pathways^[12,53,54]. No association between polymorphisms and the primary sites of SCRC were identified in the present study. In accordance with these findings, the study by Vlaykova *et al*^[41] did not find an association between polymorphisms of *GSTT1* and *GSTM1* null genotypes and the primary site. However, Wang *et al*^[41], observed an increased risk of rectal cancer in the presence of the *GSTM1* null genotype, while the *GSTT1* null genotype was associated with a risk of colon cancer.

It is worth noting that predisposition to SCRC is multifactorial and results from the interaction between allelic variants of low-penetrance genes and environmental factors such as advanced age, eating habits, and smoking and drinking habits^[3,55,56]. Therefore, the findings regarding the modulation of susceptibility to SCRC in the presence of the polymorphisms analyzed, regardless of smoking or drinking habits, reinforce the influence of these polymorphisms on the etiology of SCRC, even though they do not influence patient survival. These results may contribute to the understanding of the mechanisms involved in colorectal carcinogenesis.

In conclusion, females with advanced age are more susceptible to SCRC. The presence of the *GSTM1* null genotype is associated with an increased risk of SCRC. The *GSTM1* null genotype is associated with tumor progression. The combination of *GSTT1/GSTM1* and *GSTT1/GSTM1/GSTP1* genotypes are associated with

Table 5 Polymorphisms *GSTT1*, *GSTM1*, and *GSTP1* in relation to overall survival of colorectal cancer patients

Polymorphisms	Survival (5 yr)
<i>GSTT1</i>	
Positive	64
Negative	68
<i>GSTM1</i>	
Positive	67
Negative	63
<i>GSTP1</i> A313G	
AA	61
AG	70
GG	63

^a*P* < 0.05 vs control.

an increased risk of SCRC and tumor progression. Polymorphisms are not associated with the overall survival rate of SCRC patients.

ARTICLE HIGHLIGHTS

Research background

Colorectal cancer is the third most common cancer worldwide and develops on the inner walls of the colon and rectum. Genetic and environmental factors may increase the risk of colorectal cancer via the metabolism of carcinogens. Therefore, the evaluation of polymorphisms in genes involved in this process may help to modulate the development of colorectal cancer. Polymorphisms in genes encoding *GSTP1*, *GSTT1*, and *GSTM1* may alter enzymatic activity. This change can lead to DNA damage and deregulation of the mechanisms involved in colorectal cancer.

Research motivation

Polymorphisms in the coding genes *GSTP1*, *GSTT1*, and *GSTM1* have been studied in terms of susceptibility to diseases such as cancer. However, the literature presents conflicting results. Therefore, several studies are needed to assess and confirm the role of factors that influence changes in metabolic processes related to colorectal cancer.

Research objectives

The main objective of this study was to evaluate the influence of polymorphisms in the *GSTP1*, *GSTT1* and *GSTM1* genes on the risk of colorectal cancer. The data showed that carriers of polymorphisms in the *GSTM1* genes and the combination of *GSTT1* non-null/*GSTM1* null genotypes and *GSTT1* non-null/*GSTM1* null/*GSTP1* Val* (*with the presence of at least one polymorphic allele) constitute a risk group for sporadic colorectal cancer (SCRC), and polymorphisms in the *GSTM1* gene and the *GSTT1* non-null/*GSTM1* null combinations, *GSTT1* non-null/*GSTM1* null/*GSTP1* Val* increase the aggressiveness of the tumor. Thus, considering the high incidence of this cancer, it is important to understand the factors that lead to carcinogenesis in order to develop preventive and therapeutic strategies for the management of cancer.

Research methods

This case-control study evaluated 970 individuals, 232 cases and 738 controls, using multiplex polymerase chain reaction (PCR) and polymerase chain reaction-restriction fragment chain reaction (PCR-RFLP) polymorphism. Demographics are tabulated by percentage. The binary logistic regression model was used to evaluate the association of age, gender, smoking and eating habits with SCRC, and to evaluate the association of the Hardy-Weinberg equilibrium (HWE) with the Chi-square test. Multiple binary logistic regression, adjusted for age, gender and smoking and alcohol habits, was also used to evaluate the association between the genetic models of the polymorphisms and the development of SCRC. The dominant genotypic model was used to assess the interaction between polymorphisms and smoking habits, adjusted

for age, gender, and ethnicity, and to evaluate the interaction of polymorphisms and alcohol consumption, adjusted for age, gender and smoking, on the risk of SCRC. In addition, the Kapla-Meier curve was used to assess the overall survival of patients with SCRC.

Research results

The data showed that carriers of polymorphisms in the *GSTM1* genes and the combination of *GSTT1* non-null/*GSTM1* null genotypes and *GSTT1* non-null/*GSTM1* null/*GSTP1* Val* (*with the presence of at least one polymorphic allele) constitute a risk group for SCRC, and polymorphisms in the *GSTM1* gene and the *GSTT1* non-null/*GSTM1* null combinations, *GSTT1* non-null/*GSTM1* null/*GSTP1* Val* increase the aggressiveness of the tumor. Thus, considering the high incidence of this cancer, it is important to understand the factors that lead to carcinogenesis in order to develop preventive and therapeutic strategies for the management of cancer.

Research conclusions

Similar studies have not previously been performed in the Brazilian population. Therefore, this work is unprecedented in this population. In addition, we emphasize the importance of the association between female gender and susceptibility to SCRC as well as the survival analysis associated with the polymorphisms studied, which have not been extensively studied in the literature. Polymorphisms in the *GSTP1*, *GSTT1* and *GSTM1* genes were involved in carcinogenesis and the poor prognosis of SCRC. In the Brazilian population it was observed that females with advanced age were more susceptible to SCRC. The presence of the *GSTM1* null genotype, and the combination of *GSTT1*/*GSTM1* and *GSTT1*/*GSTM1*/*GSTP1* genotypes are associated with an increased risk of SCRC and tumor progression.

This study provides a perspective on biomarkers of GSTs related to the prognosis of SCRC that has not been extensively studied in other populations, especially the Brazilian population. These data may contribute to clinical practice. Another interesting fact was the association between females, age over 60 years and the risk of SCRC. Menopausal women (estrogen reduction) were also shown to be more susceptible to SCRC. Polymorphisms in the genes involved in the xenobiotic metabolism pathway are associated with the development and poor prognosis of SCRC.

In this study, statistical analyses were widely used, and unlike other studies, multiple logistic regression was performed to evaluate the interactions between the polymorphisms studied and variables. In addition, survival was assessed by Kaplan Meier analysis. These analyses are extremely relevant in studies involving population genetic polymorphisms.

An association between some polymorphisms of xenobiotic metabolism and the development and progression of SCRC was observed. Advanced age and female gender were associated with the development of SCRC and polymorphisms in the genes involved in the xenobiotic metabolism pathway were associated with the development and poor prognosis of SCRC. This study may contribute to GSTs being used as diagnostic and prognostic biomarkers for SCRC. These data together with the findings of other studies may contribute to the development of treatment strategies for SCRC.

Research perspectives

This study demonstrated the importance of population studies with a large sample size in research on polymorphisms. Thus, we intend to expand the sample size to validate the results and include more polymorphisms related to the xenobiotic pathways in order to better understand the roles of these pathways in SCRC carcinogenesis. Research methods such as real-time PCR, are important in order to accurately quantify the presence of polymorphisms.

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Retrospective Cohort Study

Barrett's esophagus with high grade dysplasia is associated with non-esophageal cancer

Nir Bar, Naama Schwartz, Michal Nissim, Naomi Fliss-Isacov, Shira Zelber-Sagi, Revital Kariv

Nir Bar, Naama Schwartz, Michal Nissim, Naomi Fliss-Isacov, Shira Zelber-Sagi, Revital Kariv, Department of Gastroenterology, Tel Aviv Medical Center, Tel Aviv 6423906, Israel

Nir Bar, Naama Schwartz, Michal Nissim, Naomi Fliss-Isacov, Revital Kariv, Faculty of Medicine, Tel Aviv University, Tel Aviv 6423906, Israel

Shira Zelber-Sagi, School for Public Health, University of Haifa, Haifa 31905, Israel

ORCID number: Nir Bar (0000-0002-6148-5668); Naama Schwartz (0000-0002-5238-4080); Michal Nissim (0000-0001-9423-7583); Naomi Fliss-Isacov (0000-0003-4849-0291); Shira Zelber-Sagi (0000-0002-1324-7497); Revital Kariv (0000-0002-3831-9020).

Author contributions: Kariv R designed the study; Nissim M and Bar N collected the data; Schwartz N, Fliss-Isacov N and Bar N did the statistical analysis; Bar N, Kariv R, and Zelber-Sagi S prepared the manuscript; Bar N, Kariv R, Zelber-Sagi S, Fliss-Isacov N and Schwartz N critically reviewed the manuscript.

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Correspondence to: Revital Kariv, MD, Doctor, Department of Gastroenterology, Tel Aviv Medical Center, Weitzman 6 street, Tel Aviv 6423906, Israel. revitalk@tlvmc.gov.il
Telephone: +972-3-6974458
Fax: +972-3-6974868

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Abstract

AIM

To study factors associated with esophageal and non-esophageal cancer morbidity among Barrett's esophagus (BE) patients.

METHODS

A cohort study within a single tertiary center included 386 consecutive patients with biopsy proven BE, who were recruited between 2004-2014. Endoscopic and histologic data were prospectively recorded. Cancer morbidity was obtained from the national cancer registry. Main outcomes were BE related (defined as esophagus and cardia) and non-BE related cancers (all other cancers). Cancer incidence and all-cause

mortality were compared between patients with high-grade dysplasia (HGD) and with low-grade or no dysplasia (non-HGD) using Kaplan-Meier curves and cox regression models.

RESULTS

Of the 386 patients, 12 had HGD, 7 had a BE related cancer. There were 75 (19.4%) patients with 86 cases of lifetime cancers, 76 of these cases were non-BE cancers. Seven (1.8%) and 18 (4.7%) patients had BE and non-BE incident cancers, respectively. Twelve (3.1%) patients had HGD as worst histologic result. Two (16.7%) and 16 (4.4%) incident non-BE cancers occurred in the HGD and non-HGD group, respectively. Ten-year any cancer and non-BE cancer free survival was 63% and 82% in the HGD group compared to 93% and 95% at the non-HGD group, respectively. Log-rank test for patients with more than one endoscopy, assuring longer follow up, showed a significant difference ($P < 0.001$ and $P = 0.017$ respectively). All-cause mortality was not significantly associated with BE HGD.

CONCLUSION

Patients with BE and HGD, may have a higher risk for all-cause cancer morbidity. The implications on cancer prevention recommendations should be further studied.

Key words: Barrett's esophagus; High grade dysplasia; Esophageal cancer; Upper endoscopy; Cancer morbidity

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Core tip: Barrett's esophagus (BE) is known to be associated with esophageal carcinoma (EAC) and increased all cause and cancer specific mortality, but EAC is responsible only for a minority of BE mortality cases. We found patients with high-grade dysplasia to be more prone to non-BE related cancers, on top of BE related cancers. Such information can affect the recommended extraesophageal surveillance, and contribute the debate about the cost-effectiveness of endoscopic surveillance and to health systems decision making.

Bar N, Schwartz N, Nissim M, Fliss-Isacov N, Zelber-Sagi S, Kariv R. Barrett's esophagus with high grade dysplasia is associated with non-esophageal cancer. *World J Gastroenterol* 2018; 24(39): 4472-4481 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v24/i39/4472.htm> DOI: <http://dx.doi.org/10.3748/wjg.v24.i39.4472>

INTRODUCTION

Barrett's esophagus (BE) is a premalignant condition in which intestinal metaplasia replaces normal squamous epithelium at the distal esophagus^[1,2]. BE predisposes for esophageal adenocarcinoma (EAC), and current

guidelines recommend endoscopic surveillance for early detection^[1,2] and endoscopic treatment of early esophageal neoplasia^[3-7]. BE surveillance is associated with earlier stage EAC and increased survival^[8,9]. In addition, endoscopic treatment can result in complete eradication of both dysplasia and intestinal metaplasia and a reduced risk of disease progression^[7,10-12].

Mortality in the overwhelming majority of BE patients is not related to EAC but is rather due to non-esophageal malignancies and cardiovascular disorders^[11,13,14]. All-cause mortality is higher in patients with advanced grades of BE dysplasia compared to matched controls^[11,14]. Non-esophageal cancer mortality in Danish patients with high grade dysplasia (HGD) was higher than non-dysplastic BE and matched controls, though comparing HGD to non-dysplastic BE was not an endpoint, and was not analyzed for significance^[14]. Another population based cohort study conducted in Israel showed increased prevalence of colorectal, prostate, kidney, bladder and thyroid cancer in BE patients occurring at a younger age compared to matched controls^[15]. To the best of our knowledge, no other publications examined the potential association between histologic features of BE and non-EAC cancer morbidity. Better characterization of cancer morbidity among patients with BE may identify risk factors and enable better surveillance, cancer prevention and optimal resource use^[16]. Therefore, the primary aim of the current study was to study cancer morbidity and overall mortality within a prospectively followed cohort of BE patients according to grade of dysplasia.

MATERIALS AND METHODS

Patients and definitions

All consecutive BE patients undergoing upper endoscopy at the Tel-Aviv Sourasky medical center between 2009-2014 were included, thus determining sample size. Clinical, endoscopic, and histologic data were collected from patient files in a prospective manner between 2009 and 2014. Pre-study data was retrospectively collected, as far back as 2004.

BE was defined as having a characteristic endoscopic appearance of any length, and histologic diagnosis of intestinal metaplasia with goblet cells on biopsies taken from the columnar esophageal mucosa^[1].

Study design

This is a retrospective cohort study.

Study setting

Tel-Aviv Sourasky medical center - a tertiary referral center for BE.

Data retrieval and databases used

Data collection included the following parameters for each endoscopy: BE segment length-circumferential and maximal lengths were calculated and recorded according to the Prague classification^[17]. We categorized

the BE segment length as long (BE segment measuring 3 cm and above), short (1-2.9 cm), and ultra-short (< 1 cm). Presence of endoscopic abnormalities was also recorded. Histologic results for each endoscopy were classified as no dysplasia, low grade dysplasia (LGD), HGD, intramucosal adenocarcinoma (IMC), and EAC^[18]. All biopsies with suspected dysplasia were reviewed by 2 expert GI-pathologists. If a patient had more than one dysplasia result or endoscopic report, the most severe dysplasia as well as the longest BE segment during follow up were chosen for analysis, respectively. Individual follow up was censored either by a diagnosis of cancer, at the end of the follow up period (December 2014), or death. Patient information collected included age, gender, cancer history (including type of cancer), individual number of endoscopies during the study period, and date of death.

The primary outcome of this study was non-BE cancer incidence, and secondary aims included BE related cancer and overall mortality. In order to determine the difference in cancer morbidity in patients with higher degrees of dysplasia, we compared patients who had HGD and patients with LGD or non-dysplastic BE (non-HGD group).

Cancer morbidity data was retrieved from the Israeli national cancer registry (NCR). The NCR records all incident cases of malignant neoplasms other than basal or squamous cell skin cancers. Trained registrars review available documents from hospitals, pathology labs, and death certificates from local health authorities. Upon retrieval of data from the NCR, its records were updated until December 2014.

Cancers were categorized as BE related or non-BE related. As diagnostic inaccuracies between EAC and gastric cardia adenocarcinoma are known to occur^[19], we classified them both as BE cancers. All other malignancies were recorded as non-BE cancer. For all cancer free analysis, we used cancer cases which occurred within the follow up period. We also recorded cases occurring before the first available endoscopy as pre-study cancer, and reported the total life-time cancers retrieved from the NCR.

The date of death information was retrieved from the Central Bureau of Statistics.

This study was approved by our center's institutional review board - approval number 0022-09. As data were collected from medical records throughout the study, informed consent was waived by the institutional review board.

Statistical analysis

Categorical variables were described as frequency and percentage and continuous variables were presented with mean (standard deviation) median, (range) as needed. Comparisons between patients with HGD and patients with lower degrees of dysplasia (non-HGD) were performed using Chi-square test (or Fisher's exact test) for categorical variables. For continuous variables,

the independent-sample *t*-tests (or Mann-Whitney test) were used.

For each patient, the cancer free survival time was calculated based on the first endoscopy date (*i.e.*, start date) and the first cancer date or the end of the follow-up date (December 2014) for patients who were cancer free. For non-BE cancer, we used the first non-BE cancer date for the calculation. Kaplan-Meier curve was utilized to compare survival trends, using the Log-rank test. All Kaplan-Meier analysis was done for patients with multiple endoscopies to avoid confounding. In addition, the Cox regression was used to perform univariate and multivariable regression in all patients (adjusting for potential confounders, found to be associated with HGD in the univariate analysis), displaying the hazard ratios (HRs) and adjusted HR (Adj.HR) with 95% confidence intervals (95%CI).

The statistical analyses were performed using SAS 9.4 software (SAS Institute Inc., Cary, NC, United States). Significance was set at $P < 0.05$.

RESULTS

A total of 387 patients with biopsy proven BE were included with endoscopic data starting at May 2004 until December 2014. One foreign patient with missing NCR data was excluded, leaving 386 for analysis, see Figure 1. The analysis for non-BE cancer among BE patients with or without dysplasia, included 379 patients who did not develop IMC or BE-cancer during the study follow-up. The total cohort number of endoscopies was 963, with a mean of 2.5 ± 2.0 endoscopies per patient. Two hundred sixty-eight (69.4%) were males with an overall mean age of 60.0 ± 13.1 years (Table 1). Long segment BE was found in 225 (59.7%) of patients. The worst degrees of dysplasia/neoplasia were LGD, HGD, IMC, and EAC in 19 (4.9%), 12 (3.1%), 1 (0.3%), and 6 (1.6%), respectively. Study inclusion per year is shown in Supplementary Table 1 in the supplementary section.

Seventy-five patients (19.4%) had invasive lifetime cancers, reported by the NCR database, of whom 10 (2.6%) had lifetime BE cancers. There were pre-study cancers in 50 (13%) patients, of which 3 were BE cancers (0.8%) and 47 were non-BE cancers (12.2%). Incident cancers occurred in 7 BE cancers (1.8%) patients and non-BE cancers in 18 (4.7%) patients. The pre-study cancers and incident cancers are detailed in Table 2. Of note, one of the 2 esophageal pre-study cancers was a squamous cell carcinoma and not EAC. Subjects pre-study cancers were not included in cancer outcome analysis as they occurred outside of follow up period as explained in the materials and methods section.

The HGD group included 12 patients, and the non HGD group (LGD and non-dysplastic BE) 367 patients. Comparison of these 2 groups is presented in Table 2 for demographic and endoscopic characteristics. In the HGD group 2 patients (16.7%) had non-BE incident

Table 1 Demographic, endoscopic and histologic characteristics of the study population

Patient data	All sample (<i>n</i> = 386)	HGD (<i>n</i> = 12)	Non-HGD (<i>n</i> = 367)
Age at study inclusion	60.0 (13.1) [61.1, 18.4-89.6]	66.3 (12.8) [66.6, 42.4-85.8]	59.8 (13.2) [60.6, 18.4-89.6]
Gender - male	268 (69.43)	9 (75)	253 (68.94)
Number of endoscopies per patient	2.5 (2.0) [2, 1-17]	6.6 (2.6) [7, 3-11]	2.4 (1.7) [2, 1-10] ^b
Patients with multiple endoscopies	245 (63.47%)	12 (100)	227 (61.9) ^a
Circumferential extent of BE (cm)	3.3 (3.3) [2, 0-19]	4.5 (2.92) [4.5, 1-11]	3.2 (3.23) [2, 0-19]
Maximal extent of BE (cm)	4.4 (3.2) [3, 0.2-20]	6.0 (3.22) [6, 2-14]	4.25 (3.17) [3, 0.2-20] ^a
Presence of endoscopic abnormalities	56 (14.51)	8 (66.67)	44 (11.99) ^b
Ultra-short BE segment (BE < 1 cm)	28 (7.43)	0 (0)	28 (7.8)
Short BE segment (1 cm ≤ BE < 3 cm)	124 (32.89)	2 (18.18)	122 (33.98)
Long BE segment (BE ≥ 3 cm)	225 (59.68)	9 (81.82)	209 (58.22)
Worst degree of dysplasia			
Esophageal adenocarcinoma	6 (1.6)		
Intramucosal carcinoma	1 (0.3)		
High grade dysplasia	12 (3.1)		
Low grade dysplasia	19 (4.9)		

Continuous variables were presented as mean (SD) and [median, range]. Categorical variables are presented as *n* (%). Statistical difference refers to the HGD and non-HGD groups. ^a*P* < 0.01; ^b*P* < 0.001. BE: Barrett's esophagus; HGD: High grade dysplasia; Non-HGD: Low grade or without dysplasia.

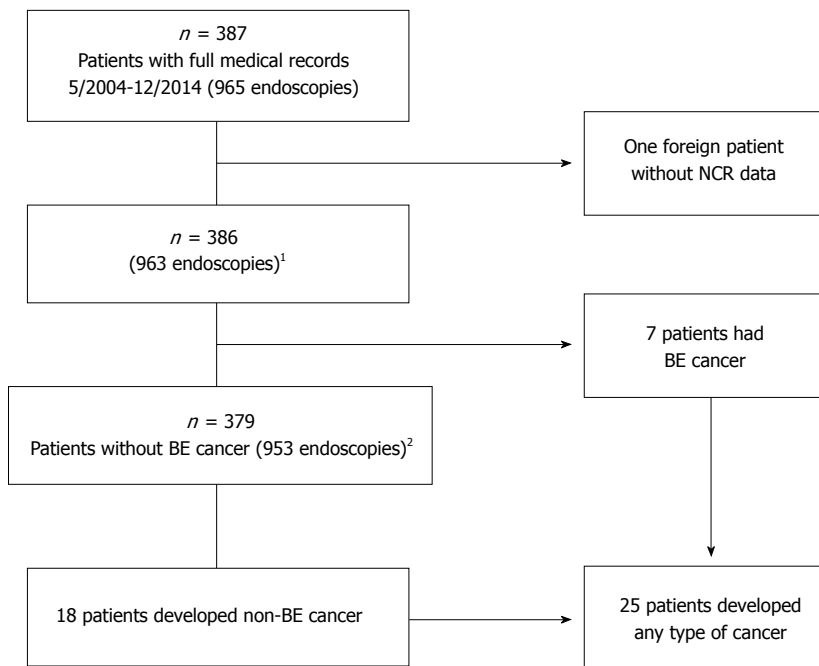


Figure 1 Study flow-chart. ¹Out of the 386 patients, 245 (63%) had more than one endoscopy; ²Out of the 379 patients, 239 (63%) had more than one endoscopy. BE: Barrett's esophagus; NCR: National Cancer Registry.

cancers (lung and pancreatic cancers), compared with 16 (4.4%) patients in the non HGD group who had 18 cancers (in patients with 2 incident cancers the earliest one was included for analysis). As expected, patients with HGD, had a higher frequency of endoscopies, higher rate of endoscopic abnormalities (*P* < 0.001), and a longer maximal extent of BE compared with non-HGD patients (*P* < 0.01).

Kaplan-Meier survival curves for cancer free and all-cause survival are presented in Figure 2. To avoid the confounding effect of the number of endoscopies and assuring longer follow-up for all patients, Kaplan Meier analysis was done on patients with at least 2 endoscopies.

For the primary endpoint, non-BE cancer free survival was worse in patients with HGD compared with non-HGD, as shown in the Kaplan-Meier curves, Figure 2A. Log-rank test *P*-value was 0.0166 (*n* = 239), The 2-year and 10-year non-BE cancer free survival rates were 91% and 82% and 98% and 95% for the in HGD group and non-HGD group, respectively, in patients with multiple endoscopies. Univariate Cox regression analysis, Table 3, showed that HGD was not a significant predictor non-BE cancer (HR = 3.40, 95%CI: 0.78-14.84, *P* = 0.104), but in multivariable Cox regression adjusting for age, cancer history and number of endoscopies, HGD was significantly associated with increased risk for non-BE cancer (Adj.HR = 8.32, 95%CI: 1.35-51.33, *P* = 0.022).

Table 2 Overall cancer cases, stratified to pre-study and incident cases *n* (%)

	Pre-study cancers	Incident cancers	Lifetime cancers
Esophagus	2 (0.5) ²	4 (1)	6 (1.6)
Cardia	2 (0.5)	3 (0.8)	5 (1.3)
Stomach	3 (0.8)	0 (0)	3 (0.8)
Colorectal cancer	9 (2.3)	1 (0.3)	10 (2.6)
Small intestine	1 (0.3)	0 (0)	1 (0.3)
Cholangiocarcinoma	1 (0.3)	0 (0)	1 (0.3)
Pancreas	0 (0)	2 (0.5)	2 (0.5)
Bladder	5 (1.3)	4 (1)	9 (2.3)
Prostate	11 (2.8)	0 (0)	11 (2.8)
Kidney	2 (0.5)	2 (0.5)	4 (1)
Hematologic cancer	8 (2.07)	4 (1)	12 (3.1)
Skin	4 (1)	2 (0.5)	6 (1.6)
Breast	4 (1)	2 (0.5)	6 (1.6)
Thyroid	3 (0.8)	0 (0)	3 (0.8)
Lung	0 (0)	2 (0.5)	2 (0.5)
Kaposi	0 (0)	1 (0.3)	1 (0.3)
Laryngeal	3 (0.8)	0 (0)	3 (0.8)
Cervical	1 (0.3)	0 (0)	1 (0.3)
Any type of cancer	59 (15.3)	27 (7)	86 in 75 (19.4) patients ¹
BE cancer	3 (0.8)	7 (1.8)	10 in 10 (2.6) patients
Non-BE cancers	56 (14.5)	20 (5.2)	76 in 65 (16.8) patients

¹There were 9 patients with 2 lifetime cancers, and one with 3 cancer cases; ²One patient had a pre-study diagnosis of esophageal squamous cell carcinoma (thus, not counted as BE related and not included in the outcome analysis for non-BE cancer free survival). BE: Barrett's esophagus.

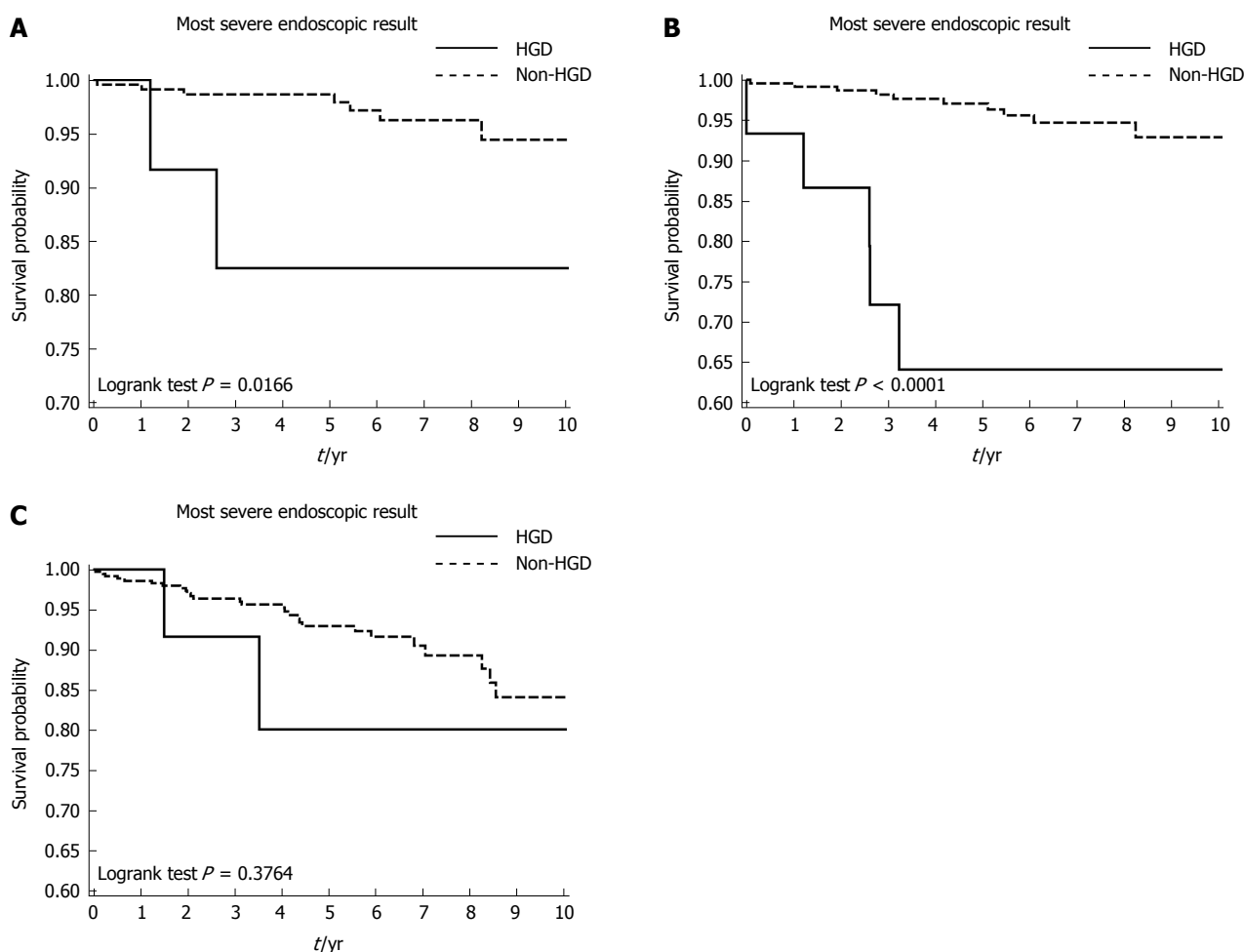


Figure 2 Kaplan Meier curves by high-grade dysplasia vs low grade or without dysplasia groups. A: Non-Barrett's esophagus (BE) cancer free survival, $n = 239$; B: Any cancer (BE and non-BE cancers) free survival, $n = 245$; C: All-cause mortality, $n = 245$. BE: Barrett's esophagus; HGD: High grade dysplasia; Non-HGD: Low grade or without dysplasia.

Table 3 Univariate and multivariable Cox regression for the prediction of non- Barrett's esophagus cancers

<i>n</i> = 379 (excluding EAC and IMC)	HR (95%CI)	Adjusted HR (95%CI)
Age at study inclusion ¹	1.11 (0.99-1.24)	1.07 (0.95-1.21)
Gender - male	2.19 (0.63-7.55)	
Number of endoscopies per patient	0.82 (0.59-1.12)	0.72 (0.50-1.03)
Presence of endoscopic abnormalities	1.19 (0.34-4.09)	
Circumferential extent of BE (cm)	0.99 (0.79-1.26)	
Maximal extent of BE (cm)	1.06 (0.92-1.23)	
Ultra-short segment (BE < 1 cm)	1	
Short segment (1 cm ≤ BE < 3 cm)	0.96 (0.11-8.24)	
Long segment (BE ≥ 3 cm)	0.99 (0.13-7.85)	
Pre-study cancer history	2.58 (0.92-7.25)	2.12 (0.73-6.17)
HGD <i>vs</i> non-HGD	3.40 (0.78-14.84)	8.32 (1.35-51.33) ^a

^a*P* < 0.05; ¹For a 3-year increase. BE: Barrett's esophagus; HGD: High grade dysplasia; Non-HGD: Low grade or without dysplasia; EAC: Esophageal adenocarcinoma; IMC: Intramucosal carcinoma.

Table 4 Univariate and multivariable Cox regression for the prediction of any cancer

<i>n</i> = 386 (including EAC and IMC)	HR (95%CI)	Adjusted HR (95%CI)
Age at study inclusion ¹	1.09 (0.99-1.20)	1.08 (0.97-1.21)
Gender - male	2.29 (0.79-6.67)	
Number of endoscopies per patient	1.11 (0.96-1.29)	0.99 (0.82-1.21)
Presence of endoscopic abnormalities	2.27 (0.95-5.44)	
Circumferential extent of BE (cm)	1.10 (0.96-1.26)	
Maximal extent of BE (cm)	1.12 (1.008-1.24) ^a	1.13 (1.000-1.27)
Ultra-short segment (BE < 1cm)	1	
Short segment (1 cm ≤ BE < 3 cm)	0.97 (0.11-8.30)	
Long segment (BE ≥ 3 cm)	1.77 (0.23-13.34)	
Pre-study cancer history	2.15 (0.86-5.39)	
HGD <i>vs</i> non-HGD	6.33 (2.37-16.91) ^b	4.28 (1.17-15.76) ^a

^a*P* < 0.05, ^b*P* < 0.01. ¹For a 3-year increase. BE: Barrett's esophagus; EAC: Esophageal adenocarcinoma; IMC: Intramucosal carcinoma; HGD: High grade dysplasia; Non-HGD: Low grade or without dysplasia.

Examining the secondary endpoints, we saw a significant difference in the any-cancer free survival time among patients with HGD and patients with non-HGD, Figure 2B. Again, the worse outcome was in the HGD group, log rank test *P* < 0.001 (*n* = 245). For all-cause cancer, the 2-year and 10-year cancer free survival were 86% and 64% in the HGD group compared to 98% and 93% at the non HGD group, respectively.

Occurrence of all-cause cancer was associated with the maximal extent of the BE segment and HGD at the univariate analysis, see Table 4. After adjusting for age, number of endoscopies and pre-study cancer history, HGD was independently associated with all-cause cancer occurrence (Adj.HR = 4.28, 95%CI: 1.17-15.76, *P* = 0.029) whereas the maximal BE segment length was borderline significant (Adj.HR = 1.13 95%CI: 1-1.27, *P* = 0.050).

As BE cancer outcome was uncommon, we compared it with the Fisher exact test and not by statistical modeling. Among the 7 incident BE-cancer cases, 3 (42%) had HGD previously documented, while among the other 379 BE patients, only 12 (3.2%) had HGD (RR = 18.6, 95%CI: 4.6-75.6, *P* = 0.002).

There were 31 (8%) patients who died out of the entire cohort during the study period. Two (22.2%) of patients with IMC/EAC, 2 (16.7%), and 27 (7.0%) in

the HGD and non-HGD groups respectively. Kaplan-Meier curve for mortality presented no association between HGD and all-cause mortality, Figure 2C. Log rank test *P* = 0.376 (*n* = 245). Cox regression analysis maintained this conclusion, after adjusting for age, cancer history and number of endoscopies for HGD (Adj. HR = 3.19, 95%CI: 0.66-15.46, *P* = 0.149).

The Kaplan-Meier curves for cancer free survival of the total cohort, including patients with a single endoscopy are shown in the online supplementary section, Supplementary Figure 1.

DISCUSSION

Our study reveals an association between BE with HGD and cancer outcome which, to the best of our knowledge, has not been reported before. Our main finding is that BE patients with HGD had a significantly higher risk of having non-BE cancer compared to patients with lower grades of dysplasia. This association was found in the group of patients who underwent more than a single endoscopy, which decreases the chance of dysplasia grade misclassification.

As expected, the known association between BE with HGD and adenocarcinoma of the esophagus or gastro-esophageal junction was also demonstrated

in this study. Since this was an uncommon event, we did not use survival analysis models to investigate the association. We did not find HGD to be associated with all-cause mortality compared to lower levels of dysplasia.

The association between BE and extra-esophageal cancers mortality has long been studied. Most studies established increased cancer mortality risk in BE compared to normal population, even after matching for other risk factors^[14,15,20-23]. In a large Danish registry study^[14], patients with BE had a 71% increased all-cause mortality compared to matched controls, while the non-esophageal cancer mortality incidence rate was increased by 77% (14.7 cases per 1000 patient years) and was the leading cause for mortality. Moreover, patients with HGD had higher non-esophageal cancer mortality rates than patients with LGD or non-dysplastic BE: HR (95%CI) were 2.47 (1.98-3.07), 1.62 (1.31-2.01), and 1.44 (1.34-1.56), respectively. However, statistical significance of the difference between groups was not reported.

Wolf *et al.*^[11] looked at patients following radiofrequency ablation, in a United States based registry. A dose response effect for all-cause mortality to baseline BE degree of dysplasia with HGD having an adjusted odds ratio (95%CI) of 2.7 (1.7-4.4) vs 1.3 (0.7-2.2) for LGD and 1.6 (0.8-3.3) for indefinite dysplasia compared with non-dysplastic BE.

Solaymani-Dodaran *et al.*^[13] showed increased cancer specific mortality rates in patients with BE, but did not stratify the population according to dysplasia grade.

The above data described mortality, and not morbidity. Due to the lack of data about non-BE cancer morbidity we aimed to correlate it with BE degree of dysplasia. Assuming patients with dysplastic BE have higher mortality rate, our findings imply that the above association may be related, at least in part, to increased cancer incidence.

The role of gastro-esophageal reflux disease in BE is clear, but what predisposes certain patients to develop BE and neoplasia is still under debate. Studies have linked various factors such as smoking^[24,25], abdominal obesity^[26,27], genetics^[28], and nutrition^[29,30] to BE and dysplasia/EAC. Most of these factors are also associated with other non-BE malignancies^[31].

The molecular basis of BE and EAC has been studied avidly, P53 and SMAD4 somatic mutations play a role in dysplastic BE and EAC development^[32-35]. P53 is also a key player in many non-esophageal neoplasia, such as colorectal cancer, prostate cancer, and melanoma^[36]. SMAD4 somatic mutations are prevalent in pancreatic cancer and colorectal cancer^[37]. This complex association of molecular and environmental factors with BE dysplasia/neoplasia and other cancers may indicate similar cancer pathways induced by similar exposures.

Our findings imply that HGD in BE may be a marker of increased risk for cancer morbidity and therefore may require extraesophageal surveillance and lifestyle modification to prevent and decrease cancer risk. As for now, it may be prudent to stringently perform

routine cancer screening tests among patients with BE and those with HGD in particular, according to age and gender and to recommend adherence to cancer protective lifestyle.

Given the low incidence of EAC mortality rates in BE patients, the risk-benefit and cost effectiveness of surveillance has been a matter of discussion, with conflicting evidence concerning EAC and mortality prevention and cost effectiveness^[9,38-40]. We show another potential motivation for BE surveillance to better define overall cancer risk.

Our study carries some limitations. Investigator initiated studies done in teaching hospitals are prone to referral bias and are also smaller in size than population-based studies, limiting generalizability, and perhaps overestimating associations. On the other hand, patients with dysplasia are usually managed in a tertiary center.

We could not ascertain how long patients had BE before study inclusion, which may have influenced the outcomes. However, these estimates are approximate at best, as BE itself may be asymptomatic and this limitation is shared by other studies.

In our study, we adjusted for age and a past history of cancer, but we were not able to adjust for other risk factors such as lifestyle parameters. Our study did not include a population with no BE as a control, but we assume based on previous studies^[14,15,20-23], that cancer rates are even lower in subjects with no BE. Death as an outcome was determined in this study according to ministry of health database, but cancer specific mortality was not available and cannot be associated with cancer morbidity. Our major limitation is the low statistical power due to the small number of patients with HGD and incident cancers, which reflects real life clinical data of an uncommon condition. In addition, the majority of patients were male, as prevalent in other studies^[41]. Gender may act as a confounder when addressing cancer morbidity (as with prostate and breast cancer). Again, this reflects reality in many centers treating patients with BE.

The advantages of our study include a prospective comprehensive 6 year follow up of a relatively large number of consecutive BE patients within a single referral center, enabling a uniform data collection and fully verified clinical, endoscopic and revised histologic data.

In conclusion, in our cohort we found an endoscopic and histologic profile comparable to other Western world data. Non-BE related malignancies were more prevalent, and significantly associated with HGD as well as BE related malignancies in comparison with non HGD BE. Our findings suggest BE patients with HGD may have a significantly higher overall risk for cancer morbidity. This may imply endoscopic surveillance for BE patients could aid in prediction of all-cause cancer risk and encourage current cancer prevention measures such as lifestyle modification and appropriate cancer screening among patients. Further characterization of cancer morbidity and mortality profile among patients with BE should follow

with large population-based studies.

ARTICLE HIGHLIGHTS

Research background

Patients with Barrett's esophagus (BE) are at risk for esophageal adenocarcinoma, and surveillance is recommended. However, non-esophageal cancer is the leading cause of death in this population. This raises questions about the focus we give to surveillance for esophageal cancer, and the need for broader cancer surveillance.

Research motivation

We wanted to better describe the non-esophageal cancer morbidity in patients with BE, and specifically in patients with high grade dysplasia (HGD). Finding that patients with HGD carry a higher non-esophageal cancer risk can direct efforts and resources for cancer prevention.

Research objectives

We aimed to describe the non-esophageal cancer morbidity in patients with BE, and to test whether patients with HGD have a higher risk as compared to low grade dysplasia. Indeed, in this study we have shown that compared to non-HGD, patients with HGD have a lower all cancer and non-BE cancer free survival. The significance of these findings is in the recognition of the importance of total cancer surveillance in these patients. In addition, by comparing non-esophageal cancer morbidity in HGD and less dysplastic BE, we show the added value of information received in surveillance endoscopies. These findings put the foundations for larger cohort studies, preferably multi-center for reaching a significant number of patients.

Research methods

Endoscopic and histologic data were collected, and cancer morbidity data were retrieved from the national cancer registry. We compared non-esophageal cancers, all cancers and mortality between patients with HGD and less dysplastic BE. Cancer free survival analysis was done.

Research results

We found patients with HGD had a worse non-BE cancer free survival and all cancer free survival. The higher frequency of non-esophageal cancer in patients with HGD raises the question as to the reason for this association. Further population based and mechanistic studies are required to further investigate these reasons.

Research conclusions

Our study shows that HGD may act as a marker for all cause cancer outcome, not just esophageal cancer. Perhaps it reflects a behavioral, environmental and genetic inclination towards malignancy. After endoscopic treatment for the dysplasia, we should focus our efforts to teach these patients about healthier lifestyle, and modifiable cancer risk factors such as smoking cessation and weight reduction. Perhaps in this population, screening for other malignancies may hold a different cost-effective profile.

Research perspectives

Patients with BE and HGD have a higher non-esophageal cancer risk, on top of esophageal cancer risk. This should be confirmed in more prospective studies and population-based studies. This may shift the focus of esophageal based surveillance to a more holistic cancer prevention program for certain patients. Future research should include larger cohorts of patients from multiple centers, with detailed endoscopic and histologic data as well as other cancer risk factors including obesity measures and lifestyle behaviors as smoking, physical activity and dietary intake to better encompass risk stratification and prevention potential.

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Retrospective Study

Agitation thrombolysis combined with catheter-directed thrombolysis for the treatment of non-cirrhotic acute portal vein thrombosis

Chao-Yang Wang, Le-Qun Wei, Huan-Zhang Niu, Wan-Qin Gao, Tong Wang, Shun-Jun Chen

Chao-Yang Wang, Le-Qun Wei, Huan-Zhang Niu, Wan-Qin Gao, Tong Wang, Shun-Jun Chen, Department of Interventional Radiology, The First Affiliated Hospital, and College of Clinical Medicine of Henan University of Science and Technology, Luoyang 471000, Henan Province, China

ORCID number: Chao-Yang Wang (0000-0002-9740-2158); Le-Qun Wei (0000-0002-0213-3216); Huan-Zhang Niu (0000-0001-9844-9778); Wan-Qin Gao (0000-0002-7450-2914); Tong Wang (0000-0003-3573-1148); Shun-Jun Chen (0000-0002-4597-9131).

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Correspondence to: Huan-Zhang Niu, MD, Chief Doctor, Department of Interventional Radiology, The First Affiliated Hospital, and College of Clinical Medicine of Henan University of Science and Technology, No. 24 Jing Hua Road, Luoyang 471000, Henan Province, China. niuhezhangsci@sina.com
Telephone: +86-379-64830771

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Abstract

AIM

To evaluate the safety and efficacy of agitation thrombolysis (AT) combined with catheter-directed thrombolysis (CDT) for the treatment of non-cirrhotic acute portal vein thrombosis (PVT).

METHODS

Nine patients with non-cirrhotic acute PVT who underwent AT combined with CDT were analyzed retrospectively. Portography was carried out *via* the transjugular intrahepatic portosystemic (commonly known as TIP) or percutaneous transhepatic (commonly known as PT) route, followed by AT combined with CDT. Complications of the procedure, and the changes in clinical symptoms, hemodynamics of the portal vein and liver function were recorded. Follow-up was scheduled at

1, 3 and 6 mo after treatment, and every 6 mo thereafter, or when the patients developed clinical symptoms related to PVT. Color Doppler ultrasound and contrast-enhanced computed tomography/magnetic resonance imaging were performed during the follow-up period to determine the condition of the portal vein.

RESULTS

AT combined with CDT was successfully performed. The portal vein was reached *via* the TIP route in 6 patients, and *via* the PT route in 3 patients. All clinical symptoms were relieved or disappeared, with the exception of 1 patient who died of intestinal necrosis 9 d after treatment. Significant differences in the changes in portal vein hemodynamics were observed, including the maximum lumen occupancy of PVT, portal vein pressure and flow velocity between pre- and post-treatment ($P < 0.05$). During the follow-up period, recurrence was observed in 1 patient at 19 mo after the procedure, and the portal vein was patent in the remaining patients.

CONCLUSION

AT combined with CDT is a safe and effective method for the treatment of non-cirrhotic acute PVT.

Key words: Agitation thrombolysis; Catheter-directed thrombolysis; Portal vein thrombosis

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Core tip: Agitation is a common phenomenon in daily life, and can accelerate the rate of solute dissolution in a solvent. As the thrombus is porous in the acute stage, it can easily be broken into smaller fragments by agitation, which increases the contact area between the thrombus and thrombolytics, and increases the speed of thrombus dissolution. According to our research, agitation thrombolysis combined with catheter-directed thrombolysis is a safe and effective method for the treatment of non-cirrhotic acute portal vein thrombosis, with a good short- to middle-term efficacy. However, the long-term efficacy of agitation thrombolysis combined with catheter-directed thrombolysis in a large population requires further investigation.

Wang CY, Wei LQ, Niu HZ, Gao WQ, Wang T, Chen SJ. Agitation thrombolysis combined with catheter-directed thrombolysis for the treatment of non-cirrhotic acute portal vein thrombosis. *World J Gastroenterol* 2018; 24(39): 4482-4488 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v24/i39/4482.htm> DOI: <http://dx.doi.org/10.3748/wjg.v24.i39.4482>

INTRODUCTION

Non-cirrhotic portal vein thrombosis (PVT) has a low

incidence with a prevalence of approximately 1%, and partial obstruction of the portal vein is often clinically imperceptible^[1]. However, complete obstruction due to acute thrombus can lead to a series of clinical symptoms, such as abdominal pain, ascites, and even intestinal necrosis. Common treatment options, including anticoagulation or indirect thrombolysis, are useful for relieving symptoms, but the outcome is unsatisfactory as most patients developed cavernous transformation of the portal vein due to incomplete recanalization^[2,3]. Catheter-directed thrombolysis (CDT) *via* the transjugular intrahepatic portosystemic (TIP) or percutaneous transhepatic (PT) route is a minimally invasive method, which can dissolve the thrombus and relieve symptoms rapidly with a lower dose of thrombolytics^[4]. In addition, agitation thrombolysis (AT) can break the thrombus into smaller fragments and accelerate the speed of thrombolysis^[5]. The objective of this study was to evaluate the safety and efficacy of AT combined with CDT for the treatment of non-cirrhotic acute PVT.

MATERIALS AND METHODS

Patients

The data from 9 patients with non-cirrhotic acute PVT who underwent AT combined with CDT in our hospital between September 2015 and December 2017 were analyzed retrospectively. The patients consisted of 3 men and 6 women, with a mean age of 47.9 ± 10.6 years. All patients met the following eligibility criteria: (1) the PVT was diagnosed definitively by color Doppler ultrasound (CDUS) and enhanced-contrast computed tomography (CT) or magnetic resonance imaging (MRI) (Figures 1A and 2A); (2) absence of massive periportal collaterals and features of liver cirrhosis in the imaging findings; and (3) excluded malignant tumor. In addition, the following information was collected for each patient before treatment: clinical symptoms, days from onset to operation, routine blood examination, liver function, etiology, the maximum lumen occupancy of PVT and the flow velocity of the portal vein measured by CDUS (Table 1).

Treatment

The procedures were performed under digital subtraction angiographic guidance (Artis zeego; Siemens, Munich, Germany). The choice of TIP or PT route depended on if the patients had ascites.

The portal vein was reached *via* the TIP route in 6 patients with ascites. A Rosch-Uchida set (Cook, Bloomington, IN, United States) was used to gain access to the portal vein branch from the hepatic vein under fluoroscopic guidance. When a 0.035-in hydrophilic guidewire (Terumo, Tokyo, Japan) reached the superior mesenteric vein or splenic vein in cooperation with a Cobra catheter (Cook), a pigtail catheter (Cook) was exchanged to perform portography (Figure 1B) and measure the portal pressure. The Rosch-Uchida sheath

Table 1 Characteristics of the included patients

Patient No.	Age (yr)	Sex	Etiology	Symptoms	Time from onset to treatment (d)
1	29	M	Myelodysplastic syndromes	Abdominal pain, abdominal distension	4
2	39	F	Protein S deficiency	Abdominal pain, abdominal distension, vomiting	11
3	43	M	Myelodysplastic syndromes	Abdominal pain, vomiting	14
4	48	F	Protein C deficiency	Abdominal pain, abdominal distension	5
5	40	F	Nephrotic syndrome	Abdominal pain, vomiting	8
6	53	M	Protein S deficiency	Abdominal pain, diarrhea	2
7	61	F	Splenectomy after trauma	Abdominal pain, abdominal distension, vomiting	10
8	36	F	Myelodysplastic syndromes	Abdominal pain, abdominal distension	5
9	57	F	Unknown	Abdominal pain	16

F: Female; M: Male.

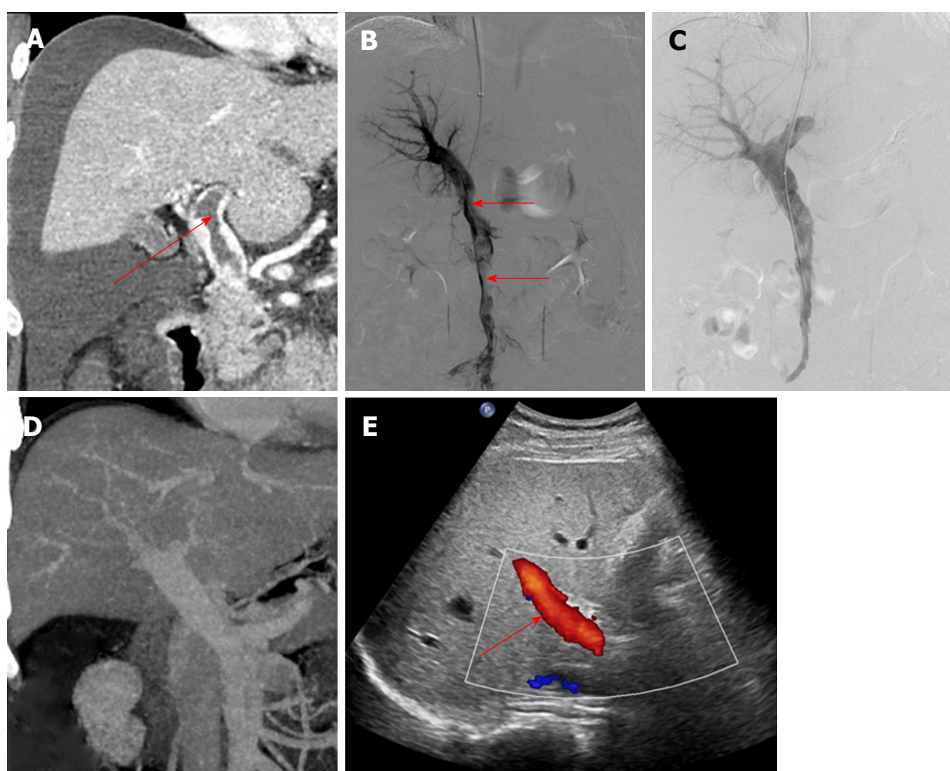


Figure 1 A 29-year-old man had acute abdominal pain and distension for 4 d. A and B: Contrast-enhanced computed tomography (CT) and portography via the transjugular intrahepatic portosystemic route showed thrombus formation in the portal vein and superior mesenteric vein (arrow) with massive ascites; C: Portography after agitation thrombolysis and catheter-directed thrombolysis showed that the filling defect in the portal vein had decreased; D: Contrast-enhanced CT after surgery showed that the thrombus had disappeared completely; E: At 6 mo after treatment, color Doppler ultrasound showed smooth blood flow in the portal vein (arrow).

was then introduced into the portal vein following intrahepatic dilatation by a 6-mm balloon catheter (Boston Scientific, Natick, MA, United States). A pigtail catheter was inserted into the thrombus through the sheath, and a guidewire with a helical tip molded *in vitro* was advanced through the catheter (Figure 3). The guidewire and pigtail catheter were pushed and drawn together, and then rotated clockwise and anticlockwise alternatively, to agitate the thrombus into smaller fragments. In addition, 2×10^5 IU urokinase (Tianjin Biochemical Pharmaceutical Co., Ltd., Tianjin,

China) was injected through the catheter by intermittent pulsatile delivery. The AT procedure was continued for approximately 10 min. The Rosch-Uchida sheath was then removed and the indwelling pigtail catheter was left in the PVT.

The portal vein was reached *via* the PT route in 3 patients without ascites. After successful puncture of a portal vein branch using a 22-gauge Chiba needle (Cook), a 6F coaxial dilator (Cook) was inserted into the portal vein. Then, a 5F sheath (Cook) and a pigtail catheter were exchanged. AT was performed after

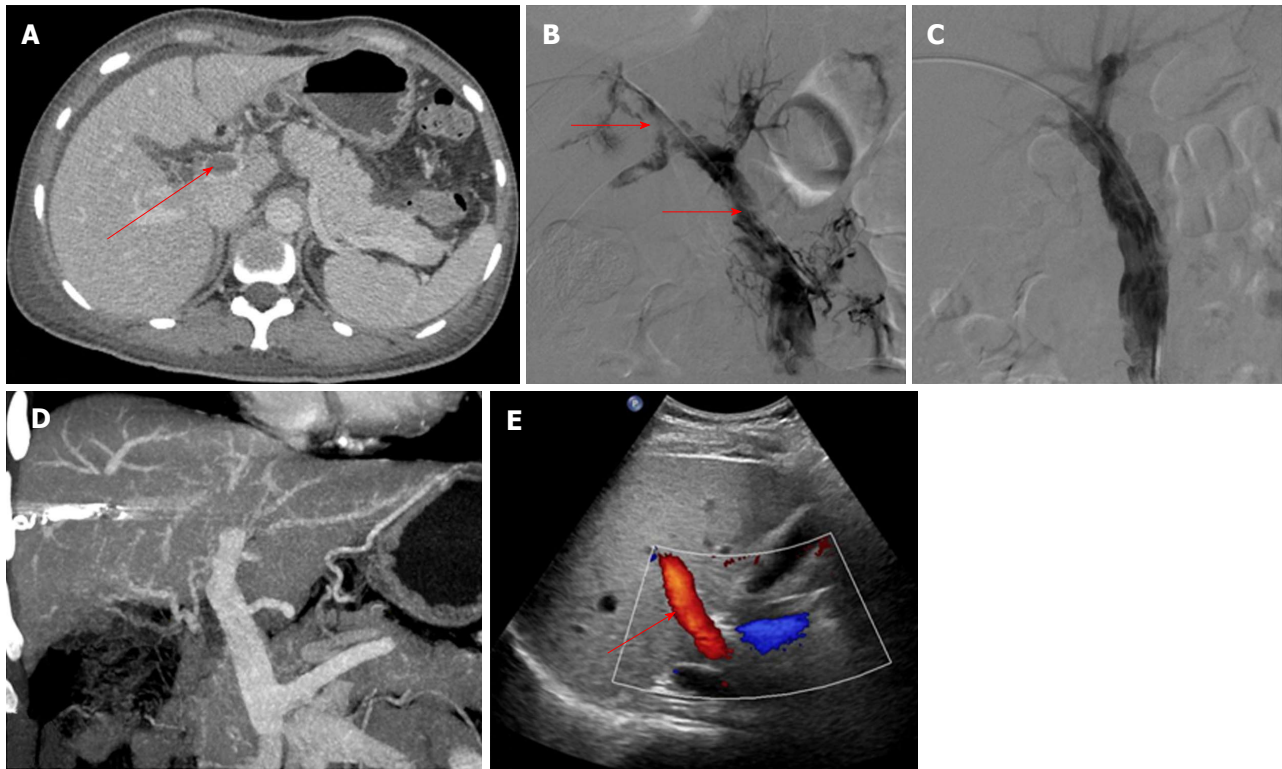


Figure 2 A 57-year-old woman had acute abdominal pain for 16 d. A and B: Contrast-enhanced computed tomography (CT) and portography via the percutaneous transhepatic route showed thrombus formation in the portal vein and superior mesenteric vein (arrow); C: Portography after agitation thrombolysis and catheter-directed thrombolysis showed that the filling defect in the portal vein had decreased; D: Contrast-enhanced CT after treatment showed that the thrombus had disappeared completely; E: At 12 mo after treatment, color Doppler ultrasound showed smooth blood flow in the portal vein (arrow).

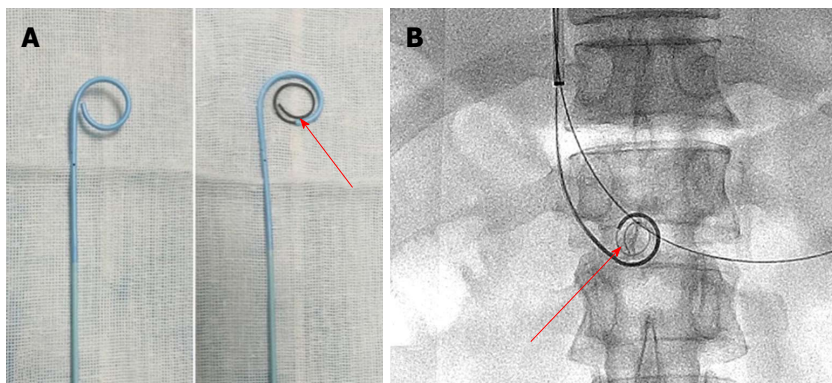


Figure 3 Pigtail catheter was inserted into the thrombus through the sheath, and a guidewire with a helical tip molded *in vitro* was advanced through the catheter. A: A pigtail catheter, and a guidewire with a helical tip (arrow) through the catheter *in vitro*; B: The catheter and guidewire in the portal vein (arrow) during agitation thrombolysis via the transjugular intrahepatic portosystemic route.

portography (Figure 2B), and the AT procedures were similar to those carried out *via* the TIP route. The indwelling pigtail catheter was left in the PVT.

Six hundred thousand IU of urokinase was infused continuously *via* the pigtail catheter each day, unless complications developed. In addition, 5000 IU of low molecular weight heparin was injected twice a day, and warfarin was administered after treatment at an initial dose of 5 mg. The low molecular weight heparin was discontinued when the international normalized ratio was maintained at 2.0-3.0, and warfarin was continued

for at least 12 mo. Portography was repeated every 24 h, and termination of CDT was based on the patency of the portal vein assessed by imaging and an obvious improvement in clinical symptoms. The portal vein pressure was measured again before the pigtail catheter was removed.

Follow-up study

Follow-up was scheduled at 1, 3 and 6 mo after treatment, and every 6 mo thereafter, or when the patients developed clinical symptoms related to PVT.

Table 2 Results of treatment and follow-up

Patient No.	Treatment route	Duration of CDT (d)	Dose of urokinase ($\times 10^6$ IU)	Complications	Follow-up time (mo) and results
1	TIP	2	1.0	Hematuria	27/patent
2	PT	2	1.4	None	23/patent
3	TIP	-	-	Death	Death
4	TIP	1	0.8	None	31/patent
5	PT	3	2.0	Hemorrhage from the puncture tract	5/patent
6	TIP	4	2.6	Subcapsular hematoma	22/reappearance
7	TIP	1	0.8	None	9/patent
8	TIP	1	0.8	None	12/patent
9	PT	3	2.0	Hemorrhage from the puncture tract	18/patent

PT: Percutaneous transhepatic; TIP: Transjugular intrahepatic portosystemic.

Table 3 Changes in liver function and portal vein hemodynamics

	Pre-treatment	Post-treatment	P value
Albumin, g/L	34.9 min	36.7 min	0.872
Alanine aminotransferase, U/L	38.2 inea	33.6 inea	0.934
Total bilirubin, sferase	17.9 lbi	17.4 lbi	0.991
Flow velocity of the portal vein, cm/s	4.2 tal	16.3 alv	< 0.05
Maximum lumen occupancy of PVT, %	78.2 occ	14.1 occ	< 0.01
Portal pressure, mmHg	31.0 alp	14.1 alp	< 0.05

PVT: Portal vein thrombosis.

Observations during follow-up mainly included clinical symptoms, routine blood examination, liver function and the condition of the portal vein evaluated by CDUS and contrast-enhanced CT/MRI. The end of follow-up was April 30, 2018.

Statistical analysis

Data are shown as mean \pm standard deviation (SD). Paired Student's *t*-test was used to determine statistically significant differences between pre- and post-treatment values. Significance was set at $P < 0.05$. Statistical analysis was performed using SPSS (version 19.0; IBM, Armonk, NY, United States) software.

RESULTS

Treatment outcome

AT combined with CDT was successful in all patients. Immediate portography after AT showed greater blood flow than pre-AT. One patient treated *via* the TIP route underwent intestinal resection as a result of congestive necrosis 5 d after the procedure; however, this patient died 9 d later. In the remaining patients, CDT was continued for 2.1 ± 1.1 d with a total dose of urokinase of $0.8\text{--}2.6 \times 10^6$ IU (Table 2). All clinical symptoms were relieved or disappeared, except in 1 patient who continued to experience abdominal distension after meals. There was a significant difference in the maximum lumen occupancy of PVT (Figures 1C and 2C), portal vein pressure and flow velocity between pre- and

post-treatment ($P < 0.05$). No significant differences in the changes in liver function, such as alanine aminotransferase, albumin and bilirubin were observed ($P > 0.05$) (Table 3).

Complications

One patient developed subcapsular hemorrhage during puncture of the portal vein *via* the TIP route and experienced a rapid heartbeat. The patient's vital signs were stable after erythrocyte transfusion, and CDT was started 3 d later. One patient *via* PT route had hematuria during CDT. These symptoms disappeared when urokinase was discontinued (Table 2).

Follow-up

The follow-up period ranged from 5 mo to 31 mo. During this period, 1 patient who had been taking warfarin for 12 mo had an irregular poor appetite at 19 months after treatment, and contrast-enhanced CT showed that cavernous transformation of the portal vein had developed in the right portal vein. In the remaining patients, the portal vein was patent and none of the clinical symptoms related to PVT reappeared (Table 2). Liver function and routine blood examination were normal in all patients during follow-up (Figures 1D and E, 2D and E).

DISCUSSION

PVT is mostly seen in liver cirrhosis, with a prevalence

ranging from 10% to 23%^[6]. Non-cirrhotic PVT rarely occurs, with a prevalence of approximately 1%, and has an intimate relationship with inherited or acquired coagulation diseases, such as primary myeloproliferative disorders, Protein C/S deficiency, and abdominal surgery^[7]. In addition to the etiology of PVT, it is particularly important to distinguish acute PVT from chronic PVT as the treatment is different. The American Association for the Study of Liver Diseases (commonly known as AASLD) describes acute PVT as the sudden formation of thrombus in the portal vein, and chronic PVT occurs when the obstructed portal vein is replaced by periportal collaterals bypassing the PVT^[8]. However, the time boundary is not mentioned. In our study, acute PVT was diagnosed by clinical symptoms and the absence of massive periportal collaterals on imaging.

For non-cirrhotic acute PVT with obvious symptoms, the aim of treatment is recanalization of the obstructed portal vein and the prevention of complications. Common treatment methods including anticoagulation or indirect thrombolysis can be useful for relieving symptoms and recanalizing the portal vein. A meta-analysis involving 353 patients showed a significantly higher rate of recanalization following anticoagulation compared with the control group, which did not receive anticoagulation (71% vs 42%; $P < 0.01$)^[2]. However, less than 20% of the patients achieved complete recanalization, and the others developed cavernous transformation of the portal vein due to incomplete recanalization, which can lead to chronic portal hypertension. Indirect thrombolysis *via* a peripheral vein or the superior mesenteric artery is less technically demanding, whereas the effect is limited as the thrombolytics are diverted from the patent branches or collaterals^[3]. In addition, a high dose of thrombolytics may increase the risk of gastrointestinal hemorrhage.

CDT is an effective treatment for acute thrombus, which can enhance the efficacy of thrombolytics at a lower dose compared to indirect thrombolysis^[4]. CDT *via* the PT route is relatively simple to perform, but it is not suitable for patients with massive ascites as it may cause hemorrhage through the PT tract during subsequent anticoagulation or thrombolysis. In addition, patients often felt pain when breathing as the indwelling catheter traversed the hepatic capsule. CDT *via* the TIP route can avoid these complications, but puncturing the portal vein from the hepatic vein is more difficult than using the PT route. Chen *et al.*^[9] reported that direct portography *via* the PT route, then a balloon catheter inflated with contrast medium to 80% of its volume positioned at the site of the bifurcation of the right and left portal veins can improve the success rate. Wang *et al.*^[10] reported the successful treatment of 12 patients with acute PVT treated with aspiration combined with CDT *via* the TIP route. The CDT time was 7.6 h. Eight patients achieved complete recanalization and four patients had partial recanalization, and AT combined with CDT was more effective than anticoagulation or indirect thrombolysis.

Agitation is a common phenomenon in daily life,

and can accelerate the rate of solute dissolution in a solvent. AT was first reported by Ding *et al.*^[5] following the successful treatment of acute inferior vein thrombus in Budd-Chiari syndrome, which showed a good long-term efficacy^[11], but has not yet been reported in the treatment of PVT. As the thrombus is porous in the acute stage, it can easily be broken into smaller fragments by agitation, which increases the contact area between the thrombus and thrombolytics, and increases the speed of thrombus dissolution. The shape of the catheter tip is important. In this study, the pigtail catheter was chosen as an agitator, as it did not injure the vessel wall and resulted in greater fragmentation of the thrombus during rotation. In addition, a guidewire with a helical tip can enhance the fracture resistance of the pigtail catheter, and increase the range of agitation. Compared with other methods, such as endovascular aspiration or mechanical thrombectomy^[12], AT is simpler and safer, with less blood loss. In our study, all the patients had a smoother blood flow after treatment than before treatment, and the duration of CDT was shorter and the rate of recanalization was higher than those reported by Wang *et al.*^[10].

There are some limitations in this study. Firstly, the study was retrospective, with a limited number of cases; therefore, the data may have been affected by various potential biases. Secondly, puncture of the portal vein is difficult to perform under fluoroscopic guidance, and required extensive experience and a good understanding of imaging data including contrast-enhanced CT/MRI; thus, the treatment is restricted to University Hospitals.

In conclusion, AT combined with CDT is a safe and effective method for the treatment of non-cirrhotic acute PVT, with a good short- to middle-term efficacy. However, the long-term efficacy of AT combined with CDT in a large population requires further investigation.

ARTICLE HIGHLIGHTS

Research background

Non-cirrhotic portal vein thrombosis (PVT) has a low incidence, with a prevalence of approximately 1%, and partial obstruction of the portal vein is often clinically imperceptible. However, complete obstruction due to acute thrombus can lead to a series of clinical symptoms, such as abdominal pain, ascites, and even intestinal necrosis. Common treatment opinions including anticoagulation or indirect thrombolysis are useful for relieving symptoms, but the outcome is unsatisfactory as most patients develop into cavernous transformation of the portal vein due to incomplete recanalization.

Research motivation

Catheter-directed thrombolysis (CDT) *via* the transjugular intrahepatic portosystemic (commonly known as TIP) or percutaneous transhepatic (PT) route is a minimally invasive method, which can dissolve the thrombus and relieve symptoms rapidly with a lower dose of thrombolytics. In addition, agitation thrombolysis (AT) can break the thrombus into smaller fragments and accelerate the speed of thrombolysis.

Research objectives

The objective of this study was to evaluate the safety and efficacy of AT combined with CDT for the treatment of non-cirrhotic acute PVT.

Research methods

Nine patients with non-cirrhotic acute PVT who underwent AT combined with CDT via TIP or percutaneous transhepatic route were analyzed retrospectively. AT had not been reported for the treatment of PVT so far. The changes in clinical symptoms, hemodynamics of the portal vein and liver function were recorded and followed up to evaluate the safety and efficacy.

Research results

According to our research, AT combined with CDT is a safe and effective method for the treatment of non-cirrhotic acute PVT, with a good short- to middle-term efficacy. However, the long-term efficacy of AT combined with CDT in a large population requires further investigation.

Research conclusions

Agitation is a common phenomenon in daily life, and can accelerate the rate of solute dissolution in a solvent. AT was first reported for the treatment of acute inferior vein thrombus in Budd-Chiari syndrome, which showed good long-term efficacy, but has not yet been reported in the treatment of PVT. As the thrombus is porous in the acute stage, it can easily be broken into smaller fragments by agitation, which increases the contact area between the thrombus and thrombolytics, and increases the speed of thrombus dissolution. Compared with other methods, such as endovascular aspiration or mechanical thrombectomy, AT is simpler and safer, with less blood loss.

Research perspectives

The shape of the catheter tip is important. In this study, the pigtail catheter was chosen as an agitator, as it did not injure the vessel wall and resulted in greater fragmentation of the thrombus during rotation. In addition, a guidewire with a helical tip can enhance the fracture resistance of the pigtail catheter, and increase the range of agitation. The future research we will focus on involves the long-term efficacy of AT combined with CDT in a large population.

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Retrospective Study

Ursodeoxycholic acid combined with percutaneous transhepatic balloon dilation for management of gallstones after elimination of common bile duct stones

Hai-Yang Chang, Chang-Jun Wang, Bin Liu, Yong-Zheng Wang, Wu-Jie Wang, Wei Wang, Dong Li, Yu-Liang Li

Hai-Yang Chang, Bin Liu, Yong-Zheng Wang, Wu-Jie Wang, Wei Wang, Dong Li, Yu-Liang Li, Department of Intervention Medicine, the Second Hospital of Shandong University, Jinan 250033, Shandong Province, China

Hai-Yang Chang, Bin Liu, Yong-Zheng Wang, Wu-Jie Wang, Wei Wang, Dong Li, Yu-Liang Li, Interventional Oncology Institute, Shandong University, Jinan 250033, Shandong Province, China

Chang-Jun Wang, Department of Radiology, Jiyang People's Hospital, Jinan 251400, Shandong Province, China

ORCID number: Hai-Yang Chang (0000-0001-8684-4877); Chang-Jun Wang (0000-0002-9390-832X); Bin Liu (0000-0003-1686-1553); Yong-Zheng Wang (0000-0001-5889-2118); Wu-Jie Wang (0000-0001-9431-7153); Wei Wang (0000-0002-2124-5328); Dong Li (0000-0002-3944-3680); Yu-Liang Li (0000-0001-8117-4317).

Author contributions: All authors helped to perform the research; Chang HY contributed to manuscript writing, statistical analysis, and manuscript critical revision; Li YL contributed to study conception and design, manuscript writing, statistical analysis, and manuscript critical revision; Wang CJ, Liu B, Wang YZ, Wang WJ, Wang W, and Li D contributed to manuscript writing.

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Correspondence to: Yu-Liang Li, MD, PhD, Chief Doctor, Doctor, Professor, Department of Intervention Medicine, The Second Hospital of Shandong University, 247 Beiyuan Road, Jinan 250033, Shandong Province, China. lyl.pro@sdu.edu.cn
Telephone: +86-531-85875927

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Abstract

AIM

To evaluate the effectiveness and safety of combined ursodeoxycholic acid and percutaneous transhepatic balloon dilation for management of gallstones after expulsion of common bile duct (CBD) stones.

METHODS

From April 2014 to May 2016, 15 consecutive patients (6 men and 9 women) aged 45-86 (mean, 69.07 ± 9.91) years suffering from CBD stones associated with gallstones were evaluated. Good gallbladder contraction function was confirmed by type B ultrasonography. Dilation of the CBD and cystic duct was detected. Percutaneous transhepatic balloon dilation of the papilla was performed, ursodeoxycholic acid was administered, and all patients had a high-fat diet. All subjects underwent repeated cholangiography, and percutaneous transhepatic removal was carried out in patients with secondary CBD stones originating from the gallbladder.

RESULTS

All patients underwent percutaneous transhepatic balloon dilation with a primary success rate of 100%. The combined therapy was successful in 86.7% of patients with concomitant CBD stones and gallstones. No remaining stones were detected in the gallbladder. Transient adverse events include abdominal pain ($n = 1$), abdominal distension ($n = 1$), and fever ($n = 1$). Complications were treated successfully *via* nonsurgical management without long-term complications. No procedure-related mortality occurred.

CONCLUSION

For patients with concomitant CBD stones and gallstones, after percutaneous transhepatic removal of primary CBD stones, oral ursodeoxycholic acid and a high-fat diet followed by percutaneous transhepatic removal of secondary CBD stones appear to be a feasible and effective option for management of gallstones.

Key words: Common bile duct stones; Gallstones; Percutaneous transhepatic removal; Ursodeoxycholic acid

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Core tip: Percutaneous transhepatic removal combined with oral ursodeoxycholic acid and a high-fat diet appears to be a feasible and safe alternative to surgery or endoscopic procedure for elimination of gallstones, especially for patients with good gallbladder contraction function, diameter of gallstones no greater than 12 mm, and dilation of the cystic duct. It also provides an alternative when operative management is not available for patients in poor condition.

Chang HY, Wang CJ, Liu B, Wang YZ, Wang WJ, Wang W, Li D, Li YL. Ursodeoxycholic acid combined with percutaneous transhepatic balloon dilation for management of gallstones after elimination of common bile duct stones. *World J Gastroenterol* 2018; 24(39): 4489-4498 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v24/i39/4489.htm> DOI: <http://dx.doi.org/10.3748/wjg.v24.i39.4489>

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INTRODUCTION

Bile duct stones are the major cause of benign biliary diseases^[1]. Surgical exploration or endoscopic intervention can be managed successfully in most common bile duct (CBD) stones^[2]. However, open surgery is contraindicated in cases with severe comorbidities, and endoscopic sphincterotomy (EST) for extraction of CBD stones in patients with prior surgically modified gastrointestinal tract may result in failure due to inviability of the papilla of Vater^[3]. Hence, percutaneous transhepatic intervention appears to be an alternative for these patients. To prevent the recurrence of CBD stones for patients with concomitant CBD stones and gallstones, subsequent cholecystectomy is the first choice after the elimination of CBD stones within 48 h^[4]. Herein, we present our experience in percutaneous transhepatic removal of stones for patients with CBD stones associated with gallstones *via* an innovative nonsurgical treatment including percutaneous transhepatic balloon dilation (PTBD) combined with oral ursodeoxycholic acid. Moreover, this study aimed to assess the efficacy and safety of this combined therapy.

MATERIALS AND METHODS

This was a retrospective study to assess the efficacy and safety of PTBD combined with ursodeoxycholic acid for removal of CBD stones associated with gallstones. The procedure was approved by the ethics committee of our institution. Written informed consent was obtained from all patients.

Patients

Fifteen consecutive patients (6 men and 9 women) aged 45-86 (mean, 69.07 ± 9.91) years, diagnosed with concomitant CBD stones and gallstones, admitted to our institution from April 2014 to May 2016 were evaluated.

Overall, 2-5 CBD stones and gallstones were detected in 15 patients, with diameters ranging from 2 to 25 mm. Eleven patients were confirmed to have concomitant CBD stones and gallstones before procedure using type B ultrasonography, enhanced computed tomography, or magnetic resonance cholangiopancreatography (MRCP), and the remaining 4 were detected by cholangiography during the removal of CBD stones. All patients suffered from fever, jaundice, abdominal discomfort, poor appetite, or vomiting.

Ultrasonography, enhanced CT, MRCP, or cholangiography were carried out to determine the diagnosis of stones (Figure 1A and B). Pancreatitis was not detected. For patients with poor condition, multiple

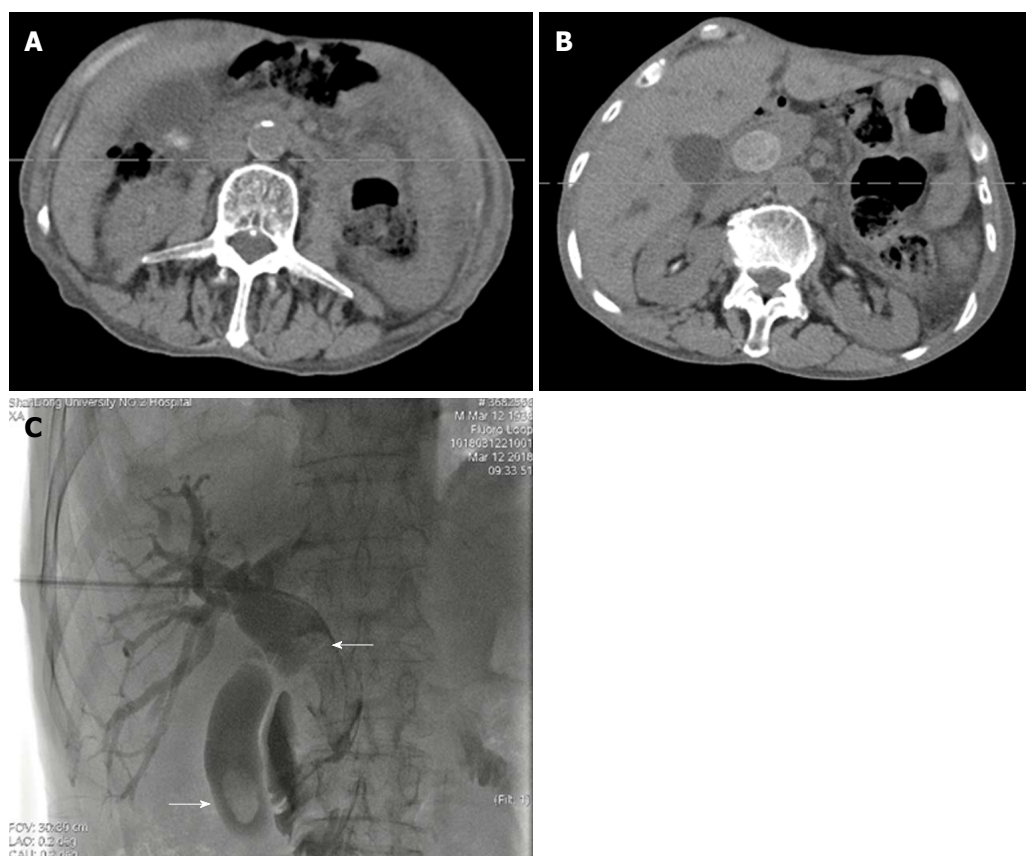


Figure 1 Computed tomography scan and cholangiography showing filling defect in the common bile duct and gallbladder (white arrow). Dilation of the common bile duct and cystic duct was detected. A and B: Ultrasonography, enhanced computed tomography, magnetic resonance cholangiopancreatography, or cholangiography was carried out to determine the diagnosis of stones; C: Advancing cholangiography was performed to detect the number, size, and location of stones.

disciplinary consultations were carried out as pre-procedure assessment.

Follow-up of patients included clinical assessment, physical examination, laboratory test and imaging evaluation for 1 year at a 3-mo interval. Technical success was defined as complete absence of CBD stones. The absence of symptoms was regarded as medical success regardless of the presence or absence of residual stones.

Procedure

After pretreatment with antibiotics (levofloxacin or cephalosporin), all patients were positioned under intravenous sedation and fluoroscopic monitoring, and a 21G Chiba needle (Neff Percutaneous Access Set, Cook Medical LLC, Bloomington, IN, United States) was used to puncture the right hepatic duct. The biliary tree was shown by injecting a contrast agent *via* the needle. A tiny guidewire (Wire Guide Diameter inch. 018, Cook Medical LLC, Bloomington, IN, United States) was introduced into the biliary system, and a sheath was inserted into the bile duct over the tiny guidewire. Advancing cholangiography was performed to detect the number, size, and location of stones (Figure 1C). A hydrophilic guidewire [150 cm in length, Terumo (China)

Holding Co., Ltd. China] was deployed in the CBD *via* the transhepatic route. A 6F to 10F sheath [Terumo (China) Holding Co., Ltd. China] was introduced into the right hepatic duct according to the balloon size to dilate the papilla of Vater. A Vert catheter (Cook Medical LLC, Bloomington, IN, United States) was introduced into the duodenum or jejunum. A stiff guidewire [260 cm in length, Terumo (China) Holding Co., Ltd. China] was passed through the catheter and papilla of Vater. An angiographic catheter balloon was inserted through the stiff guidewire and was placed across the papilla. The diameter of the balloon varied from 12 mm to 24 mm and its length was 40 mm or 60 mm depending on the size of the stones (Figure 2). The papilla was inflated gradually until the maximal pressure reached 6-8 atm. Stone-crushing device such as a basket was used in some cases with large stones. Larger balloon was inserted to dilate the papilla in patients with primary failure, and stone expulsion was performed repeatedly. Intraoperative cholangiography was performed to confirm residual stones in CBD. An 8.5F external drainage tube (Biliary Drainage Catheter, Cook Medical LLC, Bloomington, IN, United States) was deployed in the CBD for postoperative drainage and assessment of efficacy of the procedure (Figure 3).

Oral ursodeoxycholic acid (250 mg, Losan Pharma

Table 1 Baseline characteristics of patients

No.	Gender /age	CBD/gallbladder		
		Number of stones	Diameter of the largest stone (mm)	Diameter of the largest balloon (mm)
1	F/58	2/2	10/6	12/8
2	M/45	3/1	15/7	16/8
3	M/75	1/2	25/10	24/10
4	F/67	1/1	20/8	20/8
5	F/64	3/2	20/9	20/10
6	M/68	2/2	21/10	20/10
7	F/73	3/1	22/14	20/-
8	F/76	3/2	20/11	20/12
9	M/86	2/1	21/10	20/10
10	F/81	3/1	19/8	18/8
11	F/67	1/2	20/10	20/10
12	F/72	2/1	21/12	20/12
13	M/76	2/3	18/15	18/-
14	F/65	3/1	18/12	18/12
15	M/63	1/1	20/12	20/12

CBD: Common bile duct.

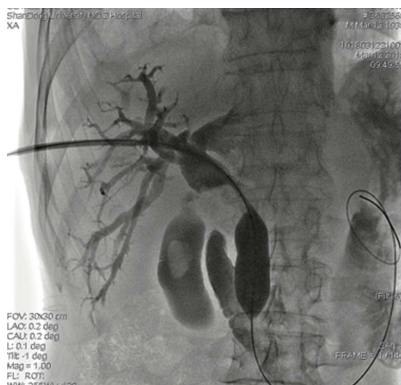


Figure 2 Dilatation of the sphincter of Oddi with a balloon catheter was performed.

GmbH) was initiated in all patients after procedure. The prescribed dose was 250 mg three times a day. After 7-10 d, repeated cholangiography *via* external drainage catheter was performed, and balloon dilation of the sphincter of Oddi and elimination of stones were carried out in patients with secondary CBD stones (Figures 4 and 5). Intraoperative cholangiography confirmed the absence of all stones and the external drainage tube was left (Figure 6A). Furthermore, 3-5 d after the procedure, cholangiography was performed again to confirm no residual of stones, and the catheter was retrieved (Figure 6B).

Statistics analysis

Data are reported as mean \pm standard deviation. Comparison of means was analyzed by the paired *t*-test. All statistical analyses were performed using IBM SPSS Statistics 24.0. *P*-values < 0.05 were defined as statistical difference for all data.

RESULTS

Table 1 shows baseline characteristics of the patients.

Table 2 shows that complete clearance of CBD stones was obtained in one session for all patients. No plastic or bare metal stent was inserted in any patients. All patients were administered subsequently with oral ursodeoxycholic acid after undergoing PTBD. Secondary CBD stones originating in the gallbladder were detected in 13 of these patients with concomitant CBD stones and gallstones. The stones were eliminated in one session in all these patients. Gallstones with reduced size still existed *in situ* in the remaining two patients. One patient with residual gallstones with symptoms was transferred to department of general surgery for laparoscopic cholecystectomy. One asymptomatic patient was discharged. Intensive long-term follow-up was essential for them. No further treatment except for observation was carried out for this patient. No evidence of retained CBD stones was detected in any patients. The technical success rate was 86.7%, and the overall medical successful rate was 93.3%.

Table 3 demonstrates the result of laboratory tests pre and postintervention. Serum alanine transaminase and total bilirubin (TBIL) levels became normal in patients with jaundice after the procedure. White blood cell (WBC) levels decreased significantly on day 14 postoperatively. However, there was no statistical difference between preoperative and postoperative values for hemoglobin and amylase.

Transient adverse effects including vomiting, chills, fever, and abdominal distension were found in a few patients after the procedure. They were cured with analgesic and antiemetic agents. No severe complications such as bile peritonitis, hemobilia, and cholangitis occurred. TBIL and WBC values of one patient complicated with fever were 63 μ mol/L and 12.21×10^9 /L, respectively, after the procedure. One patient suffered from abdominal distention and decreased hemoglobin levels from 122 g/L to 82 g/L. The WBC count increased slightly for the patient complicated with abdominal

Table 2 Operative parameters

No.	Primary technical success	Secondary technical success	Adverse events	Treatment
1	Yes	Yes	No	Medication
2	Yes	Yes	Fever	
3	Yes	Yes	No	
4	Yes	Yes	No	
5	Yes	Yes	Abdominal distension	Medication
6	Yes	Yes	No	
7	Yes	No	No	
8	Yes	Yes	No	
9	Yes	Yes	No	Medication
10	Yes	Yes	No	
11	Yes	Yes	Abdominal pain	
12	Yes	Yes	No	
13	Yes	No	No	
14	Yes	Yes	No	
15	Yes	Yes	No	

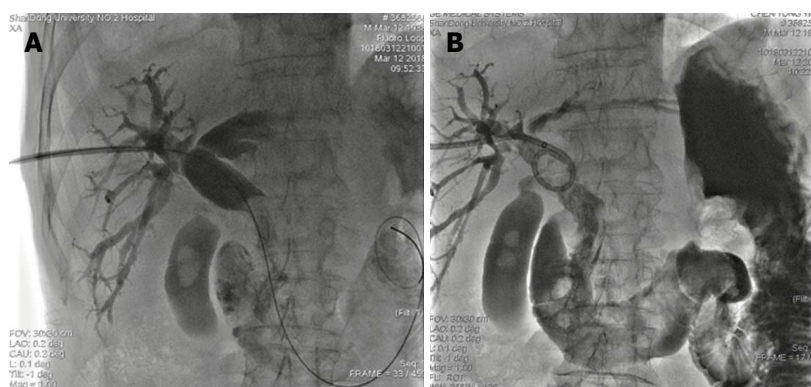


Figure 3 Common bile duct stones were expelled into the duodenum through the dilated sphincter. A and B: An 8.5F external drainage tube was deployed in the common bile duct for postoperative drainage and assessment of efficacy of the procedure.



Figure 4 Ursodeoxycholic acid was given and repeated cholangiography was performed. The secondary common bile duct stones originating from the gallbladder (white arrow) and shrunken gallbladder were detected by cholangiography.

pain. All complications were treated successfully *via* nonsurgical management without remote complications. Antibiotics (Ceftriaxone) and somatostatin were injected until the symptoms vanished. No procedure-related mortality occurred. During 1-year follow-up, no obstruction of bile ducts or recurrence of symptoms was

detected.

DISCUSSION

Bile duct stones, one of the most common digestive problems needing admission to hospital, are the major cause of benign diseases of the biliary tract, such as obstructive jaundice and cholangitis^[1,5]. It includes intrahepatic and extrahepatic bile duct stones, CBD stones and gallstones. CBD stones comprise primary and secondary stones. Secondary stones from the gallbladder and migrating into the ductal system are different from primary stones that form in the biliary tract. Primary stones may be the consequence of bacterial infection and biliary stasis. The majority of the secondary stones are cholesterol gallstones, while primary stones are mainly pigment stones^[6]. Compared to the Western population, primary stones are more prevalent in Asia^[7]. The prevalence of CBD stones in patients with symptomatic gallstones varies from 10% to 20%^[8]. In this study, 15 patients with CBD stones suffered from gallstones, of which 11 were confirmed before the procedure, while 4 patients who underwent PTBD had gallstones detected by cholangiography.

Table 3 Laboratory tests pre and post-intervention

	Pre-intervention	2 wk after intervention	<i>t</i>	<i>P</i> value
ALT (U/L)	98.93 ± 24.47	36.13 ± 8.99	10.41	< 0.001
TBIL (μmol/L)	39.40 ± 7.76	21.47 ± 12.09	6.52	< 0.001
Amylase (U/L)	80.73 ± 14.94	82.07 ± 17.77	0.34	0.741
WBC (× 10 ⁹ /L)	11.58 ± 1.45	7.65 ± 2.11	5.90	< 0.001
HGB (g/L)	122.93 ± 8.66	118.80 ± 13.39	1.52	0.150

ALT: Alanine transaminase; HGB: Hemoglobin; TBIL: Total bilirubin; WBC: White blood cell.

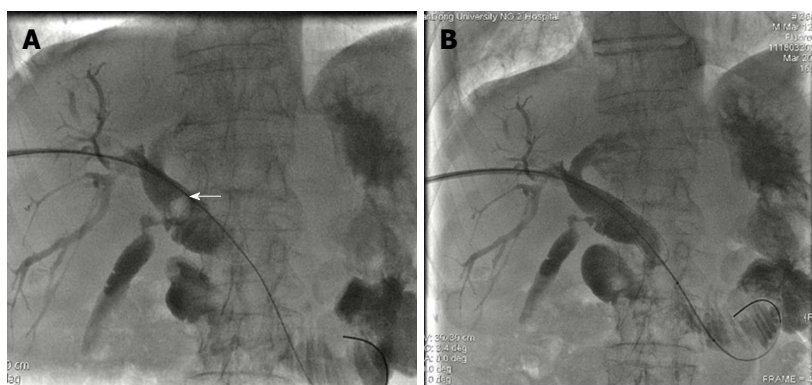


Figure 5 Secondary common bile duct stones (white arrow) were expelled into the duodenum without gallstone residual. A and B: After 7-10 d, repeated cholangiography via external drainage catheter was performed, and balloon dilation of the sphincter of Oddi and elimination of stones were carried out in patients with secondary common bile duct stones.

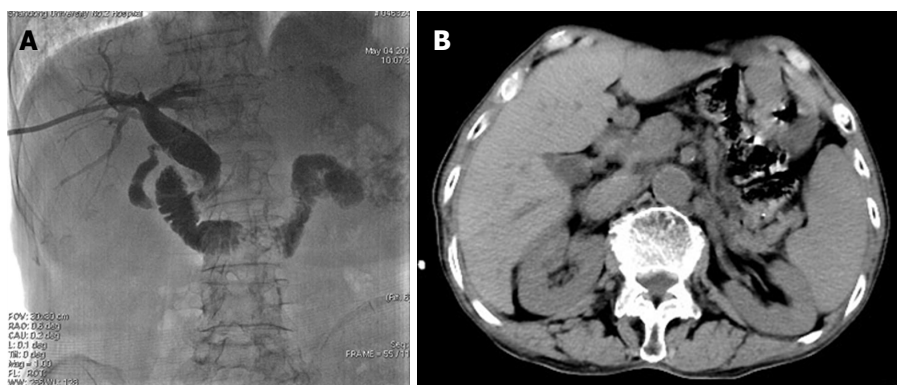


Figure 6 Computed tomography and postoperative cholangiography demonstrating that there was no residual of common bile duct stones or gallstones. A: Intraoperative cholangiography confirmed the absence of all stones and external drainage tube was left; B: Furthermore, 3-5 d after the procedure, cholangiography was performed again with no residual of stones, and the catheter was retrieved.

Many people are hospitalized for acute pancreatitis due to CBD stones that occlude the ampulla. In addition, bile duct obstruction caused by stones result in septic cholangitis. Chronic occlusion could induce secondary biliary cirrhosis. All types of CBD stones should be cured aggressively. Many management options, including open surgery, laparoscopic surgery, endoscopic and percutaneous procedure, are available for removal of CBD stones^[1,2,9-11]. Abdominal exploration with incision of the CBD and stone removal was the predominant choice a few decades ago. With technological advances and improvement of skills, various alternatives could be employed in the extraction of bile duct stones.

However, open surgery still retains its important role in the management of complicated stone disease. Laparoscopic procedure has comparable morbidity and mortality rates to open surgery. Hence, both open and laparoscopic surgery should be considered in cases unsuitable to be treated by nonsurgical options.

Endoscopic retrograde cholangiopancreatography (ERCP) was first introduced in 1968^[12]. It was accepted quickly as a feasible diagnostic and therapeutic technique for CBD stones^[13,14]. In the 1990s, EST was considered a feasible alternative for patients with serious comorbidity contraindicated to open surgery^[15,16]. It appears to be a better choice for elder patients with benign biliary

tract diseases. CBD stones could be eliminated by ERCP *via* sphincterotomy or balloon dilation^[17]. For patients requiring maintenance of papillary function, balloon dilation may be an effective and safe alternative to EST in the management of bile duct stones^[18-20]. However, open surgery is superior to ERCP for clearance of CBD stones. Compared to open surgery, ERCP necessitates increased number of procedures for each patient^[2]. Complications of ERCP with sphincterotomy include hemorrhage, papillary stenosis, pancreatitis, duodenal perforation, and recurrent stones^[21], and the complication rate ranges from 0.5%-5.4%^[22].

In the past decades, percutaneous intervention has been reported as an effective alternative to open or laparoscopic surgery and endoscopic intervention for elimination of CBD stones^[9,23,24]. Several reports indicated that transhepatic balloon dilation of papilla could be an alternative to extraction of biliary stones^[23-25]. Numerous devices, such as Dormia basket, occlusive, or cutting balloon, were introduced to improve the success rate of the technique^[25-27]. The technique success rate varies from 94.7% to 100%^[28,29]. Papillary dilation was performed using balloons with a diameter ranging from 8 mm to 20 mm^[9,28,30]. Transient adverse events, including nausea, vomiting, and abdominal pain, were observed in some cases which resolved with medication composed with analgesic and antiemetic drugs. A study by Nevzat Ozcan revealed 18 complications, including cholangitis (2.7%), subcapsular biloma (1.5%), subcapsular hemotoma (0.38%), subcapsular abscess (0.38%), bile peritonitis (0.38%), duodenal perforation (0.38%), and CBD perforation (0.38%)^[9]. Only 2 of 38 main complications were observed by Santiago Gil with complete expulsion of stones in 36 of the 38 patients. No procedure-related deaths occurred^[29]. Although a few cases were reported, ERCP for patients with prior Billroth II gastrectomy may be challenging^[26,31,32]. EST for extraction of CBD stones may lead to failure, even in experienced surgeons^[33]. For these cases, percutaneous transhepatic intervention appears to be an available and safe management for expulsion of stones^[34].

Several other methods for percutaneous expulsion of stones to the duodenum were reported. Extraction from the T-tube or existing gallbladder drain for access has been published as an effective percutaneous technique for stone expulsion^[30,35]. A novel technique of combined percutaneous transhepatic and endoscopic or laparoscopic approach also acts an important role in patients unsuitable to be treated with routine ERCP^[36-38].

Gallstones with a higher prevalence in adults may occur in all societies and races. Its increasing prevalence associates with age in both sexes, and women are involved more commonly than men^[6]. Gallstones are composed of cholesterol, calcium bilirubinate, protein, lipid, and less water. Occlusion of the gallbladder duct can cause abdominal pain, chills, fever, and jaundice. Treatment is indicated in patients with symptomatic

gallstones. Cholecystectomy is the most effective procedure for symptomatic patients^[39]. Laparoscopic, small-incision, or open cholecystectomy could be a feasible treatment in the management of gallstones. These three techniques can resolve symptoms caused by gallstones. No statistically significant differences in the outcome have been found. Although laparoscopic cholecystectomy is the most popular method, small-incision cholecystectomy has shorter operative time and appears to be less costly^[40]. However, the increased incidence of colon cancer is associated with cholecystectomy^[41]. Several nonsurgical treatments have been developed for treatment of gallstones with recurrence. Percutaneous cholecystostomy serves a role with few complications in management acute calculous cholecystitis^[42,43]. Medical treatment also plays an important role in management of gallstones. Gallstone dissolution may be achieved by oral administration of ursodeoxycholic acid which decreases biliary cholesterol secretion, increases solubility of cholesterol by formation of liquid crystals, and reduces intestinal cholesterol absorption^[39].

To prevent the recurrence of stones, for CBD stones associated with gallstones, subsequent cholecystectomy is the first choice after the elimination of the CBD stones within 48 h^[4]. Patients with suspected or proven CBD stones undergoing cholecystectomy can anticipate benefit from the perioperative management of CBD stones^[11]. Nowadays, several procedures depending on the experience of surgeons are available for treatment of combined cholecystocholedocholithiasis, such as laparoscopic treatment, simultaneous laparo-endoscopic treatment, and combined ERCP and EST with cholecystectomy^[44]. Concurrent transhepatic percutaneous balloon dilation combined with laparoscopic cholecystectomy is introduced for treatment of gallstones associated with CBD stones^[38]. Fifteen patients with concomitant CBD stones and gallstones were enrolled in our study, and the primary technical success rate was 100%. Subsequently, PTBD was performed repeatedly to expel secondary CBD stones originating in the gallbladder. Immediate complications including bile peritonitis, bile pleura effusion, hemobilia, acute pancreatitis, and duodenum perforation, were not observed in our study. All slight complications were treated successfully *via* nonsurgical management.

In our series, 15 patients with CBD stones and gallstones were enrolled and 13 of them were treated successfully *via* an innovative technique. For these patients, the strategy of treatment was as follows: First, routine PTBD was performed to eliminate the CBD stones without any difficulties. Then, all patients with good gallbladder contraction function were confirmed. Second, ursodeoxycholic acid, a kind of oral dissolution agent, was administered to patients with 250 mg for three times per day. A high-fat diet was initiated similar to that in gallbladder contraction test. Third, repeated

cholangiography was performed 7-10 d later, and 13 cases showed secondary CBD stones originating in the gallbladder retaining in the CBD. Gallstones with reduced size still existed *in situ* in the remaining two patients. For patients with secondary CBD stones, subsequently PTBD was carried out repeatedly with great care, and the stones were expelled into the duodenum. One asymptomatic patient with reduced gallstones was discharged directly with intending long-term follow-up. The remaining patient underwent cholecystectomy. Three to five days later, cholangiography demonstrated no residual stones in all patients with secondary CBD stones, and the drainage tubes were removed.

In conclusion, PTBD is an option for patients with CBD stones. Percutaneous transhepatic removal combined with oral ursodeoxycholic acid and a high-fat diet appears to be a feasible and safe alternative to surgery or endoscopic procedure for elimination of gallstones, especially for patients with good gallbladder contraction function, diameter of gallstones no greater than 12 mm, and dilation of the cystic duct. It also provides an alternative when operative management is not available for patients in poor condition.

ARTICLE HIGHLIGHTS

Research background

Bile duct stones are the most frequent cause of benign bile duct disease. The choice of management of common bile duct (CBD) stones includes surgical exploration, endoscopic intervention and percutaneous transhepatic intervention. Subsequent cholecystectomy is the first choice to prevent the recurrence of stones for patients with concomitant CBD stones and gallstones. This retrospective study aimed to evaluate the clinical efficacy and safety of ursodeoxycholic acid combined with percutaneous transhepatic balloon dilation for management of gallstones after elimination of CBD Stones.

Research motivation

Percutaneous transhepatic intervention served as an effective option for management of CBD stones in the past decades. The preferable choice of management for patients with concomitant CBD stones and gallstones is controversial.

Research objectives

The retrospective study evaluated the effectiveness and safety of a novel technique for management of gallstones after expulsion of CBD stones in terms of technical success and postoperative complications.

Research methods

Fifteen consecutive patients diagnosed with concomitant CBD stones and gallstones were evaluated. All patients underwent application of ursodeoxycholic acid combined with percutaneous transhepatic balloon dilation for management of gallstones after elimination of CBD stones. Clinical assessment, physical examination, laboratory tests and imaging were assessed in all patients. All statistics analyses were performed using SPSS 24.0. *P*-values < 0.05 were defined as statistically difference for all data.

Research results

The novel technique was successful in 86.7% of patients with concomitant CBD stones and gallstones with few postoperative complications treated successfully via nonsurgical management. It seems to be an alternative to open or laparoscopic surgery and endoscopic intervention.

Research conclusions

The present study showed that ursodeoxycholic acid combined with percutaneous transhepatic balloon dilation was secure and feasible for management of gallstones after elimination of CBD stones, especially for patients with good gallbladder contraction function, diameter of gallstone no greater than 12 mm, and dilation of the cystic duct. It also provides an alternative when operative management is not available for patients in poor condition.

Research perspectives

In case of therapeutic failure, good gallbladder contraction function or dilation of the cystic duct was not observed. However, the diameters of stones in failed cases were much greater than those of successful cases. This novel technique provides a feasible option for patients with concomitant gallstones and CBD stones. Prospective studies are needed for further confirmation.

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Retrospective Study

Postoperative survival analysis and prognostic nomogram model for patients with portal hypertension

Ya-Fei Zhang, Hong Ji, Hong-Wei Lu, Le Lu, Lei Wang, Jin-Long Wang, Yi-Ming Li

Ya-Fei Zhang, Hong Ji, Hong-Wei Lu, Le Lu, Lei Wang, Jin-Long Wang, Yi-Ming Li, Department of General Surgery, the Second Affiliated Hospital of Xi'an Jiaotong University, Xi'an 710004, Shaanxi Province, China

ORCID number: Ya-Fei Zhang (0000-0001-8223-4406); Hong Ji (0000-0002-4800-679X); Hong-Wei Lu (0000-0003-2904-9978); Le Lu (0000-0002-0529-1391); Lei Wang (0000-0001-7384-7913); Jin-Long Wang (0000-0002-3380-5425); Yi-Ming Li (0000-0002-4122-7020).

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Correspondence to: Yi-Ming Li, MD, Professor, Department of General Surgery, the Second Affiliated Hospital of Xi'an Jiaotong University, No. 157, Xiwu Road, Xi'an 710004, Shaanxi Province, China. liyiming@xjtu.edu.cn
Telephone: +86-29-87679746
Fax: +86-29-87679746

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Abstract

AIM

To analyse the postoperative survival of patients with portal hypertension and determine the factors that influence survival and construct nomograms.

METHODS

We retrospectively followed 1045 patients who underwent splenectomy plus pericardial devascularisation (SPD) between January 2002 and December 2017. Two SPD types are used in our department: splenectomy plus simplified pericardial devascularisation (SSPD) and splenectomy plus traditional pericardial devascularisation (STPD). The Kaplan-Meier method and Cox regression analysis were used to evaluate the prognostic effects of multiple parameters on overall survival (OS), disease-specific survival (DSS) and bleeding-free survival (BFS). Significant prognostic factors were combined to build nomograms to predict the survival rate of individual patients.

RESULTS

Five hundred and fifty-seven (53.30%) patients were

successfully followed with 192 in the SSPD group and 365 in the STPD group; 93 (16.70%) patients died, of whom 42 (7.54%) died due to bleeding. Postoperative bleeding was observed in 84 (15.10%) patients. The 5- and 10-year OS, DSS and BFS rates in the group of patients who underwent SSPD were not significantly different from those in patients who underwent STPD. Independent prognostic factors for OS were age, operative time, alanine transaminase level and albumin-bilirubin score. Independent prognostic factors for BFS were male sex, age, intraoperative blood loss and time to first flatus. Independent prognostic factors for DSS were the Comprehensive Complication Index and age. These characteristics were used to establish nomograms, which showed good accuracy in predicting 1-, 3- and 5-year OS and BFS.

CONCLUSION

SSPD achieves or surpasses the long-term survival effect of traditional pericardial devascularisation and is worthy of clinical promotion and application. Nomograms are effective at predicting prognosis.

Key words: Nomogram; Portal hypertension; Pericardial devascularisation; Survival analysis

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Core tip: The mortality and re-bleeding rate are still extremely high among patients with portal hypertension after splenectomy plus pericardial devascularisation. This study aimed to analyse the postoperative survival, identify risk factors, construct nomograms, and explore the clinical effect of splenectomy plus simplified pericardial devascularisation (SSPD). Five hundred and fifty-seven (53.30%) patients were successfully followed, and the results suggested that the 5- and 10-year overall survival, disease-specific survival and bleeding-free survival rates were not significantly different between patients who underwent SSPD and patients who underwent splenectomy plus traditional pericardial devascularisation. Age, operative time, alanine transaminase level and albumin-bilirubin score were independent prognostic factors influencing overall survival. Male sex, age, intraoperative blood loss and time to first flatus were independent prognostic factors influencing bleeding-free survival. Comprehensive Complication Index and age were independent prognostic factors influencing disease-specific survival.

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INTRODUCTION

Portal hypertension (PH) is mainly caused by cirrhosis, of which most cases are posthepatic cirrhosis in China and alcoholic cirrhosis in Western countries. Its clinical manifestations are splenomegaly and hypersplenism, and eventually oesophageal and gastric varices. The most important complication of PH is acute variceal bleeding, with a high mortality rate of more than 50%. And there is also a high risk of re-bleeding in surviving cases^[1,2]. Due to a difference in aetiology, the therapy of PH is different. Shunts are the main method in the West. However, in oriental countries, such as China, where patients who develop PH after hepatitis cirrhosis have poor liver function, treatment mainly involves devascularisation. Splenectomy plus pericardial devascularisation (SPD) is the main operative method used to prevent and treat PH^[3,4]. This operation does not reduce portal vein blood flow to the liver, does not damage liver function, can dislodge hypersplenism, and can effectively reduce the incidence of hepatic encephalopathy. However, splenectomy plus traditional pericardial devascularisation (STPD) is difficult and complicated, which will still cause greater tissue damage and increase the operative time, thereby increasing the damage of the liver and kidneys. By simplifying STPD, we put forward splenectomy plus simplified pericardial devascularisation (SSPD). The short-term curative effect of SSPD has been verified, but its effects on long-term survival are not clear^[5,6].

Patients with PH still face high risks of re-bleeding and death after SPD. Therefore, the best postoperative treatment must be identified to improve the prognosis of these patients, and a determination of the parameters impacting survival by examining factors related to the disease course in patients with PH is useful. Moreover, the identification of prognostic factors in patients with PH is important for estimating outcomes and determining the appropriate treatments. Nomograms have been used to integrate a variety of prognostic factors, quantify the impact of different factors on survival and visualise the results to predict the survival rate of individual patients. Nomograms have been widely used to assess patient prognosis^[7-9].

In the present study, 1045 patients with PH who underwent SPD were followed retrospectively to explore the long-term survival effect of SSPD and the prognostic factors for patients with PH, as well as construct a survival nomogram to predict the overall survival rate of patients with PH.

MATERIALS AND METHODS

Study subjects

Patients with PH presenting with oesophagogastric varices and hypersplenism who were treated in our department from January 2002 to February 2017 were

screened for this single-centre retrospective cohort study. Two SPD types are used in our department: SSPD and STPD. The surgical details are described in a previous publication^[5,6]. The inclusion criteria were: (1) patients with PH who were diagnosed with oesophagogastric varices and hypersplenism based on clinical symptoms combined with laboratory, digestive endoscopy or image examinations; (2) patients with PH classified as grade A or B according to the Child-Pugh grading criteria or Child-Pugh grade C at admission and assigned a reduced classification to preoperative Child-Pugh grade A or B after liver preservation therapy to attain appropriate surgical indications; and (3) patients who were able to tolerate general anaesthesia and had no surgical contraindications. The exclusion criteria were: (1) patients with hepatocellular carcinoma, acute heart failure, shock, or other vital organ diseases; (2) patients in an acute haemorrhagic state with unstable vital signs; and (3) patients with poor heart, lung, liver or kidney function. This research was approved by the Ethical Committee of the Second Affiliated Hospital of Xi'an Jiaotong University. All procedures were conducted in accordance with the Declaration of Helsinki from the World Medical Association and with the ethical standards of the committees responsible for human experimentation (institutional and national). The requirement for written informed patient consent was waived due to the retrospective and anonymous nature of this study; all data were used only for statistical analysis.

Data collection

Survival and postoperative bleeding conditions of the patients were monitored and recorded. The following primary data were collected: age, gender, aetiology, Charlson score, blood type, history of variceal ligation, history of abdominal surgery, smoking, drinking, history of variceal bleeding, body mass index (BMI), Child-Pugh score at admission, model for end-stage liver disease (MELD) score at admission, albumin-bilirubin (ALBI) score at admission, and Comprehensive Complication Index (CCI) score. The following laboratory data were collected at admission and during the perioperative period: White blood cell (WBC) count, haemoglobin (Hb) level, platelet count, prothrombin time (PT), international normalised ratio (INR), total bilirubin (TBIL) level, direct bilirubin (DBIL) level, alanine transaminase (ALT) level, aspartate transaminase (AST) level, albumin (ALB) level, globulin (GLB) level, serum creatinine (Scr) level, cystatin C (Cys C) level, duration of the preoperative hospital stay, duration of the postoperative hospital stay, total hospital stay, operative time, intraoperative blood loss, time to first flatus, and type of surgery. Information about the therapies used to correct a specific complication was also collected to calculate the CCI.

Calculation of CCI and ALBI, CP and MELD scores

The ALBI, CP, and MELD scores were calculated using the relevant formulas^[10-12]. The ALBI score was calculated as

follows: $\text{ALBI score} = 0.66 \times \log_{10} [\text{total bilirubin } (\mu\text{mol/L})] - 0.085 \times [\text{albumin (g/L)}]^{[10]}$. The CP score included five parameters: presence or absence of encephalopathy and ascites, serum total bilirubin level, albumin level and prothrombin time^[13]. The MELD score was calculated using the following formula: $11.2 \times \ln (\text{international normalised ratio}) + 9.57 \times \ln (\text{creatinine, mg/dL}) + 3.78 \times \ln (\text{bilirubin, mg/dL}) + 6.43 \times (\text{aetiology: 0 if cholestatic or alcoholic, 1 otherwise})^{[14]}$. Complications that occurred within 30 d after the operation were considered surgical complications and the severity of all complications was graded using the Centers for Disease Control (CDC) criteria^[15]. The CCI was calculated as the severity-weighted sum of all postoperative complications (available at <http://www.assesssurgery.com>). The CCI ranges from 0 (no complications) to 100 points (death)^[16-18].

Follow-up and survival endpoints

All included patients underwent routine follow-up examinations. Follow-up methods were mainly telephone calls or inpatient or outpatient re-examinations, and the last follow-up occurred on January 31, 2018. One of our primary endpoints of interest was overall survival (OS), which was defined as the time from surgery to death from any cause. In the analysis of OS, patients who were alive at the last follow-up date were counted as censored observations. The other primary endpoint of interest was disease-specific survival (DSS), which was defined as the time from surgery to death attributed to PH. In the DSS analysis, patients who died of other causes or were alive at the last follow-up date were counted as censored observations. Another primary endpoint was bleeding-free survival (BFS), which was defined as the time from surgery to the first appearance of initial oesophagogastric variceal bleeding. In the BFS analysis, patients who died of other causes or were alive at the last follow-up date were counted as censored observations. Univariate and multivariate Cox regression models were used to determine survival-related factors. Factors that were observed to have significant associations with survival in multivariate analyses ($P < 0.05$) were used to build the nomograms for OS and BFS.

Statistical analysis

Statistical analyses were performed using IBM SPSS statistics 22 software (SPSS Inc., Chicago, IL, United States) and R version 3.2.2 software (Institute for Statistics and Mathematics, Vienna, Austria; <http://www.r-project.org/>). Continuous variables are presented as means \pm SD, and categorical variables are presented as frequencies and percentages. Survival curves were generated using the Kaplan-Meier method, and the significance of difference in survival among selected variables was verified using the log-rank test. A univariate Cox regression analysis with an Enter method was used to estimate the relative risk (RR). A multivariate Cox regression analysis with a Forward Condition method

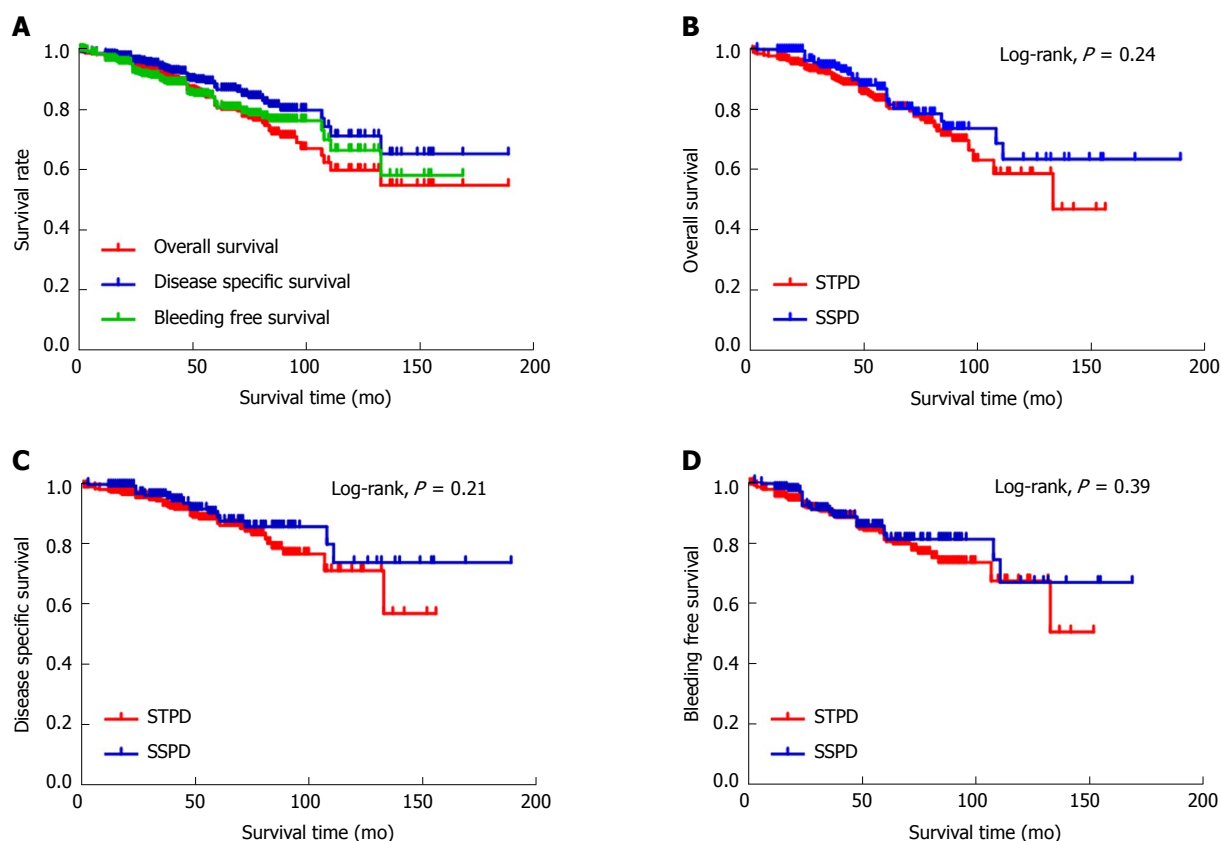


Figure 1 Kaplan-Meier survival curves for overall survival, disease-specific survival and bleeding-free survival. A: Kaplan-Meier survival curves for OS, DSS and BFS; B: Kaplan-Meier survival curves for OS stratified by the type of operation; C: Kaplan-Meier survival curves for DSS stratified by the type of operation. D: Kaplan-Meier survival curves for BFS stratified by the type of operation. OS: Overall survival; DSS: Disease-specific survival; BFS: Bleeding-free survival.

was used to estimate the RR and identify independent prognostic factors. The “rms” R library (cran.r-project.org/web/packages/rms) was used to construct nomogram models. All *P*-values were two-sided, and *P* < 0.05 was considered statistically significant.

RESULTS

Follow-up

Five hundred and fifty-seven (53.30%) patients were successfully followed, with 192 (34.47%) in the SSPD group and 283 (50.81%) males. These included 48 patients who had been followed in 2011 and were alive at that time^[5,6]. However, we were unable to reach these 48 patients now because of a change of contact or address, so we used this data as a censored data. The mean age of the patients was 48.79 ± 11.53 years. Ninety-three (16.70%) patients died, of whom 42 (7.54%), 21 (3.77%), 18 (3.23%) and 12 (2.15%) died of bleeding, liver failure, liver cancer, and cardiovascular and circulatory diseases, respectively. Postoperative bleeding was observed in 84 (15.10%) patients, and the bleeding mortality rate was 50.00%.

Kaplan-Meier survival curves for OS, DSS and BFS

Figure 1 shows the Kaplan-Meier survival curves for the OS, DSS and BFS of patients with PH. The 5-year OS

rate was 81.3% and the 10-year OS rate was 59.7%. The 5-year DSS rate was 87.3% and 10-year DSS rate was 71.0%. The 5-year BFS rate was 81.6% and 10-year BFS rate was 66.3%. Figure 1B shows the Kaplan-Meier survival curves for OS stratified by the type of operation. For the STPD group, the 5-year OS rate was 80.7% and 10-year OS rate was 58.4%. For the SSPD group, the 5-year OS rate was 82.5% and 10-year OS rate was 63.2%. The 5-year and 10-year OS rates in the SSPD group were higher than those in the STPD group, but the *P*-value of the log-rank analysis was 0.24 (*P* > 0.05), indicating that the difference was not statistically significant. Figure 1C shows the Kaplan-Meier survival curves for DSS stratified by the type of operation. For the STPD group, the 5-year DSS rate was 86.6% and 10-year DSS rate was 71.0%. For the SSPD group, the 5-year DSS rate was 88.7% and 10-year DSS rate was 73.6%. The 5-year and 10-year DSS rates in the SSPD group were higher than those in the STPD group, but the *P*-value of the log-rank analysis was 0.21 (*P* > 0.05), indicating that the difference was not statistically significant. Figure 1D shows the Kaplan-Meier survival curves for BFS stratified by the type of operation. For the STPD group, the 5-year BFS rate was 81.1% and 10-year BFS rate was 67.4%. The 5-year and 10-year BFS rates in the SSPD group were 82.6% and 66.9%, respectively. The *P*-value of the log-

Table 1 Patient demographics, laboratory information and perioperative characteristics (*n* = 319) *n* (%)

Parameter	STPD (<i>n</i> = 200)	SSPD (<i>n</i> = 119)	<i>P</i> value
Age (yr)	49.79 ± 11.14	48.92 ± 10.09	0.49
Gender (male)	93 (46.50)	54 (45.38)	0.85
Aetiology: Hepatitis B/hepatitis C/others	131 (65.50)/23 (11.50)/46 (23.00)	88 (73.95)/17 (14.29)/14 (11.76)	0.04
Charlson score: 0/1/2/3/≥ 3	105 (52.50)/54 (27.00)/21 (10.50)/ 20 (10.00)	62 (52.10)/42 (35.29)/12 (10.08)/3 (2.52)	0.06
Blood type: A/B/O/AB	57 (28.50)/62 (31.00)/59 (29.50)/22 (11.00)	36 (30.25)/33 (27.73)/36 (30.25)/14 (11.76)	0.94
History of variceal ligation	27 (13.50)	9 (7.56)	0.11
History of abdominal surgery	35(17.50)	21(17.65)	0.97
Smoking	57 (28.50)	28 (23.53)	0.33
Drinking	38 (19.00)	25 (21.01)	0.66
History of variceal bleeding	105 (52.50)	43 (36.13)	0.01
BMI	21.97 ± 3.04	21.62 ± 2.54	0.29
Child-Pugh score	6.56 ± 1.27	6.92 ± 1.29	0.02
MELD score	5.92 ± 0.40	5.97 ± 0.47	0.32
ALBI score	-2.26 ± 0.50	-2.10 ± 0.54	0.01
CCI score	18.64 ± 11.78	17.53 ± 9.53	0.38
WBC count (10 ⁹ /L)	2.79 ± 1.78	2.40 ± 1.38	0.04
Hb (g/L)	93.35 ± 24.77	94.74 ± 25.82	0.63
Platelet count (10 ⁹ /L)	49.32 ± 28.63	43.28 ± 21.20	0.05
PT (s)	13.85 ± 1.96	14.29 ± 1.82	0.04
INR	1.19 ± 0.18	1.32 ± 1.10	0.10
TBIL (mmol/L)	27.24 ± 16.22	29.37 ± 16.81	0.26
DBIL (mmol/L)	11.87 ± 7.67	12.33 ± 6.87	0.59
ALT (IU/L)	35.79 ± 55.76	38.20 ± 31.58	0.67
AST (IU/L)	43.72 ± 52.72	44.55 ± 33.76	0.88
ALB (g/L)	37.24 ± 5.50	35.61 ± 5.66	0.01
GLB (g/L)	28.36 ± 5.90	29.03 ± 5.91	0.32
Scr (mmol/L)	62.44 ± 18.02	58.71 ± 13.68	0.05
Cys C (mg/L)	1.12 ± 0.32	1.11 ± 0.25	0.71
Duration of preoperative hospital stay (d)	14.56 ± 9.43	16.08 ± 9.29	0.16
Duration of postoperative hospital stay (d)	15.75 ± 6.46	14.97 ± 5.30	0.27
Total hospital stay (d)	30.61 ± 12.20	31.77 ± 11.75	0.40
Operative time, min	139.76 ± 50.73	124.55 ± 42.49	0.00
Intraoperative blood loss (mL)	869.51 ± 692.77	591.34 ± 477.54	0.00
Time to first flatus (d)	4.69 ± 1.70	3.67 ± 1.18	0.00

BMI: Body mass index; MELD: Model for end-stage liver disease; ALBI: Albumin-bilirubin; CCI: Comprehensive Complication Index; WBC: White blood cell; Hb: Haemoglobin; PT: Prothrombin time; INR: International normalised ratio; TBIL: Total bilirubin; DBIL: Direct bilirubin; ALT: Alanine transaminase; AST: Aspartate transaminase; ALB: Albumin; GLB: Globulin; Scr: Serum creatinine; Cys C: Cystatin C; SSPD: Splenectomy plus simplified pericardial devascularisation.

rank analysis was 0.39 ($P > 0.05$), indicating that the difference was not statistically significant.

Independent prognostic factors for OS, DSS and BFS

In the follow-up analysis, we selected 319 patients with complete data to perform Cox regression analyses and to explore the prognostic factors for OS. Table 1 shows the demographics, laboratory information and perioperative characteristics of patients with PH.

As shown in Table 2, CCI; age; operative time; intraoperative blood loss; WBC, Hb, ALT, AST, and ALB levels; and Child-Pugh and ALBI scores at admission were remarkably correlated with OS in univariate Cox regression analyses ($P < 0.05$). CCI, age, intraoperative blood loss, platelet count, Scr level, Cys C level and MELD score were remarkably correlated with DSS in univariate Cox regression analyses ($P < 0.05$). Gender, age, history of variceal ligation, history of variceal bleeding, operative time, intraoperative blood loss, time to first flatus, Hb level, platelet count and GLB level at admission were remarkably correlated with BFS in univariate Cox regression analyses ($P < 0.05$).

A multivariate Cox regression analysis was used to further explore the influences of all variables that were significant in the univariate analysis. The multivariate analyses of OS showed that age, operative time, ALT levels and ALBI score were independent positive risk factors ($P < 0.05$); CCI and age were independent prognostic factors influencing DSS ($P < 0.05$), and male sex, age, intraoperative blood loss and time to first flatus were independent positive risk factors ($P < 0.05$) (Table 3).

Nomograms for predicting OS and BFS

We recruited all independent prognostic factors identified in Cox regression analysis of OS and BFS to construct the nomograms. The prognostic nomograms for 1-, 3- and 5-year OS rates are shown in Figure 2A, and nomograms for 1-, 3- and 5-year BFS rates are shown in Figure 2B. Each variable is projected upward to the value of the small ruler (Points) to get the score of each parameter. The higher the score, the worse the prognosis of survival. The sum of all small rulers is the total score (Total Points). The 1-, 3- and 5-year OS and

Table 2 Univariate Cox regression analysis of overall survival, bleeding-free survival and disease-specific survival

Parameter	OS			BFS			DSS					
	B	P value	RR	95%CI	B	P value	RR	95%CI	B	P value	RR	95%CI
Type of operation	0.16	0.62	1.18	0.62-2.23	-0.39	0.33	0.68	0.31-1.48	-0.45	0.17	0.64	0.34-1.21
CCI	0.04	0.00	1.04	1.01-1.07	0.02	0.36	1.02	0.98-1.05	0.06	0.00	1.06	1.02-1.09
Gender	0.01	0.99	1.01	0.54-1.89	0.94	0.02	2.56	1.20-5.43	0.06	0.91	1.06	0.43-2.60
Age	0.06	0.00	1.06	1.03-1.09	0.04	0.01	1.05	1.01-1.08	0.05	0.00	1.05	1.02-1.09
Aetiology	0.07	0.74	1.07	0.73-1.56	0.16	0.44	1.18	0.78-1.78	-0.16	0.43	0.86	0.58-1.26
History of variceal ligation	0.75	0.10	2.12	0.87-5.19	1.07	0.01	2.92	1.24-6.88	0.54	0.21	1.72	0.73-4.02
History of abdominal surgery	0.33	0.42	1.39	0.63-3.03	0.16	0.73	1.17	0.48-2.87	-1.28	0.20	0.28	0.04-1.96
Smoking	0.45	0.19	1.56	0.80-3.04	0.54	0.15	1.72	0.82-3.60	0.27	0.55	1.31	0.55-3.11
Drinking	0.17	0.65	1.19	0.56-2.51	0.71	0.07	2.04	0.96-4.33	-0.12	0.81	0.89	0.34-2.35
Charlson Score	0.11	0.53	1.12	0.80-1.56	0.02	0.93	1.02	0.69-1.51	0.06	0.77	1.06	0.72-1.58
History of variceal bleeding	0.07	0.82	1.08	0.57-2.03	1.02	0.01	2.77	1.30-5.90	-0.30	0.43	0.74	0.36-1.55
Blood type	-0.20	0.25	0.82	0.58-1.15	-0.15	0.41	0.86	0.60-1.23	-0.16	0.29	0.85	0.63-1.15
BMI	-0.06	0.34	0.94	0.83-1.07	0.00	0.97	1.00	0.88-1.14	-0.03	0.57	0.97	0.86-1.09
Total hospital stay	0.01	0.36	1.01	0.99-1.04	0.00	0.79	1.00	0.98-1.03	-0.03	0.26	0.97	0.93-1.02
Duration of preoperative hospital stay	0.01	0.43	1.01	0.98-1.05	0.01	0.59	1.01	0.97-1.05	0.04	0.20	1.04	0.98-1.09
Duration of postoperative hospital stay	0.03	0.20	1.03	0.98-1.09	-0.01	0.71	0.99	0.92-1.06	0.00	0.91	1.00	0.94-1.08
Operative time	0.01	0.00	1.01	1.00-1.02	0.01	0.04	1.01	1.00-1.02	0.00	0.33	1.00	1.00-1.01
Intraoperative blood loss	0.00	0.01	1.00	1.00-1.01	0.00	0.00	1.00	1.00-1.01	0.00	0.09	1.00	1.00-1.00
Time to first flatus	-0.01	0.94	0.99	0.81-1.21	0.23	0.03	1.26	1.03-1.53	-0.06	0.57	0.94	0.78-1.15
WBC count	0.20	0.03	1.22	1.02-1.45	0.15	0.13	1.16	0.96-1.41	-0.02	0.88	0.98	0.80-1.21
Hb	-0.01	0.04	0.99	0.97-1.00	-0.02	0.04	0.99	0.97-1	-0.01	0.31	0.99	0.97-1.01
Platelet count	0.01	0.08	1.01	1.00-1.02	0.01	0.01	1.01	1.00-1.02	0.01	0.05	1.01	1.00-1.03
PT	0.07	0.46	1.07	0.90-1.27	-0.08	0.44	0.92	0.74-1.14	-0.26	0.75	0.77	0.16-3.66
INR	-0.03	0.93	0.97	0.51-1.83	-0.93	0.44	0.39	0.04-4.29	-1.80	0.84	0.17	0.00-9821579.13
TBIL	0.01	0.22	1.01	0.99-1.03	-0.01	0.45	0.99	0.97-1.02	0.00	0.97	1.00	0.96-1.04
DBIL	0.02	0.20	1.02	0.99-1.06	-0.03	0.34	0.97	0.92-1.03	0.02	0.72	1.02	0.93-1.12
ALT	0.00	0.00	1.00	1.00-1.01	-0.01	0.35	0.99	0.98-1.01	0.01	0.12	1.02	1.00-1.03
AST	0.00	0.01	1.00	1.00-1.01	-0.02	0.10	0.98	0.96-1.00	-0.01	0.27	0.99	0.97-1.01
ALB	-0.11	0.00	0.90	0.84-0.96	-0.03	0.45	0.98	0.91-1.04	-0.39	0.25	0.68	0.35-1.31
GLB	0.02	0.53	1.02	0.96-1.07	-0.07	0.04	0.93	0.87-1.00	0.03	0.28	1.03	0.97-1.10
Scr	0.01	0.32	1.01	0.99-1.03	0.02	0.07	1.02	1.00-1.03	-0.08	0.01	0.93	0.87-0.98
Cys C	0.71	0.09	2.03	0.91-4.57	0.23	0.67	1.26	0.44-3.66	1.17	0.02	3.21	1.20-8.62
Child-Pugh score	0.29	0.01	1.33	1.08-1.65	0.13	0.36	1.13	0.87-1.48	0.14	0.54	1.15	0.74-1.81
MELD score	0.50	0.18	1.64	0.80-3.40	0.02	0.97	1.02	0.44-2.36	4.18	0.05	65.31	0.94-4536.32
ALBI score	1.07	0.00	2.92	1.49-5.76	0.19	0.59	1.21	0.60-2.46	-3.86	0.33	0.02	0.00-48.67

OS: Overall survival; BFS: Bleeding-free survival; CCI: Comprehensive Complication Index; BMI: Body mass index; WBC: White blood cell; Hb: Haemoglobin; PT: Prothrombin time; INR: International normalised ratio; TBIL: Total bilirubin; DBIL: Direct bilirubin; ALT: Alanine transaminase; AST: Aspartate transaminase; ALB: Albumin; GLB: Globulin; Scr: Serum creatinine; Cys C: Cystatin C; MELD: Model for end-stage liver disease; ALBI: Albumin-bilirubin; B: Regression coefficient; SE: Standard error; RR: Relative risk; CI: Confidence interval.

BFS rates can be obtained from the downward projection of the Total Points. This nomogram can predict the survival rate individually according to the different conditions of different patients, so as to improve the prediction efficiency and accuracy. The nomograms showed that the OS rates were higher for patients with younger age, patients with shorter operative time, patients with lower alanine transaminase levels and patients with lower albumin-bilirubin scores. The BFS rates were better for females,

Table 3 Multivariate Cox regression analysis of overall survival and bleeding free survival

Parameter	<i>B</i>	<i>SE</i>	<i>P</i> value	<i>RR</i>	95%CI
OS					
Age	0.06	0.02	0.00	1.06	1.03-1.09
Operative time	0.01	0.00	0.01	1.01	1.00-1.02
ALT	0.01	0.00	0.00	1.01	1.00-1.01
ALBI score	1.03	0.37	0.01	2.79	1.36-5.72
BFS					
Male	1.17	0.40	0.00	3.22	1.48-7.01
Age	0.05	0.02	0.01	1.05	1.01-1.09
Intraoperative blood loss	0.00	0.00	0.05	1.00	1.00-1.01
Time to first flatus	0.23	0.10	0.02	1.26	1.04-1.53
DSS					
CCI	0.06	0.01	0.00	1.06	1.05-1.08
Age	0.04	0.01	0.00	1.04	1.02-1.06

ALT: Alanine transaminase; ALBI: Albumin-bilirubin; B: Regression coefficient; SE: Standard error; RR: Relative risk; CI: Confidence interval.

younger patients, patients with less intraoperative blood loss and patients with less time to the first flatus. Guided by nomograms, we can better predict the prognosis based on the different characteristics of each patient.

DISCUSSION

We simplified STPD and introduced SSPD in 2002, which includes cutting and ligating of the posterior gastric vessels, suturing left gastric vessels and suturing from the lesser curvature to the lower esophageal vessels. Holding the paraoesophageal vein and cutting off the perforating vein only can effectively block the reflux of esophageal vessels, lower the portal vein pressure and ensure thorough haemostasis. On the other hand, SSPD can reduce gastric mucosal congestion, so as to reduce the incidence of PHG and prevent postoperative re-bleeding. Changing to suture and refraining from incision of seromuscular layer minimises wound injuries and reduces intraoperative blood loss. In addition, the operative time is significantly shortened, which can reduce liver injury to some extent. Higher 5- and 10-year OS and DSS rates were reported for the SSPD group than the STPD group, but the difference was not statistically significant. Moreover, the 5- and 10-year BFS rates for the SSPD group were not statistically significant compared with the STPD group. Based on these results, SSPD achieved or surpassed the long-term survival effect of STPD. However, the procedure of SSPD is simple and prone to master, with less tissue injury and inflammatory reactions. Therefore, SSPD is a good method of treating PH patients, and it can and should be promoted and applied to hospitals at different levels.

Five hundred and fifty-seven (53.30%) patients were successfully followed; 93 (16.70%) patients died, of whom 42 (7.54%) died due to bleeding. Postoperative bleeding was observed in 84 (15.10%) patients, and the bleeding mortality rate was 50.00%. Acute variceal bleeding is the most life-threatening complication of

PH, and despite the recent progress in management, this complication still occurs in approximately 20% of patients at 6 wk^[19,20]. Therefore, the risk factors for OS, DSS and BFS must be determined to accurately predict the OS and BFS of patients with PH and to conduct individualised prevention and treatment as early as possible. The multivariate analyses of OS showed that age, operative time, ALT levels and ALBI score were independent positive risk factors. Not surprisingly, age and operative time were independent positive risk factors for OS, as older age and longer operative time are always accompanied by underlying disease and more severe conditions, respectively. ALT is a sensitive marker of acute hepatocyte damage^[21], which may dramatically influence the OS. The ALBI score was recently established as an evidence-based model to assess the liver function of patients with hepatocellular carcinoma^[10] and has already been validated and proven to be more objective and precise than CP and MELD scores in predicting postoperative efficacy and survival^[22-28]. In clinical practice, we should pay more attention to older patients and patients with longer operative time and higher ALT levels and ALBI scores at admission to improve the preventative treatments for these patients during the perioperative period. According to the multivariate analyses of DSS, CCI and age were independent positive risk factors. The CCI is a novel method that mathematically integrates all complications graded by the conventional CDC criteria into one number, regardless of the number and severity of the complications, to capture the overall burden of an operation. Additionally, CCI is a continuous variable ranging from 0 (no complications) to 100 (death) points that easily quantifies complications and can be included in multifactor analyses. Thus, the CCI is the most attractive method for evaluating postoperative complications^[29]. The CCI has been applied in abdominal surgery and in the context of randomised controlled trials for patients undergoing oesophagectomy and has achieved better results^[30-32]. The multivariate analyses of

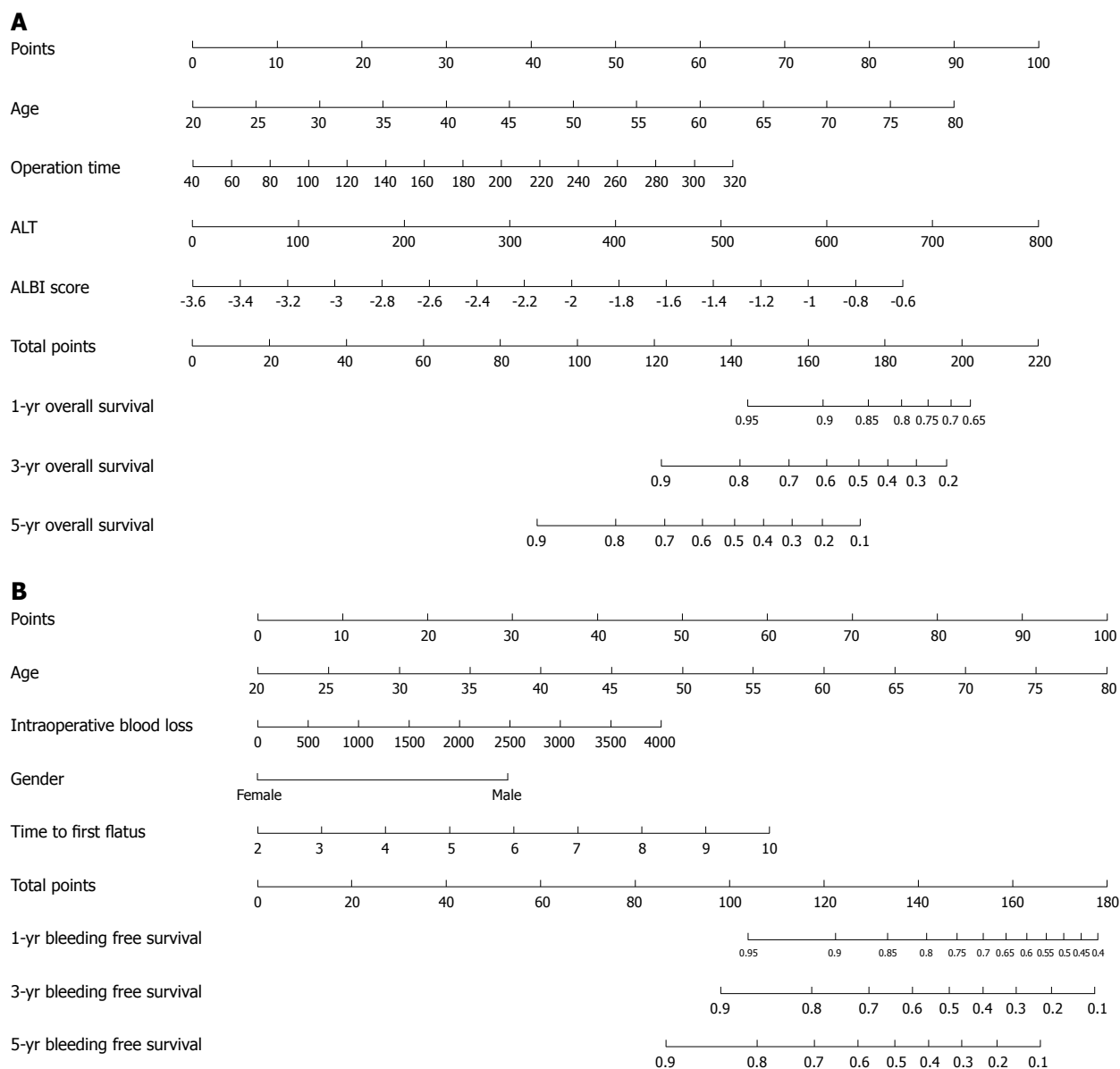


Figure 2 Nomograms for predicting 1-, 3-, and 5-year overall survival (A) and bleeding-free survival (B). ALT: Alanine transaminase; ALBI: Albumin-bilirubin score.

BFS showed that male sex, age, intraoperative blood loss and time to first flatus were independent positive risk factors. Intraoperative blood loss and time to first flatus were common factors in the perioperative evaluation index. However, male sex was an independent positive risk factor that has not been reported in previous studies. This discrepancy may be more related to the smoking and drinking history of male patients. In clinical practice, we should pay more attention to older male patients and patients with larger amounts of intraoperative bleeding and longer postoperative exhaust time. We should strengthen the measures to protect these patients during the perioperative period, pay attention to the postoperative re-examination, and perform ligation in a timely manner to prevent bleeding. In addition, a precise survival and re-bleeding prognostic tool is urgently needed to guide therapy selection for high-risk patients.

Integrating independent prognostic parameters, a nomogram can provide individualised evaluation of the clinical event incidence, such as survival rate^[7-9]. Compared with traditional methods, nomograms make predictions more quickly, conveniently and accurately. Its predictive value is better than other evaluation systems and is very important in clinical decision-making processes^[33,34]. However, the application of nomogram in PH patients has rarely been reported. In the present report, the prognostic nomograms included all significant independent factors in the Cox regression analyses of OS and BFS. According to the nomograms, the OS rates were better for younger patients, patients with shorter operative time, patients with lower ALT levels and patients with lower ALBI scores. In addition, the BFS rates were better for females, younger patients, patients with less intraoperative blood loss and patients

with less time to first flatus. Guided by nomograms, we can better predict the prognosis based on the different characteristics of each patient. To the best of our knowledge, our study is the first to construct nomograms to predict OS and BFS rates in patients with PH.

There are several limitations in the present study. First, potential bias may exist for the retrospective nature of our study^[35]. However, a randomised clinical trial may be not realizable for the reason of ethics. Second, for the research included only Chinese patients, our results may not be directly applicable to other races. In some cases, it may need to be verified. Third, this study was just conducted in a single hospital. Our results may not be fully applicable to other hospitals for the difference in treatment modalities and medical conditions. In addition, the nomograms constructed in our study lacked external validation due to the limited number of cases, which may reduce the credibility of the nomograms. Despite the aforementioned limitations, the present study has identified the prognostic factors for patients with PH after SPD and is the first to construct a nomogram to forecast the postoperative survival and re-bleeding rates of PH patients.

In summary, SSPD achieves or surpasses the long-term survival effect of STPD and is worthy of clinical promotion and application, particularly in primary hospitals. In clinical practice, we should pay more attention to males, older patients, and patients with longer operative time, patients with higher CCI scores, ALT levels and ALBI scores at admission, and patients with larger amounts of intraoperative bleeding and longer postoperative exhaust time. Nomograms are effective in predicting prognosis according to individual patient characteristics. Further large-scale prospective studies are needed to confirm our findings.

ARTICLE HIGHLIGHTS

Research background

Patients with portal hypertension (PH) still have higher re-bleeding rates and mortality after splenectomy plus pericardial devascularisation. We simplified splenectomy plus traditional pericardial devascularisation (STPD) and put forward splenectomy plus simplified pericardial devascularisation (SSPD), whose initial curative effects have been verified, but its long-term survival effects are not clear. Therefore, we need to identify the best postoperative treatment to improve the prognosis of these patients, and a determination of the underlying influencing factors is useful for estimating outcomes and determining the appropriate treatments.

Research motivation

SSPD achieves or surpasses the long-term survival outcome of STPD and is worthy of clinical promotion and application. In clinical practice, males and older patients, patients with longer operative time, patients with higher Comprehensive Complication Index (CCI), alanine transaminase (ALT) and albumin-bilirubin (ALBI) scores at admission, patients with larger amounts of intraoperative bleeding and patients with longer postoperative exhaust time should receive more attention.

Research objectives

The main aim of the retrospective research was to assess the postoperative survival rates of PH patients and identify the clinical efficacy of SSPD. Factors

influencing survival and nomograms were also identified.

Research methods

Five hundred fifty-seven (53.30%) patients were successfully followed. We performed a Kaplan-Meier analysis to construct survival curves. We also applied log-rank test to verify the significance of difference in survival rates. The risk factors were estimated using a univariate Cox regression analysis. A multivariate Cox regression analysis was used to estimate the relative risk and to identify independent prognostic factors. The "rms" R library was used to construct nomograms.

Research results

Five hundred and fifty-seven (53.30%) patients were successfully followed; 93 (16.70%) patients died, of whom 42 (7.54%) patients died due to bleeding. Postoperative bleeding was observed in 84 (15.10%) patients. There was no significant difference between SSPD and STPD in 5- and 10-year overall survival (OS), disease-specific survival (DSS) and bleeding-free survival (BFS) rates. Age, operative time, ALT level and the ALBI score were independent prognostic factors for OS. Male sex, age, intraoperative blood loss and time to the first flatus were independent prognostic factors for BFS. CCI and age were independent prognostic factors for DSS. Nomograms were established and were better at predicting 1-, 3-, and 5-year OS and BFS rates.

Research conclusions

SSPD achieves or surpasses the long-term survival outcomes of STPD, which is worthy of clinical promotion and application. In clinical practice, males, older patients, patients with longer operative time, patients with higher CCI scores, ALT levels and ALBI scores at admission, and patients with larger amounts of intraoperative bleeding and longer postoperative exhaust time should receive more attention. Nomograms are better in predicting prognosis according to individual patient characteristics.

Research perspectives

In the future, the long-term survival of patients with PH undergoing SSPD should be assessed in large-scale prospective studies.

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Observational Study

Fungal dysbiosis predicts the diagnosis of pediatric Crohn's disease

Mohammad I El Mouzan, Kirill S Korolev, Mohammad A Al Mofarreh, Rajita Menon, Harland S Winter, Ahmad A Al Sarkhy, Scot E Dowd, Ahmad M Al Barrag, Asaad A Assiri

Mohammad I El Mouzan, Ahmad A Al Sarkhy, Department of Pediatrics, King Saud University, Riyadh 11461, Saudi Arabia

Asaad A Assiri, Department of Pediatrics, Supervisor of Prince Abdullah Bin Khalid Celiac Disease Research Chair, King Saud University, Riyadh 11461, Saudi Arabia

Kirill S Korolev, Rajita Menon, Bioinformatics Program, Boston University, Boston, MA 02215, United States

Mohammad A Al Mofarreh, Al Mofarreh PolyClinic, Riyadh 11423, Saudi Arabia

Harland S Winter, MassGeneral Hospital for Children, Boston, MA 02114, United States

Scot E Dowd, MRDNA, Shallowater, TX 79363, United States

Ahmad M Al Barrag, Department of Microbiology, King Saud University, Riyadh 11461, Saudi Arabia

ORCID number: Mohammad I El Mouzan (0000-0001-8699-3143); Kirill S Korolev (0000-0003-1988-0645); Mohammad A Al Mofarreh (0000-0001-5426-9425); Rajita Menon (0000-0002-4767-0971); Harland S Winter (0000-0003-1122-4811); Ahmad A Al Sarkhy (0000-0002-1424-5784); Scot E Dowd (0000-0002-6296-1427); Ahmad M Al Barrag (0000-0002-8829-9371); Asaad A Assiri (0000-0003-3357-5794).

Author contributions: El Mouzan MI and Winter HS contributed to the conception and design of the study; Al Mofarreh MA, Al Sarkhy AA, Assiri AA and Al Barrag AM contributed to data acquisition and samples' storage; Dowd SE performed DNA extraction and fungal sequencing; Korolev KS and Menon R performed the biostatistics and bioinformatics; El Mouzan MI drafted the manuscript and all co-authors contributed to reviewing, editing and giving approval of the final manuscript.

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Correspondence to: Mohammad I El Mouzan, MD, Professor and Consultant Pediatrician, Department of Pediatrics, Gastroenterology Unit, College of Medicine, King Saud University, Riyadh 11461, Saudi Arabia. drmouzan@gmail.com

Telephone: +966-55-5479281

Fax: +966-11-4679364

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Abstract

AIM

To investigate the accuracy of fungal dysbiosis in

mucosa and stool for predicting the diagnosis of Crohn's disease (CD).

METHODS

Children were prospectively enrolled in two medical centers: one university hospital and one private gastroenterology clinic in the city of Riyadh, Kingdom of Saudi Arabia. The children with confirmed diagnosis of CD by standard guidelines were considered cases, and the others were considered non-inflammatory bowel disease controls. Mucosal and stool samples were sequenced utilizing Illumina MiSeq chemistry following the manufacturer's protocols, and abundance and diversity of fungal taxa in mucosa and stool were analyzed. Sparse logistic regression was used to predict the diagnosis of CD. The accuracy of the classifier was tested by computing the receiver operating characteristic curves with 5-fold stratified cross-validation under 100 permutations of the training data partition and the mean area under the curve (AUC) was calculated.

RESULTS

All the children were Saudi nationals. There were 15 children with CD and 20 controls. The mean age was 13.9 (range: 6.7-17.8) years for CD children and 13.9 (3.25-18.6) years for controls, and 10/15 (67%) of the CD and 13/20 (65%) of the control subjects were boys. CD locations at diagnosis were ileal (L1) in 4 and colonic (L3) in 11 children, while CD behavior was non-stricturing and non-penetrating (B1) in 12 and stricturing (B2) in 3 children. The mean AUC for the fungal dysbiosis classifier was significantly higher in stools (AUC = 0.85 ± 0.057) than in mucosa (AUC = 0.71 ± 0.067) ($P < 0.001$). Most fungal species were significantly more depleted in stools than mucosal samples, except for *Saccharomyces cerevisiae* and *S. bayanus*, which were significantly more abundant. Diversity was significantly more reduced in stools than in mucosa.

CONCLUSION

We found high AUC of fungal dysbiosis in fecal samples of children with CD, suggesting high accuracy in predicting diagnosis of CD.

Key words: Fungiome; Mycobiome; Crohn's disease; Inflammation; Saudi children

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Core tip: We found high accuracy of fungal dysbiosis in predicting diagnosis of Crohn's disease (CD), a finding similar to bacterial dysbiosis. However, the higher area under the curve for the fungal dysbiosis classifier in stool (0.85 ± 0.057) than in mucosa (0.71 ± 0.067) ($P < 0.001$), contrasts with bacterial studies, suggesting higher accuracy of stool samples. Although the clinical application of this finding is limited at present by the high cost of fungal analysis, such information is

important from a scientific viewpoint, to increase the understanding of the role of fungal flora in CD and to stimulate further studies.

El Mouzan MI, Korolev KS, Al Mofarreh MA, Menon R, Winter HS, Al Sarkhy AA, Dowd SE, Al Barrag AM, Assiri AA. Fungal dysbiosis predicts the diagnosis of pediatric Crohn's disease. *World J Gastroenterol* 2018; 24(39): 4510-4516 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v24/i39/4510.htm> DOI: <http://dx.doi.org/10.3748/wjg.v24.i39.4510>

INTRODUCTION

Inflammatory bowel diseases (IBDs), including Crohn's disease (CD) and ulcerative colitis, are chronic conditions. Their incidence is highest, with an increasing trend, in Western populations^[1,2]. However, their incidence is increasing in non-Western populations as well^[3,4]. The cause of CD remains unknown despite extensive research, and a multifactorial etiology has been suggested. In genetically-susceptible individuals, environmental triggering factors play a major role and diet and microbiota are the most relevant causative factors for children^[5]. Dietary components may act directly or through alteration of gut microbiota to initiate and maintain inflammation in susceptible subjects^[6,7]. Significant fungal dysbiosis has been demonstrated in adults and children with CD^[8-10]. Recent reports found high accuracy of bacterial dysbiosis in predicting the diagnosis of IBD in general and CD in particular^[11-13].

Despite the demonstration of fungal dysbiosis in adults and children with CD, there are no similar reports on the potential role of fungal dysbiosis in the diagnosis of CD. The objective of this report was, therefore, to evaluate the accuracy of fungal dysbiosis in stool and mucosal samples, for the diagnosis of CD in a cohort of non-Western children with new onset disease.

MATERIALS AND METHODS

Ethical considerations

This report is a portion of the main study project titled "Characteristics of inflammatory bowel disease in Saudi children". The study was reviewed and approved by the Institutional Review Board of the College of Medicine, King Saud University, Riyadh, Kingdom of Saudi Arabia (Approval number: 10/2647/IRB).

Study population, sample collection, storage, and processing

Children were prospectively enrolled in two medical centers: One university hospital and one private gastroenterology clinic in the same city of Riyadh, Kingdom of Saudi Arabia. The children were referred to these clinics for investigation of suspected IBD. The

children with confirmed diagnosis of CD by standard guidelines^[14] were considered cases and those in whom the diagnosis of CD was excluded were considered non-IBD controls. The most common final diagnoses in non-IBD controls were functional abdominal pain and polyps. Mucosal and fecal samples were collected from 15 children with confirmed CD and 20 controls without inflammation or infection. A total of 78 samples (58 from CD children and 20 from non-IBD controls) were obtained. Stool samples were collected before bowel preparation, and none afterward. Mucosal forceps biopsies were taken from various parts of the colon and ileum. All samples were put into cryovials without preservatives and transported immediately in ice to the laboratory and stored at -80 °C. At the end of the study, all samples were shipped by express mail in dry ice to MRDNA Laboratories (Shallowater, TX, United States) for microbiome analysis.

Fungal DNA extraction and sequencing

Fungal DNA was extracted using the Mobio PowerSoil kit as per the manufacturer's instructions (Mobio, Carlsbad, CA, United States). Amplicon sequencing service (bTEFAP[®]) was performed at MRDNA Laboratories and used for the fungal analysis^[15]. The internal transcribed spacer primers, ITS1F (CTTGGTCATTAGAGGAAGTAA) and ITS2R (GCTGCGTTCTTCATCGATGC), were used. A single-step 30-cycle polymerase chain reaction (PCR with HotStart *Taq* Plus Master Mix kit (Qiagen, Valencia, CA, United States) was employed. Samples were sequenced utilizing Illumina MiSeq chemistry following the manufacturer's protocols.

The Q25 sequence data derived from the sequencing process was processed using the MRDNA ribosomal and functional gene analysis pipeline (www.mrdnalab.com). Sequences were depleted of barcodes and primers, short sequences of < 150 bp were removed, and sequences with ambiguous base calls were removed. Operational taxonomic units were defined by clustering at 3% divergence (97% similarity), followed by removal of singleton sequences and chimeras. Final operational taxonomic units were taxonomically classified using BLASTn top hit analysis against a curated database derived from RDP II and NCBI (<http://rdp.cme.msu.edu> and www.ncbi.nlm.nih.gov, respectively) and compiled into each taxonomic level as both "counts" and "percentage" files.

Statistical analysis

All analyses were performed using Python and scikit-learn^[16]. Custom functions implementing the permutation test were written to detect the taxa with abundances significantly different between CD and control samples. When more than one sample was available from the same patient for analysis, the log relative abundances from these samples were averaged.

It has been shown that variations in species abundance are better captured by a log-transformed

than a linear scale, improving the statistical power^[13]. Therefore, we followed this approach. In addition, rare taxa (< 1% abundance or absent from > 50% of the samples) were removed to improve the statistical power. Statistical significance was assessed *via* a permutation test (Fisher's exact test), which yielded raw, uncorrected *P*-values. These were transformed into *q*-values (corrected *P*-value) that measure the probability of false discovery following the Benjamini Hochberg procedure^[17]. We considered associations statistically significant only when the corrected *P*-values were less than 0.05.

A linear logistic regression classifier (linearmodel. LogisticRegression) in scikit-learn, Machine Learning in Python^[16], was used to predict CD based on the subject's microbiota. The accuracy of the classifier was tested by computing the receiver operating characteristic (ROC) curve with 5-fold stratified cross-validation under 100 permutations of the training data partition. We partitioned the data into randomly assigned training and test sets 100 times; in each case, the classifier was trained on 4/5 of the data and tested on 1/5 of the data (*i.e.* 5-fold cross-validation).

Alpha diversity, a measure of genera richness (number of genera), was evaluated using the Shannon index. We used Fisher's *t*-test to determine *P*-values for alpha diversity.

The difference in community composition (Beta diversity) was quantified by the Bray-Curtis distance, which accounts for both patterns of presence-absence of taxa and changes in their relative abundances. Nonparametric multidimensional scaling (NMDS) was applied to visualize the distance between mucosa and stool samples taken from CD and control subjects. NMDS quantifies the dissimilarity in community composition between samples *via* a combination of presence-absence and absolute abundance of taxa. For the data shown, the separations were analyzed by the ANOSIM or analysis of (dis)similarity. The ANOSIM statistic compares the mean of ranked dissimilarities between groups to the mean of ranked dissimilarities within groups.

The statistical analyses in this article were performed and reviewed by the coauthors Kirill S Korolev, PhD and Rajita Menon, PhD from the Bioinformatics Program, Boston University, Boston, MA, United States.

RESULTS

Demographic data

All children were Saudi nationals. There were 15 CD children and 20 controls; mean age was 13.9 (range: 6.7-17.8) years for CD children and 13.9 (3.25-18.6) years for controls, and 10/15 (67%) of the CD and 13/20 (65%) of the control subjects were boys. At diagnosis, CD locations were ileal (L1) in 4 and ileocolonic (L3) in 11 children, while CD behavior was non-stricturing and non-penetrating (B1) in 12 and stricturing (B2) in 3 children. Controls included all patients with no evidence of IBD

Table 1 Fungal species abundance in mucosa and stool of controls

Fungal species	Mucosa abundance, %	Stool abundance, %	Ratio	P value
<i>Volvariella dunensis</i>	0.027	0.0013	0.047	0.036
<i>Lepraria humida</i>	0.015	0.0010	0.063	0.042

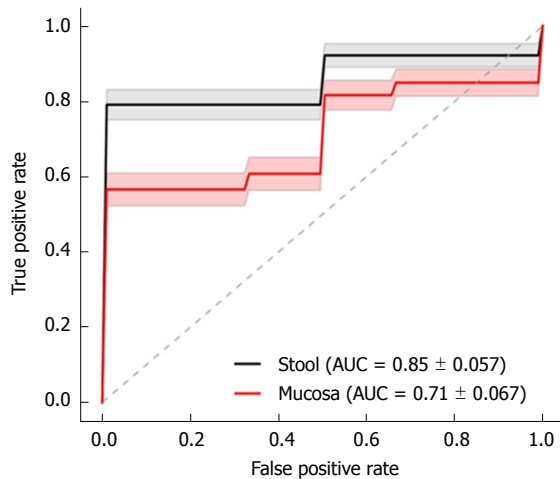


Figure 1 Dysbiosis score classification curve to distinguish between children with Crohn's disease and controls. The receiver operating characteristic (ROC) curve demonstrated the logistic regression dysbiosis classifier. The mean ROC curve for stool (solid black line) and mucosal (solid red line) dysbiosis scores in fungi cohorts is shown. The standard deviation from 100 permutations is shown in gray and light red shading. The area under the curve for stool dysbiosis is significantly higher than in mucosal dysbiosis, using a Fisher's *t*-test computed $P < 0.001$.

or other causes of inflammation. The final diagnoses in control subjects were juvenile polyps, recurrent abdominal pain, recurrent cyclic vomiting or other functional gastrointestinal disorders.

Fungal dysbiosis

In a previous report, description of fungal community structure in mucosa and stool in children with CD relative to controls indicated significantly-abundant CD-associated taxa included Psathyrellaceae ($P = 0.01$), Cortinariaceae ($P = 0.04$), Psathyrella ($P = 0.003$), and Gymnopilus ($P = 0.03$). Monilinia was significantly depleted ($P = 0.03$), whereas other depleted taxa, although not statistically significant, included Leotiomyces ($P = 0.06$), Helotiales ($P = 0.08$), and Sclerotiniaceae ($P = 0.07$)^[10].

Prediction analysis

The mean area under the ROC curve (AUC) for the fungal dysbiosis classifier is illustrated in Figure 1, indicating a significantly-higher AUC in stools (0.85 ± 0.057) than in mucosa (0.71 ± 0.067) ($P < 0.001$).

This analysis was further expanded to demonstrate the difference in abundance and diversity between mucosa and stool in controls and children with CD separately. Table 1 shows a comparison of fungal abundance between mucosa and stools in controls,

indicating that only two species, *Volvariella dunensis* ($P = 0.03$) and *Lepraria humida* ($P = 0.04$), were significantly less abundant in stool than in mucosal samples. In contrast, about 50 species were significantly less abundant in stools of children with CD ($P < 0.05$) and only two species, *Saccharomyces cerevisiae* ($P = 0.02$) and *S. bayanus* ($P = 0.001$), were significantly more abundant in stools than in mucosal samples (Table 2). Alpha diversity, as measured by the Shannon Index and illustrated in Figure 2, was different in mucosa and stool. The stool community for children with CD was more than 5 times less diverse than that of mucosa ($P = 0.0001$), whereas in controls the reduction in stool diversity was statistically not significant ($P = 0.35$). Beta diversity, as measured by the Bray-Curtis distance and visualized by the NMDS in Figure 3, shows a significant difference in fungal community separation between mucosal and stool samples ($P = 0.005$).

DISCUSSION

Microbial dysbiosis in the form of depletion of beneficial organisms, expansion of harmful organisms, and reduced microbial diversity may occur independently or concurrently and result in significant effects on immune responses^[18]. Fungal dysbiosis demonstrated in Saudi children with CD is in line with previous studies^[19,20].

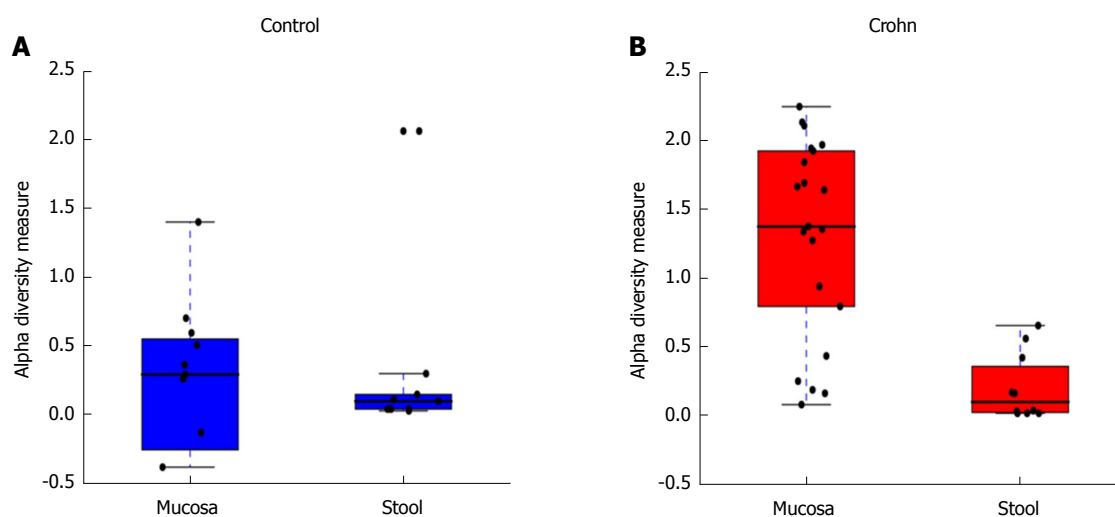
Key findings

This is the first report on the accuracy of fungal dysbiosis in mucosa and stools in predicting the diagnosis of CD in children. The main finding in this study is the high AUC for the fungal dysbiosis classifier, suggesting high accuracy in predicting the diagnosis of CD, which is in line with the high AUC for bacterial dysbiosis^[11-13]. However, the higher AUC for the fungal dysbiosis classifier is in stool (0.85 ± 0.057) rather than in mucosa (0.71 ± 0.067) ($P < 0.001$), in contrast with the higher AUC for bacterial dysbiosis in mucosal samples^[12-13]. Since this is the first report on fungal dysbiosis accuracy in predicting the diagnosis of CD, further studies are needed to clarify this result.

The finding of significant differences in a large number of species between stool and mucosa in CD children compared to much smaller numbers of species in controls reflects the degree of disturbance of the fungal community in children with CD. In this study, except for *S. cerevisiae* and *S. bayanus*, which were significantly more abundant in stool samples ($P = 0.02$ and $P = 0.003$, respectively), the significantly lower abundance of most fungal species in stool than in mucosal samples indicates

Table 2 Fungal species abundance in mucosa and stool of children with Crohn's disease

Fungal species	Mucosa abundance, %	Stool abundance, %	Ratio	P value
<i>Volvariella dunensis</i>	0.06	0.0014	0.025	< 0.001
<i>Malassezia restricta</i>	0.09	0.0044	0.049	< 0.001
<i>Ceriporia lacerate</i>	0.03	0.0022	0.065	< 0.001
<i>Cl. Cladosporioides</i>	0.11	0.004	0.035	< 0.001
<i>Trametes hirsute</i>	0.048	0.0043	0.089	< 0.001
<i>Psathyrella artemisiae</i>	0.44	0.028	0.065	< 0.001
<i>Amyloporia</i> sp	0.027	0.0012	0.046	< 0.001
<i>Irpex</i> sp	0.095	0.0019	0.02	< 0.001
<i>Bjerkandera adusta</i>	0.022	0.0011	0.049	< 0.001
<i>Lepista sordida</i>	0.041	0.0015	0.037	< 0.001
<i>Cerrena</i> sp	0.022	0.0011	0.05	< 0.001
<i>Coprinellus radians</i>	0.034	0.0011	0.032	< 0.001
<i>Phlebia acanthocystis</i>	0.022	0.0011	0.048	< 0.001
<i>Leptosphaerulina</i> sp	0.037	0.0013	0.036	< 0.001
<i>Coprinus</i> sp	0.085	0.0019	0.022	< 0.001
<i>Malassezia globosa</i>	0.028	0.0026	0.095	< 0.001
<i>Alternaria alternate</i>	0.054	0.0037	0.069	< 0.001
<i>Ramalinopsis mannii</i>	0.066	0.0018	0.027	< 0.001
<i>Saccharomyces bayanus</i>	2.5	53	21	< 0.001
<i>Trichoderma hypocre</i>	0.022	0.0014	0.067	< 0.001
<i>Aspergillus penicillioides</i>	0.11	0.0066	0.062	< 0.001
<i>Psathyrella candolleana</i>	0.063	0.0059	0.093	< 0.001
<i>Cladosporium</i> sp	0.072	0.0053	0.074	< 0.001
<i>Aspergillus</i> sp	0.064	0.0049	0.076	< 0.001
<i>Galactomyces geotrichum</i>	0.079	0.0063	0.08	< 0.001
<i>Peniophora incarnate</i>	0.011	0.0027	0.25	< 0.001
<i>Eutypella</i> sp	0.038	0.0044	0.12	< 0.001
<i>Ophiocordyceps sinensis</i>	0.059	0.004	0.068	< 0.001
<i>Nakaseomyces candida</i>	0.12	0.0058	0.049	0.019
<i>Hypocrea ceramic</i>	0.02	0.0037	0.18	0.019
<i>Saccharomyces cerevisiae</i>	0.043	0.23	5.3	0.024

**Figure 2** Alpha diversity evaluated by the Shannon Index. Comparison between mucosa and stool in children with Crohn's disease (CD) and controls. Alpha diversity is significantly lower in stool than in mucosa in CD samples ($P = 0.0001$), while the difference is not significant in control samples ($P = 0.35$).

variation in dysbiosis between stools and mucosa and supports the variable accuracy in predicting the diagnosis of CD.

It has been suggested that the presence of some fungi in the gut may reflect environmental exposure, rather than colonization of the mucosa^[21]. However, reports indicate that many fungi, such as *Candida*, *Cryptococcus*, *Malassezia*, *Saccharomyces* and *Trichosporon* spp, have

been isolated from the stool of humans^[22,23]. Furthermore, the role of fungi in the pathogenesis of IBD has been suggested based on animal models of colitis. Pattern recognition receptors in the innate immune system cells include dectin-1, dectin-2, DC-SIGN, mannose receptor, and mannose receptor lectin^[24], and mice lacking dectin-1 had increased susceptibility to experimental colitis^[25]. In addition, treatment with antifungal drugs may

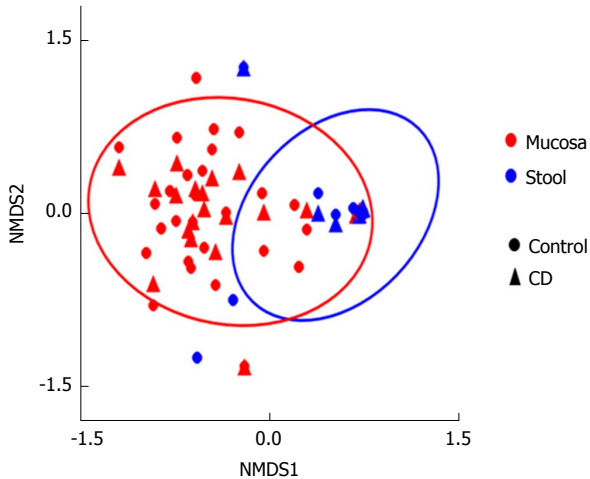


Figure 3 Beta diversity evaluated by Bray-Curtis distance and illustrated by the NMDS plot. This is a measure of beta diversity, and quantifies the similarity in abundance of taxa between samples. The ovals contain 95% of the probabilities for mucosa and stool. This plot shows a clear separation of mucosa and stool samples ($P = 0.005$). NMDS: Nonparametric multidimensional scaling; CD: Crohn's disease.

reduce the inflammation^[26].

Strengths and weaknesses of this study

The strengths of this study comprise the facts that it is the first to investigate the subject and provides inclusion of newly-diagnosed, treatment-naïve children with CD and controls from a well-defined population. Weaknesses include the relatively small number of cases which may be adequate for a first report. The controls were not completely healthy. Obviously, performing endoscopy and biopsies to exclude IBD in healthy children is unethical. Therefore, children who are free of IBD, infection and inflammation have been considered appropriate non-IBD controls.

In conclusion, the most important finding in this study is the high AUC in fecal samples of children with CD, suggesting high accuracy in predicting the diagnosis. Although, the clinical application of this finding is limited at present by the high cost of fungal analysis, such information is important from a scientific viewpoint, to increase the understanding of the role of fungal flora in CD and to stimulate further research, possibly leading to a "dysbiosis test" as a noninvasive screening tool for CD.

ARTICLE HIGHLIGHTS

Research background

Bacterial dysbiosis has been reported to predict the diagnosis of Crohn's disease (CD), but no similar reports for fungal dysbiosis exist. The study is of scientific significance to stimulate further research important for further clarification of the role of fungi in CD.

Research motivation

The role of microbiota in CD, bacterial or fungal, is of worldwide research interest. However, a key problem to be solved is whether dysbiosis is the cause or the result of inflammation. Solving this problem may facilitate discovery of

new microbiota-based treatment options. Regarding the accuracy of fungal dysbiosis in predicting the diagnosis, the main problem would be the current high cost of fungal analysis. Solving this problem could lead to development of a dysbiosis screening test for CD.

Research objectives

The objective of this study was to evaluate the accuracy of intestinal fungal dysbiosis as a predictor of CD. High accuracy was found. Future research is needed to confirm this finding and to develop low-cost fungal dysbiosis tests.

Research methods

Mucosal and stool samples were collected from children with CD at presentation and controls. Fungal DNA was extracted from these samples and sequencing was performed. Fungal abundance and diversities were determined. Fungal dysbiosis in children with CD was demonstrated. This is the first study of the accuracy of fungal dysbiosis in predicting the diagnosis of CD in children.

Research results

The main finding was the high accuracy of fungal dysbiosis in predicting diagnosis of CD. This should stimulate further research to confirm our findings and to develop a low-cost dysbiosis test.

Research conclusions

The high accuracy of fungal dysbiosis in predicting the diagnosis of CD is a new finding. The finding could lead to further research in the role of fungal dysbiosis in CD. A new theory suggests the possibility of design of a noninvasive fungal dysbiosis screening test for CD.

Research perspectives

The role of microbiota in CD may include development of a noninvasive screening test. Further research is needed to confirm the findings and to develop low-cost fungal analysis.

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Correction to “Maturity of associating liver partition and portal vein ligation for staged hepatectomy-derived liver regeneration in a rat model [*World J Gastroenterol* 2018 March 14; 24(10): 1107-1119]”

Yi-Fan Tong, Xiu-Jun Cai

Yi-Fan Tong, Xiu-Jun Cai, Department of General Surgery, Sir Run Run Shaw Hospital, School of Medicine, Zhejiang University, Hangzhou 310000, Zhejiang Province, China

ORCID number: Yi-Fan Tong (0000-0001-9028-5756); Xiu-Jun Cai (0000-0002-3615-4680).

Author contributions: Yi-Fan Tong and Xiu-Jun Cai contributed to this correction.

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Correspondence to: Xiu-Jun Cai, FRSC, MD, Professor, Surgeon, Department of General Surgery, Sir Run Run Shaw Hospital, School of Medicine, Zhejiang University, Qingchun East Road No. 3, Hangzhou 310016, Zhejiang Province, China. srsh_cxj@zju.edu.cn
Telephone: +86-571-86006605
Fax: +86-571-86006605

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CORRECTION

Correction to: Tong YF, Meng N, Chen MQ, Ying HN, Xu M, Lu B, Hong JJ, Wang YF, Cai XJ. Maturity of associating liver partition and portal vein ligation for staged hepatectomy-derived liver regeneration in a rat model (*World J Gastroenterol* 2017; 24(10): 1107-1119)^[1].

Erratum: In the “Conclusion of Abstract”, “Core tip”, “Discussion” and “Research perspectives”, the description regarding the relationship between the volumetric and functional proliferation during ALPPS-derived liver regeneration should be revised. Specifically, the sentence that reads “as the ALPPS-derived proliferation in volume lags behind the functional regeneration” should be revised to “as the ALPPS-derived functional regeneration lags behind the proliferation in volume”.

Conclusion of Abstract: The sentence “This could be convincing evidence that the stage II of ALPPS should be performed prudently in patients with marginally adequate FLR, as the ALPPS-derived proliferation in volume lags

behind the functional regeneration” should be revised to “This could be convincing evidence that stage II of ALPPS should be performed prudently in patients with marginally adequate FLR, as the ALPPS-derived functional regeneration lags behind the proliferation in volume”.

Core tip: The last sentence “as the ALPPS-derived proliferation in volume lags behind the functional regeneration” should be revised to “as the ALPPS-derived functional regeneration lags behind the proliferation in volume”.

Discussion: In line 8, the sentence “rapid increase in volume derived from ALPPS lags behind the functional proliferation” should be revised to “that ALPPS-derived functional regeneration lags behind the proliferation in volume”. In addition, the last sentence “as the ALPPS-

derived proliferation in volume lags behind the functional regeneration” should be revised to “as the ALPPS-derived functional regeneration lags behind the proliferation in volume”.

Research perspectives: In line 2, the sentence “as the ALPPS-derived proliferation in volume lags behind the functional regeneration” should be revised to “as the ALPPS-derived functional regeneration lags behind the proliferation in volume”.

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